Characterization of the various functional pathways elicited by synthetic agonists or antagonists at the melatonin MT$_1$ and MT$_2$ receptors

Céline Legros$^1$ | Clémence Dupré$^1$ | Chantal Brasseur$^1$ | Anne Bonnaud$^1$ | Damien Valour$^2$ | Preety Shabajee$^1$ | Adeline Giganti$^1$ | Olivier Nosjean$^1$ | Terrence P. Kenakin$^3$ | Jean A. Boutin$^1$

1 Pôle d’Expertise Biotechnologie, Chimie & Biologie, Institut de Recherches Servier, Croissy-sur-Seine, France
2 Pôle d’Expertise Méthodologie et Valorisation des Données, Institut de Recherches Internationales Servier, Suresnes, France
3 Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Correspondence
Céline Legros, Institut de Recherches Servier, 125 chemin de Ronde, 78290 Croissy-sur-Seine, France.
Email: celine.legros@servier.com

Present address
Olivier Nosjean and Jean A. Boutin, Institut de Recherches Internationales SERVIER, Suresnes, France

Original Article

Abstract
Melatonin is a neurohormone that translates the circadian rhythm to the peripheral organs through a series of binding sites identified as G protein-coupled receptors MT$_1$ and MT$_2$. Due to minute amounts of receptor proteins in target organs, the main tool of studies of the melatonergic system is recombinant expression of the receptors in cellular hosts. Although a number of studies exist on these receptors, studies of several signaling pathways using a large number of melatonergic compounds are rather limited. We chose to fill this gap to better describe a panel of compounds that have been only partially characterized in terms of functionality. First, we characterized HEK cells expressing MT$_1$ or MT$_2$, and several signaling routes with melatonin itself to validate the approach: GTP$_S$, cAMP production, internalization, β-arrestin recruitment, and cell morphology changes (CellKey$^8$). Second, we chose 21 compounds from our large melatonergic chemical library and characterized them using this panel of signaling pathways. Notably, antagonists were infrequent, and their functionality depended largely on the pathway studied. This will permit redefining the availability of molecular tools that can be used to better understand the in situ activity and roles of these receptors.

Keywords
biased ligands, melatonin, melatonin receptors, signaling pathways

Abbreviations: 2-[125I]-MLT, 2-[125I]-iodomelatonin; 2-Br-MLT, 2-bromомелатонин; 4P-PDOT, 4-phenyl-2-propionamidotetraline; 5HT, 5-hydroxytryptamine; 6-CI-MLT, 6-chloromelatonin; CAMP, cyclic AMP; CDS, cellular dielectric spectroscopy; D600, methoxyverapamil; DIV880, 2-[(2-iodo-4,5-dimethoxyphenyl)methyl]-4,5-dimethoxyphenyl; GTP$_S$, guanosine 5'-O-γ-thiotriphosphate; HTRF, homogeneous time-resolved fluorescence; IOP, intraocular pressure; ITC, isothermal titration calorimetry; luzindole, N-acetyl-2-benzyltryptamine; MLT, melatonin; MT$_1$, melatonin receptor 1; MT$_2$, melatonin receptor 2; PDOT, 4-phenyl-2-propionamidotetralin; S20098, N-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide; S20928, N-[2-[1-naphthyl(ethy)]cyclobutane carboxamide]; S21278, N-[2-(6-methoxy-benzimidazo[1,2-β]thieno]ethyl]acacetamide; S22153, N-[2-(5-ethylbenzothiophen-3-yl)ethyl]acetamide; S21278, N-[2-(2-iodo-5-methoxy-1H-indol-3-yl)ethyl]acetamide; S20893, N-[3-(methoxy-2-[2-(7-methoxy-1-naphthyl)propyl])acacetamide; S73893, N-[3-(methoxy-2-[2-(7-methoxy-1-naphthyl)propyl])acacetamide; S75436, 2-fluoro-N-[3-hydroxy-2-(7-methoxy-1-naphthyl)propyl]acetamide; S77834, N-[8-methoxy-10,11-dihydro-5H-dibenzo[a,d] [7]annuleno-10-yl]methyl] acetamide; S77840, 1-[8-methoxy-10,11-dihydro-5H-dibenzo[a,d] [7]annuleno-10-yl]methyl] urea; SD1881, N-[2-(6-methoxy-5-methoxy-1H-indol-3-yl)ethyl]acetamide; SD1882, N-[2-(4-iendo-5-methoxy-1H-indol-3-yl)ethyl]acetamide; SD1918, N-[2-(7-methoxy-5-methoxy-1H-indol-3-yl)ethyl]acetamide; SD21278, N-[2-(2-iodo-5-methoxy-1H-indol-3-yl)ethyl]acetamide; SD6, N-[2-(5-methoxy-1H-indol-3-yl)ethyl]iodoacetamide.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2019 The Authors. Pharmacology Research & Perspectives published by John Wiley & Sons Ltd, British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics.
INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone that regulates various biological functions in mammals through three different G protein-coupled receptors (GPCRs): MT₁, MT₂, and GPR50/Mel1c as reviewed by Jockers et al. However, GPR50 lost its capacity to bind melatonin during evolution, except in the case of the platypus, in which Mel1c binds melatonin. Melatonin is also an effective pleiotropic agent in plants. The well-described functions seem to be dependent on the activation of these receptors at low, physiological concentrations in the picomolar range. However, some doubts exist concerning the local concentration of melatonin in organs, particularly the brain, as they may be far higher than generally thought. Furthermore, melatonin has been described as a beneficial modulator of a plethora of processes, including the various side effects of agonists of melatonin and melatonin receptor ligands can transduce information inside cells (see Benleulmi-Chaachoua et al. and Ceccon et al. for further discussions). Historical approaches for studying the functionality of melatonin receptors have been animal models, such as the pigment aggregation of Xenopus laevis melanophores. More recently, by overexpressing the receptors in host cells, they were demonstrated to be coupled to pathways involving ERK1/2 and PI3K/AKT. Despite a clear and complete review of melatonergic ligands, including a long discussion on agonists and antagonists, these signaling pathways have been poorly characterized for MT₁ and MT₂.

Because these receptors are poorly expressed in vivo, it is difficult to measure the pathways in ex vivo samples. Nevertheless, several reports have demonstrated the feasibility and use of recombinant receptors expressed in host cells to describe their functionality. We stably expressed the human receptors in HEK cells and previously characterized binding in these cell lines, using a panel of melatonin ligands. Therefore, we chose to measure such functionality at MT₁ and MT₂ in the following functional systems: inhibition of the cAMP production in both agonist and antagonist modes, G protein activation by [35S]-GTP γ S binding (in the same agonistic and antagonistic modes), β-arrestin recruitment, receptor internalization, and the more global of cell shape shift (CellKey®). These last pathways were developed, assessed, and standardized using melatonin itself before 20 different ligands were tested.

