Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: http://www.tandfonline.com/loi/ienz20

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To cite this article: Miriam Carr, Andrew J. S. Knox, David G. Lloyd, Daniela M. Zisterer & Mary J. Meegan (2016): 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, Journal of Enzyme Inhibition and Medicinal Chemistry, DOI: 10.1080/14756366.2016.1210136

To link to this article: http://dx.doi.org/10.1080/14756366.2016.1210136

Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–14
 DOI: 10.1080/14756366.2016.1210136

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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 (IC_{50} = 186 nM) and ERα binding (IC_{50} = 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

History

Received 8 March 2016
Revised 26 June 2016
Accepted 4 July 2016
Published online 28 July 2016

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[1,2]. Incidence rates vary from 27 per 100 000 women in Middle Africa and Eastern Asia to 96 per 100 000 women in Western Europe[3]. 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[4].

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[5]. The first antiestrogen to show positive clinical results was tamoxifen **(1)**, (Figure 1), 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[6,7]. Tamoxifen is extensively metabolized by the human hepatic cytochrome P450 enzyme system into several metabolites including 4-hydroxytamoxifen **(2)** and 4-hydroxy-N-desmethyltamoxifen (endoxifen), **(3)**, Figure 1[8,9]. They are ∼100-fold more potent as antiestrogens than tamoxifen and are most likely contributors to the base antiproliferative activity observed with tamoxifen[10]. The metabolite norendoxifen was shown to have dual aromatase inhibitory and estrogen receptor modulatory activities[11]. Novel tamoxifen analogs that avoid CYPD6 metabolism have been recently reported[12]. The SERM raloxifene (**4**, Figure 1), a 2,3-disubstitued 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[13,14]. 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[15]. 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[16,17]. The steroid fulvestrant **(5)**, Figure 1, is also effective as second line therapy against advanced breast cancer in patients who develop resistance to tamoxifen[18]. Due to its pure antiestrogen 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

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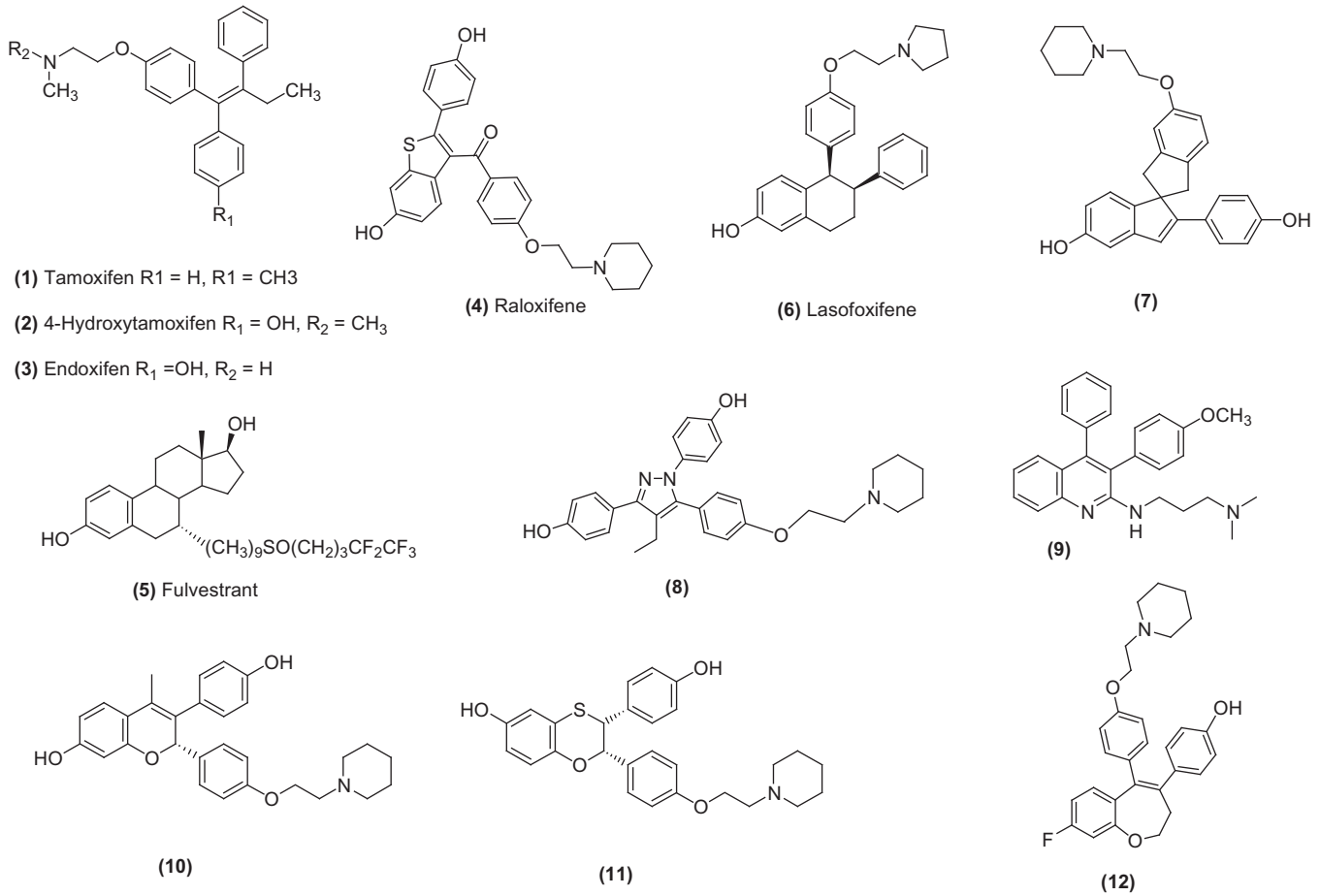


Figure 1. Structures of clinical SERMs and related ER antagonists.

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 Erα, but they exert exclusively antagonistic activity on Erβ[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], benzoxathiin **(11)**[29] and benzoxepin 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 tryptase[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 effects and ER-binding properties of a series of 1,4-diarylsubstituted azetidin-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 ERα and ERβ. 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

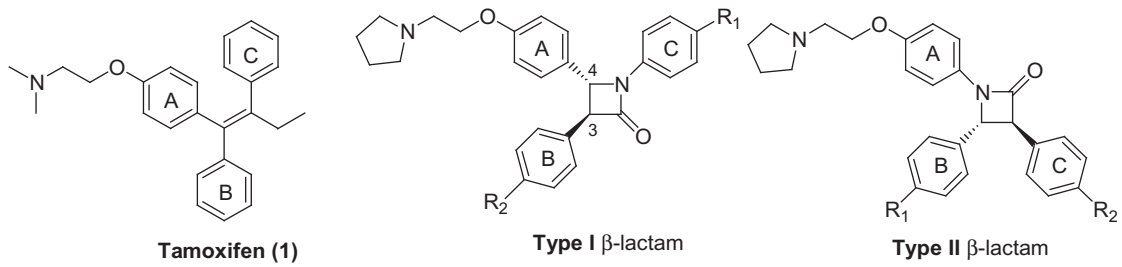


Figure 2. Structure of tamoxifen (1) and Type I and Type II β-lactam ER antagonists.

