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Synthesis of novel isoflavene–propranolol hybrids as anti-tumor agents

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ABSTRACT

Isoflavene–propranolol hybrid molecules were developed as potentially novel anti-tumour agents. Isoflavene itself has potent anti-cancer activity while propranolol can enhance anti-proliferative and anti-angiogenic properties of 5-fluorouracil and paclitaxel. The hybrids were produced via nucleophilic addition of substituted amine groups to a dioxiran intermediate, which was in turn generated from the Williamson-type reaction of isoflavene with (±)-epichlorohydrin. These analogues were tested in anti-cancer cell viability assays against SHEP neuroblastoma and MDA-MB-231 breast adenocarcinoma cell lines, and were found to exhibit potent anti-proliferative activities. These compounds also displayed anti-angiogenic and anti-proliferative effects in HMEC-1 human microvascular endothelial cell lines. Notably, the most potent hybrid molecules synthesized in this work showed enhanced potency against cancer cell lines compared to either isoflavene or propranolol alone, while retaining significant selectivity for cancer cells over MRC-5 normal lung fibroblast cells.

1. Introduction

The most frequent forms of cancer are lung, breast, colorectal, stomach and prostate cancer. It has been estimated that in 2012, prostate cancer (male) and breast cancer (female) would represent 29% of all new cancer cases, and would furthermore account for 9% and 14% of cancer-related deaths in males and females, respectively.[1]

Surgery, chemotherapy, immunotherapy and radiotherapy are some of the many treatment modalities available for cancer patients. Combinations of various therapies are commonly used to combat different types of cancer since combination therapy can show higher efficacies compared to monotherapy.[2] Combination chemotherapy typically involves the administration of two or more chemotherapeutics with distinct mechanisms of action. This allows the drugs to target different aspects of the cancer cells, as well as minimizing chances of resistance to any single drug.

Drugs are conventionally developed following a single-target approach and this has proven to be quite successful in single-target therapy. However, treating more complex multi-factorial diseases such as AIDS and cancer may require the targeting of multiple sites. This has led to an increasing interest in the development of multitarget drugs that are capable of targeting multiple sites either directly or following metabolism.[3]

One major approach to developing multitarget drugs has been termed 'chemical hybridization', whereby new compounds are generated by combining two or more known drugs (or drug pharmacophores) into a single hybrid molecule. Hybridization of biologically active molecules may not only enhance its activity and/or selectivity, but also may reduce adverse effects and overcome multi-drug resistant diseases.[4,5]

Isoflavones are a class of phytoestrogens that are naturally present in soy products. The dietary consumption of isoflavones has been associated with decreased incidences of certain cancers such as breast and lung cancer.[6,7] Genistein is the most predominant isoflavone found in soy products, and it has been shown to possess anti-cancer and anti-oxidative properties.[8] The promising biological activities of natural isoflavones have spurred the development of synthetic analogues with greater potency and bioavailability. The synthetic isoflavene derivative (**1**) shows potent anti-proliferative activity and has been investigated for the treatment of drug-resistant ovarian cancer and prostate cancer in clinical trials.[9]

Due to the rising cost of developing new pharmaceuticals, there has been an increased interest in the repositioning of existing drugs for the potential treatment of other diseases.[10] Propranolol (Fig. 1), a non-selective beta-blocker used primarily for the treatment of cardiovascular diseases, has been recently shown to enhance the anti-angiogenic and anti-proliferative properties of the

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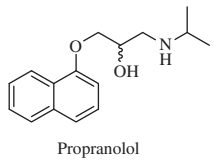


Propranolol

Figure 1. Molecular structure of propranolol.

anti-tumor agents 5-fluorouracil and paclitaxel.[11] This finding together with recent epidemiologic studies[12–14] suggested that propranolol may be used in cancer treatment to enhance the efficacy of chemotherapy.

In this paper, we discuss the synthesis, characterization and in vitro biological activities of a series of newly synthesized isoflavene–propranolol hybrids. Because the anti-cancer properties of isoflavenes are well established, this initial study focused on the development of hybrids retaining the phenyl isoflavene substituent in preference to the naphthalene unit of propranolol.

2. Results and discussion

2.1. Synthesis of disubstituted hybrid molecules

Williamson-type ether synthesis was utilized for the preparation of the dioxiran intermediate **2**. Isoflavene (**1**) was stirred with K_2CO_3 in acetone for 20 min to generate the phenoxide ions. (±)-Epichlorohydrin was added and the resulting solution was heated under reflux for 24 h (Scheme 1).

The crude product was purified by column chromatography to generate the novel dioxiran compound **2** as a mixture of stereoisomers in 63% yield. The ^{1}H NMR spectrum of compound **2** ($CDCl_3$) showed the disappearance of the two broad singlets representing the hydroxyl groups in isoflavene (**1**). The appearance of five new multiplets with an integration of two protons each in the alkyl region indicated the successful attachment of the epoxide. ^{13}C NMR with DEPT-135 indicated the appropriate number of CH and CH_2 resonances. Further confirmation of the structure of product **2** was performed through mass spectrometry and X-ray crystallography of the *S*,*R* diastereoisomer which crystallised from dichloromethane (Fig. 1).

Nucleophilic ring-opening of the epoxides was conducted by treating the dioxiran intermediate **2** with the corresponding amines in a pressure tube under nitrogen at 80–90 °C for 24 h (Scheme 2). The product was then obtained either by removal of the solvent in vacuo followed by recrystallization from EtOAc or CH_2Cl_2, or by precipitation with *n*-hexane followed by filtration. The corresponding disubstituted amine hybrid molecules **3** were obtained as a racemic mixture of diastereoisomers in moderate to good yields (Table 1).

The structure of the isopropyl analogue **3a** was established through ^{1}H and ^{13}C NMR spectroscopy and HRMS data. In the ^{1}H NMR spectrum, the appearance of two new broad singlets at δ 1.55 and 4.95 ppm with an integration of 2 protons each corresponded to the OH and NH groups, respectively. Additionally, two new doublets at δ 0.90 ppm represented the four methyl groups of the isopropyl moiety, while the CH groups appeared as a multiplet at δ 2.50 ppm. The three new carbon signals at δ 49.1 (2 × CH), δ 23.4 (2 × CH_3), and δ 23.2 (2 × CH_3) ppm were assigned to the attached isopropyl groups. Further confirmation was obtained from HRMS (+ESI) which showed the presence of a molecular ion signal at *m*/*z* 471.2843 $[M+H]^+$ corresponding to the molecular formula $C_{26}H_{36}N_2O_5$ (471.2853). The remaining analogues showed similar chemical shift patterns for the OH and NH protons and characteristic peaks in their aliphatic regions corresponding to the respective amine substituents.

