Bioorganic & Medicinal Chemistry 17 (2009) 3847–3856

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of 3,4,6-triaryl-2-pyranones as a potential new class of anti-breast cancer agents

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ARTICLE INFO

Article history:
Received 25 February 2009
Revised 15 April 2009
Accepted 16 April 2009
Available online 22 April 2009

Keywords:
Triaryl-2-pyranones
Anti-proliferative
Apotosis
DNA fragmentation

ABSTRACT

A series of 3,4,6-triaryl-2-pyranones, new class of anti-breast cancer agents, have been synthesized as a structural variants of cyclic triphenylethylenes by replacing the fused benzene ring with pendant phenyl ring to mimic the phenolic **A** ring of estradiol. Nine of these newly synthesized pyranones exhibited significant anti-proliferative activity in both ER+ve and ER−ve breast cancer cell lines. Four active non-cytotoxic compounds **5c**, **5d**, **5g** and **5h** showed specific and selective cytotoxicity and two compounds **5d** and **5h** induced significant DNA fragmentation in both MCF-7 and MDA-MB-231 cell lines. Based on RBA studies, the molecules probably act in an ER-independent mechanism. The involved pathway was observed as caspase-dependant apoptosis in MCF-7 cells. However, the particular caspases involved and the possible cellular target through which this series of compounds mediate cell death are not known.

1. Introduction

Breast cancer is one of the most commonly diagnosed cancer, comprising 23% of all the female cancers and the second leading cause of cancer deaths in women worldwide today.[1,2] Several studies have established that estrogens are predominantly involved in the initiation and proliferation of breast cancer and much efforts are now being devoted to block estrogen formation and action.[3] The most widely used strategy to treat breast cancer is to disrupt estrogen mediated cancer proliferation through targeted antagonism of estrogen receptor by antiestrogens. Tamoxifen is the best estrogen receptor modulator capable of exhibiting breast cancer specific activity,[4] but still its therapeutic potential is limited in the sense that it is not effective against ER negative tumors as it being an ER agonist and mostly acts via ER receptor. Therefore, current need is to develop novel agents active against both estrogen dependent and independent breast cancers.

Since the development of triaryl template-based tamoxifen estrogen receptor modulator,[5] substituted diarylnapthalenes[6] such as nafoxidine and lasofoxifene, diarylbenzopyrans,[7] for example, ormeloxifene, benzopyranones[8] like SP500263, benzothiophenes[9] such as raloxifene have formed the backbone of a multitude of non-steroidal estrogen agonists as well as antagonists (Fig. 1).

As part of our drug development program, we aimed to explore and develop new ER recognition motifs. Functionalized 2-pyranones are key structural motifs found in a large number of biologically important natural products[10] that form a family of active compounds with a wide range of biological activities like, anti-cancer, antimicrobial, phytotoxic and HIV-1 protease inhibitors.[11–14] Recently, a series of arylated 2-pyranones have shown cyclooxygenase-2(COX-2) inhibitory activity. One of the compounds, 3,4,6-triphenyl-2-pyranone (Fig. 1), was found to be a potent and selective COX-2 inhibitor with an $IC_{50} = 0.02$ μM.[15] Structural resemblance of 3,4,6-triaryl-2-pyranones to triarylethylene based tamoxifen and ormeloxifene, a potent SERM developed by this institute and exhibiting antifertility and antibreast cancer activities,[16] prompted us to explore this pharmacophore as potential lead for developing new anti-breast cancer agents.

The rationale to design the 2-pyranones was to build a scaffold with specific alignment of three aryl substituents based on precedented ligands or leads[17] that are suitable to estrogen receptor binding and activity. The 2-pyranone ring served as the central core and the three aryl rings were suitably aligned in the desired molecular geometry as to mimic the triaryl template of TAPs, the only difference is the presence of pendent, that is, 6-aryl ring in

Abbreviations: ER, estrogen receptor; MCF-7, Michigan cancer foundation-7; MDA-MB-231, MD Anderson metastatic breast-231; RBA, receptor binding affinity.

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† CDRI comm. No. 7469.

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doi:10.1016/j.bmc.2009.04.032

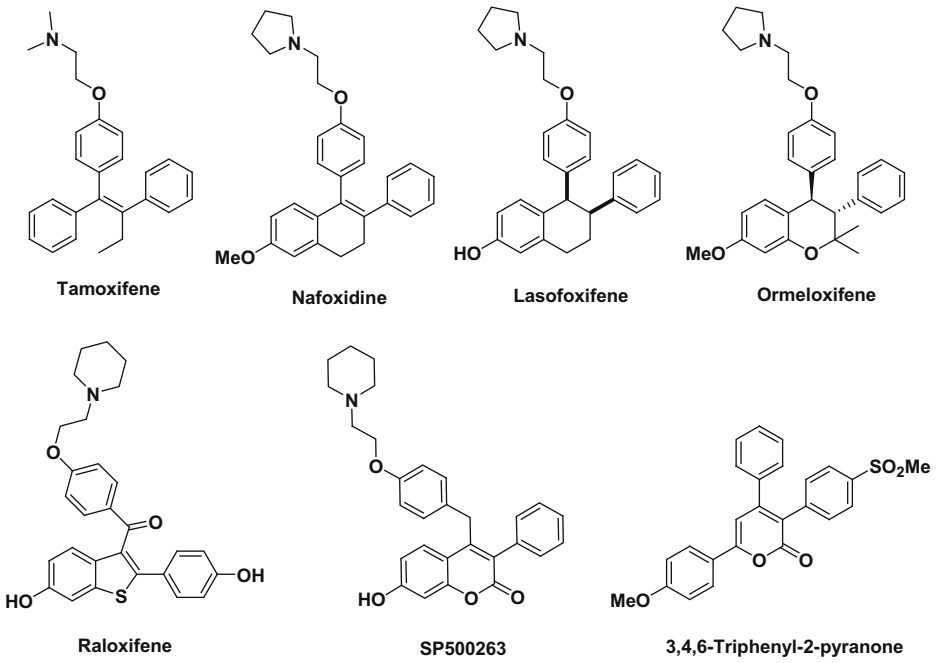


Figure 1. Structures of non-steroidal estrogen antagonists.

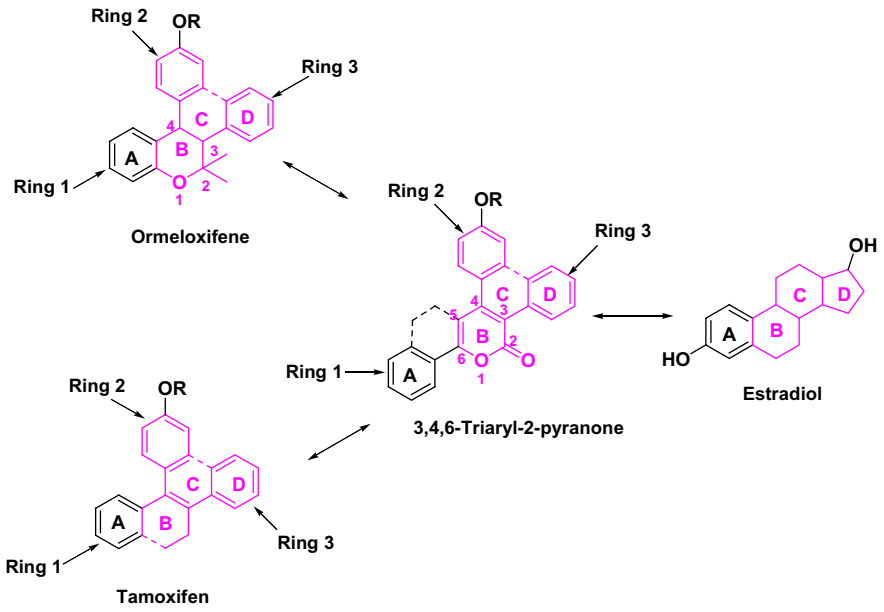


Figure 2. Structural resemblance of 3,4,6, triaryl-2-pyranones to tamoxifen, estradiol and ormeloxifene.

