

RESEARCH ARTICLE



Cite this: *RSC Med. Chem.*, 2025, 16, 4239

New melatonin biphenyl-linked scaffold targeting colorectal cancer: design, synthesis, biological, and ADME-Tox modelling studies†

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A series of melatonin biphenyl-linked conjugates was designed and synthesized using a simple, cost-effective, and environmentally friendly method. All the new compounds were evaluated for their cytotoxic or cytostatic activity against SW480 human colorectal adenocarcinoma cells. Screening at 100 μM revealed that most compounds exhibited high activity ($\geq 60\%$ inhibition), with compounds **3b**, **3h**, **4f**, **4g**, and **4i-l** also demonstrating subtle lethality. Based on these initial results, a subset of the most active hybrids was selected for further in-depth evaluation to calculate three key parameters of cell viability: GI_{50} , TGI, and LC_{50} values. The results showed that most compounds, except **3c** and **4d**, significantly outperformed the parental compound (**2** and melatonin) in inhibiting cancer cell proliferation, highlighting the efficacy of hybridization in improving cytotoxic potential. Besides, it is noticeable that hybrids **4f-l** exhibited superior activity compared to 5-FU, as evidenced by lower GI_{50} values. Although hybrids **4f** and **4g** seemed to exert the greatest activity as demonstrated in the LC_{50} values ($70.89 \pm 11.72 \mu\text{M}$ and $68.03 \pm 0.46 \mu\text{M}$, respectively), we observed that only hybrids **4j** and **4l** showed significant selectivity, as revealed by higher GI_{50} concentrations over non-malignant cells (NCM460). The observed total growth inhibition and lack of LC_{50} values in **4j** and **4l** suggest their potential for a cytostatic effect. Lastly, theoretical evaluations of drug-likeness, pharmacokinetic behaviour, and toxicological parameters suggest that the most promising hybrids, compounds **4j** and **4l**, exhibit strong potential for advancement into preclinical studies. Our findings highlight the effectiveness of a novel melatonin biphenyl-linked scaffold, with **4j** and **4l** structures in particular serving as prototypes for future innovative adjuvant drugs.

Received 10th May 2025,
Accepted 17th June 2025

DOI: 10.1039/d5md00410a

rsc.li/medchem

1. Introduction

Although colorectal cancer (CRC) is largely preventable through lifestyle modifications and early detection, it remains a significant global health challenge. In 2023, more than 53 010 individuals died from CRC, making it the second most prevalent cancer worldwide.¹ Key preventable risk factors, such as smoking, obesity, unhealthy diets, excessive alcohol consumption, and physical inactivity, contribute to the rising incidence of CRC, which has been steadily increasing since the mid-1980s.² Despite the potential for prevention, the high rates of CRC diagnoses and related deaths highlight the limitations of current public health strategies. Treatment for CRC typically involves a combination of surgery, chemotherapy, and radiation therapy, with chemotherapy

remaining the preferred option for more advanced stages, while surgery is commonly used for early-stage disease. However, therapy-resistant and severe side effects of long-term anticancer therapy have limited the use of traditional chemotherapeutics to treat colorectal cancer (CRC). As a result, there is an urgent need to accelerate the development of safer, more effective therapies to treat CRC.

In this context, melatonin (MLT, *N*-acetyl-5-methoxy-triptamine) has been extensively investigated as a potential treatment for colorectal cancer (CRC) due to its probable anticancer activity and its ability to do so without causing harm.^{3–5} It is a naturally occurring hormone produced and secreted by the pineal glands of vertebrates in response to changes in the light–dark cycle. Numerous preclinical and clinical investigations into innovative chemotherapeutics have demonstrated their capacity to inhibit the invasion and migration of cancerous cells in both the early and late stages of the disease.⁶ As a result of these efforts, many drugs based on MLT have been licensed by the Food and Drug Administration (FDA) for use in oncological interventions (Fig. 1B). To treat metastatic prostate cancer and ovarian

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d5md00410a>

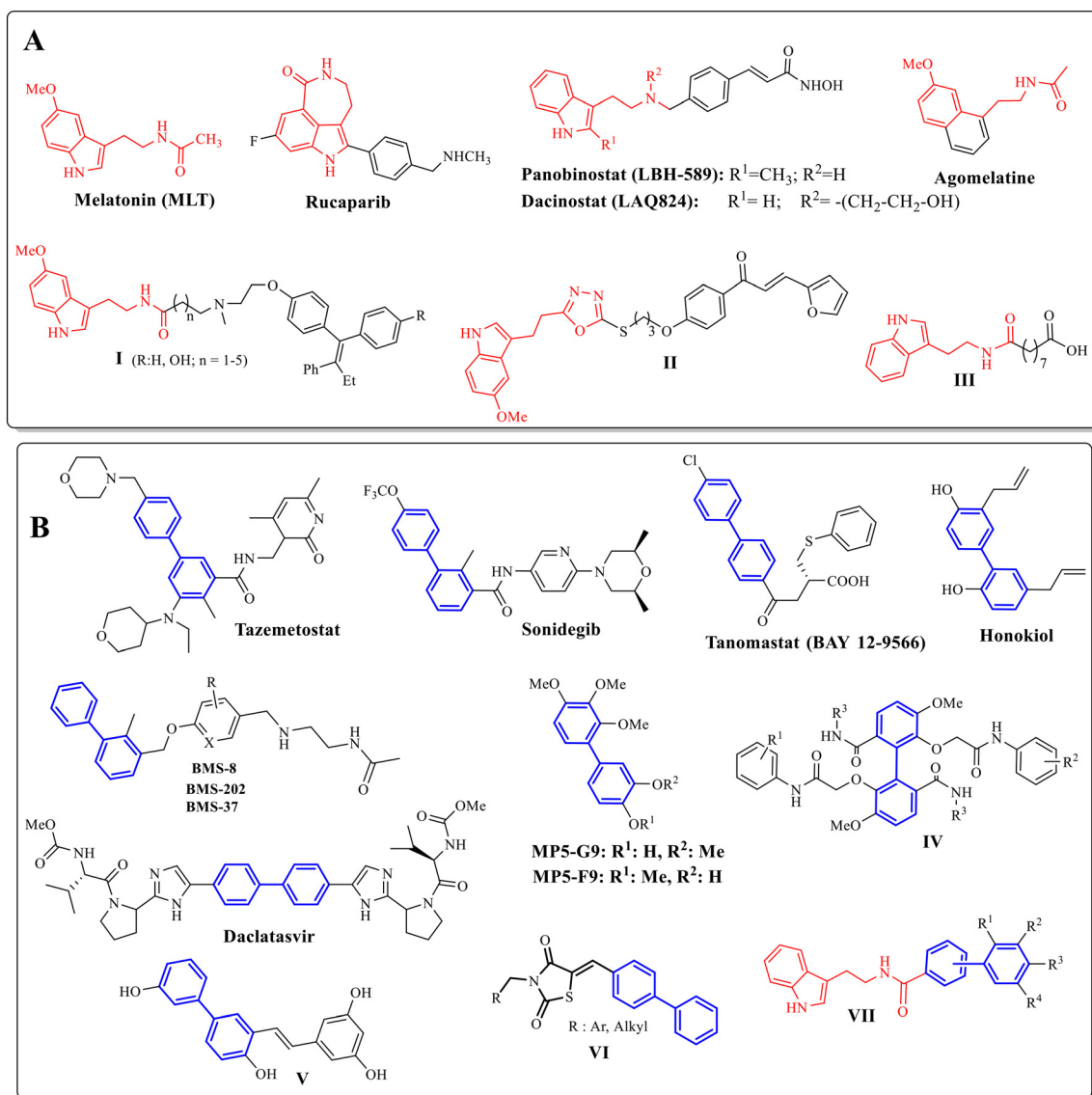


Fig. 1 Structures of effective anti-tumoral compounds bearing A) a portion of MLT (in red). B) the biphenyl framework (in blue).

carcinomas that have returned, for example, Rucaparib (Rubraca®), a conformationally restricted melatonin analog, was licensed in 2020. Dacinostat and panobinostat have recently been approved as chemotherapeutic agents for the treatment of prostate/breast cancer and multiple myeloma, respectively. Furthermore, agomelatine, a naphthalenic bioisostere of melatonin (MLT), is an FDA-approved antidepressant that has recently been proposed as a potential option for the treatment of colorectal cancer.⁷

MLT could have great potential as an anticancer drug, however, its short half-life and poor oral bioavailability (about 3–33%) due to its rapid metabolic inactivation (over 80% of melatonin is eliminated in the urine) limit its therapeutic use in both adults and children.^{8–11} Accordingly, several strategies have been optimized to enhance MLT's pharmacokinetic characteristics. For instance, novel MLT-based hybrids, derivatives, and bioisosteres have been

designed (Fig. 1A), using MLT as a foundational template for potential cancer therapies, particularly colorectal cancer. Notably, these synthesized compounds, exhibiting micromolar to picomolar potencies, have shown significant antiproliferative and cytotoxic effects against various common cancer types, often surpassing the efficacy of MLT itself. Thus, for example, MLT was associated with the FDA-approved antitumoral drug tamoxifen given the conjugates I, which in a mouse model exhibits strong anticancer action (2.2 nM to 3.0 pM).¹² A combination of oxadiazole MLT-bioisostere and chalcone II has shown a strong antiproliferative action against human colon cancer cells with an IC_{50} value around 260 nM.¹³ In addition, according to a different study, the MLT-derivative III had an IC_{50} of 6.0 nM and showed important cytotoxic activity against the human colorectal cancer HT29 cell line.¹⁴ As mentioned above, the rapid advancement in understanding the anticancer

properties of MLT, or compounds containing MLT, highlights its structure as a promising framework for designing new therapeutic anticancer candidates.

The biphenyl framework is another molecular scaffold that has attracted significant attention due to its potential anticancer properties (Fig. 1B). In medicinal chemistry, biphenyl is found in around 4.3% of all approved medications and is well-known for its great selectivity in targeting various proteins. Regarding cancer therapy, the biphenyl scaffold is found in the FDA-approved Tazemetostat, Sonidegib, and Daclatasvir, which are currently prescribed for use in patients with basal cell carcinomas and advanced solid epithelioid carcinomas. Importantly, both Tazemetostat and Sonidegib have demonstrated considerable antitumor activity in phase I/II studies against metastatic colorectal cancer cells.^{15,16} In addition, it was reported that compounds containing a biphenyl moiety have exhibited great potential as anticancer drug candidates. It is interesting to mention a phase III randomized trial of Tanomastat (BAY 12-9566) with a biphenyl matrix metalloprotease (MMP) inhibitor that revealed its strong antiangiogenic and antimetastatic properties *in vivo* in advanced ovarian cancer.¹⁷ In this context, it has recently been reported that a series of structurally simplified biphenyl derivatives of combretastatin-A4 (MP5-F9 and MP5-G9) exhibit potent antiproliferative and pro-apoptotic activity in two colorectal cancer cell lines (HCT-116, HT-29).¹⁸ Furthermore, a series of biphenyl compounds (**IV**) has demonstrated both *in vitro* and *in vivo* antitumor activity against colorectal cancer cells.¹⁹ In addition, in 2017, the pharmaceutical Bristol-Myers Squibb (BMS) reported a series of potent biphenyl compounds as candidates for cancer immunotherapy, highlighting BMS-202, BMS-8, and BMS-37, which exhibited activity at nanomolar concentrations.²⁰ Besides, further studies have shown that Honokiol, a lignan isolated from the bark of *Magnolia officinalis*, is effective in suppressing proliferation and inducing apoptosis in SW620 colorectal cancer cells.^{21,22} Finally, it was recently reported that the biphenyl-resveratrol hybrid **V** exhibited potent antitumor activity by inhibiting tumor cell migration in a scratch wound healing assay carried out on breast cancer cells (MDA-MB-231) without affecting cell viability. Additionally,

this compound demonstrated antimetastatic effects *in vivo* in a mouse pulmonary metastasis model using MDA-MB-231 cell line.²³

