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N-(Phenoxyalkyl)amides as MT_1 and MT_2 ligands: Antioxidant properties and inhibition of Ca^{2+}/CaM-dependent kinase II

Alessia Carocci [a,*], Alessia Catalano [a], Claudio Bruno [a], Angelo Lovece [a], Maria Grazia Roselli [a], Maria Maddalena Cavalluzzi [a], Francesco De Santis [b], Annalisa De Palma [b], Maria Rosaria Rusciano [c], Maddalena Illario [c], Carlo Franchini [a], Giovanni Lentini [a]

[a] Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari 'Aldo Moro', Via E. Orabona 4, 70125 Bari, Italy
[b] Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica, Università degli Studi di Bari 'Aldo Moro', Via Orabona 4, 70125 Bari, Italy
[c] Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, 80131 Napoli, Italy

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ABSTRACT

Recently a series of chiral *N*-(phenoxyalkyl)amides have been reported as potent MT_1 and MT_2 melatonergic ligands. Some of these compounds were selected and tested for their antioxidant properties by measuring their reducing effect against oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) in the DCFH-diacetate (DCFH-DA) assay. Among the tested compounds, *N*-[2-(3-methoxyphenoxy)propyl]butanamide displayed potent antioxidant activity that was stereoselective, the (*R*)-enantiomer performing as the eutomer. This compound displayed strong cytoprotective activity against H_2O_2-induced cytotoxicity resulting slightly more active than melatonin, and performed as Ca^{2+}/calmodulin-dependent kinase II (CaMKII) inhibitor, too.

1. Introduction

Melatonin (MLT, **1**, Fig. 1) is a tryptophan derivative primarily produced by the pineal gland with a marked circadian rhythm that is governed by the central circadian pacemaker in the suprachiasmatic nuclei of the hypothalamus, the highest levels occurring during the period of darkness.[1] This hormone regulates and modulates a wide variety of physiological functions. Besides the well-known chronobiotic and sleep inducing properties,[2] many other physiological effects have been ascribed to MLT, such as the modulation of cardiovascular[3] and immune[4] systems and the influence on hormone secretion and metabolism.[5] Other effects of MLT described in the literature include antitumor,[6,7] anti-inflammatory,[8] pain modulator,[9] neuroprotective,[10,11] and antioxidant[12] properties. MLT exerts its actions by multiple mechanisms. Many of its physiological actions are mediated through G-protein coupled receptors expressed in a wide variety of tissues. Cloning studies have revealed at least three MLT receptor subtypes, two of which (MT_1 and MT_2) have been found in mammals,[13,14] and are localized in different areas of the central nervous system as well as in peripheral tissues.[15,16] Moreover, a non-mammalian MLT binding site with a lower affinity profile (MT_3) has been found in hamster brain and characterized as a MLT-sensitive form of the human enzyme quinone reductase 2.[17] Many in vitro and in vivo experimental models have contributed to demonstrate the role of MLT as an efficient radical scavenger against several reactive oxygen species (ROS), for example, the hydroxyl radical, the peroxynitrite anion, the superoxide anion, and singlet oxygen.[18,19] MLT has also been shown to enhance the production and the activity of several antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, and glucose-6-phosphate dehydrogenase.[20,21] Furthermore, in vivo observations on the protective role of MLT in ischemic brain injury[22] or in animal models of Alzheimer's disease[23] emphasize the therapeutic potential of this compound as a neuroprotective agent.[24] Nevertheless, the potential application of MLT in an antioxidant therapy could be complicated by the pharmacokinetic behaviour of the molecule, which is subjected to a considerable first-pass effect and to a limited bioavailability and plasma half life after oral administration.[25] Thus, the availability of new compounds sharing the beneficial properties of MLT, but presenting different pharmacokinetic profiles could therefore be envisaged. During the past two decades, a great number of structurally different MLT receptor ligands have been reported in the literature.[26–29] Several melatonin-related compounds such as melatonin metabolites and synthetic analogues have been under investigation as antioxidants, most of them being indole-based compounds.[30,31] Recently, basing on the results obtained on a series of *N*-phenylalkyl amides by Garratt et al.,[32] we examined a series of substituted *N*-(pheno-

* Corresponding author. Tel.: +39 080 5442745; fax: +39 080 5442724.
E-mail address: alessia.carocci@uniba.it (A. Carocci).