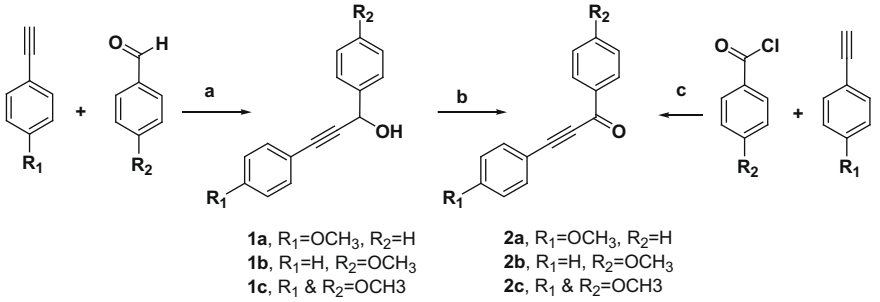
the place of fused benzopyran (Fig. 2). The essential requirement of basic amino alkyl chain for inducing the favorable conformational change to elicit the desired antagonistic effect[18,19] to facilitate interaction of ER with co-activator or co-repressor proteins was introduced at the para position of 2-ring to give the target molecules.

2. Results and discussion

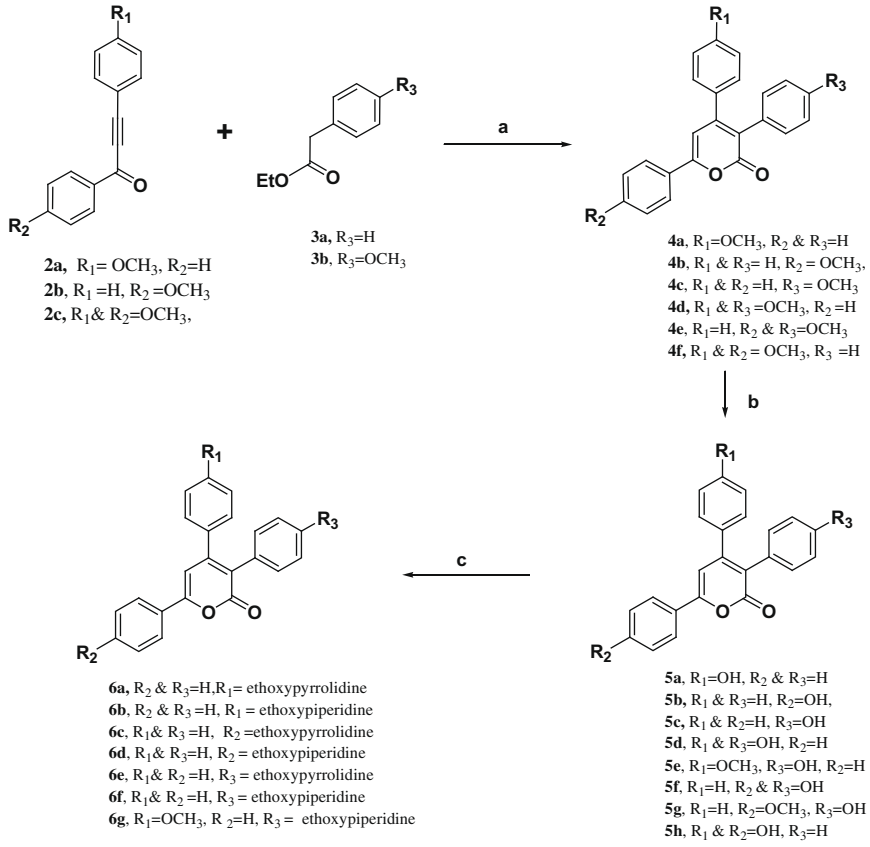
2.1. Chemistry

At the outset, 1,3-diarylpropynones (**2a–c**) were synthesised by oxidation of 1,3-diarylprop-2-yn-1-ols (**1a–c**, which in turn were achieved by condensation of phenylacetylene or 4-methoxy phenylacetylene with benzaldehyde or anisaldehyde in presence of anhyd zinc chloride) with activated manganese dioxide in 50% yield.[20,21] Since only moderate yields of 1,3-diarylpropynones could be obtained by this method, Sonogashira coupling[22] between a substituted alkyne and substituted benzoyl chloride in presence of cuprous iodide formed propynones in good yields of 70–75% (Scheme 1).

The methodology[21,23] used to prepare the regioisomeric 3,4,6-triphenyl-2-pyranones (**5a–h**) is outlined as shown in Scheme 2. Simple and 4-methoxyphenyl ethyl phenylacetate (**3a, b**) when condensed with 1,3-diarylpropynones (**2a–c**) in presence of sodium hydride at ambient temperature formed the desired methoxy



Scheme 1. Synthesis of 1,3-diarylpropynones. Reagents and conditions: (a) $ZnCl_2$, Et_3N, dry toluene, rt, 1 h (b) MnO_2, dry acetone, rt, 5 h (c) CuI, Et_3N, rt, 6 h.



Scheme 2. Synthesis of 3,4,6-triphenyl-2-pyranones and its derivatives. Reagents and conditions: (a) NaH/DMSO, 25 °C, 1 h; (b) pyridine hydrochloride, 220 °C, 30 min or HBr/gl.CH_3COOH, 120 °C; (c) 1-(2-chloroethyl)pyrrolidine/piperidine hydrochloride, K_2CO_3, dry DMF, 70 °C.

substituted 3,4,6-triphenyl-2-pyranones(**4a–f**). Demethylation of monomethoxy pyranones (**4a–c**) was carried out either with pyridine hydrochloride or HBr in glacial acetic acid. The reaction was neat and monophenolic derivatives **(5a–c)** were obtained in good yields. Dimethoxypyranone **4f** on demethylation under similar conditions formed the single dihydroxy compound **5h** in 60% yield. However, demethylation of dimethoxy pyranones **4d** and **4e** under similar conditions formed two products each: with **4d**, 3-(4-hydroxyphenyl)-4-(4-methoxyphenyl)-6-phenyl-2*H*-pyran-2-one **(5e)** was formed as the major product (75%) along with 3,4-bis(4-hydroxyphenyl)-6-phenyl-2*H*-pyran-2-one **(5d)** as the minor product (25%), obtained after purification by column chromatography, similarly **4e** on demethylation again formed two products **5g** (major) and **5f** (minor).

The position of hydroxyl groups in compounds **5e** and **5d** was confirmed by NMR spectra. The proton spectrum of **5e** showed a single resonance peak at δ 3.81 for OCH_3 and a sharp singlet at δ 7.9 for the phenolic OH. On comparison with the proton spectra of dimethoxy derivative **4d** with **5e**, the absence of peak at δ 3.78 in **5e** which corresponds to the methoxy group at the para position of 3-aryl ring confirmed the demethylation of this methoxy group. This selectivity in demethylation is due to lactone ring in conjugation with aryl rings at 4 and 6 positions, as a result, the electron density is decreased at the oxygen of these methoxy groups as compared to the para methoxy of 3-aryl ring. The attack of Lewis acid is therefore preferential at this methoxy and leads to its demethylation. The same was observed in case of **4e**, where the para methoxy of 3-aryl ring is again preferentially demethylated in comparison to para methoxy of 6-aryl ring, forming compound **5g** showing the same phenolic absorption at δ 7.9 and the dihydroxy compound **5f**. In case of demethylation of dimethoxy pyranone **4f**, only the dihydroxy compound **5h** was obtained showing phenolic absorptions a little downfield at δ 8.8 and 9.2 ppm because here both para methoxy groups on 4 and 6 aryl rings are almost equally

conjugated with the lactone ring. Finally, alkylation of phenolic derivatives **(5a–c, e)** with 1-(2-chloro ethyl) pyrrolidine/piperdine hydrochlorides formed the target molecules **(6a–g)**. All the synthesized compounds were well characterized by spectroscopic analyses.