2.2. Anti-proliferative properties of hybrid molecules

It was hypothesized that the attachment of the 1-isopropylamine-2-propanol side chain of propranolol onto the isoflavene (**1**) scaffold would furnish hybrid molecules with superior anti-proliferative and anti-angiogenic properties against cancer cells compared to either parent molecule alone. To assess the anti-proliferative properties of the synthesized hybrid molecules, their effects on cell proliferation were assessed in vitro using MDA-MB-231 triple-negative breast cancer cells, SHEP neuroblastoma cells, HMEC-1 human microvascular endothelial cells and MRC-5 human lung fibroblasts.

As shown in Figure 3, all compounds inhibited cell proliferation in a dose-dependent manner. When compared with the parental compounds, isoflavene (GI_{50} = 62.9 μM against MDA-MB-231/ GI_{50} = 8.0 μM against SHEP) and propranolol (205/109 μM) , all hybrid molecules (<6.5/<5.0 μM), with the exception of **3j** (229/96.7 μM), displayed improved anti-proliferative activity against cancer cells.

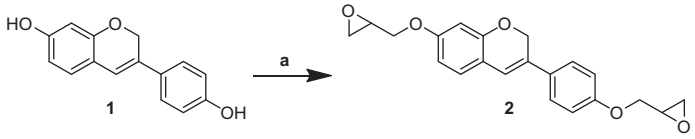
When comparing the anti-proliferative activity of individual hybrid molecules across the two different cancer cell lines, compounds **3a**, **3b**, **3c**, **3d**, and **3f**, showed no significant differences. Isoflavene, compounds **3h**, **3i** and **3j** performed significantly better against SHEP cells while compounds **3e** performed significantly better against MDA-MB-231 cells (Table 2).

When compared with the parental compounds, all hybrid molecules displayed improved anti-proliferative activity against HMEC-1 as compared to propranolol but the results were more variable when compared to isoflavene (**1**). Four compounds (**3b**, **3c**, **3d**, and **3e**) displayed significant increased anti-proliferative properties while four compounds (**3a**, **3g**, **3h**, and **3i**) showed unchanged efficacy and two compounds (**3f** and **3j**) showed decreased activity (Table 2).

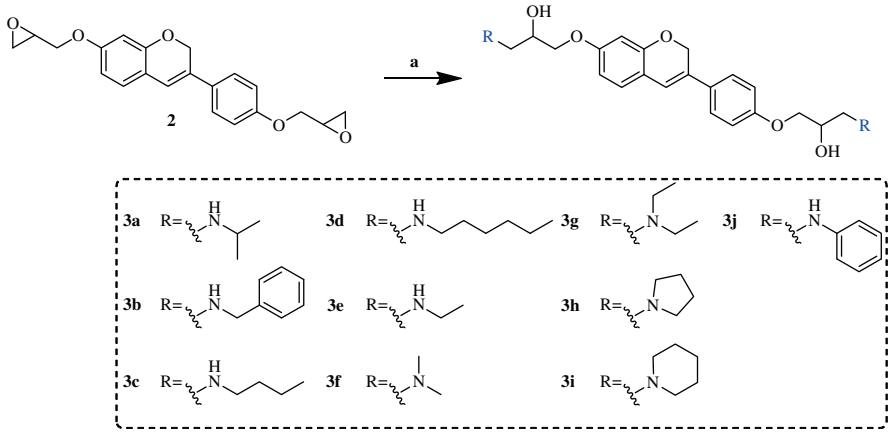
Under physiological conditions, most human cells are quiescent, which means that they are not dividing. Chemotherapy agents should be able to selectively target rapidly dividing cancerous cells while leaving the normal cells unharmed. To assess the specificity of the synthesized hybrid molecules, their toxicity against normal quiescent cells was assessed in vitro using MRC-5 lung fibroblasts (Fig. 2). The anti-proliferative GI_{50} values of the compounds against the various cell lines are summarized in Table 2.

The specificity value of the compounds was calculated as a ratio of the GI_{50} value of the compound against lung fibroblast cells to that in the cell line of interest (Table 3):

$$\text{Specificity} = \frac{GI_{50} \text{ of compound against normal cell}}{GI_{50} \text{ of compound against cell line of interest}}$$



Scheme 1. Reagents and conditions: (a) (i) K_2CO_3 (4 equiv)/acetone, rt, 20 min, and (ii) (±)-epichlorohydrin (4 equiv), reflux, 24 h.



Scheme 2. Reagents and conditions: (a) amine, 24 h, 80–90 °C.

Table 1
Yields of disubstituted hybrid molecules

	R	% Yield		R	% Yield
3a	Isopropylamine	66	**3f**	Dimethylamine	95
3b	Benzylamine	51	**3g**	Diethylamine	96
3c	*n*-Butylamine	65	**3h**	Pyrrolidine	57
3d	*n*-Hexylamine	63	**3i**	Piperidine	80
3e	Ethylamine	73	**3j**	Aniline	63

Isoflavene (**1**) recorded high specificity values against HMEC-1 (specificity value = 26.4) and SHEP (13.6) cell lines, but showed little selectivity for MDA-MB-231 cells (1.7). Compound **3j** showed a similar specificity profile to isoflavene (**1**), but with significantly reduced cytotoxicity. Meanwhile compounds **3c**, **3e–i** (1.8–5.9) exhibited broadly similar specificity values across the three different cell lines. Compounds **3d** (9.6, 9.6) and **3e** (8.1, 11.2) showed the best overall specificity values against SHEP and MDA-MB-231 cells, with ca. 10-fold higher potencies against the cancer cell lines versus the normal lung fibroblasts.

One possible explanation for the rather low anti-proliferative activity of compound **3j** against cancer cell lines may lie in the nature of its amine substituent. The aniline substituent in **3j** is significantly less basic compared to the aliphatic amine moieties found in the other hybrid molecules **3a–i**. According to the ion trapping mechanism/pH partitioning hypothesis,[15] the basic hybrid molecules **3a–i** might be more efficiently trapped in their ionized forms within the acidic intracellular environment of the cancer cells, leading to accumulation of the hybrid molecules within the cell through transmembrane equilibrium. On the other hand, the weakly basic compound **3j** would not be expected to possess the same degree of ionization within the acidic intracellular environment of the cancer cells. Therefore, molecule **3j** may readily traverse the membrane and escape out of the cells, thereby resulting in lower anti-proliferative activity for compound **3j**.

Thus, one possible hypothesis is that the nitrogen group in the hybrid molecules has to be basic in order for the compounds to show anti-proliferative effects against MDA-MB-231 and SHEP cancer cell lines. Furthermore, a weak correlation was observed between the degree of substitution on the nitrogen atom and the anti-proliferative activity of the hybrid molecules. Compounds **3a–e** bearing secondary amine groups exhibited ca. twofold higher potencies against MDA-MB-231 (GI_{50} = 1.91–2.94 μM) and SHEP (1.44–2.96 μM) cell lines compared to compounds **3f–i** that contained tertiary amine groups, which showed activities of 3.85–6.54 and 2.86–4.85 μM for MDA-MB-231 and SHEP, respectively.