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). ^{1}H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded at 27 °C on a Bruker Avance DPX 400 spectrometer (Bruker, Billerica, MA) (400.13 MHz, ^{1}H; 100.61 MHz, ^{13}C; 376.47 MHz, ^{19}F) at 20 °C in either $CDCl_3$ (internal standard tetramethylsilane (TMS)) or CD_3OD by Dr. John O'Brien and Dr. Manuel Ruether in the School of Chemistry, Trinity College Dublin. For $CDCl_3$, ^{1}H-NMR spectra were assigned relative to the TMS peak at 0.00 δ and ^{13}C-NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For CD_3OD, ^{1}H and ^{13}C-NMR spectra were assigned relative to the center peaks of the CD_3OD multiplets at 3.30 δ and 49.00 ppm, respectively. ^{19}F-NMR spectra were not calibrated. Electrospray ionization mass spectrometry (ESI-MS) was performed in the positive ion mode on a liquid chromatography time-of-flight (TOF) mass spectrometer (Micromass LCT, Waters Ltd., Manchester, UK) equipped with electrospray ionization (ES) interface operated in the positive ion mode at the High Resolution Mass Spectrometry Laboratory by Dr. Martin Feeney in the School of Chemistry, Trinity College. Mass measurement accuracies of $< \pm 5$ 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. R_f 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. Analytical high-performance liquid chromatography (HPLC) to determine the purity of the final compounds was performed using a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump, a Waters In-Line Degasser AF and a Waters 717plus Autosampler (Waters Corporation, Milford, MA). The column used was a Varian Pursuit XRs C18 reverse phase 150 × 4.6 mm chromatography column (Agilent, Santa Clara, CA). Samples were detected using a wavelength of 254 nm. All samples were analyzed using acetonitrile (70%): water (30%) over 10 min and a flow rate of 1 mL/min. Schiff bases **13a** and **13d** were prepared as previously reported[41].

*General preparation of Schiff bases **13c**, **13e**, **13 h***. A solution of the appropriately substituted aryl aldehyde (0.1 mol) and the appropriately substituted aryl amine (0.1 mol) in ethanol (50 mL) was heated to reflux for 3 h. The reaction mixture was reduced to 25 mL under vacuum, the Schiff base product crystallized from the solution. The crude product was then recrystallized from ethanol to afford the purified product.

(4-Benzyloxybenzylidene)-(4-methoxyphenyl)amine (***13c***). Preparation as above from 4-benzyloxybenzaldehyde (0.1 mol) and 4-methoxyaniline (0.1 mol). Colorless crystals, m.p. 148 °C, (88%). IR ν_{max} (KBr) cm^{-1}: 1605.9 cm^{-1} (C=N). ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H, OCH_3), 5.14 (s, 2H, OCH_2), 6.93 (d, 2H, $J = 9.0$ Hz, Ar-H), 7.08 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.24 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.41–7.46 (m, 5H, Ar-H), 7.84 (d, 2H, $J = 8.5$ Hz, Ar-H), 8.42 (s, 1H, CH=N). ^{13}C NMR ($CDCl_3$): δ 57.89, 69.63, 113.91, 114.61, 121.64, 127.06, 127.69, 128.21, 129.13, 129.86, 136.01, 144.70, 157.52, 157.60, 160.71. HRMS: Found 318.1490 (M^++H); $C_{21}H_{20}NO_2$ requires 318.1494.

4-[(4-Benzyloxyphenylimino)methyl]phenol (***13e***). Preparation as above from 4-benzyloxyaniline (0.1 mol) and 4-hydroxybenzaldehdye (0.1 mol). Yellow crystals, m.p. 214 °C, (96%). IR ν_{max} (KBr) cm^{-1}: 1607.9 cm^{-1} (C=N). ^{1}H NMR (DMSO-d_6): δ 5.07 (s, 2H, O–CH_2), 6.86 (d, 2H, $J = 8.5$ Hz, Ar-H)), 7.00 (d, 2H, $J = 8.6$ Hz, Ar-H), 7.19 (d, 2H, $J = 8.5$ Hz, 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); $C_{20}H_{18}NO_2$ requires 304.1338.

4-[(4-Benzyloxybenzylidene)amino]phenol (***13 h***). Preparation was as above from 4-benzyloxybenzaldehyde (0.1 mol) and 4-aminophenol (0.1 mol). Pale green crystals, Mp. 208 °C, (96%). IR ν_{max} (KBr) cm^{-1}: 1606.4 cm^{-1} (C=N), 3437.8 cm^{-1} (OH). ^{1}H NMR (CD_3OD): δ 5.14 (s, 2H, OCH_2), 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). ^{13}C NMR (CD_3OD): 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 304.1328 (M^++H); $C_{20}H_{18}NO_2$ 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 acetone (100 mL). Anhydrous potassium carbonate (0.16 mol, 22 g) was then added and the mixture was stirred gently for 10 min under a N_2 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-Fluorophenyl-[4-(2-pyrrolidin-1-ylethoxy)benzylidene]amine (***13f***). Preparation was as above from **13d** (5 mmol, 1.076 g). Brown oil, (37%). IR ν_{max} (film) cm^{-1}: 1624.5 cm^{-1} (CH=N). ^{1}H NMR ($CDCl_3$): δ 1.80–1.84 (m, 4H, –CH_2–CH_2–), 2.63–2.66 (m, 4H, CH_2–N–CH_2), 2.95 (t, 2H, $J = 5.8$ Hz, N–CH_2), 4.19 (t, 2H, $J = 6.0$ Hz, OCH_2), 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). ^{13}C NMR ($CDCl_3$): δ 23.08, 54.32, 54.49, 66.82, 114.34, 115.21, 115.44, 121.71, 128.59, 129.99, 147.89, 159.11, 161.14, 161.73. HRMS: Found 313.1709 (M^++H); $C_{19}H_{23}N_2OF$ requires 313.1716.

4-Benzyloxyphenyl-[4-(2-pyrrolidin-1-ylethoxy)benzylidene] amine (***13 g***). Preparation was as above from **13e** (0.02 mol, 6.067 g). Orange oil (60%). IR ν_{max} (film) cm^{-1}: 1622.3 cm^{-1} (CH=N). ^{1}H NMR ($CDCl_3$): δ 1.75 (s, br, 4H, –CH_2–CH_2–), 2.59 (s, br, 4H, –CH_2–N–CH_2–), 2.86 (t, 2H, $J = 5.8$ Hz, CH_2–N), 4.12 (t, 2H, $J = 5.8$ Hz, CH_2–N), 5.01 (s, 2H, O–CH_2), 6.94 (d, 4H,

$J = 9.1$ 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). ^{13}C NMR ($CDCl_3$): δ 23.03, 54.28, 54.66, 66.79, 69.63, 114.59, 114.61, 121.60, 127.06, 128.21, 129.24, 129.80, 136.03, 144.82, 156.78, 157.40. HRMS: Found 401.2216 (M^++H); $C_{26}H_{29}N_2O_2$ requires 401.2229.

4-Benzyloxybenzylidene-[4-(2-pyrrolidin-1-ylethoxy)phenyl] amine (***13i***). Preparation was as above from **13h** (5 mmol, 1.517 g). Orange solid, (60%), m.p. 118 °C. IR ν_{max} (KBr) cm^{-1}: 1621.6 cm^{-1} (CH=N). ^{1}H NMR ($CDCl_3$): δ 1.81–1.84 (m, 4H, –CH_2–CH_2–), 2.64–2.67 (m, 4H, CH_2–N–CH_2), 2.94 (t, 2H, $J = 5.8$ Hz, CH_2–N), 4.15 (t, 2H, $J = 5.8$ Hz, CH_2–N), 5.13 (s, 2H, O–CH_2), 6.95 (d, 2H, $J = 9.0$ Hz, Ar-H), 7.04 (d, 2H, $J = 9.0$ Hz, Ar-H), 7.20 (d, 2H, $J = 9.04$ 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). ^{13}C NMR ($CDCl_3$): δ 23.03, 54.28, 54.66, 66.79, 69.63, 114.59, 114.61, 121.60, 127.06, 128.21, 129.24, 129.80, 136.03, 144.82, 156.78, 157.40. HRMS: Found 401.2216 (M^++H); $C_{26}H_{29}N_2O_2$ requires 401.2229.