Here, we show that biased melatonin agonists can be obtained and, based on this slightly large series of compounds, a clearer image of the melatonin receptor signaling pathways can be obtained.

MATERIALS AND METHODS

2.1 Compounds

The structures of the compounds tested in the present study are reported in Figure 1. They were all described previously, as they were used as a reference set for our previous work on melatonergic systems in humans, rats, mice, and sheep. In all instances, the compounds were tested at 11 concentrations in duplicate, and at arachidonic acid production, though in a less conventional manner. Interestingly, these routes were measured in ex vivo contexts. Furthermore, the formidable complexity of the melatonin receptor interactomes as extrapolated from mostly two-hybrid kind of experiments would also augur of further difficulties in understanding the way melatonin and melatonin receptor ligands can transduce information inside cells. The diversity of the melatonin actions within the body can be explained by the influence of many factors, including melatonin levels, which fluctuate over the circadian cycle (lowest during the day, highest during the night) and over the course of a year. Although some receptor-independent actions of melatonin are evident, most of the well-described functions seem to be dependent on the activation of these receptors at low, physiological concentrations in the picomolar range. However, some doubts exist concerning the local concentration of melatonin in organs, particularly the brain, as they may be far higher than generally thought. Furthermore, melatonin has been described as a beneficial modulator of a plethora of pathological conditions, though often at very high concentrations; Boutin provides roughly 400 examples. This body of observations is surprising; therefore, we feel that the way melatonin acts in these physio-pathological conditions should be more thoroughly documented. Indeed, the mechanism of action in these reports is often poorly documented, if at all. It is important to understand how melatonin acts, including its potential targets and the pharmacology of those targets. If the main targets in these healing processes are the melatonin receptors, it would be of major interest to strictly classify the various ligands of these receptors as agonists, antagonists, or biased ligands with different types of functionality toward different downstream signaling pathways. Such studies in the past decade have led to complete analyses of the GPCR signaling pathways, including the various side effects of agonists of ghrelin receptor.

Concerning the melatonergic system, several reviews have been published over the last decade concerning its signaling pathways. MT₁ and MT₂ have been shown to be coupled to Gα₁₆ proteins, inhibiting adenylyl cyclase and the production of cAMP and cGMP, as well as calcium influx, potassium conductance, and PI3K/AKT. Despite a clear and complete review of melatonergic systems in humans, rats, mice, and sheep, in all instances, the compounds were tested at 11 concentrations in duplicate, and at
least two independent replicates were performed (different operators, different day, different membrane or cell batch preparations, and different compound solutions). In the 96-well format, four compounds were tested per plate; in the 384-well format, 14 compounds were tested per plate.

2.2 | Repetitivity

Some experiments were only run in duplicate (210 out of 2089 experiments). Because we performed an enormous number of experiments, we felt that n = 2 would be acceptable in two types of cases. (1) The compound has no activity across the 11 doses (94 experiments). We felt that the results were strong enough not to rerun a third or a fourth assay; and (2) the variation between n = 1 and n = 2 was less than 3% (106 experiments). We present all the data in Table S1.

2.3 | Statistical analysis

The pEC50 and the maximal effect (Emax) of each product were compared to the melatonin results (the natural ligand), which were taken as reference for this type of system. Same strategy was used to analyze both parameters:

i/ ANOVA was ran following a GLM fitting including two fixed factors and an interaction: parameter ~ drug + hMTgrp + drug * hMTgrp + e; and ii/each compound was compared to melatonin in post hoc tests. The real difference between the pEC50/Emax means for the compound vs melatonin was tested using a Dunnett’s test with which we obtained an adjusted P-value. The corresponding P-values were reported for pEC50/Emax when appropriate in Table S1.

2.4 | Membrane preparations

As described previously, CHO-K1 cells stably expressing human MT1 (hMT1) or hMTR were grown to confluence, harvested in PBS buffer (Gibco, Invitrogen, Saint-Aubin, France) containing 5 mmol/L EDTA, and centrifuged at 1000g for 20 minutes at 4°C. The resulting pellet was suspended in 5 mmol/L Tris/HCl (pH 7.4) containing 2 mmol/L EDTA and homogenized using Kinematicapolytron. The homogenate was then centrifuged at 20,000g for 30 minutes at 4°C and the resulting pellet suspended in 75 mmol/L Tris/HCl (pH 7.4) containing 2 mmol/L EDTA and 12.5 mmol/L MgCl2. Protein content was determined according to Bradford using the Bio-Rad kit (Bio-Rad SA, Ivry-sur-Seine, France). Aliquots of membrane preparations were stored in resuspension buffer (75 mmol/L Tris/HCl pH 7.4, 2 mmol/L EDTA, 12.5 mmol/L MgCl2) at −80°C until use.

2.5 | 2-[125I]-iodomelatonin and [35S]-GTPγS binding assays

The assays were described previously.36 Briefly, for competition experiments in CHO cells, the membranes were incubated in 250 μL binding buffer (50 mmol/L Tris/HCl pH 7.4, 5 mmol/L MgCl2) containing 20 pmol/L 2-[125I]-iodomelatonin for 2 hours at 37°C. The results were expressed as the inhibition constant (Ki), taking into account the concentration of radioligand used in each experiment. Nonspecific binding was defined using 10 μmol/L melatonin. The reaction was stopped by rapid filtration through GF/B unfiltrators, followed by three successive washes with ice-cold buffer. The data were analyzed using the program PRISM (GraphPad Software Inc.). Ki was calculated according to the Cheng–Prussoff equation: Ki = IC50/[1 + (L/Kd)], where IC50 is the half maximal inhibitory concentration and L is the concentration of 2-[125I]-MLT. For the [35S]-GTPγS binding assay, the membranes and compounds were diluted in binding buffer (20 mmol/L Hepes pH 7.4, 100 mmol/L NaCl, 3 mmol/L MgCl2, 3 μmol/L GDP) in the presence of 20 μg/mL saponin in order to enhance the agonist-induced stimulation (Audinot et al., 2002). Incubation was started by adding 0.1 mmol/L [35S]-GTPγS to the membranes and ligands in a final volume of 250 μL and allowed to continue for 60 min at room temperature. Nonspecific binding was assessed using nonradiolabeled GTPγS (10 μmol/L). Reactions were stopped by rapid filtration through GF/B unfiltrators presoaked with distilled water, followed by three successive washes with ice-cold buffer. The data were analyzed using the program PRISM to yield the half maximal effective concentration (EC50) and Emax expressed as a percentage of the effect observed with melatonin (1 μmol/L = 100%). pEC50 was calculated as −log(EC50). Both assays were historical assays in our laboratory. Importantly, none of the compounds in the selection of ligands presented any activity on naïve cells or membranes.