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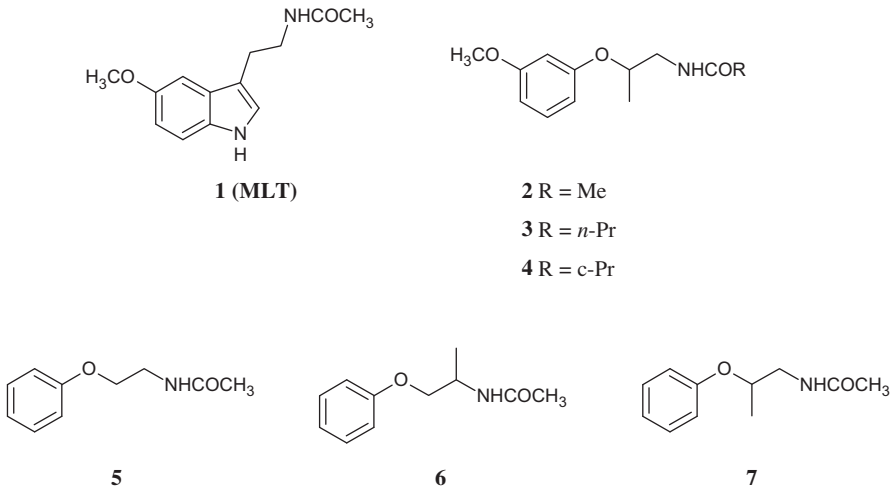

Figure 1. Set of compounds included in the study.

xyalkyl)amides as melatonergic ligands.[33] A structure–activity relationship (SAR) study around the terminal amide moiety, the alkyl chain, and the methoxy group on the aromatic ring provided compounds with nanomolar affinity for both melatonin receptor subtypes. In the present study we investigated some of these compounds (**2**–**7**, Fig. 1) as antioxidants. Furthermore, the ability of MLT to inhibit in vitro Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity at physiological concentrations has been reported.[34] CaMKII is particularly abundant in the nervous system where it is involved in a series of important patho-physiological processes because of its ability to phosphorylate a broad spectrum of substrates.[35–37] Despite its pharmacological significance, a complete characterization of the interaction between MLT and CaMKII is still lacking. Both antioxidant and CaMKII inhibiting properties might prove beneficial to neurodegenerative disorders.[38] Herein we decided to investigate the potential CaMKII inhibitory effect of our best melatonergic ligands (**3**,**4**) and MLT (**1**).

2. Results

The in vitro antioxidant activity of a series of melatonergic ligands (Fig. 1) was evaluated by means of the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) cellular-based assay by measuring the reducing effect of the test compounds against oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to the fluorescent probe 2′,7′-dichlorofluorescein (DCF) The set of compounds was constructed by selecting a series of *N*-(phenoxyalkyl)amides previously reported as MLT receptor ligands.[33] As shown in Table 1, the selected compounds are characterized by different affinities for MLT receptors with K_i values spanning four orders of magnitude.[33] We tested MLT receptor ligands with high affinity (**2**–**4**) and three compounds that behave as poor melatonergic ligands (**5**–**7**). These compounds were compared with MLT (**1**) which was chosen as the reference compound. Tests were performed on human hepatocellular liver carcinoma (HepG2) cells because they have an enhanced oxidative metabolism that causes cellular oxidative stress and/or generates reactive metabolites. Thus it may be assumed that HepG2 cells are suited to study protection against oxidative and cytotoxic effects, if any. The results of the DCFH-DA assay showed that only compound **3** significantly reduces the H_2O_2-induced oxidation, being slightly more potent than MLT. Tests carried out on the homochiral forms of **3** revealed a difference between the enantiomers being the (*R*)-isomer the eutomer. Then, in order to evaluate the potential efficacy as scavenger of stable free radicals, MLT and compounds **3**, (*R*)-**3**, and (*S*)-**3** were analyzed by observing the reactivity with 1,1-diphenyl-2-picrylhydrazyl (DPPH), a violet-coloured stable radical that absorbs strongly at 517 nm. The effect of three different concentrations (0.1–5.5 mM) of the tested compounds on their interaction with DPPH (100 μM) is shown in Table 2. Experiments were performed also with gallic acid, a naturally occurring plant phenol with antioxidative activity, as the reference substance. In agreement with what was reported in the literature,[39] MLT showed low reactivity with this radical. At a concentration of 5.5 mM the compound decreases DPPH absorption by only 31.8%. The same behaviour was observed for compounds **3**, (*R*)-**3**, and (*S*)-**3** that slightly scavenged the level of DPPH radical

