

Cyanidin-3-O-Rutinoside Exhibits ER α preferential and SERM like Modulation

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Abstract

Cyanidin-3-*O*-rutinoside (C3R) is a dietary anthocyanin with reported phytoestrogenic and anticancer properties, but the structural determinants of its ER subtype selectivity and SERM-like behaviour remain unclear. C3R exerted cytotoxic effects on ER-positive cell lines MCF-7 and T47D over triple-negative MDA-MB468, suggestive of estrogen receptor-associated mechanisms. The lower IC₅₀ value in T47D cells (predominant ER α , negligible ER β) compared to MCF-7 (established ER α / β ratio) suggests a possible preference for ER α -associated responses. C3R enhanced ER α expression and extracellular estrogen at 3h but downregulated ER α with drastically elevated extracellular estrogen at 24h in breast cancer cells. In osteosarcoma MG63 cells, ER α expression persisted at both time points. To understand this time- and cell-type-dependent structural basis of these observations, pharmacophore analysis, molecular docking, and dynamics simulations were performed. Pharmacophore modeling revealed C3R shares key features with both ER α agonists and antagonists: its flavonoid core overlays estradiol's aromatic scaffold with phenolic hydroxyl aligning to agonist-critical hydrogen-bond donors, while aromatic side chain and carbohydrate moieties map to antagonist-associated pharmacophores. C3R ranked among top-scoring ligands in both pharmacophore spaces, surpassing estradiol in agonist modelling and achieving highest antagonist score. Docking studies suggested stable binding of C3R to ER α agonist (L387, H524) and antagonist (D351, E353, R394) conformations, with poor ER β compatibility. Molecular dynamics simulations supported stable association (binding free energy – 93.7 kcal/mol), with ER α predominantly maintaining antagonist conformation while showing partial transition toward agonist state. These data provide structural explanation for C3R's dual ER α modulation observed in vitro and exhibits characteristics consistent with SERM-like activity.

1. Introduction

Cyanidin-3-*O*-rutinoside (C3R) is a widely distributed dietary anthocyanin present in berries and mulberry, known for its antioxidant, anticancer, and phytoestrogenic properties. Anthocyanins, particularly those enriched in blackcurrant such as C3R, have been reported to exert phytoestrogenic effects through interactions with estrogen receptors. Previous studies have reported that anthocyanins present in blackcurrant can stimulate the expression of estrogen-responsive genes through the activation of both ER α and ER β signaling pathways [1]. Furthermore, cyanidin-derived compounds have been shown to elicit estrogen-like responses in vitro, including activation of estrogen response elements and modulation of ER-mediated transcription [2]. Collectively, these findings highlight C3R as a bioactive phytoestrogen capable of modulating estrogen signaling pathways.

Estrogen receptor alpha (ER α) is a ligand-dependent transcription factor whose agonist or antagonist activity is determined by ligand-specific conformational changes in the ligand-binding domain, particularly the positioning of helix-12 that controls coactivator and corepressor recruitment [3]. Classical ER agonists share conserved pharmacophore features, including a phenolic ring that acts as a hydrogen-bond donor/acceptor analogous to estradiol's A-ring, and a hydrophobic core with a distal hydroxyl group mimicking the D-ring, which collectively stabilise the active conformation. By contrast,

antagonists typically retain the phenolic ring but incorporate bulky side chains that displace helix-12 and favour corepressor binding, thereby enforcing transcriptional repression [4].

Previous results shows that anthocyanins have been shown to exhibit phytoestrogenic activity via ER α -mediated signaling, with subsequent studies demonstrating partial involvement of ER β , indicating that these compounds can function as modulators of both estrogen receptor subtypes; this is consistent with the classical model of ER action, wherein ligand-dependent conformational changes, particularly the positioning of helix 12 (H12), regulate the recruitment of co-activators or co-repressors [1, 5].

The classical model of ER α action is regulated through ligand-dependent conformational changes in ER α , particularly the positioning of helix 12 (H12), which regulates whether co-activators or co-repressors has to be recruited [6]. 17 β -estradiol (E2) and other agonists are shown to stabilize H12 conformations that favours the recruitment and binding of co-activators, while antagonists displace H12, facilitating co-repressor recruitment, thus resulting in trans repression and premature termination of transcription [7].

The ligands acting as an ER α agonist display distinct pharmacophore features, such as a phenolic ring that contributes as a hydrogen bond donor or acceptor, par to estradiol's A-ring. The presence of the hydrophobic core and a hydroxyl group at the distal end, similar to the D-ring of 17- β -estradiol, stabilize the active conformation of the receptor [8]. In contrast, ER α antagonists ideally retain the phenolic ring but may incorporate a sterically hindering bulky side chain, which alters the positioning helix-12, facilitating co-repressor binding and favouring antagonism [7]. However, several known SERMs can adopt both agonistic and antagonistic conformation in a tissue-specific manner. Such tissue-specific effects are reported for Tamoxifen, which acts as an agonist in bone and endometrium at the same time antagonist in the breast [9, 10].

In silico-based studies have reported anthocyanins to act as a SERM and modulate both ERs, with independent studies reporting preference for ER β [5]. However, the structural determinants that enable C3R to engage ER α in both agonist-like and antagonist-like modes, and its selectivity relative to ER β , have not been defined. In this study, molecular docking and pharmacophore modelling were employed to delineate the pharmacophore features of C3R relative to known ER agonists and antagonists and characterise its binding modes within agonist and antagonist ER α ligand-binding domains. further we assessed the contribution of key ER α residues to C3R binding using mutagenesis-guided docking. These in silico analyses provide a structural framework for understanding C3R's SERM-like dual ER α engagement.

2. Materials and methods

2.1. In Vitro culture and treatment

The ER α -positive, human female breast adenocarcinoma cell line MCF-7 (RRID: CVCL_0031), NCCS ECACC Cat# 86012803; passage no: 22, T-47D (RRID: CVCL_0553), ATCC Cat# HTB-133, passage no: 47,

MDA-MB-468 (RRID: CVCL_0419) NCCS; passage no:36, osteosarcoma cell line MG-63 (RRID: CVCL_0426), ATCC Cat# CRL-1427, passage no: 19 were used for the current study. MCF-7, MDA MB-468 and MG-63 Cells were grown in DMEM complete media containing FBS (10%), 2 mM L-Glutamic acid 5-amide and 1% antibiotic and antimycotic. T47D cells was cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine, and maintained at 37°C in a humidified incubator with 5% CO₂. To evaluate ER α -expression, the cells were treated with different concentrations of Cyanidin 3-O-rutinoside chloride (Cat # 18719-76-1; $\geq$ 98%/Sigma) at two time points, while 17 β -Estradiol (E2) (Cat # 50-28-2; $\geq$ 98%/Sigma) was used as a positive control.

2.2. Yeast estrogen reporter assay (YES Assay)

Agonist activity of Cyanidin 3-O-rutinoside chloride was evaluated using the XenoScreen XL YES Assay Kit (Xenometrix, AG) following manufacturers protocol. The YES assay involves a recombinant *Saccharomyces cerevisiae* BJ1991 (YES strain) with human ER α gene incorporated into its genomic DNA along with a plasmid containing estrogen-responsive elements (ERE) upstream of a β -galactosidase (lacZ) reporter gene. The interaction of an agonistic-ligand to the recombinant-ER α receptor activates the receptor, inducing transcription of the lacZ gene. The expressed β -galactosidase enzyme cleaves chlorophenol red- β -D-galactopyranoside (CPRG, #59767), to a pink-coloured product. Optical density of the pink colour was measured spectrophotometrically at 570 nm, is proportional to β -galactosidase activity, providing a quantitative measure of estrogenic agonism [11].

2.3. MTT-assay for Cytotoxicity assessment

Cytotoxicity of C3R was evaluated using MTT assay [12] in estrogen receptor positive cell lines (MCF-7 and T47D) and the estrogen receptor negative cell line (MDA-MB-468). Briefly, cells were seeded at a density of 4,000 cells per well (200 μ L/well) in a 96-well plate (P21024, Abdos) and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂. Cells were pre-treated with Cyanidin 3-O-rutinoside (C3R), dissolved in DMSO, at concentrations ranging from 10 to 200 μ M, with four technical replicates, ensuring that the final DMSO concentration did not exceed 0.01%. Following treatment, cells were incubated for 24 hours under standard conditions. A final concentration of 0.01% DMSO was used as the vehicle control.

