Bioorganic Chemistry 85 (2019) 349–356

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

# O-linked melatonin dimers as bivalent ligands targeting dimeric melatonin receptors

Angeliki Karamitri[a,b,c], Mirna S. Sadek[d], Anne-Sophie Journé[a,b,c], Florence Gbahou[a,b,c], Romain Gerbier[a,b,c], Mai B. Osman[d], Samy A.M. Habib[d], Ralf Jockers[a,b,c,1], Darius P. Zlotos[d,*,1]

[a] Inserm, U1016, Institut Cochin, Paris, France
[b] CNRS UMR 8104, Paris, France
[c] Univ. Paris Descartes, Sorbonne Paris Cite, Paris, France
[d] The German University in Cairo, Department of Pharmaceutical Chemistry, New Cairo City, 11835 Cairo, Egypt

ARTICLE INFO

*Keywords:*
Melatonin
$MT_1$ receptors
$MT_2$ receptors
Bivalent ligands
Inverse agonist
BRET

ABSTRACT

A series of dimeric melatonin analogues **3a-e** obtained by connecting two melatonin molecules through the methoxy oxygen atoms with spacers spanning 16–24 atoms and the agomelatine dimer **7** were synthesized and characterized in 2-[$^{125}$-I]-iodomelatonin binding assays, bioluminescence resonance energy transfer (BRET) experiments, and in functional cAMP and β-arrestin recruitment assays at $MT_1$ and $MT_2$ receptors. The binding affinity of **3a-e** generally increased with increasing linker length. Bivalent ligands **3a-e** increased BRET signals of $MT_1$ dimers up to 3-fold compared to the monomeric control ligand indicating the simultaneous binding of the two pharmacophores to dimeric receptors. Bivalent ligands **3c** and **7** exhibited important changes in functional properties on the $G_i$/cAMP pathway but not on the β-arrestin pathway compared to their monomeric counterparts. Interestingly, **3c** (20 atoms spacer) shows inverse agonistic properties at $MT_2$ on the $G_i$/cAMP pathway. In conclusion, these findings indicate that *O*-linked melatonin dimers are promising tools to develop signaling pathway-based bivalent melatonin receptor ligands.

## 1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins, which represent major molecular targets for approved drugs [1]. There is growing evidence that GPCRs form homodimers, heterodimers and oligomers [2–4]. Because of their possible unique ligand binding profile and functional selectivity, GPCR dimers and oligomers may be promising new targets for drug development [3].

Melatonin $MT_1$ and $MT_2$ receptors are the major pharmacological targets for the neurohormone melatonin (Fig. 1) [5]. They belong to the first GPCRs whose homo- and heterodimerization have been studied by bioluminescence resonance energy transfer (BRET) [6,7] Interestingly, it has been reported that human melatonin receptors predominantly form $MT_1$/$MT_2$ heterodimers and $MT_1$ homodimers, rather than $MT_2$ homodimers [8]. Radioligand binding and BRET studies suggested that $MT_1$/$MT_2$-heterodimers contain two functional ligand binding sites which maintain their respective selectivity for $MT_1$ and $MT_2$ ligands. Formation of $MT_1$/$MT_2$ heterodimers under physiological conditions has been recently reported in the mouse retina [9]. Targeting melatonin receptor dimers, or heterodimers with other GPCRs, could open new therapeutic perspectives, for example for cancer, cardiovascular, and neurodegenerative diseases [10].

A common approach for targeting dimeric GPCRs is the use of bivalent ligands. In bivalent ligands, two pharmacophores specific for a particular GPCR are linked to each other by a spacer of a certain length ideally allowing simultaneous binding to both receptors (protomers) in a dimer [11]. The bivalent ligand approach has been applied for a variety of GPCRs including opioid [12,13], adrenergic [14,15], dopamine [16], serotonin [17,18], muscarinic [19,20], and cannabinoid [21] receptors. Recently, a bivalent ligand-induced formation of dopamine $D_2$ receptor dimers was confirmed using high-resolution cryogenic localization microscopy [22].

The development of bivalent ligands involves choosing the appropriate monomeric pharmacophore, selecting the attachment point on the pharmacophore molecule, and optimizing the length of the spacer. The functional group linking the spacer to both pharmacophores should

* Corresponding author.
*E-mail addresses:* mirna.sadek@mailbox.tu-dresden.de (M.S. Sadek), florence.gbahou@inserm.fr (F. Gbahou), ralf.jockers@inserm.fr (R. Jockers), darius.zlotos@guc.edu.eg (D.P. Zlotos).
[1] Authors sharing the last author position.

https://doi.org/10.1016/j.bioorg.2019.01.004
Received 20 August 2018; Received in revised form 30 December 2018; Accepted 2 January 2019
Available online 04 January 2019
0045-2068/ 

