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Optimisation of estrogen receptor subtype-selectivity of a 4-Aryl-4*H*-chromene scaffold previously identified by virtual screening

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Breast cancer
Cytotoxic
Knovenagel condensation
Molecular modeling

A B S T R A C T

4-Aryl-4*H*-Chromene derivatives have been previously shown to exhibit anti-proliferative, apoptotic and anti-angiogenic activity in a variety of tumor models *in vitro* and *in vivo* generally via activation of caspases through inhibition of tubulin polymerisation. We have previously identified by Virtual Screening (VS) a 4-aryl-4*H*-chromene scaffold, of which two examples were shown to bind Estrogen Receptor α and β with low nanomolar affinity and < 20-fold selectivity for α over β and low micromolar anti-proliferative activity in the MCF-7 cell line. Thus, using the 4-aryl-4*H*-chromene scaffold as a starting point, a series of compounds with a range of basic arylethers at C-4 and modifications at the C3-ester substituent of the benzopyran ring were synthesised, producing some potent ER antagonists in the MCF-7 cell line which were highly selective for ERα (compound **35**; 350-fold selectivity) or ERβ (compound **42**; 170-fold selectivity).

1. Introduction

Estrogen Receptors (ER) are hormone regulated receptors that are known to produce their long-term effects on cell structure and function *via* intracellular signalling and subsequent modulation of gene expression. In addition to genomic signalling, a rapid non-genomic signalling pathway has also been identified which is mediated by cell membrane-associated estrogen receptors. In fact a G-Protein Coupled Estrogen Receptor (GPR30/GPER) has been identified that binds estrogen, initiating a plasma membrane-initiated signaling cascade.[1–4] Focusing on 'classical' ER, two subtypes exist and are expressed in a wide-range of tissues and cells throughout the body; ER alpha (ERα) is predominantly found in the female bone, uterus, ovary (thecal cells), mammaries, adipose tissue, cardiovascular system and brain. ER beta (ERβ) is found also in the adipose tissue, cardiovascular system and brain but also in the lung, bladder, colon and granulosa cells of the ovary.[5–14] The concept of Selective Estrogen Receptor Modulation (SERM)[15] was first demonstrated with the clinical drug Tamoxifen[16,17] which was introduced in the 1970 s as a treatment for advanced breast cancer in postmenopausal women. Tamoxifen was shown to have anti-estrogenic function in the breast, however partial estrogenic effects in other target tissues such as bone and the cardiovascular system where it is beneficial but conversely harmful in the endometrium.[18–21] However a strong association between its use and the development of endometrial

Abbreviations: EI, Electron Impact; ER, Estrogen receptor; GTP, Guanidine triphosphate; HRMS, High Resolution Molecular Ion Determination; IC, Inhibitory concentration; IR, Infrared; LDB, Ligand binding domain; LBP, Ligand binding Pocket; LRMS, Low Resolution Mass Spectra; MTD, Maximum tolerated dose; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, Nuclear Magnetic Resonance; ORTEP, Oak Ridge Thermal Ellipsoid Plot; PDB, Protein Data Bank; PBS, Phosphate buffer saline; SAR, Structure-Activity Relationship; SERM, Selective Estrogen Receptor Modulation; TBDMS, *tert*-Butyldimethylchlorosilane; THF, Tetrahydrofuran; TLC, Thin Layer Chromatography; TMCS, Trimethylchlorosilane; TMS, Tetramethylsilane

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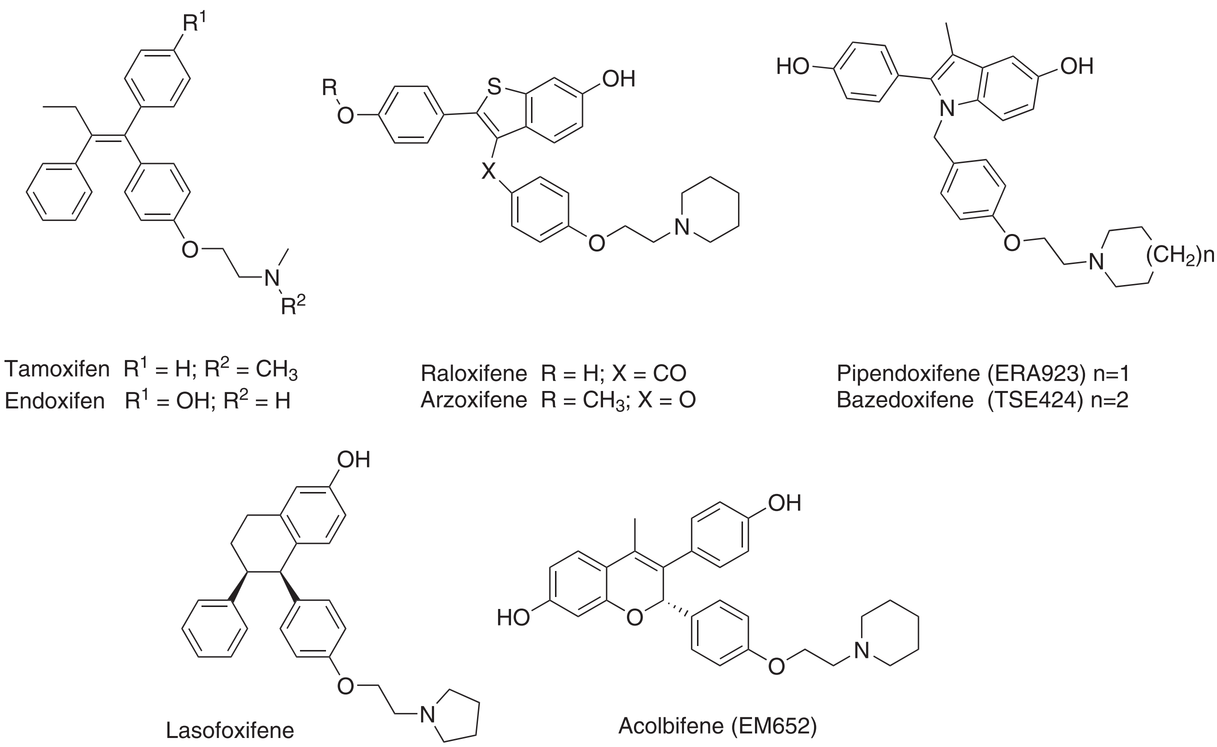


Fig. 1. Structures of selected SERM structural classes including triarylethylenes tamoxifen and endoxifen, benzothiophenes raloxifene and arzoxifene, indoles pipendoxifene and bazedoxifene, benzopyran acolbifene and the tetrahydronaphthalene lasofoxifene.

carcinoma emerged fuelling research for the discovery of alternative compounds with mixed agonistic/antagonistic profiles (Fig. 1).

It has been since hypothesised that the optimal route to the development of an 'ideal' SERM can only occur when delineation of the full array of the biological roles orchestrated by ERα or ERβ in different tissues is revealed, and this is best achieved through the design of subtype-selective ER ligands. To date, a plethora of both ERα and ERβ selective agonists and antagonists have been described (compounds **1**–**12**, Fig. 2).[22–57] The ERα agonist 16a-LE2 (**1**) has been reported to inhibit cardiac hypertrophy.[58] The synthesis and characterisation of a series of pyrazole-based compounds resulted in a > 400-fold selective agonist for ERα, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole, (**2**) (PPT).[44] Mortenson et al subsequently synthesised a library of heterocycle-based (furans, thiophenes, and pyrroles) ER agonists, the most ERα selective (65-fold) of which was compound **3**–2,4,5-tris(4-hydroxyphenyl)-3-methylfuran.[39] Several SERMs have also been reported with ERα selectivity, for example; benzothiophenes (**4**), tetrahydroisoquinolines (**5**)[59] and pyrazoles[45], with the most selective ERα antagonist to date being a pyrazole core incorporating a basic side-chain termed MPP (**6**)(~220-fold).[60] To facilitate the study of ERβ specific mechanisms of action, the first reported full agonist discovered some time ago by Katzenellenbogen and co-workers, diarylpropionitrile (**7**) (DPN), exhibited 70-fold preferential binding for ERβ over ERα and 78-fold selectivity in transcriptional assays.[37] ERB-041 (Prinaberel)(**8**), WAY-200070 and WAY-202196(**9**) were all developed by Wyeth and reported in 2004-5[61–63], followed by a series of ERβ selective benzopyrans developed by Lilly in 2006.[41,64,65] The wide range of activities of these compounds both *in vitro* and *in vivo* has made it particularly difficult to assign clinical relevance to them and has also been additionally hampered by the existence of different isoforms of ERβ. ERβ1 appears to be the isoform displaying the strongest activity, however, some variant forms may interact with ERβ1 and also with ERα presenting a challenge in understanding the full complexity of the biology of ERβ.[66] To date, very few examples of ERβ antagonists have been reported (Fig. 2). Two 30-fold ERβ selective antagonists have been described; one based on a triazine scaffold (**10**) was discovered by GSK[32] and another by researchers at Organon[67] belonging to the class of 10-aryl substituted benzo[*b*]fluorenes (**11**). The (*R,R*)-*cis*-tetrahydrochrysene (**12**) (THC) was subsequently demonstrated by the Katzenellenbogen lab to behave as a full agonist in ERα and full antagonist in ERβ[36]. Surprisingly, as THC lacks a basic side-chain, the term 'passive antagonism' was proposed for its mechanism of action supported by crystal structures of THC bound to both ERα and ERβ ligand binding domains (LBDs)[68].

We have described in earlier work the identification by *in silico* screening of a novel 4-aryl-4*H*-chromene scaffold which was shown to potently bind both ER isoforms[69]. The two 4-aryl-4*H*-chromene analogs (**13** and **14**) displayed modest ERα selectivity (20-fold) and also antiproliferative activity in an ER positive breast cancer cell line, MCF-7, (Fig. 3). Importantly, it was noted that neither analog incorporated a typical basic arylether and this formed the premise of the current study. In the present work, a series of 4-aryl-4*H*-chromene derivatives were designed and synthesised some of whch incorporated the typical basic arylethers usually required in ER antagonists (Fig 2). In addition, we have now carried out modifications at the C3 ester position of the benzopyran ring to probe potential differences in ER binding pockets thereby maximising isoform selectivity. The most active compound in the series exhibited 350-fold selectivity for ERα and interestingly one of the series showed 170-fold selectivity for ERβ. The 4-aryl-4*H*-chromene scaffold has been reported in numerous studies to possess anti-proliferative, apoptotic and anti-angiogenic activity in a variety of tumor models *in vitro* and *in vivo*, generally *via* activation of caspases through inhibition of tubulin polymerisation.[70] In addition, the inhibition of insulin-regulated aminopeptidase by related 4-aryl-4*H*-chromenes has been recently reported.[71] The 4-aryl-4*H*-chromene Crolibulin (**15**), Fig. 3, (a microtubule destabilizing agent that disrupts vascular endothelial cells, and in turn, blood flow to the tumor) is currently in Phase I/II clinical trials assessing its toxicity levels when co-administered with cisplatin and progression-free survival (PFS) in adults with anaplastic thyroid cancer (ATC).[72] We envisage that further

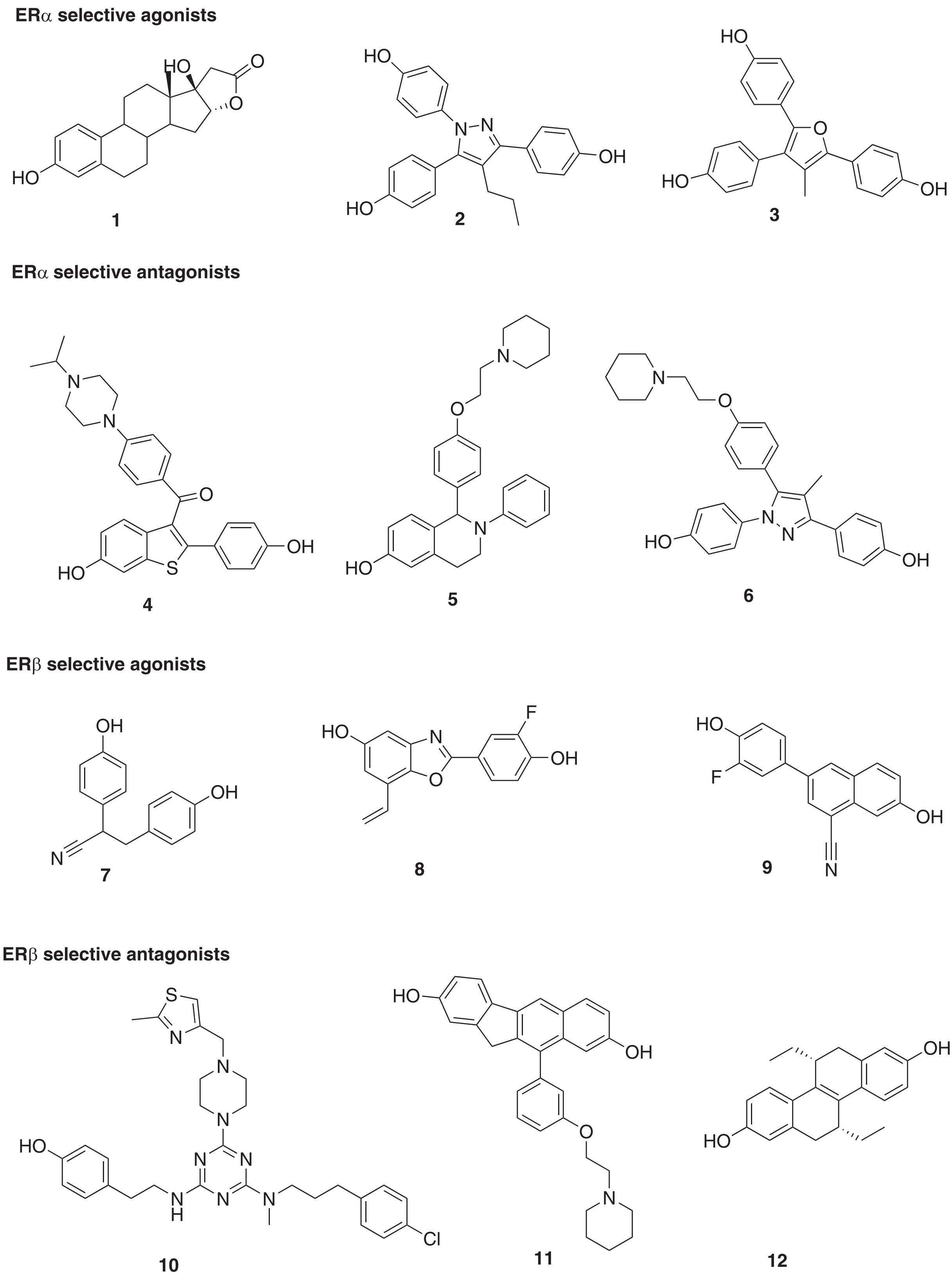


Fig. 2. Subtype selective ER ligands.

biochemical analysis of our 4-aryl-4*H*-chromene compound series could potentially lead to the developement of novel clinically relevant ER modulating compounds featuring the 4-aryl-4*H*-chromene scaffold structure.