2.4. ELISA

Estrogen levels in conditioned culture media were quantified using a Human Estrogen ELISA Kit (Cat. No. ELK8408; ELK Biotechnology) according to the manufacturer's instructions. Cells were treated with cyanidin-3-O-rutinoside (C3R) and 17 β -estradiol (E2) for 3 or 24 h, followed by collection of the conditioned media and centrifugation at 1,000 $\times$ g for 20 min. The clarified supernatants were collected and stored at -20°C or -80°C until analysis. Cells were maintained in phenol free-DMEM medium supplemented with 10% charcoal stripped fetal bovine serum to minimize interference from endogenous steroid hormones. Estrogen concentrations were determined by competitive ELISA, with absorbance measured at 450 nm and concentrations calculated from the kit provided standard curve. All samples were analysed in technical triplicates and expressed relative to the corresponding untreated controls.

2.5. Immunofluorescence and Confocal microscopy

Cells were cultured on poly-L-lysine coated coverslips and allowed to adhere overnight. Following treatment with C3R and 17 β -estradiol (E2) for 3 and 24 hours, cells were fixed with 4% paraformaldehyde and permeabilized using 0.01% Triton X-100. Non-specific binding was blocked with 5% BSA. Cells were then incubated overnight with primary antibodies against estrogen receptor α (Abcam, Cat# ab32063, RRID: AB_732249). Subsequently, Anti-Rabbit Alexa Fluor 488 (Abcam, Cat# ab150077, RRID: AB_2630356) was applied as the secondary antibody. Coverslips were mounted into microscopic glass slides using DABCO/PI mounting medium, and cells were visualized under confocal microscope [13].

2.6. Receptor and ligand preparation for in silico Analysis

Agonist-bound ligand-binding domain structures of ER α and ER β were obtained from the Protein Data Bank (PDB IDs: 1ERE for ER α and 1QKM for ER β) [14], and an antagonist-bound ER α structure was taken from PDB 6CHZ [15]. Missing residues in experimental structures were modelled using Swiss-Model [16], and the wild-type antagonist conformation of ER α was similarly rebuilt from the mutant 6CHZ structure. The molecular structure of C3R was retrieved from PubChem (ID 441674).

2.7. Docking protocol

Molecular docking was performed using the GPU-accelerated version of AutoDock4 [17]. Guided docking was conducted by centring the docking grid on the ligand-binding cavity of each receptor structure, and 500 ligand conformations were generated per calculation. The complex with the lowest predicted binding free energy (ΔG) was selected as the representative C3R–ER complex. Cavity volumes and ligand-binding pockets were analysed using ChimeraX with the pyKVFinder algorithm [18]. ER ligand-binding residues were identified based on information from the InterPro database.

To evaluate the contribution of individual residues to binding, selected ER α ligand-binding domain residues with high interaction contributions were mutated in silico using Swiss-Model¹⁶, prioritising mutations with high predicted pathogenicity scores from Alpha-missense [19]. C3R was redocked into each mutant structure with the same protocol, and changes in binding free energy and predicted inhibition constant (K_i) were calculated using Eq. (1):

$$K_i = e^{\Delta G/RT}$$

1

Where K_i is the theoretical binding affinity, ΔG is the binding free energy obtained from molecular docking calculations, R is the gas constant and T is temperature, set to 298K in this work.

2.8. Pharmacophore modelling and virtual screening

Pharmacophore modelling and virtual screening were performed using PharmacoNet [20]. Pharmacophore models were generated from agonist-bound ER α and ER β structures and antagonist-

bound ER α structures. A library of ER ligands with experimentally characterised agonist or antagonist activity was screened against these models. The PharmacoNet score quantified the extent of fit between ligand conformations and each pharmacophore model, and was used to infer agonist or antagonist-like properties. C3R conformations were mapped onto the pharmacophore models and visualised in ChimeraX [18].

2.9. Molecular Dynamics

The ER α -C3R complex was further subject to Molecular Dynamics simulations. The protein as well as the ligand was described using Gromos54a7 force field [21]. Force field parameters for the ligand were generated using Automated topology builder tool [22]. The system was solvated in a cubic box with 1.0nm edge distance, with TIP3P solvent model. The simulation system was neutralized using 0.15M NaCl. The constructed system was subject to energy minimization using steepest descent method, minimized system subject to equilibration in NVT and NPT ensembles with positional restraints. In NVT ensemble, equilibration was performed for 2ns with 1fs time step. Constant temperature was maintained at 310K using V-rescale thermostat. In NPT ensemble, equilibration was performed for 3ns with 2fs time step. Constant temperature was maintained at 310K using V-rescale thermostat, constant pressure was maintained at 1bar using Parrinello-Rahman barostat. Following the equilibration, all the positional restraints were removed and production MD simulation was performed for 100ns using the above-mentioned parameters. The bonds involving hydrogens were constrained using LINCS method [23], electro statistic interactions were calculated using Particle Mesh Ewald algorithm [24], another simulation involving same preparation protocols and parameters was performed on the ER α -LBD in absence of any ligand. All the simulation preparation and production steps were performed using GROMACS 2020 [25].

2.10. Analysis of MD simulations:

The trajectory pre-processing steps were done to remove PBC-associated artefacts using gmx trjconv tool of GROMACS. To ascertain the stability of ligand binding, minimum distance was calculated between the ligand and the protein atoms using gmx mindist tool of GROMACS [25]. Ligand RMSD was calculated after least-square fit with ligand using gmx rms tool of GROMACS [25]. Binding free energy between the ligand and the protein in the simulation trajectory was calculated using g_mmpbsa tool [26]. For the MMPBSA calculations, dielectric constant was set to 1.0.

The solvent accessibility of functionally important residues was calculated and evaluated using gmx sasa tool of GROMACS [25, 27]. The average of absolute solvent accessible surface area was calculated from both the C3R bound and apo state simulations. Relative solvent accessibility was calculated as the ratio of absolute areas from the simulation and the absolute solvent accessible surface area of the given residue 'x' in the tripeptide GxG. The tripeptide surface areas were obtained from the previous work [28]. The residues having relative accessibility ≥ 15 are considered to be accessible; those having accessibility < 15 are considered to be buried. The data on the functionally important residues was obtained from the InterPro database [29].

The conformational transition of the ER α in both the C3R-bound simulations were evaluated by RMSD calculations. C-alpha RMSD calculations were performed using two structures as references: ER α in agonist conformation (PDB:1ERE) and antagonist conformation (PDB:6CHZ). The agonist and antagonist conformations were also compared with the last frame of the ER α trajectory complexed with C3R (t=100ns) through structural superposition. From the superposition, C-alpha RMSD was calculated between the respective structures.

3. Results

3.1 Cyanidin 3- O rutinoside (C3R) Interacts with Estrogen Receptor Alpha (ER α) and can modulate the Endogenous Estrogen Secretion in vitro

Cyanidin-3-O-rutinoside (C3R), a known phytoestrogen, can act as an ER α agonist and exhibits differential expression of estrogen receptor alpha (ER α), in a time- and cell-type dependent manner

3.1.1. C3R exhibits differential ER-dependent cytotoxicity demonstrating ER α selectivity

Cytotoxicity analysis using the MTT assay demonstrated a receptor-dependent response, with significant effects observed in ER-positive breast cancer cell lines (MCF-7 and T47D), while no comparable activity was detected in the ER-negative cell line (MDA-MB-468) (shown in Fig. 1a). Notably, the significantly lower IC₅₀ value of C3R in T47D cells (with the higher ER α and barely detectable ER β expression profile) compare to MCF-7 cells (with significant ER α / β) indicates a stronger preference for ER α .

3.1.2. C3R demonstrates concentration-dependent ER α agonism in YES assay

The ER α -specific interaction of C3R was further validated using the yeast estrogen screen (YES) assay). The yeast estrogen screen (YES) assay confirmed the ER α -binding capacity of C3R, demonstrating concentration-dependent agonistic activity (Fig. 1b) C3R induced β -galactosidase expression in a dose-dependent manner, validating direct interaction with recombinant human ER α . While the agonistic response was lower in magnitude compared to 17 β -estradiol (E2), the positive dose-response relationship establishes C3R as a functional ER α ligand capable of receptor activation.

3.1.3. Differential ER α expression and localization reveal Time- and Cell Dependent agonist-antagonist transition

In ER-positive breast cancer cell lines (MCF-7 and T47D), C3R predominantly acted as an agonist at 4 h and antagonist, at 24 h, as evidenced by time-dependent differential ER α protein expression, and cytoplasm to nuclear translocation (Fig. 2a-2h). In contrast, in the osteosarcoma cell line, C3R displayed prolonged agonistic activity, as evidenced by enhanced ER α expression relative to control at all the time points (Fig. 2i-2l)

In, MCF7, ER β expression was absent at 4 h, but enhanced at 24 h corresponding to ER α declined expression. However, the antagonistic effect of C3R on ER α appears to be independent of ER β modulation. This is particularly evident in T47D cells, which are characterized by high ER α expression and minimal ER β levels. In this cellular context, C3R treatment resulted in a marked reduction in ER α expression, at 24 h in T47D without any corresponding increase in ER β levels (Supplementary Fig. 1).

