Cancer Cells Show Higher Sensitivity to Melatonin-Tamoxifen Drug Conjugates than to Combination of Melatonin and Tamoxifen

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ABSTRACT: Drug conjugates of tamoxifen and melatonin linked through the amide side chain of melatonin (**4a,4b**) were reported as promising agents for future treatment of breast cancer, possibly reversing the adverse effects of tamoxifen. Here, we report the synthesis and pharmacological evaluation of a novel series of anticancer drug conjugates linking melatonin with tamoxifen through polymethylene spacers through the ether oxygen of melatonin (**16a−c**, **19a−c**, **21**) and compare them to the previously reported amide-linked analogues **4a** and **4b**. All hybrid ligands are antagonists of estrogen receptor alpha and agonists of the melatonin MT_1 receptor with variable potencies. Several drug conjugates including the $(CH_2)_4$-linked analogues **4a** and **16a** and the $(CH_2)_6$-linked compound **16c** showed higher potency to inhibit cell viability than the combination of melatonin and tamoxifen on at least one cancer cell line including MCF-7, MDA-MB-231, and HT-1080.

	ERα IC$_{50}$ [nM]	MT$_1$ K$_i$ [nM]	Cancer cells IC$_{50}$ [μM]		
			MCF-7	MDA-MB-231	HT-1080
melatonin	-	0.33	27.9	> 100	> 100
tamoxifen	48.9	-	9.5	4.1	4.5
melatonin + tamoxifen	-	-	11.7	19.9	5.7
4a	83.2	690	2.1	7.3	6.9
16c	864	3.1	6.8	6.4	1.7

INTRODUCTION

Anticancer drug conjugates consist of two anticancer pharmacophores linked into a single chemical entity.[1] They are expected to show simultaneous and synergistic action at two distinct anticancer targets possibly leading to increased potency and efficacy, lower toxicity, fewer drug−drug interactions and improved patient compliance when compared to a combination of two single drugs.[2] However, drug conjugates represent rather challenging structures for optimization toward a favorable pharmacokinetic and/or pharmacodynamic profile.[3] In particular, connecting two pharmacophores by long spacers often generates large chemical entities with molecular mass >500 Da leading not only to reduced solubility and cell permeability, but also to decreased binding and activity at both targets. Moreover, when the effective concentration at the first target considerably differs from that at the second one (e.g nanomolar versus micromolar), an effective and safe dose might be difficult to determine as in a drug conjugate both pharmacophores are present in a fixed 1:1 ratio.

Nevertheless, in the last 25 years, the number of publications in the field continually increased. Indeed, the PubMed data search using the term "anticancer hybrids" revealed 38 reports in 1998 rising to 475 in 2023.[4] For developments in the field, the reader is referred to a recent review article.[5]

The majority of anticancer drug conjugates target breast cancer, as reviewed.[6,7] Breast cancer is the most frequent type of neoplasia among women.[8] Estrogens, such as 17β-estradiol,

are crucial for the development of female sexual features including breast growth.[9] Estrogen action is mediated by intracellular estrogen receptors alpha and beta (ERα and ERβ) that regulate gene expression by binding to DNA response elements associated with target genes.[10] While ERα is expressed at low levels in noncancerous tissues, it is overexpressed in the hormone-dependent tumors that represent 75% of breast cancer.[11] Consequently, inhibition of ERα stimulation is widely used in the pharmacotherapy of breast cancer.[12] Two classes of ER inhibitors are currently approved as breast cancer therapeutics, selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, and selective estrogen receptor downregulators (SERDs), exemplified by fulvestrant. SERMs show either antagonist or (partial) agonist action dependent on tissues. For example, tamoxifen is an antagonist in breast cancer cells but has estrogenic effects on the uterus and bones. In contrast, SERDs are pure ER antagonists in all tissues.

Antiestrogens are among the most frequent anticancer pharmacophores incorporated into drug conjugates as reviewed.[13−15] Most of these hybrids are derivatives of
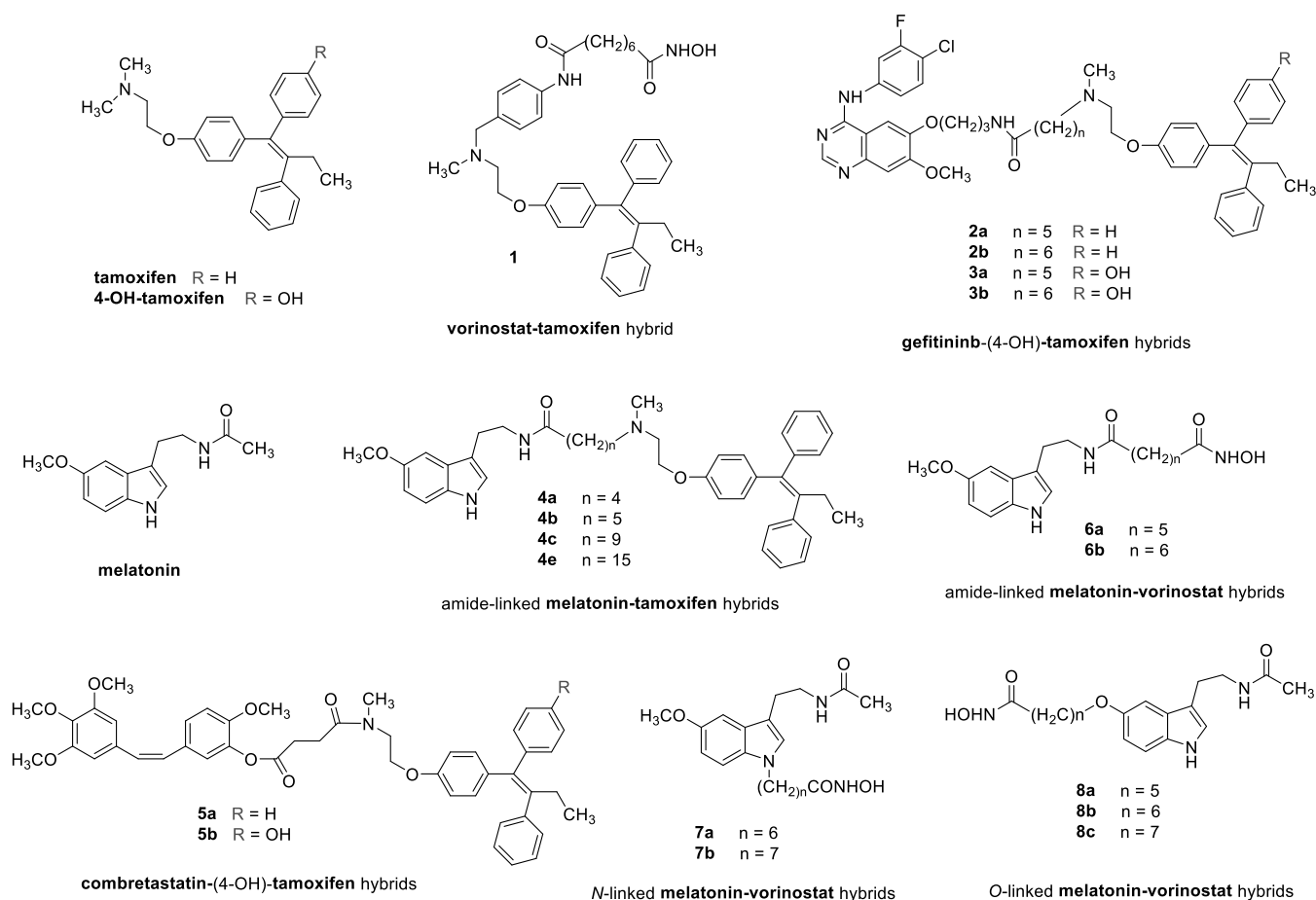
Figure 1. Structures of melatonin, tamoxifen, 4-OH-tamoxifen and of selected drug conjugates incorporating the latter drugs.

tamoxifen and of its active metabolite 4-OH-tamoxifen. Tamoxifen is the most common endocrine therapy against ER+ breast cancer. Unfortunately, its prolonged use can increase the risk of developing uterine cancer[16] and induce resistance.[17] Several reports indicated that some anticancer drugs, such as the EGFR inhibitor gefitinib,[18] the HDAC inhibitor vorinostat,[19] and the neurohormone melatonin,[20,21] may reverse acquired resistance to tamoxifen making a combination treatment possibly more effective than tamoxifen monotherapy. These studies prompted us and others to develop hybrid ligands combining tamoxifen and/or its active metabolite 4-hydroxytamoxifen with vorinostat, gefitinib and melatonin. The respective drug conjugates **1**,[22] **2a−b** and **3a− b**,[23] and **4a−e**[24,25] are shown in Figure 1.

Moreover, in drug conjugates such as **5a−b**, tamoxifen may act as a carrier selectively delivering cytotoxic agents to hormone-dependent tumor cells.[26,27]

Numerous previous publications report inhibitory effects of melatonin on cancer in general,[28] and on breast cancer in particular.[29,30] Most of the studies describe in vitro experiments using experimental models,[31] such as transformed cancer cell lines, and some report tumor-suppression in animals.[32,33] However, as recently pointed out, the conclusions from many of these studies are to be viewed with caution, especially when high supra-physiological concentrations of melatonin (>100 nM) were used, possibly leading to activation of signaling pathways that are not melatonin-specific and/or to down-regulation of melatonin-specific targets, such as MT_1 and MT_2 receptors.[34,35] Only few information is available on the

tumor-suppressing actions of melatonin in humans. Epidemiologic studies showed that light exposure at night leading to disruption of melatonin production is a risk factor for the development of breast cancer.[36−38] The few clinical trials evaluating the effect of melatonin in cancer patients have so far not generated positive outcomes.[35] None of these studies evaluated the use of melatonin in a combination therapy.

Many melatonin's anticancer actions are thought to be mediated through binding to its specific membrane associated G-protein-coupled receptors.[39,40] While two subtypes of melatonin receptors, MT_1 and MT_2, exist, the anticancer actions of the neurohormone are thought to be primarily MT_1-mediated.[41] Higher levels of MT_1 receptors were reported in malignant compared to normal human breast tissue.[42,43] MT_1 receptor overexpression enhanced the growth suppressive effect of melatonin in human breast cancer cells MCF-7.[44] Moreover, a correlation of MT_1 expression in biopsies from a variety of breast tumors (including ERα-positive and TNBC) with an improved prognosis was reported.[45,46]

The reported antiestrogen actions of melatonin in ERα-positive breast cancer cells include reduction of ERα transactivation,[47] inhibition of binding of the ER-estrogen composite to the estrogen response element of the DNA,[48] as well decreasing the activity of aromatase.[49]

Melatonin has been often incorporated in drug conjugates. However, most reports deal with the neurohormone linked to a variety of neuroprotective agents, such as tacrine,[50,51] and ferulic acid,[52] addressing neurodegenerative disorders, such as Alzheimer's disease.