2. Results

2.1. Chemistry

Many approaches to the preparation of 4-aryl-*4H*-chromenes have

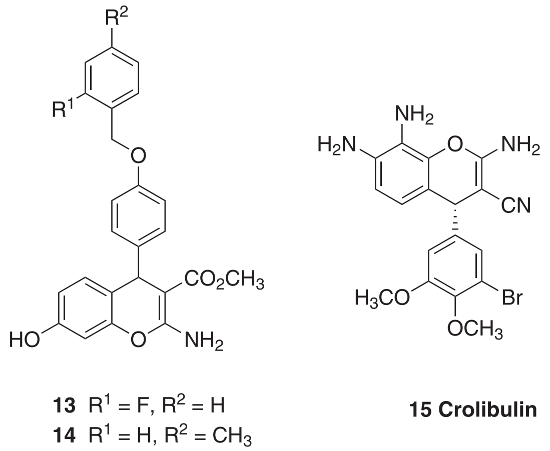
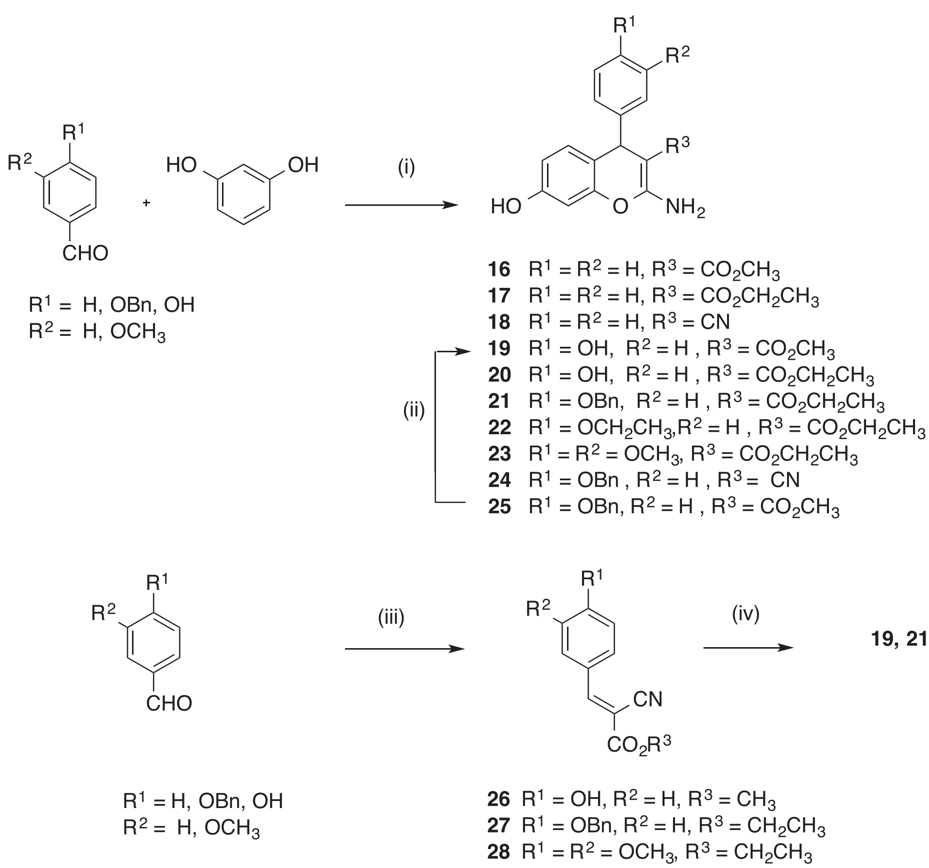


Fig. 3. 4-Aryl-*4H*-chromenes **13, 14** and Crolibulin (**15**).

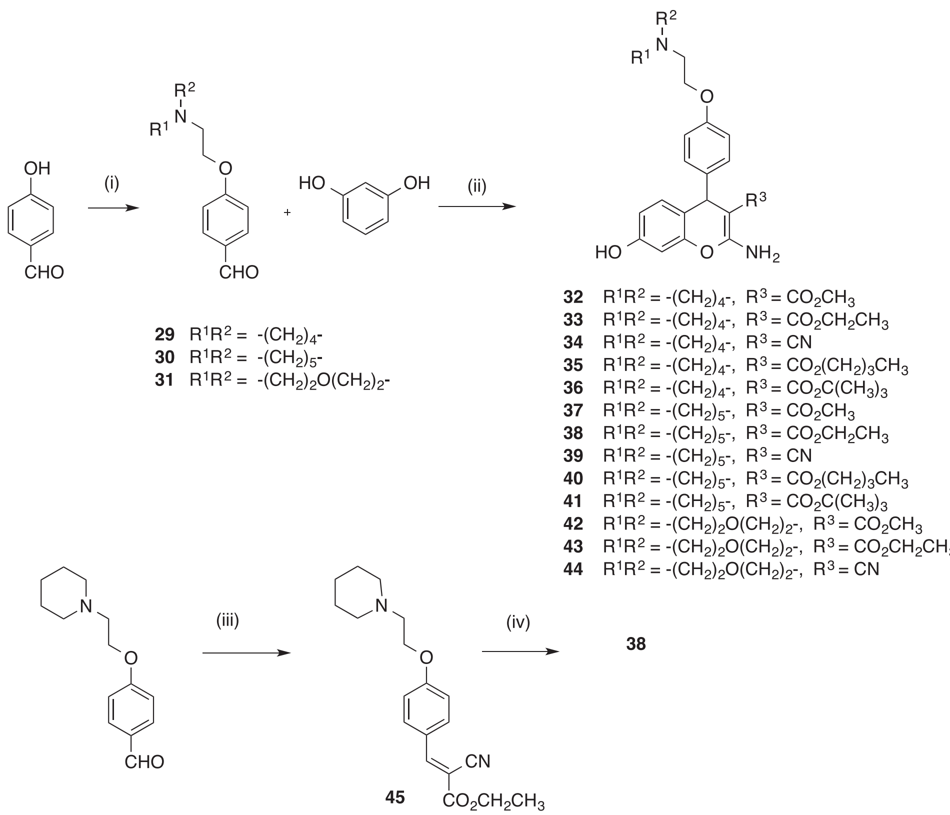
been described.[71,73–76] In the present study, the synthetic route to the 4-aryl-*4H*-chromenes (**16–25**) initially identified for the study is outlined in Scheme 1. These compounds contain methyl ester, ethyl ester or nitrile substituents at C-3 of the chromene ring and also benzyl, ethyl, methyl ether or phenol substituents on the C-4 aryl ring. The appropriate arylaldehyde is reacted with malononitrile or the cyanoacrylate ester and resorcinol in the presence of base (piperidine or triethylamine) in a one-pot synthesis. The reaction proceeds through the formation of an intermediate cyanoacrylate ester or benzylidene malonitrile by Knoevenagel condensation from the cyanoacetate ester, malonitrile and the benzaldehyde. This intermediate cyanoacrylate ester can be observed as a solid forming within ten minutes of heating the mixture. Further reaction of the resorcinol phenolic OH with the nitrile and subsequent electrophilic ring closure afforded the desired final chromene products, **16–25**. The cyanoacrylate intermediate product was isolated in the case of **26–28** and subsequently reacted with resorcinol to afford the desired products. The phenolic methyl ester **19** was also obtained from the benzyl ether **21** by hydrogenation (H_2/Pd/C) (Scheme 1).

The series of 4-aryl-*4H*-chromenes containing a basic ether substituent at C-4 (compounds **24–36**) were obtained as outlined in Scheme 2. These compounds also contain methyl, ethyl, *n*-butyl or *tert*-butyl ester or nitrile substituents at C-3 of the chromene. The benzaldehydes **29–31** were obtained by alkylation of 4-hydroxybenzaldehyde with 1-(2-chloroethyl)pyrrolidine, 1-(2-chloroethyl)piperidine or 1-(2-chloroethyl)morpholine respectively as previously reported[77] to afford the pure aldehydes in 65–97% yield. Subsequent reaction of the aldehydes **29–31** with malononitrile or the appropriate cyanoacrylate ester in the presence of piperidine or triethylamine afforded desired products **32–44** in moderate yield. The cyanoacrylate intermediate **45** was also isolated on reaction of the aldehyde **30** with ethyl cyaoacrylate and subsequently reacted with resorcinol to afford the desired product **38**.

While attempting the synthesis of the methyl esters **16, 19, 25, 32, 37 , 42** using ethanol as solvent, it was noted that the final product isolated in each case was in fact the corresponding ethyl ester **17, 20, 21, 33, 38, 43** respectively. It appeared that in the presence of ethanol, a facile transesterification was occurring. Several different solvents were then employed in an attempt to obtain the methyl ester products, e.g. methanol, ethyl acetate, acetone, dichloromethane and tetrahydrofuran. Dichloromethane was found to be successful for the synthesis of all other methyl ester compounds (**16, 19, 25, 32, 37** and **42**), however yields were low. Details of the reaction conditions for the synthesis of compounds **16–25** and **32–44** are presented in Table 1. Attempts to improve the yield using potassium carbonate or cetyltrimethylammonium chloride in aqueous conditions were not successful.[75,76] The most efficient method of preparation for the methyl



Scheme 1. Synthesis of 4-aryl-4*H*-chromenes **16–25.**



Scheme 2. Synthesis of 4-aryl-4*H*-chromenes **32–44.**

ester products was by replacing the initially used triethylamine with piperidine as the base of choice and by performing the reactions under microwave conditions.

The stability of representative examples of the target compounds **38** and **40** was determined in phosphate buffer and the half life was determined to be greater than24 h for each compound at pH values 7.4 and 9. The compounds **38** and **40** degraded at lower pH = 4, with 27% and 32% remaining after 30 min respectively. The compounds showed very high stability in human blood plasma, and the half-life was determined to be greater than 24 h.

The molecular structure of a representative example of the 4-aryl-4*H*-chromenes series, compound **22** was determined by single crystal X-ray crystallography (*S* enantiomer shown). The ORTEP diagram is displayed in Fig. 4 (thermal ellipsoids at 50% probability). It can be seen that the benzopyran ring is planar, and the aryl ring at C-4 positioned at a dihedral angle of 78.403(77) ° with respect to the benzopyran ring (Fig. 4).

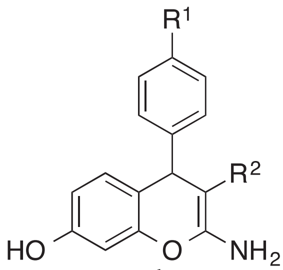
2.2. Biological results and discussion

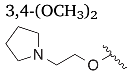
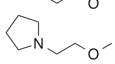
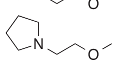
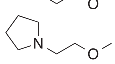
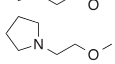
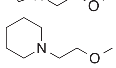
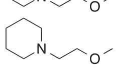
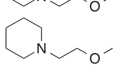
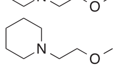
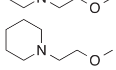
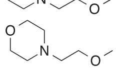
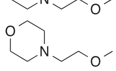
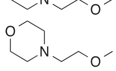
All compounds were initially evaluated for antiproliferative effects in MCF-7 cells using the MTT assay (72 h) and the results are displayed in Table 1. The most potent compound was identified as compound **41** (which contains piperidine aryl ether at C-4 and a *tert*-butyl ester at C-3 of the chromene ring) which showed comparative results to that of tamoxifen in the antiproliferative assay with IC_{50} value of 2.65 μM (IC_{50} value determined for control drug tamoxifen = 4.12 μM). Compounds **16, 21, 35** and **38** also demonstrated low microlmolar antiproliferative effects with IC_{50} values of 10.82, 4.50, 7.73 and 8.75 μM respectively. Selected compounds were concurrently tested to assess the extent of their cytotoxicity using a LDH assay. Compounds displayed negligable cytotoxicity in LDH assay with 0% cell death observed for compounds **32, 33, 37, 38, 43** and **44**.

Selected compounds from the series **16–23** and **32–44** were then screened for their binding affinity to ERα and ERβ in a fluorescence polarisation assay, with tamoxifen as a positive control. The results are displayed in Table 1. For compounds **16** and **17** tested, the methyl and ethyl ester compounds showed similar affinity to each other for ERα and ERβ. The cyano compound **18** did not show any binding affinity for ERα at concentrations up to 10 μM. Compound **19** with the phenolic substituent at C-4 showed a slightly greater affinity for ERα than the benzyloxy ether **21,** however it showed markedly less affinity for ERβ. The ethyl ether compounds **22** and the 3,4,dimethoxyether **23** having little antiproliferative activity, were not further evaluated.