Table 2
GI_{50} (μM) values of compounds

Compounds	GI_{50} (μM)			
	HMEC-1	MDA-MB-231	SHEP	MRC-5
Isoflavene (**1**)	4.1	62.9	8.0	109
Propranolol	149	205	109	150
3a	4.7	1.9	1.4	8.2
3b	0.3	2.5	2.7	5.9
3c	1.6	1.9	2.1	6.7
3d	0.7	2.9	3.0	28.3
3e	2.7	2.0	2.8	22.3
3f	9.1	6.5	4.9	23.0
3g	5.7	5.9	4.6	20.0
3h	3.9	4.1	3.6	7.3
3i	3.0	3.9	2.9	16.9
3j	8.3	229	96.7	449

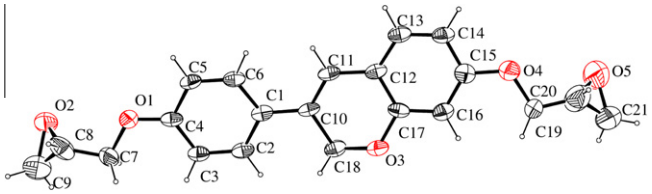


Figure 2. ORTEP diagram of 7-(oxiran-2-ylmethoxy)-3-(4-(oxiran-2-ylmethoxy)phenyl)-2*H*-chromene **2**.

2.3. Anti-angiogenic properties of the hybrid molecules

Tumour angiogenesis, which is defined as the formation of new blood vessels from pre-existing vasculature, is a key factor in cancer progression and metastasis.[16] This complex and dynamic process requires activation, proliferation, migration and morphological differentiation of endothelial cells. Inhibition of one or more of these steps may dramatically alter tumour angiogenesis and potentially slow tumour progression. To assess the anti-angiogenic properties of the synthesized hybrid molecules, their effects on endothelial cell proliferation and morphological differentiation into capillary structures were assessed in vitro using the HMEC-1 endothelial cell line.

Initially, the anti-angiogenic properties of the individual compounds were analyzed at 10 μM (Fig. 4, Table 4). Isoflavene (**1**) induced potent anti-angiogenic effects at this concentration (80 ± 6% vs untreated cells) while propranolol was observed to be inactive under the same conditions. Interestingly, the hybrid compounds

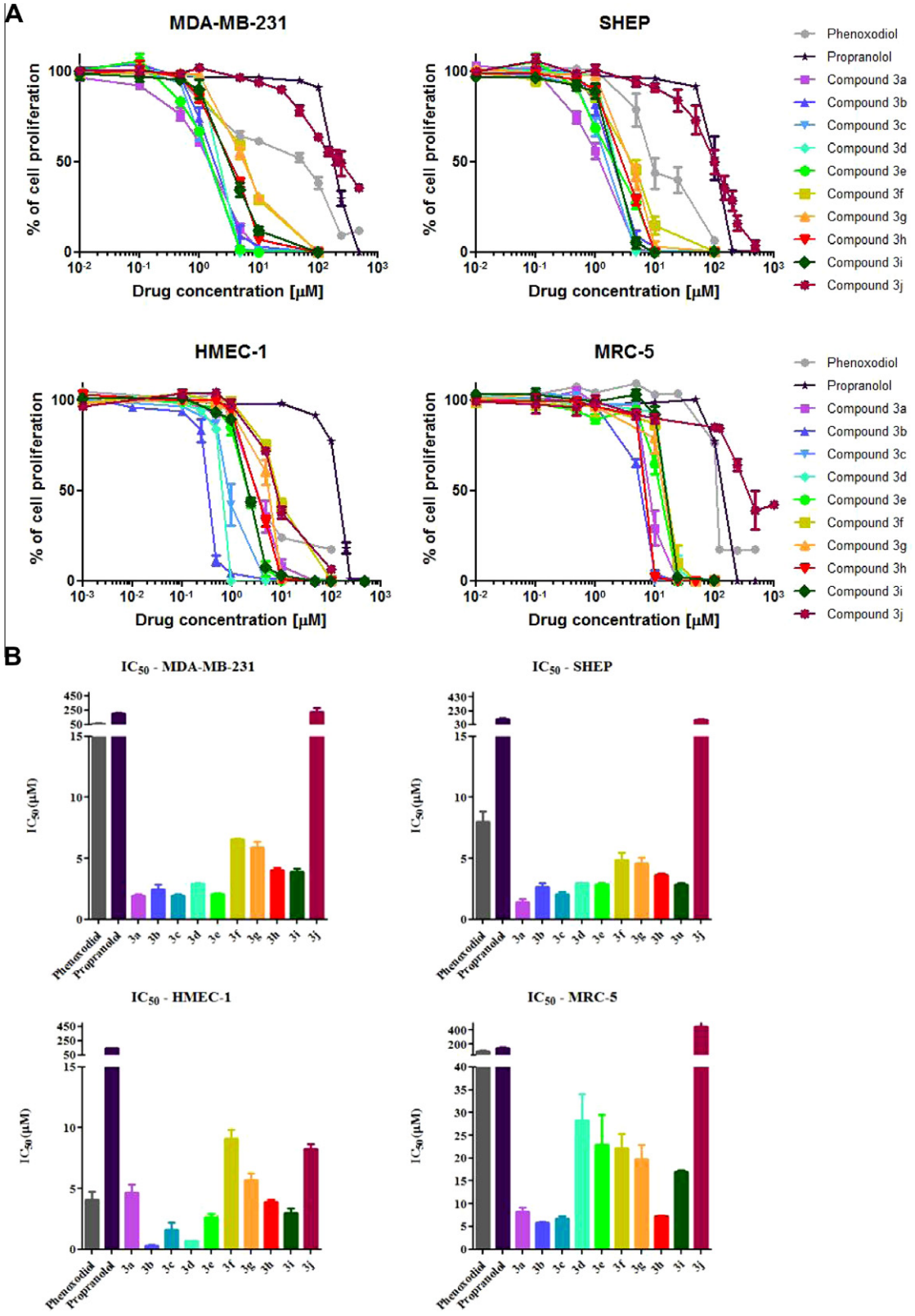


Figure 3. In vitro anti-proliferative properties of isoflavene **1**, propranolol and compounds **3a–j** against cancer, endothelial and normal cells. (A) Growth inhibition assay performed on MDA-MB-231, SHEP, HMEC-1 and MRC-5 cells using Alamar Blue after 72 h incubation with a range of drug concentrations. Points, % of cell proliferation as compared to untreated control cells, means of at least four individual experiments; bars, SE; log scale for *x* axis. (B) Histogram representation of the micro molar concentration of the compounds required to inhibit 50% of MDA-MB-231, SHEP, HMEC-1, and MRC-5 cell proliferation after 72 h drug incubation (GI_{50}). Columns, means of at least four individual experiments; bars, SE.