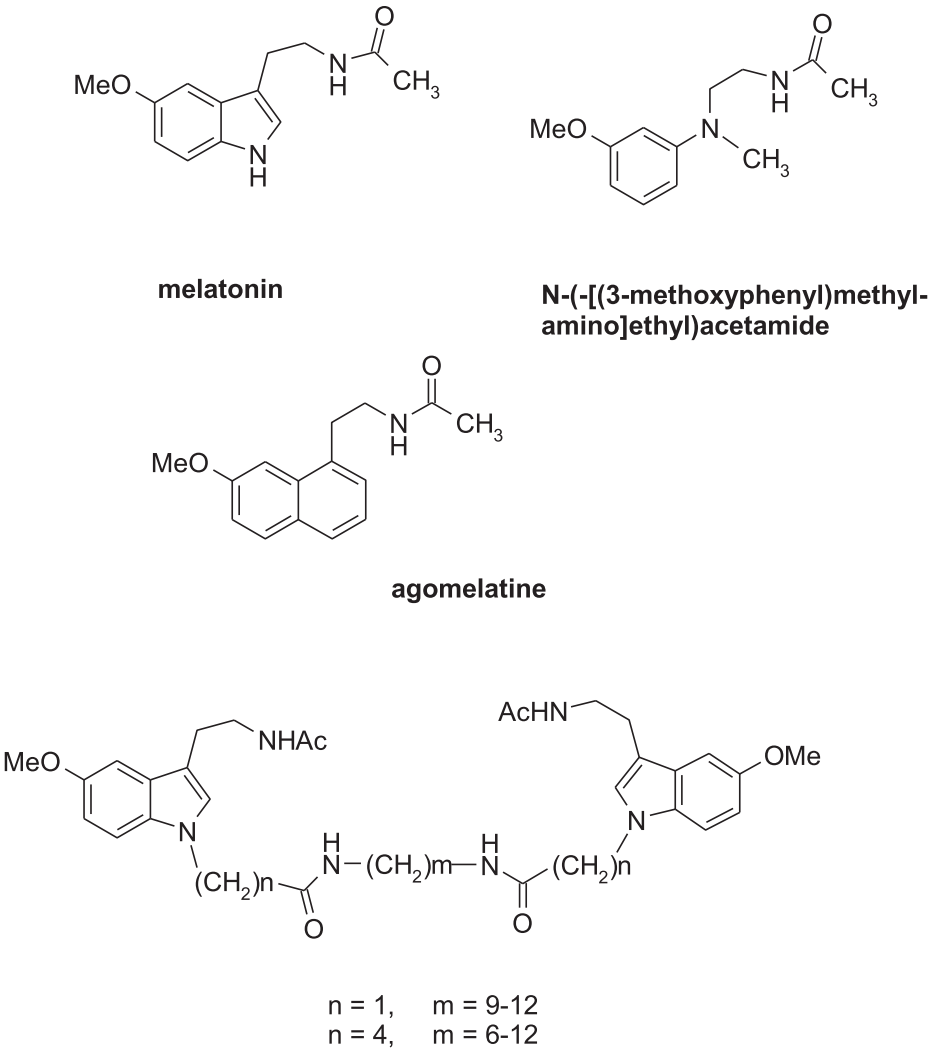
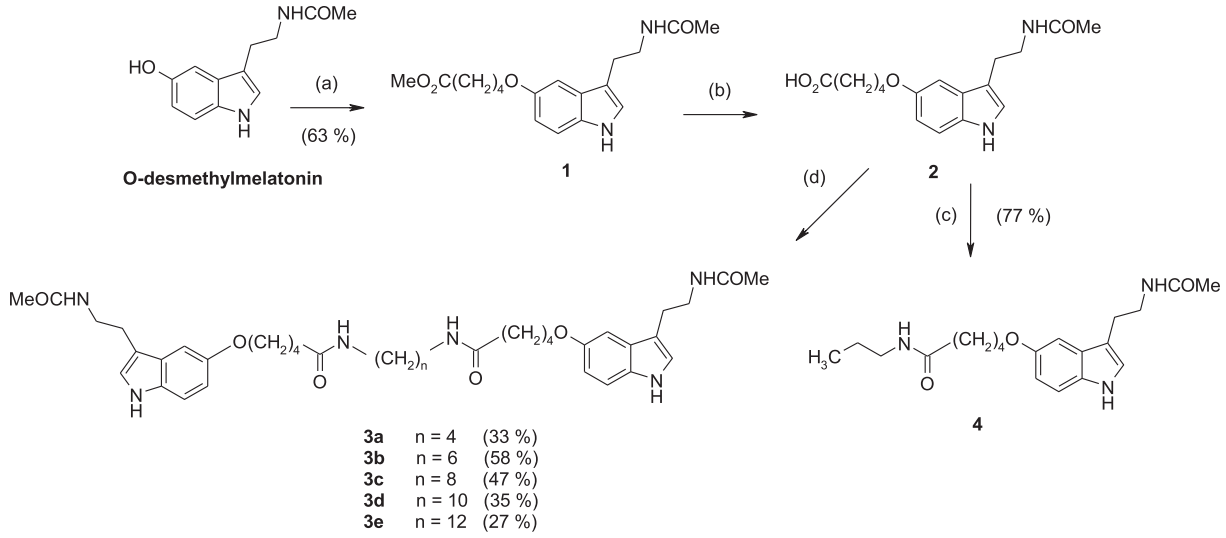



**Fig. 1.** Structures of melatonin, agomelatine, N-{2-[(3-methoxyphenyl)methylamino]ethyl}-acetamide, and the previously reported series of *N*1-linked melatonin dimers.

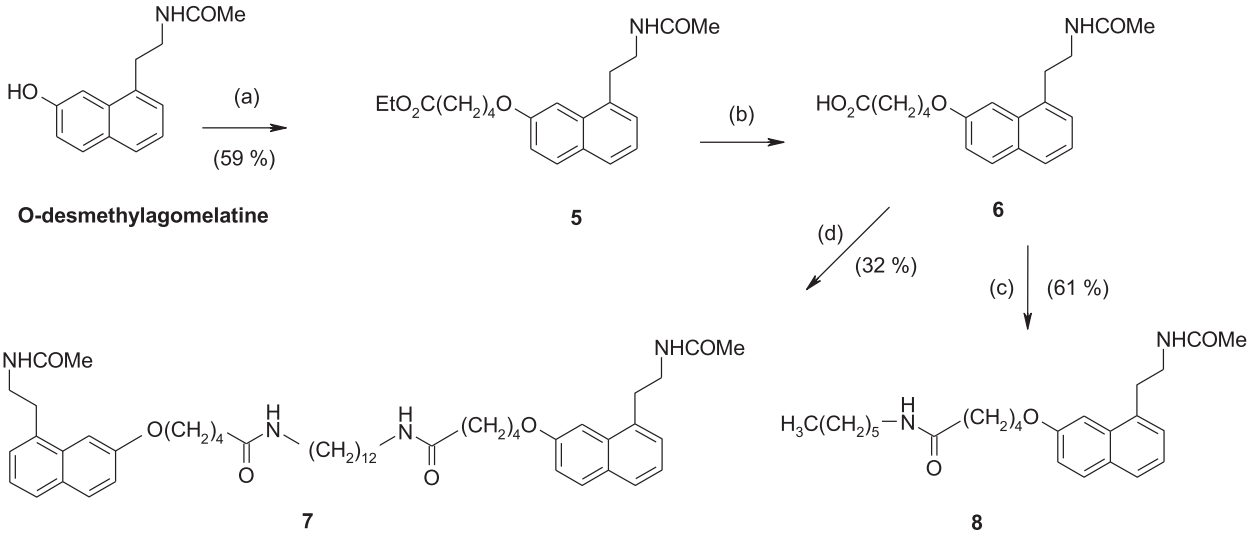
ideally not impair binding and/or potency of the protomers. The optimal length of the spacer enabling simultaneous binding to both protomers of a particular GPCR dimer should be determined by screening a series of bivalent ligands with spacers containing increasing number of atoms, and has been reported to be within the range of 18–25 atoms [23]. Moreover, pure polymethylene chains should be avoided due to possible aqueous solubility problems. Indeed, hydrophilic units, such as, amides, ethers, and/or amines have been often incorported into the middle chain of bivalent ligands.

Recently, our research groups reported the synthesis and pharmacological evaluation of the first series of *N*1-linked dimeric melatonin analogues targeting homomeric ($MT_1/MT_1$ or $MT_2/MT_2$) and heteromeric ($MT_1/MT_2$) melatonin receptors (Fig. 1) [24]. Although *N*1-substitution of melatonin is known to decrease the affinity to melatonin receptors [25], and all bivalent ligands showed indeed considerably reduced binding to $MT_1$ and $MT_2$ receptors when compared to melatonin, this series allowed a first assessment of the spacer length for bridging the protomers of dimeric melatonin receptors. While among the shorter bivalent ligands with acetamide units attached to *N*1 of melatonin, the dimeric compound with a 16-atoms spacer (Fig. 1, n = 1, m = 10) induced the maximal BRET changes at $MT_1$-homodimers, $MT_2$-homodimers and $MT_1/MT_2$-heterodimers, the bivalent ligand with a 24-atoms spacer (Fig. 1, n = 4, m = 12) showed the strongest increase in the corresponding BRET signals in the series of compounds possessing longer -$(CH_2)_4$CONH-linkers. Most importantly, ligand-induced BRET changes observed for compounds linked through spacers of 22–24 atoms could be attributed to ligand-induced conformational changes between the two protomers of $MT_1$ and $MT_2$ homo- and heterodimers indicating simultaneous binding of both pharmacophores to the two protomers of receptor dimers.