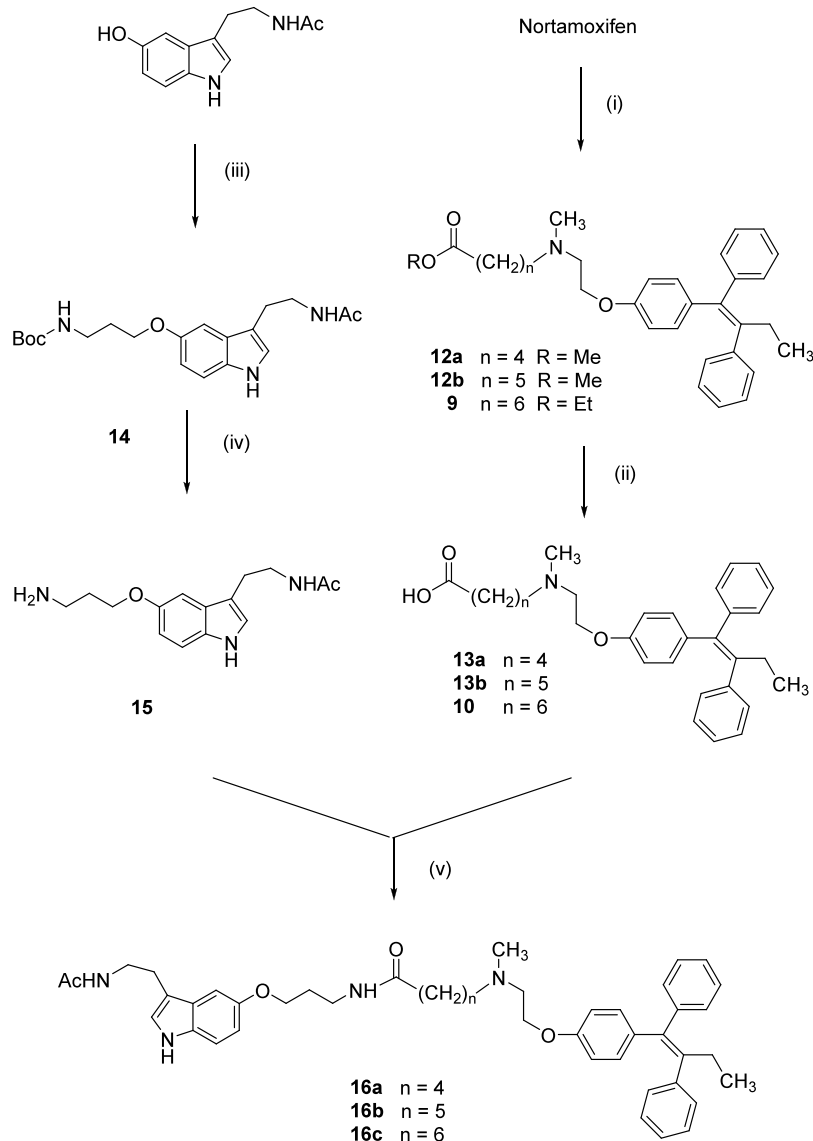**Scheme 1. Synthesis of drug conjugate 11**[a]
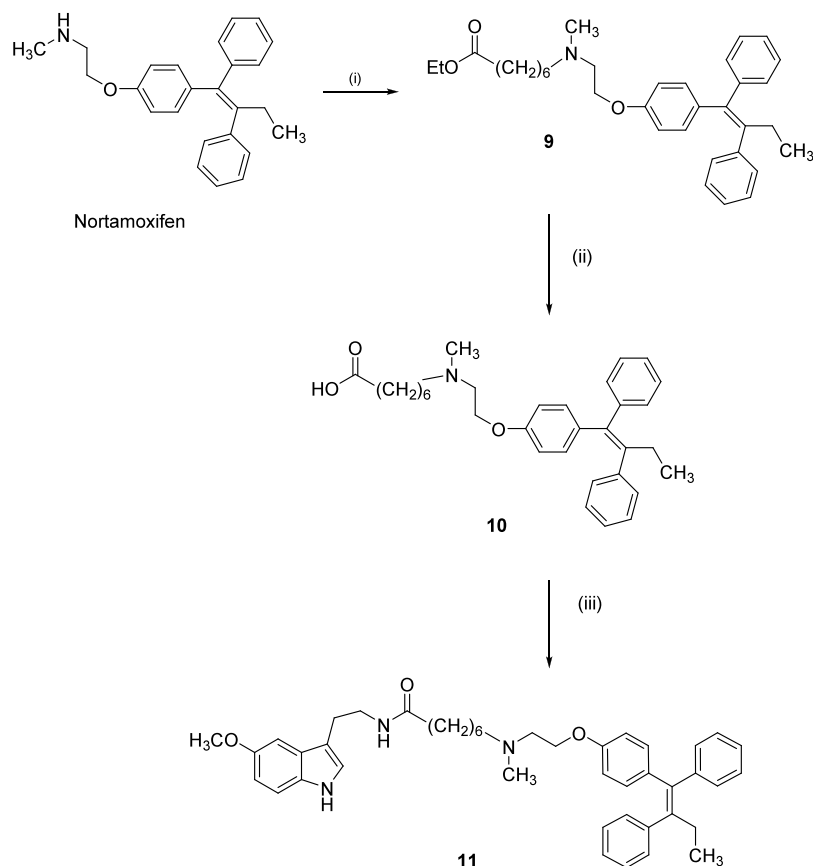
[a]Reagents and conditions: (i) 1. NaH, DMF, 2. Br(CH₂)₆CO₂Et; (ii) 1. 2M LiOH, THF, 2. 1M HCl; (iii) 1. EDCI HCl, DIPEA, HOBt, CH₂Cl₂; 2. *N*-deacetylmelatonin.

Two series of drug conjugates of melatonin with tamoxifen (compounds **4a−e**)[24,25] and with the histone deacetylase (HDAC) inhibitor vorinostat (compounds **6a−b, 7a−b, 8a−c**)[53] target cancer. Among the vorinostat-melatonin hybrids, the most potent HDAC-inhibitors **6b, 7a** and **8b** showed also the highest potency for reducing viability of MCF-7, PC-3M-Luc and HL-60 cancer cells. As these compounds displayed only weak agonist activity at melatonin MT₁ receptors, their anticancer actions are thought to be driven by HDAC inhibition.

Among the tamoxifen-melatonin hybrid ligands **4a−e**, the pharmacologically best characterized compound is the (CH₂)₅-linked analogue **4b**.[24] In competition binding experiments on ERα expressed in mouse uterus using [¹²⁵I]-estradiol, **4b** showed 5-fold higher affinity toward ERα than tamoxifen (IC₅₀ = 2.2 nM vs 10 nM, respectively). As for binding to melatonin MT₁ receptors, the hybrid ligand **4b** was also superior to the parent drug showing 3-fold higher affinity in [¹²⁵I]-iodomelatonin competition binding experiments at human MT₁ receptors expressed in CHO cells (2.8 nM vs 8.6 nM, respectively).[24] The reported higher MT₁-affinity of compound **4b** compared to the parent drug is rather surprising taking into account that according to well-established structure−activity relationships, alkyl substituents larger than propyl attached to the amide carbonyl group of melatonin reduce binding affinity to melatonin receptors.[54,55]

■ RESULTS AND DISCUSSION

Ligand Design. In the tamoxifen-melatonin hybrids **4a−e**, melatonin is attached to tamoxifen through an amide linkage of melatonin's side chain. Here, we examine a new series of tamoxifen-melatonin hybrids with linkers attached to the ether oxygen of melatonin. The ether-linked hybrids could possibly benefit from increased affinity toward melatonin MT₁ receptors as replacement of melatonin's methoxy group with bulkier alkoxy substituents is well tolerated.[56] Indeed, the crystal structures of the melatonin MT₁[57] and MT₂[58] receptors, albeit representing inactive conformations, revealed the presence of a lateral channel in the C5-binding region of melatonin that is wider in MT₁ allowing an easier accommodation of an alkyl chain attached to the ether oxygen.[59] The linkers have been chosen based on the data reported for the series of tamoxifen-gefitinib hybrids with the (CH₂)₅- and (CH₂)₆-linked analogues **2a** and **2b** (Figure 1) displaying the highest affinity and antagonist potency toward ERα. Moreover, the (CH₂)₄ and (CH₂)₅-linked melatonin-tamoxifen drug conjugates **4a** and **4b** have shown the most favorable pharmacological profile.[25] Consequently, in our *O*-linked melatonin-tamoxifen hybrids **19a−c** (Scheme 3), the parent drugs are connected by 4−6 methylene units. In an extended series of (CH₂)ₙ-linked hybrids (n = 4−6) **16a−c** (Scheme 2), an additional propylene unit is attached to the ether oxygen of melatonin. Moreover, a new melatonin side chain-linked analogue **11** (Scheme 1) with a (CH₂)₆-moiety,

Scheme 2. Synthesis of drug conjugates 16a−c[a]

[a]Reagents and conditions: (i) 1. NaH, DMF, 2. Br(CH$_2$)$_n$CO$_2$R; (ii) 1. 2M LiOH, THF, 2. 1M HCl; (iii) Cs$_2$CO$_3$, KI, MeCN, BocNH(CH$_2$)$_3$Br; (iv) Me$_3$SiBr, MeCN; (v) 1. EDCI HCl, DIPEA, HOBt, CH$_2$Cl$_2$.

and a polyethylene glycol (PEG)- linked hybrid ligand 21 (Scheme 4) have been included in our studies to extend SAR.

Synthesis. The synthetic approaches toward the novel conjugates 11, 16a−c, 19a−c, and 21 are shown in Schemes 1, 2, 3 and 4, respectively. Briefly, the amide side chain-linked hybrid 11 was prepared from nortamoxifen by N-alkylation using Br(CH$_2$)$_6$CO$_2$Et, followed by ester hydrolysis and final amide formation using N-deacetylmelatonin.[60] The ether-linked conjugates 16a-c were synthesized by amidation of the corresponding acid derivatives of tamoxifen (13a, 13b and 10) with O-(3-aminopropyl)-O-demethylmelatonin 15. As for the compounds 19a-c, the melatonin-derived acids 18a-c were coupled with nortamoxifen. Finally, the ether-linked compound 21 with three PEG units in the spacer was prepared by O-alkylation of O-demethylmelatonin[61] using the intermediate 20 that was synthesized from nortamoxifen by N-mono-alkylation using I-(CH$_2$)$_2$O(CH$_2$)$_2$O(CH$_2$)$_2$−I.

Pharmacological Characterization. The melatonin-tamoxifen drug conjugates were first evaluated for their antagonist action at ERα as their ability to antagonize the effect of 17β-estradiol in a luciferase reporter assay.[62,63] Tamoxifen and the previously reported conjugates 4a and 4b (Figure 1) were also screened as control compounds. Briefly, C3A cells were double transfected with plasmids expressing the human ERα and ERE2-TATA-luciferase (estrogen response elements) and then treated with 10 nM 17β-estradiol and the hybrid compounds. The estradiol-activated ERα binds to the ERE2 element leading to the luciferase expression. The results are shown in Table 1, the dose−response curves in Figure S1.