On addition of the basic ether substituent to the core phenol **20**, whether pyrolidine, piperidine or morpholine, greater ERα binding ability was observed. For the pyrolidine containing compounds, the methyl (**32**), *n*-butyl (**35**) and *tert*-butyl (**36**) esters all displayed similar affinity to ERα with IC_{50} binding of less than 100 nM each (e.g. 20 nM, 20 nM and 10 nM respectively). The ethyl ester (**33**) demonstrated slightly less affinity with binding of 1.41 μM. The C-3 cyano compound **34** as observed with the unsubstituted compounds had decreased affiniy for ERα (IC_{50} = 0.89 μM), and little affinity for ERβ (IC_{50} > 10 μM). All compounds showed selectivity for ERα over ERβ with significant difference noted in binding affinity, this was consistent across all compounds tested. For the compounds with the piperidine containing side chain, the methyl (**37**), ethyl (**38**) and *tert*-butyl (**41**) esters all demonstrated good affinity to ERα with ethyl ester compound **38** showing greatest activity with IC_{50} values of 20 nM and 60 nM for ERα and ERβ respectively. The *tert*-butyl ester (**41**) which was the most active in the antiproliferative assay, also demonstrated good affinity for ERα (IC_{50} = 0.090 μM) and also sub-micromolar binding to ERβ (IC_{50} = 370 μM). Once again the cyano compound **39** was the least effective in the series in the ERα and ERβ binding assays, with IC_{50} values of 1.47 and 10.42 μM respectively. Of the compounds examined containing the morpholine side-chain (**42–44**), the ethyl ester **43** showed the greatest ERα binding activity (IC_{50} = 220 nM), while the methyl ester **42** was the most effective as a ligand for ERβ (IC_{50} = 0.63 μM).

Table 1
ERα and ERβ binding effects and antiproliferative activity in MCF-7 cells of selected 4-aryl-*4H*-chromenes.



Compound	R^1	R^2	ERα IC_{50}(μM)[c,d]	ERβ IC_{50}(μM)[c,d]	β/α	Antiproliferative activity MCF-7 cells IC_{50}(μM)[a,e]
16	H	$-CO_2CH_3$	3.30 ± 0.30	1.59 ± 0.17	0.48	10.82 ± 1.15
17	H	$-CO_2CH_2CH_3$	2.36 ± 1.62	0.61 ± 0.05	0.26	> 100
18	H	−CN	> 10	8.39 ± 3.32	0.84	> 100
19	OH	$-CO_2CH_3$	0.79 ± 0.40	10.02 ± 1.39	12.67	> 100
20	OH	$-CO_2CH_2CH_3$	nd	nd	-	> 100
21	$OCH_2C_6H_5$	$-CO_2CH_2CH_3$	40.32	> 100	-	4.5
22	OCH_2CH_3	$-CO_2CH_2CH_3$	nd	nd	-	> 100
23	3,4-$(OCH_3)_2$	$-CO_2CH_2CH_3$	nd	nd	-	> 100
32		$-CO_2CH_3$	0.02 ± 0.02	0.35 ± 0.25	14.31	19.60 ± 20.78
33		$CO_2CH_2CH_3$	1.41 ± 0.40	3.10 ± 1.57	2.21	27.29 ± 23.78
34		−CN	0.89 ± 0.19	> 10	11.23	> 50
35		$-CO_2CH_2\ CH_2CH_2CH_3$	0.02 ± 0.01	6.55 ± 2.72	353.34	7.73 ± 7.54
36		$-CO_2C(CH_3)_3$	0.01 ± 0.01	0.67 ± 0.40	49.49	10.03 ± 0.48
37		$-CO_2CH_3$	0.05 ± 0.00	0.22 ± 0.14	4.77	16.2 ± 5.72
38		$-CO_2CH_2CH_3$	0.02 ± 0.00	0.06 ± 0.02	2.56	8.75 ± 4.19
39		−CN	1.47 ± 0.44	10.42 ± 8.57	7.10	23.21 ± 2.81
40		$-CO_2(CH_2)_3CH_3$	0.03 ± 0.03	2.35 ± 0.47	87.29	31.82 ± 30.54
41		$-CO_2C(CH_3)_3$	0.09 ± 0.00	0.37 ± 0.02	3.93	2.65 ± 0.15
42		$-CO_2CH_3$	109.10 ± 6.9	0.63 ± 0.03	0.0054	> 100
43		$-CO_2CH_2CH_3$	0.22 ± 0.02	2.44 ± 0.73	11.02	18.79 ± 21.29
44		−CN	13.97 ± 1.66	> 50	3.58	35.31 ± 10.30
Tamoxifen	-	-	0.070	0.170[c]	2.43	4.12 ± 0.038[b]

[a] IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 cells. Values represent the mean ± S.E.M (error values × 10^{-6}) for at least two experiments performed in triplicate.
[b] The IC_{50} value obtained for Tamoxifen is 4.12 ± 0.038 μM is in good agreement with the reported IC_{50} value for tamoxifen using the MTT assay on human MCF-7 cells [78].
[c] The ER binding values obtained are in agreement with the reported ER IC_{50} binding data for tamoxifen (ERα 60.9 nM ERβ 188 nM, Panvera/Invitrogen).
[d] Values are an average of at least nine replicate experiments, for ERα with typical standard errors below 15%, and six replicate experiments for ERβ, with typical standard errors below 15%.
[e] 0% cell death observed for compounds **32, 33, 37, 38, 43** and **44** in LDH assay at 10 μM concentration, 13.4% cell death for tamoxifen in LDH assay at 10 μM concentration.

2.3. Computational

To rationalise the subtype selectivity observed for compounds **35** (350-fold ERα selective) and **42** (170-fold ERβ selective) we carefully selected the most appropriate X-ray structure to utilise for subsequent docking experiments, through visual analysis of the binding modes made by all co-crystallised SERMs/SERDs with their respective ER isoforms. Lasofoxifene co-crystallised with ERα (PDBID 2OUZ) was considered to be the most suitable LBP to dock compound **35**, however, the electron withdrawing oxygen of the morpholino-ring of **42** has the potential to H-bond unlike the other pyrrolidine/piperidine side-chains in the chromene series. Taking this into account, we reasoned that PDBID 1R5K whose co-crystallised ligand GW5638, uses a bridging water molecule that is perfectly positioned for substitution by the morpholino-ring oxygen of **42** to enable H-bonding with the backbone amide N−H of Leu536 (H12) would be most appropriate for docking in this case. As no suitable ERβ isoforms of these structures existed, we constructed refined models of each as described in the experimental section. Briefly, the iTASSER server was used for protein structure prediction using 2OUZ and 1R5K as templates to guide the prediction and the co-ordinates of each were firstly morphed[79] to the co-ordinates of 2OUZ and 1R5K respectively followed by refinement using

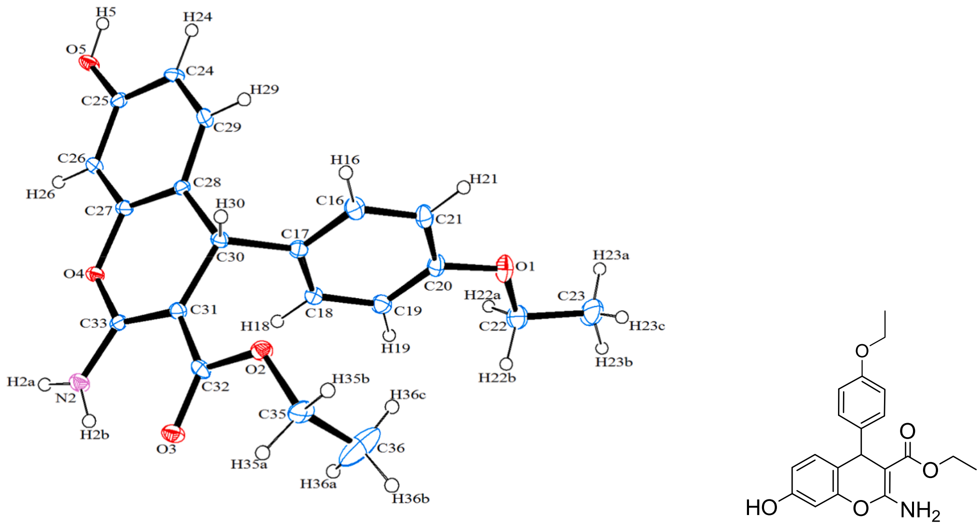


Fig. 4. ORTEP representation of the X-Ray crystal structure of **22**, ellipsoids at 50% probability.

3DRefine.[80] Reconstruction of any missing residues in 2OUZ and 1R5K was also undertaken using the iTASSER server.

Conformers of all R and S enantiomers of compound **35** and **42** were docked and in the LBP our refined models of ERα and β using FRED,[81] and scored using Chemgauss4.

Marvin pKa prediction[82] for compound **35** revealed the tertiary amine of the piperidine side-chain of compound **35** is protonated at pH7.4. Interestingly, Marvin predicted that only 15% of compound **42** would have the tertiary amine of its morpholine ring protonated at pH7.4 with the remaining > 80% predominantly neutral.

Only in the case of compound **35**, was there a clear binding preference to both ER subtypes for the R enantiomer (*see supplementary information*) with 1.6 and 1.9-fold differences observed in scoring for top scoring docked poses, indicating that enantiomeric separation of the R enantiomer might afford additional binding afffinity and selectivity. Fig. 5 highlights the key interactions made by compound **35** in both isoforms, and as is commonly observed with most Selective Estrogen Receptor Modulators (SERMs), it strongly interacts with Asp351 (3 0 3) via a salt bridge and is stabilised within the binding pocket through a series of hydrophobic interactions (e.g. with Phe404 (3 5 6), Met343 (2 9 5)). Methionine residues are relatively unique in the protein core whereby they can form hydrophobic interactions but also engage polar oxygen (e.g. carbonyl) as recently reported by Pal et al.[83] In the case of compound **35**, direct contact of the nucleophilic oxygen of the carbonyl group of **35** with divalent sulfur of methionine (Met343 and Met421) through a hypervalent nonbonded S—X interaction is apparent contributing approximately 2.5 kcal/mol for this interaction.[84] This interaction is not present in ERβ as the mutation of Met421 → Ile373 allows **35** to slightly rotate in the LBP and H-bonding with Phe346, Leu339 and Arg346 occurs rather than H-bonding to Glu353 and Arg394 observed in ERα.[85] This mutation has been reported by Nilsson et al.[11] as being critical in achieving subtype-selective ER based therapeutics. A similar trend is also seen for the bulky *tert*-butyl moiety of **36** whereby alpha selectivity is observed for the same reasons but to a lesser degree than **35** (~50-fold). Interestingly, switching from the pyrrolidinyl (**36**) to piperidinyl basic side-chain (**41**) led to a marked reduction in ERα selectivity (> 12-fold) (Table 1). (see Fig. 6).

Compound **42** (Fig. 7) is stabilised in the binding site of both ERα and ERβ via hydrogen bonding interactions with Glu353 (3 0 5) and Arg394 (3 4 6) and also through additional H-bonding contacts to the morpholino oxygen of **42** (Leu536; 2.12 Å (Val487; 2.32 Å)) and the hydroxy group of Thr347 (2 9 9) which is well positioned to H-bond with the morpholino tertiary amine, irrespective of its protonation state. Interestingly, Fig. 7 highlights the additional polar contact made between Asp303 and Tyr488 in ERβ as a result of rotation of the carboxylate group of Asp303 which potentially would reduce the

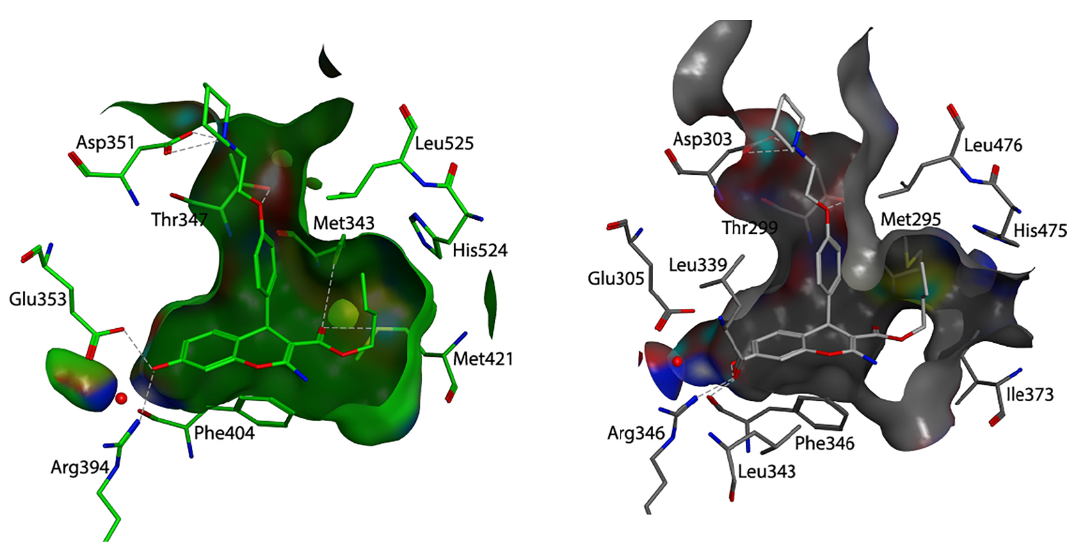


Fig. 5. Top ranked poses of Compound **35** in ERα (green) and ERβ (grey) indicating key hydrogen bonding interactions.

