Design, Synthesis and Biochemical Evaluation of Estrogen Receptor Ligand Conjugates as Tumour Targeting Agents

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Abstract: The estrogen receptors (ERα and ERβ), are ligand inducible nuclear receptors which play a key role in many cellular functions through specific gene expression regulation. The estrogen receptor is regarded as an attractive therapeutic target for hormone-dependent breast cancers. The antiestrogen drug tamoxifen is useful in the treatment of breast cancer. To develop new ER targeting agents as probes of estrogen receptor action, the synthesis and preliminary biochemical evaluation of five structurally varied estrogen receptor ligand conjugates containing the tamoxifen metabolite endoxifen are now reported. These structurally varied conjugates bind to the estrogen receptor (commonly overexpressed in breast cancer cells) and contain DNA alkylating, aromatase inhibitor and COX2 inhibitor moieties. The ER targeting group endoxifen (*E/Z* 4-hydroxy-N-desmethyltamoxifen) was selected for its ability to bind to the estrogen receptor. Compound **11** exhibited moderate antiproliferative activity IC_{50} = 1.64 μM in MCF-7 breast cancer cells, while compound **9b** demonstrated the most potent ER binding effects with IC_{50} values of 35.6 nM(ERα), 19.5 nM(ERβ).

Keywords: Estrogen receptor, Conjugates, Tamoxifen, Tumour targeting, Antiproliferative activity, Breast cancer.

INTRODUCTION

Nuclear receptors play a key role in many cellular functions through specific gene expression regulation and are targeted by a large number of both endogenous and exogenous ligands [1]. The majority of early stage breast cancers, in both pre- and postmenopausal women, are hormone-dependent [2]. Estradiol, the endogenous hormonal ligand, has a key role in the development and progression of the tumour and the estrogen receptor is regarded as an attractive target for hormone-dependent breast cancers [3-5]. Tamoxifen **1a** is widely used as an estrogen receptor antagonist in the treatment of breast cancer. However, many cancer chemotherapies lack specificity which can lead to toxicity and undesirable side effects and more selective chemotherapeutic approaches have been investigated to target tumours. For example, agents targeting nuclear receptors over-expressed in tumours can be directed to malignant tissue and result in improved chemotherapeutic treatments. In hormone-dependent cancers, such as certain breast cancers, a number of structurally varied estrogen receptor ligand conjugates have previously been investigated, attempting to take advantage of the presence of over-expressed estrogen receptor [6]. Conjugate compounds containing multiple ligands, separated by covalent linking groups, can exert a synergistic and improved selective action on the target disease [7]. Many examples of the coupling of cytotoxic agents to the steroid estradiol and related scaffolds, hexestrol, diethylstilbestrol and tamoxifen have been reported [8-10]. Conjugates of estradiol with geldanamycin [11], the indole alkaloid ellipticine [12], mitomycin C [13], doxorubicin [14, 15] and 5-fluorouracil [16] have been found to show lower ER binding affinities and variable cytotoxicity effects in ER positive breast tumour cells. Doxorubicin–formaldehyde conjugates linked to the antiestrogen 4-hydroxytamoxifen incorporating a base labile N-Mannich linking group have been reported to inhibit the growth of breast cancer cell lines with enhanced activity relative to doxorubicin [17, 18]. 17β-Estradiol platinum (II) complexes have demonstrated antiproliferative enhanced activity (when compared to cisplatinum) in ER positive and ER negative breast cancer cell lines [19, 20].

To develop new ER targeting agents as probes of estrogen receptor action, the synthesis and ER binding effects of novel conjugates containing components with known activity in the prevention of breast cancer proliferation (e.g. chlorambucil and indomethacin), linked to the tamoxifen metabolite endoxifen are now reported. The ER targeting group endoxifen (*E/Z* 4-hydroxy-N-desmethyltamoxifen, **1c**) was selected for its ability to bind to the estrogen receptor. Endoxifen has been shown to be the major metabolite of tamoxifen **1a,** and is present at greater concentrations than 4-hydroxytamoxifen **1b** in human plasma in breast cancer patients treated with tamoxifen [21].

Chlorambucil **2** is a nitrogen mustard alkylating agent used in the treatment of select chronic lymphocytic leukaemia and advanced ovarian and breast carcinomas. Alkylating agents have limited selectivity towards malignant cells over normal tissue and as a result can be quite toxic. By coupling alkylating agents such as chlorambucil, to a carrier agent such as endoxifen which targets tumour cells, the selectivity of the agents would be increased, their toxicity diminished and their efficacy improved. The synthesis of an endoxifen conjugate with indomethacin **3** was of interest as there is considerable evidence showing that prostaglandins

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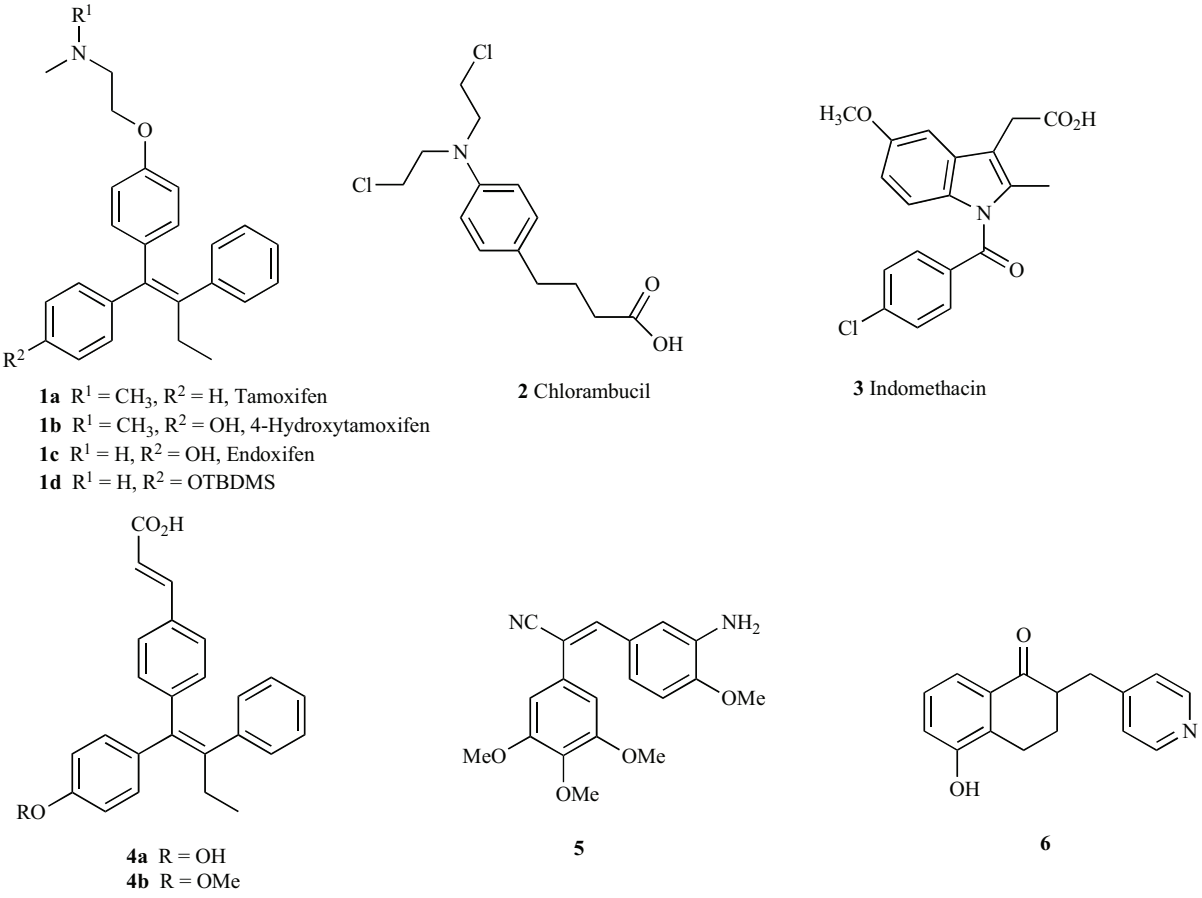


Fig. (1). Structures of estrogen receptor antagonists **1a-d** and compounds **2-6**.