In this paper, we report the synthesis and pharmacological evaluation of a novel series of melatonin dimers linked through the ether oxygen of the parent molecule. Three series of ether oxygen-linked dimeric melatonergic ligands have been previously reported. However, for all series, the spacer lengths applied (3–12 atoms) were too short for bridging dimeric receptors. Connecting two molecules of a highly potent $MT_1/MT_2$ agonist agomelatine (Fig. 1) by polymethylene spacers $(CH_2)_n$ (n = 2–6, 8, 10) led to ligands with high-affinity for the $MT_1$ receptor with the longer analogs (n = 5, 6, 8, 10) displaying the same $K_i$ values as agomelatine ($K_i$ ~ 0.05 nM) [26]. At the $MT_2$ receptor, the shorter analogues (n = 2–6) showed much lower affinity than at $MT_1$ resulting in preferential $MT_1$ binding. The most $MT_1$-selective ligands




**Scheme 1.** Reagents and conditions: (**a**) $Br(CH_2)_4CO_2Me$, $K_2CO_3$, MeCN, reflux, 24 h; (**b**) 1. LiOH, THF, $H_2O$, rt, 24 h, 2. 2 M HCl; (**c**) PyBop, HOBt, DIPEA, DMF, *n*-propylamine, rt. 24 h; (**d**) EDCI HCl, HOBt, $CH_2Cl_2$, $H_2N(CH_2)_nNH_2$, rt, 24 h.




**Scheme 2.** Reagents and conditions: (**a**) NaH, DMF, $Br(CH_2)_4CO_2Et$, rt, 24 h; (**b**) 1.KOH, THF, $H_2O$, rt, 48 h, 2. 2 M HCl; (**c**) PyBop, HOBt, DIPEA, DMF, *n*-hexylamine, rt. 24 h; (**d**) PyBop, HOBt, DIPEA, $CH_2Cl_2$, $H_2N(CH_2)_{12}NH_2$, rt, 24 h.

were agomelatine dimers linked by three and four methylene groups. The second series of oxygen-linked dimeric melatonergic ligands are analogues of the $MT_1/MT_2$ agonist N-{2-[(3-methoxyphenyl)methylamino]ethyl}-acetamide (Fig. 1) [27]. The parental molecules were linked by polymethylene spacers $(CH_2)_n$ (n = 3–6, 8, 10). The dimers showed considerably lower affinities for both $MT_1$ and $MT_2$ receptors compared to the parental molecule. Similar to the dimeric agomelatine series, the dimer with the head pharmacophores separated by a $(CH_2)_3$-spacer showed $MT_1$ selectivity ($MT_1$, $K_i$ = 20 nM; $MT_2$, $K_i$ = 2089 nM) [27]. Finally, in the series of dimeric 4-azaindole melatonin analogues, the most potent compound with a $(CH_2)_6$-spacer showed high affinity ($K_i$ = 8.5 nM) and 20-fold preference toward $MT_1$ receptors [28].

The structures of the novel *O*-linked melatonin dimers **3a-e** are shown in Scheme 1. The $(CH_2)_4CONH$-$(CH_2)_n$-$OCNH(CH_2)_4$-spacers (n = 4, 6, 8, 10, 12) correspond to 16, 18, 20, 22, and 24 atoms connecting the oxygens of both melatonin units and span the length expected for simultaneous binding of both melatonin pharmacophores to both protomers of dimeric melatonin receptors. To examine the influence of melatonin-agomelatine exchange in the series, an agomelatine dimer with a 24-atoms spacer **7** was included in our studies. Moreover, the monomeric control compounds **4** (half the molecule of **3b**) and **8** (half the molecule of agomelatine dimer **7**) were synthesized.

## 2. Results and discussion

### 2.1. Chemistry

The key starting material for the synthesis of melatonin dimers **3a-e** is *O*-desmethylmelatonin. It was prepared from commercially available 5-benzyloxyindole-3-acetonitrile according to our previously reported procedure [29]. O-alkylation of *O*-desmethylmelatonin using methyl 5-bromovalerate yielded ester **1**. After ester hydrolysis the resultant acid **2** was subjected to amide coupling with diaminoalkanes of different chain lengths $H_2N$-$(CH_2)_n$-$NH_2$ (n = 4, 6, 8, 10, 12) using EDCI HCl as a coupling reagent to give the target bivalent ligands **3a-e** (Scheme 1). The monovalent melatonin analogue **4**, whose structure represents half the molecule of the bivalent ligand **3b**, was prepared by coupling of the acid **2** with *n*-propylamine. The agomelatine dimer **7** was synthesized in four steps starting from agomelatine [30] as shown in Scheme 2. Briefly, agomelatine was subjected to ether cleavage using $BBr_3$ as

**Table 1**
Binding affinities and functional characteristics of the indicated compounds in HEK 293 cells expressing human $MT_1$ and $MT_2$ receptors.

| Cpd | $MT_1$ $pK_i$ | $MT_2$ $pK_i$ | $MT_1$ – cAMP $pEC_{50}$ ($E_{max}$) | $MT_2$ – cAMP $pEC_{50}$ ($E_{max}$) | $MT_1$ – βARR $pEC_{50}$ ($E_{max}$) | $MT_2$ – βARR $pEC_{50}$ ($E_{max}$) |
|---|---|---|---|---|---|---|
| **Melatonin** | 9.32 ± 0.45 | 9.48 ± 0.25 | 9.06 ± 0.19 (100 ± 8) | 8.57 ± 0.26 (100 ± 11) | 9.85 ± 0.10 (100 ± 4) | 9.51 ± 0.11 (100 ± 4) |
| **3a** | 5.33 ± 0.17 | 5.65 ± 0.26 | – | – | – | – |
| **3b** | 5.78 ± 0.02 | 5.60 ± 0.04 | – | – | – | – |
| **3c** | 6.00 ± 0.23 | 5.30 ± 0.13 | nd | > 5 (nd) | 6.97 ± 0.37 (39 ± 6) | 6.08 ± 0.20 (50 ± 5) |
| **3d** | 6.63 ± 0.16 | 6.71 ± 0.17 | – | – | – | – |
| **3e** | 7.19 ± 0.27 | 7.51 ± 0.22 | – | – | – | – |
| **4** | 6.33 ± 0.14 | 6.04 ± 0.29 | 6.59 ± 0.25 (93 ± 10) | 7.20 ± 0.46 (84 ± 17) | 8.18 ± 0.29 (67 ± 8) | 7.31 ± 0.19 (62 ± 5) |
| **Agomelatine** | – | – | 9.20 ± 0.11 (100 ± 5) | 10.2 ± 0.29 (100 ± 12) | 9.89 ± 0.12 (100 ± 5) | 9.78 ± 0.16 (100 ± 6) |
| **7** | 8.01 ± 0.46 | 8.61 ± 0.32 | 7.61 ± 0.29 (49 ± 7) | nd (nd) | 7.87 ± 0.15 (90 ± 6) | 7.64 ± 0.14 (56 ± 4) |
| **8** | 8.20 ± 0.71 | 8.35 ± 0.32 | 7.71 ± 0.23 (89 ± 10) | 6.75 ± 0.89 (118 ± 51) | 7.94 ± 0.26 (88 ± 11) | 7.24 ± 0.17 (78 ± 8) |