On another hand, promising cytotoxic response has been also observed in previous studies incorporating the biphenyl scaffold. Earati and colleagues designed and synthesized a novel class of thiazolidine-2,4-dione-biphenyl derivatives **VI**, confirming the structures by spectral analysis. The compounds were evaluated for *in vitro* anticancer activity against cervical, prostate, and breast cancer cells (Hela, PC3, and MDA-MB-231, respectively), using an MTT assay. Doxorubicin was employed as a positive control. Most of the synthesized biphenyl derivatives exhibited moderate to good anticancer activity. In addition, molecular docking studies targeting the epidermal growth factor receptor (EGFR) revealed two compounds that interacted favorably with key residues at the active site, like the co-crystal ligand, Erlotinib.²⁴

Given the complexity of cancer and the abnormal activation of several signaling pathways through its development, multitarget-directed ligands are considered an effective therapeutic method for treating it. One extension of this approach is the production of hybrid molecules, which have bioactive features covalently bonded. These substances provide new opportunities for the creation of more effective cancer candidates for chemotherapy.^{25,26} In this study, a novel scaffold was designed by conjugating a biphenyl moiety with the melatonin (MLT) framework (Fig. 2), yielding a single molecular entity that combines the distinct biological properties of both components. These new compounds were synthesized and spectroscopically characterized. Besides, the antiproliferative and cytotoxic potential against human colorectal cancer cells was tested. To further analyse a possible structure–activity relationship (SAR), a diverse clock-type pattern substitution at the A ring in the biphenyl structure was used. Finally, for the most potent hybrid, we computed its pharmacokinetics and toxicological profile. Here, it is worth mentioning that there are few reports in the anti-cancer field when the integration of MLT and the biphenyl cores into a unique molecular entity is informed. For instance, Jenkins and colleagues have reported that a series of tryptamine linked to a biphenyl portion at C-2, C-3 and C-4 positions **VII** with inhibitory potential against CDK4/

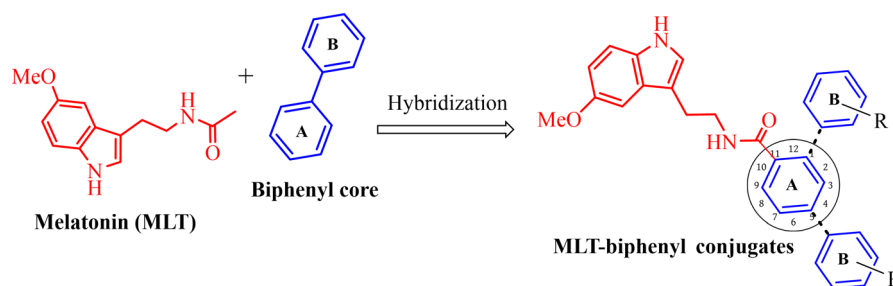
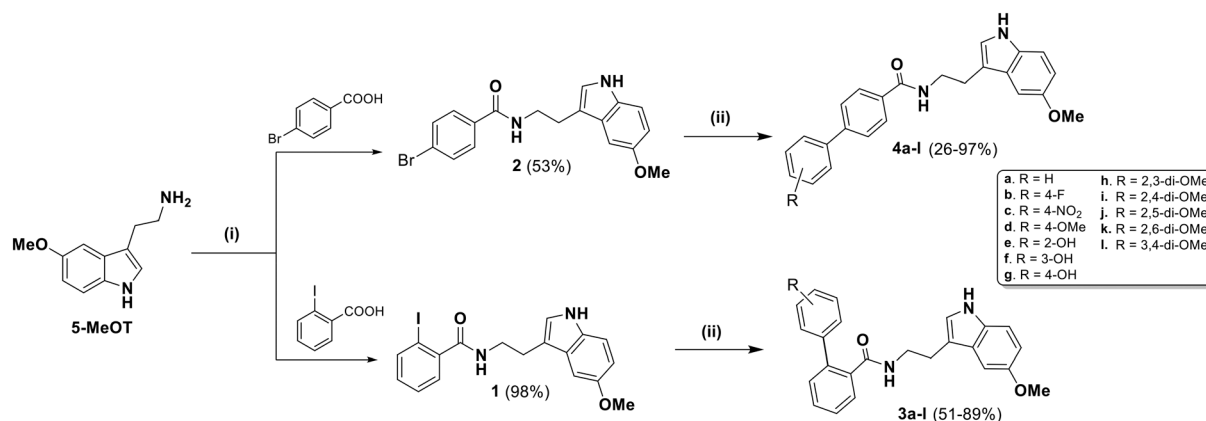


Fig. 2 Design of novel conjugates originated by merging melatonin (in red) with a biphenyl unit (in blue). A clock-type aryl substitution was used at C-2 and C-4 positions of A-ring, while different substitution patterns were attached at B-ring.



Scheme 1 Reagents and conditions (i) Et₃N, HBTU, THF, rt, 24 h; (ii) Arylboronic acids, Na₂CO₃, Ph₃P, *n*-propanol : H₂O 10 : 1, Pd(AcO)₂, US, 50 °C, 3.5 h.

cyclinD1, an anti-cancer drug target in MCF-7 breast cancer cells.²⁷ Similar compounds were investigated by Chaudhuri and Govindrao Mahale, which reported the potent antiproliferative activity of a library of tryptamine-linked biphenyl compounds against two non-small cell lung carcinoma (NSCLC) cell lines, A549 and NCI-H1299.²⁸ Other reports have considered analogous compounds, however, these references do not address anticancer activity as in the present study.^{29,30}

2. Results and discussion

2.1 Chemistry

In this work, a library of new melatonin biphenyl-linked conjugates was designed, synthesized, and biologically investigated for their anti-colorectal cancer potency.

2.1.1 Synthesis of melatonin biphenyl-linked conjugates 3a-l and 4a-l. A simple, cost-effective, and environmentally friendly methodology was employed for the synthesis of the novel melatonin biphenyl-linked hybrids. As illustrated in Scheme 1, a simple two-step sequential route was used to produce the novel conjugates. This protocol initiates with the

amide bond-coupling reaction between the commercially available 5-methoxytryptamine (5-MeOT; 1.3 equiv.) and the 2-iodobenzoic acid or 4-bromobenzoic acid (1.0 equiv.), in the presence of HBTU (*O*-(1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) and triethylamine as base at room temperature to give the amide intermediates **1** and **2**. Finally, the intermediate **1** or **2** (1.0 equiv.) was efficiently reacted under ultrasound conditions with various arylboronic acids (1.3 equiv.) under Suzuki-Miyaura cross-coupling conditions, allowing the successful preparation of the expected melatonin biphenyl-linked conjugates **3a-l** and **4a-l** in moderate to excellent yields (26–97%; Table 1). A replacement attempted for the A-phenyl ring in **3a-l** and **4a-l** was carried out, aiming at understanding the effects of this replacement on the toxicity and cytotoxic potency.^{31,32} However, in these reaction conditions, or when the reaction is carried out under different coupling conditions, the hybrids **4c** and **4k** with the 4-NO₂ and 2,6-dimethoxy substitution pattern were not obtained. Altogether, target hybrids were produced through safe, environmentally friendly, high-yielding, and straightforward experimental processes, together with commercially accessible starting materials.

Fourier transform infrared spectroscopy (FT-IR), electrospray ionization mass spectrometry (MS-ESI), ¹H-NMR, and ¹³C-NMR were used to characterize the newly synthesized hybrids.

The typical FT-IR spectra of hybrids **3a-l** and **4a-l** showed an only and intense sharp band at ~3300 cm⁻¹ which was assigned to indole -NH, and confirms the absence of free NH₂ and presence of indole-NH group. Likewise, series of bands in the region of ~3100 cm⁻¹ to 3000 cm⁻¹ can be attributed to symmetric and asymmetric C-H stretching vibration peaks, and an intense sharp band at ~1630 cm⁻¹ was due to C=O stretching frequencies of amide group. Besides, regarding the presence of aromatic fragments, vibrations peaks between ~1530 cm⁻¹ to 1438 cm⁻¹ were due to C=C aromatic stretches, as well as one peak observed at around 760 cm⁻¹ due to =C-H bending modes in the target

Table 1 Yields and the substitution pattern in all target hybrids **3a-l** and **4a-l**

Hybrid	Substitution pattern	Yield ^a (%)
3a/4a	H	89/93
3b/4b	4-F	85/97
3c/4c	4-NO ₂	74/N.A. ^c
3d/4d	4-OMe	78/36
3e/4e	2-OH	62 ^b /58
3f/4f	3-OH	65/42
3g/4g	4-OH	62/26
3h/4h	2,3-di-OMe	56/87
3i/4i	2,4-di-OMe	64/68
3j/4j	2,5-di-OMe	57/79
3k/4k	2,6-di-OMe	51/N.A. ^c
3l/4l	3,4-di-OMe	84/55

^a Yield was obtained after preparative thin layer chromatographic isolation. ^b Isolated as a mixture of atropisomers. ^c Not obtained under several coupling conditions.

hybrids. Another important peak was observed at $\sim 1336\text{ cm}^{-1}$ assigned to C–O–C stretching mode of asymmetric methoxy groups. An MS-ESI spectra analysis of hybrids **3a–l** and **4a–l** typically reveals m/z values of the molecular ion peak $[M + H]^+$ consistent with their molecular weights. In the corresponding ^1H -NMR spectra (300 MHz, CDCl_3), hybrids both methylene protons as part of the MLT moiety were observed as triplet and quartet peaks at ~ 3.6 and ~ 3.8 ppm, respectively, with a mutual coupling constant between 6.4 and 6.7 Hz. Moreover, chemical shifts around ~ 3.8 ppm (singlet) in ^1H -NMR spectra confirmed the presence of methoxy substituent groups. Downfield shifts of the ^1H -NMR spectrum of hybrids **3a–l** and **4a–l** also registered several signals assigned to the aromatic protons. On ^{13}C -NMR (75 MHz, CDCl_3) spectra of the synthesized hybrids **3a–l** and **4a–l**, a resonant signal around 169 ppm corresponding to the carbon atom in C=O amide bond, and upfield shifts of the chain methylene carbon atoms, which showed chemical shifts at $\delta = 25\text{--}40$ ppm. The aromatic ring carbons (MLT and biaryl fragments) gave resonances in the region from $\delta = 100\text{--}154$ ppm. Of note, a rigorous analysis of ^1H , ^{13}C -NMR spectra revealed that the 2-OH substituted hybrid **3e** exists as an equimolar mixture of stable atropisomers, presumably as a consequence of hindered rotation of the sigma bond linking the *ortho*-substituted biaryl in this compound. Accordingly, ^1H and ^{13}C -NMR spectra of **3e** recorded at 300 K in CDCl_3 (Fig. S10 and S11 in ESI†) showed duplicity in the majority of registered signals. In summary, the spectral data confirmed that the novel melatonin biphenyl-linked hybrids **3a–l** and **4a–l** were successfully synthesized using the proposed methodology.

2.2 Pharmacology

2.2.1 Cytotoxic and antiproliferative activity on SW480 cells. Biphenyl derivatives have attracted significant attention in drug development due to their diverse biological activities, including antimicrobial, anti-inflammatory, anti-HIV, and analgesic effects for treating neuropathic pain. Notably, their potential anticancer properties further underscore their therapeutic value. The biphenyl scaffold, recognized as a privileged structure in organic chemistry, has become a key focus for designing novel drug candidates. Furthermore, biphenyl derivatives are becoming more widely recognized for their potential to help develop novel therapeutic agents due to their wide spectrum of biological effects, especially for complex diseases like cancer and infectious disorders.^{33,34} Considering these facts, we designed and synthesized a series of biphenyl derivatives, with the subsequent evaluation *in vitro*. All compounds were screened at a starting concentration of 100 μM to determine their potential as cytotoxic or cytostatic agents against SW480 human colorectal adenocarcinoma cells, a standard 2D model for colorectal cancer research. The screening results are presented in Table 2, showing both the inhibition and lethality

percentages for each compound, offering initial insights into their impact on cell viability and proliferation. Based on the inhibition levels, the compounds were categorized into three activity groups: low ($\leq 30\%$), moderate (30–60%), and high ($\geq 60\%$). These findings indicated that most new hybrids exhibited high activity, as evidenced by the inhibition percentages higher than 60% (Fig. 3A), suggesting the potential antiproliferative effect under the conditions evaluated. Besides, compounds **3b**, **3h**, **4f**, **4g**, and **4i–l** induced a reduction in cell viability of the cells regarding the starting concentration seeded which suggests they have a certain grade of lethality, being higher in hybrids **4f** and **4g** (LC_{50} values of 62.88 ± 27.39 and $54.91 \pm 2.66\text{ }\mu\text{M}$, respectively).