The absence of ER β upregulation alongside the observed decrease in ER α in T47D indicates that the antagonistic activity of C3R is not mediated through a compensatory ER β -dependent mechanism, but rather reflects a direct inhibitory effect on ER α .

Importantly, when considered alongside the agonistic effects observed in osteosarcoma cells, this ER α -specific modulation in breast cancer cells underscores the cell-dependent and the time-dependent nature of C3R activity. Such differential modulation of ER α across distinct cellular environments highlights its ability to function as both an agonist and antagonist depending on tissue type, thereby supporting its classification as a selective estrogen receptor modulator (SERM).

3.1.4. Extracellular estrogen accumulation validates time-dependent SERM-like activity

ELISA-based quantification of estrogen in spent culture medium from MCF-7 cells revealed time-dependent modulation of extracellular estrogen dynamics by C3R treatment (Fig. 3). At 3 hours, C3R-treated cells exhibited significantly elevated extracellular estrogen levels compared to untreated controls, exceeding even E2-treated samples, which showed the lowest extracellular estrogen reflecting efficient receptor mediated estrogen clearance from the medium, characteristic of classical agonist activity. The level of estrogen observed at 3h for C3R is drastically lower than that observed at 24 h, suggesting partial agonism that shift to full antagonism at 24 h. By 24 hours, C3R treatment resulted in significantly elevated extracellular estrogen accumulation, representing the highest levels observed across all conditions and time points. This pronounced accumulation coincides with C3R-induced ER α downregulation observed by immunofluorescence, demonstrating that loss of functional receptor severely impairs cellular estrogen uptake capacity, leading to maximal extracellular estrogen retention. In contrast, both control and E2-treated cells maintained lower extracellular estrogen levels at 24 hours, indicating sustained receptor-mediated estrogen clearance. This biphasic pattern, characterized by impaired estrogen uptake despite agonist-like receptor binding at 3 hours, progressing to complete loss of uptake capacity due to receptor degradation at 24 hours, distinguishes C3R's unique SERM-like mechanism distinct from classical agonist/antagonist behaviour and validates its time-dependent dual modulation of ER α function.

The left panel shows the ELISA standard curve generated using known concentrations of the analyte, plotted as absorbance at 450 nm versus concentration (pg/ml). The fitted nonlinear regression curve (black line) was used to estimate concentrations of unknown samples. Middle and right panels show interpolated sample absorbance values for the different treatment groups—Control (red), 17- β Estradiol (E2) (green), and C3R (blue)—at 3 h and 24 h, respectively. Sample positions along the standard curve indicate their estimated analyte concentrations.

3.2. C3R–ER Interactions are Driven by Ligand-Binding Cavity Architecture and Receptor -Subtype Conformation

Due to considerable homology between ER α & ER β protein sequences (47.5% sequence identity, 71.1% similarity), and co-expression of both the receptors in the ER +ve cancer cells [30], C3R may bind to both

the ER variants. Molecular docking of C3R showed stable C3R binding to both agonist and antagonist conformations of ER α (Fig. 4a-d; Supplementary Table 2).

In the agonist conformation, C3R interacted with key estrogen-binding residues (L387, H524), whereas in the antagonist conformation, C3R engaged critical AF-2 domain residue D351 and helix-12 proximal residues (E353, R394). In contrast, though C3R bound stably with the antagonist conformation of ER β with binding energy of -10.4kcal/mol (Supplementary Table 2), the binding was highly unstable with the agonist conformation of ER β (Supplementary Table 2). To understand the root of this difference in binding energies, we mapped the cavities around the ligand binding site of respective ER α and ER β structures. Analysis of ligand binding cavities revealed that both ER α and ER β antagonist conformation have larger cavities (1228Å³ and 931Å³ respectively) compared to agonist conformations (ER α : 476Å³; ER β : 365Å³) (Supplementary Table 2). The C3R at the short helical region could adopt around both the agonist as well as antagonist conformations. This difference in the cavity volumes between agonist and antagonist conformations may explain the differences in C3R binding energies between both the conformations.

3.2.1. Mutagenesis-Guided Docking Underscores the Role of Key ER α Residues

To characterize the contribution of each of the C3R-binding residue towards the C3R-ER interactions, selected ER α mutants were modelled using in silico approaches, and C3R was docked to the mutant structures using the same protocols. Mutagenesis docking revealed that substitution of top-interacting ER α residues increased the binding free energy between C3R and ER α , shifting predicted K_i values from the nanomolar to micromolar range. Except M241D mutation, all the mutations resulted in increased binding free energy between the C3R and ER α (Supplementary table 3). These findings support the notion that C3R requires a specific ER α residue constellation to sustain dual agonist/antagonist binding.

3.2.2 C3R Shares Pharmacophore Features of both ER α Agonists and Antagonists and Exhibits Stable, MD-validated Binding to the ER α Ligand-Binding Domain

Pharmacophore modelling demonstrated that C3R shares pharmacophore features common for both ER α agonist and antagonist conformations. The flavonoid backbone of C3R precisely overlays with the aromatic ring positioning of estrogen in experimental agonist structures (Fig. 4e, f). The phenolic hydroxyl group aligns with the hydrogen-bond donor pharmacophore feature critical for agonist activity. On the other hand, C3R's aromatic side chain possess features common for the antagonists in the trainee set (Fig. 4g, h). The hydroxyl group positioning aligns with the hydrogen-bond donor pharmacophore features specific for agonists, while carbohydrate side chains align with hydrophobic pharmacophore features common for both agonistic and antagonistic conformations.

Similar trend was also observed by performing virtual screening against known ER α agonists and antagonists. Virtual Screening scores by PharmacoNet revealed that C3R has high virtual screening scores against both the agonist as well as antagonist ligands. In the agonist conformation, C3R was

ranked fourth, higher than the Estrogen, the endogenous ligand (Supplementary table 1). Similarly in the antagonist conformation, C3R was ranked in the top, demonstrating the potential of C3R to elicit both agonist as well as antagonist effects

Molecular Dynamics simulations show stable binding of C3R with ER α . We performed MD simulations to check if C3R binding with ER Ligand Binding Domain (LBD) is stable. Minimum distance calculations between the ER and C3R ligand showed that the distance remained at 0.15nm (< 0.5nm) throughout the course of the simulation (Supplementary Fig. 2), demonstrating that the C3R remained stably bound to the LBD of ER α . Similarly, C3R was found to have low RMSD ranging between 0.2-0.3nm, indicating minimal change from the initial conformation. Calculations of binding free energy through MMPBSA method further showed that C3R binding to the ER α -LBD is highly stable, with binding free energy of -93.7kcal/mol. Both the VdW and electrostatic contacts were found to have equally significant contribution to the stable binding m with energetic contribution of -68.1kcal/mol and - 92.3kcal/mol respectively (Table 1). Coactivator binding site of ER α has greater solvent accessibility upon C3R binding. We next calculated absolute solvent accessible surface area of the ligand binding residues and the residues of coactivator binding site of ER-LBD, to check if C3R binding has an impact on the solvent accessibility of these sites. We clearly observed that C3R binding resulted in globally reduced accessibility of ligand binding residues of ER α -LBD, compared to the apo state (Table 2). Particularly, the residue R394 which was accessible in the apo state (RSA of 20.1) became buried in the simulation in the holo state (C3R bound state, RSA of 13.2).

Table 1

Average of binding free energies and contribution from various interactions between ER-ligand binding domain and C3R ligand from the last 10ns of the MD simulation.

ΔG VdW kcal/mol)	ΔG Electrostatic (kcal/mol)	ΔG Solvation (kcal/mol)	ΔG total (kcal/mol)
-68.1	-92.3	+ 74.2	-93.7

But interestingly, though the ligand binding site residues have reduced accessibility upon C3R binding, certain residues of coactivator binding site are more accessible in the C3R bound state compared to the apo state. These include the residues L362, Q375 and E380. Though the overall state of accessibility remained largely unchanged (the solvent accessible residues remained accessible and vice versa), localized changes in the accessible surface area suggest possible transition towards agonist conformation, supporting the hypothesis of SERM role of C3R.

ER largely remains in antagonist conformation when bound to C3R. Docking calculations showed that C3R has a preference for binding to ER in agonist conformation. To further evaluate whether the ER α -C3R complex remained in antagonist conformation or if there is a switch towards agonist conformation, we extracted the structure of ER α -C3R complex at the end of the simulation (time=100ns) and compared the conformation with experimental agonist and antagonist structures (PDB IDs 4ERE and 6CHZ respectively). Superimposition of these structures and RMSD calculations revealed that ER α bound to

C3R remains in the antagonist conformation, as seen from the fact that agonist conformation has higher RMSD (13.5Å) compared to the antagonist conformation (7.5Å) (Fig. 5a).