Concentration–response curves were analysed by non-linear regression. Binding affinity was measured with 2-[$^{125}$I]-MLT and is expressed as mean $pK_i$ ± SEM. Agonist potency is expressed as $pEC_{50}$ ± SEM. The maximal efficacy, $E_{max}$, is expressed as a percentage of the maximal effect observed with melatonin or agomelatine (=100% ± SEM). Data are mean of at least three independent experiments, each of them performed using at least/a minimum of eight different ligand concentrations. n.d., not determined; -, not tested.

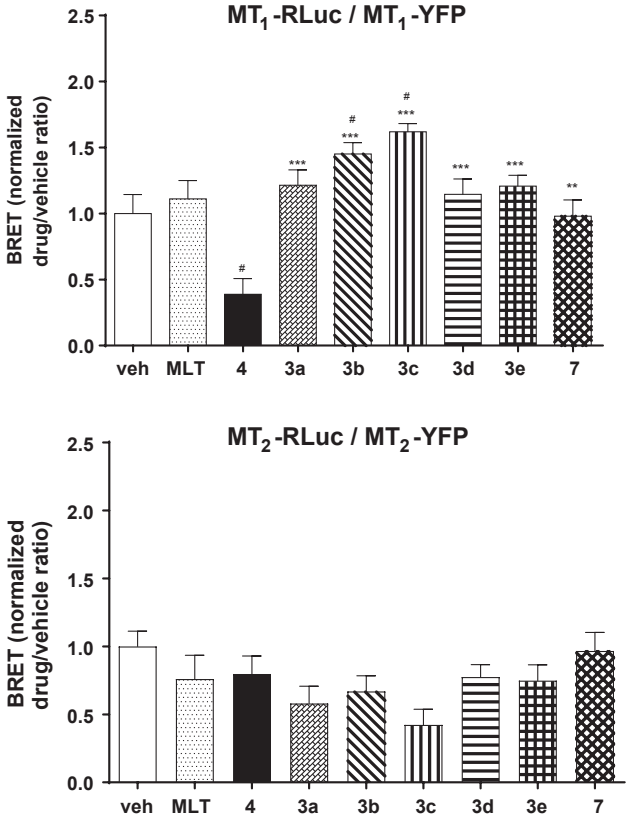



**Fig. 2.** Effect of compounds **3a-e**, **4**, **7** (10 μM) and melatonin (1 nM) on BRET signals. Ligand concentration were chosen to achieve similar receptor occupancies. Living HEK293T cells expressing the indicated BRET fusion protein couples were incubated with the indicated compounds. Results are expressed as percentage of the BRET amplitude obtained between the lowest and the highest BRET value for each experiment, and normalized to the vehicle condition. Data are means ± S.E.M. from 5 independent experiments. Differences between control compound **4** and the other compounds ($^{**}p < 0.01$; $^{***}p < 0.001$) and between vehicle and other compounds (#; veh vs 4, p = 0.005; veh vs 3b, p = 0.0168; veh vs 3c: p = 0.0012) have been evaluated with the two-tailed unpaired Student's *t*-test.

previously reported [31], and the resultant desmethylagomelatine was O-alkylated with ethyl 5-bromovalerate to give the ester **5**. Saponification using KOH yielded the acid **6** that was subjected to amide coupling with $H_2N$-$(CH_2)_{12}$-$NH_2$ to give the target bivalent ligand **7**. The monovalent agomelatine analogue **8**, whose structure represents half the molecule of the bivalent ligand **7**, was prepared by coupling of the acid **6** with *n*-hexylamine.

### 2.2. Pharmacology

#### 2.2.1. Radioligand binding studies

In order to determine the affinity of the compounds for human $MT_1$ and $MT_2$ receptors we performed competition binding experiments with 2-[$^{125}$I]iodomelatonin (2-[$^{125}$I]-MLT) in crude membranes prepared from HEK293 cells transiently expressing either receptor as previously reported [8]. Melatonin was used as the reference ligand and run in parallel. The $K_i$ values of melatonin for the $MT_1$ and $MT_2$ receptors were in the low nanomolar range as expected. All bivalent ligands **3a-e** and **7** and the monomeric control compounds **4** and **8** displayed $pK_i$ values ranging from 5 to 8 (Table 1). As already observed in the *N*1-linked series of melatonin dimers (Fig. 1) [24], none of the ligands showed selectivity toward $MT_1$ or $MT_2$ receptors. While for the previously described *N*1-linked dimeric melatonin compounds with identical spacers (Fig. 1, n = 4, m = 6, 8, 10, 12), no correlation between the length of the spacer and affinity towards $MT_1$ and $MT_2$ receptors could be found, in the present *O*-linked series **3a-e** the increasing number of atoms in the spacer led to increased $MT_1$/$MT_2$ affinity with the exception of **3c** for $MT_2$. Indeed, bivalent ligand **3e** with the longest spacer of 24 atoms connecting the ether oxygens of both melatonin pharmacophores showed the highest affinity ($MT_1$: $pK_i$ = 7.19 ± 0.27, $MT_2$: $pK_i$ = 7.51 ± 0.22). As expected, the dimeric agomelatine analogue **7** formally obtained by exchanging the melatonin units of **3e** by agomelatine moieties, showed higher affinity toward both melatonin receptors ($MT_1$: $pK_i$ = 8.01 ± 18, $MT_2$: $pK_i$ = 8.61 ± 0.32) than **3e** reflecting higher binding affinity of agomelatine compared to melatonin [26].