Significant differences among the evaluated hybrids **3a–l** and **4a–l** were identified by analyzing the substitution pattern, as shown in the bar graph (Fig. 3A), which displays the comparative inhibition (%) values of the synthesized compounds. Based on these results, a structure–activity relationship (SAR) analysis was carried out and is illustrated in Fig. 3B. In general, it was observed that shifting the aryl ring from the C-2 position (hybrids **3a–l**) to the C-4 position (hybrids **4a–l**) led to compounds with enhanced cytotoxicity. Particularly, a closed view from SAR for hybrids **3a–l**, we evidenced the following findings: 1) bioisosteric modification: substitution of a hydrogen atom (unsubstituted hybrid) with a fluorine atom at the C-4 position retains the highest cytotoxic activity. 2) Electronic effects: Incorporating a strong electron-withdrawing group such as $-\text{NO}_2$ resulted in reduced potency, whereas electron-donating substituents ($-\text{OMe}$, $-\text{OH}$) significantly enhanced cytotoxic activity from 2- to 3-fold. 3) Positional effects of $-\text{OH}$ substitution: introduction of a hydroxyl group at the C-2 position did not favor cytotoxic activity; however, substitution at the C-3 or C-4 positions resulted in markedly improved potency, exceeding 80% inhibition. 4) Dimethoxy substitution pattern: the concomitant presence of dimethoxy groups at the C-2 and C-3 positions was found to be critical for achieving high inhibitory potency. In contrast, relocating the $-\text{OMe}$ group from C-3 to C-4, C-5, or C-6 significantly reduced activity by 2- to 3-fold.

Finally, a similar analysis was performed for hybrids **4a–l**, revealing that: 1) the unsubstituted aromatic ring exhibited low cytotoxic activity, indicating limited intrinsic potency ($< 60\%$). 2) Bioisosteric substitution of a hydrogen atom with a fluorine atom at the C-4 position did not enhance cytotoxicity, maintaining a similarly low inhibitory effect ($< 60\%$). 3) Introducing electron-donating groups significantly improved cytotoxic activity, whereas electron-withdrawing substituents were associated with a pronounced decrease in potency. 4) Hydroxyl substitution at the C-2 position did not favor inhibitory activity; however, substitution at the C-3 or C-4 positions significantly improved cytotoxic efficacy, achieving the highest potency. 5) In general, dimethoxy substitution patterns were beneficial and contributed to increased inhibitory activity.

Table 2 One-dose screening in SW480 cells

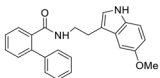
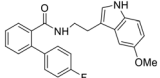
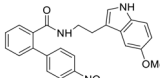
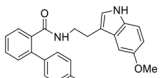
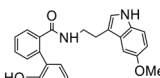
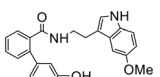
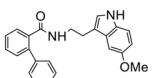
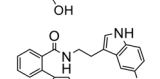
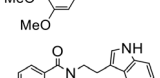
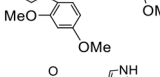
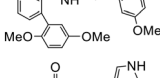
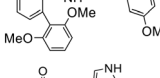
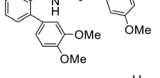
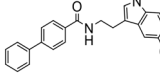
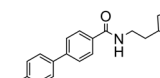
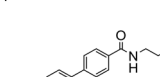
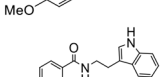
Compound	Structure	Inhibition (%) $\pm$ SD	Lethality (%) $\pm$ SD
3a		96.72 $\pm$ 2.51	N.L.
3b		100.00 $\pm$ 0.25	3.78 $\pm$ 0.25
3c		64.19 $\pm$ 0.33	N.L.
3d		91.69 $\pm$ 3.07	N.L.
3e		33.81 $\pm$ 24.96	N.L.
3f		76.59 $\pm$ 1.32	N.L.
3g		81.44 $\pm$ 8.05	N.L.
3h		100.00 $\pm$ 3.48	8.62 $\pm$ 3.48
3i		98.62 $\pm$ 13.03	N.L.
3j		78.60 $\pm$ 0.37	N.L.
3k		64.69 $\pm$ 15.76	N.L.
3l		50.59 $\pm$ 0.21	N.L.
4a		53.14 $\pm$ 1.01	N.L.
4b		49.03 $\pm$ 1.83	N.L.
4d		63.64 $\pm$ 0.84	N.L.
4e		53.07 $\pm$ 0.34	N.L.
4f		100.00 $\pm$ 27.39	62.88 $\pm$ 27.39

Table 2 (continued)

Compound	Structure	Inhibition (%) $\pm$ SD	Lethality (%) $\pm$ SD
4g		100.00 $\pm$ 2.66	54.91 $\pm$ 2.66
4h		54.73 $\pm$ 3.15	N.L.
4i		100.00 $\pm$ 13.47	31.33 $\pm$ 13.47
4j		100.00 $\pm$ 8.80	15.82 $\pm$ 8.80
4l		100.00 $\pm$ 3.61	13.22 $\pm$ 3.61

Percentage growth inhibition at a single dose assay (100 μ M). The most active hybrids are shown in bold. Values are expressed as mean $\pm$ SD. N.L. Non-lethal.

2.2.2 Five-dose screening. Considering the limitations of the current chemotherapy, the urgent need to discover new therapeutic alternatives against cancer, and the wide range of activities associated with this pharmacophore, we selected the most active compounds for further research using a complete cytotoxicity test. Those experiments included most of the hybrids, except for compounds **3e**, **3l**, **4a-b**, **4e**, and **4h**, which did not reach the threshold inhibition criteria ($\geq 60\%$) (Fig. 3A). Thus, the promising hybrid compounds (**3a-d**, **3f-k**, **4d**, **4f-g**, **4i-j**, and **4l**) were tested on SW480 and non-tumor-derived human colon epithelial cells (NCM460) across five different concentrations, based on the methodology from a previous single-dose assay. The results, presented in Table 3, allowed for the estimation of critical values, including the concentration required for 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and the concentration leading to a 50% reduction in protein levels at the end of the experiment, compared to the beginning (LC_{50}). All compounds tested in this study, except for hybrids **3c** and **4d**, demonstrated significantly better activity than the parental compound **2** and melatonin, evidenced by low GI_{50} values, providing a clear indication of the potency of each compound in inhibiting cancer cell proliferation. These findings suggest that hybridization strategies have generally improved the cytotoxic potential of these compounds. We also observed that hybrids with the C-2 substitution pattern (**3a-k**) exhibited even higher GI_{50} values than most hybrids with the C-4 substitution pattern (**4f-l**), suggesting that these changes in the structure of the first hybrids might not have been optimized for maximum activity, probably due to ineffective cellular uptake, or unfavorable interactions with cancer cell

targets. Moreover, we also evidenced that compounds **4i-l** exhibited significantly higher activity than 5-FU as evidenced by lower GI_{50} (**4i**: <6.25 , **4j**: 6.59 ± 1.18 , **4l**: <6.25 , and 5-FU: 29.69 ± 3.76). Notably, hybrids **4j** and **4l** demonstrated significant additional selectivity by showing lower activity against non-malignant cells NCM460, indicating a greater degree of cytotoxicity toward cancer cells than normal cells (5-FU = 0.75; **4j** = 1.93; **4l** = >2.22). This selectivity is an essential parameter for developing cancer therapies, as it suggests these compounds could target cancer cells more effectively while minimizing harm to healthy tissue. We also hypothesize that hybrids **4j** and **4l** may induce a cytostatic effect rather than a cytotoxic activity. This hypothesis is based on the total growth inhibition observed for these compounds, coupled with the lack of an LC_{50} value under the conditions evaluated. This characteristic could be important for their potential use as adjuvants, where they could help inhibit cancer cell growth, enhancing the efficacy of other treatments while reducing side effects, providing a promising strategy for cancer therapy.

These results are consistent with previous studies. In 2018, Duan and colleagues synthesized a series of unsymmetrical biaryl derivatives *via* a palladium-catalyzed Suzuki coupling reaction. Among the compounds evaluated, they identified a 4'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonitrile (dxy-1-175) that exhibited good anti-proliferative activity against the A549 human lung cancer cell line (IC_{50} = 5.9 μ M), disrupting tubulin polymerization through binding at the colchicine-binding site, which subsequently induced a G2/M phase cell cycle arrest.³⁵ Likewise, Cheng and colleagues in 2022 conducted a similar work based on the

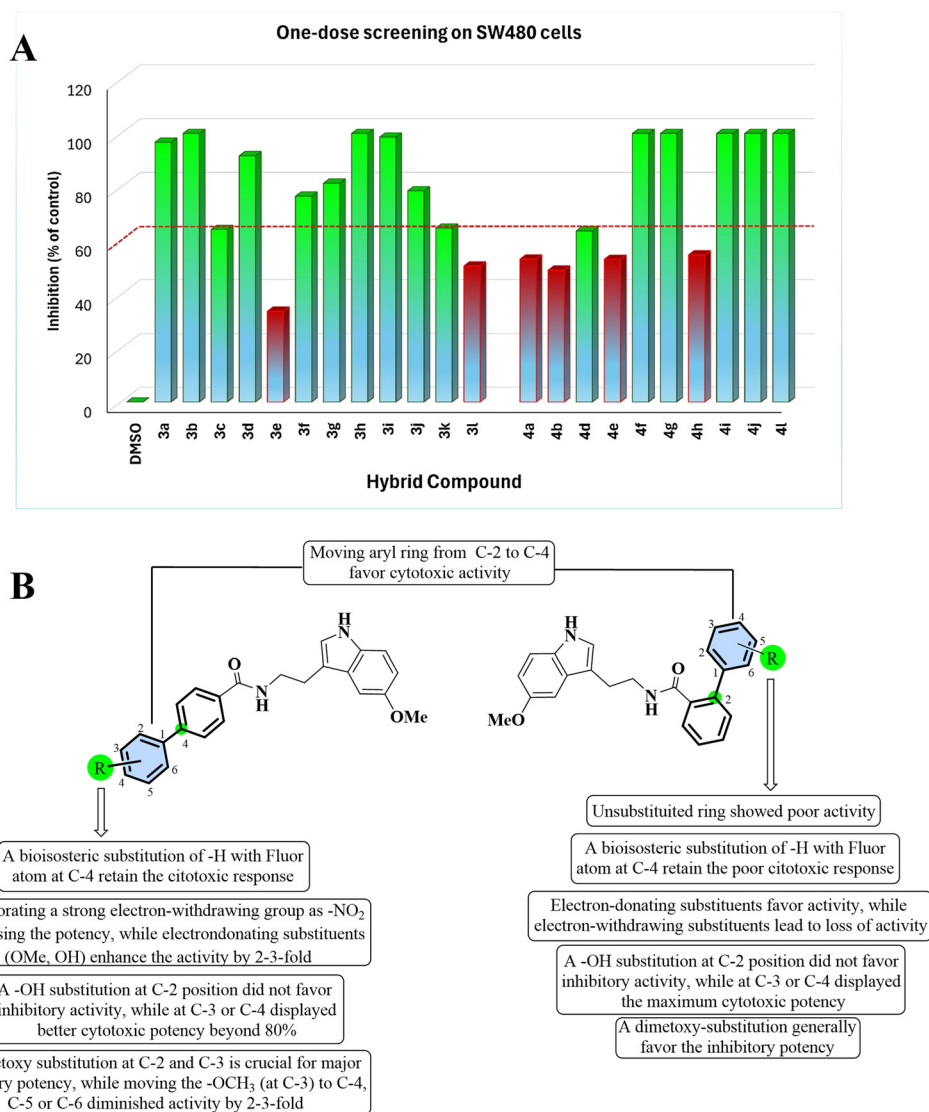


Fig. 3 A) A bar graph presenting the inhibition (%) values of the new hybrids. Green bars: the most active compounds; red bars: hybrids with low activity. The dotted red line represents the minimum activity threshold. Cells treated with DMSO served as the control group, assumed to have 100% viability (0% inhibition), and were used as the reference for calculating inhibition percentages. B) A structure-activity relationship (SAR) analysis illustrating the cytotoxic effect of hybrids **3a-l** and **4a-l**.

computationally predicted binding interactions of dxy-1-175. They designed and synthesized a series of new 4-benzoylbiphenyl analogues, identifying compounds with potent anti-proliferative activity with IC₅₀ values between 1 and 10 μ M against human lung cancer A549 cells.³³ These informed results were in good agreement to our findings when most active compounds exhibited inhibitory concentrations <6.2 μ M, strongly suggesting that hybrid compounds prepared in this work may serve as promising candidates for designing novel therapeutic strategies against colorectal cancer.