Table 2

Absolute solvent accessible surface areas and Relative Solvent Accessibilities of functionally important sites of ER ligand binding domain. Calculations were performed from 100ns MD simulations of ER-C3R complex and apo state ER, represented as holo and apo respectively. Residues transitioning from accessible to buried states between both the simulations are represented in bold.

Residue	ASA-holo (Å ²)	ASA-apo (Å ²)	RSA-holo	RSA-apo
Ligand binding site				
L346	3.0	12.1	1.7	6.7
A351	54.2	72.2	48.0	63.9
R353	7.0	18.5	2.9	7.7
L387	2.2	11.3	1.2	6.3
R394	31.9	48.5	13.2	20.1
M522	58.4	50.4	28.6	24.7
H524	57.8	46.7	29.8	24.1
Activation function				
I358	9.7	11.9	5.3	6.5
L362	98.5	58.0	46.7	27.5
Q375	22.2	6.6	11.7	3.5
V376	1.9	4.8	1.2	3.0
E380	72.2	62.2	39.5	34.0
D538	80.0	104.4	53.0	69.1
E542	98.0	154.3	53.6	84.3

Similarly, we also calculated C-alpha RMSD on the MD trajectory frames, using agonist and antagonist structures as reference. We found that ERα-LBD remained in antagonist conformation, as seen from low RMSD (~ 3Å) compared to the RMSD with agonist structure as reference (4–5Å). However, some reduction in RMSD can also be seen for the calculation with agonist structure as reference, where the initial RMSD of approximately 5Å reduced to 4Å by the end of the simulation, suggesting the possibility of transition of ERα-LBD from antagonist to agonist conformation (Fig. 5b). These observations, coupled with the insights from solvent accessibility calculations further cements the idea of SERM role of C3R.

4. Discussion

4.1. C3R Exhibits ER α -Associated and Cell Type-Dependent Biological Activity

This study provides evidence that C3R may act as a context-dependent modulator of ER α -associated responses. While previous computational based studies have suggested preferential interactions of anthocyanins with ER β [5,31]. Our findings are consistent with a potential preference for ER α -associated activity under the experimental conditions examined. Previous studies have shown that anthocyanins, including cyanidin-3-glucoside and cyanidin-3-rutinoside, exhibit higher selectivity for ER β over ER α with phytoestrogenic activity mediated primarily through ER β in endothelial cells [32,33]. However, receptor selectivity can vary substantially depending on cellular context, tissue-specific coregulator expression, and ligand-induced conformational changes [34]. Estrogen receptor (ER) dependent cytotoxicity observed in ER positive breast cancer cell lines, but not in ER negative cells (Figure 1a), suggests a potential contribution of estrogen receptor-associated pathways to the biological effects of C3R. The enhanced sensitivity of T47D cells to C3R mediated cell killing, may indicate a greater contribution of ER α -associated responses. These observations suggest that the apparent receptor preference of C3R may be influenced by cellular context and receptor–ligand interactions rather than an inherent preference for a particular ER subtype. An important observation in this study is the time- and context-dependent response associated with C3R treatment. In ER-positive breast cancer cells, early increases in ER α expression were followed by reduced ER α expression at later time points (Figure 2). Together with changes in extracellular estrogen levels, these findings may be indicative of temporally distinct effects on ER α -associated cellular responses. However, additional studies examining downstream ER transcriptional activity and co-regulator recruitment would be required to define the precise nature of these responses.

Dual agonist- and antagonist-like activity is a characteristic feature of SERMs and has been associated with ligand-induced conformational changes in helix 12 (H12) of the ER α ligand-binding domain, which determines whether coactivators or corepressors are recruited [35,36]. In contrast, in osteosarcoma cells, C3R maintains or enhances ER α expression, indicative of agonist-like effects (Figure 2). The tissue-specific agonist or antagonist activity of SERMs are thought to depend, at least in part, on differential expression of coregulatory proteins such as SRC-1 (steroid receptor coactivator-1) and NCoR (nuclear receptor corepressor) across different cell types [37,38]. The observed changes in ER α expression and extracellular estrogen levels (Figure 3, suggest that C3R may influence ER α -associated biological processes in a time-dependent manner. Collectively, these findings suggest that C3R may exhibit context-dependent biological effects that are associated with ER α status and cellular background. Further, the differential expression observed in breast cancer and osteosarcoma models are also consistent with characteristics commonly attributed to SERM-like compounds and warrant further investigation in additional experimental systems. Similar tissue-selective behaviour has been demonstrated for clinical SERMs such as tamoxifen, which acts as an ER α antagonist in breast tissue but as an agonist in bone and uterus, with these differential effects determined by tissue-specific coregulator profiles [37]. These results provide a mechanistic framework for further structural and

translational studies aimed at exploiting C3R as a targeted modulator of ER α in hormone-dependent cancers.

4.2. Ligand- and Structure-Based Analyses Suggest ER α -Preferential and SERM-Like Properties of Cyanidin-3-O-Rutinoside

The pharmacophore and docking analyses suggest that C3R possesses structural features compatible with both agonist and antagonist ER α ligand-binding environments. These observations may provide a structural basis for the experimentally observed time- and context-dependent responses. Contrast the possibility of preferential interactions with ER α and provides structural evidence consistent with SERM-like properties. to previous studies reporting that the four major anthocyanins, including cyanidin-3-rutinoside, exhibit higher binding affinity toward ER β [5], our findings demonstrate a preferential and strong binding affinity of C3R toward ER α . This is supported by data on the overall expression of ER α in T47D cells (Figure 2), a cell line characterized by high ER α expression, where C3R treatment resulted in a pronounced interaction with ER α at two different time points. The combination of an estradiol-like flavonoid core, a phenolic hydroxyl group aligned to agonist hydrogen-bond donor features, and flexible carbohydrate moieties mapping to hydrophobic pharmacophore elements allows C3R to satisfy both agonist and antagonist pharmacophore constraints. Its high PharmacoNet scores in both models and superior ranking relative to estradiol in the agonist space underscore this dual compatibility. While previous studies on anthocyanins from *Hibiscus sabdariffa* demonstrate, through molecular docking and dynamics analyses, that these compounds effectively bind to Estrogen Receptor Alpha (ER α) and form stable ligand–receptor complexes [39], our findings reveal a distinct biphasic modulation of ER α by C3R, characterized by an early agonistic effect followed by a delayed antagonistic response under physiological conditions.

The similarity in the common pharmacophore features of endogenous ligand architecture with C3R explains its ability to stabilize the active ER α conformation. These findings suggest that C3R possesses structural features compatible with both agonist and antagonist ER α binding environments. This dual-interaction capacity enables adaptation to both ER α conformations. These in silico analysis supports our experimental observations of C3R's SERM-like action, where it stably interacts with both ER α conformations, while fail s to interact with ER β 's agonist conformation with a stable binding energy.

Docking results provide a structural explanation for this duality: in the agonist conformation, C3R recapitulates canonical estrogen interactions with L387 and H524, while in the antagonist conformation it engages D351, E353 and R394 near helix-12, a region that governs coactivator versus corepressor recruitment and SERM behaviour. The larger cavity of the ER α antagonist conformation permits helix-12 to adopt multiple positions around C3R, consistent with the ability of SERMs to stabilise intermediate conformations that can be either agonistic or antagonistic in a tissue-specific manner.

While anthocyanins from *Ribes nigrum* have been reported to exert predominantly ER α -mediated phytoestrogenic (agonistic) effects, our findings reveal that Cyanidin-3-O-rutinoside exhibits a structurally driven dual agonist to antagonist binding to ER α , indicating a more complex, SERM-like mode

of receptor modulation [1]. The incompatibility of C3R with ER β agonist conformations, combined with mutagenesis-driven loss of binding affinity upon perturbing key ER α residues, supports the possibility of preferential interactions with ER α and highlights distinct structural requirements for C3R binding. Together, these findings provide structural evidence consistent with SERM-like properties of C3R with intrinsic capacity to stabilise both agonist-like and antagonist-like ER α conformations. This dual ER α engagement is consistent with experimental observations of context-dependent ER α activation versus repression and offers a mechanistic framework for understanding how dietary anthocyanins may exhibit complex, temporally and tissue-specific estrogenic effects.