#### 2.2.2. Bioluminescence resonance energy transfer studies

To study the effect of bivalent ligands on $MT_1$ and $MT_2$ homodimers, BRET experiments with the *Renilla* luciferase (Rluc) energy donor or the yellow fluorescent protein (YFP) energy acceptor fused each to the C-terminus of $MT_1$ or $MT_2$ were performed. Indicated donor-acceptor pairs were coexpressed in HEK293T cells and then incubated for 10 min

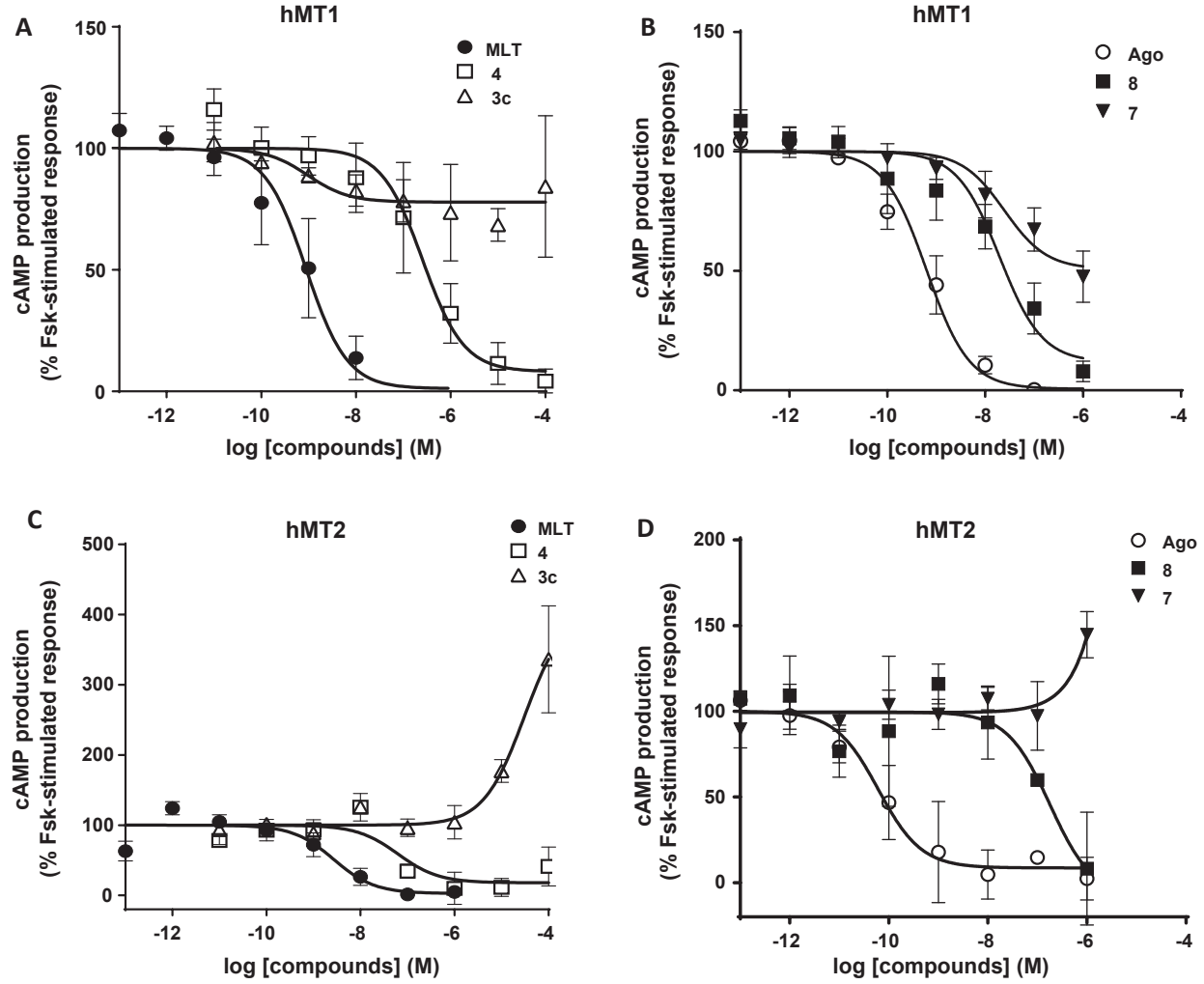



**Fig. 3.** Inhibition of cAMP production by melatonin (**MLT**), **3c**, **4,** agomelatine (**Ago**), 7 and **8** in HEK293 cells expressing $hMT_1$ (**A, B**) and $hMT_2$ (**C, D**) receptors. Data are expressed as mean ± S.E.M. from 3 to 4 independent experiments and are represented as percentage of FSK-stimulated response.

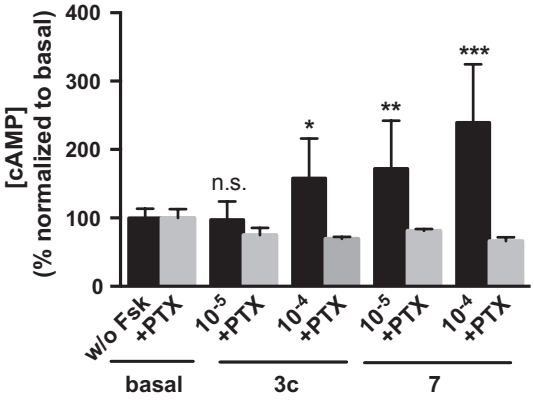



**Fig. 4.** Inhibition of cAMP production by **3c** and **7** at $10^{-5}$ and $10^{-4}$ M in HEK293 cells expressing $hMT_2$ receptors. Data are expressed as mean ± S.E.M. from 3 independent experiments and are represented as percentage of the basal response. Pertussis toxin; PTX. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$; n.s. non significant; compared to basal using one way ANOVA follow by Dunnett's test.

with either the monovalent control **4**, bivalent ligands (**3a-e**, **7**) or melatonin followed by BRET measurements 5 min after the addition of the Rluc substrate coelenterazine. Melatonin had no detectable effect on either receptor combination (Fig. 2). The monovalent control **4**, containing one melatonin pharmacophore and the linker, decreased the BRET signal for $MT_1$ homodimers, most likely by binding of its linker part to an additional, allosteric site, which then induces conformational changes between the two protomers that are detectable by BRET, as previously observed for another monomeric compound containing a linker [24]. Control **4** had no effect on $MT_2$ homodimers. For $MT_1$ homodimers, **3b** and **3c** significantly increased the BRET signal with a maximal effect for **3c** (20 atoms spacer) when compared to vehicle. When compared to the monomeric control **4,** BRET signals of all bivalent ligands **3a-e** were significantly increased with a maximal increase of > 3-fold for **3c**. BRET signals of $MT_2$ homodimers were insensitive to bivalent ligands **3a-e** with a tendency of **3c** to decrease the BRET signal. These findings indicate that $MT_1$ homodimers are particularly sensitive in detecting the binding of the series of O-linked dimeric melatonins **3a-e**, most likely by binding with both pharmacophores to the two protomers of receptor dimers as exemplified by the difference in BRET signal between control **4** and the bivalent ligands. The existence of an optimal length of the spacer (here corresponding to 20 atoms) further supports the binding of the two pharmacophores to the $MT_1$ homodimer. Exchange of melatonin pharmacophores against agomelatine pharmacophores resulted in similar BRET changes for $MT_1$ homodimers at a linker length of 24 atoms (**3e** vs. **7**). As for the $MT_2$ homodimers, the absence of ligand-induced effect might be due to the absence of simultaneous binding of the two pharmacophores to the two receptor protomers or, in case of simultaneous binding, the lack of