2.2. Biological activity

2.2.1. Anti-proliferative activity against breast cancer

The target compounds (**5a–h**) and (**6a–g**) were evaluated for anti-proliferative activity in both MCF-7 (ER+ve) and MDA-MB-231 (ER−ve) cells using MTT assay. Nine of these, **5b–d**, **g**, **h**, **6a–c** and **6g**, were found to inhibit growth of MCF-7 and MDA-MB-231 cells at IC_{50} of less than 50.0 μM. In MCF-7 cells, three compounds, **5g**, **5h** and **6a** showed maximum inhibition of cell growth with an IC_{50} of 14 μM (Table 1). In addition to MCF-7 cells, **6a** also exhibited potent cell growth inhibition in MDA-MD-231 cells with an IC_{50} of 9.0 μM, which is better than that observed with MCF-7 cells (Table 1). From these data, we may conclude that **6a** is the most effective and potent molecule for anti-proliferative activity in breast cancer cells.

2.2.2. Cytotoxicity towards normal cells

Specific killing of cancer cells without affecting normal cell growth is a key safety feature of cancer chemotherapy. The nine active compounds were therefore evaluated for possible cytotoxicity in normal cells, for example, Vero and osteoblasts and five of these compounds, **5b**, **6a–c** and **6g** induced significant cell death in both Vero and osteoblast cells (Fig. 3a and b). Compounds **6a**, **6b** and **6g** which exhibited greater anti-proliferative activity in cancer cells are thus unsuitable as potential drug candidate as they are cytotoxic in normal cells. On the other hand, compounds, **5c**, **5d**, **5g** and **5h** did not significantly affect growth of Vero and osteoblasts, suggesting that these molecules have selectivity for inducing growth inhibition of cancer cells.

2.2.3. Relative binding affinity to ER

In order to confirm if the action of active compounds is through ER, their ER binding potential was checked using competitive binding assay, employing radio labeled estradiol (^3H–E_2) as the reference compound. Briefly, the uterine cytosol (obtained from immature 20–21 days old E_2 primed rats) was incubated with ^3H-estradiol in the absence or presence of various concentrations of test compounds for 22 h at 4 °C. ^3H–E_2 bound and free fractions were separated by charcoal adsorption method. The relative binding affinity of each test compound was calculated from the graph plotted between percent bound radioactivity verses molar concentration of the test substance. At 50% inhibition, log of the competitor concentration relative to that of estradiol expressed the affinity of the test compound to ER relative to estradiol. This when multiplied with 100 gave the% value designated as RBA.

Based on RBA studies, all the compounds appeared to exhibit weak ER ligand binding affinity. The highest RBA was observed in compound **5h** (RBA, 0.272% of 17-β estradiol) which is very low in comparison to anticancer drug, tamoxifen (RBA, 2.2% of 17-β estradiol) as shown in Table 1. From these data, it can be concluded that the series is safe in terms of activating ER with some compounds showing better activity in ER−ve cell line.

We next assessed whether the molecules **5c**, **5d**, **5g** and **5h,** that specifically exert anti-proliferative activity on breast cancer cells exhibit ERα or ERβ activation. Consistent with our RBA data, Cos-7 cells transiently transfected with ERα or ERβ did not exhibit any transcriptional activation by any of the compounds when compared with E_2 treatment (Fig. 4). This further confirms that the active molecules act in an ER-independent mechanism or if at all act through ER in MCF-7 then activity is also being mediated through some non-ER target in MDA-MB-231.

Table 1
Antiproliferative activity of compounds against MCF-7 and MDA-MB-231 cell lines

Compound no.	IC_{50} (μM)		RBA (% of estradiol-17β)
	MCF-7	MDA-MB-231	
5a	38	>100	0.015
5b	21	13	0.143
5c	25	38	0.107
5d	24	27	0.150
5e	>50	>100	0.093
5f	26	>100	0.125
5g	14	17	0.03
5h	14	26	0.272
6a	14	9	0.02
6b	26	11	0.091
6c	25	34	<0.001
6d	24	>100	<0.001
6e	23	>100	0.12
6f	>50	>100	0.08
6g	16	12	0.02
Tamoxifen	10	20	2.2

2.2.4. Induction of apoptosis

We next investigated the mechanism of inhibition of cell growth of the breast cancer cells by **5c**, **5d**, **5g** and **5h** and studied whether or not apoptosis was involved in such process. This was studied by DNA fragmentation and caspase activation assays.

2.2.4.1. DNA fragmentation. DNA fragmentation was measured with BrdU-labeling (5-bromo-2-deoxyuridine) of fragmented DNA (Roche, USA) with the four active compounds, **5c**, **5d**, **5g** and **5h.** The results showed that compound **5d** at 10 μM ($p = 0.034$) and 20 μM ($p = 0.035$), **5h** at 20 μM ($p = 0.042$) induced significant DNA fragmentation in MCF-7 cell line (Fig. 5a). With the other two compounds **5c** and **5g**, the DNA fragmentation was not statistically significant. Similarly, in MDA-MB-231 cell line compounds **5d** at 20 μM ($p = 0.025$) and **5h** at 10 μM ($p = 0.044$) and at 20 μM ($p = 0.028$) induced significant DNA fragmentation (Fig. 5b). Further, the four active and non-cytotoxic compounds (**5d**, **5h**, **5c** and **5g**) also induced significant qualitative changes in percentage of apoptotic cells in treated MDA-MB-231 cells through morphological analysis for apoptosis with Hoechst staining.

2.2.4.2. Induction of caspase. Caspases are a family of cysteine proteases which sequentially get activated and are therefore used as a measure of induction of caspase-mediated apoptosis. To assess whether the apoptosis induced by these four compounds was caspase mediated, evaluation of the four active compounds showed that **5d** at 20 μM ($p = 0.033$), **5h** at 20 μM ($p = 0.018$) and **5g** at 20 μM ($p = 0.039$) significantly induced basal caspase level in MCF-7 signaling for caspase dependent apoptosis. However, **5c** caused induction of caspase only at a higher concentration of 40 μM ($p = 0.021$) (Fig. 6). Further, compounds did not induce caspase activation in MDA-MB-231 cells because DNA fragmentation and apoptosis do not always require caspase activation and induction of apoptosis may be due to oxidative stress or through apoptosis inducing factors (AIF) where caspase activation is not essential.

From the results, it is clearly observed that 3,4,6-triarylpyranones with phenolic substituent groups showed significant anti-proliferative activity, diphenolic compounds showing better activity than monophenolic molecules. Incorporation of tertiary alkoxy amino groups, like ethoxypyrrolidine or piperidine an essential requisite of SERM molecules at any of the phenolic group rendered the compound toxic or diminished the antiproliferative

