RESEARCH ARTICLE

Development of the β-lactam type molecular scaffold for selective estrogen receptor α modulator action: synthesis and cytotoxic effects in MCF-7 breast cancer cells

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Abstract

The estrogen receptors (ERα and ERβ) which are ligand inducible nuclear receptors are recognized as pharmaceutical targets for diseases such as osteoporosis and breast cancer. There is an increasing interest in the discovery of subtype Selective Estrogen Receptor Modulators (SERMs). A series of novel β-lactam compounds with estrogen receptor modulator properties have been synthesized. The antiproliferative effects of these compounds on human MCF-7 breast tumor cells are reported, together with binding affinity for the ERα and ERβ receptors. The most potent compound 15g demonstrated antiproliferative effects on MCF-7 breast tumor cells (IC50 = 186 nM) and ERα binding (IC50 = 4.3 nM) with 75-fold ERα/β receptor binding selectivity. The effect of positioning of the characteristic amine containing substituted aryl ring (on C-4 or N-1 of the β-lactam scaffold) on the antiproliferative activity and ER-binding properties of the β-lactam compounds is rationalized in a molecular modeling study.

Keywords

Antiproliferative activity, azetidin-2-one, β-lactam, breast cancer, estrogen receptor

Introduction

Breast cancer is by far the most frequent cancer among women globally, with an estimated 1.67 million new cancer cases diagnosed in 2012 ranking second overall (25% of all female cancers), and accounting for 521,817 deaths. Incidence rates vary from 27 per 100,000 women in Middle Africa and Eastern Asia to 96 per 100,000 women in Western Europe. In Europe, breast cancer is the most frequent cancer in women with 464,000 new diagnoses in 2012, which accounts for 29% of all new female cancers in Europe. One in three people in Ireland will develop cancer during their lifetime. Irish statistics note that breast cancer now accounts for 32% of all cancers in women in Ireland, with 2942 new diagnoses in 2013.

Breast cancers are classified as estrogen receptor (ER) positive or negative with 70–80% of all primary breast tumors being ER positive, which is a less aggressive type than ER negative breast cancer. The first antiestrogen to show positive clinical results was tamoxifen, a synthetic non-steroidal antiestrogen, which was approved by the Food and Drug Administration in 1977 for the treatment of women with advanced breast cancer and several years later for adjuvant treatment of primary cancer. Tamoxifen is extensively metabolized by the human hepatic cytochrome P450 enzyme system into several metabolites including 4-hydroxytamoxifen and 4-hydroxy-N-desmethyaltamoxifen (endoxifen). They are ~100-fold more potent as antiestrogens than tamoxifen and are most likely contributors to the base antiproliferative activity observed with tamoxifen. The metabolite norendoxifen was shown to have dual aromatase inhibitory and estrogen receptor modulatory activities. Novel tamoxifen analogs that avoid CYPD6 metabolism have been recently reported. The SERM raloxifene, a 2,3-disubstituted benzothiophene containing compound, is a potent antiestrogen that binds to the ER with an affinity higher than that of tamoxifen or 4-hydroxytamoxifen and equal to that of estradiol. Raloxifene was approved for the treatment of osteoporosis in 1997 and has shown modest activity in ER-positive breast cancer while lacking the increased risk for endometrial cancer associated with the use of tamoxifen. Aromatase inhibitors such as the non-steroidal agent’s letrozole and anastrozole are reported to be more efficacious than tamoxifen as a first-line therapy, and are useful for second-line therapy and against tamoxifen-resistant tumors. The steroid fulvestrant is also effective as second line therapy against advanced breast cancer in patients who develop resistance to tamoxifen. Due to its pure antiestrogenic activity, it is devoid of endometrial stimulation and therefore the risk of endometrial cancer; similarly it does not possess the positive side effects on the skeletal and cardiovascular systems of SERMs.

There are two types of ER assigned as ERα and ERβ. A comparison of the amino acid sequence of ERα and ERβ shows that they share the same functional domain architecture, and the full length residues are ~50% identical. The high homology between the DNA binding domain (96%) suggests that ERα and ERβ are expected to bind various estrogen response elements (EREs) with similar specificity and affinity and therefore interact...
with, and activate the same genes. However, because there is only 58% similarity between the ligand-binding domain, various estrogens may differentially bind to the two ERs\(^{19,20}\). ER subtypes bind some ligands with different affinity; ligands may also demonstrate different agonist or antagonist character mediated by the two receptors\(^{21}\). SERMs such as 4-hydroxytamoxifen (2) and raloxifene (4) act as partial agonists on Er\(^a\), but they exert exclusively antagonistic activity on Er\(^b\)\(^{22}\).

Due to the increased risk of endometrial cancer with the use of tamoxifen many different fixed ring structures have been developed as potential SERMs to prevent E/Z isomerization of the triarylethylene structure\(^{23}\). Some of these are illustrated in Figure 1 and include the tetrahydronaphthalene lasofoxifene (6)\(^{24}\), spiroindene (7)\(^{25}\), pyrazole (8)\(^{26}\), quinoline (9)\(^{27}\), benzopyran (10)\(^{28}\), benzoathin (11)\(^{29}\) and benzoepin ring structures (12)\(^{30}\). The recently reported benzopyranobenzoxepanes were identified as potent SERMs for the treatment of postmenopausal symptoms\(^{31}\).

We are specifically interested in the development of novel heterocyclic ring scaffolds as ER antagonists\(^{32}\). Natural and synthetic azetidinone derivatives occupy a central place among medicinally important compounds due to their diverse and interesting biological activities\(^{33}\). Their importance is no longer exclusive due to the extensive clinical use of the β-lactam antibiotics but also because of their potential as intermediates in the synthesis of other types of compounds of biological interest. The antitumour properties of β-lactams have previously been reported\(^{32,34–36}\), together with inhibitory activity against serine proteases such as prostate-specific antigen (PSA)\(^{37}\), and other serine proteases such as trypase\(^{38}\) and human leukocyte elastase (HLE)\(^{39}\).

In a study to discover subtype selective ER scaffolds, we have identified a novel estrogen receptor modulator scaffold structure containing the β-lactam ring as a potential scaffold for SERMs, and have reported the antiproliferative and ER-binding properties of a series of 1,4-diarylsulfurazidin-2-ones, where the required basic amine function was positioned on the benzylic substituent at the C-3 position\(^{32,40}\). We now report the further investigation of this novel heterocyclic core scaffold structure as ER subtype selective ligands and the subsequent synthesis of a number of structurally varied β-lactam compounds, which are substituted at N-1, C-3 and C-4 with the required aryl rings. We have evaluated the antiproliferative activity of these products in ER-positive MCF-7 human breast tumor cells and also in ER-negative MDA-MB-231 cells and have determined their relative binding affinities for ER\(^a\) and ER\(^b\). The two main β-lactam structural types now reported contain the important basic side-chain substituent positioned on the phenyl ring at the C4-position (type I) and at the N1-position (type II) as these were the optimal positions for substitution indicated from initial molecular modeling and docking studies. The general features of the β-lactam target scaffold structures selected for synthesis are illustrated in Figure 2.