2.2.3 Optical microscopy evaluation. As shown in Fig. 4, optical microscopy evaluation revealed distinct morphological changes in human SW480 adenocarcinoma cells following treatment with hybrids **4j** and **4l**, exhibiting classic features of death, such as cell shrinkage, chromatin condensation,

membrane blebbing, and nuclear fragmentation. Cytoplasmic vacuolization and detachment from the substrate were also observed, reflecting cellular stress and loss of adhesion. Importantly, we noticed that non-malignant NCM460 cells exhibited fewer effects regarding the number of cells with less structural alterations under the same treatment conditions, especially for compound **4l** which correlates with our findings about the selectivity index of these hybrids, supporting the potential of the tested compounds as candidates for further experiments in the search for new therapeutic alternatives against colorectal cancer.

2.3 Theoretical studies

2.3.1 ADME-tox modelling. To find new drug candidates, the investigation of physicochemical and pharmacokinetic

Table 3 Five-dose screening on NCM460 and SW480 cell lines

	SW480			NCM460			SI
	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)	
3a	49.87 ± 4.49 ****	74.65 ± 10.08	>100	21.85 ± 8.43	46.66 ± 11.44	55.67 ± 3.71	0.44
3b	56.83 ± 5.03 ****	90.06 ± 0.55	>100	31.71 ± 5.35	96.13 ± 45.65	>100	0.56
3c	>100	>100	>100	85.56 ± 70.42	>100	>100	<1
3d	45.79 ± 4.43 ****	61.42 ± 12.64	>100	20.65 ± 2.16	44.37 ± 6.59	>100	0.45
3f	64.49 ± 4.01 ****	>100	>100	>100	>100	>100	>1
3g	65.24 ± 2.15 ****	>100	>100	>100	>100	>100	>1
3h	55.75 ± 3.42 ****	78.64 ± 29.50	>100	26.29 ± 6.04	>100	>100	0.47
3i	71.18 ± 9.63 ****	>100	>100	51.79 ± 7.89	84.82 ± 21.17	>100	0.73
3j	55.44 ± 4.47 ****	>100	>100	>100	>100	>100	>1.80
3k	54.94 ± 5.08 ****	>100	>100	>100	>100	>100	>1.82
4d	84.40 ± 4.11	>100	>100	43.20 ± 1.96	>100	>100	0.51
4f	22.33 ± 2.25 ****	51.36 ± 0.40	70.89 ± 11.72	<6.25	39.52 ± 2.97	62.94 ± 7.87	<0.28
4g	20.24 ± 2.93 ****	43.79 ± 3.82	68.03 ± 0.46	<6.25	32.86 ± 0.87	46.00 ± 1.57	<0.31
4i	<6.25 ****	10.59 ± 4.84	>100	<6.25	>100	>100	N.C.
4j	6.59 ± 1.18 ****	14.74 ± 0.36	>100	12.73 ± 1.00	>100	>100	1.93 ****
4l	<6.25 ****	<6.25	>100	13.88 ± 1.35	>100	>100	>2.22 ****
2	95.59 ± 4.40	>100	>100	74.53 ± 3.65	99.53 ± 0.77	>100	0.78
MLT	>100	>100	>100	>100	>100	>100	N.C.
5-FU	29.69 ± 3.76	>100	>100	22.38 ± 2.30	>100	>100	0.75

Dose-response curves were used to determine the GI₅₀, TGI, and LC₅₀ values 48 hours after treatment. The mean ± standard deviation (SD) of independent experiments was used to display the data. The ratio of the GI₅₀ for NCM460 cells to the GI₅₀ for SW480 cells was used to calculate selectivity indices. 5-FU: The reference chemotherapeutic agent; melatonin (MLT) and 2: parental molecules. NC = Not calculated. *****p* ≤ 0.0001.

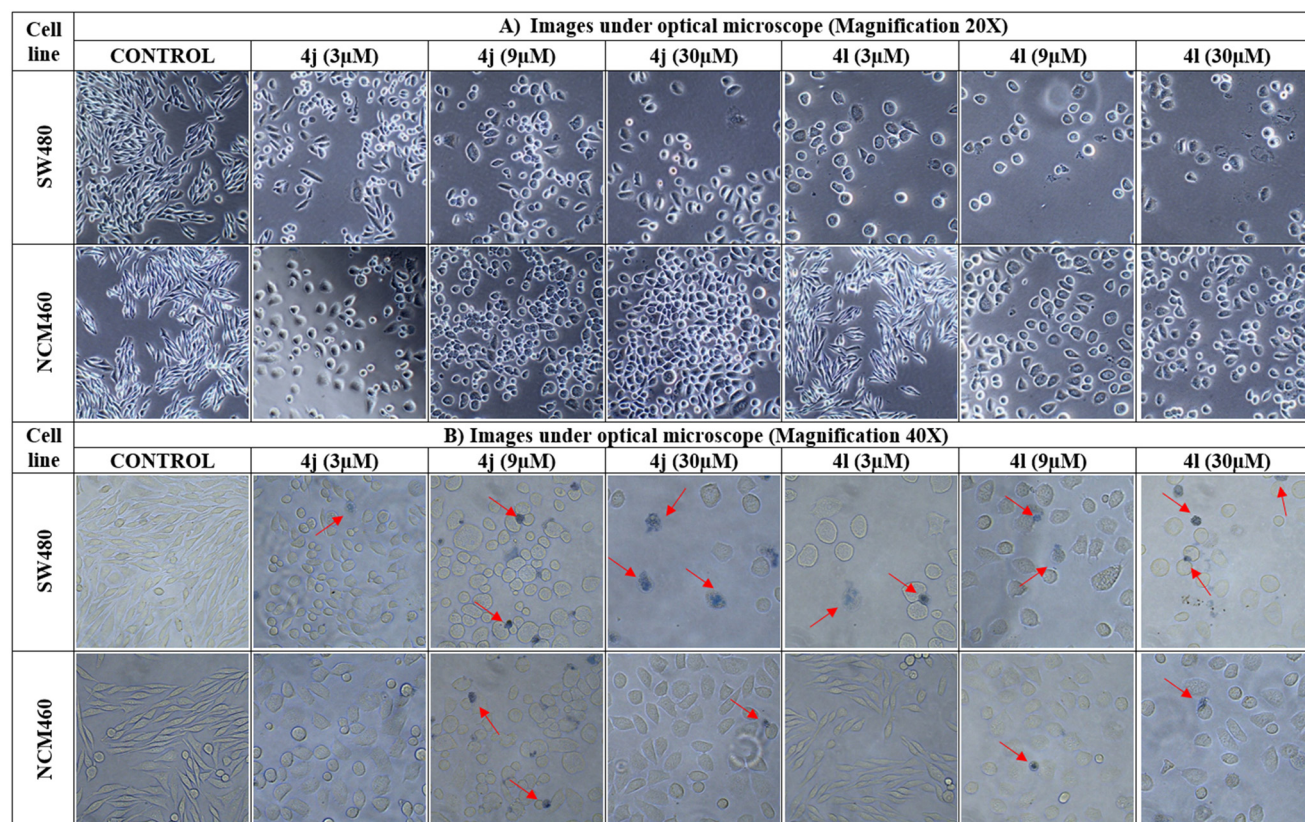


Fig. 4 Cell viability of SW480 and NCM460 cells after treatment with 4j and 4l hybrids. Cells were stained with trypan blue dye. A) Magnification 20×; B) magnification 40×. Red arrows show dead cells stained with trypan blue dye.

parameters that critically affect drug solubility, ionization, and biological activity is mandatory. In drug discovery, simple rules/filters have been incorporated for defining a drug-like prototype, which are based on simple molecular properties derived from chemical structure. Especially to develop novel anti-cancer compounds, crucial pharmacokinetics indices have been counted in the last decade, and determined successfully *via* computational resources, being a conveniently simple, cheap, and fast method. In this regard, using computer-assisted strategies, the most promising compounds **4j** and **4l** were further analyzed for their biopharmaceutical parameters, and drug-like properties allowed us to infer early on their potential as drug-like candidates. With particular interest in this work, twelve drug-like properties that define the bioavailability of a drug-candidate were computed *via* the SwissADME algorithm's and depicted in Table 4.

The analysis suggested that both **4j** and **4l** would have an optimal drug-like profile when compared to most of the oral drug medications. For instance, the lipophilicity index ($\log P_{o/w}$) was calculated in approximately 4.37, which is in good agreement with the optimal range for lipid-based formulations (−2.0 to 6.0).³⁶ Thus, we computed the percent human intestinal absorption (%GI) for hybrids **4j** and **4l**, showing an excellent >80% value, meaning that a great fraction of these hybrids would be absorbed from the gastrointestinal tract after oral administration. In agreement with this result, apparent permeability values were measured using the Caco-2/MDCK cell monolayer

models, correlating with human intestinal absorption. Thereon, calculations would suggest that **4j** and **4l** possess a remarkable apparent permeation through Caco-2/MDCK cells of 2261 and 1195 nm s^{−1}, respectively.^{37–39} In a similar scenario, we found that **4j** and **4l** would tend to an ideal topological polar surface area (TPSA) value of 72.58 Å², which means that this compound may have a greater probability to be absorbed across cell membranes upon oral intake.⁴⁰

More importantly, we computed the binding of **4j** and **4l** to human serum albumin (noted as $\log K_{HSA}$), a parameter that determines the distribution of compounds through the systemic circulation. From a therapeutic viewpoint, most of the approved drugs have values of $\log K_{HSA}$ between −1.5 and +1.5.^{41,42} For **4j** and **4l**, favorable $\log K_{HSA}$ values of 0.870 and 0.921, respectively, were computed, falling within the recommended range. On the other hand, two drug-like characteristics widely used in modern drug discovery are the fraction of sp³-hybridised carbon atoms (denoted as F_{sp^3}) and the aromatic/heteroaromatic ring count (#ArRNG). Most of the existing approved drugs satisfy a F_{sp^3} value <0.5 and possess from one to three counts for aromatic/heteroaromatic rings; beyond these desirable values result in detrimental effects on human bioavailability.^{43–46} **4j** and **4l** possess a calculated F_{sp^3} value of 0.19 and three aromatic rings, indicating that these compounds fit well within the recommended endpoints. Finally, we also computed the passage of **4j** and **4l** through the blood–brain barrier (BBB) from blood, which is a parameter that determines whether a compound can effectively enter the brain. Low brain penetration due to low blood–brain barrier (BBB) permeability could prevent potential psychiatric side effects for emerging anticancer drug candidates. In that sense, our calculations suggest that hybrids **4j** and **4l** do not cause any conspicuous psychotropic side effects.

2.3.2 Bioavailability radar model for 4j and 4l. The bioavailability radar plot is a powerful tool for helping researchers to represent molecule properties graphically in the bioavailability context.⁴⁷ Crucial molecular characteristics based on molecular size, polarity, solubility, saturation, flexibility, and lipophilicity are graphically inspected and compared to drug-like candidates. Using a radar map, we inspected eight drug-like key parameters for developing new oral pharmaceuticals. The bioavailability radar study for **4j** and **4l** was similar and is shown in Fig. 5, where the computed properties for hybrids **4j** and **4l** are depicted within the cyan zone, and the yellow color zone represents the recommended range for each parameter. It is interesting to note that calculated parameters for **4j** and **4l** met within the ideal drug-like ranges, thereby suggesting that these compounds eventually would have a great chance of eligibility for entrance into the more advanced pre-clinical trials.