Table 1
MT_1 and MT_2 melatonin receptors affinities and antioxidant potencies in the DCFH-DA assay for MLT and the set of compounds under study

Compounds	pK_i[a]		DCFH-DA[b]
	MT_1	MT_2	IC_{50} ± SEM (μM)
MLT	9.48	9.21	271.0 ± 0.1
2	8.44	8.02	>1000
3	8.38	8.43	226.4 ± 0.1
(*R*)-**3**	7.09	7.17	182.9 ± 0.2
(*S*)-**3**	8.51	8.56	>1000
4	9.03	7.89	>1000
5	4.94	5.26	>1000
6	4.92	5.42	>1000
7	5.86	5.94	>1000

[a] Ref. 33.
[b] Values are the mean of at least three determinations performed in sextuplicate.

Table 2
Effect of different concentrations (0.1–5.5 mM) of compounds **3**, (*R*)-**3**, (*S*)-**3**, and MLT on their interaction with DPPH (100 μM)

Compounds	Percentage of Interaction with DPPH[a]			
	0.1 mM	1 mM	5.5 mM	IC_{50} (μM)
3	3.22 ± 0.24	4.05 ± 0.19	11.7 ± 0.4	nd[b]
(*R*)-**3**	5.60 ± 0.25	5.10 ± 0.90	20.7 ± 1.00	nd[b]
(*S*)-**3**	2.80 ± 0.70	1.30 ± 1.00	15.1 ± 0.9	nd[b]
MLT	2.10 ± 0.70	7.80 ± 0.50	31.8 ± 0.3[c]	nd[b]
Gallic acid	97.14 ± 0.03	nd[b]	nd[b]	6.0 ± 0.3[d]

[a] Values are the mean of at least three determinations performed in quadruplicate.
[b] nd: not determined.
[c] Literature value: 40%.[39]
[d] Literature value: 1.83 ± 0.17 μM.[45]

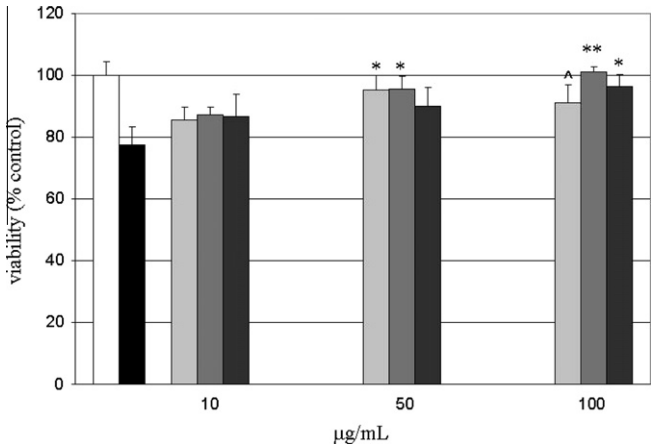

Figure 2. Cell viability in the absence of H_2O_2 (control, white histogram), in the presence of 125 μg/mL H_2O_2 (black histogram), and with H_2O_2 (125 μg/mL) and increasing concentrations of **3** (light gray), (*R*)-**3** (gray), or MLT (dark gray). No viability variation was obtained with (*S*)-**3** in the range of concentrations observed (10−100 μg/mL). Viability is expressed in percent of the number of the control viable cells; $n = 3$ independent experiments; mean ± SEM presented; $^{\wedge}P$ <0.1, $^{*}P$ <0.05, $^{**}P$ <0.01 versus H_2O_2 treated cells.

at 5.5 mM concentration by about 11.7%, 20.7%, and 15.1%, respectively. Then, the cytoprotective effect of MLT, compound **3** and its (*R*)-enantiomer, was evaluated against H_2O_2-induced cytotoxicity (Fig. 2) on HepG2 cells. Compared with MLT, both **3** and (*R*)-**3** showed higher cytoprotective activity when tested at concentrations close to their IC_{50} values. In particular, compound (*R*)-**3** showed a more pronounced action, with percentage of cell viability close to 100% at the concentration of 100 μg/mL. As far as the inhibition of CaMKII is concerned, the effects of MLT and compounds **3** and **4**, in their racemic and homochiral forms, are shown in Table 3. Interestingly, all of them exert inhibitory activities comparable to that of MLT. Results obtained with the homochiral forms of these two compounds revealed that, conceivably, the inhibition occurs in a stereoselective manner since the enantiomers slightly differ in potency, being the (*S*)-isomers the eutomers.