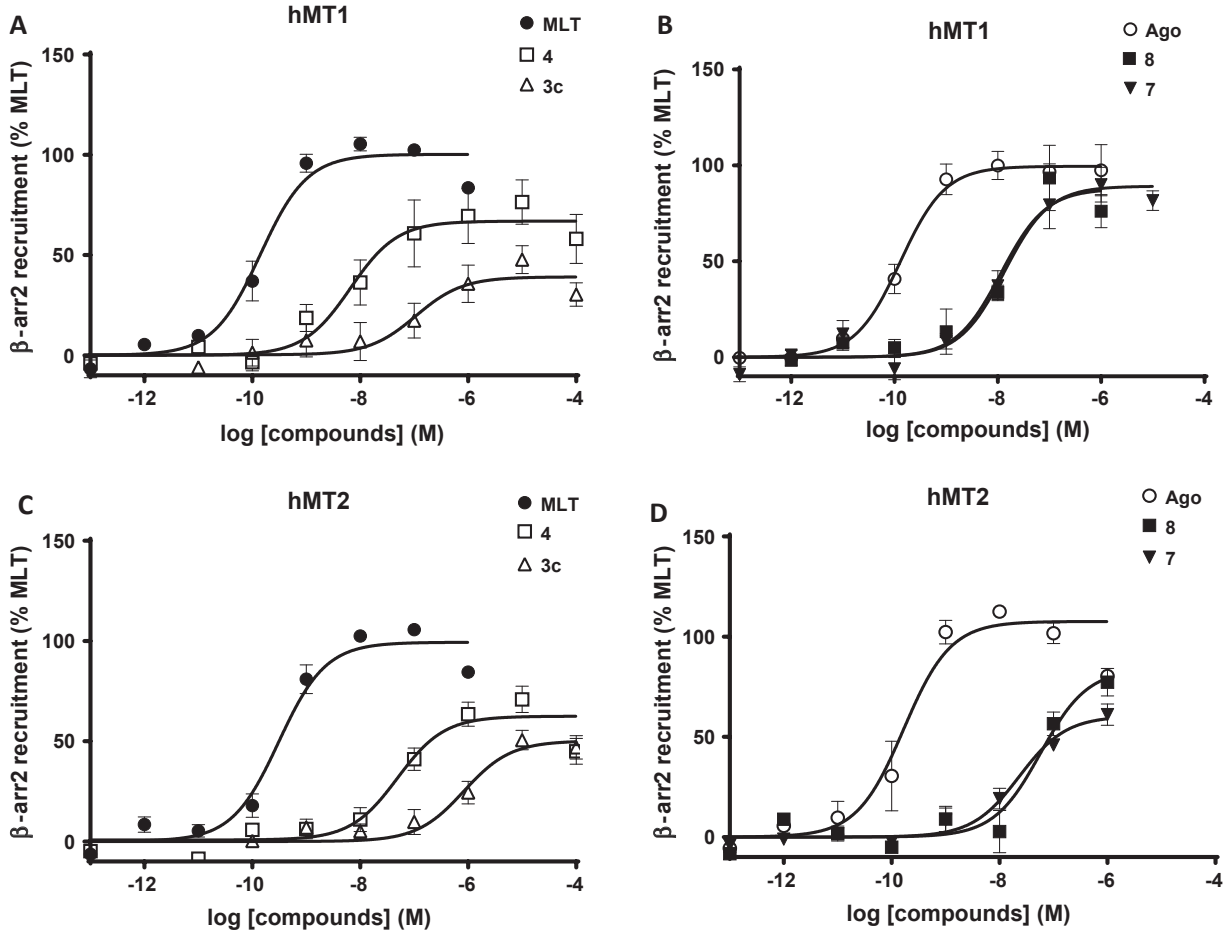



**Fig. 5.** Recruitment of β-arrestin2 to melatonin receptors by melatonin (**MLT**), **3c**, **4**, agomelatine (**Ago**), **7** and **8**. Recruitment of β-arrestin2 to $hMT_1$ (**A, B**) and $hMT_2$ (**C, D**) receptors induced by MLT, **3c, 4**, Ago, **7** and **8** compounds in HEK293 cells. Data are expressed as mean ± S.E.M. from 3 to 4 independent experiments and are represented as percentage of MLT or Ago effect.

effect on the BRET signal. These findings indicate that for *O*-linked dimeric melatonin/agomelatine analogs, $MT_2$ homodimers are less suitable for detection of ligand-induced BRET changes than their $MT_1$ counterparts.

### 2.2.3. Functional studies

The bivalent melatonin-based ligand **3c** inducing the highest BRET change in $MT_1$ homodimers, the bivalent agomelatine-based compound **7**, and the monomeric ligands **4** and **8** were subjected to functional studies at $hMT_1$ and $hMT_2$ receptors using the cAMP inhibition assay and the β-arrestin recruitment assay [32]. Both monomeric compounds, **4** and **8,** behaved as full agonists, as they inhibited forskolin-stimulated cAMP production to a similar extent as the reference compounds melatonin and agomelatine (Fig. 3) with $pEC_{50}$ values ranging from 6.60 to 7.70 (Table 1). Bivalent ligand **3c** was without effect on the $hMT_1$ receptor and behaved as inverse agonist on the $hMT_2$ receptor. Bivalent ligand **7** behaved as a partial agonist on $hMT_1$ receptors and showed a tendency for inverse agonism on $hMT_2$ receptors. To further study the effect of the bivalent ligands **3c** and **7** on the cAMP pathway we performed these studies in the absence of forskolin. Similar to the data obtained in the presence of forskolin, basal cAMP levels were also increased by **3c** and **7**. This effect was pertussis toxin sensitive indicating the involvement of Gi/o proteins (Fig. 4).

To explore the effect of the ligands on G protein-independent signaling of melatonin receptors, we monitored β-arrestin 2 recruitment. In contrast to the cAMP result, bivalent compounds **3c** and **7** behaved similar to their corresponding monomeric counterparts in the β-arrestin assay. Compounds **4** and **3c** were both partial agonist compared to melatonin with $pEC_{50}$ values between 7 and 8 (Fig. 5, Table 1). Compounds **7** and **8** were both full agonists for $hMT_1$ receptors and partial agonists for $hMT_2$ receptors compared to agomelatine with $pEC_{50}$ values between 7 and 8 (Fig. 5, Table 1). Based on these data we hypothesize that the linker (present in the monomeric compounds **4** and **8** and bivalent compounds **3c** and 7) constitute most likely the main molecular determinant converting these ligands into partial agonists for β-arrestin 2 recruitment as compared with melatonin or agomelatine in particular for $hMT_2$ receptors.

Taken together the results of the two functional assays it appears that the introduction of a second pharmacophore, as in bivalent ligands, modifies primarily the capacity of bivalent ligands to activate the $G_i$ protein-dependent signaling properties of melatonin receptors as compared to β-arrestin2 recruitment. Assuming that both pharmacophores of bivalent ligands bind to the receptor, binding of the second pharmacophore, most likely modifies receptor conformations associated with G protein coupling or activation more pronouncedly than conformations associated with β-arrestins interaction.