2.3.3 Predictive toxicological profile for 4j and 4l. In addition to pharmacokinetic studies, a set of toxicity endpoints was examined for lead-hybrids **4j** and **4l**. To reach

Table 4 Pharmacokinetic/drug-likeness properties for hit hybrids **4j** and **4l**

Property	4j	4l
MW ^a	430.1990	430.1990
TPSA ^b	72.58/72.58 ^m	72.58/72.58 ^m
<i>n</i> -RotBond	8	8
<i>n</i> -ON ^c	5	5
<i>n</i> -OHNH ^d	2	2
$\log P_{o/w}$ ^e	4.37	4.36
$\log K_{HSA}$ ^f	0.870	0.921
F_{sp^3} ^g	0.19	0.19
#ArRNG ^h	3	3
Caco-2 (nm s ^{−1}) ⁱ	2261	2261
App. MDCK (nm s ^{−1}) ^j	1195	1195
%GI ^k	>80%	>80%
BBB permeant ^l	No/inactive ^m	No/inactive ^m

^a Molecular weight distribution of traded drugs peaked in the 200–600 g mol^{−1} range. ^b Polar surface area (PSA, Å²) (<140 Å²). ^c *n*-ON number of hydrogen bond acceptors <10. ^d *n*-OHNH number of hydrogens bonds donors ≤5. ^e Octanol–water partition coefficient ($\log P_{o/w}$) (−2.0 to +6.5). ^f Binding-serum albumin ($\log K_{HSA}$) (−1.5 to +1.2). ^g Fraction of sp³ carbon atoms (optimal: F_{sp^3} <0.5). ^h The number of aromatic/heteroaromatic rings (optimal: ≤3). ⁱ Human intestinal permeation (<25 poor, >500 great). ^j Madin-Darby canine kidney (MDCK) cells permeation (<25 poor, >500 great). ^k % Human oral gastrointestinal (GI) absorption (<25% is poor, >80% is high). ^l The probability of a good BBB crossing blood–brain barrier (BBB) permeation both consist in the readout of the BOILED-Egg model ^m Calculated by ProTox 3.0.

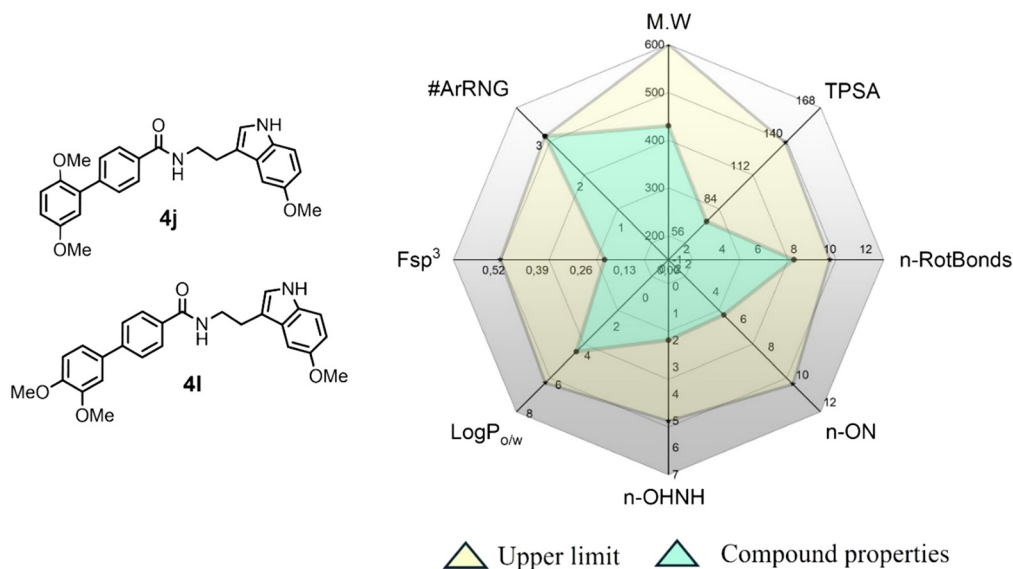


Fig. 5 Bioavailability radar diagram for promising **4j** and **4l**.

this, we used the open-source computational servers ToxTree, Pred-hERG, ProTox 3.0, TEST, OSIRIS, pkCSM, ADMET-SAR, and SwissADME⁷. Toxicological evaluations of chemicals concerning human health are essential in the preclinical development of potential lead molecules. Early estimation of these parameters by using chemoinformatics techniques offers a fast and reliable alternative to experimental methods.^{48,49} With this in mind, we early access to the toxicological knowledge on the **4j** and **4l** hybrids using an *in silico* approach, showing that these compounds would not appear to have any adverse effects or warnings as tumorigenic, mutagenic, immunotoxic, nephro/neuro/hepato/cardiotoxic, and irritant when compared to optimal toxicological reference values. Additionally, it was found that

4j and **4l** seem unlikely to cause any detrimental effect on the reproductive system, and they also do not present any structural alert associated with covalent DNA binding or other oral toxicities. Finally, after investigating potential promiscuous features (PAINS) for hybrids **4j** and **4l**, our findings suggest that the rationally designed biphenyl-MLT molecules are not prone to promiscuity.

In summary, for the first time, these novel anticancer candidates with apparently ideal pharmacokinetic profiles were designed by merging melatonin and biphenyl features into a single structural core. Given this scenario, we cautiously propose that hybrids **4j** and **4l** be considered in future challenges targeting colorectal cancer therapy (Table 5).

Table 5 *In silico* toxicological screening for the hit hybrids **4j** and **4l**

Toxicity	Toxicity risk	4j	4l
Oral toxicity	LD ₅₀ (mg kg ⁻¹) ^{a,b}	1000	1000
	Toxicity class ^{a,b}	IV	IV
Organ toxicity	Hepatotoxicity ^b	Inactive	Inactive
	Reproductive effect ^c	Green	Green
	Neurotoxicity ^d	Inactive	Inactive
	Nephrotoxicity ^a	Non-inhibitor	Non-inhibitor
	Cardiotoxicity ^a	Inactive	Inactive
	Immunotoxicity ^b	Inactive	Inactive
Toxicity endpoints	Mutagenicity ^{b,c,e}	Inactive/green/0.38	Inactive/green/0.39
	Tumorigenic ^{b,c}	Inactive/green	Inactive/green
	Irritant effect ^c	Green	Green
	hERG binding alert ^{f,g}	Non-blocker/no	Non-blocker/no
	DNA binding alert ^h	Inactive	Inactive
	PAINS alerts ⁱ	No	No

^a Predicted by Protox 3.0. LD₅₀: probable oral lethal dose (human); toxicity category IV is practically non-toxic and not an irritant. ^b Calculated by ProTox 3.0 web-based server. ^c Based-on OSIRIS property explorer green (indicates no risk or low risk), yellow (indicates medium risk), red (indicates higher toxicity risk). ^d Calculated by the SwissADME tool. ^e Calculated by The Toxicity Estimation Software Tool (TEST) from US EPA; if calculated score <0.5, then activity = negative. ^f Calculated by the Pred-hERG platform. ^g Predicted using the pkCSM web tool. ^h Calculated by the ToxTree software. ⁱ Promiscuous activity by toxic fragments.

3. Conclusions

Our study presents the design and synthesis of novel molecular hybrids that integrate structural elements from melatonin and the biphenyl core, achieved through straightforward, cost-effective, and environmentally friendly methods. Among the synthesized compounds, hybrids **4j** and **4l** demonstrated noticeable antiproliferative activity against SW480 colorectal cancer cells, with GI_{50} values of approximately 6.59 μ M and <6.25 μ M, respectively. These values indicate up to a 5-fold increase in potency compared to the reference anticancer agent 5-fluorouracil (5-FU), which exhibited a GI_{50} of 29.69 ± 3.76 μ M. More important, both compounds also displayed markedly improved selectivity, with selectivity indices (SI) of 1.93 and >2.22, respectively, in contrast to 5-FU, which while effective, it is also known for its significant toxicity (SI: 0.75). Interestingly, these hybrids appear to exert a cytostatic effect rather than a purely cytotoxic one, suggesting adjuvant potential for tumour growth suppression with reduced cellular damage. *In silico* pharmacokinetic and toxicological evaluations further suggest favourable biopharmaceutical properties and safety profiles of **4j** and **4l**, showing, for instance, optimal calculated values for TPSA (72.58 $\text{\AA}^2$), $\log P_{o/w}$ (~ 4.3), $\log K_{HSA}$ (~ 4.36), and intestinal absorption values in Caco-2 and MDCK cell models of 2261 1195 nm s^{-1} , reinforcing their potential as lead candidates for colorectal cancer therapy. The hybridization strategy adopted in this work resulted in enhanced biological activity and selectivity, likely due to the synergistic interaction between melatonin and the biphenyl scaffold. Overall, these findings highlight the potential of these hybrid molecules to develop new safer therapeutic alternatives against CRC, however, additional in-depth studies are necessary to fully validate their therapeutic potential and clinical applicability.

4. Experimental

4.1 Chemistry

Purchased from Sigma Aldrich and Alfa Aesar (USA), all chemical reagents were analytical grade and utilized without further purification. When using TLC on silica gel pre-coated chromatographic plates 60 F254 (Merck), the spots were visible under UV light ($\lambda = 254$ nm), allowing for the monitoring of reaction progress. Reactions aided by ultrasound were conducted in a Branson B1510 DTH ultrasonic cleaning bath (50 kHz, 245 W). Melting points were determined using an open capillary and the Stuart Digital Melting Point instrument (SMP10) (uncorrected). FT-IR spectra were collected in a PerkinElmer (Waltham, Massachusetts, USA) Spectrum two Fourier transform infrared spectrophotometer with attenuated total reflectance (UATR) modulus between 550 and 4000 cm^{-1} at a resolution of 4 cm^{-1} . Using tetramethylsilane (TMS) as the internal standard, the ^1H and ^{13}C NMR (CDCl_3 , 300 MHz, 75.0 MHz) spectra were captured at 25 $^{\circ}\text{C}$ in a Varian apparatus. An

electrospray ionization mass spectrometry (ESI-HRMS) was used to record high-resolution mass spectra of all hybrids.

4.1.1 General procedure for the synthesis of intermediates

1 and 2. A mixture of 2-iodobenzoic acid or 4-bromobenzoic acid (1.0 mmol) and triethylamine (4.0 mmol) in 4 mL of dry THF. After 15 min stirring at room temperature (rt), HBTU (1.5 mmol) was added and the resulting mixture stirred for an additional 10 min at rt. Finally, 5-MeOT (1.3 mmol) was incorporated into this solution and stirred for a period of 24 h at rt (reaction completion was determined by TLC). The crude reaction mixture was extracted three times with ethyl acetate, and the combined organic layers were washed with brine and dried over sodium sulphate, filtered, and concentrated. The crude product was purified on a silica gel column using a Biotage Isolera One purification system employing an *n*-hexane/ethyl acetate gradient (10% AcOEt: 90% hexane for 5 min followed by an increase to 50% AcOEt over 15 min) to give the title intermediates **1** and **2**.

2-Iodo-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)benzamide (1). Brownish solid; yield 98%; mp: 45–47 $^{\circ}\text{C}$; IR (neat): $\tilde{\nu}$ cm^{-1} = 3289 (N–H stretch), 3069–2987 (C–H stretch aromatic), 1640 (C=O stretch amide), 1585 (N–H bend), 1514–1438 (C=C stretch aromatic), 1213 (C–O–C stretch), and 746 (=C–H bend aromatic) ^1H NMR (300 MHz, CDCl_3): δ 7.87 (d, J = 7.3 Hz, 1H), 7.41–7.31 (m, 2H), 7.28 (d, J = 7.3 Hz, 1H), 7.16–7.06 (m, 3H), 6.91 (dd, J = 8.8, 2.4 Hz, 1H), 3.89 (s, 3H), 3.84 (q, J = 6.7 Hz, 2H), 3.14 (t, J = 6.7 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.5, 154.1, 142.3, 139.9, 131.6, 131.1, 128.2, 127.7, 123.2, 112.5, 112.4, 112.1, 100.5, 92.5, 56.0, 40.0, 25.2. HRMS (ESI), calcd for $\text{C}_{18}\text{H}_{17}\text{IN}_2\text{O}_2$ [$\text{M} + \text{H}$] $^{+}$: 421.0411, found: 421.0415.

4-Bromo-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)benzamide (2).⁵⁰ Off-white solid; yield 53%; mp: 129–131 $^{\circ}\text{C}$; IR (neat): $\tilde{\nu}$ cm^{-1} = 3353 (N–H stretch), 3085–2983 (C–H stretch aromatic), 1632 (C=O stretch amide), 1592 (N–H bend), 1544–1425 (C=C stretch aromatic), 1212 (C–O–C stretch), and 795 (=C–H bend aromatic) ^1H NMR (300 MHz, CDCl_3): δ 7.48–7.38 (m, 4H), 7.19 (d, J = 8.4 Hz, 1H), 6.99–6.91 (m, 2H), 6.80 (dd, J = 8.8, 2.5 Hz, 1H), 3.71 (s, 3H), 3.69 (q, J = 6.6 Hz, 2H), 2.98 (t, J = 6.6 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.5, 154.2, 133.5, 131.8, 131.6, 128.5, 127.7, 126.1, 122.9, 112.7, 112.7, 112.2, 100.4, 55.9, 40.5, 25.2.