Table 3
Specificity values of hybrid compounds

Compounds	Specificity value		
	HMEC-1	MDA-MB-231	SHEP
Isoflavene (**1**)	26.4	1.7	13.6
Propranolol	1.0	0.7	1.4
3a	1.8	4.2	5.7
3b	17.6	2.4	2.2
3c	4.2	3.5	3.3
3d	40.3	9.6	9.6
3e	8.6	11.2	8.1
3f	2.5	3.4	4.6
3g	3.5	3.4	4.3
3h	1.9	1.8	2.0
3i	5.7	4.4	5.9
3j	54.2	2.0	4.3

displayed varying anti-angiogenic properties. Compound **3d** produced comparable anti-angiogenic effects to isoflavene while compound **3c** completely suppressed HMEC-1 cell morphological differentiation and angiogenesis at 10 μM. The remaining compounds induced weak anti-angiogenic effects at the same concentration. Interestingly, while compounds **3d** and **3c** were the second and third-most cytotoxic hybrid molecules against HMEC-1 cells, respectively, the most cytotoxic hybrid molecule **3b** showed only 20% inhibition of angiogenesis at 10 μM. This tentatively suggests that the anti-proliferative effects of the hybrid molecules may be partially but not fully dependent upon their anti-angiogenic effects.

Further dose–response studies were then carried out on isoflavene (**1**) and the two most potent anti-angiogenic compounds, **3c** and **3d**. Compound **3j**, which showed 27% inhibition of angiogenesis activity at 10 μM, was also included due to its high anti-proliferative specificity towards HMEC-1 cells.

As compound **3c** induced 100% anti-angiogenic activity at 10 μM, the concentration range for **3c** was reduced to 0.1, 1, and 5 μM to allow for the evaluation of any dose–response relationship effects. The concentration range used for the remaining compounds was 1, 5 and 10 μM. As shown in Figure 4B, compound **3c** exhibited significant (32%) anti-angiogenic effects from 0.1 μM, while isoflavene **1** and compound **3d** at 1 μM significantly inhibited angiogenesis by 18% and 21%, respectively. Compound **3j** only exhibited anti-angiogenic effects (22%) at 1 μM. As expected, the anti-angiogenic potency of the molecules generally increased with compound concentration.

3. Conclusions

This study has shown that the hybridization of isoflavene (**1**) with the 1-(substituted amino)-2-propanol side chains based on propranolol could generate hybrid molecules with improved anti-angiogenic and anti-proliferative properties compared to either parent compound alone. Significantly, the most active hybrid molecules showed up to 30-fold and 4-fold improved potencies against MDA-MB-231 breast adenocarcinoma and SHEP neuroblastoma cancer cell lines, respectively. However, this improvement in anti-cancer activity was accompanied by increased toxicity towards MRC-5 normal lung fibroblast cells. The hybrid molecules **3d** and **3e** stand out as potentially promising anti-cancer agents, as they show significantly higher potency against SHEP and MDA-MB-231 cells compared to isoflavene (**1**) while retaining high specificity values (ca. 10) for the two cancer cell lines versus MRC-5.

The current compounds synthesized and used in the biological screening were a racemic mixture of diastereoisomers. Further studies can be conducted to determine if the anti-proliferative and anti-angiogenic properties of the compounds are enantiospecific or are affected by the interactions between stereoisomers. These interactions may be insignificant or may include synergistic or antagonistic effects. Furthermore, isoflavones are known to have relatively poor bioavailability due to the rapid glucuronidation of the free phenolic groups resulting in excretion of the active compound.[17] These newly synthesized hybrids do not have free phenolic groups, which might increase their bioavailability. Although the hybrids show potent anti-angiogenic properties, the precise mechanism of action is still not clear. Therefore, it would be interesting to investigate the mechanisms of action for these compounds in terms of their effects on particular metabolic or signaling pathways. These studies could provide a basis for the observed SAR of these hybrid molecules and pave the way for the rational design of new hybrids. In particular hybrid molecules with alternative or additional mechanisms of action compared to isoflavene **1** could represent promising candidates for further investigation in future in vitro and in vivo studies.

4. Experimental section

4.1. Materials and methods

All reagents and solvents were obtained from commercial sources and purified if necessary. Melting points were measured using a Mel-Temp melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained on Bruker DPX300 (300 MHz) spectrometer. Mass spectra were recorded on Micromass ZQ2000 (ESI) spectrometer. Infrared spectra were recorded with a Thermo Nicolet 370 FTIR Spectrometer using KBr discs. Column chromatography was carried out using Merck 230–400 mesh ASTM silica gel, and preparative thin layer chromatography was performed using Merck silica gel 7730 60GF^{254}.

4.1.1. 7-(Oxiran-2-ylmethoxy)-3-(4-(oxiran-2-ylmethoxy)phenyl)-2*H*-chromene (2)

Powdered K_2CO_3 (4.0 mmol) was added to a stirred solution of isoflavene **1** (1.0 mmol) in acetone (15 mL) and the mixture was stirred at room temperature for 20 minutes. (±)-Epichlorohydrin (4.0 mmol) was then added and the reaction mixture was stirred at reflux for 48 h. The reaction mixture was then filtered and the filtrate was removed in vacuo to give the crude product. The crude product was purified by column chromatography with DCM (97%) and EtOAc (3%) to obtain the titled compound **2**.

White crystalline solid: 63%; Mp 158–160 °C decomposed; UV–vis (MeOH): λ_{max} 211 (ε 36859 cm^{-1} M^{-1}), 250 (24808), 333 (40665) nm. IR (KBr): ν_{max} 3068, 3002, 2923, 2835, 1611, 1578, 1516, 1451, 1431, 1348, 1315, 1296, 1279, 1248, 1176, 1120, 1029, 970, 913, 865, 825, 771 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (2H, d, *J* 8.87 Hz, ArH), 6.98 (1H, d, *J* 8.37 Hz, ArH), 6.94 (2H, d, *J* 8.87 Hz, ArH), 6.68 (1H, s, *H*C═C), 6.49 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.46 (1H, d, *J* 2.43 Hz, ArH), 5.10 (2H, s, CH_2), 4.27–4.17 (2H, m, CH_2), 4.00–3.92 (2H, m, CH_2), 3.38–3.33 (2H, m, CH), 2.93–2.89 (2H, m, CH_2), 2.80–2.75 (2H, m, CH_2). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.33 (ArC), 158.27 (ArC), 154.38 (ArC), 130.14 (ArC), 128.65 (ArC), 127.59 (*C*═CH), 125.94 (2 × ArCH), 118.35 (HC═C), 116.98 (ArC), 114.98 (2 × ArCH), 108.04 (ArCH), 102.30 (ArCH), 68.96 (2 × CH_2), 67.38 (CH_2), 50.23 (CH), 50.18 (CH), 44.87 (CH_2), 44.82 (CH_2).HRMS (+ESI) *m*/*z* Calcd for $C_{21}H_{20}O_5Na$ $(M+Na)^+$ 375.1208. Found 375.1203