1-(4-Methoxyphenyl)-3-phenyl-4-[4-(2-pyrrolidin-1-ylethoxy)-phenyl]azetidin-2-one (***15a***).

Preparation was as above from **14c** (0.4 mmol, 0.138 g). Yellow oil, (30%), IR ν_{max} (film) cm^{-1}: 1747.4 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.82–1.84 (m, 4H, –CH_2–CH_2–), 2.67 (s, br, 4H, CH_2–N–CH_2), 2.91 (t, 2H, $J = 6.0$ Hz, N–CH_2–C), 3.76 (s, 3H, OCH_3), 4.12 (t, 2H, $J = 5.8$ Hz, OCH_2), 4.24 (d, 1H, $J = 2.0$ Hz, H-3), 4.86 (d, 1H, $J = 2.0$ Hz, H-4), 6.81 (d, 2H, $J = 9.0$ Hz, Ar-H), 6.98 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.28–7.4 (m, 9H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 23.01, 53.75, 54.26, 54.99, 63.10, 64.74, 66.57, 113.85, 114.79, 118.09, 126.81, 127.02, 127.35, 128.54, 129.02, 130.61, 134.50, 155.61, 164.75. HRMS: Found 443.2333 (M^++H); $C_{28}H_{31}N_2O_3$ requires 443.2335.

[4-(tert-Butyldimethylsilanyloxy)benzylidene]-(4-methoxyphenyl) amine (***13b***). To a suspension of the phenol **13a** (0.02 mol, 4.54 g) and dimethyl-*tert*-butylchlorosilane (0.024 mol) in dry dichloromethane (60 mL) was added 1,8-diazobicyclo[5.4.0] undec-7-ene (DBU) (0.032 mol). The resulting mixture was stirred at room temperature until complete as monitored on TLC. The solution was then diluted with dichloromethane (80 mL) and washed with water (60 mL), 0.1M HCl (60 mL) and finally with saturated aqueous $NaHCO_3$ (60 mL). The organic layer was dried using anhydrous sodium sulfate and the solvent removed by evaporation under reduced pressure to afford the product as orange crystals, (58%), m.p. 210 °C. This product was used in the following reactions without further purification. IR ν_{max} (KBr) cm^{-1}: 1605.3 cm^{-1} (CH=N). ^{1}H NMR ($CDCl_3$): δ 0.28 (s, 6H, Si-$(CH_3)_2$), 1.04 (s, 9H, Si-C-$(CH_3)_3$), 3.77 (s, 3H, O-CH_3), 6.94 (d, 4H, $J = 8.9$ Hz, Ar-H), 7.22 (d, 2H, $J = 8.9$ Hz, Ar-H), 7.83 (d, 2H, $J = 8.9$ Hz, Ar-H), 8.38 (s, 1H, –CH=N). ^{13}C NMR ($CDCl_3$): δ – 4.80, 17.82, 25.38, 54.79, 113.89, 119.95, 121.72, 129.72, 144.66, 157.24, 157.59, 157.99.

General preparation of 3-substituted azetidin-2-ones ***14a***, ***14b***, ***15b-e***, ***15 h–i.*** A solution consisting of the appropriate acetyl chloride (7.5 mmol, 0.99 mL) in dry dichloromethane (50 mL) was added dropwise to a stirring solution containing the appropriate imine (5 mmol) and triethylamine (15 mmol, 2.091 mL) in dry dichloromethane (50 mL) at reflux. The solution was heated at reflux for 10 h and then cooled, washed with saturated sodium bicarbonate solution (50 mL), dilute HCl (10%, 50 mL) and brine (50 mL). The organic layer was dried and the solvent was evaporated *in vacuo*. The product was obtained by column chromatography as required over silica gel (eluent: dicholoromethane).

4-[4-tert-Butyldimethylsilanyloxy)phenyl]-1-(4-methoxyphenyl)-3-phenylazetidin-2-one (**14a**). Preparation was as above from **13b** (6 mmol, 2.046 g) and phenylacetyl chloride (6 mmol, 0.79 mL). Brown oil, (78%). This compound was used without further purification in the next experiment. IR ν_{max} (film) cm^{-1}: 1750.1 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 0.28 (s, 6H, Si–$(CH_3)_2$), 1.03 (s, 9H, Si–C–$(CH_3)_3$), 3.70 (s, 3H, O–CH_3), 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-Benzyloxyphenyl)-1-(4-methoxyphenyl)-3-phenylazetidin-2-one (**14b**). 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 ν_{max} (KBr) cm^{-1}: 1735.2 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 3.69 (s, 3H, O-CH_3), 4.19 (d, 1H, $J = 2.5$ Hz, H-3), 4.82 (d, 1H, $J = 2.5$ Hz, H-4), 6.74–6.77 (m, 2H, Ar-H), 6.93–6.99 (m, 2H, Ar-H), 7.15–7.28 (m, 9H, Ar-H), 7.30–7.36 (m, 5H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 55.02, 63.10, 64.76, 69.67, 113.90, 113.93, 114.62, 115.10, 118.15, 126.62, 127.69, 127.06, 127.69, 127.93, 129.25, 129.90, 130.60, 134.50, 136.25, 155.67, 157.57, 164.82.

1-(4-Fluorophenyl)-3-phenyl)-4-[4-(2-pyrrolidin-1-ylethoxy)-phenyl]azetidin-2-one ***(15b).*** Preparation was as above from **13f** (2 mmol, 0.624 g). Orange oil, (23%). IR ν_{max} (film) cm^{-1}: 1751.2 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.82 (s, br, 4H, –CH_2–CH_2–), 2.66 (s, br, 4H, –CH_2–N–CH_2–), 2.91 (t, 2H, $J = 5.8$ Hz, CH_2–N), 4.14 (t, 2H, $J = 5.8$ Hz, CH_2–C), 4.26 (d, 1H, $J = 2.0$ Hz, H-3), 4.87 (d, 1H, $J = 2.0$ Hz, H-4), 6.93 (2xd overlapping, 4H, $J = 7.3$ Hz, Ar-H), 7.26–7.39 (m, 9H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 23.01, 54.23, 54.49, 63.20, 64.91, 65.54, 114.89, 115.31, 118.16, 126.80, 126.97, 127.46, 128.60, 133.27, 133.30, 134.20, 157.42, 158.76, 165.08. HRMS: Found 431.2131 (M^++H); $C_{27}H_{28}FN_2O_2$ requires 431.2135.

1-(4-Fluorophenyl)-3-(4-methoxyphenyl)-4-[4-(2-pyrrolidin-1-ylethoxy) phenyl]azetidin-2-one (***15c***). Preparation was as above from **13f** (2 mmol, 0.624 g). Orange oil, (21%). IR ν_{max} (film) cm^{-1}: 1747.8 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.84 (s, br, 4H, –CH_2–CH_2–), 2.77 (m, 4H, –CH_2–N–CH_2–), 2.99 (t, 2H, $J = 5.5$ Hz, CH_2–N), 3.79 (s, 3H, O–CH_3), 4.16 (t, 2H, $J = 5.5$ Hz, CH_2–C), 4.20 (d, 1H, $J = 2.5$ Hz, H-3), 4.82 (d, 1H, $J = 2.5$ Hz, H-4), 6.89–6.97 (m, 6H, Ar-H), 7.20–7.31 (m, 6H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 22.90, 53.99, 54.16, 54.88, 63.53, 64.37, 65.90, 113.99, 114.85, 115.52, 118.16, 126.19, 128.14, 133.31, 158.46, 158.82, 159.81, 165.52 (C=O, C_2). HRMS: Found 461.2218 (M^++H); $C_{28}H_{30}FN_2O_3$ requires 461.2218.