All tamoxifen-derived compounds retain the antagonist effect of the parent drug with variable potencies. The (CH$_2$)$_5$-linked analogues 4b and 19b show the highest antagonist activity and are equipotent to tamoxifen with IC$_{50}$ values ∼50 nM. Interestingly, while both tamoxifen and hybrid ligand 4b possess a basic amino group, the latter is incorporated into an amide moiety in compound 19b indicating that a basic nitrogen is not necessary for antiestrogenic action. Truncation or elongation of the linker

Scheme 3. Synthesis of drug conjugates 19a–c[a]

17a n = 4 R = Me
17b n = 5 R = Me
17c n = 6 R = Et

18a n = 4
18b n = 5
18c n = 6

19a n = 4
19b n = 5
19c n = 6
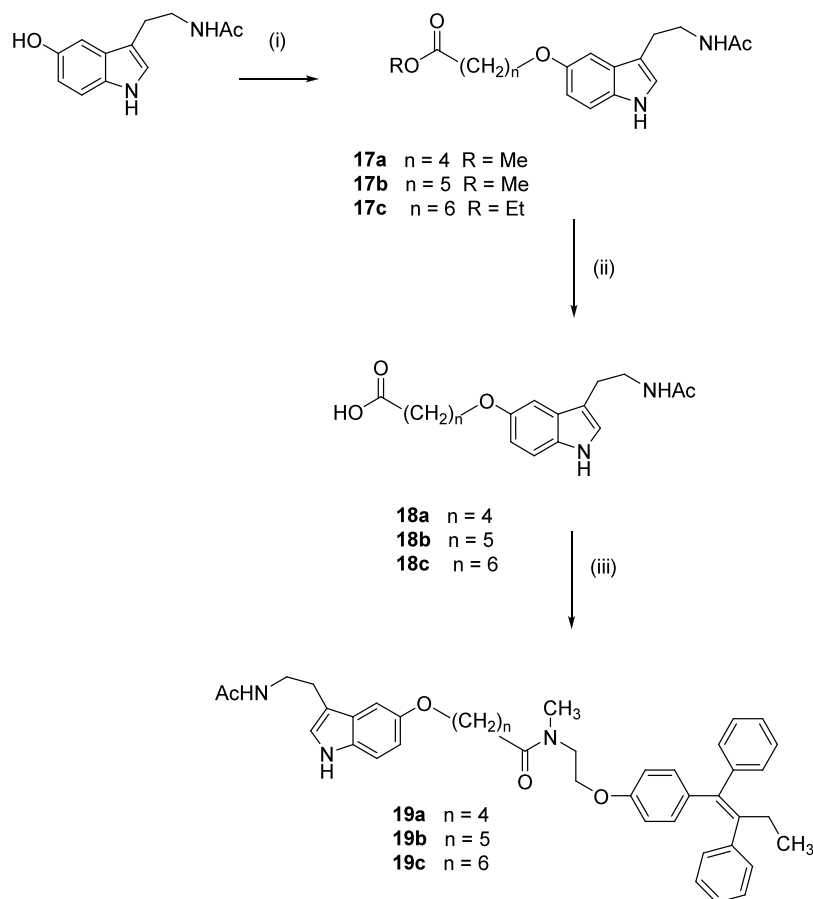
[a]Reagents and conditions: (i) 1. Cs_2CO_3, KI, MeCN, $Br(CH_2)_nCO_2R$; (ii) 1. 2M LiOH, THF, 2. 1M HCl; (iii) 1. EDCI HCl, DIPEA, HOBt, CH_2Cl_2, 2. nortamoxifen.

by one (CH_2)-group in both **4b** and **19b** resulted in an average 2-fold reduction of their antagonist activity with IC_{50} values ~100 nM for the respective compounds **4a, 11, 19a** and **19c**. A further elongation of the linker by a $(CH_2)_3$-chain attached to melatonin's ether oxygen present in conjugates **16a–c** is detrimental for antagonist action. Among these compounds, **16b** with a $(CH_2)_5$-chain is, again, the most potent ERα-antagonist (EC_{50} = 141 nM). As for the PEG-linked analogue **21**, its linker is also longer than in the most active compounds **4b** and **19b** leading to reduced antagonist potency (IC_{50} = 195 nM).

As discussed in the Introduction section, many anticancer actions of melatonin are believed to be mediated by MT_1 receptors. To examine whether our drug conjugates activate MT_1 receptors, they were screened for their binding affinity and agonist potency in radioligand displacement experiments using 2-[^{125}I]-melatonin[64] and in functional assays on HEK293 cells expressing human MT_1 receptors.[65] The results are shown in Table 2.

The hybrid ligand with the highest MT_1-affinity is the *O*-$(CH_2)_6$-linked analogue **19c** showing an impressive single digit nanomolar binding constant K_i = 2.8 nM. Shortening of the spacer by one or two methylene units (**19b** and **19a**) caused a dramatic, > 1,000-fold drop in binding affinity (Table 2). The findings indicate that $(CH_2)_4$- and $(CH_2)_5$-linkers supposedly accommodated in the lateral channel of the MT_1 receptor are not long enough to allow the tamoxifen unit to protrude from

the other side of the channel into the lipid-bilayer exposed receptor region (see binding mode of a bitopic ligand with two agomelatine units connected by an ethoxyethane spacer[57]). On the contrary, elongation of the spacer by a $(CH_2)_3$-chain attached to melatonin's ether oxygen present in hybrid ligands **16a–c** is well-tolerated with the best analogue **16c** (K_i = 3.1 nM) displaying a binding affinity comparable to that of **19c**. Among the melatonin side chain-linked analogues **4a, 4b** and **11**, the highest-affinity compound is again the $(CH_2)_6$-linked hybrid **11** (K_i = 88 nM).

The functional effects of melatonin-tamoxifen hybrids were then evaluated on different signaling pathways associated with MT_1 receptors. In the cAMP accumulation assay, all hybrids, excepted compound **21**, behave as full agonists as they decreased forskolin-stimulated cAMP production at a similar extend as melatonin (Figure S2B). Surprisingly, EC_{50} values for compounds **16c** and **19c** with the highest affinity are right-shifted by 2 logs compared to their K_i values (Table 2). Of note, for melatonin K_i and EC_{50} values are in the same concentration range. This observation might hint toward a biased effect of compounds **16c** and **19c**.[66,67] To verify this hypothesis, we evaluated the effects of the hybrid compounds on ß-arrestin recruitment and ERK activation in cell expressing MT_1. The pEC_{50} values for ß-arrestin recruitment were right-shifted compared to the pK_i values similar to the cAMP assay (Figure S2C and Table 2). In contrast, pEC_{50} values for ERK phosphorylation were more similar to pKi values. Compound

Scheme 4. Synthesis of Drug Conjugate 21[a]

Nortamoxifen

20

21
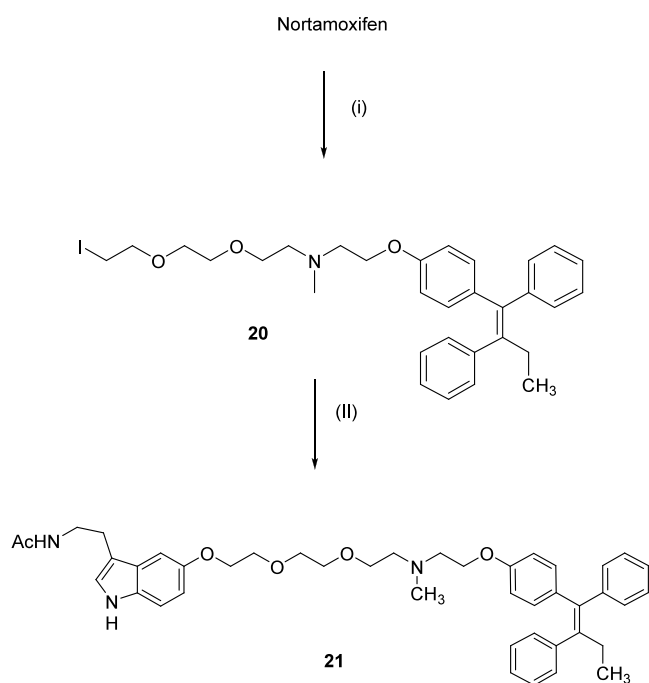
[a]Reagents and conditions: (i) Cs_2CO_3, CH_3CN, 1,2-bis(2-iodoethoxy) ethane, rt, 24 h; (ii) O-demethylmelatonin, Cs_2CO_3, CH_3CN, 24 h, rt.

Table 1. Human Estrogen Receptor α Antagonist Activity Determined in a Luciferase Reporter Assay

compound	IC$_{50}$ [nM][a]	CI 95% [nM][b]
tamoxifen	48.9	26.9−88.7
4a	83.2	40.4−171.5
4b	52.1	27.5−98.9
11	109.0	59.8−198.5
16a	1610	603.1−4300
16b	141.1	65.5−303.8
16c[c]	863.9	515.5−1448
19a	117.9	71.2−195.3
19b	54.8	36.2−83.0
19c	139.9	52.5−372.8
21[c]	194.8	90.2−420.8

[a]Inhibition of receptor activation relative to 10 nM 17β-estradiol (E2) corresponding to 100% activation. Values represent the mean of three independent experiments (SEM < 15%). [b]Upper and lower limits of the confidence interval for IC$_{50}$ values. [c]Also showing agonistic activity in the luciferase reporter assay; at 100 nM concentration, **16c** up to 6.7-fold, **21** up to 4.6-fold over vehicle control.

19a has no effect in both assays and compound **21** has a clear partial effect for ß-arrestin recruitment (Figure S2C). Moreover, several compounds **16a−c** and **4a** show a bias for ERK1/2 signaling over inhibition of cAMP production.

The hybrid ligands were further screened for their inhibitory effects on the viability of different cancer cell lines including ERα-positive breast cancer cells (MCF-7), ERα-negative breast cancer cells (MDA-MB-231), and the fibrosarcoma cell line HT-1080 (see Supporting Information, Table S1 for cell line characteristics). Among these cancer cell lines, luminal breast cancer cells (MCF-7) show high expression of melatonin MT$_1$ receptors while triple-negative breast cancer (TNBC) cell line

MDA-MB-231 manifests low MT$_1$-expression level.[45] The IC$_{50}$-values determined by MTT assay are compiled in Table 3, additional statistical evaluations in Supporting Information, Tables S2−S4 and dose−response curves in Figure S3.

Melatonin and tamoxifen were reported to affect survival of luminal breast cancer cell lines. Sensitivities to tamoxifen in luminal breast cancer cells can be explained by expression of ERα and ERß,[68] in TNBC cells by ERß and even in other cell types by the multiple off-target effects of this drug, i.e. ER-independent mechanisms of action.[69,70] Of note, MCF-7 cells showed a higher IC$_{50}$ value for tamoxifen treatment compared to the other lines, which can be possibly explained by the fact that MCF-7 cells are devoid of caspase 3 expression[71] and therefore less sensitive to apoptosis induction. In support of such resistance mechanisms in MCF-7, examining the tamoxifen response of the as well ERα- and ERß-positive, but caspase 3 expressing luminal breast cancer cell line T47D, revealed an IC$_{50}$-value of 3.1 μM, i.e. below the other values (Table S5, Supporting Information). As for the effect of melatonin, comparatively high sensitivity was observed in MCF-7 cells reflecting elevated expression levels of melatonin MT$_1$ receptors in this cell type.[45] Accordingly, the luminal breast cancer line MCF-7 and the TNBC line MDA-MB-231 are interesting breast cancer models to screen sensitization by combined tamoxifen and melatonin treatments or dual inhibitory drug treatments.

Combined treatments were performed with the breast cancer cell lines MCF-7 and MDA-MB-231 in comparison with the nonbreast cancer cell line HT-1080, and resulted in a similar IC$_{50}$-value as for tamoxifen in MCF-7 cells and intermediate IC$_{50}$ values in the other cancer cell lines. When comparing IC$_{50}$ values for combined melatonin and tamoxifen treatments with dual inhibitory drug treatments of MCF-7, MDA-MB-231, and HT-1080, we found that cells can all be sensitized by at least one dual inhibitory drug. Thus, MCF-7 cells can be sensitized by **4a** (3.5-fold) and **16a** (2.6-fold) with statistical significance ($p < 0.05$), MDA-MB-231 by **4a** (4.8-fold), **11** (6.6-fold), **16c** (5.5-fold), and **19a** (3.0-fold), and HT-1080 by **4a** (3.1-fold), **11** (3.8-fold), **16c** (12.6-fold), and **21** (3.6-fold). However, in MDA-MB-231 cells the IC$_{50}$ values for **4a**, **11**, **16c**, and **19a** were comparable to tamoxifen treatments. As for HT-1080 cells, the IC$_{50}$ values for **4a**, **11**, and **21** are also similar to that of tamoxifen.