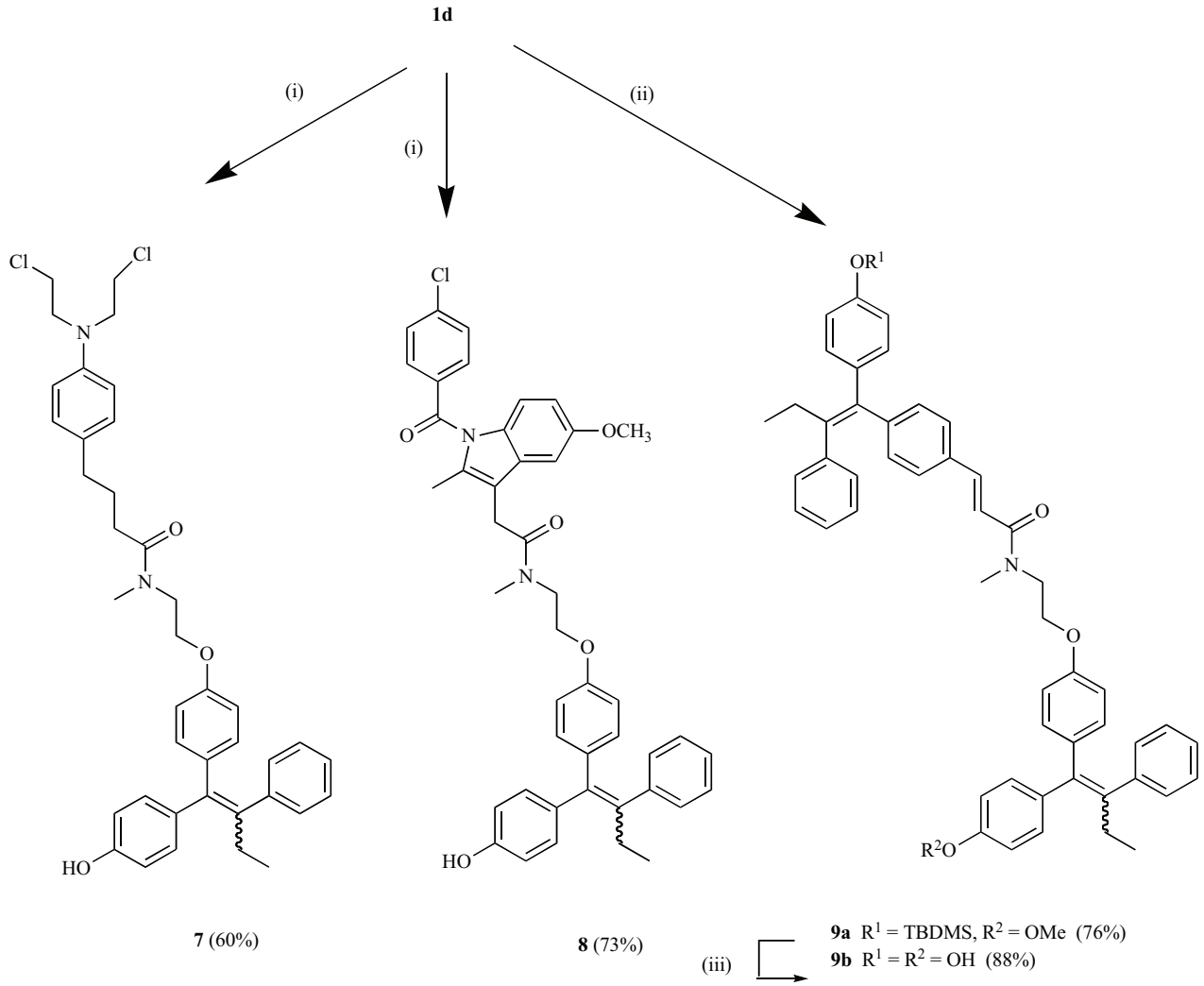
(PGs) play an important role in breast cancer development and growth [22] and there has been interest in a possible role of COX inhibitors such as indomethacin in the prevention of malignancy. Cyclooxygenase-2 (COX-2) is overexpressed in several epithelial tumours, including breast cancer. The COX-2 inhibitor indomethacin **3** has been shown to induce apoptosis and reduce the proliferation rate of HT-29 colon cancer [23], and MCF-7 cancer cell lines [24] and also exerts a synergistic effect when combined with other chemotherapeutic drugs such as doxorubicin and vincristine [25]. We wished to determine if the indomethacin fragment of the conjugate, covalently bound *via* an amide linkage to endoxifen, would act as a bulky side chain to displace the helix-12 in the ER binding site resulting in increased (or pure) antagonist activity.

A dual ER-ligand conjugate was also synthesised comprising of the fragments endoxifen **1c** and GW7604, **4a**, a selective estrogen receptor downregulator [26], in which the dimethylaminoethoxy group of tamoxifen is replaced by an acrylate side chain. A secondary binding site exists on the ER, also known as the antiestrogen binding site (AEBS) that demonstrates high affinity binding of triarylethylene antiestrogens (i.e. specifically tamoxifen and its hydroxy derivatives) which is not in competition with the binding of estrogens [27, 28]. By synthesising a bivalent ER-ligand conjugate we wish to probe the possibility of binding in both the ER and AEBS sites concurrently. The binding properties of bivalent ER targeting ligands based on the nonsteroidal estrogen hexestrol have been reported by Bergmann [29]. We wished to investigate any antagonistic effect that the presence of one ER-ligand covalently linked to a second ER-ligand would have on antiproliferative activity through the displacement of Helix-12 in the ER binding site.

The acrylonitrile combretastatin analogue **5** was chosen for conjugation with endoxifen **1c** as an example of a potent antitubulin drug with cytotoxic effects in against murine Colon 26 adenocarcinoma [30]. A conjugate incorporating the tetralone aromatase inhibitor **6** [31] was also examined. Inhibitors of aromatase enzyme reduce the production of estrogen thus decreasing the stimulation of breast tumours, hence developing multitargeting agents may result in more selective drug delivery to the tumour target site.

CHEMISTRY

Endoxifen **1c** was synthesised [17], and used as the protected *tert*-butyldimethylsilyl ether **1d** in the subsequent coupling reactions. The chlorambucil conjugate **7** was obtained by the reaction of chlorambucil **2** with the silyl-protected endoxifen ligand **1d** *via* a DCC/DMAP coupling reaction, immediately followed by the TBAF deprotection step (Scheme **1**) to afford a 1:1 *E/Z* isomeric mixture of the desired product **7** (60 % yield). Previous work has demonstrated that *p*-hydroxy substituted triarylbutenes may isomerise under cell culture conditions, having little impact on ER binding activity [32, 33]. In the ^{13}C-NMR spectrum,