## 3. Conclusions

In summary, *O*-linked melatonin dimers are valuable pharmacological tools for exploring the impact of melatonin receptor dimerization and receptor function. BRET experiments at $MT_1$ homodimers indicated simultaneous binding of the bivalent ligands to the two protomers with the strongest BRET changes (> 3-fold higher than for the monomeric

control ligand) observed for the analogue **3c** possessing a 20 atoms spacer. This increased BRET of **3c** was accompanied by the loss of the agonistic property observed for the monomeric **4** on $MT_1$ homodimers. While the BRET signal of $MT_2$ homodimers was not modified upon binding of **3c**, functional studies on the cAMP pathway showed a clear shift from agonistic to inverse agonistic activity of the monomeric **4** to the bivalent **3c** suggestive of binding of both pharmacophores of **3c** to $MT_2$ homodimers. The difficulty to detect BRET changes for **3c** in $MT_2$ homodimers also illustrates that not all conformational changes are readily detectable by a given BRET sensor. Accordingly, it is impossible to predict the functional effect based on the magnitude of the BRET signal. Indeed, the amplitude of the BRET signal is a measure of the relative position and orientation of the BRET donor and acceptor and its modification depends on the receptor conformation. Although the amplitude of the BRET changes is not necessarily correlated with the amplitude of the conformational change, BRET is however able to detect relative changes within a series of compounds such as the determination of the maximal linker length for the current and previous series of bivalent ligands [24]. The marked decrease in BRET signal in the presence of the monomeric control compound **4** at $MT_1$ homodimers suggests that the linker might have an additional function apart from defining the distance between the two pharmacophores, namely the induction of a conformational change between the two protomers, most likely by binding to an allosteric binding site close to the orthosteric binding site. This second function is likely to exist also in the bivalent compounds in the $MT_1$ homodimer as similar affinities for mono- and bivalent compounds (pKi ~ 6) suggest a similar pose of the pharmacophore and the linker position.

Our data confirm previous observations that binding of both pharmacophores of bivalent ligands does not necessarily go along with an increased binding affinity and that the binding affinity does not correlate with the amplitude of the BRET signal [17,24]. Although it is difficult to draw any conclusions about a possible correlation between BRET changes and functional properties of the presented compounds based on the current data, it is intriguing that compound **3c** shows the most differential BRET change between $MT_1$ and $MT_2$ homodimers correlating with a switch of compound **3c** from a partial agonist at $MT_1$ to inverse agonist at $MT_2$. Future studies will be necessary to explore this aspect in more detail.

The increased cAMP production of compounds **3c** and **7** at $MT_2$ receptors in the presence and absence of FSK suggests an inverse agonistic activity of these two compounds on this Gi-dependent pathway. Alternatively, it could be also an indication for Gs activation of the $MT_2$ receptor by these ligands. As the effect was pertussis toxin-sensitive, a Gi-dependent mechanism seems more likely.

Altogether, the *O*-linked melatonin dimers are more suitable for investigating receptor dimerization than the previously reported *N*1-linked analogues [24]. *O*-linked melatonin dimers show profoundly modified functional properties compared to their monomeric counterparts including compound **3c**, which has inverse agonistic activity on the cAMP pathway. The findings are important for the design of melatonin receptor inverse agonists, a class of ligands that has been only poorly described for melatonin receptors [33,34].

## Acknowledgments

The authors thank Prof. Ulrike Holzgrabe, University of Würzburg for her support and Jan Glaser, University of Würzburg for recording ESI mass spectra. Mirna Sadek appreciates financial support from the German Academic Exchange Service DAAD. Ralf Jockers was supported by the Agence Nationale de la Recherche (ANR-2011-BSV1-012-01 “MLT2D”, ANR-2011-META “MELA-BETES”, ANR-12-RPIB-0016, ANR-16-CE18-0013 et ANR-15-CE14-0025-02), the Fondation pour la Recherche Médicale (Equipe FRM DEQ20130326503), France Alzheimer, la Fondation Philippe Chatrier, Servier, the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Centre National de la Recherche Scientifique (CNRS).

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.004.