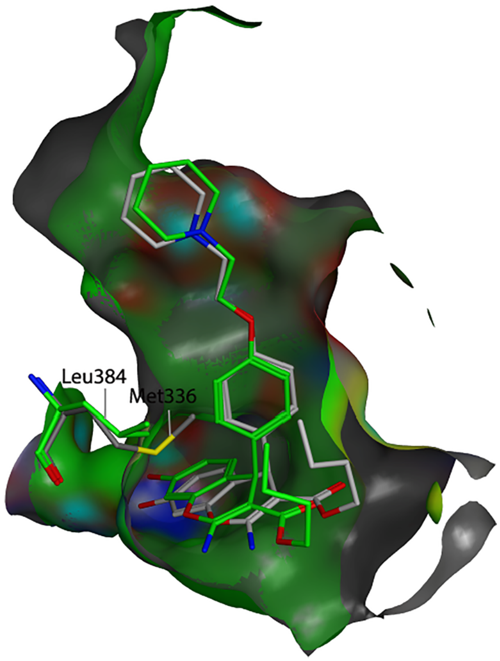


Fig. 6. Side-view of top ranked poses of Compound **35** in ERα (green) and ERβ (grey) indicating shift in orientation due to Leu384 → Met336 mutation.

conformational entropy of the ligand bound state of **42**, translating to an increase in binding affinity.

3. Conclusion

We have previously demonstrated the utility of the 4-aryl-4*H*-chromene scaffold as a potent modulator of ER activity. Although two 4-aryl-4*H*-chromene analogues displayed anti-proliferative activity in the ER positive MCF-7 cell line, ERα/β selectivity was modest.[69] Following on from these findings, the rationale that 4-aryl-4*H*-chromene analogues incorporating a typical basic side-chain could improve ERα/β selectivity was explored. Alongside this approach, modifications at the 3-position of the benzopyran ring were also investigated with a view to maximising ER isoform selectivity *via* probing of binding pocket topology. Several of these compounds possessed potent ER binding activity indicative of potential ER antagonistic effects, the most active compound (**35**) displayed 350-fold selectivity for ERα, whilst another (**42**) showed 170-fold selectivity for ERβ. Our computational study suggests that compound **35** achieves its selectivity in a completely different manner to compound **42.** In the case of compound **35**, selectivity is achieved through exploitation of the differing LBP size of each isoform which forms as a result of the mutational differences (Met421 → Ile373) and results in a hypervalent nonbonded S—X interaction with Met343 and Met421 in ERα only. Increasing the size of the carboxylate alkyl chain increases the binding affinity and selectivity for ERα. Compound **42** appears to exhibit differences in interaction potential to H12 of ERβ via Asp303 (H3) H-bonding to Tyr488 (H12) which may stabilize the ligand bound state and reduce its conformational entropy in order to achieve this selectivity.

4. Experimental section

4.1. Chemistry

Uncorrected melting points were measured on a Gallenkamp apparatus. Infra-red (IR) spectra were recorded on a Perkin Elmer FT-IR Paragon 1000 spectrometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 27 °C on a Brucker DPX 400 spectrometer (400.13 MHz, ^{1}H; 100.61 MHz, ^{13}C; 376.47 MHz, ^{19}F) in either $CDCl_3$ (internal standard tetramethylsilane (TMS)) or CD_3OD or DMSO-d_6. 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. Coupling constants are reported in Hertz. For ^{1}H NMR assignments, chemical shifts are reported: shift value (number of protons, description of absorption, coupling constant(s) where applicable). Low resolution mass spectra were run on a Hewlett-Packard 5973 MSD GC–MS system in an electron impact mode. Electrospray ionisation mass spectrometry (ESI-MS) was performed in the positive ion mode on a liquid chromatography time-of-flight mass spectrometer (Micromass LCT, Waters Ltd., Manchester, UK). The samples were introduced into the ion source by an LC system (Waters Alliance 2795, Waters Corporation, USA) in acetonitrile : water (60:40 %v/v) at 200 μL/min. The capillary voltage of the mass spectrometer was at 3 kV. The sample cone (de-clustering) voltage was set at 40 V.

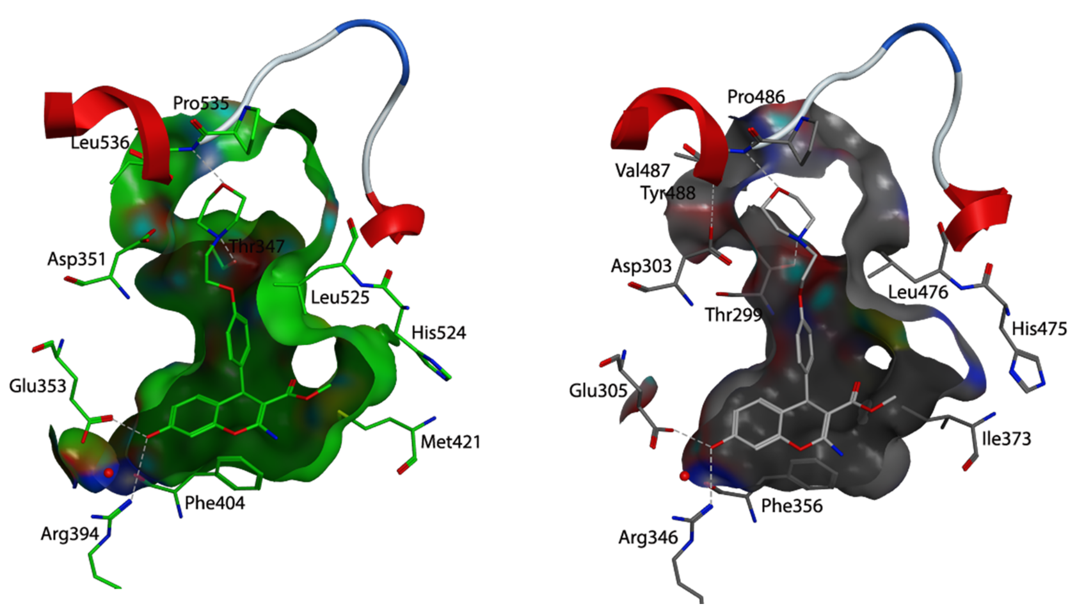


Fig. 7. Top ranked poses of Compound **42** (R Enantiomer) in ERα (green) and ERβ (grey) indicating key interactions.

For exact mass determination, the instrument was externally calibrated for the mass range *m/z* 100 to *m/z* 1000. A lock (reference) mass (*m/z* 556.2771) was used. Mass measurement accuracies of < ± 5 ppm were obtained. R_f values are quoted for thin layer chromatography 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 aluminium oxide, (activated, neutral, Brockmann I, 50 mesh) or Aldrich aluminium oxide, (activated, acidic, Brockmann I, 50 mesh). Chromatographic separations were also carried out on Biotage SP4 instrument. All products isolated were homogenous on TLC. Microwave experiments were carried out with in the Biotage initiator and Discover CEM microwave synthesisers.

4.1.1. General procedures for synthesis of benzopyrans.

Method A: To the appropriate aldehyde (benzaldehyde, 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde or 4-(2-piperidin-1-ylethoxy)benzaldehyde) (10 mmol, 1 equiv) was added the cyanoacetate ester (10 mmol, 1 equiv) or malononitrile (10 mmol, 1 equiv) and resorcinol (10 mmol, 1 equiv). Ethanol (or dichloromethane for methyl ester) (30 mL) was added and the reaction was heated briefly to reflux. The solution was then cooled and triethylamine (0.5 mL) was added dropwise, and the reaction solution was then heated at reflux for 4 h. Solvent was removed *in vacuo* and the product was recrystallized in ethanol or methanol (for the methyl esters). ***Method B:*** To the appropriate aldehyde (4-hydroxybenzaldehyde, 4-benzyloxybenzaldehyde, 4-ethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde, 4-(2-piperidin-1-ylethoxy)benzaldehyde or 4-(2-morpholin-1-ylethoxy)benzaldehyde)) (10 mmol, 1 equiv) was added the cyanoacetate ester (10 mmol, 1 equiv) or malononitrile (10 mmol, 1 equiv) and resorcinol (10 mmol, 1 equiv). Ethanol (dichloromethane for methyl ester) (30 mL) was added and the reaction was heated briefly to reflux. The solution was then cooled and piperidine (0.5 mL) was added dropwise and the reaction solution was then heated at reflux for 4 h. The solvent was removed *in vacuo* and product was recrystallized in ethanol (or methanol for methyl esters). ***Method C:*** To the appropriate aldehyde (4-benzyloxybenzaldehyde, 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde, 4-(2-piperidin-1-ylethoxy)benzaldehyde or 4-(2-morpholin-1-yl)ethoxybenzaldehyde, (5 mmol, 1 equiv) was added the cyanoacetate ester (5 mmol, 1 equiv) or malononitrile (5 mmol, 1 equiv) together with resorcinol (5 mmol, 1 equiv). Ethanol (10 mL), (dichloromethane for methyl esters) was added and the reaction heated in the microwave reactor for 5 min. The solution was then cooled and piperidine (0.25 mL) was added dropwise and the reaction solution was then heated in the microwave reactor for a further 30 min. The solvent was removed *in vacuo* and the product was recrystallized from ethanol (or methanol for the methyl esters).

4.1.1.1. 2-Amino-7-hydroxy-4-phenyl-4H-chromeme-3-carboxylic acid methyl ester (16).. Preparation following method C from benzaldehyde (5 mmol, 0.51 mL), resorcinol (5 mmol, 550 mg) and methyl cyanoacetate (5 mmol, 0.50 g). Yield 5.5%, yellow powder, M.p. 231 °C. IR υ_{max} (KBr) cm^{-1}: 3417.5, 3305.5 (NH_2), 1664.7 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.55 (s, 3H, O-CH_3), 4.88 (s, 1H, CH), 6.50 (d, 1H, *J* = 2.52 Hz, Ar—H), 6.55 (dd, 1H, *J* = 2.52 Hz, 8.4 Hz, Ar—H), 7.00 (d, 1H, *J* = 8.56 Hz, Ar—H), 7.06–7.09 (m, 1H, Ar—H), 7.20–7.23 (m, 4H, Ar—H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.1 (CH,), 49.4 (O-CH_3), 78.1 (C), 101.8 (CH), 111.6 (CH), 117.3 (C), 125.3 (CH), 126.7 (CH), 127.7 (CH), 129.5 (CH), 149.2 (C), 156.3 (C), 160.8(C), 168.7(C=O), 168.8 (C-NH_2). HRMS: Found 320.0898; $C_{17}H_{15}NO_4Na$ requires 320.0899(M^+ + Na).

4.1.1.2. 2-Amino-7-hydroxy-4-phenyl-4H-chromeme-3-carboxylic acid ethyl ester (17).. Preparation following Method A from benzaldehyde (10 mmol, 1.01 mL), resorcinol (1.10 g) and ethyl cyanoacetate (10 mmol, 1.13 g). Yield 17%, white powder, M.p. 243 °C. IR υ_{max} (KBr) cm^{-1}: 3306.4, 3300.0 (NH_2), 1662.8 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.11 (t, 3H, *J* = 7.02 Hz, —CH_3), 2.06 (m, 2H, NH_2), 4.00 (q, 2H, *J* = 7.01 Hz, CH_2), 4.89 (s, 1H, CH), 6.51 (d, 1H, *J* = 2.64 Hz, Ar—H), 6.54 (dd, 1H, *J* = 2.52 Hz, 6.00 Hz, Ar—H), 7.00 (d, 1H, *J* = 8.00 Hz, Ar—H), 7.08–7.11 (m, 1H, Ar—H), 7.19–7.25 (m, 4H, Ar—H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.33 (CH_3), 39.21 (CH), δ 58.18 (CH_2), 77.01 (C), 101.84 (CH), 111.59 (CH), 117.12 (C), 125.24 (CH), 126.87 (CH), 127.55 (CH), 129.58 (CH), 148.48 (C), 149.12 (C), 156.30 (C), 160.59(C-NH_2), 168.37(C=O). HRMS: Found 334.1042; $C_{18}H_{17}NO_4Na$ requires 334.1055(M^+ + Na).

4.1.1.3. 2-Amino-7-hydroxy-4-phenyl-4H-chromeme-3-carbonitrile (18). Preparation following Method A from benzaldehyde (10 mmol, 1.01 mL), resorcinol (1.10 g, 10 mmol) and malononitrile (10 mmol, 0.66 g). Yield 56%, Yellow powder, M.p. 246 °C[73]. IR υ_{max} (KBr) cm^{-1}: 3401.1 (OH), 3336.3, 3218.99 (NH_2), 2180.9 (CN), 1638.46, 1624.90(C=C) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.54 (s(br), 2H, NH_2), 2.76 (s(br), 1H, OH), 4.68 (s, 1H, CH), 6.51 (d, 1H, *J* = 2.00 Hz, Ar—H), 6.57 (dd, 1H, *J* = 2.34 Hz, 8.76 Hz, Ar—H), 6.86 (d, 1H, *J* = 8.76 Hz, Ar—H), 7.22–7.25 (m, 3H, Ar—H), 7.31 (d, 2H, *J* = 7.6 Hz, Ar—H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.21 (CH), 58.21 (C), 101.96 (CH), 111.99 (CH), 113.79 (CN), 118.99 (C), 126.26 (CH), 127.19 (CH), 128.08 (CH), 129.71 (CH), 145.88 (C), 148.89 (C), 156.83(C—O), 159.59 (C-NH_2). HRMS: $C_{16}H_{12}N_2NaO_2$ requires 287.0796, found 287.0794(M^+ + Na).