1-(4-Benzyloxyphenyl)-3-phenyl-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl] azetidin-2-one (***15d***). Preparation was as above from **13 g** (3 mmol, 1.202 g). Orange oil, (61%), IR ν_{max} (film) cm^{-1}: 1744.9 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.86 (s, br, 4H, –CH_2–CH_2–), 2.77 (s, br, 4H, –CH_2–N–CH_2–), 2.99–3.04 (m, 2H, CH_2–N), 4.19–4.21 (m, 2H, CH_2–C), 4.18 (d, 1H, $J = 2.5$ Hz, H-3), 4.84 (d, 1H, $J = 2.5$ Hz, H-4), 5.06 (s, 2H, CH_2), 6.86 (d, 2H, $J = 9.0$ Hz, Ar-H), 6.96–7.01 (m, 4H, Ar-H), 7.18 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.27–7.44 (m, 10H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 22.89, 53.71, 54.12, 63.07, 64.69, 66.34, 69.79, 114.60, 115.08, 118.10, 126.85, 127.02, 128.55, 132.10, 134.45, 136.21, 158.64, 165.78. HRMS: Found 519.2648 (M^++H); $C_{34}H_{35}N_2O_3$ requires 519.2648.

1-(4-Benzyloxyphenyl)-3-(4-methoxyphenyl)-4-[4-(2-pyrrolidin-1-ylethoxy) phenyl] azetidin-2-one (***15e***). Preparation was as above from **13 g** (3 mmol, 1.202 g). Brown oil, (33%). IR ν_{max} (film) cm^{-1}: 1744.2 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.80 (s, br, 4H, –CH_2–CH_2–), 2.65 (s, br, 4H, –CH_2–N–CH_2–), 2.89 (t, 2H, $J = 5.8$ Hz, CH_2–N), 3.76 (s, 3H, O–CH_3), 4.11 (t, 2H, $J = 5.8$ Hz, CH_2–C), 4.16 (d, 1H, $J = 2.0$ Hz, H-3), 4.78 (d, 1H, $J = 2.0$ Hz, H-4), 4.96 (s, 2H, CH_2), 6.84–6.92 (m, 6H, Ar-H), 7.23 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.26–7.38 (m, 9H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 22.97, 54.17, 54.41, 54.88, 63.47, 64.23, 66.29,

69.77, 113.97, 114.79, 114.90, 118.12, 126.52, 126.82, 127.02, 127.56, 128.15, 128.20, 129.11, 130.85, 136.40, 154.80, 158.53, 158.76, 165.28. HRMS: Found 549.2777 (M^++H); $C_{35}H_{37}N_2O_4$ requires 549.2753.

4-(4-Benzyloxyphenyl)-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 ν_{max} (film) cm^{-1}: 1746.6 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.81 (m, 4H, –CH_2–CH_2–), 2.66 (m, 4H, –CH_2–N–CH_2–), 2.89 (t, 2H, $J = 5.8$ Hz, CH_2–N), 4.05 (t, 2H, $J = 5.8$ Hz, CH_2–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). ^{13}C NMR ($CDCl_3$): 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, 158.64, 164.79. HRMS: Found 519.2641 (M^++H); $C_{34}H_{35}N_2O_3$ requires 519.2648.

4-(4-Benzyloxyphenyl)-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 ν_{max} (film) cm^{-1}: 1741.9 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($CDCl_3$): δ 1.82 (s, br, 4H, –CH_2–CH_2–), 2.69 (s, br, 4H, –CH_2–N–CH_2–), 2.92 (t, 2H, $J = 5.4$ Hz, CH_2–N), 3.80 (s, 3H, OCH_3), 4.05 (t, 2H, $J = 5.8$ Hz, CH_2–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-CH_2), 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, 5H, Ar-H). ^{13}C NMR ($CDCl_3$): 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, 127.22, 127.48, 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); $C_{35}H_{37}N_2O_4$ 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 avoid decomposition of the β-lactam ring. The reaction mixture was then diluted with ethyl acetate (100 mL) and quenched with 10% HCl (100 mL). The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The organic layers were combined and then washed with water (100 mL) and brine (100 mL) and dried with anhydrous sodium sulfate. The solvent was removed by evaporation under reduced pressure, to afford the product as a yellow oil (50%). IR ν_{max} (film) cm^{-1}: 1735.6 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($(CD_3)_2CO$): δ 3.65 (s, 3H, O–CH_3), 4.21 (d, 1H, $J = 2.5$ Hz, H-3), 4.97 (d, 1H, $J = 2.0$ Hz, H-4), 6.77–6.83 (m, 4H, Ar-H), 7.24-7.32 (m, 9H, Ar-H), 9.78 (s, 1H, OH). ^{13}C NMR ($(CD_3)_2CO$): δ 54.34, 62.41, 64.58, 113.72, 115.45, 118.02, 127.09, 127.29, 128.16, 128.38, 130.83, 131.50, 135.13, 155.66, 157.30, 164.34. HRMS: Found 368.1260 (M^++Na); $C_{22}H_{19}NO_3Na$ requires 368.1263.

4-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)-3-phenylazetidin-2-one (***14c***). The benzyloxy protected compound **14b** (2 mmol) was dissolved in ethanol: ethyl acetate, (50 mL, 1:1) and hydrogenated over 10% palladium on carbon (1.2 g) for 2 h. The reaction was carefully monitored by TLC. The catalyst was filtered, the solvent was removed under vacuum and residue was purified by column chromatography on silica gel (eluent: dichloromethane, methanol) to afford the product as a yellow oil, (70%). IR ν_{max} (film) cm^{-1}: 1735.6 cm^{-1} (C=O, β-lactam). ^{1}H NMR ($(CD_3)_2CO$): δ 3.65 (s, 3H, O–CH_3), 4.21 (d, 1H, $J = 2.3$ Hz, H-3), 4.97 (d, 1H, $J = 2.3$ Hz, H-4), 6.77–6.83 (m, 4H, Ar-H), 7.24-7.73 (m, 9H, Ar-H), 9.78 (s, 1H, OH). ^{13}C NMR ($(CD_3)_2CO$): δ 54.34, 62.41, 64.58, 113.72, 115.45, 118.02, 127.09, 127.29, 128.16, 128.38, 130.83, 131.50, 135.13, 155.66, 157.30, 164.34. HRMS: Found 368.1260 (M^++Na); $C_{22}H_{19}NO_3Na$ requires 368.1263.

1-(4-Hydroxyphenyl)-3-phenyl)-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl]azetidin-2-one (***15f***). Preparation was as above from **15d** (1 mmol, 0.518 g). Yellow oil, (50%). IR ν_{max} (film) cm^{-1}: 1738.82 cm^{-1} (C=O, β-lactam), 3434.80 cm^{-1} (OH). ^{1}H NMR ($CDCl_3$): δ 1.89 (s, br, 4H, –CH_2–CH_2–), 2.83 (s, br, 4H, –CH_2–N–CH_2–), 3.14-3.16 (m, 2H, CH_2–N), 4.20 (d, 1H, $J = 2.2$ Hz, H-3), 4.23–4.29 (m, 2H, CH_2–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). ^{13}C NMR ($CDCl_3$): δ 22.83, 54.13, 54.22, 63.01, 64.59, 66.09, 114.40, 115.63, 118.35, 126.91, 127.03, 128.57, 129.51, 129.62, 131.58, 134.42, 153.18, 157.99, 164.75. HRMS: Found 429.2184 (M^++H); $C_{27}H_{29}N_2O_3$ requires 429.2178.