MD simulations supported stable association of C3R with ER α throughout the simulation period. While numerous ligand binding residues were buried in the MD simulations, we found that some residues of coactivator binding site are more accessible. This led us to hypothesize that C3R binding may permit limited transitions between agonist-like and antagonist-like ER α conformational states. RMSD calculations also showed putative transition towards agonistic conformation, since the RMSD using agonist structure as the reference reduced from 5Å at the start of the simulation to 4Å towards the end of the simulation, though the RMSD with antagonist structure as the reference remained at 3Å for all the simulation. We also compared the structure of the last frame of the simulation trajectory, with the experimental ER α LBD structures in antagonist and agonist conformations (PDB IDs 1ERE and 6CHZ). This comparison also showed closer orientation of ER α trajectory snapshot with respect to the antagonist conformation as opposed to agonist conformation, based on the orientation of the C-terminal helix that forms the activation function. However, the C-terminal helix has a slight tilt towards the agonist conformation, suggesting that possibility of partial transition of ER α towards an agonist-like conformation. These observations are consistent with previous studies on the SERM potential of Cyanidin-3-Rutenoside [1,5]. Although the receptor predominantly retained an antagonist-like conformation, modest changes in RMSD relative to the agonist structure suggest the possibility of limited conformational flexibility. The primary limitation of this work is the limited sampling by the MD simulations, which is restricted to 100ns. However, we are able to predict a stable association of C3R binding with the ER α -LBD and suggest limited conformational flexibility toward an agonist-like state.

Declarations

Author's contributions:

D.L.M. designed the study and the experimental framework, interpreted the results, and critically revised and finalized the manuscript. S.M.N. carried out the in vitro experiments, performed data analysis, and prepared the initial draft of the manuscript. R.M.R. conducted and interpreted the molecular docking and MD simulation studies. S.M. and H.K. assisted with the experimental work and contributed to data analysis. All data were generated internally. All authors reviewed and approved the final version of the manuscript and take responsibility for the integrity and accuracy of the work.

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Data Availability Statement:

The following PDB structures were utilized for the in silico analysis: 1ERE (<https://www.rcsb.org/structure/1ERE>), 1QKM (<https://www.rcsb.org/structure/1QKM>), 6CHZ (<https://www.rcsb.org/structure/6CHZ>). The PubChem compound CID: 441674 was used as a structure source for Cyanidin-3-O-Rutenoside for docking studies (<https://pubchem.ncbi.nlm.nih.gov/compound/441674>). The domain definitions and the functional residue boundaries used during the MD trajectory analysis was obtained from the Human Estrogen Receptor-1 entry on InterPro database (<https://www.ebi.ac.uk/interpro/protein/UniProt/Q9SAD4/>). Trajectory and analysis data from the Molecular Dynamics simulations will be made available by the corresponding authors upon request.

Conflicts of interest

Authors declare that there are no conflicts of interest associated with this work.

Ethics and Consent to Participate: Not applicable

Consent to Publish declarations: Not applicable

References

1. Nanashima, N., Horie, K., Tomisawa, T., Chiba, M., Nakano, M., Fujita, T., Maeda, H., Kitajima, M., Takamagi, S., Uchiyama, D., Watanabe, J., Nakamura, T., & Kato, Y. (2015). Phytoestrogenic activity of blackcurrant (*Ribes nigrum*) anthocyanins is mediated through estrogen receptor alpha. *Molecular nutrition & food research*, 59(12), 2419–2431. <https://doi.org/10.1002/mnfr.201500479>
2. Matsumura, A., Ghosh, A., Pope, G. S., & Darbre, P. D. (2005). Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *The Journal of steroid biochemistry and molecular biology*, 94(5), 431–443. <https://doi.org/10.1016/j.jsbmb.2004.12.041>
3. Lidia Nieto, Inga M. Tharun, Mark Balk, Hans Wienk, Rolf Boelens, Christian Ottmann, Lech-Gustav Milroy, and Luc Brunsveld. Estrogen Receptor Folding Modulates cSrc Kinase SH2 Interaction via a Helical Binding Mode. *ACS Chemical Biology* 2015 10 (11), 2624-2632 DOI: 10.1021/acscchembio.5b00568
4. Lee S, Barron MG (2017) Structure-Based Understanding of Binding Affinity and Mode of Estrogen Receptor α Agonists and Antagonists. *PLoS ONE* 12(1): e0169607. <https://doi.org/10.1371/journal.pone.0169607>
5. Nanashima, N., Horie, K., & Maeda, H. (2017). Phytoestrogenic Activity of Blackcurrant Anthocyanins Is Partially Mediated through Estrogen Receptor Beta. *Molecules* (Basel, Switzerland), 23(1), 74.

<https://doi.org/10.3390/molecules23010074>

6. Batista, M. R., & Martínez, L. (2013). Dynamics of nuclear receptor Helix-12 switch of transcription activation by modeling time-resolved fluorescence anisotropy decays. *Biophysical journal*, 105(7), 1670–1680. <https://doi.org/10.1016/j.bpj.2013.07.032>
7. Min J, Nwachukwu JC, Min CK, Njeri JW, Srinivasan S, Rangarajan ES, Nettles CC, Sanabria Guillen V, Ziegler Y, Yan S, Carlson KE, Hou Y, Kim SH, Novick S, Pascal BD, Houtman R, Griffin PR, Izard T, Katzenellenbogen BS, Katzenellenbogen JA, Nettles KW. Dual-mechanism estrogen receptor inhibitors. *Proc Natl Acad Sci U S A*. 2021 Aug 31;118(35):e2101657118. doi: 10.1073/pnas.2101657118. PMID: 34452998; PMCID: PMC8536401.
8. Ng, H. W., Zhang, W., Shu, M., Luo, H., Ge, W., Perkins, R., Tong, W., & Hong, H. (2014). Competitive molecular docking approach for predicting estrogen receptor subtype α agonists and antagonists. *BMC bioinformatics*, 15 Suppl 11(Suppl 11), S4. <https://doi.org/10.1186/1471-2105-15-S11-S4>.
9. Matsushima-Nishiwaki R, Yamada N, Hattori Y, Hosokawa Y, Tachi J, Hori T, Kozawa O. SERMs (selective estrogen receptor modulator), acting as estrogen receptor β agonists in hepatocellular carcinoma cells, inhibit the transforming growth factor- α -induced migration via specific inhibition of AKT signaling pathway. *PLoS One*. 2022 Jan 10;17(1):e0262485. doi: 10.1371/journal.pone.0262485. PMID: 35007301; PMCID: PMC8746762.
10. Chang, B.Y., Kim, S.A., Malla, B. et al. The Effect of Selective Estrogen Receptor Modulators (SERMs) on the Tamoxifen Resistant Breast Cancer Cells. *Toxicol Res*. 27, 85–93 (2011). <https://doi.org/10.5487/TR.2011.27.2.085>
11. Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem*. 1996 Mar 1;15(3):241–8.
12. Shaheer K, Prabhu BS, Ali HS, Lakshmanan-M D. Breast cancer cells are sensitized by piperine to radiotherapy through estrogen receptor- α mediated modulation of a key NHEJ repair protein- DNA-PK. *Phytomedicine Int J Phytother Phytopharm*. 2024 Jan; 122:155126.
13. Bouris P, Skandalis SS, Piperigkou Z, Afratis N, Karamanou K, Aletras AJ, et al. Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. *Matrix Biol J Int Soc Matrix Biol*. 2015 Apr; 43:42–60.
14. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A., & Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, 389(6652), 753–758. <https://doi.org/10.1038/39645>
15. Puyang, X., Furman, C., Zheng, G. Z., Wu, Z. J., Banka, D., Aithal, K., Agoulnik, S., Bolduc, D. M., Buonamici, S., Caleb, B., Das, S., Eckley, S., Fekkes, P., Hao, M. H., Hart, A., Houtman, R., Irwin, S., Joshi, J. J., Karr, C., Kim, A., ... Korpai, M. (2018). Discovery of Selective Estrogen Receptor Covalent Antagonists for the Treatment of ER α WT and ER α MUT Breast Cancer. *Cancer discovery*, 8(9), 1176–1193. <https://doi.org/10.1158/2159-8290.CD-17-1229>
16. Andrew Waterhouse, Martino Bertoni, Stefan Bienert, Gabriel Studer, Gerardo Tauriello, Rafal Gumienny, Florian T Heer, Tjaart A P de Beer, Christine Rempfer, Lorenza Bordoli, Rosalba Lepore,

Torsten Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, *Nucleic Acids Research*, Volume 46, Issue W1, 2 July 2018, Pages W296–W303, <https://doi.org/10.1093/nar/gky427>