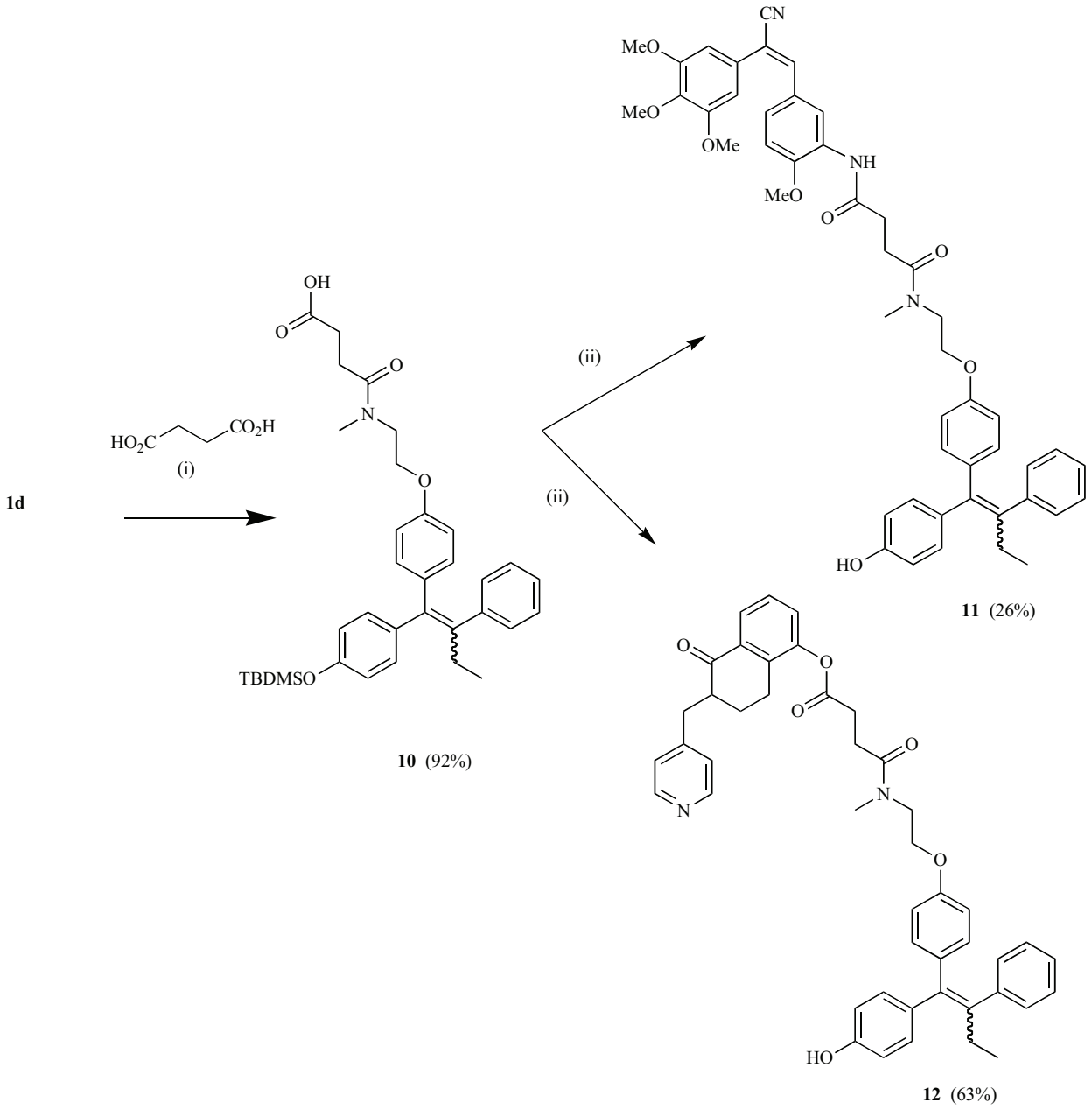


Scheme 1. Synthesis of conjugates **7, 8, 9a** and **9b**.

Reagents and conditions: (i) (a) DCC, DMAP, **2** or **3**, CH_2Cl_2, rt, 24h (b) TBAF, THF, rt, 24h (c) CH_2Cl_2, 10 % HCl, rt, 5min; (ii) DCC, DMAP, **4b**, CH_2Cl_2, rt, 24h (iii) (a) BBr_3, CH_2Cl_2, -78°, 45min, then rt, 3h (b) TBAF, THF, rt, 24h (c) 10 % HCl, CH_2Cl_2, rt, 5min.

the amide carbon is observed at 173.2 ppm while in the IR spectrum, the amide signal is observed at 1723.9 cm^{-1} and the broad hydroxyl signal at 3401.5 cm^{-1}. The conjugate **8** was synthesised by reaction of indomethacin **3** with the silyl-protected endoxifen ligand **1d** *via* a DCC/DMAP coupling reaction, immediately followed by the TBAF deprotection step (Scheme **1**) to afford a 1:1 *E*/*Z* isomeric mixture of the desired product **8** in 73 % yield. The main diagnostic signal of the coupled conjugate is observed in the ^{13}C-NMR spectra, where three amide carbons (due to the *E*- and *Z*-isomers of conjugate and also the indomethacin moiety) are observed at 171.82 ppm, 171.88 ppm, 172.22 ppm. In the IR spectrum, the amide signal is observed at 1724.5 $cm^{-1}$and the broad hydroxyl signal at 3327.2 cm^{-1}. The GW7604 analogue **4b** [34] was coupled with **1d** using a DCC/DMAP reaction (Scheme **1**) to afford the protected-conjugate product **9a** (76 %). Following demethylation with boron tribromide and subsequent silyl deprotection steps, the product **9b** was afforded in a 1:1 *E*/*Z* isomeric mixture (88 % yield).

For the synthesis of the endoxifen conjugates with **5** and **6**, the protected endoxifen was first reacted with succinic acid to afford the endoxifen-succinimide **10**, (Scheme **2**). The *cis*-acrylonitrile **6** was prepared following the literature method [30], and was reacted with endoxifen analogue **10** *via* a DCC/HOBt coupling, immediately followed by the TBAF deprotection step (Scheme **2**) to afford a 1:1 *E*/*Z* isomeric mixture of the product **11** (26 % yield). The two amide carbons of the linker fragment are observed at 171.48 and 173.27 ppm in the ^{13}C-NMR spectrum of the coupled conjugate **11** with the nitrile, amide and hydroxyl signals observed at 2208.6, 1734.8 and 3401.5 cm^{-1} in the IR spectrum. The conjugate **12** was obtained by reaction of the aromatase inhibitor **5** and the protected endoxifen ligand **10** *via* a DCC/HOBt coupling reaction, followed by the TBAF deprotection step to afford a 1:1 *E*/*Z* isomeric mixture of the desired product **12** in 63 % yield, (Scheme **2**). The coupled conjugate is observed in the ^{13}C-NMR spectrum, where the ester and amide carbons are identified at 171.45 and 172.00 ppm and the carbonyl group of the aromatase inhibitor fragment at 198.52 ppm. In the IR spectrum, the ester signal



Scheme 2. Synthesis of conjugates **11** and **12**.

Reagents and conditions:(i) CH_2Cl_2, rt, 16h (ii) (a) DCC, HOBt, **5** or **6**, CH_2Cl_2, rt, 24h (b) TBAF, THF, rt, 24h (c) 10 % HCl, CH_2Cl_2, rt, 5min.

is observed at ν1738.0 cm^{-1}, the amide signal at ν1631.2 cm^{-1} and the broad hydroxyl signal at ν3435.0 cm^{-1}.

BIOLOGICAL RESULTS AND DISCUSSION

The conjugates in this series contain an ER-ligand component (i.e. endoxifen) linked *via* amide and/or ester linkages, with or without a succinic acid linker group, to another compound with bioactivity towards cancers and tumours. The subset of bioactive compounds include an aromatase inhibitor, an alkylating agent (chlorambucil), a tubulin targeting agent, an ER antagonist and the NSAID indomethacin (shown to have synergistic effects when used in combination with the DNA intercalating drugs doxorubicin). Therefore, some structural comparison may highlight possible mechanistic features regarding the functionality of the conjugates.

The antiproliferative and cytotoxicity data for the conjugates evaluated in the ER positive MCF-7 breast cancer cells are shown in Table **1**. The data obtained for this series of conjugates ranged from low micromolar antiproliferative IC_{50} values to IC_{50} values greater than 50 μM. All the conjugates displayed low cytotoxicity levels at 10 μM in the lactate dehydrogenase assay [35]. The endoxifen-acrylonitrile conjugate **11** displays the most potent antiproliferative activity of the series, with IC_{50} = 1.64 μM,

Table 1. Antiproliferative and ER Binding Affinities for Conjugates 7, 8, 9b, 11 and 12

Compound	MCF-7 IC_{50} (μM) [a]	Cytotoxicity 10 μM[c]	ER α IC_{50} (nM)[d]	ER β IC_{50} (nM) [d]	Selectivity α/ β ratio	RBA[f] (ERα)	RBA[f] (ERβ)
7	30.3	0	490	415	0.85	1	1
8	11.6	0	78.9	200	2.5	7	3
9b	>50	0	35.6	19.5	0.55	16	29
11	1.64	1.7	524	269	0.51	1	2
12	12.6	0	51.4	51.6	1.00	11	11
Tamoxifen	4.12[b]	13.4	70 [e]	170 [e]	2.42	8.14	2.97

[a]IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 cells. Values represent the mean ± S.E.M (error values x 10^{-6}) for three experiments performed in triplicate.