1-(4-Hydroxyphenyl)-3-(4-methoxyphenyl)-4-[4-(2-pyrrolidin-1-ylethoxy) phenyl]azetidin-2-one (***15g***)**.** Preparation was as above from **15e** (0.7 mmol, 0.384 g). Yellow oil, (78%). IR ν_{max} (film) cm^{-1}: 1737.6 cm^{-1} (C=O, β-lactam), 3387.0 cm^{-1} (OH). ^{1}H NMR ($CDCl_3$): δ 1.85 (s, br, 4H, –CH_2–CH_2–), 2.84 (s, br, 4H, –CH_2–N–CH_2–), 3.04 (t, 2H, $J = 5.3$ Hz, CH_2–N), 3.77 (s, 3H, O–CH_3), 4.11 (d, 1H, $J = 2.0$ Hz, H-3), 4.12 (t, 2H, $J = 5.5$ Hz, CH_2–C), 4.75 (d, 1H, $J = 2.0$ Hz, H-4), 4.78 (bs, 1H, OH), 6.69 (d, 2H, $J = 8.6$ Hz, Ar-H), 6.82 (2 × d, overlapping, 4H, $J = 8.8$ Hz, Ar-H), 7.14-7.20 (m, 6H, Ar-H). ^{13}C NMR ($CDCl_3$): δ 22.75, 54.03, 54.12, 54.88, 63.41, 63.96, 65.11, 113.95, 114.69, 115.55, 118.36, 126.42, 126.86, 128.18, 129.38, 153.34, 158.01, 158.72, 165.35. HRMS: Found 459.2276 (M^++H); $C_{28}H_{31}N_2O_4$ requires 459.2284.

4-(4-Hydroxyphenyl)-3-phenyl)-1-[4-(2-pyrrolidin-1-ylethoxy)-phenyl] azetidin-2-one (***15j***). Preparation was as above from **15 h** (0.11 mmol, 0.057 g). Yellow oil, (37%). IR ν_{max} (film) cm^{-1}: 1745.1 cm^{-1} (C=O, β-lactam), 3437.5 cm^{-1} (OH). ^{1}H NMR ($CDCl_3$): δ 1.88 (s, br, 4H, –CH_2–CH_2–), 2.95 (s, br, 4H, –CH_2–N–CH_2–), 3.11 (bs, 2H, CH_2–N), 4.07 (bs, 2H, O–CH_2), 4.21 (d, 1H, $J = 1.8$ Hz, H-3), 4.80 (d, 1H, $J = 1.8$ Hz, H-4), 5.06 (bs, 1H, OH), 6.65 (d, 2H, $J = 8.8$ Hz, Ar-H), 6.77 (d, 2H, $J = 8.8$ Hz, Ar-H), 7.15 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.25 (d, 2H, $J = 8.8$ Hz, (Ar-H), 7.30–7.36 (m, 5H, Ar-H). HRMS: Found 429.2177 (M^++H); $C_{27}H_{29}N_2O_4$ requires 429.2178.

4-(4-Hydroxyphenyl)-3–(4-methoxyphenyl)-1-[4-(2-pyrrolidin-1-ylethoxy) phenyl]azetidin-2-one (***15k***). Preparation was as above from **15i** (0.153 mmol, 0.084 g). Orange oil, (15%). IR ν_{max} (film) cm^{-1}: 1740.0 cm^{-1} (C=O, β-lactam), 3233.2 cm^{-1} (OH). ^{1}H NMR ($CDCl_3$): δ 1.91 (s, br, 4H, –CH_2–CH_2–), 2.93 (s, br, 4H, –CH_2–N–CH_2–), 3.12 (s, br, 2H, CH_2–N), 3.81 (s, 3H, O–CH_3), 4.12 (t, 2H, $J = 5.23$ Hz, CH_2–C), 4.18 (d, 1H, $J = 2.2$ Hz, H-3), 4.78 (d, 1H, $J = 2.24$ Hz, H-4), 5.13 (bs, 1H, OH), 6.74 (d, 2H, $J = 9.0$ Hz, Ar-H), 6.81 (d, 2H, $J = 8.0$ Hz Ar-H), 6.91 (d, 2H, $J = 8.5$ Hz, Ar-H). 7.18 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.23–7.24 (m. 4H Ar-H). HRMS: Found 459.2294 (M^++H); $C_{28}H_{31}N_2O_4$ requires 459.2284.

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 cultured in Eagles Minimum Essential (MEM) medium in a 95% air/5% CO_2 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% CO_2. 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 0.5×10^4 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 0.5×10^4 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 0.5×10^4 cells per well into a 96-well plate (200 μL per well) and incubated at 37 °C in air supplemented with 5% CO_2 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[42] 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% NaN_3 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 mM) 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.[43], 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 confluent 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 2.5×10^4 cells/well. Test compounds were dissolved in ethanol at 10^{-3}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% CO_2 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 $MgCl_2$ 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[44] (4-hydroxytamoxifen co-crystallised with ERα) was downloaded from the Protein Data Bank (PDB) was utilized for all ERα dockings and also as a template for creation of a homology model of ERβ. The model was constructed in an automated fashion using EsyPred3D.[45] Hydrogens were added to both structures using MOE[46] (Chemical Computing Group, Montreal, QC, Canada) and their positions minimized with the AMBER99 force field[47].

Docking/scoring

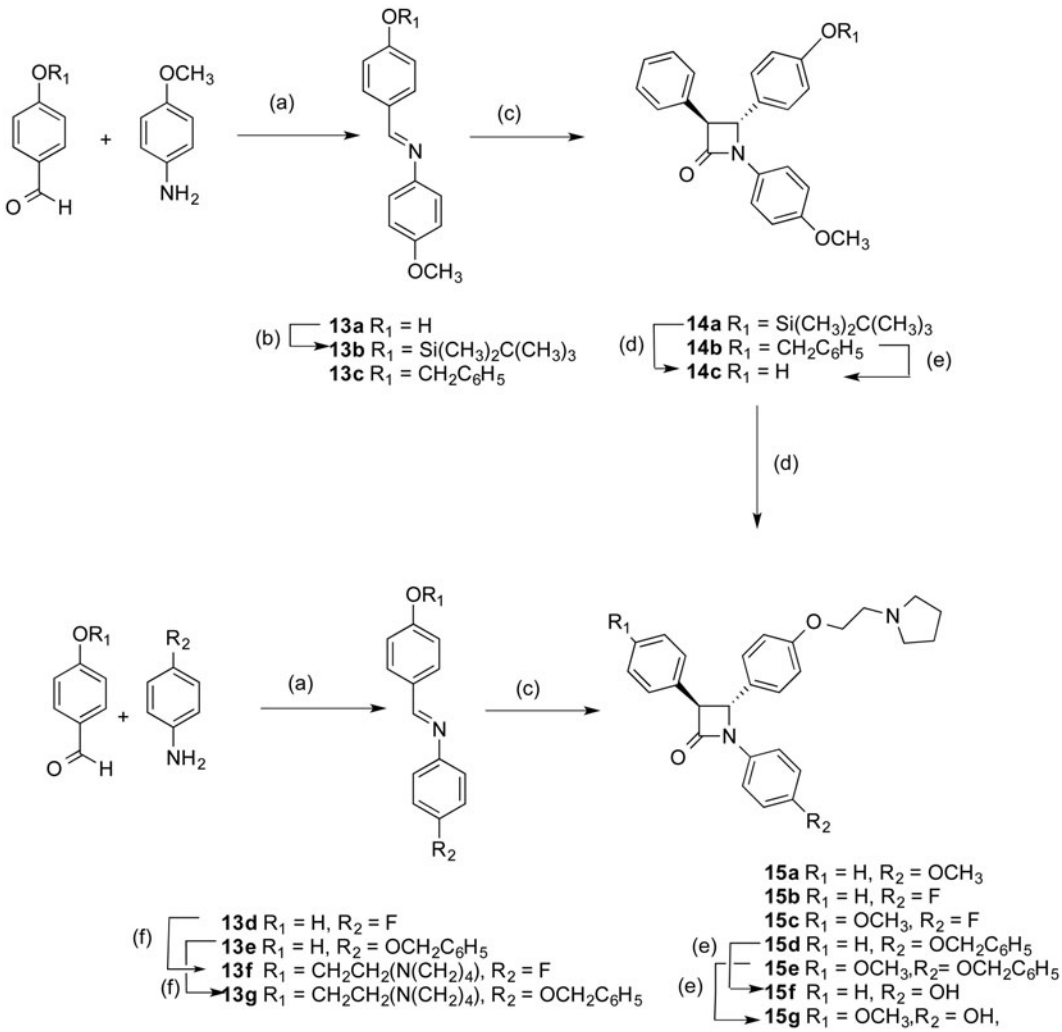
50 conformations of both compounds **15g** and **15k** were built using OMEGAv2.2.1[48] and docked using FREDv2.2.3[49]

(Openeye Scientific Software, Santa Fe, NM) retaining all default settings and employing the Chemgauss3 scoring function[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)[46] to allow side-chain repositioning.