Most importantly, synergistic effects of dual inhibitory molecules that go beyond single as well as combined treatments with melatonin and tamoxifen were indeed observable with the luminal MCF-7 cell line engaging **4a** or **16a** as well as with HT-1080 cells when applying **16c**. From this, we conclude that these dual inhibitory drugs may indeed serve to break resistance mechanisms such as in MCF-7 characterized by dampened apoptosis.

Interestingly, the strongest inhibitors of cancer cell viability are not the most potent compounds at both targets simultaneously. For example, compound **16c** with low micromolar activity in all three cancer cell lines (IC$_{50}$ = 2−7 μM) shows indeed high affinity (K_i = 3.1 nM) and good agonist potency (EC$_{50}$ = 98 nM) in the ERK assay at melatonin MT$_1$ receptors, while its antagonist action at ERα is one of the lowest in the whole series (IC$_{50}$ = 863 nM). On the contrary, compound **4a** with an anticancer profile similar to that of **16c** is a potent ERα-antagonist (IC$_{50}$ = 83 nM) and a low-affinity weak MT$_1$-agonist (K_i = 690 nM, EC$_{50}$ >10 μM in cAMP and β-arrestin recruitment assays). Interestingly, several

Table 2. Affinity and Potency of Melatonin-Tamoxifen Hybrid Compounds on the hMT₁ Receptor Stably Expressed in HEK293 Cells[a]

	hMT$_1$			
	2-[^{125I}]MLT binding assay	cAMP accumulation	ß-arrestin recruitment	ERK activation
	K_i(nM)	EC$_{50}$(nM)	EC$_{50}$(nM)	EC$_{50}$(nM)
Compound	(pK_i± S.E.M.)	pEC$_{50}$ ± S.E.M.	pEC$_{50}$ ± S.E.M.	pEC$_{50}$ ± S.E.M.
MLT	0.33	0.64	1.18	0.11
	(9.45 ± 0.10)	(10.3 ± 0.06)	(8.97 ± 0.05)	(10.1 ± 0.07)
4a	690	>10 000	>10 000	253
	(6.23 ± 0.27)	(5.60 ± 0.45)	(5.01 ± 0.06)	(6.66 ± 0.24)
4b	1856	761	>10 000	807
	(5.79 ± 0.29)	(6.13 ± 0.08)	(4.16 ± 0.61)	(6.42 ± 0.38)
11	88	198	3939	308
	(7.07 ± 0.13)	(6.77 ± 0.16)	(5.44 ± 0.08)	(7.19 ± 0.77)
16a	438	>10 000	>10 000	247
	(6.39 ± 0.18)	(5.12 ± 0.22)	(5.17 ± 0.22)	(6.88 ± 0.26)
16b	22	987	999	16
	(7.70 ± 0.23)	(6.06 ± 0.16)	(6.02 ± 0.06)	(7.86 ± 0.11)
16c	3.1	223	914	98
	(8.54 ± 0.19)	(6.68 ± 0.11)	(6.25 ± 0.19)	(7.28 ± 0.35)
19a	>10 000	>10 000	9141	−
	(>5)	(5.05 ± 0.28)	(6.01 ± 0.65)	(−)
19b	3147	389	4664	178
	(5.53 ± 0.19)	(6.43 ± 0.10)	(5.41 ± 0.12)	(6.92 ± 0.23)
19c	2.8	223	2234	161
	(8.58 ± 0.19)	(6.68 ± 0.11)	(5.97 ± 0.28)	(7.21 ± 0.22)
21	6.5	74.3	197	36
	(8.21 ± 0.15)	(7.20 ± 0.19)	(6.86 ± 0.18)	7.55 ± 0.20

[a]pK_i and pEC$_{50}$ values are calculated from the concentration-response curves (supporting information, Figure S2).

compounds with high affinity for MT$_1$ seem to be biased toward the ERK1/2 pathway (i.e., **4a**, **16a−c**) while showing a lower potency for the cAMP pathway. Activation of the ERK1/2 pathway likely participates in the effect of these compounds on cell proliferation. These findings indicate that simultaneous high activity at both targets is not solely responsible for anticancer actions of our drug conjugates and other cellular effects might be involved. The latter conclusion is supported by the fact that, at most cancer cell lines, **4a** and **16c** are more potent than a 1:1 combination of the parent drugs, although the parent drugs show considerably higher activity at the respective target.

CONCLUSIONS

A series of drug conjugates connecting the neurohormone melatonin with tamoxifen using melatonin's side chain (**4a**, **4b**, **11**) and its ether oxygen (**16a−c**, **19a−c**, **21**) as attachment points for the linker were synthesized and pharmacologically evaluated for inhibition of radioligand binding and cAMP accumulation at melatonin MT$_1$ receptors, for antagonist action at estrogen receptor alpha and for inhibition of cell viability at three types of cancer cells MCF-7, MDA-MB-231 and HT-1080. All hybrid ligands are antagonists of estrogen receptor alpha and agonists of melatonin MT$_1$ receptor with variable potencies. Most importantly, several drug conjugates including the (CH$_2$)$_4$-linked analogues **4a** and **16a** and the (CH$_2$)$_6$-linked compound **16c** showed higher potency to inhibit cell viability than melatonin, tamoxifen and combination of both drugs at least in one cancer cell line indicating a synergistic dual-inhibitory anticancer action.

EXPERIMENTAL SECTION

Chemistry. *General Remarks.* All starting materials, reagents, and solvents (technical and HPLC grade) were purchased from Sigma-Aldrich, Schnelldorf, Germany. Dry solvents used in synthesis were distilled over a molecular sieve with a 4 Å pore size. Only distilled water was used during synthesis or workup. Millipore water was used for HPLC and LC/MS runs. Analytical thin layer chromatography (TLC) was used for reaction monitoring on silica gel 60 F254 (Merck, Darmstadt, Germany) or basic ALOX UV$_{254}$ (Machery-Nagel, Düren, Deutschland) glass plates. Non-UV-active compounds were viewed in an I$_2$ chamber or by means of a suitable staining reagent. For silica gel column chromatography, silica gel 60 (SiO$_2$, 0.063−0.2 mm) was purchased from Merck (Darmstadt, Deutschland). For basic ALOX column chromatography, CHROMABOND Alox B was purchased from Machery-Nagel (Düren, Deutschland) (high purity basic aluminum oxide, pore volume: 0.90 mL/g, particle size: 60−150 μm, pH: 9.5 ± 0.5). The mass spectra of all final compounds were acquired on a Shimadzu LC/MS-2020 instrument (Hilden, Germany) using Electrospray ionization (ESI), DGU-20A3R degassing unit, an LC20AB liquid chromatograph, and an SPDA-20A UV−vis detector. The ^{1}H (400.13 MHz) and ^{13}C (100.61 MHz) NMR spectra were recorded on a Bruker Avance 400 Ultra Shield spectrometer (Bruker Biospin, Ettlingen, Germany). Residual undeuterated solvent signals were used as reference (^{1}H, ^{13}C): DMSO-d_6 at 2.50 ppm, 39.5 ppm; CD$_3$OD = 3.31 ppm, 49.0 ppm; CDCl$_3$ = 7.26 ppm, 77.2 ppm. Coupling constants (*J*-values) are given in Hertz. All signals of the final compounds were confirmed using two-dimensional NMR experiments (HH−COSY and

Table 3. Sensitivity of Cancer Cell Lines to Single, Combined, and Dual Inhibitory Drug Treatments Determined by the MTT Assay[a]

	IC$_{50}$[μM](p value for IC$_{50}$compound versus IC$_{50}$MLT+TAM)		
Compound	MCF-7	MDA-MB-231	HT-1080
MLT	27.9	n.a.	n.a.
	(0.0059)		
TAM	9.5	4.1	4.5
	(0.5090)	(0.0002)	(<0.0001)
MLT+TAM	7.3	35.0	21.4
4a	2.1	7.3	6.9
	(0.0243)	(0.0037)	(0.0062)
4b	4.6	15.8	14.6
	(0.4372)	(0.0711)	(0.2493)
11	12.4	5.3	5.7
	(0.1821)	(0.0001)	(0.0004)
16a	2.8	n.a.	n.a.
	(0.0489)		
16b	n.a.	31.2	14.8
		(0.8347)	(0.2465)
16c	6.8	6.4	1.7
	(0.8272)	(0.0045)	(<0.0001)
19a	13.0	11.6	26.3
	(0.1499)	(0.0045)	(0.6658)
19c	55.0	n.a.	n.a.
	(0.0982)		
21	21.3	27.6	5.9
	(0.0387)	(0.6716)	(0.0006)

[a]Values were calculated from the combined viability data obtained in at least two independent experiments with duplicates each. MLT, melatonin; TAM, tamoxifen; IC$_{50}$, inhibitory concentration 50; n.a. = not applicable, when IC$_{50}$ remained undefined, i.e. > 100μM, in at least one experiment.

HMQC). The multiplicities of the resonance signal are represented with the following symbols: s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quintet, and m = multiplet. The purity of the final compounds was determined on an analytical Shimadzu HPLC-MS instrument (Hilden, Germany) equipped with a DGU-20A3R degassing unit, an LC20AB liquid chromatograph, and an SPD20A UV/vis detector. The stationary phase was a Synergi fusion-RP (150 × 4.6 mm, 4 μm) column (Phenomenex, Aschaffenburg, Germany). The following gradient elution was conducted: solvent A: water with 0.1% formic acid, solvent B: MeOH with 0.1% formic acid. Solvent A from 0% to 100% in 13 min, then 100% A maintained for 5 min, followed by a decrease of A from 100% to 5% in 1 min, and finally maintaining 5% of A for 4 min. The flow rate was adjusted to 1.0 mL/min and injection volume to 5 μL. UV detection was performed at 254 nm. ^{1}H NMR and ^{13}C NMR spectra and HPLC traces for all drug conjugates are shown in the Supporting Information, Figures S4 and S5, respectively.

Ethyl (Z)-7-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)-ethyl)-(methyl)amino)heptanoate (9). An ice cooled suspension of N-demethyltamoxifen (450 mg, 1.26 mmol) in dry DMF (20 mL) under argon was treated with NaH (60% dispersion in mineral oil) (36.3 mg, 1.51 mmol). After stirring for 15 min, ethyl 6-bromohexanoate (0.29 mL, 1.51 mmol) was added dropwise and then the reaction was allowed to stir until the TLC indicated full consumption of the N-

demethyltamoxifen (~24h). Upon completion, the reaction was quenched with brine (15 mL) and extracted using EtOAc (3 × 15 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, evaporated under reduced pressure, and purified using column chromatography on silica gel (eluent: EtOAc/MeOH 10:2) to yield 64% (412 mg) of **9** as colorless oil. LC-MS (ESI)*m/z*: 514.55 [M + H]$^+$; HPLC t$_{ret}$ = 9.448 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.92 (t, J = 7.4 Hz, 3H), 1.22−1.36 (m, 2H), 1.61 (dt, J = 14.8, 7.4 Hz, 2H), 1.48 (dt, J = 14.4, 7.3 Hz, 2H), 2.22−2.32 (m, 2H), 2.37−2.49 (m, 2H), 2.75 (t, J = 5.9 Hz, 2H), 3.95 (t, J = 5.9 Hz, 2H), 4.11 (q, J = 7.1 Hz, 2H), 6.54 (d, J = 8.7 Hz, 2H), 6.76 (d, J = 8.7 Hz, 2H), 7.09−7.19 (m, 5H), 7.23−7.28 (m, 3H), 7.32−7.36 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.72, 14.40, 25.04, 26.81, 27.20, 29.16, 34.43, 42.76, 56.01, 58.02, 60.32, 65.67, 113.51, 126.14, 126.65, 128.00, 128.23, 129.60, 129.84, 131.99, 135.73, 138.39, 141.48, 142.56, 143.96, 156.80, 173.93.