[b]The IC_{50} value obtained for Tamoxifen using the MTT assay is 4.12± 0.038 μM, with cytotoxicity value 13.4 % (10 μM) is in good agreement with the reported IC_{50} value for tamoxifen on human MCF-7 cells, (reference 36).

[c]Lactate Dehydrogenase assay: Following treatment of the cells, the amount of LDH was determined using LDH assay kit from Promega. Data is presented as % cell lysis at compound concentration of 10 μM (reference 35).

[d]Values are an average of at least nine replicate experiments, for ERα with typical standard errors below 15%, and six replicate experiments for ERβ, with typical standard errors below 15%.

[e]The ER binding values obtained are in agreement with the reported ER IC_{50} binding data for tamoxifen (ERα 60.9 nM ERβ188 nM, Panvera/Invitrogen).

[f]The RBA (Relative Binding Affinity value, %) is calculated as the ratio of the binding IC_{50} value for the reference ligand (estradiol) divided by the binding IC_{50} value for the selected ligand, multiplied by 100.

comparable to activity of the acid **6** (IC_{50} = 0.83 μM) in this cell line, with low cytotoxicity in the LDH assay. The endoxifen conjugates of chlorambucil **7**, indomethacin **8** and the aromatase inhibitor **12** displayed moderate antiproliferative activity when compared to tamoxifen [36]. The IC_{50} values for these conjugates were in the range of 11.6 - 30.3 μM and displayed virtually no cytotoxicity at 10 μM in the lactate dehydrogenase assay [35]. The aromatase inhibitor conjugate **12** contains an ester linkage between the tetralone and the succinate linker fragment which may be enzymatically hydrolysed *in vivo,* thus possibly exerting a dual-effect through aromatase inhibition and ER-antagonism. The dual ER-ligand conjugate **9b** containing the amide linking groups, did not display significant antiproliferative activity, with an IC_{50} value greater than 50 μM, resulting in negligible cytotoxicity. The presence of the amide linkage may restrict the conformation of this conjugates unfavourably. This suggests that presence of the bulky ligand side chain in this case has a detrimental effect on the antiproliferative activity of either the endoxifen or GW7604 ER-ligand fragment at the ER site. As the conjugate **9b** contains an amide linkage in the scaffold, it is possible that **9b** may not be easily hydrolysed *in vivo* and could act as a possible pure ER antagonist due to the large bulky side chain group.

The conjugates were investigated for their ERα and ERβ binding abilities in a fluorescence polarization based-competitor assay. The ER-binding data for these selected conjugates is reported in Table **1**. In order to function as ER-targeting agents, the conjugates should display some binding affinity towards the receptor target. All the conjugates display binding affinities for the ERα and ERβ with IC_{50} values below 1 μM, confirming their ability to act as ER-targeting agents. The acrylonitrile conjugate **11** and chlorambucil conjugate **7** showed the least affinity for either ER isoform yet still demonstrated sub-micromolar competitive binding IC_{50} values towards ERα (96 and 490 nM respectively) and ERβ (415 and 61 nM respectively). The indomethacin conjugate **8** and aromatase inhibitor conjugate **12**, which did not demonstrate potent antiproliferative activity, display impressive binding affinities towards ERα and ERβ with competitive binding IC_{50} values in the range 50 – 200 nM. The acrylonitrile conjugate **11** displayed the lowest ER binding of the series with IC_{50} of 524 nM(ERα) and 269 nM(ERβ). The bivalent ER-ligand conjugate **9b** which also did not display significant antiproliferative activity, demonstrated potent competitive binding with IC_{50} values of 35.6 nM (ERα) and 19.5 nM (ERβ). This may suggest that these conjugates bind in orientations or sites which as a result do not displace helix-12 in the ER, hence, do not display a significant antagonistic activity *in vitro*. The phenolic hydroxy group present on the endoxifen fragment can interact favourably with residues Glu353, Arg394 and His524 in the ER binding site through hydrogen bonding which can explain the high binding affinity for all conjugates which make them useful probes for the estrogen receptor action.

The relative binding affinity (RBA) of the estrogen receptor ligands is reported. Estradiol is the reference ligand, with 100 % binding value. Using the IC_{50} binding values obtained for estradiol for ERα (5.7 nM) and ERβ (5.6 nM), the relative binding affinities of the selected conjugates were calculated (Table **1**). As the estrogen receptor has between 1000 and 10000 molecules per cell and calculations on the basis of the number of receptors per cell and the possible drug concentration show that the relative binding affinity (RBA) value should be at least 1% of that of estradiol for effective activity [12]. All of the conjugates investigated in the ER competitor binding assays had RBA values greater than or equal to 1%. The bivalent ER conjugate **9b** demonstrates impressive RBA values of 16 for ERα and 29 for ERβ.

CONCLUSION

We have synthesised a new series of estrogen receptor ligand conjugates, which contain components with known activity in the prevention of breast cancer proliferation

covalently linked to the tamoxifen metabolite endoxifen. Preliminary *in vitro* evaluation of the antiproliferative and cytotoxic activities of the compounds in MCF-7 breast cancer cells, together with determination of the ER binding indicated that some of these estrogen receptor ligand conjugate molecules may be useful as probes of estrogen receptor action and may also result in a more selective and targeted delivery of non-specific agents such as chlorambucil to breast tumour cells. The bivalent ER conjugate **9b** demonstrates potent ER binding with IC_{50} values of 35.6 nM(ERα), 19.5 nM(ERβ) and RBA values of 16 (ERα) and 29 (ERβ) and provides a template scaffold for the development of further ER ligands.

EXPERIMENTAL SECTION

General

Uncorrected melting points were measured on a Stuart SMP11 apparatus. Infra-red spectra were recorded on a Perkin Elmer Paragon 100 Spectrometer. Band positions are given as cm^{-1}. Solid and resin samples were analysed by KBr disc, while oils were analysed as neat films on NaCl plates. ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded at 20°C on a Bruker DPX 400 spectrometer (400.13 MHz, ^{1}H; 100.61 MHz, ^{13}C) in $CDCl_3$, with internal standard trimethylsilane (TMS), or CD_3OD. ^{1}H-NMR spectra were assigned relative to the TMS peak at 0.00. ^{13}C NMR spectra were assigned relative to the centre peak of the $CDCl_3$ triplet at 77.00 ppm. Coupling constants are reported in Hertz (Hz). High resolution molecular ion determinations (HRMS) were acquired on a Micro mass spectrometer (E.I. Mode) at the Department of Chemistry, Trinity College Dublin and a Micro mass spectrometer (E.I. Mode) at the Centre for Synthesis and Chemical Biology, University College Dublin. Thin layer chromatography (TLC) was carried out using Merck F-254 silica plates. Flash chromatography was carried out on Merck Kieselgel $60F_{254}$ silica. Solvents were dried according to the standard protocols.

[2-(4-{(E/Z)-1-[4-(tert-Butyldimethylsilanyloxy)phenyl]-2-phenylbut-1-enyl}phenoxy)ethyl] methylamine (1d)

(4-{(*E/Z*)-1-[4-(2-Bromoethoxy)phenyl]-2-phenylbut-1-enyl}phenoxy)-*tert*-butyldimethylsilane (0.54 g, 1.00 mmol) was reacted with methylamine in anhydrous tetrahydrofuran (20 mL) in a sealed tube at 60 °C under pressure while stirring for 48 – 72 h, [17]. After this time the reaction container was allowed sufficient time to cool, and then sodium carbonate/sodium hydrogen carbonate pH 10 buffer solution (50 mL) was added. The mixture was then extracted with CH_2Cl_2 (3 x 50 mL). The organic phases were combined, dried over sodium sulfate and the solvent evaporated *in vacuo* to afford a crude product which was then purified *via* flash chromatography over silica gel (CH_2Cl_2:MeOH, 4:1) to afford the product as a brown oil (0.38 g, 78 %, *Z/E* = 1:1.3). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.13 (s, 0.57 x 6H, $Si(CH_3)_2$), 0.26 (s, 0.43 x 6H, $Si(CH_3)_2$), 0.95 – 1.03 (m, 12H, $(CH_3)_3$, CH_3), 2.45 – 2.53 (m, 5H, NCH_3, CH_2), 2.89 (s, 0.43 x 2H, CH_2), 2.99 (s, 0.57 x 2H, CH_2), 3.36 (s, 1H, NH), 3.95 (t, 0.43 x 2H, *J* = 5.0 Hz, CH_2), 4.11 (t, 0.57 x 2H, *J* = 5.0 Hz, CH_2), 6.50 – 7.20 (m, 13H, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$): (*Z/E* mixture) δ -4.91, -4.80, 13.21, 13.24, 17.75, 25.24, 25.26, 28.47, 28.60, 35.39, 35.26, 49.47, 50.03, 50.12, 65.73, 65.99, 112.80, 113.54, 118.54, 119.12, 125.44, 125.48, 127.33, 127.41, 129.25, 130.12, 130.20, 131.43, 131.57, 135.68, 136.02, 136.06, 136.35, 137.44, 137.53, 140.59, 140.65, 142.11, 142.19, 153.06, 153.84, 156.10, 156.94. IR: ν_{max} (KBr) cm^{-1}: 3340.9, 2956.9, 2930.0, 2857.0, 1605.5. HRMS (EI): Found 488.2980 (M^{+}+H), $C_{31}H_{42}NO_2Si$ requires 488.2985.