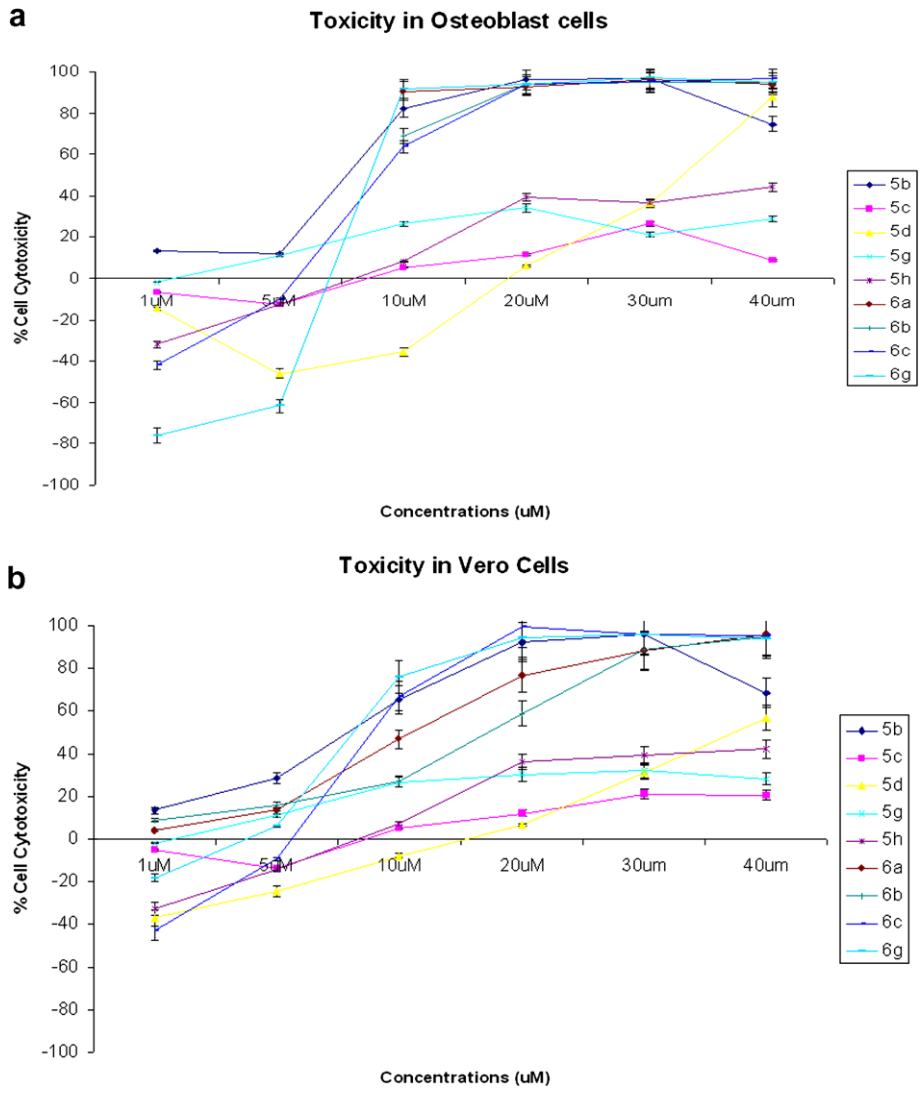


Figure 3. Cytotoxicity of pyranone compounds towards normal cells. (a) Vero cell lines and (b) primary cultured osteoblasts were treated with active pyranone compounds at varying concentrations for 12 h and induced cell death was measured with MTT assay. The data presented are average of three independent experiments.

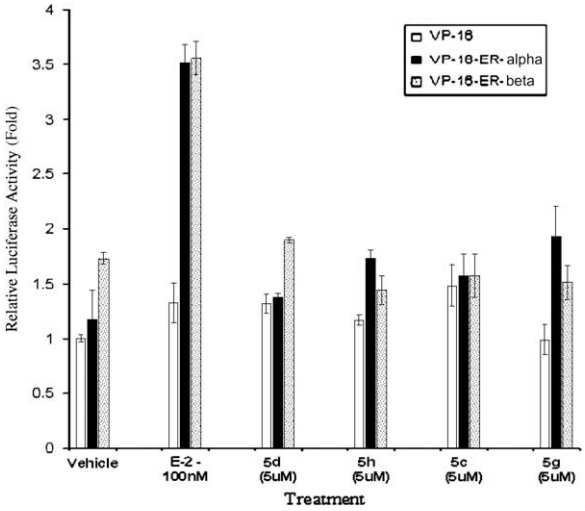


Figure 4. Trans-activation of ERα and ERβ in Cos-7. COS-7 cells were transfected with plasmids and treated with compounds as described in experimental section. Results represent mean ± SD of relative luciferase activities obtained from three independent experiments.

activity, thereby giving credence to the low ER binding and an ER independent pathway.

Furthermore, to rationalize the activity profile, we performed comparative docking study of computationally energy minimized conformation of active molecule **5h** with structurally similar drug, 4-hydroxy tamoxifen (OHT) and 17β-estradiol in estrogen receptor (ER α). The modeling study was carried out in molecular operating environment (MOE) software using a systematic conformational search with energy minimization. The crystal structure of estrogen receptor ligand binding domain (LBD) in complex with the endogenous estrogen, 17β-estradiol, 4-hydroxytamoxifen and reference protein coordinates used for structural alignment were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home, entry codes 1ERE and 3ERT, respectively).

Figure 7a and b, shows the orientation and interaction of the active molecule **5h** within the ER cavity in comparison to 4-hydroxy tamoxifen and 17β-estradiol. As is evident from Figure 7a, of the three aryl rings in **5h**, 3 and 4-aryl rings are more favorably aligned with the aryls of 4-hydroxy tamoxifen as rationalized. Deviation of the pendant aryl ring from the planarity of the stilbene core of tamoxifen and also with that of 17β-estradiol (Fig. 7b) may be responsible for weak binding interaction of the ligand with the

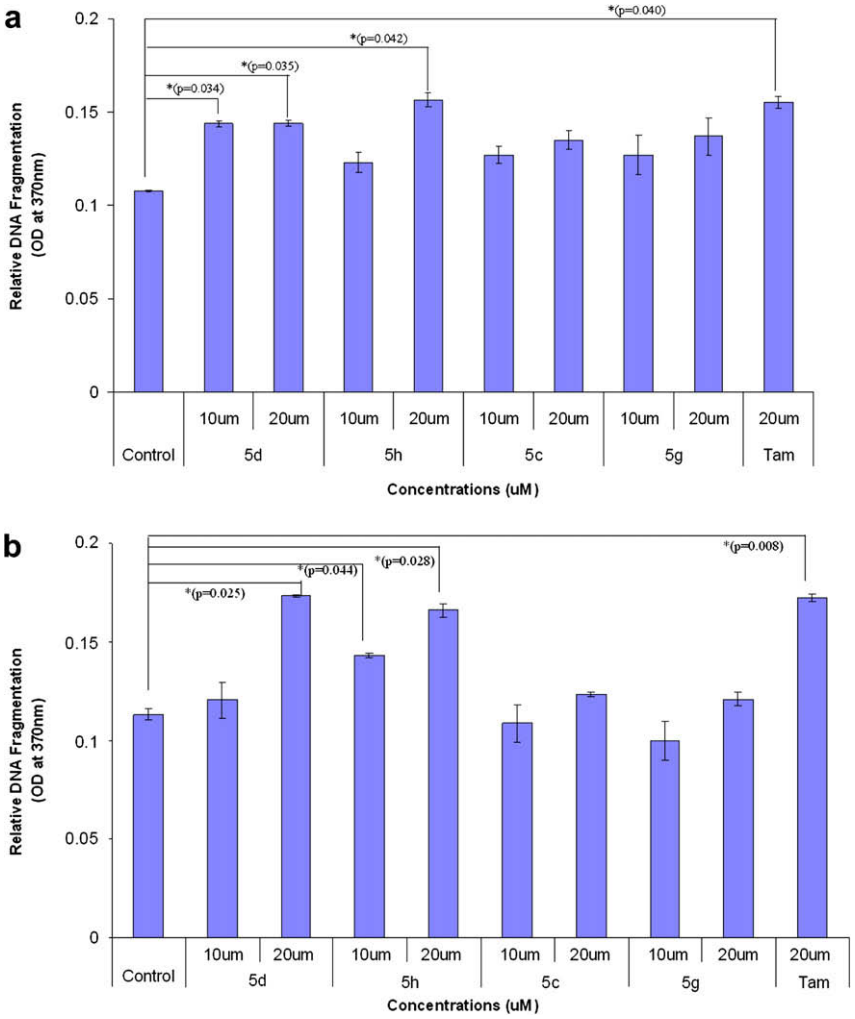


Figure 5. DNA Fragmentation induced in breast cancer cells. (a) MCF-7 and (b) MDA-MB-231. Cells were treated with 10, 20 and 30 μM concentrations of pyranone compounds for 12 h and labeled fragmented DNA was measured at OD 370 nm. Positive and negative reference controls were used as per manufacturer's instruction. Data presented are mean of duplicates from two independent experiments.