(Z)-7-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino)heptanoic acid (10). To a cooled solution of the intermediate **9** (412 mg, 0.802 mmol) in THF (20 mL), 2 M LiOH aqueous solution was added dropwise. The mixture was left to stir for 24 h. until TLC indicated complete conversion. The reaction mixture was allowed to cool to room temperature and was then neutralized using 1 M aqueous HCl solution until reaching pH 6. The product was extracted using DCM (3 × 15 mL) and the combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to afford **10** in almost quantitative yield of 98% (380 mg) as a yellow oil. The crude product, confirmed by LC-MS , was directly used in the next step without any further purification. LC−MS (ESI) *m/z*: 486.60 [M + H]$^+$; HPLC t$_{ret}$ = 9.124 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.85 (t, J = 7.4 Hz, 3H), 1.17−1.29 (m, 2H), 1.43−1.58 (m, 2H), 2.14 (t, J = 7.0 Hz, 2H), 2.32−2.40 (m, 2H), 2.42 (s, 3H), 2.55−2.68 (m, 2H), 2.97 (t, J = 4.5 Hz, 2H), 4.01 (t, J = 5.1 Hz, 2H), 6.44 (d, J = 8.6 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 7.01−7.12 (m, 5H), 7.14−7.22 (m, 3H), 7.24−7.29 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.71, 25.47, 25.58, 27.07, 29.14, 29.18, 35.76, 54.78, 57.14, 63.92, 40.95, 113.48, 126.22, 126.70, 128.04, 128.26, 129.59, 129.83, 132.09, 136.22, 138.25, 141.69, 142.49, 143.86, 156.14, 173.86.

(Z)-N-(3-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-propyl)-7-((2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino) heptanamide (11). To a stirred solution of the crude acid **10** (92 mg, 0.189 mmol) dissolved in dry DCM (15 mL) under an argon atmosphere. DIPEA (0.097 mL, 0.57 mmol), EDCI HCl (43.5 mg, 0.227 mmol) and HOBt (30.69 mg, 0.227 mmol) were added portionwise, and the reaction was left to stir for 10−15 min, followed by dropwise addition of a solution of N-deacetylmelatonin (36 mg, 0.189 mmol) in dry DCM (3 mL) and stirring at room temperature was continued until TLC indicated full consumption of the reacting amine (24 h). A saturated aqueous solution of NH$_4$Cl (20 mL) was added, and the aqueous layer was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with saturated aqueous NaHCO$_3$ solution and brine (20 mL each), dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. Purification of the crude residue was carried out via silica gel column chromatography (DCM/MeOH,10:1) to yield 92% (115 mg) of **11** as a yellow oil. LC−MS (ESI) *m/z*: 658.35 [M + H]$^+$; HPLC t$_{ret}$ = 12.801 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.92 (t, J = 7.4 Hz, 3H), 1.18−1.29 (m, 4H), 1.35−1.47 (m, 2H), 1.51−1.64 (m, 2H),

2.08 (t, J = 7.5 Hz, 2H), 2.28 (s, 3H), 2.35−2.39 (m, 2H), 2.46 (q, J = 7.4 Hz, 2H), 2.71 (t, J = 5.9 Hz, 2H), 2.93 (t, J = 6.6 Hz, 2H), 3.59 (q, J = 6.4 Hz, 2H), 3.85 (s, 3H), 3.93 (t, J = 5.9 Hz, 2H), 5.53 (t, J = 5.2 Hz, 1H), 6.54 (d, J = 8.7 Hz, 2H), 6.77 (d, J = 8.7 Hz, 2H), 6.85 (dd, J = 8.8, 2.2 Hz, 1H), 6.98 (d, J = 2.0 Hz, 1H), 7.03 (d, J = 2.2 Hz, 1H), 7.08−7.19 (m, 5H), 7.22−7.28 (m, 4H), 7.32−7.36 (m, 2H), 8.41 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.70, 25.41, 25.76, 29.14, 27.10, 27.15, 36.86, 39.53, 42.89, 56.07, 56.25, 58.20, 65.77, 100.60, 112.19, 112.45, 112.62, 113.50, 123.09, 126.13, 126.64, 127.82, 127.98, 128.22, 129.57, 129.81, 131.75, 131.97, 135.72, 138.34, 141.49, 142.53, 143.92, 154.14, 156.81, 173.17.

General Procedure for the Synthesis of 12a−b. Under argon, an indicated amount of NaH (60% dispersion in mineral oil; 1.2 equiv) was added to a stirred solution of N-demethyltamoxifen (a specified amount) in dry DMF (ca. 0.1 M) at 0 °C. Stirring was continued for 15 min, and the respective alkylating agent Br(CH$_2$)$_n$CO$_2$R (1.2 equiv; n = 4, 5, 6; R = CH$_3$, C$_2$H$_5$) was added dropwise to the reaction mixture. The cooling bath was removed and stirring was continued for 24 h. Water (30 mL) was added, and the mixture was extracted with EtOAc (4 × 20 mL). The combined organic layers were washed with water (7 × 30 mL), dried over sodium sulfate and the solvent was removed under vacuum. The crude products were purified using column chromatography on silica gel (eluent: EtOAc/MeOH, 10:2).

Methyl (Z)-5-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)-ethyl)-(methyl)amino)pentanoate (12a). **12a** (380 mg, 67.4%) was obtained from 433 mg (1.211 mmol) of N-desmethyltamoxifen, 34.9 mg (1.45 mmol) of 60% NaH, and 0.21 mL (1.45 mmol) of Br(CH$_2$)$_4$CO$_2$Me as a colorless oil. LC−MS (ESI) m/z: 473 [M + H]$^+$; HPLC t$_{ret}$ = 8.417 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.94 (t, 3H, J = 7.4 Hz), 1.59−1.68 (m, 2H), 1.47−1.56 (m, 2H), 2.31 (s, 3H), 2.32−2.35 (m, 2H), 2.42−2.51 (m, 4H), 2.75 (t, 2H, J = 5.9 Hz), 3.66 (s, 3H), 3.95 (t, 2H, J = 5.9 Hz), 6.52−6.58 (m, 2H), 6.75−6.81 (m, 2H), 7.11−7.21 (m, 5H), 7.23−7.30 (m, 3H), 7.32−7.38 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.70, 22.86, 26.62, 29.12, 33.97, 42.83, 51.59, 56.14, 57.67, 65.78, 113.50, 126.12, 126.63, 127.98, 128.21, 129.58, 129.81, 131.97, 135.70, 138.37, 141.45, 142.54, 143.93, 156.79, 174.08.

Methyl (Z)-6-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)-ethyl)-(methyl)amino)hexanoate (12b). **12b** (140 mg, 68%) was obtained from 152 mg (0.425 mmol) of N-demethyltamoxifen, 12.2 mg (0.51 mmol) of 60% NaH, and 0.08 mL (0.51 mmol) of Br(CH$_2$)$_5$CO$_2$CH$_3$ as a colorless oil. LC−MS (ESI) m/z: 486.55 [M + H]$^+$; HPLC t$_{ret}$ = 8.783 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.85 (t, J = 7.4 Hz, 3H), 1.52−1.61 (m, 6H), 2.21−2.24 (m, 2H), 2.25 (s, 3H), 2.34−2.43 (m, 4H), 2.70 (t, J = 5.8 Hz, 2H), 3.57 (s, 3H), 3.89 (t, J = 5.9 Hz, 2H), 6.44−6.49 (m, 2H), 6.67−6.71 (m, 2H), 7.02−7.12 (m, 5H), 7.14−7.21 (m, 3H), 7.24−7.29 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.68, 24.93, 26.50, 27.00, 29.11, 34.35, 42.60, 51.57, 55.90, 57.77, 65.49, 113.47, 126.12, 126.62, 127.97, 128.20, 129.56, 129.80, 131.97, 135.76, 138.34, 141.47, 142.52, 143.91, 156.69, 173.76.

General Procedure for the Synthesis of 13a−b. An aqueous LiOH solution (2M, 10 mL) was dropwise added to a solution of the specified amount of the corresponding esters **12a−b** in THF (25 mL). After stirring for 24 h, the solution was acidified using an aqueous solution (1M) HCl under ice−water cooling. The product was extracted with DCM (3 × 20 mL), the organic phases were combined, and

dried over anhydrous sodium sulfate and the solvent was evaporated under vacuum to give the crude acid that was pure enough to be used in the next step without further purification.

(Z)-5-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino)pentanoic acid (13a). **13a** (276 mg, 97%) was obtained from **12a** (292 mg, 0.619 mmol), as a yellow oil. LC−MS (ESI) m/z: 458.15 [M + H]$^+$; HPLC t$_{ret}$ = 9.276 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.85 (t, J = 7.4 Hz, 3H), 1.49−1.51 (m, 2H), 1.58−1.69 (m, 2H), 2.17 (t, J = 6.3 Hz, 2H), 2.38 (q, J = 7.4 Hz, 2H), 2.59 (s, 3H), 2.81−2.88 (m, 2H), 3.13−3.21 (m, 2H), 4.04−4.13 (m, 2H), 6.44 (d, J = 8.7 Hz, 2H), 6.71 (d, J = 8.7 Hz, 2H), 7.01−7.12 (m, 5H), 7.13−7.21 (m, 3H), 7.23−7.29 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.55, 22.59, 23.94, 29.04, 34.85, 40.47, 54.29, 56.37, 62.81, 113.39, 126.15, 126.60, 127.93, 128.15, 129.40, 129.64, 131.99, 136.48, 137.96, 141.75, 142.25, 143.61, 155.48, 177.57.

(Z)-6-((2-(4-(1,2-Diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino)hexanoic acid (13b). **13b** (114 mg, 97%) was obtained from **12b** (121 mg, 0.249 mmol), as a yellow oil. LC−MS (ESI) m/z: 472.15 [M + H]$^+$; HPLC t$_{ret}$ = 9.343 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.84 (t, J = 7.4 Hz, 3H), 1.22−1.30 (m, 2H), 1.46−1.57 (m, 2H), 1.57−1.70 (m, 2H), 2.16 (t, J = 7.2 Hz, 2H), 2.37 (q, J = 7.4 Hz, 2H), 2.60 (s, 3H), 2.78−2.88 (m, 2H), 3.11−3.23 (m, 2H), 4.05−4.17 (m, 2H), 6.44 (d, J = 8.8 Hz, 2H), 6.71 (d, J = 8.8 Hz, 2H), 7.01−7.11 (m, 5H), 7.13−7.20 (m, 3H), 7.23−7.30 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.64, 24.05, 24.77, 26.35, 29.14, 34.97, 40.67, 54.46, 56.48, 63.07, 113.47, 126.24, 126.70, 128.02, 128.25, 129.52, 129.76, 132.12, 136.62, 138.05, 141.89, 142.35, 143.71, 155.58, 177.77.

tert-Butyl (3-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-propyl) carbamate (14). A mixture of O-demethylmelatonin (100 mg, 0.458 mmol), CsCO$_3$ (298.6 mg, 0.916 mmol), 3-(Boc-amino) propyl bromide (163.66 mg, 0.687 mmol), and a catalytic amount of KI (15 mg) in dry MeCN (25 mL) was heated under reflux for 12 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure and the residue was purified using column chromatography on silica gel (eluate DCM/MeOH, 10:0.75)to yield 55% (94 mg) of **14** as brown oil. LC−MS (ESI) m/z: 376.10 [M + H]$^+$; HPLC t$_{ret}$ = 8.597 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.48 (s, 9H), 1.95 (s, 3H), 2.00 (dd, J = 12.4, 6.1 Hz, 2H), 2.94 (t, J = 6.8 Hz, 2H), 3.36−3.39 (m, 2H), 3.58 (dd, J = 12.8, 6.6 Hz, 2H), 4.08 (t, J = 6.0 Hz, 2H), 4.97 (s, 1H), 5.78 (s, 1H), 6.86 (dd, J = 8.6, 2.2 Hz, 1H), 7.02 (d, J = 2.0 Hz, 1H), 7.06 (d, J = 2.2 Hz, 1H), 7.29 (d, J = 8.6 Hz, 1H), 8.44 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 23.46, 25.42, 28.55, 29.80, 38.35, 39.89, 66.97, 101.89, 112.15 112.64, 112.87, 123.07, 127.85, 131.88, 153.19, 156.25, 170.33.