4-{4-[Bis-(2-chloroethyl)amino]phenyl}-N-(2-{4-[(E/Z)-1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenoxy}ethyl)-N-methylbutyramide (7)

Chlorambucil **7** (0.06 g, 0.21 mmol), DCC (0.04 g, 0.21 mmol) and DMAP (0.03, 0.21 mmol) were stirred for 10 mins in anhydrous CH_2Cl_2 (4 mL). A solution of the amine **1d** (0.10 g, 0.21 mmol) in dry CH_2Cl_2 (4 mL) was added to the reaction mixture and then allowed stir for 24 h under nitrogen at room temperature. Reaction was monitored *via* TLC (CH_2Cl_2:MeOH, 4:1) until no more starting materials were visible. The reaction mixture was diluted to 15 mL with anhydrous CH_2Cl_2 and filtered to remove DCU. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 3 mL anhydrous THF and stirred under a nitrogen atmosphere. A solution of 0.1 M TBAF (0.21 mL, 0.21 mmol) was added to the mixture and allowed stir for 24 h. The mixture was evaporated to dryness under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with 10 % HCl solution. The resulting organic phase was dried over sodium sulfate and evaporated to dryness under vacuum. The residue was purified *via* flash chromatography (CH_2Cl_2:MeOH, 20:1) to yield an isomeric mixture of the product **7** as a brown oil (81.0 mg, 60 %, *E/Z* = 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91- 0.96 (m, 3H, CH_3), 1.29 - 2.63 (m, 8H, 4xCH_2), 2.97 - 3.15 (m, 3H, NCH_3), 3.60 - 4.16 (m, 12H, 3xCH_2N, CH_2O, 2xCH_2Cl), 6.45 – 7.18 (m, 17H, ArH), OH not observed. ^{13}C-NMR (100 MHz, $CDCl_3$), (*Z/E* mixture): δ 13.30, 24.45, 25.12, 25.22, 16.15, 26.49, 28.48, 29.26, 29.26, 29.88, 32.27, 32.76, 33.42, 33.66, 37.25, 40.13, 44.94, 47.61, 48.73, 53.12, 111.68, 112.69, 113.37, 113.99, 114.51, 114.65, 118.16, 128.65, 129.24, 130.21, 130.34, 131.59, 133.71, 136.64, 133.71, 136.64, 143.81, 154.11, 154.67, 156.85, 173.23. IR: ν_{max} (KBr) cm^{-1}: 3401.5, 2929.6, 2850.2, 1723.9, 1609.5. HRMS (EI): Found 659.2803 (M^{+}+H), $C_{39}H_{45}Cl_2N_2O_3$ requires 659.2807.

2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-(2-{4-[(E/Z)-1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenoxy}ethyl)-N-methylacetamide (8)

Indomethacin **3** (0.33 g, 0.91 mmol), DCC (0.19 g, 0.91 mmol) and DMAP (0.11, 0.91 mmol) were stirred for 10 mins in anhydryous CH_2Cl_2 (7 mL). A solution of the amine **1d** (0.45 g, 0.91 mmol) in dry CH_2Cl_2 (8 mL) was added to the reaction mixture and then allowed stir for 24 h under nitrogen at room temperature. Reaction was monitored *via* TLC (CH_2Cl_2:MeOH, 4:1) until no more starting materials were visible. The reaction mixture was diluted to 15 mL with anhydrous CH_2Cl_2 and filtered to remove DCU. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 3 mL anhydrous THF and stirred under a nitrogen atmosphere. A solution of 0.1 M TBAF (0.91 mL, 0.91 mmol) was added to the mixture and allowed

stir for 24 h. The mixture was evaporated to dryness under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with 10 % HCl solution. The resulting organic phase was dried over sodium sulfate and evaporated to dryness under vacuum. The residue was purified *via* flash chromatography (CH_2Cl_2:MeOH, 20:1) to yield an isomeric mixture of the product **8** as a brown oil (472 mg, 73 %, *E/Z* = 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.93 - 0.97 (m, 3H, endoxifen CH_3), 2.21 – 2.29 (m, 3H, indomethacin CH_3), 2.48 - 2.53 (m, 2H, CH_2), 2.97 - 3.18 (m, 3H, NCH_3), 3.69 - 4.16 (m, 9H, OCH_3, CH_2N, CH_2O, $COCH_2$), 6.44 – 7.19 (m, 20H, ArH), OH not observed. ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ 11.42, 11.46, 13.14, 13.25, 28.63, 29.28, 30.53, 30.58, 33.89, 36.07, 37.54, 48.12, 48.82, 55.36, 55.43, 64.50, 64.92, 65.96, 66.21, 99.73, 99.82, 99.89, 103.67, 103.73, 104.20, 110.28, 110.33, 112.77, 113.49, 113.94, 114.62, 125.43, 127.40, 128.37, 129.33, 129.73, 129.80, 130.15, 130.26, 131.51, 131.59, 132.54, 132.69, 132.76, 134.68, 135.08, 136.23, 137.40, 137.51, 140.27, 140.45, 142.29. 142.32, 153.49, 153.51, 153.86, 154.75, 155.61, 155.93, 156.74, 171.82, 171.88, 172.22. IR: ν_{max} (KBr) cm^{-1}: 3327.2, 2929.2, 2851.9, 1724.5, 1626.2. HRMS (EI): Found 735.2620(M^++Na), $C_{44}H_{41}ClN_2O_5Na$ requires 735.2601.

(E)-N-[2-(4-{(E/Z)-1-[4-(tert-Butyldimethylsilanyloxy)-phenyl]-2-phenylbut-1-enyl}-phenoxy)ethyl]-3-{4-[(E/Z)-1-(4-methoxyphenyl)-2-phenylbut-1-enyl]phenyl}-N-methylacrylamide (9a)

The acid **4b** [34] (0.13 g, 0.34 mmol, 1 eq.), DCC (0.07 g, 0.34 mmol, 1 eq.), DMAP (0.04 g, 0.34 mmol, 1 eq.) was stirred in 5 mL of anhydrous CH_2Cl_2 for 10 mins. **1d** (0.166 g, 0.34 mmol, 1 eq.) was added to the reaction mixture and allowed stir at room temperature for 24 h under nitrogen atmosphere. After which time the reaction was diluted with 20 mL CH_2Cl_2 and filtered to remove insoluble solids. The solvent was removed *in vacuo* and the residue was purified *via* flash chromatography (CH_2Cl_2:MeOH, 20:1) to afford the product **9a** as a resin (220 mg, yield = 76 %, *E/Z* = 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.12 – 0.25 (m, 6H, $Si(CH_3)_2$), 0.94 – 1.02 (m, 15H, CH_3 ,$SiC(CH_3)_3$), 2.49 – 2.53 (m, 4H, CH_2), 3.04 – 4.33 (m, 10H, NCH_3, NCH_2, OCH_2, OCH_3), 6.49 – 7.77 (m, 28H, ArH, CH=CH). ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ -4.91, -4.80, 13.19, 17.76, 25.25, 28.61, 33.54, 36.06, 48.56, 54.57, 112.39, 112.69, 113.14, 113.47, 116.41, 118.52, 119.10, 125.42, 125.73, 125.86, 126.55, 127.30, 127.48, 129.20, 129.26, 129.54, 130.80, 131.39, 131.51, 134.64, 136.23, 142.12, 145.03, 156.44, 157.16, 162.10. IR: ν_{max} (KBr) cm^{-1}: 3430.0, 3326.2, 2928.7, 2850.8, 1625.3, 1575.8. HRMS (EI): Found 876.4424, $C_{57}H_{63}NO_4SiNa$ requires 876.4424.