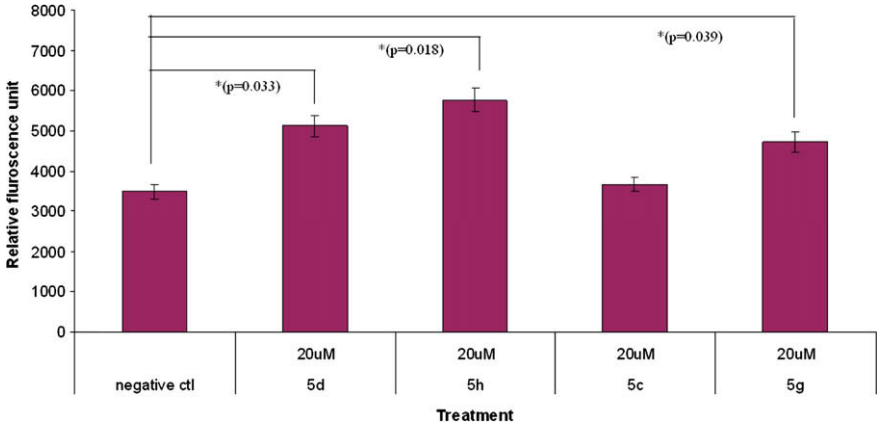


Figure 6. Induction of caspase in MCF-7. Cells were treated with compounds for 12 h at various concentrations, and then incubated with DEVD-rhodamine-110. Fluorescence from the activated caspases-mediated release of rhodamine-110 was measured at 560 nm. Data presented are mean of duplicates from two independent experiments. Compounds did not induce caspase activation in MDA-MB-231cells (data not shown).

salient amino acids essential for receptor binding affinity. However, the similarity in orientation of aryl bearing the antagonistic side chain of 4-hydroxy tamoxifen over the 4-aryl ring of **5h** may conform for the antagonistic profile shown in MCF-7 cells.

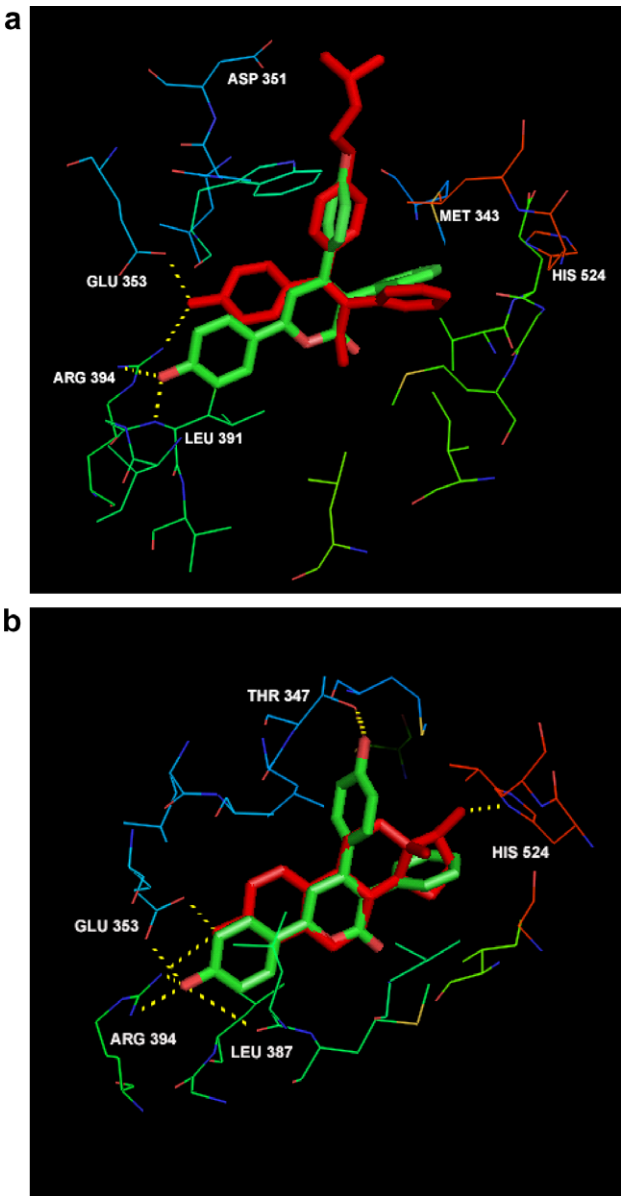


Figure 7. Comparative docking orientation and predicted receptor interactions. (a) Compound **5h** (green), 4-hydroxytamoxifen (OHT, red) and ERα; (b) compound **5h** (green), 17β-estradiol (red) and ERα (PDB code 3ERE).

3. Conclusion

This is the first report highlighting the significance of 3,4,6-triaryl pyranones as potential antiproliferative agents in breast cancer cells. This is confirmed by pharmacological results which show that compounds **5c**, **5d**, **5g** and **5h** exhibit specific and selective cytotoxicity against both ER positive and ER negative breast cancer cell lines. Their low relative binding affinity for ERα and failure to cause trans-activation of both ERα and ERβ clearly demonstrate their ER-independent nature of action in inducing cell death. Two compounds **5d** and **5h** induced significant DNA fragmentation in both MCF-7 and MDA-MB-231 cell lines and the induction of cell death by these compounds in MCF-7 cells is probably through caspase-dependant apoptosis. The active compounds could be speculated to employ some non-ER dependant pathway, the identification of the exact mechanism of action or cellular target utilized by these compounds are under investigation.

4. Experimental

4.1. General

All the glass apparatus were oven dried prior to use. Melting points were determined on COMPLAB melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer FT-IR RXI spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX-200 (200 MHz for ^{1}H and at 50 MHz for ^{13}C) or DRX-300 (300 MHz for ^{1}H and at 75 MHz for ^{13}C) spectrometers using $CDCl_3$ as solvent. Tetramethylsilane served as an internal standard in ^{1}H NMR and $CDCl_3$ in ^{13}C NMR spectra. (FAB-MS) spectra were obtained on a JEOL SX 102 spectrometer using argon/xenon (6 kV, 10 mA) as the FAB gas. Glycerol or *m*-nitro benzyl alcohol was used as matrix. High-resolution electron impact mass spectra (HR-EIMS) were obtained on a JEOL MS route 600H instrument. Reactions were monitored on precoated silica gel TLC plates (Merck). Detecting agents used (for TLC) were iodine vapors and or spraying with an aqueous solution of ceric sulfate followed by heating at 150 °C. Column chromatography was performed over silica gel (60–120 Mesh) procured from Qualigens (India) using freshly distilled solvents. All chemicals and reagents were obtained from Aldrich (Milwaukee, WI), Lancaster (England), or Spectrochem (India) and were used without further purification.