3. Discussion

We recently reported the synthesis of a series of chiral *N*-(phenoxyalkyl)amides that behave as melatonergic agonists with affinity towards MT_1 and MT_2 receptors in the range of nM.[33] Some of these compounds were studied in vitro as antioxidants. Surprisingly, among the tested compounds, despite slight structural differences, only compound **3** showed antioxidant activity being slightly more active than MLT when tested in the DCFH-DA assay. These results may allow us to rule out a possible interference of MT_1 and MT_2 receptors on the antioxidant activity shown by **3**, since both compounds **3** and **4**, which differ only for the acyl moiety, are high affinity melatonergic agonists. Interestingly, the antioxidant activity of compound **3** occurs in a stereoselective manner, since a significant difference between its enantiomers was observed; in particular the (*R*)-enantiomer behaves as the eutomer. Conceivably, this result may suggest that the antioxidant activity of **3** is specific; it should stem from the interaction with at least one more macromolecular target involved in the pharmacological outcome at the pharmacodynamic or pharmacokinetic level. Then, in order to have a better insight on the mechanism of the antioxidant capacity of compound **3** and its *R*-enantiomer, DPPH-free radical scavenging activity has been determined. From the results appears evident that the antioxidant activity of compounds **3** and (*R*)-**3** is not accompanied by radical scavenging ability, since these compounds, as well as MLT, showed only a weak DPPH inhibition activity pattern. Thus, the antioxidant profiles of **3** and (*R*)-**3** seem to be similar to that of MLT, whose direct antioxidant activity is only limited.[39] Results on CaMKII inhibitory activity for our best $MT_{1/2}$ ligands (**3** and **4**) revealed that they share inhibitory properties with MLT. The inhibition occurs in a stereoselective manner, the (*S*)-enantiomers being the eutomers. It is not possible to speculate whether there is a relationship between the results in the antioxidant tests and on the CaMKII inhibition system. In fact, compound **3** displayed modest stereoselectivity in the DCFH-DA assay. The pattern of stereoselectivity—R > S—was opposite to that observed in both CaMKII inhibition and MLT receptor binding assays.

Table 3
Inhibition of CaMKII activity by MLT and its analogues[a]

Compounds	IC_{50} ± SEM (μM)
MLT	128 ± 11
3	122 ± 28
(*R*)-**3**	129 ± 8
(*S*)-**3**	77 ± 14
4	107 ± 18
(*R*)-**4**	129 ± 8
(*S*)-**4**	117 ± 4

[a] CaM was used at 5 μM, values represent the mean of three independent determinations.

4. Conclusions

The panel of selected in vitro assays allowed us to characterize a MLT agonist, compound (*R*)-**3**, as an antioxidant, which proved to be slightly more potent than MLT with a cytoprotective effect against H_2O_2-induced cytotoxicity as high as that of MLT. While being structurally specific, these activities should not pass through MLT receptor activation. (*R*)-**3** also inhibited in vitro CaMKII. Both antioxidant and CaMKII inhibiting properties might prove beneficial to neurodegenerative disorders; indeed, ROS are considered plausible targets for neurodegeneration prevention.[38] Thus, it should be very useful to obtain a MLT analogue that shows antioxidant capability and, at the same time inhibit CaMKII, since this is, in turn, an important source of oxidative stress. Thus, (*R*)-**3**, which presents these characteristics, should be a valid candidate for further investigations in order to achieve new insights on possible mechanism of action of this class of compounds.

5. Experimental

5.1. Chemicals

Compounds **2**–**7** were reported previously by this group.[33] MLT is commercially available (Sigma–Aldrich, Milan, Italy).

5.2. Culture cells, H_2O_2-induced stress

Human hepatocellular liver carcinoma (HepG2) cells (Sigma–Aldrich, St. Louis, MO) were cultured in DMEM-Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), L-glutamine (2 mM) (Sigma–Aldrich, St. Louis, MO), penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) (Sigma–Aldrich, St. Louis, MO) and incubated at 37 °C in a humid atmosphere of 5% CO_2. For experiments, the cells were grown to 70% confluence, seeded for experiment and treated with H_2O_2 (Sigma–Aldrich, St. Louis, MO) in serum free media for the indicated times.