(E)-N-(2-{4-[(E/Z)-1-(4-Hydroxyphenyl)-2-phenylbut-1-enyl]phenoxy}ethyl)-3-{4-[(E/Z)-1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenyl}-N-methylacrylamide (9b)

A solution of **9a** (0.18 g, 0.21 mmol, 1 eq.) was stirred in anhydrous CH_2Cl_2 (5 mL). The mixture was cooled to -78 °C and 1.0 M boron tribromide solution (0.84 mL, 0.84 mmol, 4 eq.) was added slowly to the reaction mixture. The reaction was allowed stir at -78 °C for 45 minutes then allow return to room temperature while stirred for three hours. The reaction was quenched through the addition of 3 mL of methanol. The mixture was evaporated to dryness *in vacuo* and the residue was dissolved in 5 mL of anhydrous THF. To the solution, 1.0M TBAF in THF (0.50 mL, 0.50 mmol, 2.5eq.) was added. The reaction was allowed stir overnight. The reaction was diluted with 20 mL of THF and washed with 10 % HCl solution. The organic layer was separated, dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was purified *via* flash chromatography (CH_2Cl_2:MeOH, 20:1) to afford the product **9b** as a resin (135 mg, yield = 88 %, *E/Z* = 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91 – 0.96 (m, 6H, CH_3), 2.44 - 2.55 (m, 4H, CH_2), 2.91 – 3.22 (m, 3H, NCH_3), 3.65 – 4.41 (6H, NCH_2, OCH_2, 2xOH), 6.51 – 7.35 (m, 28H, ArH, CH=CH). ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ 13.60, 13.68, 24.90, 25.57, 28.93, 29.03, 30.99, 33.88, 49.26, 65.92, 68.11, 113.98, 114.33, 114.94, 115.06, 115.31, 116.56, 125.82, 126.99, 127.28, 127.81, 127.90, 128.04, 129.52, 129.67, 129.76, 130.02, 130.70, 130.85, 131.31, 132.06, 132.10, 135.54, 135.89, 138.06, 140.63, 142.79, 153.99, 154.95, 157.13. IR: ν_{max} (KBr) cm^{-1}: 3326.6, 2928.9, 2850.8, 1626.2, 1575.2. HRMS (MALDI-TOF): Found 726.3588; $C_{50}H_{48}NO_4$ requires 726.3583(M^++H).

N-[2-(4-{(E/Z)-1-[4-(tert-Butyldimethylsilanyloxy)phenyl]-2-phenylbut-1-enyl}phenoxy)ethyl]-N-methylsuccinamic acid (10)

The amine **1d** (0.20 g, 0.41 mmol, *E/Z* = 1:1) and succinic anhydride (0.04 g, 0.41 mmol) were dissolved in 5ml of dry CH_2Cl_2. The reaction was allowed stir at room temperature for 16 h. Reaction was monitored *via* TLC (CH_2Cl_2:MeOH = 4:1). Reaction mixture was worked up *via* the addition of CH_2Cl_2 (10 mL), washed with 1M NaOH solution (10 mL). The aqueous phase was extracted with CH_2Cl_2 (10 mL x3). The combined organic layers were acidified with dilute HCl dropwise, washed with water (10 mL) and brine (10 mL), dried over sodium sulfate and then evaporated to dryness *in vacuo* to afford an isomeric mixture (*E:Z* = 1:1) of the product (222 mg, 92%) as a light brown oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.12 – 0.25 (m, 6H, $Si(CH_3)_2$), 0.94 – 1.02 (m, 12H, $SiC(CH_3)_3$, CH_3), 2.48 – 2.52 (m, 2H, CH_2), 2.65 - 2.87 (m, 4H, succinic CH_2), 3.00 - 3.22 (m, 3H, NCH_3), 3.70 – 4.17 (m, 4H, CH_2N, CH_2O), 6.49 – 7.20 (m, 13H, ArH), COOH not observed. ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ -4.92, -4.80, 13.18, 13.22, 17.75, 25.22, 25.24, 28.46, 28.60, 37.04, 37.10, 47.91, 65.78, 66.12, 112.65, 113.42, 118.53, 119.10, 125.46, 127.31, 127.39, 127.43, 129.26, 130.09, 130.22, 130.30, 131.38, 131.57, 131.65, 135.73, 136.20, 136.27, 137.37, 137.44, 140.65, 142.08, 153.06, 156.68, 172.69, 176.78; IR: ν_{max} (KBr) cm^{-1}: 3435.7, 2927.5, 1696.5, 1624.0, 1603.6. HRMS (EI): Found 610.2972 $(M+Na)^+$, $C_{35}H_{45}NO_5SiNa$ requires 610.2965.

N-{5-[2-Cyano-2-(3,4,5-trimethoxyphenyl)vinyl]-2-methoxyphenyl}-N'-(2-{4-[(E/Z)-1-(4-hydroxyphenyl)-2-phenylbut-1-enyl]phenoxy}ethyl)-N'-methylsuccinamide (11)

The protected endoxifen succinic acid linker compound **10** (0.10 g, 0.17 mmol, *E:Z* = 1:1), dicyclohexylcarbodiimide (0.04 g, 0.17 mmol) and 1-hydroxybenzotriazole hydrate (0.02 g, 0.17 mmol) were dissolved in dry CH_2Cl_2 (5 mL) under a N_2 environment. The mixture was allowed to stir for 20 minutes before adding a solution of the *cis*-acrylonitrile **6** (0.06 g, 0.17 mmol) in dry CH_2Cl_2 (3 mL). The reaction was

allowed stir at room temperature for 24 h until no starting material was visible by TLC (CH_2Cl_2:MeOH, 4:1). The reaction mixture was filtered to remove the dicyclohexylurea byproduct. The mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 3 mL anhydrous THF and stirred under a nitrogen atmosphere. A quantity of 0.1 M TBAF (0.20 mL, 0.02 mmol) was added to the mixture and allowed stir for 24 h. The mixture was evaporated to dryness under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with 10 % HCl solution. The resulting organic phase was dried over sodium sulfate and evaporated to dryness under vacuum. The residue was purified *via* flash chromatography on silica gel (CH_2Cl_2:MeOH, 20:1) to yield an isomeric mixture (*E:Z* = 1:1) of the product **12** (35 mg, 26 %). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.87 – 0.95 (m, 3H, CH_3), 2.44 – 3.23 (m, 9H, NCH_3, CH_2, 2xsuccinic-CH_2), 3.65 – 4.28 (m, 16H, NCH_2, 4xOCH_3, OCH_2), 6.33 – 7.80 (m, 20H, ArH, C=CH, NH), OH not observed. ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ 13.18, 14.24, 17.93, 27.87, 27.92, 28.55, 28.80, 29.25, 31.14, 36.20, 36.96, 47.62, 51.43, 55.70, 58.03, 66.26, 105.43, 106.13, 112.79, 113.40, 114.06, 114.57, 114.73, 115.03, 127.12, 127.37, 128.13, 128.52, 128.66, 129.25, 129.54, 130.21, 130.57, 130.64, 131.24, 131.56, 131.83, 132.14, 134.85, 162.32, 171.48, 173.27. IR: ν_{max} (KBr) cm^{-1}: 3467.8, 2208.6, 1734.8, 1636.3, 1508.9. HRMS (MALDI-TOF):. Found 808.3398; $C_{47}H_{49}N_2O_8K$ requires 808.3126.