N-(2-(5-(3-Aminopropoxy)-1H-indol-3-yl)ethyl)acetamide (15). (CH$_3$)$_3$SiBr (0.105 mL, 0.799 mmol) was added dropwise under ice cooling to a solution of **14** (100 mg, 0.266 mmol) in dry CH$_3$CN (3 mL). After stirring for 1 h. at room temperature,MeOH (15 mL) was added to the reaction and the resulting mixture was concentrated in vacuo to yield 91% (67 mg) of the corresponding amine that was used for the next step without any further purifications. LC−MS (ESI) m/z: 276.30 [M + H]$^+$; HPLC t$_{ret}$ = 5.170 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.95 (s, 3H), 2.17−2.04 (m, 2H), 2.92 (t, J = 7.3 Hz, 2H), 3.09 (t, J = 7.1 Hz, 2H), 3.47 (t, J = 7.4 Hz, 2H), 4.15 (t, J = 5.9 Hz, 2H), 6.81 (dd, J = 8.7, 2.2 Hz, 1H), 7.07 (s, 1H), 7.14 (d, J = 2.2 Hz, 1H), 7.25 (d, J = 8.7 Hz, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 22.65, 26.31, 30.49, 39.37, 41.48,

67.57, 102.75, 112.92, 113.01, 113.08, 124.32, 129.13, 133.64, 153.84, 173.28.

General Procedure for the Synthesis of Compounds 16a–c. The specified amount of the crude acid (**10**, **13a**,-**13b**) was dissolved in dry DMF (40 mL) under an argon atmosphere. A specified amount of DIPEA, HOBt, and EDCI HCl was added under ice−water cooling and the mixture was stirred for 15 min followed by a dropwise addition of a solution of **15** (a specified amount) in dry DMF (5 mL). Cooling was removed and the reaction mixture was stirred for 24 h. The solvent was removed under vacuum and the residue was subjected to column chromatography using DCM/MeOH (10:1) to yield the hybrid ligands.

(Z)-N-(3-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-propyl)-5-((2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino) pentanamide (16a). **16a** (51 mg, 54%) was obtained as a yellow oil according to the general procedure using acid **13a** (60 mg, 0.131 mmol), DIPEA (0.067 mL, 0.39 mmol), HOBt (21.3 mg, 0.157 mmol), EDCI HCl (30.16 mg, 0.157 mmol) and **15** (36.06 mg, 0.131 mmol). LC−MS (ESI) m/z: 715.25 $[M + H]^+$; HPLC t_{ret} = 9.191 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.94 (t, J = 7.4 Hz, 3H), 1.44−1.55 (m, 2H), 1.69−1.58 (m, 2H), 1.93 (s, 3H), 2.01 (dt, J = 12.3, 6.1 Hz, 2H), 2.19 (t, J = 7.3 Hz, 2H), 2.29 (s, 3H), 2.38−2.52 (m, 4H), 2.73 (t, J = 5.7 Hz, 2H), 2.93 (t, J = 6.9 Hz, 2H), 3.44−3.50 (m, 2H), 3.57 (dd, J = 12.9, 6.7 Hz, 2H), 3.92 (t, J = 5.8 Hz, 2H), 4.09 (t, J = 5.8 Hz, 2H), 5.76 (s, 1H), 6.24 (s, 1H), 6.53 (d, J = 8.6 Hz, 2H), 6.78 (d, J = 8.6 Hz, 2H), 6.84 (dd, J = 8.7, 2.2 Hz, 1H), 6.99 (d, J = 2.0 Hz, 1H), 7.06 (d, J = 2.2 Hz, 1H), 7.10−7.20 (m, 5H), 7.23−7.30 (m, 4H), 7.34−7.38 (m, 2H), 8.20 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.70, 23.51, 23.69, 25.52, 26.50, 29.14, 29.18, 36.54, 37.64, 39.93, 42.66, 56.15, 57.68, 65.54, 67.53, 101.93, 112.21, 112.77, 112.86, 113.48 123.06, 126.16, 126.66, 127.92, 128.00, 128.24, 129.57, 129.82, 131.86, 131.99, 135.82, 138.31, 141.57, 142.54, 143.91, 153.07, 156.68, 170.27, 173.08.

(Z)-N-(3-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-propyl)-6-((2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino)hexanamide (16b). **16b** (73 mg, 58%) was obtained as a yellow oil according to the general procedure using acid **13b** (82 mg, 0.174 mmol), DIPEA (0.089 mL, 0.52 mmol), HOBt (28.19 mg, 0.209 mmol), EDCI HCl (40 mg, 0.209 mmol) and **15** (47.81 mg, 0.174 mmol). LC−MS (ESI) m/z: 729.35 $[M + H]^+$; HPLC t_{ret} = 9.202 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.86 (t, J = 7.4 Hz, 3H), 1.37−1.20 (m, 2H), 1.56−1.66 (m, 4H), 1.88 (s, 3H), 1.94 (p, J = 6.4 Hz, 2H), 2.18 (t, J = 7.2 Hz, 2H), 2.40 (q, J = 7.4 Hz, 2H), 2.75 (s, 3H), 2.79−2.93 (m, 4H), 3.33−3.46 (m, 6H), 3.99 (t, J = 6.1 Hz, 2H), 4.08−4.11 (m, 2H), 6.57−6.62 (m, 2H), 6.76−6.81 (m, 2H), 6.73 (dd, J = 8.7, 2.2 Hz, 1H), 7.00 (s, 1H), 7.02−7.14 (m, 6H), 7.15−7.26 (m, 4H), 7.27−7.34 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.80, 22.68, 24.68, 26.18, 26.28, 26.81, 29.88, 30.27, 36.52, 37.66, 41.20, 41.49, 55.84, 57.56, 62.78, 67.38, 102.50, 112.98, 113.08, 113.21, 114.55, 124.29, 127.23, 127.78, 128.95, 129.10, 129.25, 130.38, 130.85, 133.11, 133.43, 138.16, 139.60, 143.15, 143.61, 144.86, 154.14, 156.98, 173.28, 175.79.

(Z)-N-(3-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-propyl)-7-((2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-(methyl)amino) heptanamide (16c). **16c** (59 mg, 58%) was obtained as a yellow oil according to the general procedure using acid **10** (67 mg, 0.138 mmol), DIPEA (0.071 mL, 0.414 mmol), HOBt (22.37 mg, 0.166 mmol), EDCI HCl (31.73 mg,

0.166 mmol) and **15** (37.99 mg, 0.138 mmol). LC−MS (ESI) m/z: 743.30 $[M + H]^+$; HPLC t_{ret} = 9.205 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.85 (t, J = 7.4 Hz, 3H), 1.85 (s, 3H), 1.16−1.27 (m, 4H), 1.35 (dt, J = 14.6, 7.4 Hz, 2H), 1.53 (dt, J = 15.1, 7.6 Hz, 2H), 1.90−1.99 (m, 2H), 2.04−2.11 (m, 2H), 2.18 (s, 3H), 2.26−2.32 (m, 2H), 2.38 (q, J = 7.4 Hz, 2H), 2.61 (t, J = 6.0 Hz, 2H), 2.85 (t, J = 6.9 Hz, 2H), 3.41 (dd, J = 12.3, 6.2 Hz, 2H), 3.49 (dd, J = 12.9, 6.8 Hz, 2H), 3.83 (t, J = 6.0 Hz, 2H), 4.03 (t, J = 5.9 Hz, 2H), 5.58 (s, 1H), 5.96 (s, 1H), 6.43−6.49 (m, 2H), 6.67−6.71 (m, 2H), 6.76 (dd, J = 8.8, 2.3 Hz, 1H), 6.92 (d, J = 2.2 Hz, 1H), 6.99 (d, J = 2.3 Hz, 1H), 7.01−7.12 (m, 5H), 7.14−7.21 (m, 4H), 7.24−7.29 (m, 2H), 8.02 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 13.72, 23.55, 25.54, 25.85, 27.09, 27.28, 29.15, 29.16, 29.33, 36.94, 37.74, 39.93, 42.92, 56.25, 58.26, 65.86, 67.69, 101.97, 112.22, 112.89, 112.96, 113.52, 123.05, 126.15, 126.66, 127.96, 128.00, 128.24, 129.60, 129.84, 131.87, 131.98, 135.71, 138.37, 141.51, 142.57, 143.96, 153.12, 156.87, 170.22, 173.27.

General Procedure for the Synthesis of Compounds 17a–c. A mixture of O-demethylmelatonin (1 equiv), Cs$_2$CO$_3$ (2 equiv), the respective alkylating agent (1.5 equiv), and a catalytic amount of KI (10 mg) in dry MeCN (20 mL) was heated under reflux under argon atmosphere for 8 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure and the residue was purified using column chromatography on silica gel (eluent DCM/MeOH,10:0.5).

Methyl 5-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy) pentanoate (17a). **17a** (180 mg, 79%) was obtained as a colorless oil according to the general procedure using O-demethylme-latonin (150 mg, 0.687 mmol), Cs$_2$CO$_3$ (448 mg, 1.37 mmol), and Br(CH$_2$)$_4$CO$_2$Me (0.15 mL, 1.03 mmol). The spectral data of **17a** are in accordance with those previously reported.[25]

Methyl 6-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-hexanoate (17b). **17b** (260 mg, 82%) was obtained as a colorless oil according to the general procedure using O-demethylmelatonin (200 mg, 0.916 mmol), Cs$_2$CO$_3$ (597 mg, 1.83 mmol), and Br(CH$_2$)$_5$CO$_2$Me (0.218 mL, 1.37 mmol). LC−MS (ESI) m/z: 347.20 $[M + H]^+$; HPLC t_{ret} = 11.047 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.92 (s, 3H), 1.48−1.58 (m, 2H), 1.76−1.68 (m, 2H), 1.78−1.86 (m, 2H), 2.36 (t, J = 7.5 Hz, 2H), 2.93 (t, J = 6.7 Hz, 2H), 3.58 (dd, J = 12.7, 6.5 Hz, 2H), 3.67 (s, 3H), 4.00 (t, J = 6.4 Hz, 2H), 5.58 (s, 1H), 6.86 (dd, J = 8.8, 2.2 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 7.02 (d, J = 2.2 Hz, 1H), 7.30−7.18 (m, 1H), 8.08 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 23.54, 24.89, 25.43, 25.91, 29.30, 34.17, 39.86, 51.63, 68.71, 101.85, 112.06, 112.83, 113.09, 122.90, 127.92, 131.74, 153.60, 170.19, 174.30.