The synthesis of 3-(4-methoxyphenyl)-1-phenyl-prop-2-yn-1-one(2a), 1-(4-methoxyphenyl)-3-phenyl-prop-2-yn-1-one(2b), and 1,3-bis(4-methoxyphenyl)-prop-2-yn-1-one(2c) were prepared following the reported procedure.[21]

4.1.1. 4-(4-Methoxyphenyl)-3,6-diphenyl-pyran-2-one (4a)

To a suspension of NaH (60% oil dispersion, 32.8 mg, 1.2 mmol) in dry DMSO (5 mL) was added a solution of the ethyl phenyl acetate (3**a**, 164 mg, 1 mmol) and the reaction mixture was stirred for 15 min. at rt. A solution of **2a** (236 mg, 1 mmol) in DMSO (1 mL) was added dropwise to the above solution and the mixture was stirred at rt for 1 h. On completion, the reaction mixture was quenched with 1 N HCl, the solid precipitated was filtered, washed with water and crystallized from ethanol to afford yellow colored crystals of **4a**, yield (80%), mp 192–195 °C, IR (KBr) ν_{max}: 1705, 1450 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.8 (s, 3H, OCH_3), 6.77 (d, J = 8.4 Hz, 2H), 6.80 (s, 1H), 7.15 (d, J = 8.4 Hz, 2H), 7.22–7.28 (m, 5H, ArH), 7.49–7.51 (m, 3H, ArH), 7.87–7.92 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$): δ 55.66, 105.41, 123.56, 126.02, 128.13, 128.40, 128.81, 129.12, 129.39, 131.19, 131.30, 131.79, 134.23, 138.22, 153.18, 158.69, 163.15; MS(ESI) m /z 355 (M^++1).

4.1.2. 6-(4-Methoxyphenyl)-3,4-diphenyl-pyran-2-one (4b)

Yellow solid; yield (78%); mp 180–182 °C; IR (KBr) ν_{max}: 1716, 1442 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 3H, OCH_3), 6.73 (s, 1H), 6.94 (d, J = 8.7 Hz, 2H), 7.20–7.26 (m, 10H, ArH), 7.90 (d, J = 8.7 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 55.88, 103.99, 114.30, 122.79, 124.34, 127.72, 127.96, 128.35, 128.75, 129.10, 131.35, 134.42, 138.42, 153.55, 158.88, 162.15, 163.29; MS(ESI) m /z 354(M^+).

4.1.3. 3-(4-Methoxyphenyl)-4,6-diphenyl-pyran-2-one (4c)

Yellow solid; yield (77%); mp 203–205 °C; IR (KBr) ν_{max}: 1708, 1454 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.77 (s, 3H, OCH_3), 6.77 (d, J = 8.6 Hz, 2H), 6.83 (s, 1H), 7.13 (d, J = 8.7 Hz, 2H), 7.22–7.26 (m, 5H, ArH), 7.42–7.47 (m, 3H, ArH), 7.90 (d, J = 8.8 Hz, 2H);.^{13}C NMR ($CDCl_3$): δ 55.58, 105.49, 109.30, 123.21, 125.94, 128.86, 129.03, 129.11, 129.35, 131.05, 131.68, 132.42, 132.56, 152.25, 158.53, 159.43, 163.15; MS (ESI) m/z 355 (M^++H).

4.1.4. 3,4-Bis-(4-methoxyphenyl)-6-phenyl-pyran-2-one (4d)

Yellow solid; yield (75%), mp 210–212 °C; IR (KBr) ν_{max}: 1685, 1415, 1440 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.78 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 6.70–6.80 (m, 4H+1H), 6.81–6.83 (m, 2H, ArH), 7.42–7.46 (m, 3H, ArH), 7.87 (d, J = 8.8 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 55.60, 55.69, 105.5, 114.0, 114.2, 122.46, 125.9, 126.73, 129.32, 130.7, 130.89, 131.94, 132.53, 138.68, 152.07, 158.08, 159.32, 160.25, 163.58; MS (ESI) m/z: 385 (M^++H).

4.1.5. 3,6-Bis-(4-methoxyphenyl)-4-phenyl-pyran-2-one (4e)

Yellow solid; yield (80%); mp 180–182 °C; IR (KBr) ν_{max}: 1709, 1465, 1441 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.79 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.71 (s, 1H), 6.73 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 7.22–7.26 (m, 7H, ArH), 7.84 (d, J = 8.8 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 55.57, 55.86, 104.06, 113.87, 114.00, 122.00, 124.42, 126.53, 127.63, 128.80, 128.92, 129.08, 132.57, 138.68, 152.90, 158.46, 159.30, 162.04, 163.56; MS (ESI) m/z 385 (M^++H).

4.1.6. 4,6-Bis-(4-methoxyphenyl)-3-phenyl-pyran-2-one (4f)

Yellow solid; yield (85%); mp 195–197 °C; IR (KBr) ν_{max}: 1699, 1466, 1417 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.79 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.82 (s, 1H), 6.84 (d, J = 8.5 Hz, 2H), 7.15 (d, J = 8.4 Hz, 2H), 7.39–7.44 (m, 7H, ArH), 7.89 (d, J = 8.8 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 55.69, 55.85, 105.63, 114.27, 114.94, 125.94, 127.97, 128.41, 128.99, 129.34, 130.75, 131.00, 132.59, 122.41, 127.04, 131.93, 137.31, 152.15, 158.11, 158.61, 160.29, 163.55; MS (ESI) m/z 385 (M^++H).

4.1.7. 4-(4-Hydroxyphenyl)-3,6-diphenyl-pyran-2-one (5a)

A mixture of **4a** and pyridine hydrochloride (1:3 w/w) was heated at 200 °C for 2 h, cooled, diluted with water, and extracted with EtOAc. The organic layer was washed once with an equal volume of 1 N HCl followed by water, dried (Na_2SO_4), and concentrated to obtain the crude product. It was purified over a short column of silica gel to give pure **5a** as yellow solid, yield (70%); mp >250 °C; IR (KBr) ν_{max}: 3405, 1680 cm^{-1}; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 6.63 (d, J = 8.4 Hz, 2H), 6.9 (s, 1H), 7.0 (d, J = 8.4 Hz, 2H), 7.14–7.19 (m, 5H, ArH), 7.44–7.49 (m, 3H, ArH), 7.87–7.91 (m, 2H, ArH), 8.56 (s, OH); MS (ESI) m/z 341 (M^++1).

4.1.8. 6-(4-Hydroxyphenyl)-3,4-diphenyl-pyran-2-one (5b)

Yellow solid; yield (67%); mp >250 °C; IR (KBr) ν_{max}: 3438, 1670 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 6.52 (d, J = 8.7 Hz, 2H), 6.7 (s, 1H), 7.26 (d, J = 8.7 Hz, 2H), 7.35–7.39(m, 5H, ArH), 7.40–7.43 (m, 3H, ArH), 7.92–8.01 (m, 2H, ArH), 8.62 (s, OH); MS (ESI) m/z 341 (M^++1).

4.1.9. 3-(4-Hydroxyphenyl)-4,6-diphenyl-pyran-2-one (5c)

Yellow solid; yield (72%); mp >250 °C; IR (KBr) ν_{max}: 3430, 1684 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 6.70 (d, J = 8.7 Hz, 2H), 7.0 (s, 1H), 7.08 (d, J = 8.7 Hz, 2H), 7.29–7.31 (m, 5H, ArH), 7.54–7.56 (m, 3H, ArH), 8.01–8.02 (m, 2H, ArH), 8.42 (s, OH); MS (ESI) m/z 341(M^++1).

4.1.10. 3,4-Bis-(4-hydroxyphenyl)-6-phenyl-pyran-2-one (5d)

Yellow solid; yield (70%); mp >250 °C; IR (KBr) ν_{max}: 3332, 1680 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 6.77–6.83 (m, 4H), 7.10–7.13 (m, 3H), 7.20–7.23 (m, 2H, ArH), 7.55–7.58 (m, 3H, ArH), 8.00–8.04 (m, 2H, ArH), 8.58 (s, OH), 8.87 (s, OH); MS (ESI) m/z 357(M^++1), HRMS (ESI): calculated 356.1049 and measured mass 356.1025.