4.1.2. General procedure for nucleophilic ring-opening for formation of hybrid molecule (3a–j)

Dioxiran **2** (0.30 mmol) was added to the corresponding amines (3 mL) in a pressure tube held under a nitrogen atmosphere. The mixture was stirred for 24 h at 80–90 °C and the product was

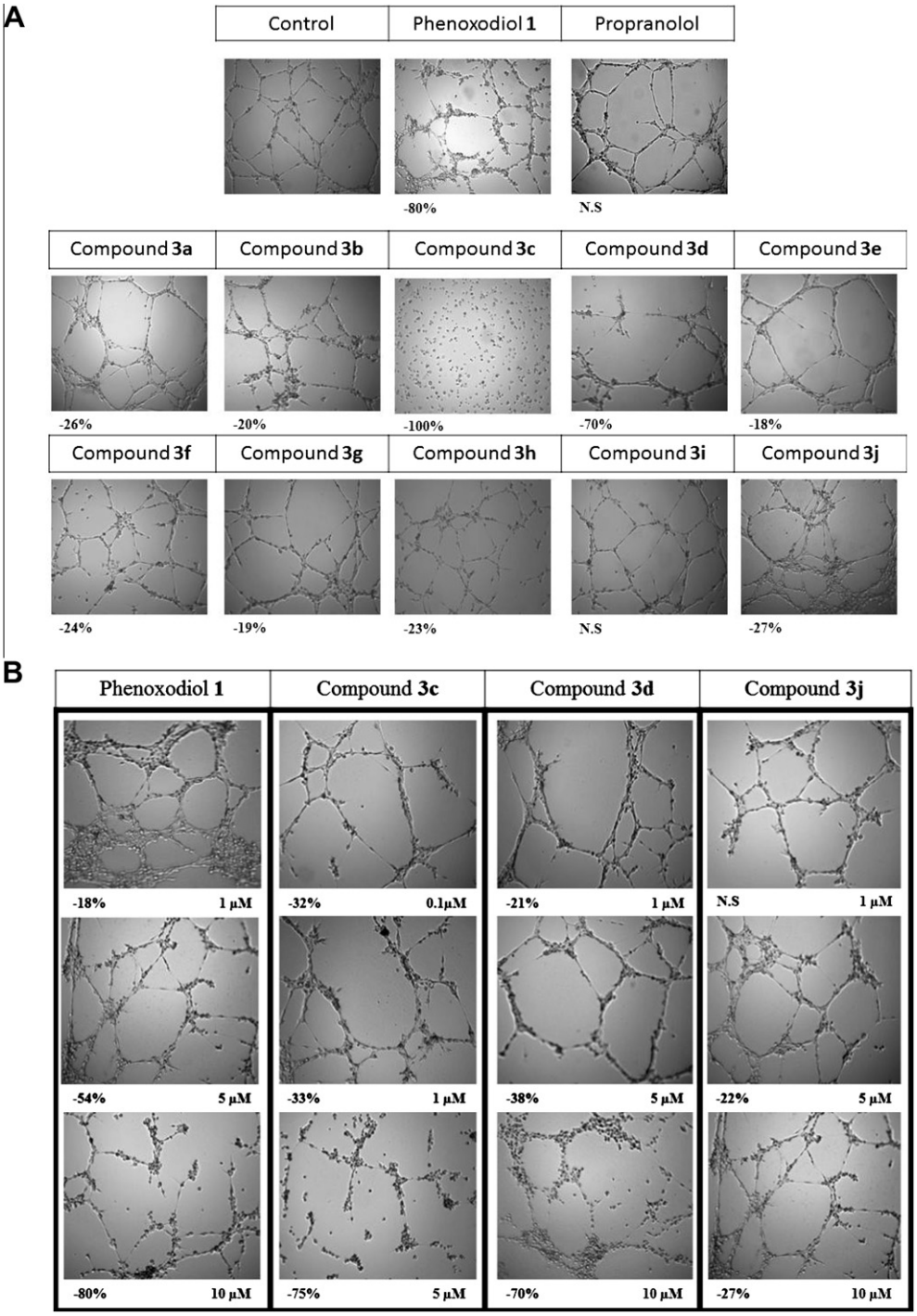


Figure 4. In vitro anti-angiogenic properties of isoflavene 1, propranolol and compounds **3a–j**. Representative photographs of HMEC-1 cells in Matrigel™ assays. (A) HMEC-1 cells were seeded on Matrigel™ for 5 min and then treated with 10 μM of compound. Photographs were taken after 8 h of drug incubation. (B) HMEC-1 cells were seeded on Matrigel™ and then treated with either 0.1, 1, 5 or 10 μM of compound. Photographs were taken after 8 h of drug incubation. Vascular structures were imaged on a Zeiss Axiovert 200M using a 5× objective lens. Inset, % of inhibition as compared to untreated control cells (N.S. = not statistically significant).

obtained either by removal of solvent in vacuo followed by recrystallization from an appropriate solvent (EtOAc or DCM/*n*-hexane), or by precipitation with *n*-hexane followed by filtration to give the desired product.

4.1.3. 1-(4-(7-(2-Hydroxy-3-(isopropylamino)propoxy)-2*H*-chromen-3-yl)phenoxy)-3-(isopropylamino)propan-2-ol (3a)

White solid Yield: 66%; Mp 129–131 °C. UV–vis (MeOH): λ_{max} 211 (ε 23487 cm^{-1} M^{-1}), 250 (15573), 334 (26140) nm; IR (KBr): ν_{max} 3321, 2966, 2924, 2869, 1618, 1577, 1514, 1456, 1383, 1341, 1317, 1285, 1250, 1171, 1135, 1114, 1024, 829 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.10 (2H, s, CH_2), 4.95 (2H, br s, 2 × OH), 4.0–3.85 (6H, m, 2 × OCH_2, 2 × CH), 2.72–2.54 (6H, m, 2 × NHCH_2, 2 × NH*CH*), 1.54 (2H, br s, 2 × NH), 0.99 (6H, d, 2 × CH_3), 0.97 (6H, d, 2 × CH_3); ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.59 (ArC), 158.52 (ArC), 154.39 (ArC), 129.97 (ArC), 128.61 (ArCH), 127.54 (*C*═CH), 125.92 (2 × ArCH), 118.31 (HC═C), 116.91 (ArC), 114.91

Table 4
Anti-angiogenic inhibition effect of compounds

Compounds	Anti-angiogenic inhibition effect at 10 μM (%)
Isoflavene (**1**)	80
Propranolol	Not significant
3a	26
3b	20
3c	100
3d	70
3e	18
3f	24
3g	19
3h	23
3i	Not significant
3j	27

(2 × ArCH), 107.99 (ArCH), 102.27 (ArCH), 70.79 (2 × CH_2), 68.62 (2 × CH), 67.42 (CH_2), 49.32 (2 × CH_2), 49.06 (2 × CH), 23.36 (2 × CH_3), 23.22 (2 × CH_3); HRMS (+ESI) *m*/*z* Calcd For $C_{26}H_{36}N_2O_5$ $(M+H)^+$ 471.2853. Found 471.2843.