4.1.2 General procedure for the synthesis of target hybrids

3a–l and 4a–l. A mixture of **1** or **2** (1.0 mmol), the corresponding arylboronic acid (1.30 mmol), triphenylphosphine (0.10 mmol), Na_2CO_3 (7.0 mmol), palladium(II) acetate ($\text{Pd}(\text{AcO})_2$) (0.05 mmol) in 4 mL of *n*-propanol- H_2O (10:1) was sonicated (Branson B1510 DTH ultrasound cleaning bath) under an inert argon atmosphere at 50 $^{\circ}\text{C}$ for 3.5 h (reaction completion was determined by TLC). After completion of the reaction, the medium was filtered through celite pad and the filtrate was extracted three times with ethyl acetate, and the combined organic layers washed with brine and dried over sodium sulphate, filtered, and concentrated. The crude product was purified by preparative TLC using EtOAc/petroleum ether (3/1) as an

eluent to afford pure the desired hybrids in moderate to excellent yields.

N-(2-(5-Methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3a**).^{30,51,52} Off-white solid; yield 89%; mp: 47–49 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3270 (N–H stretch), 3061–2928 (C–H stretch aromatic), 1635 (C=O stretch amide), 1524–1483 (C=C stretch aromatic), 1597 (N–H bend), 1215 (C–O–C stretch), and 744 (=C–H bend aromatic). ¹H NMR (300 MHz, CDCl₃): δ 7.71 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.49 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.44 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.44–7.31 (m, 6H), 7.25 (d, *J* = 8.8 Hz, 1H), 6.94 (d, *J* = 2.4 Hz, 1H), 6.88 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.61 (d, *J* = 2.4 Hz, 1H), 3.86 (s, 3H), 3.57 (q, *J* = 6.8 Hz, 2H), 2.69 (t, *J* = 6.8 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.6, 154.0, 140.2, 139.3, 135.9, 131.6, 130.2, 130.1, 128.8, 128.7, 128.5, 127.7, 127.6, 127.5, 122.6, 112.4, 112.2, 112.0, 100.5, 77.5, 77.1, 76.7, 55.9, 39.6, 24.7. HRMS (ESI), calcd for C₂₄H₂₂N₂O₂ [M + H]⁺: 371.1741, found: 371.1738. Purity: 98.61%.

4'-Fluoro-*N*-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3b**). Pale yellow solid; yield 85%; mp: 82–84 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3281 (N–H stretch), 3061–2999 (C–H stretch aromatic), 1636 (C=O stretch amide), 1598 (N–H bend), 1512–1439 (C=C stretch aromatic), 1214 (C–O–C stretch), and 760 (=C–H bend aromatic). ¹H NMR (300 MHz, CDCl₃): δ 7.64 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.46 (td, *J* = 7.5, 1.5 Hz, 1H), 7.40 (td, *J* = 7.5, 1.5 Hz, 1H), 7.32–7.27 (m, 3H), 7.24 (d, *J* = 8.8 Hz, 1H), 7.01 (t, *J* = 8.8 Hz, 2H), 6.93 (d, *J* = 2.5 Hz, 1H), 6.87 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.71 (d, *J* = 2.5 Hz, 1H), 3.82 (s, 3H), 3.57 (q, *J* = 6.8 Hz, 2H), 2.74 (t, *J* = 6.8 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.5, 162.47 (d, *J*_{C–F} = 247.4 Hz), 154.1, 138.3, 136.2, 136.1, 135.9, 131.5, 130.3, 130.2, 130.2, 130.1, 128.7, 127.7, 127.5, 122.5, 115.4 (d, *J*_{C–F} = 21.6 Hz), 115.3, 112.5, 112.2, 112.0, 100.3, 55.9, 39.8, 24.8. HRMS (ESI), calcd for C₂₄H₂₁FN₂O₂ [M + H]⁺: 389.1629, found: 389.1634. Purity: 97.91%.

N-(2-(5-Methoxy-1*H*-indol-3-yl)ethyl)-4'-nitro-[1,1'-biphenyl]-2-carboxamide (**3c**). Pale yellow solid; yield 74%; mp: 98–100 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3287 (N–H stretch), 3073–2940 (C–H stretch aromatic), 1640 (C=O stretch amide), 1595 (N–H bend), 1514–1438 (C=C stretch aromatic), 1213 (C–O–C stretch), and 746 (=C–H bend aromatic). ¹H NMR (300 MHz, CDCl₃): δ 8.09 (d, *J* = 8.8 Hz, 2H), 7.64 (dd, *J* = 7.1, 1.9 Hz, 1H), 7.54 (td, *J* = 7.5, 1.9 Hz, 1H), 7.48 (td, *J* = 7.5, 1.9 Hz, 1H), 7.41 (d, *J* = 8.8 Hz, 2H), 7.34 (dd, *J* = 7.1, 1.9 Hz, 1H), 7.30 (d, *J* = 1.9 Hz, 1H), 6.95–6.85 (m, 2H), 6.80 (d, *J* = 2.4 Hz, 1H), 3.82 (s, 3H), 3.62 (q, *J* = 6.5 Hz, 2H), 2.86 (t, *J* = 6.5 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.0, 154.1, 147.0, 146.8, 137.4, 136.1, 131.5, 130.4, 130.1, 129.3, 128.8, 128.5, 127.5, 123.5, 122.7, 112.6, 112.2, 112.0, 100.2, 55.9, 40.2, 24.6. HRMS (ESI), calcd for C₂₄H₂₁N₃O₄ [M + H]⁺: 416.1578, found: 416.1580. Purity: 99.11%.

4'-Methoxy-*N*-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3d**). Pale yellow solid; yield 78%; mp: 70–72 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3293 (N–H stretch), 3060–2995 (C–H stretch aromatic), 1633 (C=O stretch amide), 1611 (N–H bend), 1516–1441 (C=C stretch aromatic), 1213 (C–O–C stretch), and 761

(=C–H bend aromatic). ¹H NMR (300 MHz, CDCl₃): δ 7.69 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.48 (td, *J* = 7.5, 1.6 Hz, 1H), 7.41 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.35 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.32 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 1H), 6.94 (dd, *J* = 8.7, 2.5 Hz, 1H), 6.92 (d, *J* = 8.7 Hz, 2H), 6.88 (dd, *J* = 8.7, 2.5 Hz, 1H), 6.67 (d, *J* = 2.5 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.60 (q, *J* = 6.7 Hz, 2H), 2.75 (t, *J* = 6.7 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.8, 159.3, 154.0, 139.0, 135.7, 132.5, 131.5, 130.2, 130.1, 129.9, 128.8, 127.5, 127.2, 122.6, 114.0, 112.4, 112.3, 112.0, 100.4, 55.9, 55.4, 39.6, 24.8. HRMS (ESI), calcd for C₂₅H₂₄N₂O₃ [M + H]⁺: 401.1839, found: 401.1844. Purity: 97.36%.

2'-Hydroxy-*N*-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3e**). Brownish solid; yield 62%; mp: 82–84 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3297 (N–H stretch), 3063–2995 (C–H stretch aromatic), 1638 (C=O stretch amide), 1580 (N–H bend), 1530–1439 (C=C stretch aromatic), 1215 (C–O–C stretch), and 796 (=C–H bend aromatic). Mixture of atropisomers. Atropisomer A: ¹H NMR (300 MHz, CDCl₃): δ 7.74 (d, *J* = 7.7 Hz, 1H), 7.59 (d, *J* = 7.0 Hz, 2H), 7.29 (t, *J* = 7.7 Hz, 2H), 7.22 (d, *J* = 7.0 Hz, 1H), 7.21 (d, *J* = 2.1 Hz, 1H), 7.19–7.18 (m, 1H), 7.17 (d, *J* = 8.9 Hz, 1H), 7.01–6.98 (m, 2H), 6.81–6.79 (m, 1H), 3.76 (s, 3H), 3.73 (q, *J* = 6.4 Hz, 2H), 3.01 (t, *J* = 6.4 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.3 (C=O), 153.4, 143.7, 139.5, 135.2, 131.9, 131.5, 131.1, 128.7, 128.4, 128.1, 128.0, 127.6, 123.9, 123.8, 112.5, 112.2, 111.9, 111.5, 100.6, 94.1, 55.8 (OCH₃), 40.7 (N–CH₂CH₂), 25.6 (N–CH₂CH₂). Atropisomer B: ¹H NMR (300 MHz, CDCl₃): δ 7.74 (d, *J* = 7.7 Hz, 1H), 7.59 (d, *J* = 7.0 Hz, 2H), 7.38 (t, *J* = 7.7 Hz, 2H), 7.22 (d, *J* = 7.0 Hz, 1H), 7.21 (d, *J* = 2.1 Hz, 1H), 7.21–7.17 (m, 1H), 7.17 (d, *J* = 8.9 Hz, 1H), 6.97–6.94 (m, 2H), 6.79–6.76 (m, 1H), 3.73 (q, *J* = 6.4 Hz, 2H), 3.70 (s, 3H), 2.98 (t, *J* = 6.4 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 166.5 (C=O), 153.4, 143.7, 139.5, 135.2, 131.8, 131.5, 131.1, 128.7, 128.4, 128.1, 128.0, 127.6, 123.9, 123.8, 112.5, 112.2, 111.9, 111.5, 100.6, 94.1, 55.7 (OCH₃), 40.5 (N–CH₂CH₂), 25.5 (N–CH₂CH₂). HRMS (ESI), calcd for C₂₄H₂₂N₂O₃ [M + H]⁺: 387.1682, found: 387.1684. Purity: 99.86%.

3'-Hydroxy-*N*-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3f**). Off-white solid; yield 65%; mp: 74–76 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3286 (N–H stretch), 3067–2940 (C–H stretch aromatic), 1628 (C=O stretch amide), 1583 (N–H bend), 1527–1437 (C=C stretch aromatic), 1211 (C–O–C stretch), and 758 (=C–H bend aromatic). ¹H NMR (300 MHz, CDCl₃): δ 7.52–7.43 (m, 1H), 7.42–7.27 (m, 3H), 7.22 (d, *J* = 8.8 Hz, 1H), 7.16 (t, *J* = 7.8 Hz, 1H), 7.05 (d, *J* = 2.4 Hz, 1H), 7.00 (d, *J* = 2.4 Hz, 1H), 6.86 (d, *J* = 2.4 Hz, 1H), 6.83 (d, *J* = 7.8 Hz, 1H), 6.73 (td, *J* = 8.8, 2.4 Hz, 2H), 3.33 (s, 3H), 2.69–2.64 (m, 2H), 2.50 (t, *J* = 6.0 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): δ 169.5, 157.6, 153.4, 142.0, 139.6, 137.9, 131.8, 130.0, 129.6, 129.5, 128.0, 127.9, 127.4, 123.5, 119.7, 116.0, 114.6, 112.4, 112.0, 111.5, 100.6, 55.8 (OCH₃), 40.0, 25.2. HRMS (ESI), calcd for C₂₄H₂₂N₂O₃ [M + H]⁺: 387.1681, found: 387.1685. Purity: 99.36%.

4'-Hydroxy-*N*-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (**3g**). Off-white solid; yield 62%; mp: 187–189 °C; IR (neat): $\tilde{\nu}$ cm⁻¹ = 3303 (N–H stretch), 3059–2941 (C–H stretch aromatic), 1637 (C=O stretch amide), 1613 (N–H bend), 1520–1435 (C=C stretch aromatic), 1218 (C–O–C stretch), and 761

(=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.49–7.40 (m, 1H), 7.38–7.30 (m, 3H), 7.27–7.20 (m, 3H), 7.06 (d, J = 2.5 Hz, 1H), 7.00 (d, J = 2.5 Hz, 1H), 6.78 (d, J = 8.6 Hz, 2H), 6.72 (dd, J = 8.8, 2.5 Hz, 1H), 3.76 (s, 3H), 3.35 (q, J = 6.6 Hz, 2H), 2.69 (t, J = 6.6 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.8, 157.4, 153.4, 139.4, 137.7, 131.8, 131.3, 130.0, 129.6, 128.0, 127.9, 126.7, 123.6, 115.5, 112.5, 111.9, 111.5, 100.5, 55.8, 40.0, 25.3. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 387.1677, found: 387.1682. Purity: 98.84%.