4.1.11. 3-(4-Hydroxyphenyl)-4-(4-methoxyphenyl)-6-phenyl-pyran-2-one (5e)

Yellow solid; yield (68%); mp >250 °C; IR (KBr) ν_{max}: 3426, 1688, 1455 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 3.79 (s, 3H), 6.69 (s, 1H), 6.74–6.76 (m, 4H, ArH), 7.42–7.45 (m, 3H, ArH), 7.46–7.48 (m, 2H, ArH), 7.79 (s, 1H, OH); MS (ESI) m/z 371 (M^++1).

4.1.12. 3,6-Bis-(4-hydroxyphenyl)-4-phenyl-pyran-2-one (5f)

Yellow solid; yield (71%); mp >250 °C; IR (KBr) ν_{max}: 3407, 1684 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 6.72–6.78 (m, 4H, ArH), 7.04–7.08 (m, 3H, ArH), 7.14–7.17 (m, 2H, ArH), 7.50–7.56 (m, 3H, ArH), 7.98–8.01 (m, 2H, ArH), 8.48 (s, 1H), 8.77 (s, 1H); MS (ESI) m/z 357 (M^++1).

4.1.13. 3-(4-Hydroxyphenyl)-6-(4-methoxyphenyl)-4-phenyl-pyran-2-one (5g)

Yellow solid; yield (70%); mp >250 °C; IR (KBr) ν_{max}: 3405, 1683, 1432 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 3.89 (s, 3H), 6.73 (s, 1H), 6.71 (d, J = 8.0 Hz, 2H), 7.09 (d, J = 8.0 Hz, 2H), 7.12–7.17 (m, 2H, ArH), 7.20–7.38 (m, 3H, ArH), 7.70 (d, J = 8.0 Hz, 2H), 7.89 (d, J = 8.0 Hz, 2H); MS(ESI) m/z 371 (M^++1), HRMS (ESI): calculated 370.1205 and measured mass 370.1205.

4.1.14. 4,6-Bis-(4-hydroxyphenyl)-3-phenyl-pyran-2-one (5h)

Yellow solid; yield (63%), mp >250 °C; IR (KBr) ν_{max}: 3426, 1670 cm^{-1}; ^1H NMR (CD_3COCD_3): δ 6.72 (d, J = 8.7 Hz, 2H), 6.91 (s, 1H), 6.99 (d, J = 8.7 Hz, 2H), 7.11 (d, J = 8.7 Hz, 2H), 7.20–7.23 (m, 5H, ArH), 7.88 (d, J = 8.7 Hz, 2H), 8.8 (s, 1H, OH), 9.2 (s, 1H, OH); MS(ESI) m/z 357 (M^++1), HRMS (ESI): calculated 356.1049 and measured mass 356.1075.

4.1.15. 3,6-Diphenyl-4[4-(2-pyrrolidin-1-yl-ethoxy)phenyl]pyran-2-one (6a)

A mixture of **5a** (1 mmol), 2-pyrrolidinoethyl chloride hydrochloride (1.5 mmol), anhydrous K_2CO_3 (2.5 mmol) in DMF (10 mL) was heated at 70 °C for overnight. On completion the reaction was cooled and filtered. The filtrate was concentrated in vacuo and subjected to chromatography over a column of basic alumina to furnish **6a**, yellow solid; yield (63%); mp 117–119 °C; ^1H NMR ($CDCl_3$): δ 1.75–1.83 (m, 4H), 2.59–2.63 (m, 4H), 2.89 (t, 2H), 4.08 (t, 2H), 6.78 (d, J = 8.2 Hz, 2H), 6.85 (s, 1H), 7.12 (d, J = 8.2 Hz, 2H,), 7.24–7.30 (m, 5H, ArH), 7.47–7.49 (m, 3H, ArH), 7.19 (d, J = 8.2 Hz, 2H); ^{13}C NMR: δ 21.31, 54.72, 54.97, 64.22, 105.26, 112.07, 119.48, 125.77, 126.67, 129.01, 129.72, 151.20, 156.92, 159.23; MS (ESI) m/z 438(M^++H). Anal. Calcd for $C_{29}H_{27}NO_3$: C, 79.61; H, 6.22; N, 3.20. Found: C, 79.49; H, 6.32; N, 3.18.

4.1.16. 3,6-Diphenyl-4[4-(2-piperidin-1-yl-ethoxy)-phenyl]-pyran-2-one (6b)

Yellow solid; yield (70%); mp 147–149 °C; ^1H NMR ($CDCl_3$): δ 1.43–1.46 (m, 6H), 2.45–2.49 (m, 4H), 2.77(t, 2H), 4.09 (t, 2H), 6.78 (d, J = 8.8 Hz, 2H), 6.83 (s, 1H), 7.11 (d, J = 8.8 Hz, 2H), 7.23–7.26 (m, 5H, ArH), 7.46–7.48 (m, 3H, ArH), 7.91 (d, J = 8.8 Hz, 2H); ^{13}C NMR: δ 21.92, 53.11, 61.98, 98.73, 103.86, 112.80, 113.27, 114.05, 122.43, 124.43, 125.99, 126.53, 127.05, 127.33, 127.45, 127.68, 127.95, 129.95, 130.03, 130.87, 131.14, 136.46, 151.38, 155.33; MS (ESI) m/z 452 (M^++H). Anal. Calcd for $C_{30}H_{29}NO_3$: C, 79.80; H, 6.47; N, 3.10. Found: C, 79.69; H, 6.38; N, 3.16.

4.1.17. 3,4-Diphenyl-6-[4-(2-pyrrolidin-1-yl-ethoxy)phenyl]-pyran-2-one (6c)

Yellow solid; yield (65%); mp 121–123 °C; ^1H NMR ($CDCl_3$): δ 1.79–1.84 (m, 4H), 2.61–2.67 (m, 4H), 2.97 (t, 2H), 4.21 (t, 2H), 6.73 (s, 1H), 6.99 (d, J = 8.8 Hz, 2H), 7.16–7.25 (m, 10H, ArH), 7.28 (d, J = 8.8 Hz, 2H); ^{13}C NMR: δ 22.24, 53.45, 65.98, 103.55, 113.70, 120.67, 122.76, 125.99, 126.61, 127.02, 127.37, 129.64, 132.74, 136.78, 151.80, 157.21, 159.73, 161.52; MS (ESI) m/z 438

(M^++H). Anal. Calcd for $C_{29}H_{27}NO_3$: C, 79.61; H, 6.22; N, 3.20. Found: C, 79.72; H, 6.14; N, 3.32.

4.1.18. 3,4-Diphenyl-6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-pyran-2-one (6d)

Yellow solid; yield (69%); mp 180–182 °C; ^{1}H NMR ($CDCl_3$): δ 1.46–1.50 (m, 2H), 1.52–1.68 (m, 4H), 2.56–2.59 (m, 4H), 2.82(t, 2H), 4.12 (t, 2H), 6.77 (d, J = 8.6 Hz, 2H), 6.82 (s, 1H), 7.23–7.33 (m, 3H, ArH), 7.47–7.49 (m, 7H, ArH), 7.90 (d, J = 8.6 Hz, 2H). ^{13}C NMR: δ 23.49, 54.75, 54.97, 67.12, 105.02, 114.39, 125.59, 127.58, 128.08, 128.98, 129.86, 130.31, 130.66, 130.88, 134.21, 152.26, 158.08, 159.27, 162.65; MS (ESI) m/z 452(M^++H). Anal. Calcd for $C_{30}H_{29}NO_3$: C, 79.80; H, 6.47; N, 3.10. Found: C, 79.73; H, 6.34; N, 3.21.