17. Santos-Martins, D., Solis-Vasquez, L., Tillack, A. F., Sanner, M. F., Koch, A., & Forli, S. (2021). Accelerating AutoDock4 with GPUs and Gradient-Based Local Search. *Journal of chemical theory and computation*, 17(2), 1060–1073. <https://doi.org/10.1021/acs.jctc.0c01006>
18. Pettersen, E. F., Goddard, T. D., Huang, C. C., Meng, E. C., Couch, G. S., Croll, T. I., Morris, J. H., & Ferrin, T. E. (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein science: a publication of the Protein Society*, 30(1), 70–82. <https://doi.org/10.1002/pro.3943>
19. Cheng, J., Novati, G., Pan, J., Bycroft, C., Žemgulytė, A., Applebaum, T., Pritzel, A., Wong, L. H., Zielinski, M., Sargeant, T., Schneider, R. G., Senior, A. W., Jumper, J., Hassabis, D., Kohli, P., & Avsec, Ž. (2023). Accurate proteome-wide missense variant effect prediction with AlphaMissense. *Science (New York, N.Y.)*, 381(6664), eadg7492. <https://doi.org/10.1126/science.adg7492>
20. Seo, S., & Kim, W. Y. (2024). PharmacoNet: deep learning-guided pharmacophore modeling for ultra-large-scale virtual screening. *Chemical science*, 15(46), 19473–19487. <https://doi.org/10.1039/d4sc04854g>
21. Schmid, N., Eichenberger, A. P., Choutko, A., Riniker, S., Winger, M., Mark, A. E., & van Gunsteren, W. F. (2011). Definition and testing of the GROMOS force-field versions 54A7 and 54B7. *European biophysics journal: EBJ*, 40(7), 843–856. <https://doi.org/10.1007/s00249-011-0700-9>
22. Malde, A. K., Zuo, L., Breeze, M., Stroet, M., Poger, D., Nair, P. C., Oostenbrink, C., & Mark, A. E. (2011). An Automated Force Field Topology Builder (ATB) and Repository: Version 1.0. *Journal of chemical theory and computation*, 7(12), 4026–4037. <https://doi.org/10.1021/ct200196m>
23. Hess B. (2008). P-LINCS: A Parallel Linear Constraint Solver for Molecular Simulation. *Journal of chemical theory and computation*, 4(1), 116–122. <https://doi.org/10.1021/ct700200b>
24. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., & Pedersen, L. G. (1995). A smooth particle mesh Ewald method. *The Journal of chemical physics*, 103(19), 8577-8593.
25. Páll, S., Zhmurov, A., Bauer, P., Abraham, M., Lundborg, M., Gray, A., Hess, B., & Lindahl, E. (2020). Heterogeneous parallelization and acceleration of molecular dynamics simulations in GROMACS. *The Journal of chemical physics*, 153(13), 134110. <https://doi.org/10.1063/5.0018516>
26. Kumari, R., Kumar, R., Open Source Drug Discovery Consortium, & Lynn, A. (2014). g_mmpbsa--a GROMACS tool for high-throughput MM-PBSA calculations. *Journal of chemical information and modeling*, 54(7), 1951–1962. <https://doi.org/10.1021/ci500020m>
27. Eisenhaber, F., Lijnzaad, P., Argos, P., Sander, C. and Scharf, M. (1995), The double cubic lattice method: Efficient approaches to numerical integration of surface area and volume and to dot surface contouring of molecular assemblies. *J. Comput. Chem.*, 16: 273-284. <https://doi.org/10.1002/jcc.540160303>

28. Miller, S., Janin, J., Lesk, A. M., & Chothia, C. (1987). Interior and surface of monomeric proteins. *Journal of molecular biology*, 196(3), 641–656. [https://doi.org/10.1016/0022-2836\(87\)90038-6](https://doi.org/10.1016/0022-2836(87)90038-6)
29. Blum, M., Andreeva, A., Florentino, L. C., Chuguransky, S. R., Grego, T., Hobbs, E., Pinto, B. L., Orr, A., Paysan-Lafosse, T., Ponamareva, I., Salazar, G. A., Bordin, N., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D. H., Letunic, I., Llinares-López, F., Marchler-Bauer, A., ... Bateman, A. (2025). InterPro: the protein sequence classification resource in 2025. *Nucleic acids research*, 53(D1), D444–D456. <https://doi.org/10.1093/nar/gkae1082>
30. Oueslati, M., Bittaieb, I., Sassi, N., Jemaa, A. B., Gamoudi, A., Rahal, K., & Oueslati, R. (2017). ER α and ER β co-expression: An indicator of aggressive tumors and hormonal sensitivity. *Oncology letters*, 14(2), 1675–1682. <https://doi.org/10.3892/ol.2017.6314>
31. Jiang L, Li H, Kalari KR, Zhang C, Li Y, Li L, Wang L, Xue X, Cai Y, Jen J, Larson NB, Weinshilboum RM, Goetz MP, Wang L. Cyanidin-3-o-glucoside pharmacologically inhibits tumorigenesis via estrogen receptor β in melanoma mice. *FrontOncol*.2019;9:1110.
32. Huang W, Zhu Y, Li C, Sui Z, Min W. Effect of blueberry anthocyanins malvidin and glycosides on the antioxidant properties in endothelial cells. *Oxid Med Cell Longev*. 2016;2016:159180
33. Huang W, Zhu Y, Li C, Sui Z, Min W. Phytoestrogenic effects of blackcurrant anthocyanins increased endothelial nitric oxide synthase (eNOS) expression in human endothelial cells and ovariectomized rats. *Nutrients*. 2019;11(4):654
34. Maximov PY, Lee TM, Jordan VC. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. *Curr Clin Pharmacol*. 2013;8(2):135-55.
35. Fanning SW, Jeselsohn R, Dharmarajan V, Mayne CG, Karimi M, Buchwalter G, Houtman R, Toy W, Fowler CE, Han R, Lainé M, Carlson KE, Martin TA, Nowak KJ, Nwachukwu JC, Hosfield DJ, Chandarlapaty S, Katzenellenbogen JA, Greene GL, Shen Y, Brown M, Park BK, Rastinejad F. The SERM/SERD basedoxifene disrupts ESR1 helix 12 to overcome acquired hormone resistance in breast cancer cells. *eLife*. 2018;7:e37161.
36. McDougal DP, Pederick JL, Novick SJ, Jovcevski B, Warrender AK, Pascal BD, Griffin PR, Bruning JB. A ternary switch model governing ER α ligand binding domain conformation. *Nat Commun*. 2025 Nov 24;16(1):10363. doi: 10.1038/s41467-025-65323-9. PMID: 41285747; PMCID: PMC12644489.
37. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev*. 2004;25(1):45-71
38. Maximov PY, McDaniel RE, Jordan VC. Selective estrogen receptor modulators: tissue specificity and clinical implications. *Clin Interv Aging*. 2014;9:1437-52
39. Laskar, Y. B., Mazumder, P. B., & Talukdar, A. D. (2023). Hibiscus sabdariffa anthocyanins are potential modulators of estrogen receptor alpha activity with favourable toxicology: a computational analysis using molecular docking, ADME/Tox prediction, 2D/3D QSAR and molecular dynamics simulation. *Journal of biomolecular structure & dynamics*, 41(2), 611–633. <https://doi.org/10.1080/07391102.2021.2009914>