2',3'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (3h). Off-white solid; yield 56%; mp: 143–145 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3233 (N–H stretch), 3082–2990 (C–H stretch aromatic), 1646 (C=O stretch amide), 1589 (N–H bend), 1556–1440 (C=C stretch aromatic), 1217 (C–O–C stretch), and 789 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.77 (dd, J = 7.6, 2.6 Hz, 1H), 7.50–7.43 (m, 2H), 7.32–7.28 (m, 1H), 7.25 (d, J = 8.8 Hz, 1H), 7.01 (t, J = 7.6 Hz, 1H), 6.98 (d, J = 2.6 Hz, 1H), 6.91–6.85 (m, 2H), 6.82 (dd, J = 7.6, 2.6 Hz, 1H), 6.78 (dd, J = 7.6, 1.6 Hz, 1H), 3.88 (s, 3H), 3.86 (s, 3H), 3.59 (s, 3H), 3.55 (q, J = 6.4 Hz, 2H), 2.68 (t, J = 6.4 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.1, 153.9, 152.7, 145.9, 136.4, 135.6, 134.9, 131.6, 130.6, 129.6, 128.5, 127.8, 127.6, 124.2, 122.7, 122.5, 112.5, 112.2, 111.9, 100.5, 60.7, 55.9, 55.9, 39.5, 24.9. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.5229, found: 431.5233. Purity: 98.29%.

2',4'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (3i). Pale yellow solid; yield 64%; mp: 64–66 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3283 (N–H stretch), 3055–2992 (C–H stretch aromatic), 1644 (C=O stretch amide), 1608 (N–H bend), 1508–1457 (C=C stretch aromatic), 1205 (C–O–C stretch), and 763 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.80 (dd, J = 7.5, 1.6 Hz, 1H), 7.47 (td, J = 7.5, 1.6 Hz, 1H), 7.41 (td, J = 7.5, 1.6 Hz, 1H), 7.29–7.23 (m, 2H), 7.12 (d, J = 8.3 Hz, 1H), 6.98 (d, J = 2.5 Hz, 1H), 6.89 (dd, J = 8.8, 2.5 Hz, 1H), 6.77 (d, J = 2.5 Hz, 1H), 6.53 (dd, J = 8.3, 2.4 Hz, 1H), 6.42 (d, J = 2.4 Hz, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 3.59 (s, 3H), 3.57 (t, J = 6.5 Hz, 2H), 2.73 (t, J = 6.5 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.4, 160.9, 157.3, 154.0, 136.4, 135.6, 131.5, 131.2, 131.1, 130.0, 128.5, 127.6, 127.4, 122.5, 122.0, 112.5, 112.4, 111.9, 104.7, 100.4, 98.5, 55.9, 55.5, 55.2, 39.6, 25.0. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.5182, found: 431.5185. Purity: 99.27%.

2',5'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (3j). Off-white solid; yield 57%; mp: 65–67 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3286 (N–H stretch), 3066–2940 (C–H stretch aromatic), 1639 (C=O stretch amide), 1586 (N–H bend), 1505–1438 (C=C stretch aromatic), 1214 (C–O–C stretch), and 794 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.49–7.41 (m, 2H), 7.39 (d, J = 7.0 Hz, 1H), 7.29 (d, J = 7.9 Hz, 1H), 7.22 (d, J = 8.9 Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 6.98 (d, J = 2.5 Hz, 1H), 6.90 (t, J = 8.7 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 6.80 (d, J = 2.9 Hz, 1H), 6.72 (dd, J = 8.7, 2.5 Hz, 1H), 3.75 (s, 3H), 3.71 (s, 3H), 3.67 (q, J = 6.9 Hz, 2H), 3.60 (s, 3H), 2.67 (t, J = 6.9 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.0, 153.4, 153.3, 150.7, 138.3, 136.7, 131.8, 131.3, 130.7, 129.5, 127.9, 127.7, 127.4, 123.6, 116.9, 113.5, 112.5, 112.3, 112.0,

111.4, 100.5, 56.0, 55.8, 55.8, 39.7, 25.4. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.5212, found: 431.5208. Purity: 99.53%.

2',6'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (3k). Off-white solid; yield 51%; mp: 71–74 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3272 (N–H stretch), 3079–2978 (C–H stretch aromatic), 1643 (C=O stretch amide), 1589 (N–H bend), 1521–1433 (C=C stretch aromatic), 1248 (C–O–C stretch), and 786 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.52–7.43 (m, 2H), 7.40 (d, J = 7.5 Hz, 1H), 7.33 (t, J = 7.5 Hz, 1H), 7.26–7.22 (m, 1H), 7.22–7.18 (m, 1H), 7.14 (d, J = 7.5 Hz, 1H), 7.03 (s, 1H), 6.96 (d, J = 2.6 Hz, 1H), 6.71 (dt, J = 8.4, 1.4 Hz, 1H), 6.66 (dd, J = 8.4, 1.4 Hz, 1H), 3.74 (s, 3H), 3.59 (s, 6H), 3.31 (q, J = 7.0 Hz, 2H), 2.62 (t, J = 7.0 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 168.7, 157.4, 153.4, 138.3, 133.0, 132.4, 131.8, 129.3, 129.3, 127.9, 127.7, 127.1, 123.5, 118.2, 112.5, 111.9, 111.5, 104.6, 100.5, 55.9, 55.8, 39.9, 25.5. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.4928, found: 431.4932. Purity: 97.95%.

3',4'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-2-carboxamide (3l). Pale yellow solid; yield 84%; mp: 70–73 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3286 (N–H stretch), 3064–2975 (C–H stretch aromatic), 1636 (C=O stretch amide), 1587 (N–H bend), 1518–1437 (C=C stretch aromatic), 1212 (C–O–C stretch), and 757 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.72 (dd, J = 7.5, 1.8 Hz, 1H), 7.48 (td, J = 7.5, 1.8 Hz, 1H), 7.41 (td, J = 7.5, 1.8 Hz, 1H), 7.36 (dd, J = 7.5, 1.8 Hz, 1H), 7.26 (d, J = 8.8 Hz, 1H), 6.98–6.93 (m, 2H), 6.92 (s, 1H), 6.86 (td, J = 8.8, 2.6 Hz, 2H), 6.62 (d, J = 2.6 Hz, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.86 (s, 3H), 3.60 (q, J = 6.5 Hz, 2H), 2.77 (t, J = 6.5 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 169.8, 154.0, 148.9, 148.7, 139.1, 135.7, 132.9, 131.5, 130.1, 130.0, 128.8, 127.5, 127.4, 122.5, 121.1, 112.4, 112.2, 112.0, 111.9, 111.2, 100.3, 56.1, 56.0, 55.9, 39.7, 24.7. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.5116, found: 431.5119. Purity: 99.36%.

N-(2-(5-Methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4a).²⁹ Pale yellow solid; yield 93%; mp: 190–192 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3227 (N–H stretch), 3018–2999 (C–H stretch aromatic), 1621 (C=O stretch amide), 1582 (N–H bend), 1539–1447 (C=C stretch aromatic), 1215 (C–O–C stretch), and 747 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.95 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 8.3 Hz, 2H), 7.73 (d, J = 7.4 Hz, 2H), 7.49 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 1H), 7.23 (d, J = 8.7 Hz, 1H), 7.15 (d, J = 1.9 Hz, 1H), 7.07 (d, J = 2.3 Hz, 1H), 6.71 (dd, J = 8.7, 2.3 Hz, 1H), 3.72 (s, 3H), 3.56 (q, J = 7.3 Hz, 2H), 2.94 (t, J = 7.3 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.3, 153.4, 143.1, 139.6, 133.9, 131.8, 129.5, 128.5, 128.3, 128.1, 127.3, 126.9, 123.8, 112.5, 112.2, 111.5, 100.6, 55.7, 40.7, 25.6. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_2$ $[\text{M} + \text{H}]^+$: 371.1767, found: 471.1771. Purity: 98.73%.

4-Fluoro-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4b). Pale brown solid; yield 97%; mp: 120–122 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3292 (N–H stretch), 3125–2984 (C–H stretch aromatic), 1620 (C=O stretch amide), 1595 (N–H bend), 1538–1454 (C=C stretch aromatic), 1214 (C–O–C

stretch), and 767 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.95 (d, J = 8.3 Hz, 2H), 7.75 (d, J = 8.3 Hz, 2H), 7.67 (d, J = 8.7 Hz, 1H), 7.32 (t, J = 8.7 Hz, 2H), 7.23 (d, J = 8.7 Hz, 1H), 7.18–7.10 (m, 2H), 7.06 (dd, J = 7.2, 2.2 Hz, 1H), 6.72 (dd, J = 8.7, 2.2 Hz, 1H), 3.73 (s, 3H), 3.56 (q, J = 7.5 Hz, 2H), 2.94 (t, J = 7.5 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.2, 153.4, 142.0, 136.9, 133.9, 131.8, 131.7, 129.8, 129.4, 129.3, 128.3, 128.1, 126.9, 123.8, 116.4, 116.2, 114.6, 112.5, 112.2, 111.5, 100.6, 55.7, 40.8, 25.6. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{21}\text{FN}_2\text{O}_2$ $[\text{M} + \text{H}]^+$: 389.1677, found: 389.1680. Purity: 96.58%.

4'-Methoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4d). Pale brown solid; yield 36%; mp: 80–72 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3315 (N–H stretch), 3055–2999 (C–H stretch aromatic), 1638 (C=O stretch amide), 1591 (N–H bend), 1536–1456 (C=C stretch aromatic), 1215 (C–O–C stretch), and 754 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.80 (d, J = 8.6 Hz, 2H), 7.73 (d, J = 8.6 Hz, 2H), 7.67 (d, J = 8.6 Hz, 2H), 7.22 (d, J = 8.7 Hz, 1H), 7.13 (d, J = 2.1 Hz, 1H), 7.04 (d, J = 2.3 Hz, 1H), 6.88 (d, J = 8.7 Hz, 2H), 6.71 (dd, J = 8.7, 2.3 Hz, 1H), 3.75 (s, 3H), 3.72 (s, 3H), 3.53 (q, J = 7.5 Hz, 2H), 2.93 (t, J = 7.5 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 165.6, 161.4, 153.4, 136.3, 134.2, 131.8, 131.7, 129.8, 128.1, 125.2, 123.8, 113.4, 112.5, 112.1, 111.5, 100.6, 55.7, 55.3, 40.8, 25.5. HRMS (ESI), calcd for $\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 401.1584, found: 401.1589. Purity: 97.09%.

2'-Hydroxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4e). Off-white solid; yield 58%; mp: 81–83 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3282 (N–H stretch), 3085–2958 (C–H stretch aromatic), 1616 (C=O stretch amide), 1601 (N–H bend), 1546–1437 (C=C stretch aromatic), 1216 (C–O–C stretch), and 754 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.88 (d, J = 8.3 Hz, 2H), 7.58–7.50 (m, 2H), 7.21 (dd, J = 7.6, 1.6 Hz, 1H), 7.24 (d, J = 8.7 Hz, 1H), 7.20 (dd, J = 7.6, 1.6 Hz, 1H), 7.15 (s, 1H), 7.08 (d, J = 2.4 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 6.89 (t, J = 7.6 Hz, 1H), 6.72 (dd, J = 8.7, 2.4 Hz, 1H), 3.73 (s, 3H), 3.56 (q, J = 6.9 Hz, 2H), 2.95 (t, J = 6.9 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.5, 155.0, 153.4, 141.8, 133.8, 133.1, 132.6, 132.5, 132.4, 132.0, 131.9, 131.8, 130.8, 129.5, 129.3, 129.3, 129.2, 128.1, 127.3, 123.8, 119.9, 116.6, 112.5, 112.2, 111.5, 100.6, 55.7, 40.7, 25.7. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 387.1711, found: 387.1715. Purity: 98.61%.