4.1.19. 4,6-Diphenyl-3-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-pyran-2-one (6e)

Yellow solid; yield (60%); mp 110–112 °C; ^{1}H NMR ($CDCl_3$): δ 1.80–1.83 (m, 4H), 2.60–2.66 (m, 4H), 2.75 (t, 2H), 4.06 (t, 2H), 6.74 (d, J = 8.8 Hz, 2H), 6.82 (s, 1H), 7.13 (d, J = 8.8 Hz, 2H), 7.25–7.28 (m, 5H, ArH), 7.39–7.43 (m, 3H, ArH), 7.90 (d, J = 8.8 Hz, 2H); ^{13}C NMR: δ 22.36, 23.22, 27.7, 28.26, 53.57, 65.88, 103.68, 113.82, 120.86, 122.92, 126.02, 126.73, 127.13, 127.56, 129.86, 132.85, 136.96, 152.52, 157.36, 159.84, 161.65; MS(ESI) m/z 438(M^++H); HRMS (ESI): calculated 437.2481 and measured mass 437.2443.

4.1.20. 4,6-Diphenyl-3-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-pyran-2-one (6f)

Yellow solid; yield (71%); mp 150–152 °C; ^{1}H NMR ($CDCl_3$): δ 1.44–1.49 (m, 2H), 1.59–1.64 (m, 4H), 2.57 (t, 2H), 2.81 (t, 2H), 4.12 (t, 2H), 6.71 (d, J = 8.7 Hz, 2H), 6.78 (m, 2H, ArH), 6.84 (s, 1H), 7.28–7.30 (m, 5H, ArH), 7.44–7.48 (m, 3H, ArH), 7.92 (d, J = 8.8 Hz, 2H); ^{13}C NMR: δ 22.02, 51.07, 60.86, 101.62, 105.52, 113.06, 114.72, 115.02, 120.23, 125.54, 126.07, 127.00, 128.22, 129.43, 129.47, 129.75, 129.89, 129.97, 133.25, 133.78, 134.14, 138.52, 153.24, 156.89; MS (ESI) m/z 452(M^++H); HRMS (ESI): calculated 451.2147 and measured mass 451.2148.

4.1.21. 4-(4-Methoxy-phenyl)-6-phenyl-3[4-(2-piperidin-1-yl-ethoxy)-phenyl]-pyran-2-one (6g)

Oil, yield (70%); ^{1}H NMR ($CDCl_3$): δ 1.55–1.59 (m, 2H), 1.61–1.64 (m, 4H), 2.47–2.52 (m, 4H), 2.79 (t, 2H), 3.81 (s, 3H), 4.12 (t, 2H), 6.68–6.72 (m, 5H, ArH), 6.80 (s, 1H), 7.08–7.15 (m, 4H, ArH), 7.42–7.48 (m, 3H, ArH), 7.79–7.84 (m, 2H, ArH); ^{13}C NMR: δ 23.94, 25.53, 30.88, 55.25, 57.68, 65.49, 105.10, 113.86, 114.25, 121.98, 125.49, 126.50, 128.90, 130.03, 130.29, 130.54, 131.52, 132.12, 151.73, 157.65, 158.03, 159.88, 163.10; MS(ESI) m/z 482 (M^++H) Anal. Calcd for $C_{31}H_{31}NO_4$: C, 77.31; H, 6.49; N, 2.91. Found: C, 77.22; H, 6.59; N, 2.78.

4.2. Experimental protocols

4.2.1. MTT assay in Breast cancer cell line

The antiproliferative activities of the compounds were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay.[24] 1×10^4 cells/well were seeded in 100 μl DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium. After 48 h of incubation, media were removed and to each well 10 μl MTT (5 mg/mL) was added and the plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μl of DMSO and absorbance at 540 nm wavelength was recorded.

4.2.2. Cytotoxicity assay in Vero cell line and osteoblast

Cytotoxic effects of the compounds were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay.[25] 1×10^4 cells/well were seeded in 100 μl DMEM supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium. After 48 h of incubation, media were removed and to each well 10 μl MTT (5 mg/mL) was added and the plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μl of DMSO and absorbance at 540 nm wavelength were recorded.

4.2.3. Dual-luciferase reporter assay

40,000 cells Cos-7cells were seeded in 24-well plates 24 h before transfection. Cells were co-transfected with 200 ng of reporter plasmid (pGL3-Gal4Tk-Luc), 200 ng of expression plasmid (pVP-16/VP-16-ERα/VP-16-ERβ), 200 ng of co-activator (pM-Tif-2), 100 ng of pRL-CMV, 100 ng of pEGFPC-1, using transfection reagent (Lipofectamine-2000). After 24-h posttransfection, cells were harvested for luciferase reporter assay using the dual-luciferase reporter assay system.[26] The activated luciferase activity was normalized to that of the internal control Renilla luciferase activity by pRL-CMV as relative luciferase unit as well as by GFP and fold of relative luciferase activation unit were compared to VP-16 control transfection. All observations were performed as duplicate in three independent experiments.

4.2.4. DNA fragmentations assay

MCF cells were labeled with BrdU by incubating overnight. Then the labeled cells were incubated for 24 h in the presence of compound for test or without for untreated control. At the end of the incubation, cells were treated with lysis buffer for 30 min at rt, centrifuged at 250g for 10 min and supernatant was prepared. The anti-DNA antibody was adsorbed onto the wells of a microplate for 1h at rt. Supernatant of the cell lysate was added to the pre-adsorbed microplate. BrdU-labeled DNA fragments in the sample bind to the immobilized anti-DNA antibody. The immunocomplex of BrdU-labeled DNA-fragments were fixed by microwave irradiation for 5 min for the accessibility of the BrdU antigen. Then peroxidase conjugated anti-BrdU antibodies were added for reacting with the BrdU-labeled DNA to form an immuno-complex by incubating 90 min. The bound anti-BrdU-conjugates in the immuno-complex were then measured using a peroxidase substrate in ELISA reader.

4.2.5. Morphological analysis for apoptosis with Hoechst staining

Cells were seeded at a density of 10,000 cells over 18-mm coverslips and incubated for 24 h. Then, the medium was replaced, and cells were treated with 10 μM of compounds for 18 h. Cells treated with vehicle (0.001% DMSO) were included as controls for all experiments. After overnight treatment, Hoechst 33342 (Sigma–Aldrich) were added to medium at a concentration of 0.5 μg/ml containing 4% paraformaldehyde. After incubation for 30 min at 37 °C, cells from each dish were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic cells based on their relative fluorescence and nuclear fragmentation.

4.2.6. Caspase assay

The homogeneous caspase assay kit (Roche, Germany) that includes caspases 2, 3, 6, 7, 8, 9 and 10 was used to measure caspase activities after apoptosis induction according to manufacturer's instructions. Camptothecin treated U937 cells were used as positive control. MCF-7 cells were cultured under serum-deprived con-

ditions for 4 h. Compound treatment was given for 24 h at various concentrations. Cells were then incubated with DEVD-rhodamine-110((tetra-peptide sequence 'aspartic acid–glutamic acid–valine–aspartic acid' recognized by caspases).Upon cleavage of the rhodamine substrate by activated caspases, fluorescence from the released rhodamine-110 was measured.

4.2.7. Data analysis

All data were expressed as mean ± SD with at least three separate experiments. The data were compared using Microsoft Excel or Prism statistical program, with Student's *t*-test for unpaired variants. Values of $P < 0.05$ were regarded as statistically significant.

Acknowledgments

The authors, Ravi Shankar is thankful to CSIR for SRF fellowship and Uma Sharan Singh is grateful to Ministry of Health and Family Welfare for financial assistance. All are thankful to SAIF for spectroscopic analyses of the compounds. The authors are also thankful to Dr. S. Sanyal. DTDD, CDRI, for providing constructs lab facility and guidance for conducting Dual luciferace reporter assay for ER transactivation, Dr. N. Chattopadhyay, Head, Endocrinology for his critical review of manuscript and suggestions, Dr. Y.S. Prabhakar, MPC, for comparative computational studies and Dr. Brijesh Kumar, SAIF, for HRMS data.

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