Ethyl 7-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-heptanoate (17c). **17c** (200 mg, 83%) was obtained as a colorless oil according to the general procedure using O-demethylmelatonin (140 mg, 0.641 mmol), Cs$_2$CO$_3$ (418 mg, 1.28 mmol) and Br(CH$_2$)$_6$CO$_2$CH$_2$CH$_3$ (0.19 mL, 0.962 mmol). LC−MS (ESI) m/z: 375.35 $[M + H]^+$; HPLC t_{ret} = 9.410 min. ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.25 (t, J = 7.1 Hz, 3H), 1.35−1.44 (m, 2H), 1.45−1.55 (m, 2H), 1.63−1.70 (m, 2H), 1.74−1.86 (m, 2H), 1.92 (s, 3H), 2.31 (t, J = 7.5 Hz, 2H), 2.92 (t, J = 6.7 Hz, 2H), 3.57 (q, J = 6.6 Hz, 2H), 3.99 (t, J = 6.5 Hz, 2H), 4.12 (q, J = 7.1 Hz, 2H), 5.65 (s, 1H), 6.85 (dd, J = 8.8, 2.3 Hz, 1H), 6.99 (d, J = 2.3 Hz, 1H), 7.02 (d, J = 2.3 Hz, 1H), 7.21−7.25 (m, 1H), 8.22 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 14.36, 23.49, 25.03, 25.40, 25.95, 29.04, 29.43,

34.43, 39.86, 60.33, 68.89, 101.81, 112.06, 112.70, 113.03, 122.92, 127.89, 131.73, 153.59, 170.24, 173.99.

General Procedure for the Synthesis of Compounds 18a–c. An aqueous LiOH solution (2N, 10 mL, 0.83 mmol) was dropwise added to a solution of the specified amount of the corresponding esters **17a–c** in THF (30 mL). The reaction mixture was stirred at room temperature for 24 h. and then concentrated under reduced pressure to give a residue which was taken up by H_2O (20 mL), cooled to 0 °C, and acidified with 2 N HCl. The precipitate was filtered, washed out with H_2O, and dried under a vacuum. The filtrate was extracted with DCM and the organic phases were combined, washed with H_2O, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was combined with the dried precipitate and used directly for the next step without further purification.

5-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)pentanoic Acid (18a). **18a** (170 mg, 99%) was obtained according to the general procedure using **17a** (180 mg, 0.542 mmol), as a colorless oil. The spectral data of **18a** are in accordance with those previously reported.[25]

6-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)hexanoic Acid (18b). **18b** (245 mg, 98%) was obtained according to the general procedure using **17b** (260 mg, 0.751 mmol), as a colorless oil. LC–MS (ESI) *m/z*: 333.15 $[M + H]^+$; HPLC t_{ret} = 10.185 min. ^1H NMR (400 MHz, $CDCl_3$) δ 1.80 (s, 3H), 1.39–1.50 (m, 2H), 1.54–1.61 (m, 2H), 1.69–1.74 (m, 2H), 2.24 (t, *J* = 7.3 Hz, 2H), 2.76 (t, *J* = 7.4 Hz, 2H), 3.26–3.33 (m, 2H), 3.94 (t, *J* = 6.4 Hz, 2H), 6.70 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.00 (d, *J* = 2.2 Hz, 1H), 7.08 (d, *J* = 1.9 Hz, 1H), 7.20 (d, *J* = 8.7 Hz, 1H), 7.90 (t, *J* = 5.2 Hz, 1H), 10.60 (s, 1H), 11.97 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.68, 24.34, 25.24, 25.30, 28.73, 33.66, 38.89, 67.84, 101.27, 111.45, 111.88, 111.63, 123.21, 127.57, 131.40, 152.25, 168.96, 174.44.

7-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)heptanoic Acid (18c). **18c** (183 mg, 99%) was obtained according to the general procedure using **17c** (200 mg, 0.534 mmol), as a colorless oil. LC–MS (ESI) *m/z*: 347.30 $[M + H]^+$; HPLC t_{ret} = 8.538 min. ^1H NMR (400 MHz, $CDCl_3$) δ 1.81 (s, 3H), 1.31–1.38 (m, 2H), 1.40–1.48 (m, 2H), 1.49–1.61 (m, 2H), 1.64–1.77 (m, 2H), 2.22 (t, *J* = 7.3 Hz, 2H), 2.76 (t, *J* = 7.4 Hz, 2H), 3.23–3.37 (m, 2H), 3.94 (t, *J* = 6.4 Hz, 2H), 6.71 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.01 (d, *J* = 2.1 Hz, 1H), 7.09 (s, 1H), 7.21 (d, *J* = 8.7 Hz, 1H), 7.91 (t, *J* = 5.3 Hz, 1H), 10.60 (s, 1H), 11.96 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.70, 24.48, 25.41, 25.26, 28.38, 28.88, 33.60, 38.89, 67.89, 101.28, 111.49, 111.66, 111.91, 123.23, 127.60, 131.42, 152.30, 169.02, 174.45.

General Procedure for the Synthesis of Drug Conjugates 19a–c. The specified amount of the respective crude acid (**18a–c**) was dissolved in dry DMF (40 mL) under an argon atmosphere. Specified amounts of DIPEA, HOBt, and EDCI HCl were added under ice–water cooling and the mixture was stirred for 15 min followed by a dropwise addition of a solution of *N*-demethyltamoxifen (a specified amount) in dry DMF (5 mL). Cooling was removed and the reaction mixture was stirred for 24 h. The solvent was removed under vacuum and the residue was subjected to column chromatography ($CHCl_3$/ $MeOH/NH_3$, 20:1:0.1) to yield the hybrid ligand.

(Z)-5-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-N-(2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-N-methylpenta-namide (19a–c). **19a** (35 mg, 34%) was obtained according to the general procedure using acid **18a** (50 mg, 0.157 mmol),

DIPEA (0.08 mL, 0.47 mmol), HOBt (25.46 mg, 0.188 mmol), EDCI HCl (36.1 mg, 0.188 mmol) and *N*-demethyltamoxifen (56.2 mg, 0.157 mmol), as a yellow oil. LC–MS (ESI) *m/z*: 658.25 $[M + H]^+$; HPLC t_{ret} = 11.026 min. ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, *J* = 7.3 Hz, 3H), 1.91–1.75 (m, 4H), 1.97 (s, 3H), 2.89–2.99 (m, 2H), 2.38–2.48 (m, 4H), 3.08 (s, 3H), 3.46–3.72 (m, 4H), 3.87–4.13 (m, 4H), 6.46–6.52 (m, 2H), 6.74–6.79 (m, 2H), 6.80–6.84 (m, 1H), 6.96–7.03 (m, 1H), 7.07–7.20 (m, 5H), 7.21–7.29 (m, 4H), 7.30–7.38 (m, 2H), 8.07 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.69, 21.86, 23.26, 25.36, 29.16, 29.21, 33.25, 37.61, 40.03, 48.08, 66.52, 68.69, 101.91, 112.10, 112.67, 113.07, 113.38, 122.98, 126.21, 126.67, 127.89, 128.00, 128.25, 129.57, 129.82, 131.74, 132.04, 135.88, 138.30, 141.59, 142.55, 143.89, 153.48, 156.61, 170.61, 173.29.

(Z)-6-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-N-(2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-N-methylhexa-namide (19b). **19b** (45 mg, 37%) was obtained according to the general procedure using acid **18b** (60 mg, 0.181 mmol), DIPEA (0.09 mL, 0.54 mmol), HOBt (29.3 mg, 0.217 mmol), EDCI HCl (34.6 mg, 0.217 mmol) and *N*-demethyltamoxifen (64.53 mg, 0.181 mmol), as a yellow oil. LC–MS (ESI) *m/z*: 672.25 $[M + H]^+$; HPLC t_{ret} = 11.084 min. ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, *J* = 7.4 Hz, 3H), 1.45–1.57 (m, 2H), 1.65–1.76 (m, 2H), 1.77–1.88 (m, 2H), 1.92 (s, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.39–2.48 (m, 2H), 2.86–2.98 (m, 2H), 3.07 (s, 3H), 3.52–3.69 (m, 2H), 3.93 (t, *J* = 5.4 Hz, 2H), 3.99 (t, *J* = 6.1 Hz, 2H), 5.65 (s, 1H), 6.48–6.54 (m, 2H), 6.75–6.80 (m, 2H), 6.82–6.87 (m, 1H), 6.98–7.03 (m, 2H), 7.07–7.19 (m, 5H), 7.20–7.29 (m, 4H), 7.31–7.34 (m, 2H), 8.13 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.69, 23.52, 24.84, 25.44, 26.12, 29.15, 29.43, 33.57, 37.58, 39.85, 48.03, 68.77, 66.57, 101.85, 112.05, 112.79, 113.05, 113.38, 122.91, 126.17, 126.66, 127.91, 127.99, 128.23, 128.57, 129.82, 131.72, 132.03, 135.85, 138.31, 141.57, 142.55, 143.89, 153.57, 156.64, 170.18, 173.29.

(Z)-7-((3-(2-Acetamidoethyl)-1H-indol-5-yl)oxy)-N-(2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)-N-methylhepta-namide (19c). **19c** (50 mg, 42%) was obtained according to the general procedure using acid **18c** (60 mg, 0.173 mmol), DIPEA (0.09 mL, 0.52 mmol), HOBt (28.1 mg, 0.208 mmol), EDCI HCl (39.84 mg, 0.208 mmol) and *N*-demethyltamoxifen (61.9 mg, 0.173 mmol), as a yellow oil. LC–MS (ESI) *m/z*: 686.30 $[M + H]^+$; HPLC t_{ret} = 11.183 min. ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, *J* = 7.4 Hz, 3H), 1.39–1.44 (m, 2H), 1.47–1.55 (m, 2H), 1.58–1.73 (m, 2H), 1.86–1.75 (m, 2H), 1.92 (s, 3H), 2.28 (t, *J* = 7.6 Hz, 2H), 2.42–2.49 (m, 2H), 2.98–2.84 (m, 2H), 3.06 (s, 3H), 3.55–3.67 (m, 4H), 3.92–4.00 (m, 4H), 5.62 (s, 1H), 6.57–6.47 (m, 2H), 6.81–6.72 (m, 2H), 6.86 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.99–7.02 (m, 2H), 7.09–7.19 (m, 5H), 7.21–7.29 (m, 4H), 7.31–7.38 (m, 2H), 8.06 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.70, 23.54, 25.00, 25.43, 26.10, 29.16, 29.32, 29.46, 33.56, 37.60, 39.85, 48.02, 66.61, 68.91, 101.85, 112.04, 112.84, 113.14, 113.38, 122.88, 126.17, 126.67, 127.92, 128.00, 128.24, 129.57, 129.82, 131.70, 132.04, 135.84, 138.32, 141.57, 142.56, 143.90, 153.65, 156.65, 170.18, 173.41.