**Experimental section**

**Chemistry**

All reagents were commercially available and were used without further purification unless otherwise indicated. Tetrahydrofuran (THF) was distilled immediately prior to use from Na/Benzophenone under a slight positive pressure of nitrogen, toluene was dried by distillation from sodium and stored on
activated molecular sieves (4 Å) and dichloromethane was dried by distillation from calcium hydride prior to use. Uncorrected melting points were measured on a Gallenkamp apparatus. Infrared (IR) spectra were recorded as thin film on NaCl plates, or as potassium bromide discs on a Perkin Elmer FT-IR Spectrum 100 spectrometer (Perkin Elmer, Waltham, MA). 1H, 13C and 19F nuclear magnetic resonance (NMR) spectra were recorded at 27 °C on a Bruker Avance DPX 400 spectrometer (Bruker, Billerica, MA) (400.13 MHz, 1H; 100.61 MHz, 13C; 376.47 MHz, 19F) at 20 °C in either CDCl3 (internal standard tetramethylsilane (TMS)) or CD3OD by Dr. John O’Brien and Dr. Manuel Ruether in the School of Chemistry, Trinity College Dublin. For CDCl3, 1H-NMR spectra were assigned relative to the TMS peak at 0.00 ppm, and 13C-NMR spectra were assigned relative to the center peaks of the CD3OD triplets at 33.30 ppm and 49.00 ppm, respectively. 19F-NMR spectra were not calibrated. Electrospray ionization mass spectrometry (ESI-MS) was performed in the positive ion mode on a liquid chromatography-ionization (ES) interface operated in the positive ion mode at a Waters Ltd., Manchester, UK) equipped with electrospray ionization (ESI) mass spectrometry (ESI-MS) and a Waters 2487 Dual Wavelength Absorbance detector, a Waters 2487 Triple Quad detector, and a Waters 2487 Surveyor pump. All measurement accuracies of ±5 ppm were obtained. Low resolution mass spectra (LRMS) were acquired on a Hewlett-Packard 5973 MSD GC-MS system (Hewlett-Packard, Palo Alto, CA) in electron impact (EI) mode. Rf values are quoted for thin layer chromatography (TLC) on silica gel Merck F-254 plates, unless otherwise stated. Flash column chromatography was carried out on Merck Kieselgel 60 (particle size 0.040–0.063 mm), Aldrich aluminum oxide, (activated, neutral, Brockmann I, 50 mesh) or Aldrich aluminum oxide, (activated, acidic, Brockmann I, 50 mesh). All products isolated were homogenous on TLC. General preparation of Schiff bases 13c, 13e, 13i, 13j, 13l, 13m and 13n. The appropriate phenol (10 mmol) was dissolved in dry acetonitrile (100 mL). Anhydrous potassium carbonate (0.16 mol, 22 g) was then added and the mixture was stirred gently for 10 min under a N2 atmosphere. 1-(2-Chloroethyl)pyrrolidine hydrochloride (40 mmol, 5.78 g) was then added and the reaction was refluxed until reaction was complete when monitored by TLC. On completion, the solution was filtered and the solvent was removed under reduced pressure.

4-[4-Benzoxoxyphenyl(1H-1-yl)ethoxy]benzylidene]amine (13j). Preparation was as above from 4-benzoyloxybenzaldehyde (0.1 mol) and 4-aminophenol (0.1 mol). Pale green crystals,Mp. 208 °C, (96%). IR νmax (KBr) cm−1: 1607.9 cm−1 (C=N). 1H NMR (CD3OD): δ 5.14 (s, 2H, O–CH2), 6.86 (d, 2H, J = 8.6 Hz, Ar–H), 7.06 (d, 2H, J = 9.0 Hz, Ar–H), 7.14 (m, 2H, Ar–H), 7.34–7.46 (m, 5H, Ar–H), 7.84 (d, 2H, J = 9.0 Hz, Ar–H), 8.40 (s, 1H, CH=–N). 13C NMR (CD3OD): δ 69.25, 114.34, 114.91, 121.36, 126.78, 127.16, 127.70, 129.58, 136.11, 144.25, 153.94, 155.53, 158.11. HRMS: Found 304.1336 (M+H); C9H10N2O requires 304.1338.

4-[4-Benzoxoxyphenyl]imino)methylphenyl]thione (13e). Preparation was as above from 4-benzoxoxyaniline (0.1 mol) and 4-hydroxybenzaldehyde (0.1 mol). Yellow crystals, mp 214 °C, (96%). IR νmax (KBr) cm−1: 1607.9 cm−1 (C=N). 1H NMR (DMSO-d6): δ 5.07 (s, 2H, O–CH2), 6.86 (d, 2H, J = 8.6 Hz, Ar–H), 7.06 (d, 2H, J = 9.0 Hz, Ar–H), 7.19 (m, 2H, Ar–H), 7.30–7.44 (m, 5H, Ar–H), 7.71 (d, 2H, J = 8.5 Hz, Ar–H), 8.43 (s, 1H, CH=–N). HRMS: Found 304.1336 (M+H); C9H10N2O requires 304.1338.

General procedure for alkylation of phenols: preparation of 13f–g, 13i and 15a. The appropriate phenol (10 mmol) was dissolved in dry acetonitrile (100 mL). Anhydrous potassium carbonate (0.16 mol, 22 g) was then added and the mixture was stirred gently for 10 min under a N2 atmosphere. 1-(2-Chloroethyl)pyrrolidine hydrochloride (40 mmol, 5.78 g) was then added and the reaction was refuxed until reaction was complete when monitored by TLC. On completion, the solution was filtered and the solvent was removed under reduced pressure. 4-Fluorophenyl-[4-(2-pyridin-1-yl)ethyl]benzylidene]amine (13f). Preparation was as above from 13d (5 mmol, 1.076 g). Brown oil, (37%). IR νmax (film) cm−1: 1624.5 cm−1 (CH=–N=). 1H NMR (CDCl3): δ 1.80–1.84 (m, 4H, –CH2–CH2–), 2.63–2.66 (m, 4H, CH2–N–CH2), 2.95 (t, 2H, J = 5.8 Hz, N–CH2), 4.19 (t, 2H, J = 6.0 Hz, OCH2), 7.01 (d, 2H, J = 9.0 Hz, Ar–H), 7.04 (d, 2H, J = 8.5 Hz, Ar–H), 7.15 (m, 2H, Ar–H), 7.83 (d, 2H, J = 8.5 Hz, Ar–H), 8.36 (s, 1H, –CH=–N). 13C NMR (CDCl3): δ 23.08, 54.32, 54.49, 66.82, 114.34, 115.41, 121.71, 128.59, 129.99, 147.89, 159.11, 161.14, 161.73. HRMS: Found 313.1709 (M+H); C9H13N2O requires 313.1716.