3'-Hydroxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4f). Pale-yellow solid; yield 42%; mp: 98–100 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3317 (N–H stretch), 3067–2987 (C–H stretch aromatic), 1633 (C=O stretch amide), 1588 (N–H bend), 1542–1438 (C=C stretch aromatic), 1215 (C–O–C stretch), and 767 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.93 (d, J = 8.3 Hz, 2H), 7.69 (d, J = 8.3 Hz, 2H), 7.28 (t, J = 7.9 Hz, 1H), 7.24 (d, J = 8.7 Hz, 1H), 7.15 (d, J = 1.6 Hz, 1H), 7.13 (d, J = 7.9 Hz, 1H), 7.09 (s, 1H), 7.08 (d, J = 2.4 Hz, 1H), 6.82 (dd, J = 7.9, 2.1 Hz, 1H), 6.72 (dd, J = 8.7, 2.4 Hz, 1H), 3.73 (s, 3H), 3.56 (q, J = 7.4 Hz, 2H), 2.95 (t, J = 7.4 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.3, 158.4, 153.4, 143.2, 141.1, 133.9, 131.8, 130.5, 128.3, 128.1, 126.8, 123.8, 118.0, 115.5, 114.1, 112.5, 112.2,

111.5, 100.6, 55.7, 40.5, 25.7. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 387.1718, found: 387.1721. Purity: 98.15%.

4'-Hydroxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4g). Pale-yellow solid; yield 26%; mp: 188–190 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3238 (N–H stretch), 3116–2968 (C–H stretch aromatic), 1621 (C=O stretch amide), 1604 (N–H bend), 1544–1440 (C=C stretch aromatic), 1210 (C–O–C stretch), and 770 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.90 (d, J = 8.3 Hz, 2H), 7.67 (d, J = 8.3 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H), 7.23 (d, J = 8.7 Hz, 1H), 7.15 (s, 1H), 7.08 (d, J = 2.2 Hz, 1H), 6.88 (d, J = 8.5 Hz, 2H), 6.72 (dd, J = 8.7, 2.2 Hz, 1H), 3.73 (s, 3H), 3.56 (q, J = 6.9 Hz, 2H), 2.94 (t, J = 6.9 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 166.3, 158.2, 153.4, 143.1, 132.8, 131.8, 130.2, 128.4, 128.2, 128.1, 126.0, 116.3, 112.2, 55.8, 40.8, 25.7. HRMS (ESI), calcd for $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 387.1720, found: 387.1724. Purity: 97.50%.

2',3'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4h). Pale yellow solid; yield 87%; mp: 70–72 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3295 (N–H stretch), 3104–2980 (C–H stretch aromatic), 1635 (C=O stretch amide), 1578 (N–H bend), 1535–1470 (C=C stretch aromatic), 1214 (C–O–C stretch), and 765 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.64 (d, J = 8.3 Hz, 2H), 7.49 (d, J = 8.3 Hz, 2H), 7.18 (s, 1H), 7.04 (t, J = 8.2 Hz, 1H), 7.00–6.96 (m, 2H), 6.87 (dd, J = 8.2, 1.4 Hz, 1H), 6.84 (dd, J = 7.7, 1.4 Hz, 1H), 6.79 (dd, J = 8.8, 2.3 Hz, 1H), 3.83 (s, 3H), 3.74 (q, J = 6.4 Hz, 2H), 3.71 (s, 3H), 3.48 (s, 3H), 3.01 (t, J = 6.4 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 167.4, 154.2, 153.2, 146.5, 141.5, 134.9, 133.2, 132.1, 131.6, 129.5, 127.8, 126.7, 124.3, 123.0, 122.4, 112.8, 112.7, 112.1, 112.1, 100.4, 60.7, 56.0, 55.9, 40.4, 25.4. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.1985, found: 431.1987. Purity: 97.74%.

2',4'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4i). Pale yellow solid; yield 68%; mp: 110–112 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3275 (N–H stretch), 3101–2994 (C–H stretch aromatic), 1610 (C=O stretch amide), 1581 (N–H bend), 1559–1453 (C=C stretch aromatic), 1208 (C–O–C stretch), and 798 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.71 (d, J = 8.5 Hz, 2H), 7.54 (d, J = 8.5 Hz, 2H), 7.28 (s, 1H), 7.25 (d, J = 8.2 Hz, 1H), 7.08 (dd, J = 7.0, 2.4 Hz, 2H), 6.90 (dd, J = 8.8, 2.4 Hz, 1H), 6.62–6.54 (m, 2H), 3.87 (s, 3H), 3.83 (q, J = 6.6 Hz, 2H), 3.81 (s, 3H), 3.81 (s, 3H), 3.09 (t, J = 6.6 Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 167.4, 160.8, 157.5, 154.2, 141.7, 132.4, 131.6, 131.3, 129.5, 127.8, 126.6, 123.0, 122.4, 112.9, 112.6, 112.1, 104.8, 100.4, 99.1, 55.9, 55.6, 55.5, 40.3, 25.4. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.1980, found: 431.1984. Purity: 98.17%.

2',5'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4j). Off-white solid; yield 79%; mp: 139–141 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3413 (N–H stretch), 3126–2993 (C–H stretch aromatic), 1633 (C=O stretch amide), 1580 (N–H bend), 1537–1441 (C=C stretch aromatic), 1216 (C–O–C stretch), and 798 (C-H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.73 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H), 7.28 (s, 1H), 7.08 (dd, J = 8.3, 2.3 Hz, 2H), 6.96–6.92 (m, 1H), 6.92–6.85 (m, 3H), 3.84 (q, J = 6.6 Hz, 2H), 3.82 (s, 3H), 3.82 (s,

3H), 3.76 (s, 3H), 3.09 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 167.4, 154.2, 153.8, 150.7, 141.7, 133.1, 131.6, 130.5, 129.6, 127.8, 126.6, 123.0, 116.6, 113.7, 112.8, 112.8, 112.6, 112.1, 100.4, 56.3, 55.9, 40.4, 25.4. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.1981, found: 431.1985. Purity: 99.60%.

3',4'-Dimethoxy-N-(2-(5-methoxy-1H-indol-3-yl)ethyl)-[1,1'-biphenyl]-4-carboxamide (4l). Pale brown solid; yield 55%; mp: 153–155 °C; IR (neat): $\tilde{\nu}$ cm^{-1} = 3274 (N–H stretch), 3094–2999 (C–H stretch aromatic), 1608 (C=O stretch amide), 1591 (N–H bend), 1555–1449 (C=C stretch aromatic), 1218 (C–O–C stretch), and 795 (=C–H bend aromatic). ^1H NMR (300 MHz, CDCl_3): δ 7.75 (d, $J = 8.3$ Hz, 2H), 7.58 (d, $J = 8.3$ Hz, 2H), 7.30–7.27 (m, 1H), 7.17 (dd, $J = 8.3, 1.9$ Hz, 1H), 7.11 (d, $J = 1.9$ Hz, 1H), 7.10–7.05 (m, 2H), 6.97 (d, $J = 8.3$ Hz, 1H), 6.90 (dd, $J = 8.8, 2.3$ Hz, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.83 (q, $J = 6.6$ Hz, 2H), 3.81 (s, 3H), 3.10 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (CDCl_3 , 75 MHz): δ 167.2, 154.2, 149.3, 149.2, 144.0, 132.9, 132.8, 132.1, 131.6, 127.8, 127.4, 126.8, 123.0, 119.7, 112.9, 112.6, 112.1, 111.5, 110.3, 100.4, 56.0, 55.9, 40.4, 31.0. HRMS (ESI), calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 431.1987, found: 431.1990. Purity: 98.91%.

4.2 Biological assays

4.2.1 Cell lines, culture media, and treatments. The human colorectal adenocarcinoma cell line SW480 and non-malignant cells NCM460 were the basis for biological assays. These cell lines were cultivated in Dulbecco's Modified Eagle Medium (DMEM), enriched with 5% heat-inactivated fetal bovine serum and a 1% antibiotic solution (comprising streptomycin and penicillin) to maintain an aseptic environment and inhibit microbial growth. The cultures were maintained under optimal physiological conditions, specifically at 37 °C within a humidified chamber containing 5% CO_2 , to promote cell viability and preserve cellular integrity. All compounds were solubilized in DMSO before each biological evaluation, ensuring complete solubility in the organic solvent and maintaining a concentration lower than 1% in the culture media.^{53,54} For all experiments, the parental controls (molecule 2 and melatonin) and the standard chemotherapeutic agent 5-fluorouracil (5-FU) were included for comparative purposes. In addition, untreated internal controls were included to ensure the safety of the solvent control (DMSO).

4.2.2 One-dose screening. The initial evaluation of the synthesized hybrids was conducted at a single concentration, employing a modified version of the methodology established by the National Cancer Institute.^{55,56} This screening exclusively involved malignant cells, specifically the SW480 line. Initially, the cells were plated in 96-well plates and allowed to attach for 24 hours. Following this incubation, the test compounds were introduced into the culture media at a final concentration of 100 μM . The endpoint calculations were determined using the sulforhodamine B (SRB) assay, a colorimetric method that quantifies total cellular protein in

adherent cells. In this procedure, cells were fixed with 50% (v/v) trichloroacetic acid (Merck) and subsequently solubilized using a tris-base solution (10 mM). The growth percentages of the treated cells were calculated in relation to the vehicle control, and the results for each tested compound were documented.⁵⁷

4.2.3 Five-dose assay: cytotoxic and antiproliferative activity. In the subsequent phase of the investigation, a series of serial dilutions ranging from 100 μM to 6.25 μM was utilized for the five-dose assay. This phase focused on identifying the most effective hybrid molecule, specifically selecting compounds with inhibition percentages exceeding 60% in the initial single-dose screening. Both malignant and non-malignant cell lines were incorporated to assess the selectivity indices of the tested compounds. The sulforhodamine B (SRB) staining method was applied 48 hours post-treatment, following the same procedural conditions previously outlined.^{58–60}

4.2.4 Optical microscopy evaluation. Cells were seeded in 24-well plates and incubated for 24 hours before the addition of the treatments. After that, hybrids were incorporated into the culture media at three different concentrations (3, 9, and 30 μM) around the IC_{50} value, and then they were incubated for 48 hours. By the end of the treatment, cells were stained with trypan blue (0.4%) and kept in 1 $\times$ PBS. Cells were observed with 20 $\times$ and 40 $\times$ objectives of Motic AE31 Elite inverted microscope. The representative photos for each well were captured from different areas of a well using the camera Moticam ProS5. Live cells (unstained with trypan blue) and dead cells (stained with trypan blue dye). Experiments were performed in three replicates.

4.2.5 Data analysis and statistics. All quantitative experiments were analyzed using GraphPad Prism (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA) for Windows. Data are expressed as the mean $\pm$ standard deviation (SD), derived from two independent biological replicates, each consisting of three technical repetitions to ensure reproducibility and reliability. Dose-response curves were modeled based on the resulting datasets, enabling the calculation of key pharmacological endpoints: GI_{50} (the concentration required to inhibit 50% of cell growth), TGI (the concentration at which complete growth inhibition occurs), and LC_{50} (the concentration that induces a 50% reduction in protein levels at the end of the treatment, relative to initial levels). The Shapiro–Wilk test was used to assess normal distribution. To find statistical significance between treatment groups and controls, one-way analysis of variance (ANOVA) was carried out, considering a p -value lower than 0.05. Comparisons were performed with careful consideration of the specific treatment, compound concentrations, and cell lines used in the experimental design.^{61,62}

4.2.6 In silico ADME-tox studies. Free available platforms such as SwissADME,⁶³ OSIRIS,⁶⁴ T.E.S.T Version 5.1.2,⁶⁵ ProTox-II,⁶⁶ Pred-Herg,⁶⁷ pkCSM,⁶⁸ ToxTree⁶⁹ and ADMET-SAR⁷⁰ were used to estimate biopharmaceutical relevant parameters for most promising hybrids **4j** and **4l**.

Data availability

The data supporting this article contains the experimental details, computational methods, physicochemical, and spectral characterization details of compounds ($^1\text{H-NMR}$, $^{13}\text{C-NMR}$, mass spectra, HPLC analysis).

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This research was funded by the University of Antioquia and the Ministry of Science MINCIENCIAS, through the Program: NanoBioCáncer 2.0 GAT 2.0. Código: 121092092332, grant: 621-2022, project number 92355.

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