Figures

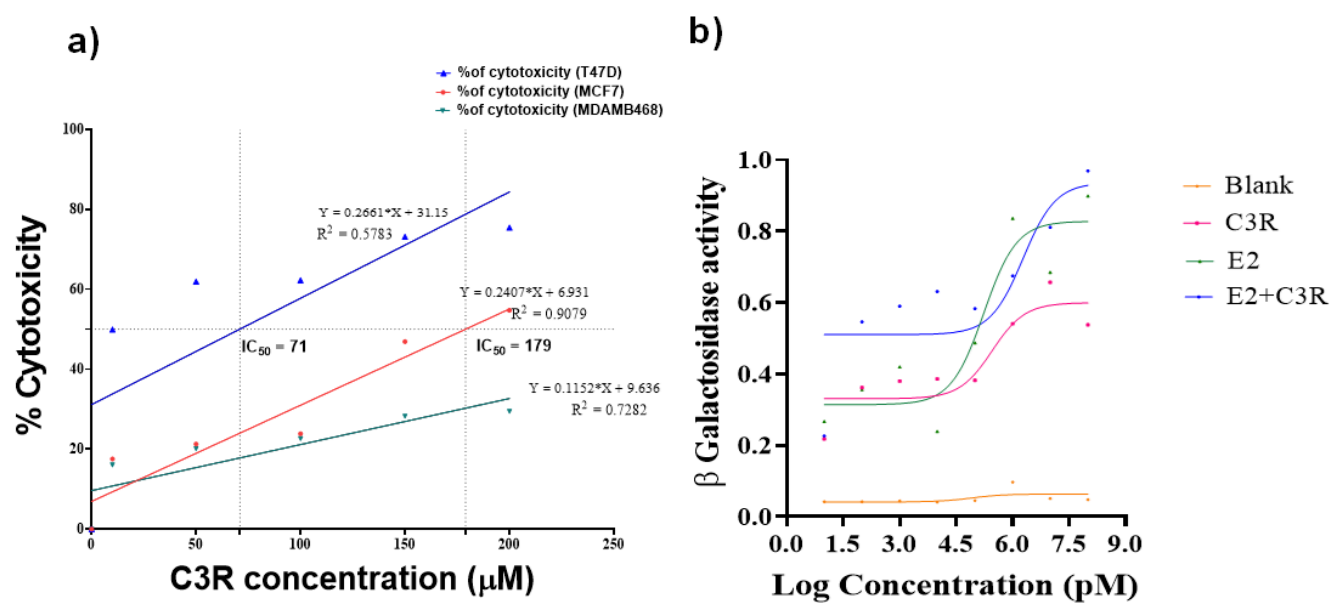


Figure 1

Cytotoxic and Estrogen Receptor α Agonistic effect of C3R in Breast Cancer cells

a) Effect of Cyanidin-3-O-rutinoside (C3R) on the viability of breast cancer cells. Cell viability of MCF-7, T47D, and MDA-MB-468 cells following 24 h treatment with increasing concentrations of C3R (10–200 μM) was assessed using the MTT assay. The IC_{50} values of all three cell lines were plotted in a single graph for comparative analysis. **b)** Agonistic activity of Cyanidin-3-O-rutinoside (C3R) toward human Estrogen Receptor α (ER α) evaluated using Yeast Estrogen Screen (YES) reporter assay. ER α -mediated transcriptional activation following C3R treatment was determined by measuring β -galactosidase (β -Gal) expression in recombinant yeast cells expressing human ER α .

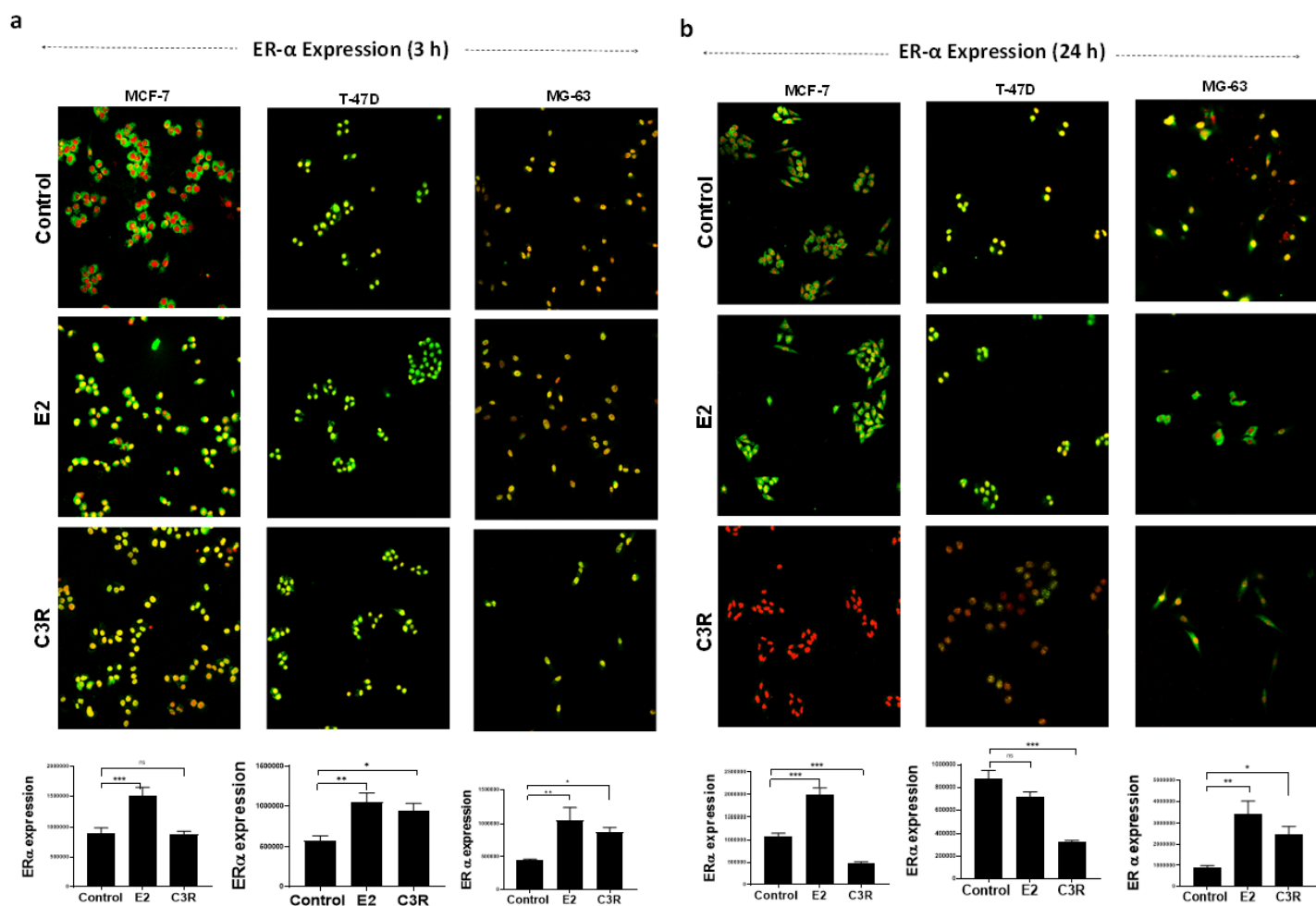


Figure 2

Estrogen Receptor α protein expression in Breast cancer cells and Osteosarcoma cells after C3R treatment

Confocal images showing the expression of ER α at **a)** 3 h and **b)** 24 h treatment with C3R in MCF-7, T-47D and MG-63 cells by 20X objective. Bar graph shows the total ER α expression in MCF-7, T-47D and MG-63 cells after 3 h and 24 h treatment with C3R (mean $\pm$ SEM, n = 3; ***P < 0.001, **P < 0.01, *P < 0.05, ns-non-significant).

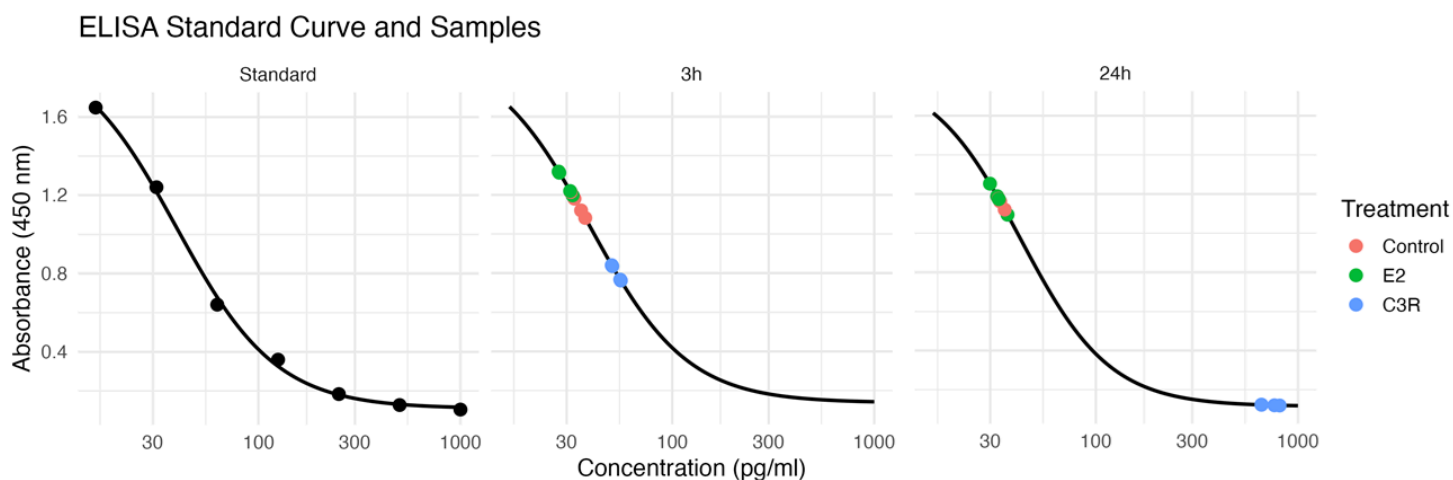


Figure 3

ELISA standard curve and sample interpolation for different treatment groups at 3 h and 24 h.