4.1.1.4. Ethyl 2-amino-7-hydroxy-4-(4-hydroxyphenyl)-4H-chromene-3-carboxylate 20.. Preparation following the general method B above from ethylcyanoacetate (10 mmol, 1.13 g), resorcinol (1.10 g, 10 mmol) and 4-hydroxybenzaldehyde (10 mmol, 1.22 g). The product was obtained as a dark brown solid which purified by flash column chromatography over silica gel (eluent: CH_2Cl_2 – MeOH; 85:15) and then recrystalised from ethanol. Yield: 1.04 g, 32%, M.p. 228-230°C[86]. IR υ_{max} (KBr): 3614.49, 3595.36, (OH), 3485.10, 3357.31 (NH_2), 1659.71 (C=O), 1629.41 (C=C), 1630.48 (C=C), 1591.36, 1538.14 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.98 (t, 3H, *J* = 7.2, CH_3), 4.28–4.47 (m, 2H, OCH_2), 4.68 (s, 1H, CH), 6.13–6.23 (m, 3H, Ar—H), 6.61–6.68 (m, 2H, Ar—H), 6.85–6.99 (m, 2H, Ar—H), 8.14 (s (br), 1H, OH), 9.15 (s (br), 2H, NH_2). ^{13}C NMR (101 MHz, DMSO-d_6): δ 14.31 (CH_3), 56.03 (CH), 58.49 (CH_2), 76.97 (C), 101.96 (CH), 102.44 (CH), 106.17 (CH), 114.75 (C), 127.81 (CH), 129.70 (CH), 132.74 (C), 152.56 (C—O), 154.93 (C-OH), 155.24 (C-OH), 158.44 (O—C—N), 160.88 (C=O). HRMS: $C_{18}H_{17}NNaO_5$ requires 350.1004, found 350.0997(M^+ + Na).

4.1.1.5. 2-Amino-4-(4-benzyloxyphenyl)-7-hydroxy-4H-chromene-3-carboxylic acid ethyl ester 21. Preparation following the general method B above from ethyl cyanoacetate (10 mmol, 1.13 g), resorcinol (1.10 g, 10 mmol) and 4-benzyloxybenzaldehyde (10 mmol, 2.12 g). The product was obtained as yellow powder which was recrystallised from ethanol. Yield: 1.54 g, 37%. Mp 192-194°C. IR υ_{max} (KBr): 3421.76 (OH), 3304.84, 3248.32 (NH_2), 1659.05 (C=O), 1610.67 (C=C), 1598.45 (C=C), 1503.32, 1454.01, 1311.69 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.10 (t, 3H, *J* = 7 Hz, CH_3), 2.07 (s (br), 2H, NH_2), 3.96 (q, 2H, *J* = 7.04 Hz, OCH_2), 4.75 (s, 1H, CH), 5.01 (s, 2H, OCH_2), 6.43 (d, 1H, *J* = 6.52 Hz, Ar—H), 6.47 (d, 1H, *J* = 8.04 Hz, Ar—H),4 (d, 1H, *J* = 8.52 Hz, Ar—H), 6.94 (d, 2H, *J* = 8.52 Hz, Ar—H), 7.23 (d, 2H, *J* = 8 *Hz*, Ar—H), 7.31 (d, 1H, *J* = 7.04 Hz, Ar—H), 7.34–7.39 (m, 2H, Ar—H), 7.42 (d, 1H, *J* = 6.76 Hz, Ar—H), OH not observed. ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.78 (CH_3), 38.58 (CH), 58.19 (CH_2), 69.58 (CH_2), 77.56 (C), 102.46 (CH), 112.53 (CH), 114.80 (C), 117.53 (CH), 128.08 (CH), 128.35 (CH), 128.85 (CH), 130.24 (CH), 137.68 (C), 141.58 (C), 149.51 (C—O), 156.89 (C-OH), 157.09 (C—O), 161.39 (O—C—N), 168.87 (C=O). HRMS: $C_{25}H_{23}NNaO_5$ requires 440.1474, found 440.1463 (M^+ + Na).

4.1.1.6. 2-Amino-4-(4-ethoxyphenyl)-7-hydroxy-4H-chromene-3-

carboxylic acid ethyl ester 22. Preparation following the general method B above from ethyl cyanoacetate (10 mmol, 1.13 g), resorcinol (1.10 g, 10 mmol) and 4-ethoxybenzaldehyde (10 mmol, 1.50 g). The product was obtained as yellow crystals following recrystallisation from ethanol, yield 0.78 g, 22%. M.p. 258-263°C. IR υ_{max} (KBr): 3563.54 (OH), 3431.59, 3419.15 (NH_2), 1660.91 (C=O), 1634.86 (C=C), 1613.89 (C=C), 1583.43, 1568,42, 1458.83 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.05 (t, 3H, J = 7.04 Hz, CH_3), 1.25 (t, 3H, J = 7.02 Hz, CH_3), 3.93–4.18 (m, 4H, 2xOCH_2), 4.72 (s, 1H, CH), 6.42–6.50 (m, 2H, Ar—H), 6.73 (d, 2H, J = 9.04 Hz, Ar—H), 6.94 (d, 2H, J = 8.04 Hz, Ar—H), 7.01 (d, 1H, J = 8.12 Hz, Ar—H), OH not observed, NH_2 not observed. ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.31 (CH_3), 14.68 (CH_3), 38.09 (CH), 58.51 (CH_2), 62.79 (CH_2), 76.81 (C), 101.97 (CH), 112.04 (CH), 113.91 (CH), 117.13 (C), 127.84 (CH), 129.76 (CH), 140.71 (C), 149.02 (C—O), 156.53 (C-OH), 160.91 (O—C—N), 168.40 (C=O). HRMS: $C_{20}H_{22}NO_5$ requires 356.1498, found 356.1488(M^+ +H).

4.1.1.7. Ethyl 2-amino-7-hydroxy-4-(3,4-dimethoxyphenyl)-4H-chromene-3-carboxylate 23. (*E*)-Ethyl 2-cyano-3-(3,4-dimethoxyphenyl)acrylate **28** (10 mmol, 2.62 g) and resorcinol (1.10 g, 10 mmol) were dissolved in ethanol and the reaction mixture was briefly heated to reflux. Piperidine (0.5 mL) was added dropwise and the reaction solution was heated at reflux for 4 h. following the general method B above. The solvent was removed *in vacuo* and product was recrystallized from ethanol. Yield: 0.07 g, 2%, yellow crystals, M.p. 284-288°C [71]. IR υ_{max} (KBr): 3488.34 (OH), 3431.11, 3412.48 (NH_2), 1681.40 (C=C), 1642.76 (C=C), 1629.45 (C=O), 1562.75 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.09 (t, 3H, J = 7.04 Hz, CH_3), 3.66 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.95 (q, 2H, J = 7.04 Hz, OCH_2), 4.73 (s, 1H, CH), 6.42 (s, 1H, Ar—H), 6.46 (dd, 1H, J = 2.48, J = 7.02 Hz, Ar—H), 6.55 (d, 1H, J = 9.04 Hz, Ar—H), 6.77 (d, 2H, J = 9.04 Hz, Ar—H), 6.99 (d, 1H, J = 8.52 Hz, Ar—H), 7.55 (s (br), 2H, NH_2), 9.57 (s (br), 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.36 (CH_3), 38.45 (CH), 55.40 (CH_3), 58.53 (CH_2), 76.72 (C), 101.97 (CH), 110.23 (CH), 111.15 (CH), 111.86 (C), 117.04 (CH), 118.64 (CH), 129.73 (CH), 141.44 (C), 146.83 (C-OCH_3), 148.19 (C-OCH_3), 149.03 (C—O), 156.60 (C—O), 160.97 (O—C—N), 168.43 (C=O). HRMS: $C_{20}H_{21}NNaO_6$ requires 394.1267, found 394.1262(M^+ +Na).

4.1.1.8. 2-Amino-4-(4-benzyloxyphenyl)-7-hydroxy-4H-chromene-3-carbonitrile (24). Preparation following the general method B above from malononitrile (0.66 g, 10 mmol) and resorcinol (1.1 g, 10 mmol) and 4-benxyloxybenzaldehyde (2.12 g, 10 mmol). The product was obtained as a pink solid, 2.08 g (56%) following recrystallisation from ethanol. M.p. 264–266 °C. IR υ_{max}: 3258.49 (OH), 3032.13 (CH), 2191.16 (CN), 1637.44 (C=C) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ9.71 (s, 1H, OH), 7.24–7.51 (m, 5H, ArH), 7.11 (d, J = 8.54 Hz, 2H, ArH), 6.96 (d, J = 8.54 Hz, 2H, ArH), 6.75–6.89 (m, 3H, ArH), 6.41–6.60 (m, 2H, ArH), 5.05 (s, 2H, CH_2), 4.59 (s, 1H, CH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.2 (O—C—N), 157.2 (C—O), 157.1 (C—O), 148.8 (C—O), 138.8 (C), 137.1 (C), 130.0 (CH), 128.5 (CH), 128.4 (CH), 127.8 (CH), 127.7 (CH), 120.8 (CN), 114.8 (CH), 114.1 (CH), 112.4 (C), 102.2 (CH), 69.3 (CH_2), 56.7 (C), 39.3 (CH). HRMS: $C_{23}H_{17}N_2O_3$ requires 369.1239; found 369.1232 $[M-H]^+$.

4.1.1.9. 2-Amino-4-(4-benzyloxyphenyl)-7-hydroxy-4H-chromeme-3-carboxylic acid methyl ester (25). Preparation as above using Method C from 4-benzyloxybenzaldehyde (10 mmol, 2.12 g), resorcinol (1.10 g, 10 mmol) and methyl cyanoacetate, (10 mmol, 0.99 g). Yield 46%, cream powder, M.p. 216–218 °C. IR υ_{max} (KBr) cm^{-1}: 3415.4, 3302.9 (NH_2), 1660.7 (C=O) cm^{-1}. ^1H NMR (400 MHz, Acetone-d_6): δ 3.57 (s, 3H, O-CH_3), 4.84 (s, 1H, CH), 5.04 (s, 2H, CH_2), 6.50 (d, 1H, J = 2.04 Hz, Ar—H), 6.55 (dd, 1H, J = 2.26 Hz, 6.04 Hz, Ar—H), 6.85 (d, 2H, J = 8.52 Hz, Ar—H), 7.00 (d, 1H, J = 8.56 Hz, Ar—H), 7.14 (d, 2H, J = 8.56 Hz, Ar—H), 7.31 (d, 1H, J = 7.04 Hz, Ar—H), 7.37 (t, 2H, J = 7.78 Hz, Ar—H), 7.44 (d, 2H, J = 7.88 Hz, Ar—H). ^{13}C NMR (100 MHz, Acetone-d_6): δ 38.2 (CH), 49.4 (O-CH_3), 68.9 (O-CH_2), 77.4 (C), 101.8 (CH), 111.6 (CH), 113.9 (CH), 117.6 (C), 127.1 (CH), 127.6 (CH), 127.9 (CH), 129.4 (CH), 137.2 (C), 140.8 (C), 149.2 (C), 156.2 (C), 160.6 (C—O), 160.7 (C-NH_2), 168.7 (C=O). HRMS: Found 426.1297; $C_{24}H_{21}NO_5Na$ requires 426.1317(M^+ +Na).

4.1.2. 2-Amino-7-hydroxy-4-(4-hydroxyphenyl)-4H-chromeme-3-carboxylic acid methyl ester (19)

2-Amino-4-(4-benzyloxyphenyl)-7-hydroxy-4*H*-chromene-3-carboxylic acid methyl ester **(25)** (1.6 mmol, 0.77 g) was dissolved in ethyl acetate (20 mL) and Pd/C (10%, 0.8 g) was added. The mixture was stirred under an atmosphere of hydrogen for 12 h until reaction was complete as monitored by TLC. The catalyst was removed by filtrations and the residue was purified by chromatography to afford the product as a waxy solid, IR: KBr υ_{max}: 3415.4, 3302.9 (NH_2), 1660.7 (C=O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.57 (s, 3H, O-CH_3), 4.79 (s, 1H, CH), 6.49 (d, 1H, J = 2.00 Hz, Ar—H), 6.56 (dd, 1H, J = 2.02 Hz, 6.00 Hz, Ar—H), 6.69 (d, 2H, J = 8.52 Hz, Ar—H), 6.99–7.04 (m, 3H, Ar—H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.2 (CH), 49.4 (O-CH_3), 68.9 (O-CH_2), 77.4 (C), 101.8 (CH), 111.6 (CH), 113.9 (CH), 117.6 (C), 127.1 (CH), 127.6 (CH), 127.9 (CH), 129.4 (CH), 137.2 (C), 140.8 (C), 149.2 (C), 156.2 (C), 160.6 (C—O), 160.7 (C-NH_2), 168.7 (C=O). Found 336.0852; $C_{17}H_{15}NO_5Na$ requires 336.0848(M^+ +Na).

4.1.3. General procedure for synthesis of cyanoacrylate esters 26–28

A solution of methyl or ethyl cyanoacetate (10 mmol) and the appropriate arylaldehyde (10 mmol) in methanol 30 mL was heated briefly to reflux. The solution was cooled and piperidine (0.5 mL) was added dropwise and the reaction solution was then heated at reflux for 4 h. The solution was cooled and the solvent was removed *in vacuo* and product was recrystallized from ethanol.

4.1.3.1. (E)-Methyl 2-cyano-3-(4-hydroxyphenyl)acrylate 26. A mixture of methyl cyanoacetate (10 mmol, 1.13 g) and 4-hydroxybenzaldehyde (10 mmol, 1.22 g) was melted under vacuum in a Kugelrohr short-path vacuum distillation apparatus at 170 °C for 40 min. The product was then dried at 80 °C and then recrystallized from ethanol as beige coloured crystals. Yield: 1.802 g, 90%, Mp: 208–210 °C.[69] IR v_{max} (KBr): 3415.50 (OH), 2224.80 (CN), 1725.19 (C=O), 1589.33 (C=C) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): 3.78 (s, 3H, OCH_3), 6.91 (d, J = 8.54 Hz, 2H), 7.94 (d, J = 9.16, Hz, 2H), 8.18 (s, 1H, CH=C). ^{13}C NMR (100 MHz, $CDCl_3$): δ 51.99 (OCH_3), 97.62 (C), 115.53 (CN), 115.88 (CH), 123.06 (C), 133.50 (CH), 153.89 (CH), 162.08 (C-OH), 162.76 (C=O).