4-Benzoxoxyphenyl-[4-(2-pyridin-1-yl)ethyl]benzylidene]amine (13g). Preparation was as above from 13e (0.02 mol, 0.607 g). Orange oil (60%). IR νmax (film) cm−1: 1622.3 cm−1 (CH=–N=). 1H NMR (CDCl3): δ 1.75 (s, br, 4H, –CH2–CH2–), 2.59 (s, br, 4H, –CH2–N–CH2), 2.86 (t, 2H, J = 5.8 Hz, CH2–N), 4.12 (t, 2H, J = 5.8 Hz, CH2–N), 5.01 (s, 2H, O–CH2), 6.94 (d, 4H, J = 8.5 Hz, Ar–H), 7.15 (m, 2H, Ar–H), 7.83 (d, 2H, J = 8.5 Hz, Ar–H), 8.36 (s, 1H, –CH=–N). 13C NMR (CDCl3): δ 23.08, 54.32, 54.49, 66.82, 114.34, 115.41, 121.71, 128.59, 129.99, 147.89, 159.11, 161.14, 161.73. HRMS: Found 313.1709 (M+H); C9H13N2O requires 313.1716.
J = 9.1 Hz, Ar-H), 7.15 (d, 2H, J = 9.4 Hz, Ar-H), 7.36 (m, 3H, Ar-H), 7.39 (d, 2H, J = 8.5 Hz, Ar-H), 7.80 (d, 2H, J = 8.5 Hz, Ar-H), 8.35 (s, 1H, –CH=N–). 13C NMR (CDCl3): δ 203.0, 54.28, 54.66, 67.69, 69.63, 114.59, 114.61, 121.60, 127.06, 128.21, 129.24, 129.80, 130.06, 143.82, 156.78, 157.40. HRMS: Found 401.2216 (M+;H); C26H29NO3S requires 401.2229.

4-Benzoxypyridine-3-(4-(2-pyrrolidin-1-yloxy)phenyl)azetidin-2-one (14b). Brown oil (78%). This compound was used without further purification in the next experiment. IR νmax (film) cm⁻¹: 1750.1 cm⁻¹ (C=O, β-lactam). 1H NMR (CDCl3): δ 0.28 (s, 6H, CH(–CH3)2), 1.03 (s, 3H, Si–C(–CH3)), 3.70 (s, 3H, O–CH3), 4.21 (d, 1H, J = 2.0 Hz, H-3), 4.97 (d, 2H, J = 2.0 Hz, H-4), 6.97–7.42 (m, 13H, Ar-H).

4-(4-Benzoxypyridin-1-yl)-3-phenylazetidin-2-one (15b). Preparation was as above from 13c (0.02 mol, 6.347 g) and phenylacetyl chloride (0.02 mol, 2.63 mL). Brown solid, (78%). This compound was used without further purification in the next experiment. IR νmax (film) cm⁻¹: 1731.5 cm⁻¹ (C=O, β-lactam). 1H NMR (CDCl3): δ 1.82 (s, br, 4H, –CH2–CH2–), 2.66 (s, br, 4H, –CH2–N–CH2–), 2.91 (t, 2H, J = 5.8 Hz, CH2–N–CH2–), 4.14 (t, 2H, J = 5.8 Hz, CH2–N), 5.13 (s, 2H, O–CH3), 6.95 (d, 2H, J = 9.0 Hz, Ar-H), 7.04 (d, 2H, J = 9.0 Hz, Ar-H), 7.20 (d, 2H, J = 9.0 Hz, Ar-H), 7.34–7.46 (m, 5H, Ar-H), 7.81 (d, 2H, J = 6.52 Hz, Ar-H), 8.40 (s, 1H, –CH=N–). 13C NMR (CDCl3): δ 23.03, 54.28, 54.66, 67.69, 69.63, 114.59, 114.61, 121.60, 127.06, 128.21, 129.24, 129.80, 130.06, 143.82, 156.78, 157.40. HRMS: Found 443.2333 (M+;H); C23H23NO3 requires 443.2335.

| Chemical Structure | Preparation | Data | Notes |
|--------------------|-------------|------|-------|
| 2,2-Dimethyl-3-phenyl-1-(4-methoxyphenyl)-4-(4-(2-pyrrolidin-1-yloxy)phenyl)azetidin-2-one (15b) | Preparation was as above from 13c (0.02 mol, 6.347 g) and phenylacetyl chloride (0.02 mol, 2.63 mL). Brown solid, (78%). This compound was used without further purification in the next experiment. IR νmax (film) cm⁻¹: 1750.1 cm⁻¹ (C=O, β-lactam). 1H NMR (CDCl3): δ 1.82 (s, br, 4H, –CH2–CH2–), 2.66 (s, br, 4H, –CH2–N–CH2–), 2.91 (t, 2H, J = 5.8 Hz, CH2–N–CH2–), 4.14 (t, 2H, J = 5.8 Hz, CH2–N), 5.13 (s, 2H, O–CH3), 6.95 (d, 2H, J = 9.0 Hz, Ar-H), 7.04 (d, 2H, J = 9.0 Hz, Ar-H), 7.20 (d, 2H, J = 9.0 Hz, Ar-H), 7.34–7.46 (m, 5H, Ar-H), 7.81 (d, 2H, J = 6.52 Hz, Ar-H), 8.40 (s, 1H, –CH=N–). 13C NMR (CDCl3): δ 23.03, 54.28, 54.66, 67.69, 69.63, 114.59, 114.61, 121.60, 127.06, 128.21, 129.24, 129.80, 130.06, 143.82, 156.78, 157.40. HRMS: Found 443.2333 (M+;H); C23H23NO3 requires 443.2335. | | |
Development of β-lactam type molecular scaffold

4-(4-Benzoylphenyl)-3-phenyl-1-[4-(2-pyrrolidin-1-ylethoxy)phenyl]azetidin-2-one (15h). Preparation was as above from 13i (5 mmol, 2.088 g). Orange oil, (14%). IR νmax (film) cm⁻¹: 1746.6 (-C=O, β-lactam). ¹H NMR ((CD₃)₂CO): δ 1.81 (s, 4H, -CH₂-CH₂-), 2.66 (m, 4H, -CH₂-N-CH₂-), 2.89 (t, 2H, J = 5.8 Hz, CH₂-N), 4.05 (t, 2H, J = 5.8 Hz, CH₂-C), 4.22 (d, 1H, J = 2.5 Hz, H-3), 4.85 (d, 1H, J = 2.5 Hz, H-4), 6.80 (m, 2H, Ar-H), 7.00 (d, 2H, J = 9.0 Hz, Ar-H), 7.41-7.48 (m, 14H, Ar-H). ¹³C NMR (CDCl₃): δ 22.91, 54.02, 63.07, 64.75, 65.01, 69.66, 114.60, 115.08, 118.10, 126.85, 128.55, 132.10, 134.45, 136.21, 136.58, 164.79. HRMS: Found 519.2641 (M⁺+H); C₃₉H₃₇N₂O₃ requires 519.2648.

4-(4-Benzoylphenyl)-3-(4-methoxyphenyl)-1-[4-(2-pyrrolidin-1-ylethoxy)phenyl]azetidin-2-one (15i). Preparation was as above from 13i (3 mmol, 1.253 g). Brown oil, (10%). IR νmax (film) cm⁻¹: 1741.9 (C=O, β-lactam). ¹H NMR ((CD₃)₂CO): δ 1.82 (s, 4H, -CH₂-CH₂-), 2.69 (s, br, 4H, -CH₂-N-CH₂-), 2.92 (t, 2H, J = 5.4 Hz, CH₂-N), 3.80 (s, 3H, OCH₃), 4.05 (t, 2H, J = 5.8 Hz, CH₂-C), 4.17 (d, 1H, J = 2.0 Hz, H-3), 4.79 (d, 1H, J = 2.0 Hz, H-4), 5.05 (s, 2H, O-CH₃), 6.81 (d, 2H, J = 9.2 Hz, Ar-H), 6.91 (d, 2H, J = 8.8 Hz, Ar-H), 6.99 (d, 2H, J = 8.4 Hz, Ar-H), 7.23 (d, 2H, J = 8.0 Hz, Ar-H), 7.28 (d, 2H, J = 8.0 Hz, Ar-H), 7.39 (d, 2H, J = 7.6 Hz, Ar-H), 7.40-7.44 (m, 4H, Ar-H). ¹³C NMR (CDCl₃): δ 23.36, 54.51, 54.77, 55.31, 63.85, 64.66, 70.06, 114.36, 115.01, 115.46, 118.49, 126.96, 129.66, 127.22, 128.08, 128.60, 129.72, 130.24, 131.22, 136.65, 155.08, 159.00, 159.17, 165.59. HRMS: Found 549.2764 (M⁺+H); C₃₂H₂₆N₂O₄ requires 549.2753.