4.1.4. 1-(Benzylamino)-3-(4-(7-(3-(benzylamino)-2-hydroxypropoxy)-2H-chromen-3-yl)phenoxy)propan-2-ol (3b)

White powder, Yield: 51%. Mp 164–166 °C. UV–vis (MeOH): λ_{max} 208 (ε 25218 cm^{-1} M^{-1}), 251 (10861), 335 (18701) nm. IR (KBr): ν_{max} 3416, 3061, 3029, 2922, 2867, 1617, 1578, 1514, 1455, 1346, 1318, 1284, 1250, 1171, 1134, 1047, 1026, 831, 746, 697 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.35–7.19 (10H, m, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH) 5.10 (2H, s, CH_2), 4.98 (2H, bt, 2 × OH), 4.01–3.94 (2H, m, 2 × CH), 3.93–3.84 (4H, m, 2 × OCH_2), 3.72 (4H, s, 2 × $BnCH_2$), 2.73–2.54 (4H, m, 2 × $NHCH_2$), 2.16 (2H, br s, 2 × NH). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.53 (ArC), 158.47 (ArC), 154.39 (ArC), 140.11 (2 × ArC), 130.01 (ArC), 128.66 (4×ArCH), 128.24 (6×ArCH), 127.55 (*C*═CH), 127.32 (2 × ArCH), 125.92 (ArC), 118.32 (HC═C), 114.92 (2 × ArCH), 107.99 (ArCH), 102.28 (ArCH), 70.66 (2 × CH_2), 68.52 (2 × CH), 67.41 (CH_2), 53.98 (2 × CH_2), 51.27 (2 × CH_2). HRMS (+ESI) *m*/*z* Calcd for $C_{34}H_{38}N_2O_5$ $(M+H)^+$ 567.2853. Found 567.2839.

4.1.5. 1-(Butylamino)-3-(4-(7-(3-(butylamino)-2-hydroxypropoxy)-2*H*-chromen-3-yl)phenoxy)propan-2-ol (3c)

White powder, Yield: 65%. Mp 163–165 °C. UV–vis (MeOH): λ_{max} 202 (ε 16705 cm^{-1} M^{-1}), 250 (8477), 333 (14212) nm. IR (KBr): ν_{max} 3318, 2957, 2926, 2872, 1618, 1577, 1514, 1457, 1380, 1347, 1318, 1284, 1250, 1171, 1136, 1040, 1013, 876, 830, 811 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.10 (2H, s, CH_2), 4.01–3.82 (6H, m, 2 × OCH_2, 2 × CH), 2.73–2.66 (2H, m, CH_2), 2.64–2.60 (2H, m, CH_2), 2.56 (4H, t, 2 × CH_2), 1.40 (4H, sextet, 2 × CH_2), 1.30 (4H, sextet, 2 × CH_2), 0.88 (6H, t, 2 × CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.59 (ArC), 158.53 (ArC), 154.39 (ArC), 129.97 (ArC), 128.61 (ArCH), 127.54 (*C*═CH), 125.92 (2 × ArCH), 118.31 (HC═C), 116.85 (ArC), 114.91 (2 × ArCH), 108.00 (ArCH), 102.27 (ArCH), 70.79 (2 × CH_2), 68.28 (2 × CH), 67.42 (CH_2), 51.83 (2 × CH_2), 49.72 (2 × CH_2), 32.49 (2 × CH_2), 20.52 (2 × CH_2), 14.14 (2 × CH_3). HRMS (+ESI) *m*/*z* Calcd for $(M+H)^+$ $C_{29}H_{43}N_2O_5$ 499.3166. Found 499.3155.

4.1.6. 1-(Hexylamino)-3-(4-(7-(3-(hexylamino)-2-hydroxypropoxy)-2*H*-chromen-3-yl)phenoxy)propan-2-ol (3d)

White powder, Yield: 63%. Mp 162–164 °C. UV–vis (MeOH): λ_{max} 212 (ε 15811 cm^{-1} M^{-1}), 250 (10984), 333 (18806) nm. IR (KBr): ν_{max} 3321, 3133, 2955, 2924, 2924, 2854, 1619, 1577, 1515, 1458, 1348, 1318, 1285, 1253, 1171, 1137, 1108, 1035, 1011, 877, 832, 8112 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH) 5.10 (2H, s, CH_2), 4.94 (2H, br s, 2 × OH), 4.00–3.90 (2H, m, 2 × CH), 3.89–3.82 (4H, m, 2 × OCH_2), 2.68–2.55 (4H, m, 2 × CH_2), 2.54–2.45 (4H, m, 2 × CH_2), 1.71 (2H, br s, 2 × NH), 1.41–1.35 (4H, m, 2 × CH_2), 1.28–1.25 (12H, m, 6×CH_2), 0.86 (6H, t, 2 × CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.60 (ArC), 158.53 (ArC), 154.39 (ArC), 129.97 (ArC), 128.61 (ArCH), 127.54 (*C*═CH), 125.91 (2 × ArCH), 118.31 (HC═C), 116.84 (ArC), 114.91 (2 × ArCH), 108.00 (ArCH), 102.27 (ArCH), 70.78 (CH_2), 70.74 (CH_2), 68.29 (2 × CH), 67.41 (CH_2), 51.81 (2 × CH_2), 50.06 (2 × CH_2), 31.90 (2 × CH_2), 30.34 (2 × CH_2), 27.08 (2 × CH_2), 22.77 (2 × CH_2), 14.20 (2 × CH_3). HRMS (+ESI) *m*/*z* Calcd for $(M+H)^+$ $C_{32}H_{48}N_2O_5$ 555.3793. Found 555.3779.