4.1.3.2. (E)-Ethyl 2-cyano-3-(4-benzyloxyphenyl)acrylate 27. Following the procedure outlined above, a solution of ethyl cyanoacetate (10 mmol, 1.13 g) and 4-benzyloxybenzaldehyde (10 mmol, 2.12 g) in benzene (20 mL) was heated briefly to reflux and then cooled. Piperidine (0.99 mL) was added dropwise and the reaction solution was then heated at reflux for 4 h. The solution was washed with HCl (10%) and then extracted with dichloromethane. The solvent was removed *in vacuo* and the product was recrystallized from ethanol as cream coloured crystals. Yield: 2.53 g, 83%, M.p. 99–100 °C [87]. IRv_{max} (KBr): 2221.96 (CN), 1714.07 (C=O), 1688.82 (C=C), 1563.74, 1473.48 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): 1.40 (t, J = 7.02 Hz, 3H, CH_3), 4.38 (q, J = 7.12 Hz, 2H, CH_2), 5.16 (s, 2H, CH_2), 7.07 (d, J = 9.16 Hz, 2H, ArH), 7.32–7.47 (m, 5H, ArH), 8.01 (d, J = 8.54 Hz, 2H, ArH), 8.18 (s, 1H, CH=C).

4.1.3.3. (E)-Ethyl 2-cyano-3-(3,4-dimethoxyphenyl)acrylate 28. Following the procedure outlined above, a solution of ethyl cyanoacetate (10 mmol, 1.13 g) and 3,4-dimethoxybenzaldehyde (10 mmol, 1.66 g) in ethanol 30 mL was heated briefly to reflux and then cooled. Piperidine (0.5 mL) was then added dropwise and the reaction solution heated at reflux for

4 h. The solvent was removed *in vacuo* and product was recrystallized from ethanol to afford the product as yellow crystals. Yield: 0.34 g, 13%, M.p. 160 °C [41]. IR υ_{max} (KBr): 2244.68 (CN), 1673.71 (C=C), 1643.64 (C=O), 1563.74, 1473.48 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.94 (t, 3H, J = 7.14 Hz, CH_3), 3.72 (s, 6H, 2xOCH_3), 3.86 (q, 2H, J = 7.04, CH_2), 6.38 (d, 1H, J = 2.28, Ar—H), 6.45 – 6.51 (m, 1H, Ar—H), 7.18–7.27 (m, 1H, Ar—H), 8.46 (d, 1H, J = 6.98, CH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.33 (CH_3), 58.85 (OCH_3), 62.03 (CH_2), 78.01 (C), 106.63 (CH), 112.53 (CN), 120.75 (CH), 128.77 (C), 148.70 (C-OCH_3), 149.77 (C-OCH_3), 157.73 (CH), 168.72 (C=O). HRMS: $C_{14}H_{16}NO_4$ requires 262.1079, found 262.1086(M^+ +H).

4.1.4. General procedure for preparation of 29–31

4-Hydroxybenzaldehyde (16 mmol (1 equiv)) was dissolved in ethyl acetate (20 mL). To this solution was added anhydrous potassium carbonate (18 mmol, 2.50 g) (1.125 equiv) and *tetra-n*-butylammonium bromide (0.054 g, 0.2 mmol). The mixture was then heated at reflux for 10 min and the appropriate basic ether was then added (32 mmol, 2 equiv). [The basic ethers 1-(2-chloroethyl)pyrrolidine, 1-(2-chloroethyl)piperidine and 1-(2-chloroethyl) morpholine were obtained as their HCl salt and were washed with water (45 mL) and sodium hydroxide (10.5 g) in toluene (6 mL) to extract the free base for use in the reaction]. The reaction mixture was heated at reflux until reaction was complete as monitored by thin layer chromatography. On completion, the solution was filtered and the solvent was removed under reduced pressure to afford product, which was used in the following reactions without further purification.

4.1.4.1. 4-(2-Pyrrolidin-1-ylethoxy)benzaldehyde (29). The preparation was according to the general procedure above. The product was isolated as orange oil[77] yield 65%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.59 (s, 4H, $(CH_2)_2$), 2.41 (s, 4H, CH_2—N—CH_2), 2.70 (2H, t, J = 5.78 Hz, –CH_2), 3.96 (t, J = 5.78 Hz, 2H, O-CH_2), 6.79 (d, 2H, J = 8.56 Hz, Ar—H), 7.58 (d, 2H, J = 8.52 Hz, Ar—H), 9.64 (s, 1H, CHO).

4.1.4.2. 4-(2-Piperidin-1-ylethoxy)benzaldehyde (30). The preparation was according to the general procedure above (32 mmol scale). The product was isolated as orange coloured oil, (5.42 g, 73%)[77]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.53–1.55 (m, 6H, 3xCH_2), 2.51–2.66 (m, 6H, CH_2), 4.29–4.36 (m, 2H, CH_2), 7.13 (d, 2H, J = 8.0 Hz, Ar—H), 7.85 (d, 2H, J = 8.0 Hz, Ar—H), 9.89 (s, 1H, CHO).

4.1.4.3. 4-(2-Morpholin-1-ylethoxy)benzaldehyde (31). The preparation was according to the general procedure above and the product was isolated as an orange coloured oil (97%)[77]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.60 (m, 4H, 2xCH_2), 2.68 (s, 2H, CH_2), 3.56 (m, 4H, 2xCH_2), 4.05 (m, 2H, 2xO-CH_2), 6.86 (m, 2H, Ar—H), 7.67 (m, 2H, Ar—H), 9.71 (s, 1H, CHO).

4.1.4.4. 2-Amino-7-hydroxy-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic methyl ester (32). Preparation was as described above from 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde (**29**) (10 mmol, 2.19 g), resorcinol (1.10 g, 10 mmol) and methyl cyanoacetate (10 mmol, 0.99 g), using general Method C. Yield 10%, M.p. 167 °C, yellow crystals. IR ν_{max} (KBr) cm^{-1}: 3413.53, 3296.89 (NH_2), 1701.9(C=O), 1656.4, 1610.6(C=C) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.74–1.77 (m, 4H, C-$(CH_2)_2$-C), 2.64–2.67 (m, 4H, CH_2—N—CH_2), 2.87–2.90 (t, 2H, J = 5.88 Hz, CH_2—N), 3.57 (s, 3H, O-CH_3), 4.05–4.08 (t, 2H, J = 5.90 Hz, CH_2—N), 4.83 (s, 1H, CH), 6.50–6.51 (d, 2H, J = 2.52 Hz, Ar—H), 6.54–6.57 (dd, 1H, J = 2.36 Hz, 6.04 Hz, Ar—H), 6.77–6.79 (d, 2H, J = 8.76 Hz, Ar—H), 6.99–7.02 (m, 1H, Ar—H), 7.11–7.13 (d, 2H, J = 8.52 Hz, Ar—H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.5 (Ar-C), 156.9 (Ar-C), 149.4 (Ar-C), 141.0 (Ar-C), 128.0 (Ar-CH), 117.6 (Ar-CH), 114.4 (Ar-CH), 112.4 (Ar-CH), 102.4 (Ar-CH), 77.0 (-C-), 66.9 (NCH_2), 54.7 (OCH_3), 54.3 (NCH_2), 40.37 (CH), 38.3 (CH_2), 23.4 (CH_2). HRMS: Found 411.1927; $C_{23}H_{27}N_2O_5$ requires 411.1920(M^+ +H).

4.1.4.5. 2-Amino-7-hydroxy-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic acid ethyl ester (33). Preparation was as described above from 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde (**29**) (10 mmol, 2.19 g), resorcinol (1.10 g, 10 mmol) and ethyl cyanoacetate (10 mmol, 1.13 g) using Method B. Purification was achieved by flash column chromatography over silica gel (eluent: dichloromethane:methanol; 9:1) to afford the product as a yellow gel, yield 10%. IR υ_{max} (film) 1671.15 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (t, 3H, J = 7.28 Hz, –CH_3), 1.76–1.90 (m, 4H, C-$(CH_2)_2$-C), 2.03–2.07 (m, 4H, CH_2—N—CH_2), 2.93–2.94 (t, 2H, J = 5.78 Hz, CH_2—N), 4.01–4.06 (q, 2H, J = 7.02 Hz, CH_2—N), 4.22–4.24 (t, 2H, J = 4.76 Hz, O-CH_2), 4.80 (d, 1H, CH), 5.12 (bs, 1H, OH), 6.47–6.49 (d, 2H, J = 3 Hz, Ar—H), 6.85–6.90 (dd, 3H, J = 5.04 Hz, 8.78 Hz, Ar—H), 7.14 (d, 2H, J = 9.04 Hz, Ar—H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.09 (CH_3), 22.14 (CH_2), 38.40 (CH), 53.32 (CH_2), 53.84 (CH_2), 58.53 (CH_2), 62.56 (CH_2), 77.09 (C), 101.73 (Ar-CH), 111.44 (Ar-CH), 113.62 (Ar-CH), 116.96 (ArC), 127.76 (Ar-CH), 129.22 (Ar-C), 131.38 (Ar-C), 129.88 (Ar-CH), 138.48 (Ar-C), 148.92 (Ar-C), 155.48 (Ar—C—O), 156.12 (Ar—C—O), 162.50 (Ar—C—N), 169.209 (C=O). HRMS: Found 425.2081; $C_{24}H_{29}N_2O_5$ requires 425.2076(M^+ +H).

4.1.4.6. 2-Amino-7-hydroxy-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-4H-chromeme-3-carbonitrile (34). Preparation following General Method B from 4-(2-pyrrolidin-1-ylethoxy)benzaldehyde (**29**) (1.3 mmol, 0.285 g), resorcinol (143 mg, 1.30 mmol) and malononitrile (1.3 mmol, 85.9 mg). Yield 20.4%, M.p. 181 °C, cream powder. IR υ_{max} (KBr) 3439.6, 3360.1 (NH_2), 2185.1 (CN) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.74 (s, 4H, C-$(CH_2)_2$-C), 2.64 (t, 4H, J = 5.10 Hz, CH_2—N—CH_2), 2.80 (t, 2H, J = 6.02 Hz, CH_2—N), 4.01 (t, 2H, J = 6.04 Hz, CH_2—N), 4.55 (s, 1H, CH), 6.10 (bs, 1H, OH), 6.38 (d, 1H, J = 2.36 Hz, Ar—H), 6.46 (dd, 1H, J = 2.56 Hz, Ar—H), 6.78 (d, 1H, J = 8.48 Hz, Ar—H), 6.85 (d, 2H, J = 4.96 Hz, Ar—H), 7.13–7.15 (d, 2H, J = 8.52 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.12 (CH_2), 26.45 (CH_2), 38.15 (CH), 46.66 (CH_2), 53.97 (CH_2), 54.35 (CH_2), 56.52 (C), 66.63 (CH_2), 102.09 (Ar-CH), 106.15 (CH), 112.35 (CH), 113.94 (C), 114.40 (CH), 120.75 (CH), 128.42 (CH), 129.88 (CH), 138.48 (C), 148.76 (C), 157.13 (C), 157.22 (C), 158.49 (C—O), 160.09 (C—N). HRMS: Found 378.1801; $C_{22}H_{24}N_3O_3$ requires 378.1818(M^+ +H).

4.1.4.7. 2-Amino-7-hydroxy-4-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic n-butyl ester (35). Preparation following the general Method A from 4-(2-pyrrolidine-1-yl-ethoxy)-benzaldehyde **(29)** (2.5 mmol, 0.547 g), resorcinol (2.5 mmol, 280 mg), and *n*-butyl cyanoacetate (2.5 mmol, 353 mg). Yield 14.2%, yellow powder. IR υ_{max} (KBr) cm^{-1}. 3407.44, 3294.68 (NH_2), 1673.91 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.78 (t, 1H, J = 7.54 Hz, CH_2), 1.06 (t, 3H, J = 3.52 Hz, CH_3), 1.40 (m, 1H, CH_2), 1.66 (bs, 4H, $(CH_2)_2$), 2.51 (d, 6H, J = 11.04 Hz, $(CH_2)_3$), 2.73 (t, 2H, J = 5.76 Hz, N—CH_2), 3.71 (m, 4H, 2 × O-CH_2), 4.73 (s, 1H, CH), 6.42 (d, 1H, J = 2.17 Hz, Ar—H), 6.48 (dd, 1H, J = 2.24 Hz, 6.04 Hz, Ar—H), 6.78 (d, 2H, J = 8.52 Hz, Ar—H), 6.99 (d, 1H, J = 8.28 Hz, Ar—H), 7.03 (m, 2H, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.32 (CH_3), 18.56 (CH_2), 23.10 (CH_2), 30.50 (CH_2), 38.10 (CH), δ 53.95 (CH_3), 54.30 (CH_2), 54.32 (CH_2), 58.52 (CH_2), 62.20 (CH_2), 66.50 (CH_2), 76.81 (C), 101.97 (CH), 112.04 (CH), 113.98 (CH), 117.05 (C), 127.74 (CH), 127.86 (CH), 129.75 (CH), 140.83 (C), 148.85 (CH), 149.02 (C), 156.51 (C), 156.61 (C), 160.91 (C), 160.99 (C), 168.49 (C=O). HRMS: Found 453.2393; $C_{26}H_{33}N_2O_5$ requires 453.2389(M^+ +H).