4-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)-3-phenylazetidin-2-one (14c). To a suspension of the protected phenol 14a (10 mmol) in THF (50 ml) was added tetrabutylammonium fluoride (1M, 1.5 equivalents). The solution was stirred in an ice bath for 15 min to afford the product as a yellow oil (50%). IR νmax (film cm⁻¹: 1737.8 (-C=O, β-lactam), 3434.0 (-OH). ¹H NMR (CDCl₃): δ 1.89 (s, br, 4H, -CH₂-CH₂-), 2.83 (s, br, 4H, -CH₂-N-CH₂-), 3.14-3.16 (m, 2H, CH₂-N), 4.20 (d, 1H, J = 2.2 Hz, H-3), 4.23-4.29 (m, 2H, CH₂-C), 4.83 (d, 1H, J = 2.2 Hz, H-4), 6.71 (d, 2H, J = 9.0 Hz, Ar-H), 6.87 (d, 2H, J = 8.5 Hz Ar-H), 7.01 (d, 2H, J = 8.5 Hz Ar-H), 7.19 (d, 2H, J = 9.0 Hz, Ar-H), 7.26-7.39 (m, 5H, Ar-H). ¹³C NMR (CDCl₃): δ 22.83, 54.13, 54.52, 63.01, 64.59, 66.69, 114.40, 115.63, 118.35, 125.91, 127.03, 128.57, 129.51, 129.62, 131.58, 134.42, 153.18, 157.99, 164.75. HRMS: Found 429.2184 (M⁺+H); C₂₉H₂₃N₂O₄ requires 429.2178.

1-(4-Hydroxyphenyl)-3-(4-methylphenyl)-1-[4-(2-pyrrolidin-1-ylethoxy)phenyl]azetidin-2-one (15j). Preparation was as above from 15d (1 mmol, 0.518 g). Yellow oil, (50%). IR νmax (film) cm⁻¹: 1738.91 (-C=O, β-lactam), 3434.0 (-OH). ¹H NMR (CDCl₃): δ 1.89 (s, br, 4H, -CH₂-CH₂-), 2.83 (s, br, 4H, -CH₂-N-CH₂-), 3.14-3.16 (m, 2H, CH₂-N), 4.20 (d, 1H, J = 2.2 Hz, H-3), 4.23-4.29 (m, 2H, CH₂-C), 4.83 (d, 1H, J = 2.2 Hz, H-4), 6.71 (d, 2H, J = 9.0 Hz, Ar-H), 6.87 (d, 2H, J = 8.5 Hz Ar-H), 7.01 (d, 2H, J = 8.5 Hz Ar-H), 7.19 (d, 2H, J = 9.0 Hz, Ar-H), 7.26-7.39 (m, 5H, Ar-H). ¹³C NMR (CDCl₃): δ 22.83, 54.13, 54.52, 63.01, 64.59, 66.69, 114.40, 115.63, 118.35, 125.91, 127.03, 128.57, 129.51, 129.62, 131.58, 134.42, 153.18, 157.99, 164.75. HRMS: Found 429.2184 (M⁺+H); C₂₉H₂₃N₂O₄ requires 429.2178.

MTT assay procedure

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 tumor cell line MCF-7 was cultivated in Eagles Minimum Essential (MEM) medium in a 95% air/5% CO₂ atmosphere supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 µg/mL penicillin/streptomycin. The medium was further supplemented with 1% non-essential amino acids. MDA-MB-231 cells are human breast adenocarcinoma cells, and representative of ER-negative breast cancer. They were grown as monolayer cultures at 37 °C, under a humidified atmosphere of air supplemented with 5% CO₂. The cells were
maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2 mM l-glutamine and 100 µg/mL penicillin/streptomycin. This medium contained phenol red, which is suspected to have estrogen-effects. However, as this assay was only concerned with anti-proliferative effects of the compounds, and these were compared to control cells grown in the same media, it was not deemed necessary to remove the phenol red prior to the assay being conducted. Similarly, the serum was not stripped with charcoal so estrogen levels were not reduced in this assay.

Cells were trypsinized and seeded at a density of $0.5 \times 10^4$ cells/well 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, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). This vehicle had no adverse effect on the cells. The culture medium was then removed and the cells were 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, solubilization was begun through the addition of 200 µL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough color 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. Effect of compounds on MCF-7 cells treated with estradiol: MCF-7 cells were trypsinised and seeded at a density of $0.5 \times 10^4$ cells/well into a 96-well plate as described above. The cells were then treated with 1 µL of test compound and 1 µL of estradiol with (starting concentration is 1 in 200 dilution), which had been pre-prepared as stock solutions in ethanol to furnish the final concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The antiproliferative assay is then completed in the same manner as described above.

**Cytotoxicity studies**

Human MCF-7 breast cancer cells or human MDA-MB-231 breast cancer cells were plated at a density of $0.5 \times 10^4$ cells per well into a 96-well plate (200 µL per well) and incubated at 37°C in air supplemented with 5% CO2 for 24 h. Cells 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, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). Following incubation, 20 µL of lysis solution was added to one row of wells to act as a 100% lysis control. After 30 min, 50 µL aliquots of medium were removed from all wells and placed in a clean 96-well plate. Cytotoxicity was determined using the LDH assay kit obtained from Promega (Madison, WI), following the manufacturer’s instructions for use. A 50 µL per well LDH substrate mixture was added and the plate left in darkness at room temperature for equilibrium. Stop solution (50 µL) was added to all the wells and absorbance read at 490 nM. Data were presented following calculation of percentage cell lysis versus concentration of subject compound.

**Estrogen receptor binding assay**

ERα and ERβ fluorescence polarization based competitor assay kits were obtained from Panvera at Invitrogen Life Technologies, Carlsbad, CA. The recombinant ER (insect expressed, full length, untagged human ER obtained from recombinant baculovirus-infected insect cells) and the fluorescent estrogen ligand were removed from the −80°C freezer and thawed on ice for 1 h prior to use. The fluorescent estrogen ligand (2 nM) was added to the ER (40 nM for ERα and 30 nM for ERβ) and screening buffer (100 mM potassium phosphate (pH 7.4), 100 µg/ml BGG, 0.02% NaN₃) was added to make up to a final volume that was dependent on the number of tubes used (number of tubes (e.g. 50) × volume of complex in each tube (50 µL) = total volume (e.g. 2500 µL)). Test compound (1 µL, concentration range 100 nM–1 M) was added to 49 µL screening buffer in each borosilicate tube (6 mm diameter). To this, 50 µL of the fluorescent estrogen/ER complex was added to make up a final volume of 100 µL.