2.6 | HTRF cAMP assay

Cellular cAMP production was measured using cAMP dynamic HTRF kits (Cisbio Bioassays) according to the manufacturer’s instructions. CHO-K1 cells stably expressing hMT1 or hMTR were grown to confluence, harvested in PBS buffer containing 5 mmol/L EDTA, and centrifuged at 100g for 10 minutes at 4°C. The cell pellet was resuspended in HAMF12 medium containing 0.5 mmol/L IBMX at a concentration of 2 million cells/mL. Incubation was started by adding 5 μmol/L forskolin (15 μL/well), 30 000 cells/well and the compounds of interest (15 μL/well, DMSO 1.7%) in a final volume of 60 μL. The incubation was allowed to continue for 20 minutes at 37°C. Next, 15 μL of cAMP-d2 conjugate and 15 μL of anti-cAMP-EuK conjugate in lysis buffer were incubated for 30 minutes at room temperature. The fluorescence intensity was measured at 340 nm excitation and 665 and 620 nm emission on an Envision (PerkinElmer). The TR-FRET 665 nm/620 nm ratio, which is inversely proportional to the production of cAMP, was used to determine the cAMP response. Nonspecific binding was assessed using 100 μmol/L nonlabeled cAMP. The data were analyzed in PRISM to yield the EC50 and Emax. No significant results were obtained with any of the compounds when they were tested in naïve cells.

2.7 | β-Arrestin

β-Arrestin recruitment was monitored using PathHunter CHO-K1 MT1 or hMTR/β-arrestin cell lines from DiscoveRx. Cells were cultured at 37°C in a 5% CO2 atmosphere in F12-GlutaMAX medium (Thermo Fisher Scientific, France) supplemented with 10% heat-inactivated
fetal bovine serum. The expression of engineered MT₁ or MT₂ and β-arrestin was maintained by antibiotic selection (hygromycin B 300 µg/mL, genetin 800 µg/mL). For assays, cells were washed in PBS, resuspended using Cell Dissociation Solution (Sigma-Aldrich, France), and seeded onto Cellstar 384-well microplates (Greiner Bio-one, France) at 6500 cells/well in 20 µL PathHunter Cell Plating Reagent, and then incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. After compound incubation (in 1.5% DMSO for a final volume of 25 µL) for 90 minutes at 37°C in a 5% CO₂ atmosphere, PathHunter detection reagent was added and the Enzyme Fragment Complementation reaction carried out for 120 minutes with shaking at room temperature. Chemiluminescence was measured on a MicroBeta TriLux (PerkinElmer, France). We assessed and validated these methodologies for melatonin receptors (Dupré et al, 2018). No significant results were obtained with any of the compounds when they were tested in naïve cells.

2.8 | Receptor internalization

We previously assessed and validated these methodologies for melatonin receptors.30

MT₁, DLD-1 cells (DiscoverX) were cultivated in RPMI 1640 Glutamax medium (Invitrogen) with 250 µg/mL hygromycin B (Invitrogen), 0.5 µg/mL puromycin (Sigma), and 10% fetal calf serum (Lonza, Belgium). Cells were grown in flasks until confluence, then detached using cell dissociation buffer solution (Sigma), and centrifuged for 10 minutes at 900 rpm. The supernatant was removed, cells collected in Cell Plating 25 solution (DiscoverX), and adjusted to 750,000 cells/mL. The suspension was dispensed in 20-µL aliquots (i.e., 15,000 cells) in a 384-well plate (Greiner, France). Cells were incubated overnight at 37°C in a 5% CO₂ atmosphere. Compounds were then added in 5 µL aliquots and incubated for 3 hours at 37°C in a 5% CO₂ atmosphere. The reaction was revealed by adding revelation reagent from the Path Hunter Detection Kit (DiscoverX) for 1 hour at room temperature. The principle is based on Enzyme Fragment Complementation (EFC) developed by DiscoverX. MT₁ was combined with prolinc PK, and endosomal protein was coupled to EA. When MT₁ was internalized by the endosome, EA and PK built up β-galactosidase, which reacts with its specific substrate, creating luminescence. The signal was read on an EnVision plate reader (PerkinElmer). No significant results were obtained with any of the compounds when they were tested in naïve cells.

MT₂, U2O-S cells (DiscoverX) were cultivated in EMEM Glutamax medium (ATCC© 30-2003) with 250 µg/mL hygromycin B (Invitrogen, France), 0.25 µg/mL puromycin (Sigma), and 10% fetal calf serum (Lonza). Cells were grown in flasks until confluence, detached using cell dissociation buffer solution (Sigma), and centrifuged for 10 minutes at 900 rpm. The supernatant was removed, cells collected in Cell Plating 5 solution (DiscoverX), and adjusted to 250,000 cells/mL. The suspension was dispensed in 20-µL aliquots (i.e., 5000 cells) in a 384-well plate (Greiner). Cells were incubated overnight at 37°C in a 5% CO₂ atmosphere. Compounds were then added in 5-µL aliquots and incubated for 3 hour at 37°C in a 5% CO₂ atmosphere. The reaction was revealed by adding revelation reagent from the Path Hunter Detection Kit (DiscoverX) for 1 hour at room temperature. As with MT₁, the principle is based on EFC developed by DiscoverX. MT₂ was combined with prolinc PK, and endosomal protein was coupled with EA. When MT₂ was internalized by the endosome, EA and PK built up β-galactosidase, which reacts with its specific substrate, creating luminescence. The signal was read on an EnVision plate reader (PerkinElmer). No significant results were obtained with any of the compounds when they were tested in naïve cells.