4.1.4.8. 2-Amino-7-hydroxy-4-[4-(2-pyrolidine-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic tert-butyl ester (36). Preparation following general method A above from 4-(2-pyrrolidine-1-yl-ethoxy) benzaldehyde **(29)** (2.5 mmol, 0.547 g), resorcinol (2.5 mmol, 280 mg) and *tert*-butyl cyanoacetate (2.5 mmol, 353 mg). Yield 63%, white powder. IR υ_{max} (KBr) cm^{-1}: 3407.03, 3291.65 (NH_2), 1672.28 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.36 (s, 9H, $(CH_3)_3$), 1.78 (s, 4H, $(CH_2)_2$), 2.47 (bs, 4H, $(CH_2)_2$), 2.72 (t, 2H, J = 6.04 Hz, CH_2), 3.97 (t, 2H, J = 5.78 Hz, CH_2), 4.65 (s, 1H, CH), 6.39 (d, 1H, J = 2.48 Hz, Ar—H), 6.44 (dd, 1H, J = 2.26 Hz, 8.52 Hz, Ar—H), 6.77 (d, 2H, J = 8.52 Hz, Ar—H), 6.93 (d, 1H, J = 8.56 Hz, Ar—H), 7.01 (d, 2H, J = 8.52 Hz, Ar—H). 7.48 (s, 2H, NH_2), 9.57 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.11 (CH_2), 28.10 (CH_3), 38.58 (CH), 53.99 (CH_2), 54.34 (CH_2), 56.03 (CH_2), 66.59 (CH_2), 77.86 (C), 78.01 (C), 101.90 (CH), 111.92 (CH), 113.90 (CH), 116.93 (C), 127.88 (CH), 129.82 (CH), 141.19 (C), 148.85 (C), 156.46 (C), 156.52 (C), 160.81 (C), 168.50 (C=O). HRMS: Found 453.2393; $C_{26}H_{33}N_2O_5$ requires 453.2389(M^+ + H).

4.1.4.9. 2-Amino-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic methyl ester (37). Preparation following the general method C above from 4-(2-piperidine-1-ylethoxy)-benzaldehyde (**30**) (5 mmol, 1.16 g) and methyl cyanoacetate (5 mmol, 495 mg) and resorcinol (5 mmol, 560 mg). Yield 25%, M.p. 180–181 °C, yellow powder. IR υ_{max} (KBr) cm^{-1}: 3400.00, 3299.51 (NH_2), 1660.34 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.35 (d, 2H, J = 4.88 Hz, CH_2), 1.46 (t, 4H, J = 5.38 Hz, $(CH_2)_2$), 2.39 (bs, 4H, $(CH_2)_2$), 2.51 (t, 2H, J = 1.96 Hz, CH_2), 3.51 (s, 3H, O-CH_3), 3.96 (t, 2H, J = 5.86 Hz, CH_2), 4.75 (s, 1H, CH), 6.44 (d, 1H, J = 2.44 Hz, Ar—H), 6.48 (dd, 1H, J = 2.20 Hz, 6.36 Hz, Ar—H), 6.78 (d, 2H, J = 8.80 Hz, Ar—H), 6.99 (d, 1H, J = 8.32 Hz, Ar—H), 7.03 (d, 2H, J = 8.80 Hz, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.39 (CH_2), 26.03 (CH_2), 38.47 (CH), 50.86 (CH_3), 54.85 (CH_2), 57.89 (CH_2), 65.85 (CH_2), 77.07 (C), 102.50 (CH), 112.52 (CH), 114.58 (CH), 117.79 (C), 128.12 (CH), 130.07 (CH), 141.08 (C), 149.54 (C), 157.08 (C), 161.64 (C), 169.20 (C=O). HRMS: Found 425.2082; $C_{24}H_{29}N_2O_5$ requires 425.2076(M^+ + H).

4.1.4.10. 2-Amino-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic ethyl ester (38). (i) Following the method outlined above for **28**, a solution of ethyl cyanoacetate (10 mmol, 1.13 g) and 4-(2-piperidin-1-ylethoxy)benzaldehyde **(30)** (10 mmol, 2.33 g) in ethanol (30 mL) was heated briefly to reflux and then cooled. Piperidine (0.5 mL) was then added dropwise and the reaction solution heated at reflux for 4 h. The solvent was removed *in vacuo* to afford the product (*E*)-ethyl 2-cyano-3-[4-(2-piperidin-1-ylethoxy)phenyl]acrylate **45** which was recrystallized from ethanol as orange coloured crystals and used immediately in the next reaction. Yield: 334 mg, 10.2%, M.p. 88–90 °C. IR υ_{max} (KBr): 2213(CN), 1702(C=O), 1610(C=C) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.06 (s, 1H, CH=C), 7.92 (d, J = 9.16 Hz, 2H, ArH), 6.86 (d, J = 8.54 Hz, 2H, ArH), 4.34 (q, J = 7.12 Hz, 2H, OCH_2), 3.45 (br. s., 4H, 2xCH_2), 1.69 (br. s., 6H, 3xCH_2), 1.38 (t, J = 7.32 Hz, 3H, $OCH_2\underline{CH_3}$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.26 (CH_3), 24.31 (CH_2), 25.32 (CH_2), 48.10 (CH_2), 61.90 (CH_2), 94.64 (C=CH), 113.24 (CH), 117.38 (CN), 120.08 (C), 134.06 (CH), 154.01 (C), 154.21 (CH=C), 164.14 (C=O). (ii) Preparation following the general method B above from (*E*)-ethyl 2-cyano-3-[4-(2-piperidin-1-ylethoxy)phenyl]acrylate **45** (10 mmol, 2.33 g) and resorcinol (10 mmol, 1.13 g). Yield 20%, M.p. 176–178 °C, yellow powder. IR υ_{max} (KBr) cm^{-1}: 3415.04, 3293.18 (NH_2), 1672.17 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.04 (m, 9H, CH_3, CH_2), 1.98 (m, 4H, CH_2), 2.78 (2H, CH_2), 3.94 (t, 4H, J = 6.13 Hz, CH_2), 4.74 (s, 1H, CH), 6.44 (d, 1H, J = 2.44 Hz, Ar—H), 6.48 (dd, 1H, J = 2.44 Hz, 5.88 Hz, Ar—H), 6.69 (d, 2H, J = 8.80 Hz, Ar—H), 6.88 (d, 1H, J = 8.32 Hz, Ar—H), 7.02 (d, 2H, J = 6.84 Hz, Ar—H), 7.09 (d, 1H, J = 8.80 Hz, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.32 (CH_3), 23.92 (CH_2), 25.55 (CH_2), 38.09 (CH), 54.39 (CH_2), 57.41 (CH_2), 58.52 (CH_2), 65.39 (CH_2), 76.81 (C), 101.99 (CH), 112.05 (CH), 114.02 (CH), 117.12 (C), 127.85 (CH), 129.75 (CH), 140.82(C), 149.02 (C), 156.53 (C), 156.60 (C), 160.91 (C), 168.39 (C=O). HRMS: Found 439.2227; $C_{25}H_{31}N_2O_5$, requires 439.2233(M^+ + H).

4.1.4.11. 2-Amino-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-4H-chromeme-3-carbonitrile (39). Preparation as described for **38** above from 4-(2-piperidine-1-ylethoxy)benzaldehyde (**30**) (5 mmol, 1.16 g), malononitrile (5 mmol, 330 mg) and resorcinol (5 mmol, 560 mg) following method A. Yield 35%, M.p. 194–196 °C, beige powder. IR υ_{max} (KBr) cm^{-1}: 3401.16, 3300.00 (NH_2), 2186.78 (CN) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.43 (m, 2H, CH_2), 1.56 (m, 4H, CH_2), 2.48 (m, 4H, CH_2), 2.68 (t, 2H, J = 6.02 Hz, N—CH_2), 4.07 (t, 2H, J = 6.04 Hz, O-CH_2), 4.63 (s, 1H, CH), 6.52 (d, 1H, J = 2.48 Hz, Ar—H), 6.60 (dd, 1H, J = 2.52 Hz, 6.00 Hz, Ar—H), 6.78 (s, 1H, Ar—H), 6.87 (dd, 2H, J = 2.34 Hz, 4.48 Hz, Ar—H), 7.15 (m, 2H, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.39 (CH_2), 26.03 (CH_2), 38.47 (CH), 50.86 (CH_3), 54.85 (CH_2), 57.89 (CH_2), 65.85 (CH_2), 77.07 (C), 102.50 (CH), 112.52 (CH), 114.58 (CH), 117.79 (C), 128.12 (CH), 130.07 (CH), 141.08 (C), 149.54 (C), 157.08 (C), 161.64 (C), 169.20 (C=O). HRMS: Found 392.1957; $C_{23}H_{26}N_3O_3$ requires 392.1974(M^+ + H).

4.1.4.12. 2-Amino-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic n-butyl ester (40). Preparation as described for **39** above from 4-(2-piperidine-1-ylethoxy)benzaldehyde (**30**) (5 mmol, 1.16 g), *n*-butylcyanoacetate (5 mmol, 705.8 mg) and resorcinol (5 mmol, 560 mg) using general method A. Yield 13%, M.p. 158 °C, yellow powder. IR υ_{max} (KBr) cm^{-1}: 3415.93, 3294.68 (NH_2), 1672.10 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.06 (t, 3H, J = 7.02 Hz, CH_2), 1.45 (m, 8H, $(CH_2)_4$), 2.40 (bs, 4H, $(CH_2)_2$), 2.51 (s, 2H, CH_2), 2.60 (t, 2H, J = 5.52 Hz, N—CH_2), 3.95 (m, 4H, $(CH_2)_2$), 4.73 (s, 1H, CH), 6.42 (d, 1H, J = 2.48 Hz, Ar—H), 6.46 (dd, 1H, J = 2.28 Hz, 6.00 Hz, Ar—H), 6.78 (d, 2H, J = 8.52 Hz, Ar—H), 6.93 (d, 1H, J = 8.52 Hz, Ar—H), 7.01 (d, 2H, J = 8.52 Hz, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.32 (CH_3), 23.88 (CH_2), 25.50 (CH_2), 30.51 (CH_2), 38.10 (CH), 54.36 (CH_3), 56.03 (CH_2), 57.36 (CH_2), 58.52 (CH_2), 62.20 (CH_2), 65.35 (CH_2), 76.82 (C), 101.99 (CH), 106.17 (CH), 112.06 (CH), 114.03 (CH), 117.09 (C), 127.73 (CH), 129.74 (CH), 140.84 (C), 149.02 (C), 156.52 (C), 158.45 (C), 160.91 (C), 168.39 (C=O). HRMS: Found 467.2558; $C_{27}H_{35}N_2O_5$ requires 467.2546(M^+ + H).

4.1.4.13. 2-Amino-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-4H-chromeme-3-carboxylic tert-butyl ester (41). Preparation as described for **39** above from 4-(2-piperidine-1-ylethoxy)benzaldehyde (**30**) (2.5 mmol, 0.583 g), *tert*-butycyanoacetate (2.5 mmol, 360 mg) and resorcinol (5 mmol, 560 mg) using general Method A. Yield 27%, colourless oil. IR υ_{max} (KBr) cm^{-1}: 3415.98, 3289.85 (NH_2), 1672.28 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.27 (s, 9H, $(CH_3)_3$), 1.36 (d, 2H, J = 5.28 Hz, CH_2), 1.48 (m, 4H, $(CH_2)_2$), 2.51 (bs, 4H, $(CH_2)_2$), 2.58 (t, 2H, J = 5.84 Hz, N—CH_2), 3.96 (t, 2H, J = 5.84 Hz, CH_2), 4.65 (s, 1H, CH), 6.39 (d, 1H, J = 2.36 Hz, Ar—H), 6.45 (dd, 1H, J = 2.32 Hz, 6.16 Hz, Ar—H), 6.77 (d, 2H, J = 8.48 Hz, Ar—H), 6.93 (d, 1H, J = 8.48 Hz, Ar—H), 7.01 (d, 2H, J = 8.76 Hz, Ar—H). 7.48 (s, 2H, NH_2), 9.56 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.93 (CH_2), 25.56 (CH_2), 28.11 (CH_3), 38.58 (CH), 54.42 (CH_2), 57.40 (CH_2), 65.46 (CH_2), 77.86 (C), 78.02 (C), 101.90 (CH), 111.92 (CH), 113.95 (CH), 116.94 (C), 127.87 (CH), 129.83 (CH), 141.18 (C), 148.85 (C), 156.47 (C), 156.52 (C), 160.52 (C), 168.50 (C=O). HRMS: Found 467.2551; $C_{27}H_{35}N_2O_5$ requires 467.2546(M^+ + H).

4.1.4.14. 2-Amino-7-hydroxy-4-[4-(2-morpholin-4-ylethoxy)phenyl]-4H-chromeme-3-carboxylic methyl ester (42). Preparation as described for **39** above from 4-(2-morpholine-1-ylethoxy)benzaldehyde (**31**) (5 mmol, 1.176 g), methyl cyanoacetate methyl cyanoacetate (5 mmol, 495 mg) and resorcinol (5 mmol, 560 mg) using general method C. Yield 226 mg, 10.6% as a brown oil. IR υ_{max} (KBr) cm^{-1}: 3432.55, 3367.14 (NH_2), 1663.47 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.07 (t, 3H, J = 7.02 Hz, CH_3), 2.42 (s, 4H, $(CH_2)_2$), 2.62 (t, 2H, J = 5.52 Hz, CH_2), 3.54 (t, 4H, J = 4.52 Hz, CH_2), 3.96 (m, 4H, 2xCH_2), 4.72 (s, 1H, CH), 6.41 (d, 1H, J = 2.00 Hz, Ar—H), 6.46 (dd, 1H, J = 2.26 Hz, 6.04 Hz, Ar—H), 6.78 (d, 1H, J = 8.52 Hz, Ar—H), 6.93 (d, 2H, J = 8.04 Hz, Ar—H), 7.01 (d, 2H, J = 8.52 Hz, Ar—H), 7.60 (s(br), 2H, NH_2), 9.65 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.32 (CH_3), 38.11 (CH), 53.60 (CH_2), 57.04 (CH_2), 58.53 (CH_2), 65.13 (CH_2), 66.14 (CH_2), 76.81 (C), 101.99 (CH), 112.06 (CH), 114.03 (CH), 117.11 (C), 127.87 (CH), 129.76 (CH), 140.89 (C), 149.03 (C), 156.48 (C), 156.62 (C), 160.91 (C), 168.40 (C=O). HRMS: Found 427.1873; $C_{23}H_{27}N_2O_6$ requires 427.1869(M^+ +H).