**Estrogenic activity: alkaline phosphatase assay**

Following the procedure of Littlefield et al., human Ishikawa cells were maintained in Eagle’s Minimum Essential Medium (MEM containing 10% v/v fetal bovine serum (FBS) and supplemented with 100 U/mL penicillin and 10 µg/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. Twenty four hours before the start of the experiment, near confluence cells were changed to an estrogen-free basal medium (EFBM), A 1:1 mixture of phenol-free Ham’s F-12 and Dulbecco’s Modified Eagles Medium, together with the supplements listed above, and 5% calf serum, stripped of endogenous estrogens with dextran-coated charcoal. On the day of the experiment, cells were harvested with 0.25% trypsin and plated in 96-well flat bottomed microtitre plates in EFBM at a density of 2.5 × 10⁴ cells/well. Test compounds were dissolved in ethanol at 10⁻⁷ M, diluted with EFBM (final concentration of ethanol 0.1%) and filter sterilized. After addition of the test compounds, (plated in 50 µL, added estradiol in 50 µL and blank medium to give a final volume 150 µL) the cells were incubated at 37°C in a humidified atmosphere containing 95%air/5% CO₂ for 72 h. All experimental values were obtained in triplicate. The microtitre plates were then inverted and the growth medium removed. The plates were then rinsed by gentle immersion and swirling in 2 L of PBS (0.15M NaCl, 10 mM sodium phosphate, pH 7.4). The plates were removed from the container, the residual saline in the plate was not removed, and the wash was repeated. The buffered saline was then shaken out, and the plate blotted on paper towel. The covers were replaced and the plates were placed at −80°C for at least 15 min, and then thawed at room temperature for 5–10 min. The plates were then placed on ice and 50 µL ice cold solution containing 50 mM p-nitrophenyl phosphate, 0.24 mM MgCl₂ and 1M diethanolamine (pH 9.8) was added. The plates were warmed to room temperature (time zero), and the yellow color from the production of p-nitrophenol was allowed to develop. The plates were monitored at 405 nm until maximum stimulation of the cells showed an absorbance of ∼1.2.

**Computational procedures: protein preparation**

PDB entry 3ERT (4-hydroxytamoxifen co-crystallised with ERx) was downloaded from the Protein Data Bank (PDB) was utilized for all ERx dockings and also as a template for creation of a homology model of ERβ. The model was constructed in an automated fashion using EsyPred3D. Hydrogens were added to both structures using MOE (Chemical Computing Group, Montreal, QC, Canada) and their positions minimized with the AMBER99 force field.

**Docking/scoring**

50 conformations of both compounds 15g and 15k were built using OMEGAv2.2.1 and docked using FREDv2.2.3.
(Openeye Scientific Software, Santa Fe, NM) retaining all default settings and employing the Chemgauss3 scoring function\textsuperscript{50} for prioritizing correct binding orientations. The active site of top docked poses of each compound was minimized and refined using LigX (Chemical Computing Group)\textsuperscript{46} to allow side-chain repositioning.

**Results and discussion**

**Chemistry**

The synthesis of the Type I series of \( \beta \)-lactam compounds which contain the basic pyrrolidinylethoxy substituent located on the C-4 aryl ring is illustrated in Scheme 1. The Staudinger reaction requiring a cycloaddition reaction of a ketene with an imine is the most commonly used method for synthesis of 1,3,4-trisubstituted \( \beta \)-lactams\textsuperscript{34,51}. The initial target compound \( 15a \), containing a methyl ether on the N-1 aryl ring (which is important for interaction with His524 in the ligand binding domain of the estrogen receptor), was synthesized using this reaction. The required Schiff base \( 13a \) was prepared in high yield (88\%) by condensation of 4-hydroxybenzaldehyde with 4-methoxyaniline. To avoid difficulties in the following cycloaddition reaction, the phenol was protected as a tert-butyldimethylsilyl ether using tert-butyldimethylsilyl chloride and the base DBU to afford the protected Schiff base \( 13b \). The \( \beta \)-lactam product \( 14a \) was obtained by subsequent Staudinger reaction of imine \( 13b \) with phenylacetyl chloride in the presence of triethylamine. To optimize the yield of the \( \beta \)-lactam product, a number of reaction conditions were investigated. \( \beta \)-Lactam \( 14a \) was initially obtained successfully using DMF as the solvent\textsuperscript{52}. This method resulted in exclusive synthesis of the \textit{trans} product in a moderate yield (45\%), (one enantiomer only shown for the products \( 14a-c \) and \( 15a-g \) in Scheme 1). However, the reaction required over 24-h reflux so an alternative method was investigated. When toluene was used as the solvent and the acid chloride was added dropwise to a refluxing solution of appropriate imine and triethylamine\textsuperscript{53}, an improved yield (65\%) was obtained with exclusive isolation of the \textit{trans} product. Finally, reaction conditions were optimized using dichloromethane as the solvent and with the dropwise addition of the acid chloride to a mixture of the imine \( 13b \) and triethylamine, initially at \(-20^\circ\text{C}\textsuperscript{54}\). The \textit{trans} product \( 14a \) was again isolated exclusively and an improved yield of 78\% obtained. This method was then employed for all subsequent Staudinger reactions. As indicated, all three methods of reaction above yielded the \textit{trans} \( \beta \)-lactam product, with the stereochemistry of the \( \beta \)-lactam deduced from the coupling constants of the C-3 and C-4 protons and were found to be in the region 1–3 Hz. The \textit{cis}-\( \beta \)-lactams have larger coupling constants (5–6 Hz) than the \textit{trans}-\( \beta \)-lactams which are usually 1–3 Hz\textsuperscript{34,52}. Stereoselectivity in the Staudinger reaction that depends on a number of experimental factors including structure of the imine and acid chloride, sequence of reagent addition, solvent, temperature and organic amine base\textsuperscript{34}.

Scheme 1. Synthesis of Type I \( \beta \)-lactam ER antagonist compounds (one enantiomer shown). Scheme reagents and conditions: (a) Ethanol, reflux; (b) tert-Butyldimethylsilyl chloride, DBU, DCM; (c) Phenylacetyl chloride, (CH\(_3\)CH\(_2\))\(_3\)N, DCM; (d) TBAF, THF; (e) H\(_2\), 10% Pd/C, ethyl acetate:ethanol; (f) K\(_2\)CO\(_3\), 1-(2-chloroethyl)pyrrolidine, acetone.
In general, when the acyl chloride is added dropwise, preferably at low temperature, to a solution of imine and a tertiary amine such as triethylamine, the cis cycloadduct is the major or exclusive stereoisomer detected. In contrast, when the tertiary base is added to a mixture of imine and acyl chloride, mixtures of cis and trans cycloadducts are obtained, in which the trans is the major or exclusive product\textsuperscript{14,45}. However, in our synthesis, using all the above conditions with reaction temperatures varied from 20 to 150 °C, the trans isomer was always exclusively formed. We also observed a similar stereochemical outcome with 4-methoxyphthaloyl chloride and 4-benzoxyphenacyl chloride in this reaction indicating that the specific acid chloride is an important variable in determining the stereochemical selectivity. Alonso et al reported synthesis of a mixture of cis and trans isomers when tetrahydrofurol chloride was the acid component\textsuperscript{49}. Phthalimidoacetyl chloride and crotonyl chloride are other reported acid chlorides, which form exclusively trans β-lactams.