2.9 | Cellular dielectric spectroscopy

Cellular dielectric spectroscopy (CDS) provides information on ligand-induced cell shape modifications, which are measured through modification of the whole-cell electrical impedance.37,38 Being a whole-cell technology, the influence of ligand action on this parameter leads to complex images, resulting in the superimposition of several pathways induced by the same ligand and the same receptor, and each leading to different shape modifications. A schematic of these pathways is provided in Scheme 1 for a global overview of melatonin receptor functionality. The impedance measurement assay used CHO-K1 cells expressing hMT₁ and hMT₂. We previously described this approach in our work on sheep melatonin receptor cloning,32 and it was previously assessed and used by our group with the human receptors.30 The approach was utilized here with minor modifications. Briefly, 40,000 cells per well were plated on MDS Analytical 96-well assay plates with embedded electrodes (MDS Analytical). The plates were incubated at 37°C in a 6% CO₂ atmosphere for 48 hour. Prior to the measurement, the cells were washed three times with Hanks balanced salt solution (0.1% BSA, 20 mm HEPES, pH 7.4). The cells were left to equilibrate at 28°C for 30 minutes. Impedance was measured on a CellKey instrument (MDS Analytical). The signal was recorded for 5 minutes before the addition of the compounds and 15 minutes thereafter. The cells in each well were stimulated once with a single concentration of compound. The resulting data were expressed as the maximal signal corrected for the baseline and represented as a percentage of the full agonist effect (melatonin, which served as a positive control in these experiments). Naïve CHO-K1 cells were used as controls in these experiments, as they did not show any signal after melatonin treatment. We previously assessed and validated these methodologies for melatonin receptors.30

2.10 | Biased agonism calculation

The technique described by Kenakin10,39,40 was applied to our data. Briefly, log(max/EC50) values were calculated from the data gathered in each assay. These values were then subtracted from the log(max/EC50) value of melatonin, the natural ligand for each assay, to obtain the Δlog(max/EC50), which was used to build the radar plots.
3 | RESULTS

3.1 | Signaling pathways

We chose to develop and assess six pathways in addition to having the binding assay as a reference for comparisons with our previous work on the human melatonin receptors. Briefly, cAMP(+/-) is the inhibition of the adenylate cyclase via G and cAMP(-) is the capacity of an antagonist to impair the melatonin-induced inhibition of adenylate cyclase. Here, melatonin was used as the reference and full agonist. GTPγS can be taken as a test to measure the capacity of an agonist to recruit G protein (GTPγS(+)) or for an antagonist to inhibit the agonist-induced G protein recruitment (GTPγS(-)) in the presence of melatonin. The measurement of β-arrestin permits an evaluation of the capacity of a ligand, theoretically agonist only, to facilitate desensitization and internalization of GPCRs, interrupting the signal induced by the ligand/receptor association. This step is the first preceding internalization. Obviously, internalization is a measure of the capacity of a ligand to internalize the receptor, making it unavailable.

Finally, CDS will give information on the ligand-induced cell shape modification measured through modification of the whole-cell electrical impedance. Being a whole-cell technology, the influence of the ligand action on this parameter leads to complex images, resulting in the superimposition of several pathways induced by the same ligand and the same receptor, and each leading to different shape modifications. Due to the enormous number of data points generated during these studies, we had to make the choice not to present the data curves, but rather to present them as numerical data on binding, [35S]GTPγS agonist mode, [35S]GTPγS antagonist mode, cAMP agonist mode, and cAMP antagonist mode in the Table S1. To visualize the data, we present histograms (Figures 2-5) showing the profile of some typical compounds.

3.2 | The natural agonist: melatonin

Our first observation was that melatonin leads to a positive result (agonism at low concentration) in every evaluated pathway. This is important because it indicates that the host cells possess all of the necessary proteins for these signaling pathways. When considering the profile of melatonin, the five pathways measured in an agonistic mode had a pEC50 in the range of 9 to 10 and were almost similar in all cases, regardless of the pathway (Figure 2). Furthermore, activation of the receptor by its natural agonist led to its internalization after activation of the β-arrestin pathway, as expected.

3.3 | Nonselective ligands at MT₁ and MT₂: 2-iodomelatonin and S20928

Historically, in addition to the first synthesis of melatonin analogs, a series of compounds modified by a halogen atom were synthesized and characterized by their affinity at the receptors (the chemistry of melatonin receptor pharmacology has been reviewed by Zlotos et al. and Rivara et al.). We present the results obtained with 2-iodomelatonin because it is an historical compound and the most used ligand in melatoninergic pharmacology (Figure 3A). The profile of the compound is very similar to that of melatonin itself. Only minute differences were seen between the
results obtained with this ligand and the tritiated melatonin. The affinities (pEC50) were between 9 and 11 and, except for GTPyS binding, the efficiency at both receptors was close to the efficiency observed with melatonin itself. GTPyS binding shows that the ligand could be considered a superagonist, as its efficiency was above 100% (130 and 170% at MT1 and MT2, respectively). Because our main goal for decades has been to find alternate powerful agonists at the melatonin receptors, we have multiple examples of compounds with identical profiles: 2-bromomelatonin, 6-chloromelatonin, SD6, S75436, SD1881, S27128, S21278, and S20098 (Agomelatine®) (Figures S1). All of these compounds act as agonists at both receptors, with similar affinities in the nanomolar range. Furthermore, they are almost as potent as melatonin, the natural ligand.

S20928 has been depicted as a mixed agonist/antagonist at MT2. Despite a “poor” affinity in the 100 nmol/L range for the receptor, the compound induces the binding of GTPyS at MT2, but not at MT1. Similar to 4P-PDOT, S20928 fully inhibits the melatonin-induced GTPyS binding at MT1, whereas it slightly inhibits it at MT2. In line with this behavior, S20928 poorly inhibits cAMP production (less than 25% with pKi = 5.78) at MT1, but inhibits 50% of the production of cAMP at MT2 with a pKi similar to its pK (Figure 3B). However, despite its capacity to recruit β-arrestin at MT2, S20928 does not induce internalization at either receptor, suggesting a “preferred” antagonist behavior.
3.4 | Nonselective antagonists at MT₁ and MT₂: 4P-PDOT and luzindole

Historically, one of the first reported antagonists at MT₁ and MT₂ was 4P-PDOT.45 Figure 4A shows the results we obtained with this compound. The molecule clearly behaves like a partial agonist at both receptor subtypes; it inhibits cAMP production with an efficacy of nearly 50% of melatonin for MT₁ and 90% of melatonin for MT₂, though with a rather poorer pKᵢ at MT₁ than at MT₂ (6.85 vs 8.97, respectively). Nevertheless, 4P-PDOT does not induce GTPγS binding at MT₁, but does at MT₂, though with low efficacy (34%). Surprisingly, 4P-PDOT inhibits the melanin-induced binding of GTPγS more potently at MT₁ (100%) than at MT₂ (49%). Unexpectedly, at both receptors, 4P-PDOT inhibited cAMP production 50% (at MT₁) and 100% (MT₂) on its own, acting as a full (MT₂) or partial (MT₁) agonist. Even more complex, whenever 4P-PDOT is tested as an antagonist at both receptors, it acts as a poor antagonist at MT₁ (48%) and is completely inactive at MT₂. It may be a typical partial agonist, acting as an agonist depending on the nature of the pathways, a typical situation of biased antagonism. Furthermore, 4P-PDOT induced the recruitment of β-arrestin at concentrations similar to its binding affinities at each receptor, but provoked the internalization of MT₂ only, with an efficacy of approximately 50% of melatonin. In contrast to the initial data and in line with our previous observations, 4P-PDOT was not selective for MT₂, as it had a non-negligible affinity for MT₁ (in the 100 nmol/L range), though with poorer efficacy than melatonin.