4.1.7. 1-(Ethylamino)-3-(4-(7-(3-(ethylamino)-2-hydroxypropoxy)-2*H*-chromen-3-yl)phenoxy)propan-2-ol (3e)

White powder, Yield: 76%. Mp 144–146 °C. UV–vis (MeOH): λ_{max} 201 (ε 21081 cm^{-1} M^{-1}), 251 (11305), 334 (17420) nm. IR (KBr): ν_{max} 3319, 2963, 2928, 2863, 1618, 1577, 1514, 1457, 1386, 1347, 1317, 1284, 1251, 1170, 1135, 1042, 1012, 874, 830, 809 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.10 (2H, s, CH_2), 4.95 (2H, br s, 2 × OH), 4.00–3.83 (6H, m, 2 × OCH_2, 2 × CH), 2.65–2.54 (8H, m, 4×CH_2), 1.87 (2H, br s, 2 × NH), 1.02 (6H, t, 2 × CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.58 (ArC), 158.51 (ArCH), 154.40 (ArC), 129.97 (ArC), 128.61 (ArC), 127.55 (*C*═CH), 125.91 (2 × ArCH), 118.40 (HC═C), 116.85 (ArC), 114.91 (2 × ArCH), 107.99 (ArCH), 102.27 (ArCH), 70.79 (CH_2), 70.74 (CH_2), 68.37 (2 × CH), 67.39 (CH_2), 51.66 (2 × CH_2), 44.25 (2 × CH_2), 15.56 (2 × CH_3). HRMS (+ESI) *m*/*z* Calcd for $(M+H)^+$ $C_{25}H_{35}N_2O_5$ 443.2540. Found 443.2529.

4.1.8. 1-(Dimethylamino)-3-(4-(7-(3-(dimethylamino)-2-hydroxypropoxy)-2*H*-chromen-3-yl)phenoxy)propan-2-ol (3f)

White powder, Yield: 95%. Mp 109–111 °C. UV–vis (MeOH): λ_{max} 211 (ε 29289 cm^{-1} M^{-1}), 250 (18587), 334 (31340) nm. IR (KBr): ν_{max} 3384, 2936, 2826, 2779, 1616, 1577, 1514, 1460, 1387, 1346, 1317, 1284, 1251, 1170, 1135, 1117, 1042, 1009, 974, 876, 828 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.22 (2H, br s, 2 × OH), 5.10 (2H, s, CH_2), 4.00–3.83 (6H, m, 2 × OCH_2, 2 × CH), 2.47–2.41 (2H, m, CH_2), 2.40–2.36 (2H, m, CH_2), 2.35 (12H, s, 4×CH_3). ^{13}C NMR (300 MHz, DMSO-d_6): δ 159.37 (ArC), 158.37 (ArC), 153.75 (ArC), 128.51 (ArC), 127.90 (ArCH), 127.16 (*C*═CH), 125.28 (2 × ArCH), 117.60 (HC═C), 116.07 (ArC), 114.31 (2 × ArCH), 107.39 (ArCH), 101.70 (ArCH), 71.01 (CH_2), 70.94 (CH_2), 66.56 (CH_2), 66.40 (CH), 66.35 (CH), 46.15 (CH_3), 45.77 (CH_3), 45.59 (CH_3), 45.56 (CH_3). HRMS (+ESI) *m*/*z* Calcd for $C_{25}H_{35}N_2O_5$ $(M+H)^+$ 443.2541. Found 443.2532.

4.1.9. 1-(Diethylamino)-3-(4-(7-(3-(diethylamino)-2-hydroxypropoxy)-2*H*-chromen-3-yl)phenoxy)propan-2-ol (3g)

Light brown powder, Yield: 96%. Mp 53–55 °C. UV–vis (MeOH): λ_{max} 201 (ε 23487 cm^{-1} M^{-1}), 250 (12666), 335 (20644) nm. IR (KBr): ν_{max} 3406, 2967, 2923, 2863, 1616, 1577, 1514, 1456, 1385, 1346, 1317, 1284, 1250, 1203, 1166, 1133, 1087, 1034, 979, 872, 828, 806 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.10 (2H, s, CH_2), 4.76

(2H, br s, 2 × OH), 4.03–3.93 (6H, m, 2 × OCH_2, 2 × CH), 2.72–2.49 (12H, m, 6×CH_2), 1.06 (12H, t, 4×CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.74 (ArC), 158.66 (ArC), 154.34 (ArC), 129.85 (ArC), 128.56 (ArCH), 127.49 (*C*═CH), 125.85 (2 × ArCH) 118.27 (H*C*═C), 116.74 (ArC), 114.90 (2 × ArCH), 108.04 (ArCH), 102.22 (ArCH), 70.77 (CH_2), 70.73 (CH_2), 67.42 (CH_2), 65.96 (CH), 65.95 (CH), 55.99 (2 × CH_2), 47.32 (4×CH_2), 12.12 (4×CH_3). HRMS (+ESI) *m*/*z* Calcd for $C_{29}H_{43}N_2O_5$ $(M+H)^+$ 499.3172. Found 499.3156.

4.1.10. 1-(4-(7-(2-Hydroxy-3-(pyrrolidin-1-yl)propoxy)-2*H*-chromen-3-yl)phenoxy)-3-(pyrrolidin-1-yl)propan-2-ol (3h)

Light brown powder, Yield: 57%. Mp 130–132 °C. UV–vis (MeOH): λ_{max} 212 (ε 12959 cm^{-1} M^{-1}), 250 (8013), 335 (13948) nm. IR (KBr): ν_{max} 3375, 2963, 2872, 2801, 1615, 1577, 1514, 1485, 1348, 1317, 1284, 1250, 1170, 1135, 1116, 1085, 1040, 889, 829 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH) 5.10 (2H, s, CH_2), 4.90 (2H, br s, 2 × OH), 4.12–4.06 (2H, m, 2 × CH), 3.99 (4H, d, 2 × CH_2), 2.88–2.81 (2H, m, CH_2), 2.79–2.73 (4H, m, 2 × CH_2), 2.59–2.53 (6H, m, 3×CH_2), 1.82–1.80 (8H, m, 4×CH_2). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.65 (ArC), 158.58 (ArC), 154.37 (ArC), 129.93 (ArC), 128.60 (ArCH), 127.52 (*C*═CH), 125.89 (2 × ArCH), 118.30 (H*C*═C), 116.81 (ArC), 114.92 (2 × ArCH), 108.04 (ArCH), 102.26 (ArCH), 70.69 (CH_2), 70.64 (CH_2), 67.42 (CH_2), 67.37 (2 × CH), 58.62 (2 × CH_2), 54.38 (4×CH_2), 23.80 (4×CH_2). HRMS (+ESI) *m*/*z* Calcd for $C_{28}H_{40}N_2O_5$ $(M+H)^+$ 495.2854. Found 495.2840.