Removal of the silyl protecting group from 14a using tetrabutylammonium fluoride (TBAF) resulted in the isolation of the phenolic 14c (50%), which was confirmed in the IR spectrum that shows a carbonyl absorption at \(\nu = 1735.6\) cm\(^{-1}\) and a broad absorption corresponding to the hydroxy group at \(\nu = 3300.0\) cm\(^{-1}\). In the \(^1\)H NMR spectrum, the doublet signals at \(\delta = 4.22\) and \(\delta = 4.98\) (\(J = 2.26\) Hz) are assigned to H-3 and H-4 respectively, indicating the trans β-lactam stereochemistry.

The alternative method used for the preparation of 14c involves the use of a benzyl ether as the protecting group in place of the silyl ether (Scheme 1), which can be easily removed using catalytic hydrogenolysis. Catalytic hydrogenation has been reported under ambient pressure of hydrogen at 50 °C in methanol with Pd/C as the catalyst to remove the benzoxyl group without any effect on the β-lactam ring\textsuperscript{34,54,55}. However, care must be taken to avoid over-hydrogenation as C-4-N-1 bond cleavage has been reported to proceed by palladium catalyzed hydrogenolysis when an aryl substituent is located at the C-4 position i.e. a benzylic carbon\textsuperscript{56,57}. The Schiff base 13c (obtained following the condensation of 4-methoxyaniline with 4-benzyloxynaldehyde) was reacted with phenylacetyl chloride under Staudinger conditions to afford the benzoxyl protected product 14b, (78%). The IR spectrum contained the characteristic β-lactam carbonyl absorption band at \(\nu = 1735\) cm\(^{-1}\) while the \(^1\)H NMR spectrum confirmed the trans β-lactam isomer with H-3 and H-4 observed as coupled doublets at \(\delta = 4.19\) and \(\delta = 4.81\), respectively, (\(J = 2.26\) Hz). Careful removal of the benzyl protecting group from 14b by hydrogenation yielded 14c (70%). Alkylation of the phenolic 14c with 1-(2-chloroethyl)pyrrolidine affords the required product 15a (30%).

Fluoro-substituted tamoxifen and cyclofenol derivatives have been investigated as ER-imaging agents for breast cancer\textsuperscript{58,59} and we have previously reported the potent ER-binding properties of fluorne-containing benzoepine type ER antagonists\textsuperscript{30}. We now wished to examine the effect of the inclusion of the lipophilic fluorne substituent on the ER activity of the β-lactam ER antagonist compounds 15b and 15c. The Schiff base 13d, obtained in 58% by the standard method (Scheme 1), was directly alkylated with 1-(2-chloroethyl)pyrrolidine hydrochloride to yield 13f which was used without further purification in the subsequent reactions. Treatment of 13f with phenylacetyl chloride under the usual Staudinger reaction conditions afforded the trans β-lactam product 15b, (Scheme 1). The \(^1\)H NMR spectrum indicated two coupled doublets at \(\delta = 4.27\) and \(\delta = 4.87\) (\(J_{4,5} = 2.04\) Hz) assigned to H-3 and H-4, respectively. 15c was similarly prepared from 13f and 4-methoxyphenacyl chloride.

The requirement for a phenolic substituent in many ER antagonists such as 4-hydroxytamoxifen\textsuperscript{4} and raloxifene is significant for successful binding to the ER as shown by interactions with Glu353 and Arg394. Therefore, in the present work it was critical to include a phenolic substituent group on the N-1 aryl ring of the Type-I β-lactam products to provide a 4-hydroxytamoxifen analog containing the β-lactam ring. Synthesis of 15f by direct demethylation of 15a proved unsuccessful using several different reagents including ethanethiol and boron trifluoride-methyl sulfide; in both cases resulting in degradation of the β-lactam ring. An alternative method of synthesis of 15f and 15g was pursued which involved the preparation of a Schiff base 13g with both the required benzyl protected phenol and the basic side-chain are in position. This protected Schiff base was then treated with the relevant acid chloride to form the β-lactam which could then be deprotected by hydrogenation to yield the free phenol as required on the N-1 aryl ring. Schiff base 13e was prepared in 96% yield by condensation of 4-hydroxybenzaldehyde with 4-benzyloxyaniline and was then alkylated with 1-(2-chloroethyl)pyrrolidine hydrochloride to afford 13g (60%). Subsequent treatment of 13g with phenylacetyl chloride under the usual Staudinger conditions resulted in the isolation of the protected β-lactam 15d (61%). The \(^1\)H NMR spectrum of 15d showed characteristic β-lactam doublet signals for H-3 at \(\delta = 4.18\) and H-4 at \(\delta = 4.85\), \((J_{3,4} = 2.52\) Hz). The benzoxyl group was carefully removed by hydrogenation yielding 15f (50%); the \(^1\)H NMR spectrum confirmed the presence of the trans β-lactam ring with H-3 at \(\delta = 4.20\) and H-4 at \(\delta = 4.84\), \((J_{3,4} = 2.24\) Hz). Compound 15g was synthesized in a similar reaction sequence. The protected Schiff base 13g was treated with 4-methoxyphenacyl chloride resulting in the isolation of 15e (33%). Subsequent removal of the benzyl protecting group from 15e by careful hydrogenation yields the phenolic β-lactam product 15g in 78% yield, with no evidence of ring hydrogenolysis (Scheme 1).

The synthetic route used for the Type II β-lactams (containing the basic side chain substituent on N1 aryl ring) is shown in Scheme 2, which again employs the Staudinger reaction for β-lactam formation. The phenolic Schiff base 13h (obtained in 96% yield on reaction of 4-benzyloxynaldehyde with 4-aminophenol) was treated with 1-(2-chloroethyl)pyrrolidine hydrochloride yielding the alkylated product 13i (60%). Reaction of 13i with phenylacetyl chloride and 4-methoxyphenacyl chloride under the usual Staudinger reaction conditions afforded the trans β-lactams 15h (15%) and 15i (33%) as confirmed by \(^1\)H NMR spectra: 15h \((J_{3,4} = 2.5\) Hz) and 15i \((J_{3,4} = 2.0\) Hz). Removal of the benzyl protecting group from 15h and 15i by hydrogenation yielded the required phenolic products 15j and 15k respectively, (one enantiomer shown for compounds 15h–15k, Scheme 2).

Antiproliferative activity in MCF-7 and MDA-MB-231 breast cancer cells

The β-lactam compounds prepared above were evaluated in a series of in vitro assays which determined their antiproliferative activity in ER positive MCF-7 and ER negative MDA-MB-231 breast cancer cell lines and also their affinity for the estrogen receptor and estrogenic effects in Ishikawa cells.