N-(2-{4-[(E/Z)-1-(4-Hydroxyphenyl)-2-phenylbut-1-enyl] phenoxy}ethyl)-N-methyl-succinamic acid 5-oxo-6-pyridin-4-ylmethyl-5,6,7,8-tetrahydronaphthalen-1-yl ester (12)

The acid **10** (1 equivalent, 0.25 mmol), DCC (1 equivalent, 0.25 mmol, 0.05 g) and HOBt (1 equivalent, 0.25 mmol, 0.03 g) were suspended in 3 mL of anhydrous CH_2Cl_2 and stirred for 10 minutes under a nitrogen atmosphere. The phenol **5** was then dissolved in 3 mL of anhydrous DCM and slowly added to the mixture *via* syringe. Reaction was allowed stir for 24 h. Reaction was monitored *via* TLC (CH_2Cl_2:MeOH, 4:1) until no more starting materials were visible. The reaction mixture was diluted to 15 mL with anhydrous CH_2Cl_2 and filtered to remove DCU. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 3 mL anhydrous THF and stirred under a nitrogen atmosphere. A solution of 0.1 M TBAF (2 equivalents) was added to the mixture and allowed stir for 24 h. The mixture was evaporated to dryness under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with 10 % HCl solution. The resulting organic phase was dried over sodium sulfate and evaporated to dryness under vacuum. The residue was purified *via* flash chromatography (CH_2Cl_2:MeOH, 20:1) to yield an isomeric mixture of the product as a brown oil (112 mg, 63%, *E/Z* = 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.99 (t, J = 7.5Hz, 3H, CH_3), 1.27 - 1.43 (m, 2H, CH_2), 1.74 (m, 2H, CH_2), 2.50 (m, 2H, CH_2), 2.64 – 2.87 (m, 4H, CH_2), 3.17 – 3.03 (m, 5H, CH_2, NCH_3), 3.45 (dd, 1H, *J* = 4 Hz, 13.5 Hz, CH), 3.66 (m, 1H, CH_2N), 3.77 (m, 1H, CH_2N), 3.97 (m, 1H, CH_2O), 4.12 (m, 1H, CH_2O), 6.51 (m, 1H, ArH), 6.70 (m, 1H, ArH), 6.77 (d, 1H, ArH, *J* = 8 Hz), 6.85 (m, 2H, ArH), 7.12 (m, 7H, ArH), 7.17 (s, br, 2H, ArH), 7.36 (s, 2H, ArH), 7.62 (d, 1H, ArH, *J* = 8 Hz), 7.71 (d, 1H, ArH, *J* = 5.0 Hz), 8.63 (d, 2H, pyridine-H, *J* = 5.2 Hz), OH not observed. ^{13}C-NMR (100 MHz, $CDCl_3$), (*E/Z* mixture): δ 13.09, 19.53, 21.24, 21.89, 24.26, 24.89, 25.03, 26.17, 26.96, 28.57, 28.86, 29.25, 32.45, 34.81, 47.66, 47.84, 52.38, 112.64, 113.36, 113.43, 113.96, 114.66, 118.40, 119.20, 119.30, 119.55, 125.42, 125.54, 126.91, 127.37, 129.25, 130.04, 130.20, 130.40, 131.59, 132.01, 133.02, 133.71, 139.04, 148.43, 149.02, 153.49, 153.80, 171.45, 172.00, 198.52. IR: ν_{max} (KBr) cm^{-1}: 3435.0, 2930.7, 1738.0, 1631.2, 1605.9. HRMS (MALDI-TOF): Found 709.3290; $C_{44}H_{45}N_2O_6$ requires 709.3278(M^++H).

Biochemistry

Estrogen Receptor (ER) Competitor Assay

ERα and ERβ fluorescence polarization based-competitor assay kits were obtained from Invitrogen. The recombinant ER and the fluorescent estrogen ligand were removed from the -80°C freezer and thawed on ice for one-hour prior to use. The assay was performed using a protocol described by the manufacturer. The fluorescent estrogen (2 nM) was added to the ER (30 nM for ERα and 20 nM for ERβ), screening buffer (100 nM potassium phosphate (pH 7.4), 100 μg/ml BGG, 0.02 M NaN_3) was added to make up a final volume that was dependent on the number of tubes used. Test compound, 1 μL, in varying concentrations, was added to 49 μL screening buffer in 96-well black plates, 50 μL of the fluorescent estrogen/ER complex was added to make up the total volume to 100 μL. A vehicle control contained 1 % DMSO (v/v). A negative control contained 50 μL of screening buffer and 50 μL of fluorescent estrogen/ER complex. This control was used to determine the polarization value when no competitor was present (theoretical maximum polarization). 1 μL of 1 mM estradiol (final concentration 10 μM) was used as complete displacement (minimum polarization value). The tubes were incubated in the dark at room temperature for 2 hours and were mixed by shaking on a plate shaker. The polarization instrument contained 485 nM excitation and 530 nM emission interference filters.

Cell Proliferation Assays

All assays were performed in triplicate for the determination of mean values reported. Compounds were assayed as the free bases isolated from reaction. The human breast tumour cell line MCF-7 was cultured in Eagles Minimum Essential (MEM) medium in a 95% O_2/5% CO_2 atmosphere supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin The medium was further supplemented with 1% non-essential amino acids. Cells were trypsinised and seeded at a density of 2.5 x 10^4 cells/mL into a 96-well plate and incubated for 24 h. After this time they were treated with 2 μl volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the final concentration range of study, 1nM-100 μM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The culture medium was then removed and the cells washed with 100 μL phosphate buffer saline (PBS) and 50 μL of 1 mg/mL MTT solution was added. Cells were incubated for 2 h in darkness at 37°C. At this point solubilisation was begun through the addition of 200 μL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough

colour diffusion before reading the absorbance at 595 nm. The absorbance value of control cells (vehicle treated) was set to 100 % cell viability and from this graphs of absorbance versus cell density per well were prepared to assess cell viability and from these, graphs of percentage cell viability versus concentration of subject compound were drawn.

Lactate Dehydrogenase (LDH) Assay

Cytotoxicity was determined using the Cyto-Tox 96 non-radioactive cytotoxicity assay by Promega [37]. The assay quantitatively measures lactate dehydrogenase (LDH) a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatant is measured in a 30 minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. MCF-7 and MDA-MB-231 cells were seeded in 96-well plates, incubated for 24 hours and then treated with compounds as above. After 72 hours 20 µl of lysis solution (10X) was added to the 'blank' wells, they were then left for 1 hour to ensure 100 % death, 50 µL was removed from each well and transferred into a new 96-well plate for use in the LDH assay. 50 µL of substrate mix from the LDH assay kit was added and the plate was placed in the dark at room temperature for 30 minutes. After this period, 50 µL of stop solution was added to each well before reading the absorbance at a wavelength of 490 nm using a Dynatech MR5000 plate reader. Percentage death was calculated at 10 µM.

CONFLICT OF INTEREST

Declared none.

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REFERENCES

[1] Gronemeyer, H.; Gustafsson, J. A.; Laudet, V. Principles for modulation of the nuclear receptor superfamily. *Nat. Rev. Drug Discov.*, **2004**, *3*, 950-964.

[2] Pasqualini, J. R.; Chetrite, G. S. Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. *J. Steroid Biochem. Mol. Biol.*, **2005**, *93*, 221-236.

[3] Ascenzi, P.; Bocedi, A.; Marino, M. Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol. Aspects Med.*, **2006**, *27*, 299-402.

[4] Brzozowski, A. M.; Pike, A. C.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J. A.; Carlquist, M.; Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, **1997**, *389*, 753-758.

[5] Pike, A. C. W. Lessons learnt from structural studies of the oestrogen receptor. *Best Pract. Res. Cl. En.*, **2006**, *20*, 1-14.

[6] Keely, N. O.; Meegan, M. J. Targeting tumors using estrogen receptor ligand conjugates. *Curr. Cancer Drug Targets,* **2009**, *9*, 370-380.

[7] Morphy, R.; Rankovic, Z. Designed multiple ligands. An emerging drug discovery paradigm. *J. Med. Chem.*, **2005**, *48*, 6523-6543.

[8] Krohn, K.; Kulikowski, K.; Leclercq, G. Diethylstilbestrol-linked cytotoxic agents: synthesis and binding affinity for estrogen receptors. *J. Med. Chem.*, **1989**, *32*, 1532-1538.