Luzindole is an antagonist at MT₂ initially described by Dubocovich46 and later in more details.45 Figure 4B clearly shows the profile of this compound. Similarly to S20928, luzindole inhibits cAMP production in both agonist modes at MT₂, with 70% the of melatonin efficiency. In the antagonism mode, luzindole presents a pKB of nearly 7 for an Emax of 55%. If it is a partial agonist at MT₂, particularly because it recruits β-arrestin and internalizes the receptor with a pEC50 in the 10 nmol/L range, it seems to act as a pure antagonist at MT₁, though in the micromolar range. Luzindole does not recruit β-arrestin, but induces the internalization of MT₂, a feature reported for a few other receptors (Paing et al, 2002; Chen et al, 2004; van Koppen et al, 2004; Giebing et al, 2005). In summary, luzindole is an antagonist at MT₁, and an antagonist at MT₂ for the recruitment of G proteins but an agonist for the cAMP route.

3.5 | Selective ligands at MT₁: D600 (±) and at MT₂: DIV880

Figure 5A shows the results obtained with D600 (gallopamil, see structure in Figure 1). This compound is one of the rare selective antagonists described at MT₁. A powerful anti-calcium compound derived from verapamil, D600 is completely nonspecific (toward nonmelatoninergic targets) but had unique selectivity toward MT₁, as demonstrated previously.47 Despite an affinity for MT₁ in the low micromolar range (pKᵢ = 7), D600 acted as a clear antagonist in the cAMP production and GTPγS binding tests with pEC50s in the micromolar range, as expected. Unsurprisingly, D600 did not induce the recruitment of β-arrestin or internalization of the receptors.

As described previously,48,49 DIV880 is a mixed antagonist/partial agonist with binding affinity pEC50s of approximately 10 nmol/L for MT₂ and 1 µmol/L for MT₁. Figure 5B shows the results obtained with this compound: it is able to antagonize melatonin-induced GTPγS binding, but not the inhibition of melatonin-induced cAMP production at MT₁. Concerning the MT₂ profile, it is typical of an agonist, with recruitment of β-arrestin, internalization, induction of GTPγS binding, and inhibition of cAMP production. These features are not intuitively consistent with the behavior of an antagonist, but rather a biased agonist.50

| Pharmacological parameters (pEC50, pKi, pIC50) | (A) | (B) |
|-----------------------------------------------|-----|-----|
| **hMT₁**                                      |     |     |
| pEC50, pKi, pIC50                             |     |     |
| 4-P-P-DOT                                     |     |     |
| hMT₁, pEC50, pKi, pIC50                       |     |     |
| hMT₂, pEC50, pKi, pIC50                       |     |     |
| **Luzindole**                                 |     |     |
| hMT₁, pEC50, pKi, pIC50                       |     |     |
| hMT₂, pEC50, pKi, pIC50                       |     |     |

**FIGURE 4** pKi/pEC50/pIC50 values and percentage of effect/inhibition (normalization vs melatonin) for 4-P-P-DOT and luzindole in all hMT₁ and hMT₂ assays. Assays include 2-[125I]-iodomelatonin binding, [35SGTPγS] and cAMP production, in agonist and antagonist modes, internalization, and β-arrestin assays. Data are presented as mean ± SD, at least n = 2. The color code corresponds to the different assays. The Emax is presented as a figure on the top of the histograms when applied. Details can be found in Table S1.
3.6 | β-Arrestin and internalization

Because these techniques are less explored than the previously presented techniques, we decided to present typical profiles for β-arrestin and internalization. The typical data obtained with melatonin, a full agonist at both melatonin receptors, are presented for β-arrestin recruitment in Figure S2, as well as the data obtained in the same assay with S20928, a reported antagonist, and 4P-PD0, an historical compound found to be an antagonist at MT2. S20928 behaved as an antagonist at both receptor subtypes; it did not recruit β-arrestin. Therefore, melatonin and S20928 behaved as expected; the agonist recruited the receptors and led to internalization (vide infra), whereas the antagonist did not. As suspected previously, for 4P-PD0, the compound was a partial agonist at MT1 with relatively poor affinity at this receptor, but behaved as a full agonist at MT2, considering cAMP production inhibition, with a shift in the IC50 leftward compared to melatonin (with a pEC50 of 8.7, see Figure 4 and Table S1).

Because β-arrestin recruitment is theoretically the first step to internalization and subsequent desensitization (although counter examples have been reported), and because it is not a widely used assay or reported parameter, we present some examples of curves obtained with this internalization test. Figure S3 shows the findings with the natural agonist, melatonin, which completely induces the internalization of both receptor subtypes. S27128, a strong MT2 agonist, also exhibited subsequent internalization of both receptor subtypes, albeit with right shifts in the concentration at 50% of melatonin. Finally, S77840, a poor antagonist, particularly at MT1, exhibited a lack of capacity to provoke internalization at both receptor subtypes.

We also tested the whole series of compounds regarding the capacity to elicit a response at both receptors. The numerical results of these experiments are given in Table S1. A few interesting situations should be pointed out. For example, DIV880 demonstrated a capacity to recruit β-arrestin at MT1 with 50% of melatonin activity at 1 µmol/L and at MT2 with 100% of melatonin activity. This can be compared to the internalization process, which does not occur at MT1, but was almost 70% at MT2. Considering that these steps are generally linked, only compounds able to recruit at least 100% of the protein will elicit internalization. Similar situations occur with S27128, with complete recruitment of β-arrestin at MT1 leading to internalization, whereas partial (75%) recruitment at MT2 was not enough to elicit internalization.

3.7 | Cellular electrical impedance

The notions behind measuring cellular electrical impedance are not clear. It is associated with elicitation of the receptor(s) by the compound, usually in agonist mode. This leads to the activation of several pathways and the redistribution of proteins in the cytoskeleton, leading to a change in the shape/form of the cells, changing its electrical impedance as a function of its redistribution and, therefore, of the agonist concentration. The nature of the concentration/impedance relationship curve depends on the nature of the actual proteins neo-synthesized in this particular context. The main reference curves and shapes recorded with this approach are described at the vendor’s website (http://photos.labwrench.com/equipmentManuals/4980-1677.pdf).