4.1.11. 1-(4-(7-(2-Hydroxy-3-(piperidin-1-yl)propoxy)-2*H*-chromen-3-yl)phenoxy)-3-(piperidin-1-yl)propan-2-ol (3i)

White powder, Yield: 80%. Mp 151–153 °C. UV–vis (MeOH): λ_{max} 202 (ε 38939 cm^{-1} M^{-1}), 250 (21996), 333 (36326) nm. IR (KBr): ν_{max} 3412, 2933, 2852, 2802, 1615, 1577, 1514, 1455, 1440, 1385, 1317, 1283, 1250, 1169, 1116, 1039, 990, 875, 828, 809 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.06 (1H, d, *J* 8.37 Hz, ArH), 6.96 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.50 (1H, dd, *J* 8.37, 2.43 Hz, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH) 5.10 (2H, s, CH_2), 4.90 (2H, bt, 2 × OH), 4.10–4.04 (2H, m, CH), 3.97 (4H, d, 2 × CH_2), 2.62–2.50 (4H, m, 2 × CH_2), 2.49–2.47 (4H, m, 2 × CH_2), 2.39–2.38 (4H, m, 2 × CH_2), 1.62–1.56 (8H, m, 4×CH_2), 1.49–1.44 (4H, m, 2 × CH_2). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.73 (ArC), 158.65 (ArC), 154.35 (ArC), 129.88 (ArC), 128.58 (ArCH), 127.50 (*C*═CH), 125.87 (2 × ArCH), 118.29 (H*C*═C), 116.76 (ArC), 114.92 (2 × ArCH), 108.07 (ArCH), 102.24 (ArCH), 70.74 (CH_2), 70.69 (CH_2), 67.42 (CH_2), 65.42 (2 × CH), 61.21 (2 × CH_2), 54.87 (4×CH_2), 26.26 (4×CH_2), 24.38 (2 × CH_2). HRMS (+ESI) *m*/*z* Calcd for $C_{31}H_{43}N_2O_5$ $(M+H)^+$ 523.3166. Found 523.3147.

4.1.12. 1-(4-(7-(2-Hydroxy-3-(phenylamino)propoxy)-2*H*-chromen-3-yl)phenoxy)-3-(phenylamino)propan-2-ol (3j)

Light grey powder, Yield: 63%. Mp 126–128 °C. UV–vis (MeOH): λ_{max} 202 (ε 100724 cm^{-1} M^{-1}), 247 (59070), 333 (43809) nm. IR (KBr): ν_{max} 3396, 3050, 2926, 2869, 1604, 1578, 1511, 1456, 1434, 1316, 1275, 1248, 1169, 1132, 1115, 1032, 827, 751, 694 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/DMSO-d_6): δ 7.46 (2H, d, *J* 8.87 Hz, ArH), 7.09–7.03 (5H, m, ArH), 6.98 (1H, d, *J* 8.87 Hz, ArH), 6.88 (1H, s, *H*C═C), 6.61 (4H, dt, *J* 8.8 Hz, ArH), 6.54–6.50 (3H, m, ArH), 6.43 (1H, d, *J* 2.43 Hz ArH), 5.55 (2H, bq, 2 × NH), 5.16 (2H, bt, 2 × OH), 5.10 (2H, s, CH_2), 4.01–3.91 (6H, m, 2 × CH_2, 2 × CH), 3.26–3.17 (2H, m, CH_2), 3.12–3.04 (2H, m, CH_2). ^{13}C NMR (300 MHz, $CDCl_3$): δ 159.31 (ArC), 158.26 (ArC), 154.47 (ArC), 148.19 (ArC), 130.23 (ArC), 129.51 (4×ArCH), 128.69 (ArCH), 127.66 (*C*═CH), 126.03 (2 × ArCH), 118.40 (ArCH), 118.29 (H*C*═C), 118.23 (ArCH), 117.08 (ArC), 114.93 (2 × ArCH), 113.44 (4×ArCH), 107.93 (ArCH), 102.33 (ArCH), 70.39 (CH_2), 70.35 (CH_2), 68.95 (CH), 68.91 (CH), 67.42 (CH_2), 46.74 (2 × CH_2). HRMS (+ESI) *m*/*z* Calcd for $C_{33}H_{34}N_2O_5Na$ $(M+Na)^+$ 561.2360. Found 561.2346.

4.2. Biological experiments

4.2.1. Cell biology techniques

The human microvascular endothelial cell line HMEC-1 was cultured on 0.1% gelatin-coated flask using MCDB-131 medium (Invitrogen, Mount Waverley, Australia) supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin and 2 mM L-glutamine (PSG), 1 μg/mL hydrocortisone and 10 ng/mL epithelial growth factor (BioScientific, Gymea, Australia). The human neuroblastoma cell line, SHEP, and human breast cancer cell line, MDA-MB-231, were cultured in DMEM medium (Invitrogen) supplemented with 10% FCS, 1% PSG. Human lung fibroblasts, MRC-5, were cultured in MEM medium (Invitrogen) supplemented with 10% FCS, 1% PSG, 2% sodium bicarbonate, 1% non-essential amino acids and 1% sodium pyruvate. All cell lines were maintained at 37 °C in 5% CO_2 as an adherent monolayer and were passaged upon reaching confluence by standard cell culture techniques.

4.2.2. Cell viability assays

HMEC-1, SHEP and MDA-MB-231 cells were seeded at 3750, 6000 and 6000 cells per well in 96-well plates respectively to ensure sustained exponential growth for 4 days. MRC-5 cells were seeded at 30,000 cells per well in 96-well plates to ensure full confluence (quiescence). Cells were treated 24 h after seeding with a range of concentrations from 0.1 to 1000 μM of compounds **3a**–**j**. After 72 h drug incubation, the metabolic activity was detected by spectrophotometric analysis by assessing the absorbance of Alamar blue as previously described by Pasquier et. al.[11] Cell proliferation was determined and expressed as a percentage of untreated control cells. The determination of GI_{50} values was performed using GraphPad Prism 5 (San Diego, CA, USA).

4.2.3. Anti-angiogensis assay using Matrigel™

The anti-angiogenic properties of the compounds were determined by using the Matrigel™ assay, as previously described by Pasquier et. al.[11,18] Briefly, 24-well plates were coated at 4 °C with 250 μL of Matrigel™ solution, which was then allowed to solidify at 37 °C for 1 h before cell seeding. HMEC-1 cells were seeded at 100,000 cells per well and allowed to adhere for 5 min before treatment was initiated. Cells were treated with 0.1 to 10 μM of the compounds. Photographs were taken after 8 h of drug incubation using the 5× objective of an Axiovert 200 M fluorescent microscope coupled to an AxioCamMR3 camera driven by AxioVision 4.8 software (Carl Zeiss, North Ryde, Australia). The total surface area of capillary tubes formed was measured in 5 view fields per well using AxioVision 4.8 software to quantitatively evaluate the anti-angiogenic properties of the compounds.

4.2.4. Statistical analysis

All in vitro experiments were performed at least in quadruplicate and statistical significance was determined using the two-sided Student's *t*-test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc).

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