Results and discussion

Chemistry

The synthesis of the Type I series of β-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 β-lactams[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 β-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 β-lactam product, a number of reaction conditions were investigated. β-Lactam **14a** was initially obtained successfully using DMF as the solvent[52]. This method resulted in exclusive synthesis of the *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[53], an improved yield (65%) was obtained with exclusive isolation of the *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 $-20\,^{\circ}C$[34]. The *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 *trans* β-lactam product, with the stereochemistry of the β-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 *cis*-β-lactams have larger coupling constants (5–6 Hz) than the *trans*-β-lactams which are usually 1–3 Hz[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[34].



Scheme 1. Synthesis of Type I β-lactam ER antagonist compounds (one enantiomer shown). Scheme reagents and conditions: (a) Ethanol, reflux; (b) tert-Butyldimethylsilyl chloride, DBU, DCM; (c) Phenylacetyl chloride, $(CH_3CH_2)_3N$, DCM; (d) TBAF, THF; (e) H_2, 10% Pd/C, ethyl acetate:ethanol; (f) K_2CO_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 acetyl chloride, mixtures of *cis* and *trans* cycloadducts are obtained, in which the *trans* is the major or exclusive product[34,51]. 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-methoxyphenylacetyl chloride and 4-benzyloxyphenylacetyl 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 tetrahydrofuroyl chloride was the acid component[53]. 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 ν 1735.6 cm^{-1} and a broad absorption corresponding to the hydroxy group at ν 3300.0 cm^{-1}. In the ^{1}H NMR spectrum, coupled doublet signals at δ 4.22 and δ 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 benzyloxy group without any effect on the β-lactam ring[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.[56,57] The Schiff base **13c** (obtained following the condensation of 4-methoxyaniline with 4-benzyloxybenzaldehyde) was reacted with phenylacetyl chloride under Staudinger conditions to afford the benxyloxy protected product **14b**, (78%). The IR spectrum contained the characteristic β-lactam carbonyl absorption band at ν1735 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 δ 4.19 and δ 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[58,59] and we have previously reported the potent ER-binding properties of fluorine-containing benzoxepine type ER antagonists[30]. We now wished to examine the effect of the inclusion of the lipophilic fluorine 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 δ 4.27 and δ 4.87 ($J_{3,4} = 2.04$ Hz) assigned to H-3 and H-4, respectively. **15c** was similarly prepared from **13f** and 4-methoxyphenylacetyl chloride

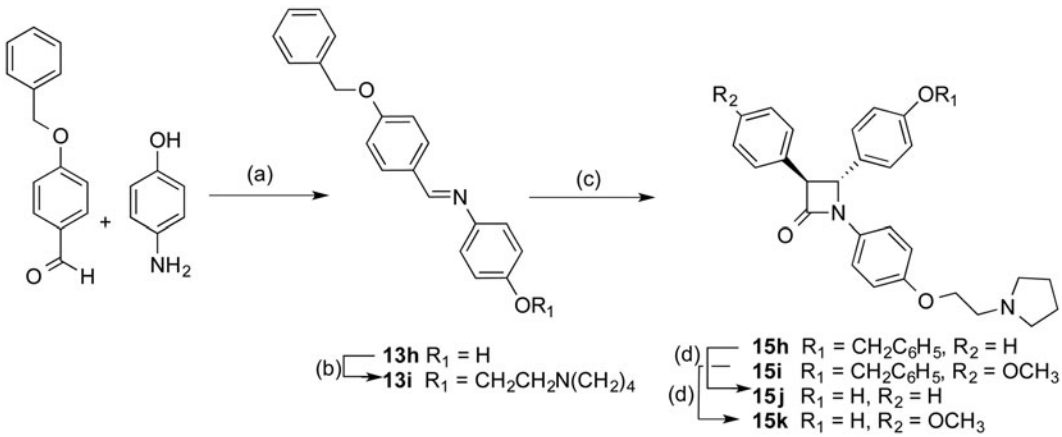
The requirement for a phenolic substituent in many ER antagonists such as 4-hydroxytamoxifen[4] and raloxifene is significant for successful binding to the ER as shown by interactions with Glu353 and Arg394. Therefore, in the present work it was critical to include a phenolic substituent group on the N-1 aryl ring of the Type-1 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 δ 4.18 and H-4 at δ 4.85, ($J_{3,4} = 2.52$ Hz). The benzyloxy 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 δ 4.20 and H-4 at δ 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-methoxyphenylacetyl 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-benzyloxybenzaldehyde 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-methoxyphenylacetyl 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

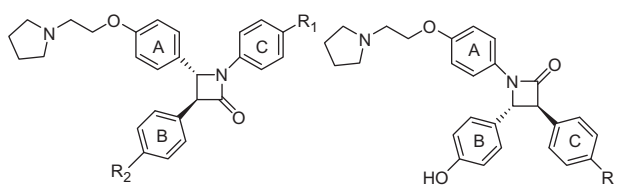
The β-lactam compounds prepared above were evaluated in a series of *in vitro* assays which determined their antiproliferative activity in ER positive MCF-7 and ER negative MDA-MB-231 breast cancer cell lines and also their affinity for the estrogen receptor and estrogenic effects in Ishikawa cells.

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[60,61]. Many of the compounds (e.g. **15a**, **15f**, **15g** and **15j**) have IC_{50} values in the range 0.185–7.54 μM in MCF-7 cells, but have significantly higher IC_{50} values in MDA-MB-231 cells (11–40 μM), a result



Scheme 2. Synthesis of Type II β-lactam ER antagonist compounds (one enantiomer shown). Scheme reagents and conditions: (a) Ethanol, reflux; (b) K_2CO_3, 1-(2-chloroethyl)pyrrolidine, acetone; (c) Phenylacetyl chloride, $(CH_3CH_2)_3N$, DCM; (d) H_2, 10% Pd/C, ethyl acetate: ethanol.

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 IC_{50} (μM)*	Cytotoxicity 10 μM§	MDA-MB-231 IC_{50} (μM)*	Cytotoxicity (%) 10 μM§	ER α IC_{50} (μM)‖	ER β IC_{50} (μM)‖	α/β ratio
15a	R_1=OCH_3 R_2=H	6.22	1	12.77	5.3	1.70	15.49	9
15b	R_1=F R_2=H	4.82	12	12.7	0	1.05	>100 μM	>95
15c	R_1=F R_2=OCH_3	3.49	26	4.62	25	0.23	1.64	7
15f	R_1=OH R_2=H	0.519	4	43.08	0	0.060	0.66	11
15g	R_1=OH R_2=OCH_3	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 = OCH_3	3.30	0	5.03	4.8	3.04	>50 μM	>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

*IC_{50} 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 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[60].