4.1.4.15. 2-Amino-7-hydroxy-4-[4-(2-morpholin-4-ylethoxy)phenyl]-4H-chromeme-3-carboxylic ethyl ester (43). Preparation as described for **39** above following the general Method B from 4-(2-morpholine-1-ylethoxy)benzaldehyde (**31**) (5 mmol, 1.176 g), ethyl cyanoacetate (5 mmol, 565 mg) and resorcinol (5 mmol, 560 mg). Yield 24%, M.p. 144–146 °C, yellow powder. IR υ_{max} (KBr) cm^{-1}: 3425.00, 3285.02 (NH_2), 1674.22 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.07 (t, 3H, J = 7.02 Hz, CH_3), 2.42 (s, 4H, $(CH_2)_2$), 2.62 (t, 2H, J = 5.52 Hz, CH_2), 3.54 (t, 4H, J = 4.52 Hz, CH_2), 3.96 (m, 4H, CH_2), 4.72 (s, 1H, CH), 6.41 (d, 1H, J = 2.00 Hz, Ar—H), 6.46 (dd, 1H, J = 2.26 Hz, 6.04 Hz, Ar—H), 6.78 (d, 1H, J = 8.52 Hz, Ar—H), 6.93 (d, 2H, J = 8.04 Hz, Ar—H), 7.01 (d, 2H, J = 8.52 Hz, Ar—H), 7.60 (bs, 2H, NH_2), 9.65 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.32 (CH_3), 38.11 (CH), 53.60 (CH_2), 57.04 (CH_2), 58.53 (CH_2), 65.13 (CH_2), 66.14 (CH_2), 76.81 (C), 101.99 (CH), 112.06 (CH), 114.03 (CH), 117.11 (C), 127.87 (CH), 129.76 (CH), 140.89 (C), 149.03 (C), 156.48 (C), 156.62 (C), 160.91 (C), 168.40 (C=O). HRMS: Found 441.2044; $C_{24}H_{29}N_2O_6$ requires 441.2026(M^+ +H).

4.1.4.16. 2-Amino-7-hydroxy-4-[4-(2-morpholin-4-ylethoxy)phenyl]-4H-chromeme-3-carbonitrile (44). Preparation was as described for **39** above from 4-(2-morpholine-1-ylethoxy)benzaldehyde (**31**) (5 mmol, 1.176 g), malononitrile (5 mmol, 330 mg) and resorcinol (5 mmol, 560 mg) following the general method B. Yield 51%, M.p. 176 °C, brown powder. IR υ_{max} (KBr) cm^{-1}: 3400.0, 3333.3 (NH_2), 2187.7 (CN) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.36 (m, 4H, CH_2), 2.57 (t, 2H, J = 5.76 Hz, CH_2), 3.36 (m, 4H, CH_2), 3.95 (t, 2H, J = 5.76 Hz, CH_2), 4.29 (bs, 2H, NH_2), 4.47 (s, 1H, CH), 6.31 (d, 1H, J = 2.04 Hz, Ar—H), 6.39 (dd, 1H, J = 2.50 Hz, 6.04 Hz, Ar—H), 6.69 (d, 1H, J = 8.04 Hz, Ar—H), 6.77 (t, 3H, J = 8.28 Hz, Ar—H), 6.97 (d, 1H, J = 8.52 Hz, Ar—H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 37.8 (CH), 53.61 (CH_2), 56.04 (CH_2), 56.53 (C), 65.21 (CH_2), 66.16 (CH_2), 102.10 (CH), 112.31 (CH), 114.05 (C), 114.45 (CH), 120.73 (C), 128.41 (CH), 129.91 (CH), 138.54 (C), 148.76 (C), 156.96 (C), 157.19 (C), 160.08 (C=O). HRMS: Found 394.1760; $C_{22}H_{24}N_3O_4$ requires 394.1767(M^+ +H).

4.2. Stability study for compounds 38 and 40

Analytical high-performance liquid chromatography (HPLC) stability studies were performed using a Symmetry® column (C_{18}, 5 μm, 4.6 × 150 mm), a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump and a Waters 717plus Autosampler. Samples were detected at wavelength of 254 nm. All samples were analysed using acetonitrile (80%): water (20%) as the mobile phase over 10 min and a flow rate of 1 mL/min. Stock solutions are prepared by dissolving 5 mg of compound **38** and **40** in 10 mL of mobile phase. Phosphate buffers at the desired pH values (4, 7.4, and 9) were prepared in accordance with the British Pharmacopoeia monograph 2016. 30 μL of stock solution was diluted with 1 mL of appropriate buffer, shaken and injected immediately. Samples were withdrawn and analysed at time intervals of t = 0 min, 5 min, 30 min, 60 min, 90 min, 120 min and 21 h.

4.3. Plasma stability study for compounds 38 and 40

Analytical high-performance liquid chromatography (HPLC) stability studies for plasma samples were performed using a Waters HPLC 2965–2487 system with a Hypersil Gold column; l = 0.15 m, i.d. = 4.6 mm, C18, 5 μm with flow rate: 1.0 mL/min, sample temperature: 25 °C, column temperature: 25 °C; mobile phase H_2O:ACN:MeOH. *Method 1*: (10% DMSO): PBS (1.6 mL) added to blood plasma (2.0 mL) and pre-warmed to 37 °C in a water bath. To this solution, 400 μL of 1.0 mg/mL stock of compounds **38** and **41** was added. Immediately a 100 μL aliquot was withdrawn and added to 900 μL acetonitrile giving a final injection concentration of 0.01 mg/mL. The samples were then centrifuged at 5000 rpm for 10 min and filtered through a 0.2 μm filter and injected according to the HPLC conditions listed above. Further samples were taken in the same manner every 15 min for the first hour and hourly thereafter up to 3 h. A final sample was taken after 24 h.

4.4. Biochemical evaluation of activity

4.4.1. Antiproliferative studies

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 cancer cell line MCF-7 was cultured in Eagles minimum essential medium in air supplemented with 5% CO_2 atmosphere with 10% fetal calf serum, 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin The medium was supplemented with 1% non-essential amino acids.

Cells were trypsinised and seeded at a density of 2.5 × 10^4 cells/mL into a 96-well plate and incubated at 37 °C, in air supplemented with 5% CO_2 atmosphere 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). The culture medium was then removed and the cells washed with 100 μL phosphate buffer saline (PBS) and 50 μL of 1 mg/mL MTT solution was added. Cells were incubated for 2 h in darkness at 37 °C. At this point solubilisation was begun through the addition of 200 μL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough colour diffusion before reading the absorbance at 595 nm. The absorbance value of control cells (vehicle treated) was set to 100% cell viability and from this graphs of absorbance versus cell density per well were prepared to assess cell viability and from these, graphs of percentage cell viability versus concentration of subject compound were drawn.

4.4.2. Lactate dehydrogenase assay for measurement of cytotoxicity

Cytotoxicity was determined using the CytoTox 96 nonradioactive cytotoxicity assay (Promega) following the manufacturer's protocol. In this assay, the release of cytoplasmic lactate dehydrogenase (LDH) is used as a measure of cell lysis. MCF-7 cells were seeded at a density of 1 × 10^4 cells/well in a 96-well plate and incubated for 24 h. The cells were then dosed with 2 μL volumes of the test compounds, over the concentration range 1 nM–50 μM. After dosing, the plates are incubated for 72 h. Control wells contained the equivalent volume of the vehicle, ethanol (1% v/v). After incubation, 30 μL of medium is removed from each well and transferred to a fresh 96-well plate. To this, 30 μL of substrate mix from the Promega cytotoxicity assay kit was added. The plate was left in darkness for 20 min at room temperature. Stop solution (30 μL) was added to the wells. The plates were read at a wavelength of 490 nm using an automated *Molecular Devices* micro-plate reader. A positive control of 100% lysis was determined for a set of untreated cells, which were lysed by the addition of lysis solution to the medium 45 min prior to harvesting. The percentage cell lysis was calculated for the treated cells relative to the control data. Assays where repeated in three experiments performed in triplicate (unless otherwise stated) and reported results represent the mean value ± standard error mean. The data was processed using PRISM(Version 4. Graphpad Software, Inc., 2236 Avenida de la Playa, La Jolla, CA 92037, USA).

4.4.3. Estrogen Receptor binding

ERα and ERβ fluorescence polarization based competitor assay kits were obtained from Panvera at Invitrogen Life Technologies, Invitrogen Corporation, 5791 Van Allen Way, PO Box 6482, Carlsbad, California 92008. 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 one hour 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 to 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. A vehicle control contained 1% (v/v) of ethanol; a negative control was used to determine the theoretical maximum polarization (50 µL of screening buffer and 50 µL of fluorescent estrogen/ER complex). The tubes were incubated in the dark at room temperature for 2 h and were mixed by shaking on a plate-shaker. Polarization values were measured on a Beacon single-tube fluorescent polarization instrument fitted with 485 nm excitation and 530 nm emission interference filters. For ERα and ERβ, graphs of anisotropy (mA) versus compound concentration were obtained for the determination of IC_{50} values.

4.5. Computational procedures

All compounds were drawn in Accelrys Draw v4.1[88] with absolute stereochemistry assigned for both R and S enantiomers. Enantiomers were protonated at pH7.4 with MarvinView and subsequently converted to 3D using Molconvert (version v15.6.29.0).[82] 10 conformers of each 3D structure were subsequently enumerated using OMEGA (version 2.5.1.4)[89,90].

To establish a reliable model of ERβ, as no X-ray crystal structure of human ERβ in an antagonist conformation currently exists, the sequence from Uniprot[91] entry Q92731 (ESR2_HUMAN) was downloaded and submitted to the iTASSER server.[92,93] PDB entry 1R5K was specified as a template to guide the structure prediction. The resulting model was submitted to the Yale Protein Multichain Morphing Server[79] to transform the atomic co-ordinates to those of PDB entry 1R5K. Finally, 3Drefine[80,94,95] was used to refine our model of ERβ. PDB ID 2OUZ was selected for modeling of the compound series in ERα as the co-crystallised ligand (Lasofoxifene) was structurally similar to compound **35**. The iTASSER server was also used to model any missing residues with 2OUZ used to guide the structural prediction and again 3Drefine[80,94,95] was used to refine our model of ERα. All conformers were docked using FRED (OEDocking version 3.0.1)[81,96] into the both Ligand Binding Pockets (LBPs). Energy minimisation of the top scoring docked pose for all enantiomers was performed using the Amber12:EHT force field (gradient of 0.05 kcal/mol) as implemented in MOE (version 2012.10).[97]

4.6. X-Ray crystallography

Crystals of compound **22** were obtained by slow crystallisation from a dilute solution of methanol over a period of 4–8 weeks. The data for the crystal structures **22** were collected on a Rigaku Saturn 724 CCD Diffractometer. A suitable crystal from each crystal compound was selected and mounted using inert oil on a 0.3 mm diameter glass fiber tip or loop and placed on the goniometer head in a 150 K N_2 gas stream. Each data set was collected using Crystalclear-SM 1.4.0 software. Data integration, reduction and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution and refinement were obtained using Bruker Shelxtl Ver. 6.14 software.[98] Each structure was solved with Direct Methods using the SHELXTL program and refined against IF2I with the program XL from SHELX-97 using all data. Non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed into geometrically calculated positions and refined using a riding model.

Crystal data for **22** are as follows: Empirical formula: $C_{20}H_{21}NO_5$, M = 355.38, T = 108(2) K, Wavelength 0.71075 Å, Crystal system: Monoclinic, Space group: P 2/c, Unit cell dimensions: a = 16.982(7) Å, b = 5.340(2) Å, c = 20.683(9) Å, α = 90°. β = 91.189(6)°, γ = 90°. Volume: 1875.2(13) $Å^3$ Z: 4, Density (calculated): 1.259 Mg/m^3, Absorption coefficient: 0.091 mm^{-1}; F(0 0 0): 752; Crystal size: 0.240 × 0.210 × 0.180 mm^3; Theta range for data collection: 2.328 to 24.999°. Index ranges $-20 \le h \le 20$, $-5 \le k \le 6$, $-23 \le l \le 24$; Reflections collected: 13677, Independent reflections: 3240 [R (int) = 0.0641]; Completeness to theta = 25.242°'; 95.2%; Absorption correction: Semi-empirical from equivalents, max. and min. transmission: 1.0000 and 0.6725, Refinement method: Full-matrix least-squares on F^2, Data/restraints / parameters: 3240 / 0 / 238, Goodness-of-fit on F^2 :1.173, Final R indices: [I > 2sigma(I)]: R1 = 0.0840, w*R*2 = 0.2055, R indices (all data): R1 = 0.1059, w*R*2 = 0.2203, Extinction coefficient: n/a, Largest diff. peak and hole: 0.216 and −0.253 e.$Å^{-3}$. CCDC deposition CCDC 1014637.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.115261.

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