The difficulties recording and analyzing these curves are due to the pure profiles being rare, as they often result from a mixture of several pathways. Figure 6A depicts the kind of records obtained with the methodology, as exemplified by four compounds: melatonin and S20928 at both receptors, SD1882 at MT1, and S77840 at MT2. The various panels were recorded at various concentrations of each compound (from 10 Pmol/L to 10 µmol/L – top to bottom). In Figure 6B, we constructed the sigmoid curves by plotting the inflection points of the curve obtained at every concentration of the product. If the shapes of the curves do not really provide any readable...
information about the pathway recruited in these cells, the inflexion curves show that the maximal effect was obtained for melatonin, an equilibrated agonist at both MT\textsubscript{1} and MT\textsubscript{2} at 10 nmol/L. A different observation was made with S20928 at MT\textsubscript{1} and S77840 at MT\textsubscript{2}, which failed to elicit any significant shift in shape. For SD1882 at MT\textsubscript{1} and S20928 at MT\textsubscript{2}, maximal effects were observed at concentrations in the 100 nmol/L range, in line with their respective binding affinities (Table S1). The numerical data (IC\textsubscript{50} and E\textsubscript{max}) for the 19 compounds at both receptors are provided in Table S8.

### 3.8 | Representation of biased agonism for ligands at MT\textsubscript{1} and MT\textsubscript{2}

Following our previous work\textsuperscript{10} we calculated the various parameters necessary to construct radar plots for the representation of biased agonism of melatonin receptor ligands (Tables S2 and S3). The vertices of the radar plot represent the Δlog(max/EC\textsubscript{50}) values, namely the log(max/EC\textsubscript{50})\textsuperscript{est compound}−log(max/EC\textsubscript{50})\textsubscript{MLT}. Therefore, negative log values (points within the shaded area, Figure 7) represent agonists that are less active than melatonin at hMT\textsubscript{1} or hMT\textsubscript{2}. For example, only 2Br-MLT, 2I-MLT, and SD6 (in the GTP\textsubscript{γ}S assay) were more active than melatonin in the majority of assays (Figure 7A); all other agonists in all assays were equiactive or less active than MLT. Similarly, for MT\textsubscript{2} (Figure 7B), the same compounds (2Br-MLT, 2I-MLT, and SD6) plus S20098 (Agomelatin\textsuperscript{®}) were slightly more active than MLT; the majority of the other compounds were equiactive or less active than melatonin in these assays.

Finally, Figure 7C depicts the selectivity of the receptors for the compounds. The vertices of the radar plot represent log(max/EC\textsubscript{50}) values, which specify the relative activity of the agonists (normalized to melatonin in each assay) at each receptor subtype in the form of ΔΔlog(max/EC\textsubscript{50}). The indices represent Δlog(max/EC\textsubscript{50})\textsubscript{hMT1}−Δlog(max/EC\textsubscript{50})\textsubscript{hMT2}. For example, S27128 was less active than melatonin at hMT\textsubscript{1} and more active than melatonin at hMT\textsubscript{2}, indicating hMT\textsubscript{2} selectivity and resulting in a
negative $\Delta \Delta \log(\text{max/EC}_{50})$ value in the plot. Thus, values within the shaded area represent hMT$_2$ selectivity, and any values outside of the shaded area represent hMT$_1$ selectivity. The question of the significance of data obtained on weak agonists is an important one, because it was claimed that the method is only valid if the hill slope does not significantly deviate from 1. In fact, the procedure we use is remarkably forgiving for deviations of the slope. Specifically, there are significant differences between Log(tau/KA) and Log(max/EC$_{50}$) only for weak agonists with intrinsic activities <30% of melatonin and for those with slopes <0.5. So we checked the compound/test in which both those parameters were concerned. We found 7 cases: DIV880/GTP$_\gamma$S at MT$_1$; S77840/GTP$_\gamma$S at MT$_2$; Luzindole/GTP$_\gamma$S at MT$_2$; S20928/cAMP at MT$_1$; and S73893/cAMP at MT$_2$; S73893/Internalization at MT$_1$: for those, the hill slope was not different from 1 ± 0.15%, as we used an automatic system that prevents to validate the results by sending an alert in case the hill slope is below 0.85 or above 1.15; and the seventh: S20928/β-arrestin at slope was 0.74.

**FIGURE 7** Biased ligand radar plot representations of agonists. Data represent $\Delta \log(\text{max/EC}_{50})$ values, which compare the agonism of the compound to melatonin for the particular signaling pathway. (A) MT$_1$ receptor, (B) MT$_2$ receptor. (C) Radar plot representation of $\Delta \Delta \log(\text{max/EC}_{50})$ values indicating receptor selectivity of MT$_2$ over MT$_1$. GTP$_\gamma$S, green traces; β-arrestin, blue traces; internalization, pink traces; cAMP, red traces; and Cellkey® , black traces. The corresponding individual calculation can be found in Tables S2 and S3.

4 | DISCUSSION

As discussed in several recent publications, we should attempt to find biased ligands at GPCRs despite obvious difficulties translating the in vitro ligand action into an in vivo context.$^{56}$ If the a priori rationale is not clear, except for having a cohort of diverse compounds with various capabilities, biased ligands may have interesting activities in various pathologies. One thing still missing in the melatonin domain is a better understanding of the various ways melatonin acts through its protein targets, particularly its receptors. In order to add to the already available tools, it seemed necessary to characterize the melatonergic receptor pathways. In other domains, the interplay between different signaling pathways and their interrelationships are apparently better known, as well as the physiological or pathological response elicited/translated into in vivo situations.

In view of the details obtained here for these compounds and signaling pathways, the “antagonist” notion is at best vague, if not completely misleading. In general terms, the notion of antagonism...
| Compound            | MT<sub>1</sub> Radioligand | IUPHAR affinity<sup>b</sup> | IUPHAR<sup>b</sup> | Affinity | Our data | MT<sub>2</sub> Radioligand | IUPHAR affinity | IUPHAR<sup>b</sup> | Affinity | Our data | Affinity |
|---------------------|-----------------------------|-----------------------------|---------------------|----------|---------|-----------------------------|-------------------|-------------------|----------|---------|---------|
| Melatonin           | 10.23                       | 9.1                         | ago                 | 9.65     | ago     | 9.87                        | 9.4               | ago               | 9.4      | ago     | 9.49    |
| 2-Bromomelatonin    | 10.82                       | ago                         |                     |          |         | super ago                   | 9.94              |               |          |         |         |
| 6-Chloromelatonin   | 7.9                         | 8.73                        | ago                 | 9.4      | ago     | 9.4                         | ago               | ago               | 9.56     |         |         |
| D600                | 7.04                        | antago                      |                     |          |         |                             |                   |                   |          |         |         |
| 2-Iodomelatonin     | 10.44                       | 9.9                         | ago                 | 10.53    | ago     | 9.8                         | 9.7               | ago               | super ago | 9.86    |         |
| 4P-PDOT             | 6.2                         | antago                      | 6.85               | partial ago | 8.8     | antago                      | partial ago       | partial ago       | 8.97     |         |         |
| DIV880              | ~6                          |                             | 6.27               | NA       | 8.02    | partial ago                 | partial ago       | partial ago       | 7.9      |         |         |
| Luzindole           | 6.2                         | antago                      | 6.59               | antago   | 7.6     | antago                      | partial ago       | partial ago       | 7.57     |         |         |
| Agomelatine         | 10                          | ago                         | 9.66               | ago      | 9.9     | ago                         | ago               | 9.87              |         |         |         |
| S20928              | antago                      |                             | 6.77               | NA       | 7.6     | antago                      | ??                | 7.1               |         |         |         |
| S21278              | 6.22                        | NA                          |                     |          |         | 6.22                        | ago               | 6.22              |         |         |         |