‡The IC_{50} value obtained for Tamoxifen in MDA-MB-231 cells (20 μM) is in agreement with reported values for tamoxifen in MDA-MB-231 cells[63,64].

§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 μM[42].

‖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 IC_{50} binding data for tamoxifen (ERα 60.9 nM ERβ188 nM, Panvera/Invitrogen).

**Work by Seeger et al.[61].

††No cytotoxic effect could be demonstrated for 4-hydroxytamoxifen in MDA-MB-321 cells[60].

which is not unexpected. However, compounds **15c** and **15k** unusually show moderate potency in MDA-MB-231 cell line (IC_{50} = 4.62 μM, 5.03 μM, respectively). The most potent compound in the series examined in MCF-7 cells is **15g** (IC_{50} 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 LBD[14]. 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 cells[62,63]. The cytotoxic effect of

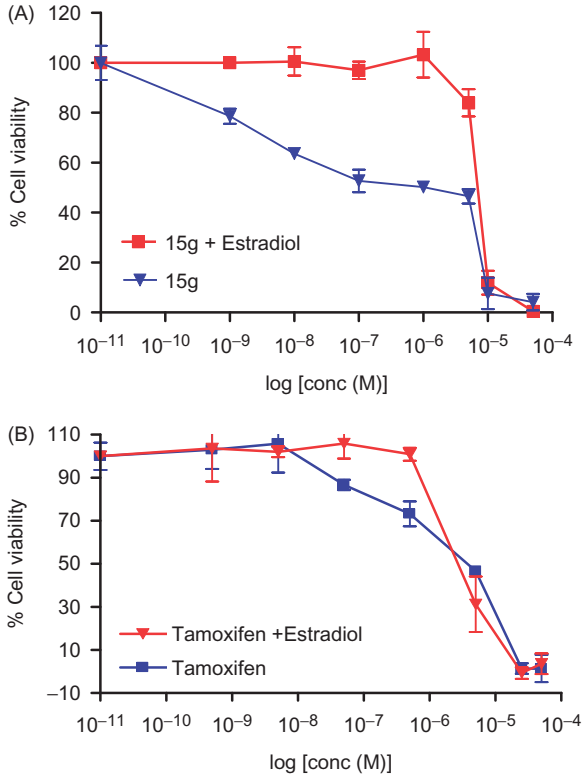


Figure 3. Effect of compounds **15g** and tamoxifen (**1**) (control) on the inhibition of proliferation of MCF-7 breast cancer cells in the absence and presence of Estradiol. MCF-7 cells were seeded at a density of 2.5×10^4 cells per well in 96-well plates. The plates were left for 24 h to allow the 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 **(1)** (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–100μM, 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 **(1)** (control) in the absence and presence of estradiol (50nM); inhibited proliferation of MCF-7 cells.

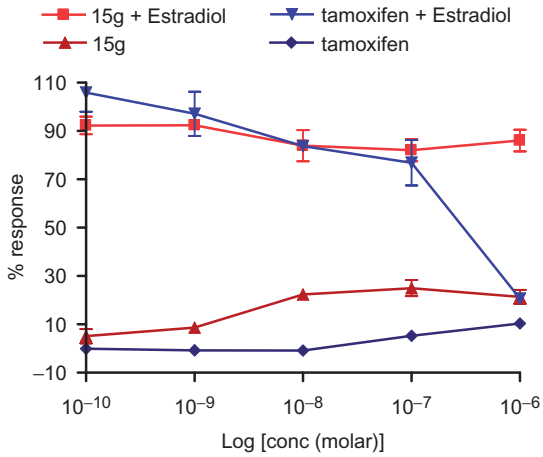


Figure 4. Effects of compound **15g** and tamoxifen (control) in Ishikawa cells (endometrial cancer cells) in the presence and absence of estradiol. Cells were plated in 96-well flat bottomed microtitre plates in EFBM at a density of 2.5×10^4 cells/well. Test compound **15g** and tamoxifen (control) were dissolved in ethanol at 10^{-3} M, diluted with EFBM (final concentration of ethanol 0.1%) and filter sterilized. After addition of the test compound, (plated in 50 μL, added estradiol (1 nM) in 50 μL, and blank medium to give a final volume of 150 μL), the cells were incubated for 72 h. 50 μL ice cold solution containing 50 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$ and 1M diethanolamine (pH 9.8) was added to the cooled plates, (0°C). The production of *p*-nitrophenol was monitored at 405nm until maximum stimulation of the cells showed an absorbance of ~1.2. Values represent the mean ± S.E.M (error values) for three experiments performed in triplicate.

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[64] 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 IC_{50} value increased from 0.186 μM to 9.17 μM, (Figure 3A). A similar result is also obtained for compound **15f** (IC_{50} values increased from 0.519 to 27.85 μM). Compound **15k** (Type II) demonstrated a small increase in IC_{50} 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[65]. Tamoxifen and 4-hydroxytamoxifen have been shown to have an estradiol reversible and estradiol 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[62]. 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; (IC_{50} 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.

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 (IC_{50} ERα = 4.3 nM, ERβ = 322 nM) and significantly more potent than both tamoxifen (IC_{50} ERα = 70 nM) and 4-hydroxytamoxifen (IC_{50} ERα = 40 nM) for ERα. This result also correlated with the antiproliferative activity of **15g** in the MCF-7 cell line (IC_{50} = 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 IC_{50} 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 IC_{50} of 3.30 μM. The IC_{50} value for **15k** in the MDA-MB-231 cell line is 5.03 μM indicating that in this case

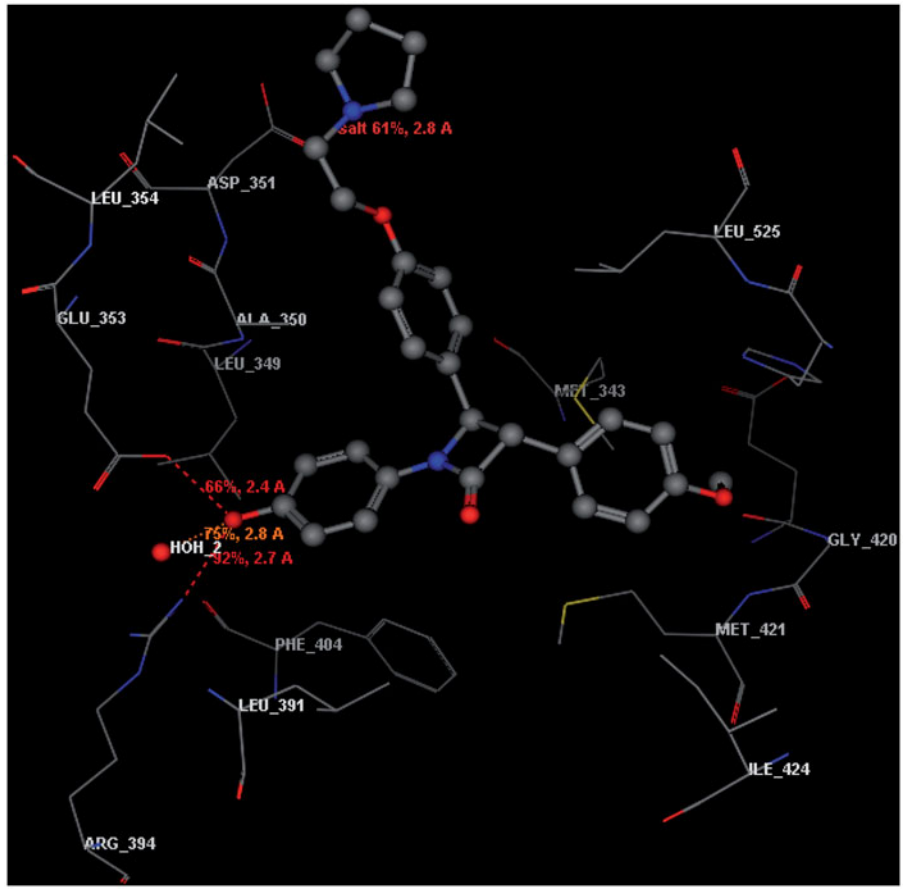


Figure 5. Key anti-estrogenic interactions are observed in the docking of **15g** in ERα. Hydrogen bonding between the C-ring 3-OH substituent and Glu353, Arg394, HOH is depicted. A salt bridge between the basic side chain and Asp351 is also formed.