Compounds 15a–15c, 15f–g and 15j–k were initially screened for their antiproliferative activity using the ER expressing (ER-dependent) MCF-7 human breast cancer cell line and the ER-independent MDA-MB-231 human breast cancer cell line. Table 1 shows the antiproliferative effects of type I and type II β-lactams in both MCF-7 and MDA-MB-231 cell lines. The majority of the compounds show anti-proliferative effects at concentrations similar to that of the positive control tamoxifen\textsuperscript{60,61}. Many of the compounds (e.g. 15a, 15f, 15g and 15j) have IC\textsubscript{50} values in the range 0.185–7.54 μM in MCF-7 cells, but have significantly higher IC\textsubscript{50} values in MDA-MB-231 cells (11–40 μM), a result
which is not unexpected. However, compounds 15c and 15k unusually show moderate potency in MDA-MB-231 cell line (IC50 = 4.62 μM, 5.03 μM, respectively). The most potent compound in the series examined in MCF-7 cells is 15g (IC50 value = 0.185 μM), representative of the Type-I structure, containing the phenolic substitution in Ring C, which would be required for interaction with the Asp351 of the estrogen receptor LBD14. This indicates that the possible mechanism of action of the compound is mediated through binding to the estrogen receptor. Tamoxifen shows some antiproliferative effects in MDA-MB-231 ER-negative cell lines at much higher concentrations (approx. 20 μM) than in MCF-7 cells62,63. The cytotoxic effect of

Table 1. Antiproliferative and cytotoxic effects for compounds 15a–c, 15f–g and 15j–k in MCF-7 and MDA-MB-231 cells; ERα and ERβ binding affinities for compounds 15a–c, 15f–g and 15j–k.

| Compound | MCF-7 IC50 (μM)* | Cytotoxicity (%) | MDA-MB-231 IC50 (μM)* | Cytotoxicity (%) | ERα IC50 (μM)|| ERβ IC50 (μM)|| α/β ratio |
|----------|------------------|-----------------|------------------------|-----------------|---------------|----------------|----------------|------------|
| 15a      | R1=OCH3          | 6.22            | 1                      | 12.77           | 5.3           | 1.70           | 15.49          | 9          |
| 15b      | R1=F             | 4.82            | 12                     | 12.7            | 0             | 1.05           | >100μM         | >95        |
| 15c      | R1=F             | 3.49            | 26                     | 4.62            | 25            | 0.23           | 1.64           | 7          |
| 15f      | R1=OH            | 0.519           | 4                      | 43.08           | 0             | 0.060          | 0.66           | 11         |
| 15g      | R1=OH            | 0.186           | 4.9                    | 19.65           | 3.5           | 0.0043         | 0.32           | 75         |
| 15j      | R=H              | 7.54            | 11                     | 17.54           | 2             | 0.21           | 2.31           | 11         |
| 15k      | R=OCH3           | 3.30            | 0                      | 5.03            | 4.8           | 3.04           | >50μM          | >16        |
| Tamoxifen |                  | 4.12†           | 13.4                   | 20†             | 0             | 0.070#         | 0.170#         | 2.42       |
| 4-Hydroxy-tamoxifen | –              | 0.107           | 0                      | 18**           | -†           | 0.040          | 0.020          | 0.5        |

*IC50 values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 or MDA-MB-231 cells. Values represent the mean ± SEM (error values × 10−6) for three experiments performed in triplicate.
†The IC50 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 IC50 value for tamoxifen on human MCF-7 cells60.
‡The IC50 value obtained for Tamoxifen in MDA-MB-231 cells (20 μM) is in agreement with reported values for tamoxifen in MDA-MB-231 cells63,64.
§Lactate dehydrogenase assay: following treatment of the cells, the amount of LDH was determined using LDH assay kit from Promega. Data are presented as % cell lysis at compound concentration of 10 μM12.
||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%.
#The ER binding values obtained are in agreement with the reported ER IC50 binding data for tamoxifen (ERα 60.9 nM ERβ 188 nM, Panvera/Invitrogen).
**Work by Seeger et al.61.
††No cytotoxic effect could be demonstrated for 4-hydroxytamoxifen in MDA-MB-321 cells60.

which is not expected. However, compounds 15c and 15k unusually show moderate potency in MDA-MB-231 cell line (IC50 = 4.62 μM, 5.03 μM, respectively). The most potent compound in the series examined in MCF-7 cells is 15g (IC50 value = 0.185 μM), representative of the Type-I structure, containing the phenolic substitution in Ring C, which would be required for interaction with the Asp351 of the estrogen receptor LBD14. This indicates that the possible mechanism of action of the compound is mediated through binding to the estrogen receptor. Tamoxifen shows some antiproliferative effects in MDA-MB-231 ER-negative cell lines at much higher concentrations (approx. 20 μM) than in MCF-7 cells62,63. The cytotoxic effect of
inhibition of proliferation of MCF-7 breast cancer cells in the absence and presence of estradiol. MCF-7 cells were seeded at a density of $2.5 \times 10^4$ cells per well in 96-well plates. The plates were left for 24 h to allow the cells to adhere to the surface of the wells. A range of concentrations (0.01 nM–50 μM) of the compound were added in triplicate and the cells were left for another 72 h. An MTT assay was performed to determine the level of anti-proliferation. (A, B) are representative of results for 15g, and tamoxifen (I) (control); the values represent the mean ± S.E.M (error values) for three experiments performed in triplicate. Effect of compounds on MCF-7 cells treated with estradiol – cells were treated with 1 μl of test compound and 1 μl of estradiol, 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). (A) Compound 15g in the absence and presence of estradiol (50nM); inhibited proliferation of MCF-7 cells. (B) Tamoxifen (I) (control) in the absence and presence of estradiol (50nM); inhibited proliferation of MCF-7 cells.

irreversible components to their inhibition of cell proliferation in vitro, with the former being highly correlated with affinity for ER. The antiproliferative effect appears to be estradiol reversible with the cytotoxic effect being irreversible. This effect is seen at the higher ER antagonist concentrations, and is demonstrated for tamoxifen where estradiol is shown to reverse the antagonist effect at lower concentrations of tamoxifen, but not at higher concentrations; (IC50 value = 4.22 μM for tamoxifen in the presence of estradiol (50 nM), Figure 3B). Compounds 15g and 15f show estradiol–reversible effects at concentrations higher than tamoxifen, indicating that they have less estradiol–irreversible effects than tamoxifen, and suggesting that the main mechanism of action of these β-lactam compounds is through affinity for the ER.

these β-lactam compounds in MCF-7 cells as determined in the lactose dehydrogenase (LDH) assay (Table 1) is also lower than that of tamoxifen with the exception of compound 15c, which resulted in 26% and 25% toxicity in MCF-7 and MDA-MB-231 cell lines, respectively. This cytotoxic action may explain the equal antiproliferative effects in both MCF-7 and MDA-MB-231 cell lines observed for compound 15c.

The antiproliferative effect of 15g (Type I) in MCF-7 cells was significantly reduced with the addition of estradiol (50 nM), with IC50 value increased from 0.186 μM to 9.17 μM, (Figure 3A). A similar result is also obtained for compound 15f (IC50 values increased from 0.519 to 27.85 μM). Compound 15k (Type II) demonstrated a small increase in IC50 value on addition of estradiol (50 nM) from 3.30 to 8.29 μM. These results support the indication that the antiproliferative effects for compounds 15f and 15g are a result of interaction of the compounds with the ER and therefore preventing estrogen mediated proliferation. Reversal of antiestrogen-mediated cell growth antagonism by estradiol has been suggested to indicate the degree to which antagonism is mediated through ER. Tamoxifen and 4-hydroxytamoxifen have been shown to have an estradiol reversible and estradiol mediated through ER.