| Compound            | Radioligand | IUPHAR affinity | IUPHAR<sup>b</sup> | Affinity | Our data | Radioligand | IUPHAR affinity | IUPHAR<sup>b</sup> | Affinity | Our data | Affinity |
|---------------------|-------------|-----------------|---------------------|----------|---------|-------------|-------------------|-------------------|----------|---------|---------|
| S22153              | 7.8         | antago          | 8.01               | partial ago | 8.1     | partial ago | ago               | 8.1               |          |         |         |
| S27128              | 8.92        | ago             |                     |          |         | 8.92        | ago               | 9.17              |          |         |         |
| S70254              | ~6           | 7.97            | NA                 | 8.73     | 9.6     | partial ago | partial ago       | 9.1               |          |         |         |
| S73893              | 8.36         | partial ago     |                     |          |         |             |                   |                   |          |         | 8.11    |
| S75436              | 8.04         | ago             |                     |          |         | 8.04        | ago               | 8.95              |          |         |         |
| SD1881              | 8.84         | ago             |                     |          |         | 8.84        | ago               | 8.61              |          |         |         |
| SD1882              | 7.76         | partial ago     |                     |          |         | 7.76        | partial ago       | 7.99              |          |         |         |
| SD1918              | 7.34         | partial ago     |                     |          |         | 7.34        | partial ago       | 7.32              |          |         |         |
| SD6                 | 9.94         | 10.9            | ago                 | 9.29     | ago     | 9.89        | 10.2              | ago               | 9.31     |         |         |

<sup>a</sup>Against 2-[^125]Iiodomelatonin.

<sup>b</sup>From IUPHAR database (http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=39); the color code correspond to the nature of the functionality: ■: superagonist; ▼, partial agonist; ●, agonist; □, antagonist. NA, not applicable.
in a given system, particularly regarding drug candidates (with receptors still being a major family of targets for marketed drugs), is essential for the therapeutic role of the compound(s). If it were understood upfront that the antagonistic property of a given candidate at a given receptor is biased to a point that only some of the signaling pathways are antagonized, it would give a completely different picture of the potential therapeutic benefit of such a drug in a given disease context, maybe up to a point where it can also give an idea of the reverse effect of a given compound.

The various data currently available on the melatonin receptor ligands presented here are summarized in Table 1. The data were taken from the IUPHAR website, and we further completed the panel of functional ligands with new entities.

It is obvious from Figure 7 that most analogs are less active as agonists at MT$_1$ and MT$_2$ than melatonin (note vertices inside the shaded area), and that there is some difference within pathways regarding $\Delta$Log(max/EC$_{50}$) values. Values of $\Delta$Log(max/EC$_{50}$) provided the relative activities of the compounds vs melatonin for each receptor and were then used to derive estimates of receptor selectivity through $\Delta$Log(max/EC$_{50}$) (Figure 7C). There are some notable exceptions to the pathway uniformity in which the calculated receptor selectivity was more pronounced for some pathways than for others (i.e., S20098, SD1881, S21278, and SD1882). Recently, kinetics was shown to be an important factor in the measurement and quantification of signaling bias$^{32}$; thus, a caveat to the present data must be that only a single time point has been used to assess bias. However, to date, no study has shown that such kinetic effects actually reverse biased estimates, but only affect the actual magnitude of the ratio.

The discovery of biased antagonists has been a source of worries, as molecules may act as antagonists in one way and agonists in another, leading to false hope that antagonists may be “universal” from this point of view. Furthermore, recent reviews$^{39,40,56,57}$ explicitly described the many outcomes of biased ligands. The final effect of a given agonist may be far more complex than initially anticipated, as it can be biased, but it can also be tissue-dependent, as the various G protein isoforms that support the functionality of receptors are not equally expressed in all tissues and organs. Furthermore, it is clear that this situation with various G proteins differentially expressed in different tissues may also have an impact on experiments in cells, particularly HEK or CHO cells, which stably express GPCRs. Interestingly, the fact that all of the systems measured in the present study were responsive to melatonin strongly suggests that the cells were equipped with the ad hoc G proteins and functional systems. Other systems, such as those described for the melatoninergic systems,$^{18}$ may not be present in this cell line, and further explorations of signaling pathways in this system will require appropriate positive controls.

Nevertheless, GPCR functionality seems to be clearer than soluble receptor pharmacology, such as that of glucocorticoids,$^{58}$ PPAR,$^{59}$ and rev-erb $\alpha,$$^{60}$ for which the combination of chromatin modeling factors, comodulators, corepressors, coactivators, and other cofactors renders a picture several orders of magnitude more complex than that of GPCRs, the coregulation of which seems to not involve other proteins despite the terrible lack of knowledge of the role(s) of the >300 proteins involved in the melatonin receptor interactome.$^{18}$

Finally, we want to stress that the present work was not aimed at describing the behavior of any particular compounds among the set of compounds we chose to use. The goal was rather to lay the basis for studying the possible pathways that are melatonin receptor-dependent. The present body of work provides a complete analysis of the capacity of ligands at these receptors to behave as biased agonists or antagonists. The work clearly indicates that, as stated previously, many more pathways have been described, sometimes in a superficial manner, for these or other GPCRs. One will now be able to consider new ligands in light of possible pathways, which would permit a better understanding of the capacity of melatonin to interfere with so many pathological situations and to provide the tools for finding an adequate alternative to melatonin whenever the healing process is melatonin receptor-dependent.

DATA REPOSITORY

All the primary data are gathered in Table S1 of the Supporting information section.