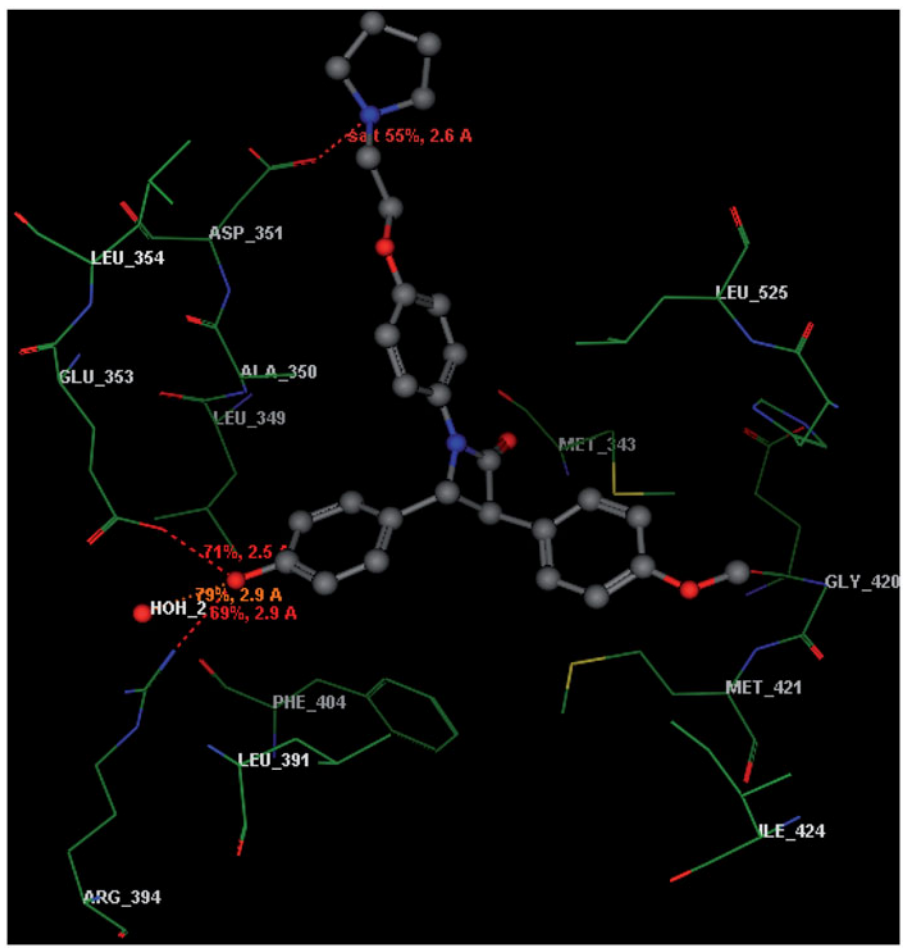


Figure 6. Key anti-estrogenic interactions are also observed in the docking of **15k** in ERα. Hydrogen bonding between the B-ring 3-OH substituent and Glu353, Arg394, HOH is depicted. A salt bridge between the basic side chain and Asp351 is also formed.

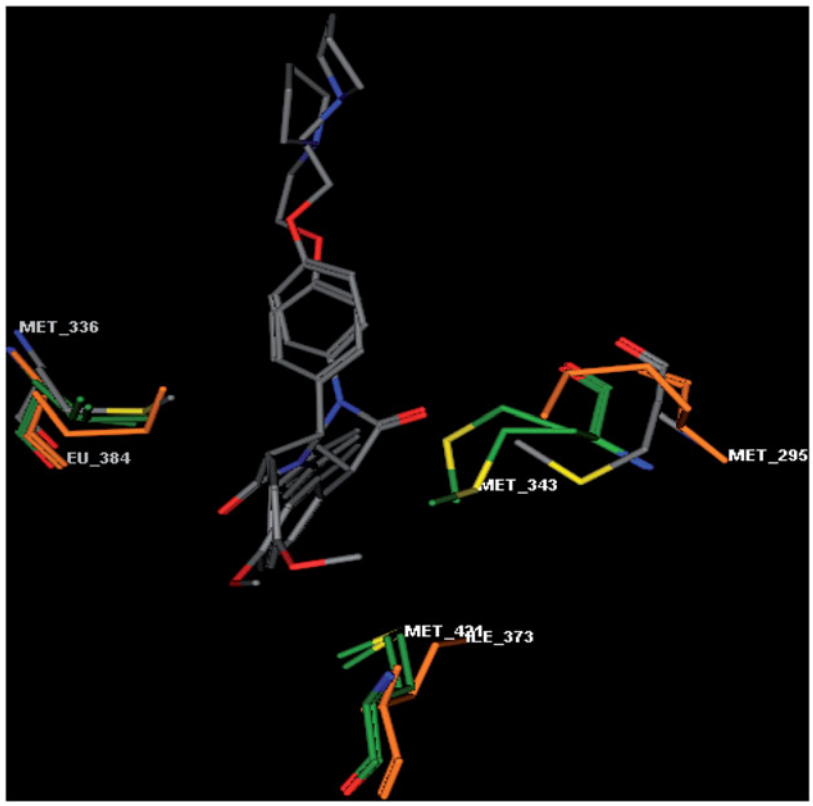


Figure 7. Docked position of **15g** and **15k** in both ERα/β superimposed by backbone. Residues Met336, met295 and Ile373 are from dockings in ERβ. Residues of ERβ depicted in orange for docking of **15k**. Residues Leu384, Met343, Met421 are from dockings in ERα.

anti-proliferation does not seem to be mediated through the ER. (Addition of estradiol (50 nM) with compound **15k** in MCF-7 cells produced only a small increase in the IC_{50} value). This indicates that the antiproliferative effect is not estradiol reversible and therefore compound **15k** does not appear to act solely as an ER antagonist, but may also have an alternative mode of action. Although, the main mechanism of action of tamoxifen is primarily mediated through the ER, studies have shown non-ER mediated mechanisms are present including the involvement of certain signaling proteins, proto-oncogenes and transforming growth factor β in tamoxifen-mediated apoptosis[66].

All of the Type I β-lactam compounds examined also show a preference for ERα binding over ERβ. In the case of the most potent example **15 g**, the α:β binding selectivity ratio is 75:1 and for **15b** no binding to ERβ was observed at concentrations of up to 100 μM. Tamoxifen demonstrates almost equal binding affinity for ERα and ERβ. Type II compounds **15j** and **15k** also shows competitive binding to ERα in excess of ERβ, while compound **15k** shows no binding to ERβ up to 50 μM (see Supplementary Information: Figure S1: Effect of compounds **15a**, **15f** , **15g**, **15k**, **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 a binding affinities for compounds **15a**, **15f**, **15g**, **15k**, **15j**; Figure S4: Estrogen receptor b 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[43]. Enmark et al have reported that Ishikawa cells contain both ERα and ERβ receptors but with ERα being far in excess[67]. 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 stimulatory 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%[68].

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[44]. 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[45]. 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 by 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 α or β 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 number of novel β-lactam compounds designed as potential estrogen receptor ligands, which demonstrate antiproliferative 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 $IC_{50} = 4.3$ 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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Supplementary material available online