Estrogen receptor binding studies

The affinity for the ER of the β-lactam compounds 15a–15c, 15f–g and 15j–k is confirmed through estrogen receptor binding studies. Table 1 shows the results of the competitive ER binding assay for β-lactam SERM Types I and II with both ERα and ERβ. While the level of affinity to the ER varies depending on the aryl substituents present, compounds of structural Type I appear to have better binding ability than compounds of structural type II. Of the Type I compounds, 15g established the most potent binding to the ER (IC50 ERα = 4.3 nM, ERβ = 322 nM) and significantly more potent than both tamoxifen (IC50 ERα = 70 nM) and 4-hydroxytamoxifen (IC50 ERα = 40 nM) for ERα. This result also correlated with the antiproliferative activity of 15g in the MCF-7 cell line (IC50 = 185 nM). The fluoro-substituted β-lactam 15c indicated some improved ERα and ERβ interaction when compared to the methoxy substituted product 15a, (Table 1).

The IC50 value for ERα binding of the Type II compound 15k (3.04 μM) is much greater than tamoxifen or that of the type I β-lactam compounds 15f and 15g. However, the moderate antiproliferative effect in MCF-7 cells is very similar to 15c with an IC50 of 3.30 μM. The IC50 value for 15k in the MDA-MB-231 cell line is 5.03 μM indicating that in this case
Figure 5. Key anti-estrogenic interactions are observed in the docking of 15g in ERz. Hydrogen bonding between the C-ring 3-OH substituent and Glu353, Arg394, HOH is depicted. A salt bridge between the basic side chain and Asp351 is also formed.

Figure 6. Key anti-estrogenic interactions are also observed in the docking of 15k in ERz. Hydrogen bonding between the B-ring 3-OH substituent and Glu353, Arg394, HOH is depicted. A salt bridge between the basic side chain and Asp351 is also formed.
Figure 7. Docked position of 15g and 15k in both ERα/β superimposed by backbone. Residues Met336, met295 and Ile373 are from dockings in ERβ. Residues of ERβ depicted in orange for docking of 15k. Residues Leu384, Met343, Met421 are from dockings in ERα. Information: Figure S1: Effect of compounds 15a and 15j on the inhibition of proliferation of MCF-7 breast cancer cells; Figure S2: Effect of compounds 15a, 15f, 15g, 15k, 15j on the inhibition of proliferation of MDA-MB-231 breast cancer cells; Figure S3: Estrogen receptor binding affinities for compounds 15a, 15f, 15g, 15k, 15j; Figure S4: Estrogen receptor binding affinities for compounds 15a, 15f, 15g, 15k, 15j.

Antiestrogenic activity in Ishikawa cells

The estrogen stimulation and antagonistic properties of the most active ER compound 15g was determined in an estrogen bioassay which is based on the stimulation of alkaline phosphatase (AP) in the Ishikawa human endometrial adenocarcinoma cell line. Enemark et al have reported that Ishikawa cells contain both ERα and ERβ receptors but with ERα being far in excess. Compound 15g was examined as an estrogen antagonist by its effect on the inhibition of estradiol stimulation in the Ishikawa cells in a dose-dependent manner. (Figure 4). The estrogenic stimulatory property of this compound was also monitored in Ishikawa cells by measuring the stimulation of alkaline phosphatase (AP) in these cells in the absence of estradiol. 15g showed little ability to inhibit the effect of estradiol in Ishikawa cells at concentrations of up to 1 μM, however, at the same time 15g itself shows a higher level of estrogen stimulation (21% at 1 μM concentration) when compared to tamoxifen, (10% stimulation at 1 μM concentration). This may indicate that the stimulating effect is due to both estradiol and 15g and is not indicative of the inability of estradiol to be displaced by the β-lactam compound. Other known anti-estrogens also show estrogen stimulation in Ishikawa cells with reports having shown that 4-hydroxytamoxifen stimulates AP activity to a level 47% of that of estradiol and the SERM lasofoxifene increases AP activity by 18%.

Molecular modeling studies of novel β-lactam compounds

To rationalize the observed ERα/β affinity of Type I and Type II compounds, a semi-flexible ligand receptor docking study of compounds 15g and 15k was undertaken. The PDB entry 3ERT was used (ERα co-crystallized with 4-hydroxytamoxifen) to examine the ERα binding mode of these compounds. Figures 5 and 6 illustrate the docked binding poses of compounds 15g and 15k respectively in the binding site of ERα. It is immediately evident that both compounds adopt a typical antiestrogenic orientation in the ligand binding site. Key hydrogen bonding interactions between the phenolic group of the ligands and Glu353, Arg394 and bridging water are observed. A salt bridge is also formed between the basic side chain nitrogens and Asp351 of Helix-12 (15g – 2.8 Å, 15k – 2.6 Å). As there is currently no antiestrogen co-crystallized in both isoforms of ERα and ERβ, it was deemed necessary to construct a homology model of ERβ using PDB entry 3ERT as a template. This process ensured that bias of ligand induced residue motion would be reduced and the process would provide more realistic dockings when used in.
conjunction with our semi-flexible docking approach. Figure 7 illustrates the docked positions of both compounds 15g and 15k in ERα/β superimposed on backbone. It is firstly apparent that the β-lactam ring of 15g lies in close proximity to the ERα Leu384/ ERβ Met336 residue mutation and favorable interaction with Leu384 could account for some of the ERα selectivity observed (≈75-fold). For the case of 15k (Type II structure), a different binding pose occurs compared with 15g whereby the β-lactam ring carbonyl oxygen of 15k is positioned towards ERα residue Met343 (ERβ Met295) making a favorable interaction, whilst Met295 seems to reposition itself so as not to cause any steric hindrance. It would appear for the most part that the significant ERα selectivity observed is mainly a result of the tighter packing of 15g in the active site of ERα by surrounding residue side chains.

Conclusion

There is currently much interest in the discovery of novel molecular scaffolds with SERM profile properties which could be suitable for development of new therapies for the treatment of breast cancer, osteoporosis, and related hormone-dependent conditions. Both raloxifene and tamoxifen are good preventive choices for treatment of postmenopausal women with elevated risk for breast cancer. Because of the known importance of ERα as a pharmaceutical target and also the potential importance of ERβ, molecules that act as agonists or antagonists selectively or ER subtypes are currently being investigated for their therapeutic potential. ERα predominates in the breast and in reproductive tissues such as the uterus, whereas ERβ is the principal subtype in the ovary and certain regions of the brain. We have synthesized a novel of β-lactam compounds designed as potential estrogen receptor ligands, which demonstrate anti-proliferative activity against the MCF-7 human breast cancer cell line. The compounds also demonstrate good affinity for the estrogen receptor and selectivity for ERα. The most potent antiproliferative compound 15g having Type I structural scaffold, demonstrated ERα binding with IC₅₀ ≈ 4.3 nM and relative binding affinity ERα/ERβ of 75:1. Further biochemical studies will determine the effects of these novel analogs on ERE transcription and ERα stability in MCF-7 cells, and will determine the mechanistic differences between their activity and that of tamoxifen.

Declaration of interest

The authors report no declarations of interest. This work was supported through funding from the Trinity College IITAC research initiative (HEA PRTLI, Cycle 3).

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