ORCID

Jean A. Boutin https://orcid.org/0000-0003-0068-7204

REFERENCES

1. Jockers R, Delagrange P, Dubocovich ML, et al. Update on melatonin receptors: IUPHAR Review 20. Br J Pharmacol. 2016;173:2702-2725.
2. Gautier C, Guenin S-P, Riest-Fery I, et al. Characterization of the Mel1c melatoninergic receptor in platyfish (Ornithorhynchus anatinus). PLoS ONE. 2018;13:e0191904.
3. Hardeland R. Melatonin in plants and other phototrophs: advances and gaps concerning the diversity of functions. J Exp Bot. 2015;66:627-646.
4. Li YD, Smith DG, Hardeland R, et al. Melatonin receptor genes in vertebrates. Int J Mol Sci. 2013;14:11208-11223.
5. Bonnefont-Rousselot D, Collin F. Melatonin: action as antioxidant and potential applications in human disease and aging. Toxicology. 2010;278:55-67.
6. Bonnefont-Rousselot D, Collin F, Jore D, Gardés-Albert M. Reaction mechanism of melatonin oxidation by reactive oxygen species in vitro. J Pineal Res. 2011;50:328-335.
7. Legros C, Chesneau D, Boutin JA, Barc C, Malpax B. Melatonin from cerebrospinal fluid but not from blood reaches sheep cerebral tissues under physiological conditions. J Neuroendocrinol. 2014;26:151-163.
8. Boutin JA. Quinone reductase 2 as a promising target of melatonin therapeutic actions. Expert Opin Ther Targets. 2016;20:303-317.
9. Boutin JA. How can molecular pharmacology help understand the multiple actions of melatonin: 20 years of research and trends. In: Manuela Drăgoi C, Crenguţa Nicolae A, eds. Melatonin · Molecular Biology, Clinical and Pharmaceutical Approaches. London, UK: IntechOpen; 2018.
10. Kenakin T. Functional selectivity and biased receptor signaling. J Pharmacol Exp Ther. 2011;336:296-302.
11. Maudsley S. G protein-coupled receptor biased agonism: development towards future selective therapeutics. *Mini Rev Med Chem*. 2012;12:803.

12. Klein Herenbrink C, Sykes DA, Donthamsetti P, et al. The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun*. 2016;7:10842.

13. M’Kadmi C, Leyris J-P, Onfroy L, et al. Agonism, antagonism, and inverse agonism bias at the ghrelin receptor signaling. *J Biol Chem*. 2015;290:27021-27039.

14. Jockers R, Maurice P, Boutin JA, Delagrange P. Melatonin receptors, heterodimerization, signal transduction and binding sites: what’s new? *Br J Pharmacol*. 2008;154:1182-1195.

15. Pandi-Perumal SR, Trakht I, Srinivasan V, et al. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Prog Neurobiol*. 2008;85:335-353.

16. Legros C, Brasseur C, Delagrange P, Melatonin receptors: ligands, models, oligomers, and therapeutic potential. *J Med Chem*. 2014;57:3161-3185.

17. Zlotos DP, Jockers R, Concen E, Rivara S, Witt-Endery PA. MT1 and MT2 melatonin receptors: ligands, models, oligomers, and therapeutic potential. *J Med Chem*. 2014;57:3161-3185.

18. Concen E, Oishi A, Jockers R. Melatonin receptors: molecular pharmacology and signalling in the context of system bias. *Br J Pharmacol*. 2018;175:3263-3280.

19. Cogé F, Guerin SP, Fery I, et al. The end of a myth: cloning and characterization of the ovine melatonin MT2 receptor. *Br J Pharmacol*. 2009;158:1248-1262.

20. Devavry S, Legros C, Brasseur C, et al. Molecular pharmacology of the mouse melatonin receptors MT1 and MT2. *Eur J Pharmacol*. 2016;777:15-21.

21. Nordic MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.

22. Audinot V, Bonnau A, Grandcolas L, et al. Molecular cloning and pharmacological characterization of rat melatonin MT1 and MT2 receptors. *Biochem Pharmacol*. 2008;75:2007-2019.

23. Audinot V, Bonnau A, Grandcolas L, et al. Molecular cloning and pharmacological characterization of rat melatonin MT2 receptor. *Br J Pharmacol*. 2008;158:1248-1262.

24. Devavry S, Legros C, Brasseur C, et al. Molecular pharmacology of the mouse melatonin receptors MT1 and MT2. *Eur J Pharmacol*. 2013;694:1045-1049.
promise and the challenges—a medicinal chemistry perspective. Med Res Rev. 2014;34:1286-1330.

51. McDonald PH, Lefkowitz RJ. Beta-Arrestins: new roles in regulating heptahelical receptors’ functions. Cell Signal. 2001;13:683-689.

52. Paing MM, Stutts AB, Kohout TA, Lefkowitz RJ, Trejo J. beta-Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. J Biol Chem. 2002;277:1292-1300.

53. Chen Z, Gaudreau R, Le Gouill C, Rola-Pleszczynski M, Stanková J. Agonist-induced internalization of leukotriene B(4) receptor 1 requires G-protein-coupled receptor kinase 2 but not arrestins. Mol Pharmacol. 2004;66:377-386.

54. van Koppen CJ, Jakobs KH. Arrestin-independent internalization of G protein-coupled receptors. Mol Pharmacol. 2004;66:365-367.

55. Giebing G, Tölle M, Jürgensen J, et al. Arrestin-independent internalization and recycling of the urotensin receptor contribute to long-lasting urotensin II-mediated vasoconstriction. Circ Res. 2005;97:707-715.

56. Luttrell LM, Maudsley S, Gesty-Palmer D. Translating in vitro ligand bias into in vivo efficacy. Cell Signal. 2018;41:46-55.

57. Kenakin T. Is the quest for signaling bias worth the effort? Mol Pharmacol. 2018;93:266-269.

58. Chow CC, Simons SS. An approach to greater specificity for glucocorticoids. Front Endocrinol (Lausanne). 2018;9:76.

59. Hsia EY, Goodson ML, Zou JX, Privalsky ML, Chen H-W. Nuclear receptor coregulators as a new paradigm for therapeutic targeting. Adv Drug Deliv Rev. 2010;62:1227-1237.

60. Kojetin DJ, Burris TP. REV-ERB and ROR nuclear receptors as drug targets. Nat Rev Drug Discov. 2014;13:197-216.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Legros C, Dupré C, Brasseur C, et al. Characterization of the various functional pathways elicited by synthetic agonists or antagonists at the melatonin MT$_1$ and MT$_2$ receptors. Pharmacol Res Perspect. 2020;e00539. https://doi.org/10.1002/prp2.539