The left panel shows the ELISA standard curve generated using known concentrations of the analyte, plotted as absorbance at 450 nm versus concentration (pg/ml). The fitted nonlinear regression curve (black line) was used to estimate concentrations of unknown samples. Middle and right panels show interpolated sample absorbance values for the different treatment groups—Control (red), 17- β Estradiol (E2) (green), and C3R (blue)—at 3 h and 24 h, respectively. Sample positions along the standard curve indicate their estimated analyte concentrations.

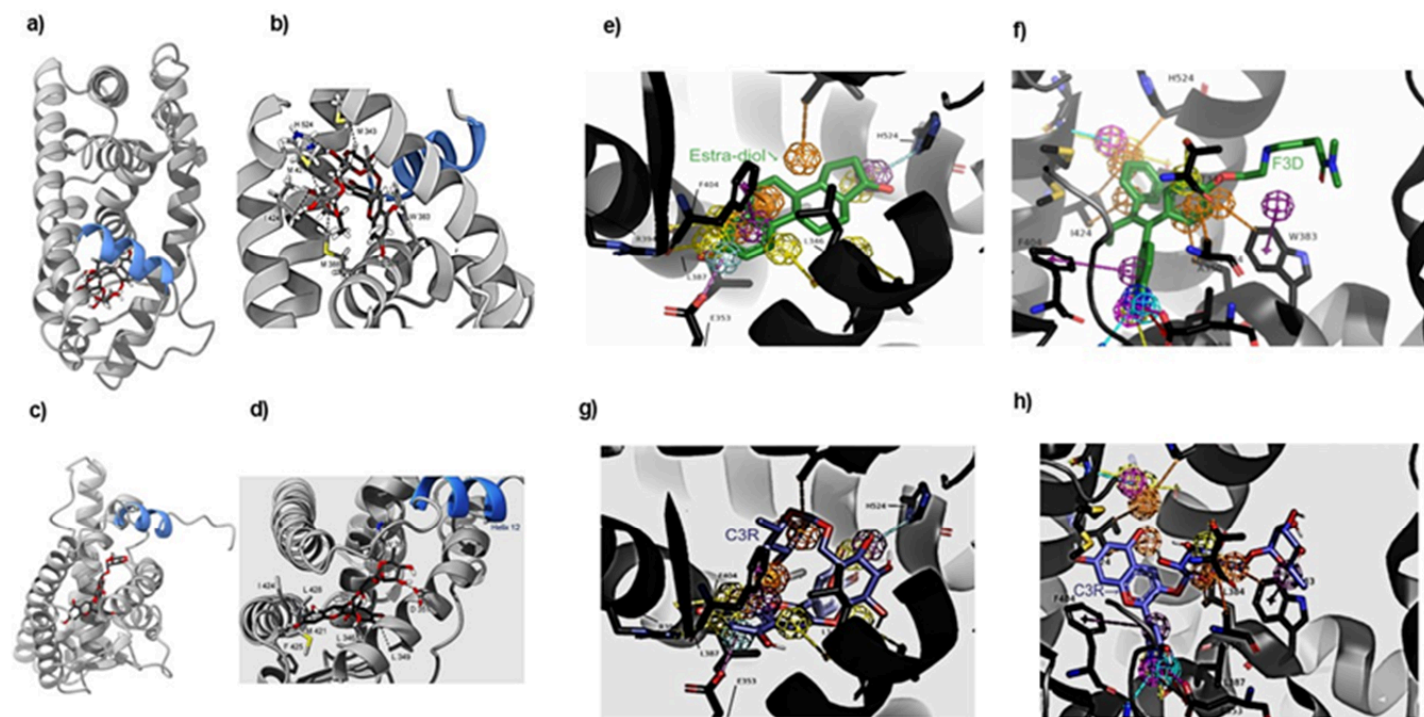


Figure 4

Molecular Docking and Pharmacophore Analysis of Cyanidin-3-O-rutinoside Interaction with Agonist and Antagonist Conformations of Estrogen Receptor α

a) C3R bound ER α (PDB : 1ERE) in agonistic conformation showing the orientation of H12 (blue) in closed conformation **b)** C3R showing H-bond interactions with the residues of LBD of 1ERE **c)** C3R bound ER α (PDB:1ERR) in antagonistic conformation showing the orientation of H12 (blue) in open conformation. **d)** C3R showing H-bond interactions with the residues of LBD of 1ERR **e)** Representation of pharmacophore model of ER ligand binding domains estradiol bound to ER α in Agonist conformation **f)** Pharmacophore features of ER antagonist H3B-9224 bound to the Antagonist conformation of ER α , **g)** Top-ranked C3R conformation superimposed over the agonist structure and the corresponding pharmacophore model, **h)** Top-ranked C3R conformation superimposed over the antagonist structure and the corresponding pharmacophore model. The pharmacophore features are coloured as follows: Orange: Hydrophobic; Magenta: Aromatic; Cyan: H-bond donor; Yellow; Halogen bond acceptors; Navy Blue: H-bond acceptor. The experimental structures are represented as green sticks, and C3R structure is represented as blue sticks.

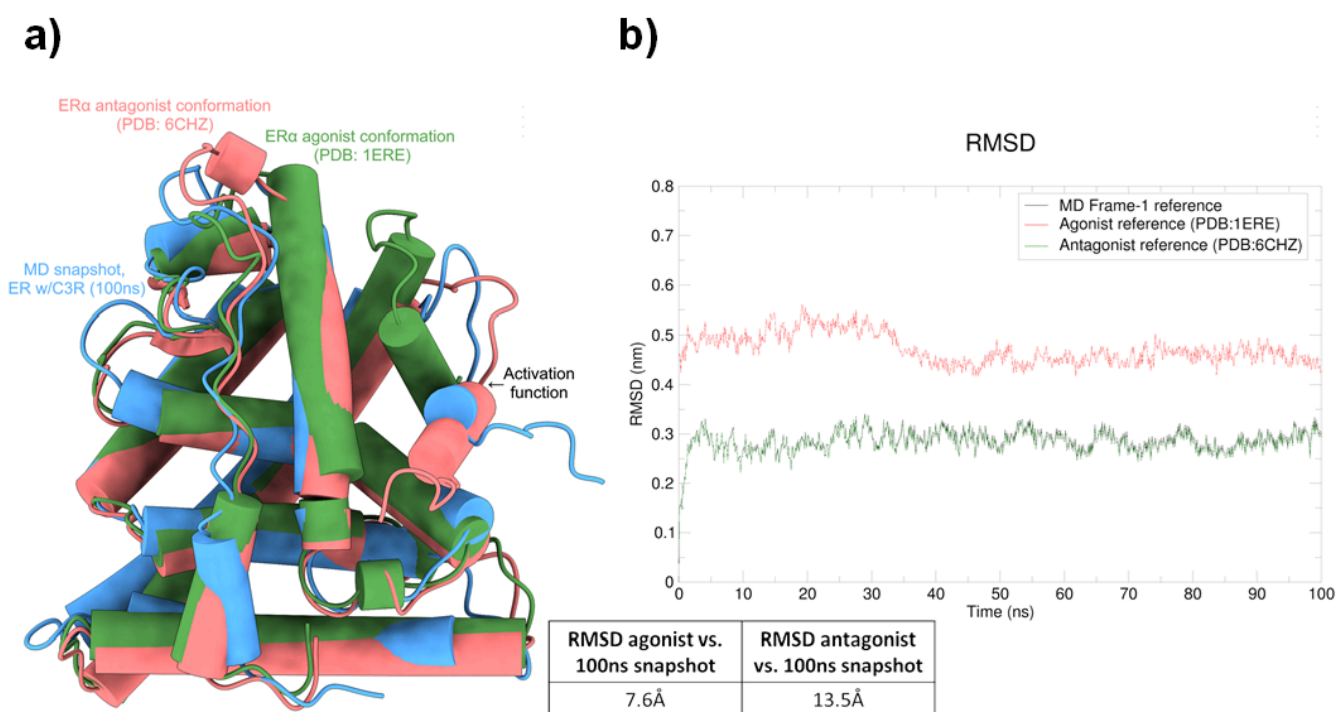


Figure 5

Molecular Dynamics Analysis of ERα and C3R interaction in Agonist and Antagonist Conformations

(a) Superimposition of final snapshots from the MD trajectory of ER-C3R complex with the experimental structure of ER-ligand binding domain in antagonist conformation (PDB: 6CHZ) and agonist conformation (PDB: 1ERE). The RMSD between the MD snapshot and respective structures are represented in the accompanying table. (b) C-alpha RMSD of ER-ligand binding domain from the ER-C3R complex MD trajectory, calculated with agonist structure and antagonist structures as reference.

Supplementary Files

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