[9] Kohle, H.; Krohn, K.; Leclercq, G. Hexestrol-linked cytotoxic agents: synthesis and binding affinity for estrogen receptors. *J. Med Chem.*, **1989**, *32*, 1538-1547.

[10] Sharma, U.; Marquis, J. C; Dinaut, A. N; Hillier, S. M.; Fedeles, B.; Rye, P. T.; Essigmann, J. M.; Croy, R. G. Design, synthesis, and evaluation of estradiol-linked genotoxicants as anti-cancer agents. *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 3829-3833.

[11] Kuduk, S. D.; Zheng, F. F.; Sepp-Lorenzino, L.; Rosen, N.; Danishefsky, S. J. Synthesis and evaluation of geldanamycin-estradiol hybrids. *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 1233-1238.

[12] Devraj, R.; Barrett, J. F.; Fernandez, J. A.; Katzenellenbogen, J. A.; Cushman, M. Design, synthesis, and biological evaluation of ellipticine-estradiol conjugates. *J. Med. Chem.*, **1996**, *39*, 3367-3374.

[13] Ishiki, N.; Onishi, H.; Machida, Y. Evaluation of antitumor and toxic side effects of mitomycin C-estradiol conjugates. *Int. J. Pharm.*, **2004**, *279*, 81-93.

[14] Kasiotis, K. M.; Magiatis, P.; Pratsinis, H.; Skaltsounis, A.; Abadji, V.; Charalambous, A.; Moutsatsou, P.; Haroutounian, S. A. Synthesis and biological evaluation of novel daunorubicin-estrogen conjugates. *Steroids*, **2001**, *66*, 785-791.

[15] Fenick, D. J.; Taatjes, D. J.; Koch, T. H. Doxoform and Daunoform: anthracycline-formaldehyde conjugates toxic to resistant tumor cells. *J. Med. Chem.*, **1997**, *40*, 2452-2461.

[16] Ali, H.; Ahmed, N.; Tessier, G.; van Lier, J. E. Synthesis and biological activities of nucleoside-estradiol conjugates. *Bioorg. Med. Chem. Lett.*, **2006**, *16*, 317-319.

[17] Burke, P. J.; Koch, T. H. Design, synthesis, and biological evaluation of doxorubicin-formaldehyde conjugates targeted to breast cancer cells. *J. Med. Chem.*, **2004**, *47*, 1193-1206.

[18] Burke, P. J.; Kalet, B. T; Koch, T. H. Antiestrogen binding site and estrogen receptor mediate uptake and distribution of 4-hydroxytamoxifen-targeted doxorubicin-formaldehyde conjugate in breast cancer cells. *J. Med. Chem.*, **2004**, *47*, 6509-6518.

[19] Descoteaux, C.; Provencher-Mandeville, J.; Mathieu, I.; Perron, V.; Mandal, S. K.; Asselin, E.; Berube, G. Synthesis of 17beta-estradiol platinum(II) complexes: biological evaluation on breast cancer cell line. *Bioorg. Med. Chem. Lett.*, **2003**, *13*, 3927-3931.

[20] Perron, V.; Rabouin, D.; Asselin, E.; Parent, S.; Gaudreault, R. C.; Berube, G. Synthesis of 17beta-estradiol-linked platinum(II) complexes and their cytocidal activity on estrogen-dependent and -independent breast tumor cells. *Bioorg. Chem.*, **2005**, *33*, 1-15.

[21] Lim, Y. C.; Desta, Z.; Flockhart, D. A.; Skaar, T. C. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother. Pharmacol.*, **2005**, *55*, 471-478.

[22] Mazhar, D.; Ang, R.; Waxman, J. COX inhibitors and breast cancer. *Br. J. Cancer*, **2006**, *94*, 346-350.

[23] Kapitanovic, S.; Cacev, T.; Antica, M.; Kralj, M.; Cavric, G.; Pavelic, K.; Spaventi, R. Effect of indomethacin on E-cadherin and beta-catenin expression in HT-29 colon cancer cells. *Exp. Mol. Pathol.*, **2006**, *80*, 91-96.

[24] Planchon, P.; Veber, N.; Magnien, V.; Prevost, G.; Starzec, A. B.; Israel, L. Evidence for separate mechanisms of antiproliferative action of indomethacin and prostaglandin on MCF-7 breast cancer cells. *Life Sci.*, **1995**, *57*, 1233-1240.

[25] Duffy, C. P.; Elliott, C. J.; O'Connor, R. A.; Heenan, M. M.; Coyle, S.; Cleary, I. M.; Kavanagh, K.; Verhaegen, S.; O'Loughlin, C. M.; NicAmhlaoibh, R.; Clynes, M. Enhancement of chemotherapeutic drug toxicity to human tumour cells *in vitro* by a subset of non-steroidal anti-inflammatory drugs (NSAIDs). *Eur. J. Cancer*, **1998**, *34*, 1250-1259.

[26] Willson, T. M.; Henke, B. R.; Momtahen, T. M.; Charifson, P. S.; Batchelor, K. W.; Lubahn, D. B.; Moore, L. B.; Oliver, B. B.; Sauls, H. R.; Triantafillou, J. A.; Wolfe, S. G.; Baer, P. G. 3-[4-(1,2-Diphenylbut-1-enyl)phenyl]acrylic acid: a non-steroidal estrogen with functional selectivity for bone over uterus in rats. *J. Med. Chem.*, **1994**, *37*, 1550-1552.

[27] Faye, J. C.; Jozan, S.; Redeuilh, G.; Baulieu, E. E.; Bayard, F. Physicochemical and genetic evidence for specific antiestrogen binding sites. *Proc. Natl. Acad. Sci. U. S. A.,* **1983**, *80*, 3158-3162.

[28] Wang, Y.; Chirgadze, N. Y.; Briggs, S. L.; Khan, S.; Jensen, E. V.; Burris, T. P. A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A.,* **2006**, *103*, 9908-9911.

[29] Bergmann, K. E.; Wooge, C. H.; Carlson, K. E.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. Bivalent ligands as probes of estrogen receptor action. *J. Steroid Biochem. Mol. Biol.*, **1994**, *49,* 139-152.

[30] Ohsumi, K.; Nakagawa, R.; Fukuda, Y.; Hatanaka, T.; Morinaga, Y.; Nihei, Y.; Ohishi, K.; Suga, Y.; Akiyama, Y.; Tsuji, T. Novel combretastatin analogues effective against murine solid tumors: design and structure-activity relationships. *J. Med. Chem.*, **1998**, *41*, 3022-3032.

[31] Bayer, H.; Batzl, C.; Hartmann, R. W.; Mannschreck, A. New aromatase inhibitors. Synthesis and biological activity of pyridyl-substituted tetralone derivatives. *J. Med. Chem.,* **1991**, *34*, 2685-2691.

[32] Katzenellenbogen, J. A.; Carlson, K. E.; Katzenellenbogen, B. S. Facile geometric isomerization of phenolic non-steroidal estrogens and antiestrogens: limitations to the interpretation of experiments characterizing the activity of individual isomers. *J. Steroid Biochem.*, **1985**, *22*, 589-596.

[33] Maximov, P. Y.; Myers, C. B.; Curpan, R. F.; Lewis-Wambi, J. S.; Jordan, V. C. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J. Med. Chem.*, **2010**, *53*, 3273-3283.

[34] Eaddy, J. F.; Heyer, D.; Katamreddy, S. R.; Martin, M. T.; McClure, M. S.; Randhawa, A. S.; Samano, V.; Ray, J. A. Preparation of acyloxydiphenylbutenylcinnamates as estrogen receptor modulator prodrugs. PCT Int. Appl. **2005**, WO 2005033056, A2 20050414 2005.

[35] Bidlack, J. M.; Lockshin, R. A. Evolution of LDH isozymes during programmed cell death. *Comp. Biochem. Physiol. B,* **1976***, 55*, 161-166.

[36] Reddel, R. R.; Murphy, L. C.; Hall, R. E.; Sutherland, R. L. Differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen. *Cancer Res.,* **1985**, *45*, 1525-1531.

[37] Allen, M. J.; Rushton, N. Use of the CytoTox 96™ Assay in Routine Biocompatibility Testing *in vitro*. *Promega Notes,* **1994**, *45,* 7-10.