(Z)-2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)-N-(2-(2-(2-iodoethoxy)ethoxy)ethyl)-N-methylethan-1-amine (20). A solution of 1,2-bis (2-iodoethoxy) ethane (0.31 mL, 1.68 mmol) was added dropwise to a stirred suspension of N-desmethyltamoxifen (200 mg, 0.559 mmol) and Cs_2CO_3 (364.6 mg, 1.12 mmol) in dry MeCN (20 mL) under argon

atmosphere. The mixture was stirred at room temperature for 24 h. Afterward, the reaction mixture was filtered, the filtrate was evaporated under reduced pressure and the residue was purified using column chromatography (silica gel, $CHCl_3$/CH_3OH, 10:0.5) to yield 66% (220 mg) of **20** as a colorless oil. LC−MS (ESI) m/z: 600.45 $[M + H]^+$; HPLC t_{ret} = 9.628 min. ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, J = 7.4 Hz, 3H), 2.35 (s, 3H), 2.45 (q, J = 7.4 Hz, 2H), 2.68 (t, J = 5.9 Hz, 2H), 2.79 (t, J = 6.0 Hz, 2H), 3.14−3.28 (m, 2H), 3.53−3.66 (m, 6H), 3.66−3.81 (m, 2H), 3.94 (t, J = 6.0 Hz, 2H), 6.49−6.57 (m, 2H), 6.72−6.79 (m, 2H), 7.07−7.20 (m, 5H), 7.21−7.30 (m, 3H), 7.30−7.38 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 3.03, 13.71, 29.13, 43.57, 57.27, 56.69, 65.91, 69.58, 70.35, 70.54, 72.09, 113.50, 126.12, 126.62, 127.98, 128.21, 129.58, 129.82, 131.95, 135.63, 138.38, 141.43, 142.55, 143.94, 156.86.

(Z)-N-(2-(5-(2-(2-(2-((2-(4-(1,2-Diphenylbut-1-en-1-yl)-phenoxy)ethyl)(methyl)amino) ethoxy)ethoxy)ethoxy)-1H-indol-3-yl)ethyl)acetamide (21). O-demethylmelatonin (0.05 mg, 0.23 mmol) and Cs_2CO_3 (149.3 mg, 0.46 mmol) were dissolved in dry CH_3CN (25 mL) under argon. After 15 min, a solution of **20** (151.1 mg, 0.25 mmol) in dry MeCN (5 mL) was added dropwise to the stirred suspension. The mixture was stirred at room temperature for 24 h. Afterward, the reaction mixture was filtered, the filtrate was evaporated under reduced pressure and the residue was purified using column chromatography (silica gel, $CHCl_3$/CH_3OH, 10:1) to yield 25% (41 mg) of **21**. LC−MS (ESI) m/z: 690.75 $[M + H]^+$; HPLC t_{ret} = 9.371 min. ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, J = 7.4 Hz, 3H), 1.91 (s, 3H), 2.42−2.48 (m, 5H), 2.77−3.04 (m, 6H), 3.55 (dd, J = 12.8, 6.6 Hz, 2H), 3.63−3.65 (m, 2H), 3.70−3.72 (m, 4H), 3.80−3.88 (m, 2H), 3.96−4.00 (m, 2H), 4.11−4.19 (m, 2H), 5.68 (s, 1H), 6.43−6.48 (m, 2H), 6.71−6.76 (m, 2H), 6.85 (dd, J = 8.8, 2.2 Hz, 1H), 6.97 (d, J = 1.6 Hz, 1H), 7.06 (d, J = 2.2 Hz, 1H), 7.10−7.21 (m, 5H), 7.22−7.29 (m, 4H), 7.33−7.36 (m, 2H), 7.88 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.71, 23.56, 25.47, 29.17, 39.84, 43.01, 64.78, 68.60, 70.15, 70.58, 70.80, 102.26, 112.10, 112.96, 113.09, 113.51, 122.97, 126.22, 126.69, 127.92, 128.05, 128.27, 129.61, 129.85, 131.84, 132.01, 138.28, 141.64, 142.54, 143.16, 143.93, 153.33, 170.22.

Biological Assays. ERα Luciferase Reporter Assay. Activation assays for human ERα (NR3A1, FL) were performed in C3A cells cultured on 96-well plates in phenol red-free DMEM (Gibco 11,880, Invitrogen), containing 5% charcoal stripped FBS (Biowest S181F), 2 mM ʟ-glutamine (Biowest X0550) and 1% antibiotics (Biowest L0022) at a density of 6.25×10^4 cells/cm². The cells were transfected with 20 ng pCMVβ, 5 ng pSG5-hERα, and 75 ng pGL3-ERE2-TATA-luciferase using calcium phosphate precipitation essentially as described before.[62,63] Four hours after transfection, the medium was replaced with fresh DMEM complemented with 5% charcoal stripped FBS (Biowest S181F) and including DMSO (Sigma D8418, 0.1% v/v) or EtOH (0.1% v/v), positive control 17β-estradiol (E2, Sigma E2758), and the test compounds. After 24 h of exposure, the cells were disrupted in lysis buffer (25 mM glycylglycine, 15 mM MgSO4, 4 mM EGTA, 1% Triton X-100, pH 7.8, supplemented with dithiothreitol and phenylmethyl sulfonyl fluoride) and analyzed for luciferase and β-galactosidase activities using a Victor2 multiplate reader. The raw luciferase activities were normalized to β-galactosidase activities to control for differences in transfection efficiency and cell loss to generate relative luminescence units. Hybrid compounds did not activate the

singly transfected C3A cell-line with only the luciferase plasmid. Relative luminescence units were normalized to the DMSO treated samples and compared with the E2 (10 nM) treated positive control, expressed as activity %. Data are presented as mean ± SEM (at least n = 2), generated from three experimental replicates. Normalized values were fitted to a nonlinear model (four parameters) and relative IC_{50} values were calculated using GraphPad Prism v9.3

2-[125I]-Iodomelatonin Binding Assay. Membranes from HEK293 cells stably expressing human MT_1 were prepared as previously described and used in a radioligand binding assay.[64] Briefly, the competition binding assay was performed by simultaneous incubation of 2-[125I]-melatonin (100 pM) and increasing concentration of melatonin or compounds. The assay was carried out in 120 min at 37 °C followed by a rapid filtration through GF/C Glass fiber filters (Whatman). Filter-retained radioactivity was determined with a gamma-counter LB2111 (Berthold Technologies). K_i values were calculated from IC_{50} values using the Cheng & Prussof formula: $K_i = IC50/[1 + (L/K_d)]$ where L represents the ligand concentration and K_d the dissociation constant. K_d values were 367 pM for MT_1 receptors. IC_{50} values were calculated from GraphPad Prism software by fitting data on a nonlinear regression analysis.

Accumulative cAMP Assay. The cyclic AMP assay was performed as previously described.[72] Briefly, stable HEK293 cells expressing human MT1 were dispensed into a 384-well plate (4000 cells per well) and stimulated with 1 μM forskolin alone or in the presence of increasing concentrations of melatonin or compound, incubated for 30 min at room temperature in stimulation buffer supplemented with 1 mM IBMX (Sigma-Aldrich). Cells were then lysed and, cAMP content was determined with the cAMP-Gi kit following the supplier's instruction (PerkinElmer/Cisbio). The plate was read using the Infinite F500 Tecan microplate reader. Data were fitted by nonlinear regression to determine EC50 and Emax values using GraphPad Prism software.

β-Arrestin2 Recruitment Assay. β-arrestin2 recruitment was measured by BRET in HEK293 cells that transiently coexpressed MT_1-Rluc8 and YPF-β-arrestin2-YFP. BRET assay was performed as previously described.[65] Briefly, cells are washed with PBS and 10 μL of the cell-permeant substrate for *Renilla* luciferase (Rluc), coelenterazine h, are added to each well at room temperature, in order to have a final 5 μM concentration. Ligands are added 5 min after the addition of the Rluc substrate, and plate reading was performed after other 5 min. BRET signal was determined by calculating the ratio of the light emitted at 535 ± 20 nm over the light emitted at 485 ± 20 nm, using a Mithras LB940 instrument (Berthold, Bad Wildbad, Germany) and filters with the appropriate band-pass. Data were fitted by nonlinear regression using GraphPad Prism software.

ERK Activation Measurement. Intracellular phospho-ERK1/2 was measured on HEK293T cells stably expressing human MT_1 receptor using the AlphaLISA Surefire pERK kit (ALSU-PERK-A500, PerkinElmer, Waltham, MA) as previously described.[73] Briefly, cells were starved overnight before being stimulated with increasing concentrations of ligands for 5 min at 37 °C 5%CO2 to generate full concentration−response curves. Cellular lysates were generated by adding the lysis buffer and 4 μL of each cellular lysates used for the assay according to the supplier instructions. The signal was detected using the Tecan Infinite M1000 PRO microplate reader

(Tecan Group, Ltd., Männedorf, Switzerland) with excitation at 680 nm (α-laser) and emission at 520−620 nm. Data were analyzed using GraphPad Prism software (La Jolla, CA, USA) and fitted by nonlinear regression to determine the EC_{50} values.

Cell Lines. MCF-7 (provided by American Type Culture Collection, ATCC, Manassas, Virginia, USA), MDA-MB-231 and HT-1080 (provided by University Clinic Ulm, Germany) were cultured in DMEM with L-glutamine (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), 10% FBS (Pan Biotech, Aidenbach, Germany), 1.2% L-glutamine (Gibco/Thermo Fisher Scientific), 1.0% Penicillin-Streptomycin-Glutamine (100x) (Gibco/Thermo Fisher Scientific), 1.0% MEM NEAA (nonessential amino acid) (Gibco/Thermo Fisher Scientific). Cells were cultured in a humid 5% CO_2 incubator at 37 °C. Cell lines were authenticated independently by Microsynth AG, Balgach, Switzerland, and Cell Line Services (CLS) GmbH, Eppelheim, Germany. Cell cultures were regularly examined for mycoplasma contamination by PCR.

Determination of Survival. Cell survival was determined by MTT assay (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich/Merck, St. Louis, MO, USA), as described previously.[74] First, the cells were seeded in 96-well plates at a densitiy of 2×10^4 cells per well. The cells were then treated on day two, four and seven with melatonin (Sigma-Aldrich/Merck, St. Louis, MO, USA), tamoxifen, **4a, 4b, 11, 16a−c, 19a, 19c** or **21** at 12 different treatment concentrations increasing up to 5 mM in fresh culture medium each. At the end of treatment, on day nine, the MTT assay was performed. First, the MTT was dissolved in 1x PBS (Gibco/Thermo Fisher Scientific) to an end concentration of 5 mg/mL. This MTT solution was diluted in DMEM 1× without phenol red (Gibco/Thermo Fisher Scientific) to a final concentration of 1 mg/mL. 100 μL of this solution were added to each well and then incubated for 2.5 h at 37 °C. MTT was removed after the incubation time and the cells were resuspended in 200 μL 5% HCl/95% isopropanol (Sigma-Aldrich/Merck). The plates were shaken for 10 min and the optical densitiy measured at 570 nm with a Tecan Sunrise Photometer (Tecan, Crailsheim, Germany). The MTT experiments were conducted in duplicates and repeated once each. The IC50 value was calculated for each cell line.

Graphic presentation of cell viability curves was performed using GraphPadPrism version 9 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using extra sum of-squares F-test, nonlinear fit. Statistical significances ($p < 0.05$) of differences between mean IC_{50} values for unpaired, nonparametric data were first determined via Kruskal−Wallis test followed by a two-tailed Mann−Whitney-U test in case of statistical significance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c08881.

> ^{1}H NMR and ^{13}C NMR spectra for novel drug conjugates, LC-MS traces and ESI mass spectra for novel drug conjugates, dose−response curves for all biological assays, characteristics of cancer cell lines, statistical comparison between IC_{50}-values for treatments on various cancer cells, sensitivity of T47D cells to melatonin, tamoxifen and combined treatment. (PDF)

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Notes

The authors declare no competing financial interest.

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