5.3. Cell viability assay

HepG2 cell viability was assessed by using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported.[40–42] Briefly, viable cells (10^4/well) were plated into a sterile 96-Well Cell Culture Cluster (Corning, NY 14831) and, after removing the medium, were incubated with a final 125 μM H_2O_2/well in DMEM for 10 min. Then cells were washed twice with Phosphate Buffered Saline (PBS, Sigma–Aldrich, Milan, Italy) and incubated for 2 h at 37 °C in 5% CO_2 with different concentrations of test compounds. At the end of incubation, the culture medium was replaced by a solution of MTT 0.5 mg/mL (Sigma–Aldrich, Milan, Italy) in PBS. After 2 h incubation at 37 °C in 5% CO_2 this solution was removed and 200 μL of DMSO was added to each well. Absorbance values were measured at 570 nm using a Victor V^3 plate reader (PerkinElmer) and DMSO medium was used as blank solution. Results are expressed as the percentage of MTT reduction respect to absorbance of control cells. All experiments were carried out in sextuplicate and were repeated twice.

5.4. Dichlorofluorescein assay

Generation of ROS was monitored using an oxidation-sensitive fluorescent probe, 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA, D6665; Sigma–Aldrich, St. Louis, MO) by slightly modifying the procedure reported by Wang and James.[43] Briefly, viable HepG2 cells (10^4/well) were plated into a sterile black Culture Plate™ 96F wells (PerkinElmer) 1 day before the experiments. After removing the medium, the cells were incubated with a final 125 μM H_2O_2/well in DMEM. After 10 min, cells were washed twice with PBS, and incubated for 2 h at 37 °C in 5% CO_2 with different concentrations of test compounds. Medium was removed and cells were incubated with final 5 mM DCFH-DA in DMSO at 37 °C in 5% CO_2 in dark for an additional 30 min and then were washed twice with PBS. The fluorescence intensity was measured directly in each well at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm by multiwells fluorescence Victor V^3 plate reader (PerkinElmer). Each experiment was performed three times in sextuplicate.

5.5. DPPH radical scavenging assay

The method of Blois[44,45] was adopted. The assay was carried out in 96-well microplates. Briefly, a methanol DPPH solution (100 μM) was mixed with different concentrations (0.1 mM, 1 mM, 5.5 mM) of test compounds and incubated for 20 min at room temperature in the dark. After this time, 200 μL were placed in a 96-well microplate and the absorbance of DPPH radical was measured at 570 nm in a spectrometric plate reader (Victor D Perkin Elmer). All reaction mixtures were prepared in quadruplicate, and at least three independent runs were performed for each sample. The antioxidant activity was determined as the RSA% (radical scavenging activity), calculated as follows: $RSA\% = 100[(A_o - A_i)/A_o) \times 100$, where A_o and A_i are the DPPH absorbance in absence and in presence of added compound concentration i, respectively. Data are expressed as means.

5.6. CaMKII activity assay

CaMKII activity was tested on Autocamtide in the presence of the tested compounds. In a first reaction step, active recombinant full-length CaMKII (Signal Chem, La Jolla) was incubated for 30 min at 30 °C with 1 mmol/L $CaCl_2$ and 1 μmol/L CaM in 50 μL of a reaction mixture (50 mmol/L HEPES pH 7.5, 10 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol (DTT), 100 nmol/L microcystin, 0.1 mmol/L non-radiolabeled ATP).[46] In a second reaction step, a 10 μL aliquot from the first reaction was then incubated with 25 mM EGTA, 0.2 μCi/μl of Easy Tides Adenosine 5′-triphosphate [γ^{32}P]-ATP (Perkin Elmer) and 0.5 mM Autocamtide,[47] in the presence of the tested compounds (at different concentrations) in order to determine the effects of the compounds on CaMKII activity on its substrate Autocamtide. AntCaNtide at a concentration of 5 μM was used as positive control of CaMKII inhibition. The reaction was carried out for 30 min at 30 °C, then 20 μL aliquots of the reaction mixture were spotted onto Whatman P-81 phosphocellulose paper. EGTA was added to quantify CaMKII autonomous activity. Dried filters were counted on a Beckman LS 6000 scintillation counter.

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