## References

[1] A.S. Hauser, M.M. Attwood, M. Rask-Andersen, H.B. Schiöth, D.E. Gloriam, Trends in GPCR drug discovery: new agents, targets and indications, Nat. Rev. Drug Discov. 16 (2017) 829–842.
[2] G. Milligan, G protein-coupled receptor dimerization: function and ligand pharmacology, Mol. Pharmacol. 66 (2004) 1–7.
[3] S. Ferré, V. Casadó, A. Lakshmi, M. Filizola, R. Jockers, M.J. Lohse, G. Milligan, J.-P. Pin, X. Guitart, G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives, Pharmacol. Rev. 66 (2014) 413–434.
[4] K. Tadagaki, R. Jockers, M. Kamal, History and biological significance of GPCR heteromerization in the neuroendocrine system, Neuroendocrinology 95 (2012) 223–231.
[5] R. Jockers, P. Delagrange, M.I. Dubocovich, R.P. Markus, N. Renault, G. Tosini, E. Cecon, D.P. Zlotos, Update on melatonin receptors: IUPHAR Review 20, Br. J. Pharmacol. 173 (2016) 2702–2725.
[6] M.A. Ayoub, C. Couturier, E. Lucas-Meunier, S. Angers, P. Fossier, M. Bouvier, R. Jockers, Monitoring of ligand-independent dimerization and ligand-induced conformational changes of melatonin receptors in living cells by bioluminescence resonance energy transfer, J. Biol. Chem. 277 (2002) 21522–21528.
[7] R. Jockers, P. Maurice, J.A. Boutin, P. Delagrange, Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? Br. J. Pharmacol. 154 (2008) 1182–1195.
[8] M.A. Ayoub, A. Levoye, P. Delagrange, R. Jockers, Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers, Mol. Pharmacol. 66 (2004) 312–321.
[9] K. Baba, A. Benleulmi-Chaachoua, A.-S. Journé, M. Kamal, J.L. Guillaume, S. Dussaud, F. Gbahou, K. Fettou, C. Liu, S. Contreras-Alcantra, R. Jockers, G. Tossini, Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function, Sci. Signal 6 (2013) ra89.
[10] D.P. Zlotos, R. Jockers, E. Cecon, S. Rivara, P.A. Witt-Enderby, MT1 and MT2 melatonin receptors: ligands, models, oligomers, and therapeutic potential, J. Med. Chem. 57 (2014) 3161–3185.
[11] J. Shonberg, P.J. Scammells, B. Capuano, Design strategies for bivalent ligands targeting GPCRs, ChemMedChem 6 (2011) 963–974.
[12] P.S. Portoghese, From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes, J. Med. Chem. 44 (2001) 2259–2269.
[13] X. Peng, J.L. Neumeyer, Kappa receptor bivalent ligands, Curr. Top. Med. Chem. 7 (2007) 363–373.
[14] W. Zheng, L. Lei, S. Lalchandani, G. Sun, D.R. Feller, D.D. Miller, Yohimbine dimers exhibiting binding selectivities for human alpha2a- versus alpha2b-adrenergic receptors, Bioorg. Med. Chem. Lett. 10 (2000) 627–630.
[15] S.G. Lalchandani, L. Lei, W. Zheng, M.M. Suni, B.M. Moore, S.B. Liggett, D.D. Miller, D.R. Feller, Yohimbine dimers exhibiting selectivity for the human alpha 2C-adrenoceptor subtype, J. Pharmacol. Exp. Ther. 303 (2002) 979–984.
[16] A.P. Tamiz, B.C. Bandyopadhyay, J. Zhang, J.L. Flippen-Anderson, M. Zhang, C.Z. Wang, K.M. Johnson, S. Tella, A.P. Kozikowski, Pharmacological and behavioral analysis of the effects of some bivalent ligand-based monoamine reuptake inhibitors, J. Med. Chem. 44 (2001) 1615–1622.
[17] O. Russo, M. Berthouze, M. Giner, J.-L. Soulier, L. Rivail, S. Sicsic, F. Lezoualc'h, R. Jockers, I. Berque-Bestel, Synthesis of specific bivalent probes that functionally interact with 5-HT(4) receptor dimers, J. Med. Chem. 50 (2007) 4482–4492.
[18] P.C. Meltzer, O. Kryatova, D.P. Pham-Huu, P. Donovan, A. Janowsky, The synthesis of bivalent 2beta-carbomethoxy-3beta-(3,4-dichlorophenyl)-8-heterobicyclo[3.2.1] octanes as probes for proximal binding sites on the dopamine and serotonin transporters, Bioorg. Med. Chem. 16 (2008) 1832–1841.
[19] A. Christopoulos, M.K. Grant, N. Ayoubzadeh, O.N. Kim, P. Sauerberg, L. Jeppesen, E.E. El-Fakahany, Synthesis and pharmacological evaluation of dimeric muscarinic acetylcholine receptor agonists, J. Pharmacol. Exp. Ther. 298 (2001) 1260–1268.
[20] W.G. Rajeswaran, Y. Cao, X.P. Huang, M.E. Wroblewski, T. Colclough, S. Lee, F. Liu, P.I. Nagy, J. Ellis, B.A. Levine, K.H. Nocka, W.S. Messer Jr., Design, synthesis, and biological characterization of bivalent 1-methyl-1,2,5,6-tetrahydropyridyl-1,2,5-thiadiazole derivatives as selective muscarinic agonists, J. Med. Chem. 44 (2001) 4563–4576.
[21] Y. Zhang, A. Gilliam, R. Maitra, M.I. Damaj, J.M. Tajuba, H.H. Seltzman, B.F. Thomas, Synthesis and biological evaluation of bivalent ligands for the cannabinoid 1 receptor, J. Med. Chem. 53 (2010) 7048–7060.
[22] A. Tabor, S. Weisenburger, A. Banerjee, N. Purkayastha, J.M. Kaindl, H. Hübner, L. Wei, T.W. Grömer, J. Kornhuber, N. Tschammer, N.J. Birdsall, G.I. Mashanov, V. Sandoghdar, P. Gmeiner, Visualization and ligand-induced modulation of dopamine receptor dimerization at the single molecule level, Sci. Rep. 6 (2016) 33233.
[23] I. Berque-Bestel, F. Lezoualć'h, R. Jockers, Bivalent ligands as specific pharmacological tools for G protein-coupled receptor dimers, Curr. Drug Discov. Technol. 5 (2008) 312–318.
[24] A.-S. Journé, S.A.M. Habib, B.R. Dodda, M.N.F. Morcos, M.S. Sadek, S.A.A. Tadros,

P.A. Witt-Enderby, R. Jockers, D.P. Zlotos, *N*1-linked melatonin dimers as bivalent ligands targeting dimeric melatonin receptors, Med. Chem.Commun. 5 (2014) 792–796.
[25] D.P. Zlotos, Recent progress in the development of agonists and antagonists for melatonin receptors, Curr. Med. Chem. 9 (2012) 3532–3549.
[26] C. Descamps-Francois, S. Yous, P. Chavatte, V. Audinot, A. Bonnaud, J.A. Boutin, P. Delagrange, C. Bennejean, P. Renard, D. Lesieur, Design and synthesis of naphthalenic dimers as selective MT1 melatoninergic ligands, J. Med. Chem. 46 (2003) 1127–1129.
[27] G. Spadoni, A. Bedini, P. Orlando, S. Lucarini, G. Tarzia, M. Mor, S. Rivara, V. Lucini, M. Pannacci, F. Scaglione, Bivalent ligand approach on N-{2-[(3-methoxyphenyl)methylamino]-ethyl}acetamide: synthesis, binding affinity and intrinsic activity for MT(1) and MT(2) melatonin receptors, Bioorg. Med. Chem. 19 (2011) 4910–4916.
[28] C. Larraya, J. Guillard, P. Renard, V. Audinot, J.A. Boutin, P. Delagrange, C. Bennejean, M.C. Viaud-Massuard, Preparation of 4-azaindole and 7-azaindole dimers with a bisalkoxyalkyl spacer in order to preferentially target melatonin MT1 receptors over melatonin MT2 receptors, Eur. J. Med. Chem. 39 (2004) 512–526.
[29] C. Markl, W.P. Clafshenkel, M.I. Attia, S. Sethi, P.A. Witt-Enderby, D.P. Zlotos, N-Acetyl-5-arylalkoxytryptamine analogs: probing the melatonin receptors for MT(1)–selectivity, Arch. Pharm. (Weinheim) 344 (2011) 666–674.
[30] C. Markl, D.P. Zlotos, A Novel Synthesis of the Antidepressant Agomelatine, Synthesis (2011) 79–82.
[31] D.P. Zlotos, N.M. Riad, M.B. Osman, B.R. Dodda, P.A. Witt-Enderby, Novel difluoroacetamide analogues of agomelatine and melatonin: probing the melatonin receptors for $MT_1$selectivity, Med. Chem.Commun. 6 (2015) 1340–1344.
[32] F. Gbahou, E. Cecon, G. Viault, R. Gerbier, F. Jean-Alphonse, A. Karamitri, G. Guillaumet, P. Delagrange, R. Friedlander, J.P. Vilardaga, F. Suzenet, R. Jockers, Design and validation of the first cell-impermeant melatonin receptor agonist, Br. J. Pharmacol. 174 (2017) 2409–2421.
[33] S. Devavry, C. Legros, C. Brasseur, P. Delegrange, G. Spadoni, W. Cohen, B. Malpaux, J.A. Boutin, O. Nosjean, Description of the constitutive activity of cloned human melatonin receptors hMT(1) and hMT(2) and discovery of inverse agonists, J. Pineal Res. 53 (2012) 29–37.
[34] F. Roka, L. Brydon, M. Waldhoer, A.D. Strosberg, M. Freissmuth, R. Jockers, C. Nanoff, Tight association of the human Mel(1a)-melatonin receptor and G(i): precoupling and constitutive activity, Mol. Pharmacol. 56 (1999) 1014–1024.