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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis and biological evaluation of hydrazone derivatives as anti-proliferative agents

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Abstract A series of triaryl-substituted hydrazones as structural acyclic prototypes were synthesized and screened for anti-proliferative activity against breast (Michigan cancer foundation-7 and MD Anderson metastatic breast-231) and uterine cancer (Ishikawa) cell lines. Two compounds were found to be the most active, **5e** showed the maximum inhibition of both functional estrogen receptor containing Michigan cancer foundation-7 cells (IC_{50}: 7.8 μM) and Ishikawa cells (IC_{50}: 7.3 μM) whereas, compound **5i** was selectively most active against ER-negative MD Anderson metastatic breast-231 cells (IC_{50}: 4.7 μM). The inhibitory effect of **5e** in breast cancer and uterine cancer cells was due to ER antagonistic action, also supported by molecular docking studies.

Keywords Hydrazones · Estrogen receptor · Antagonists · Anti-proliferative · Anti-cancer

Introduction

The discovery and identification of compounds with desired potency, and selective cytotoxicity against cancer cells that may lead to the development of new anti-cancer drugs remains an important objective of medicinal chemists. Hydrazones represent an important class of compounds known to possess, antimicrobial, anticonvulsant, analgesic, anti-inflammatory, anti-platelet, anti-tubercular and anti-tumor activities (Kumar and Narasimhan 2013). Some biologically active hydrazones **1–7** are shown in Fig. 1. The 2, 4-dinitrophenyl hydrazones of diaryl ketones have been reported to possess antiestrogenic and antineoplastic activities. One of the compound A-007, (4,4'-dihydrobenzophenone-2,4-dinitrophenyl-hydrazone (**5**) is in phase II clinical trials (Morgan et al. 2003). These hydrazones (**6**) are reported to have pseudo ring structure that stereo-chemically occupies the estrogenic region of the receptor thereby inhibiting it from undergoing allosteric change to prevent transcription of estrogenic information to the cell nucleus (Pandey et al. 2002; Singh et al. 2008). Small molecules like guanylhydrazone **7** have been reported to block estrogen receptor (ER) activity through a non-traditional mechanism, by directly interfering with coactivator binding to agonist-liganded ER (LaFrate et al. 2008).

From a study of the estrogen-ligand pharmacophore model (Agatonovic-Kustrin and Turner 2008), subtle changes in the spatial conformation of the triarylethylene core lead to some major pharmacological changes. This hypothesis has led to the development of many different ER agonists/antagonists, the most promising being the triaryl pyrazole-based ER antagonists (Stauffer et al. 2001, 2000; Sun et al. 1999). These observations prompted us to design and investigate the hydrazone pharmacophore as the acyclic prototype of triaryl substituted pyrazoles, and to build a

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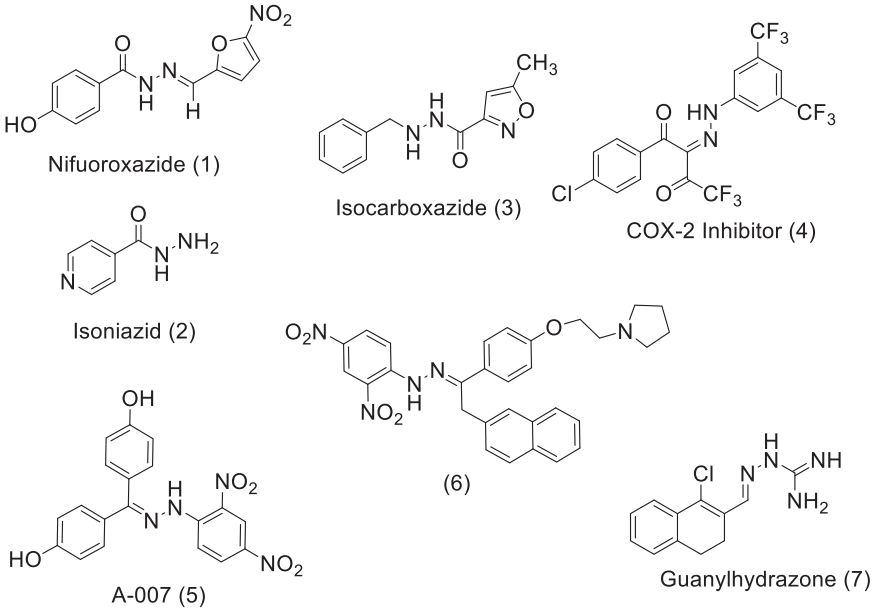


Fig. 1 Biologically active hydrazones **1–7**

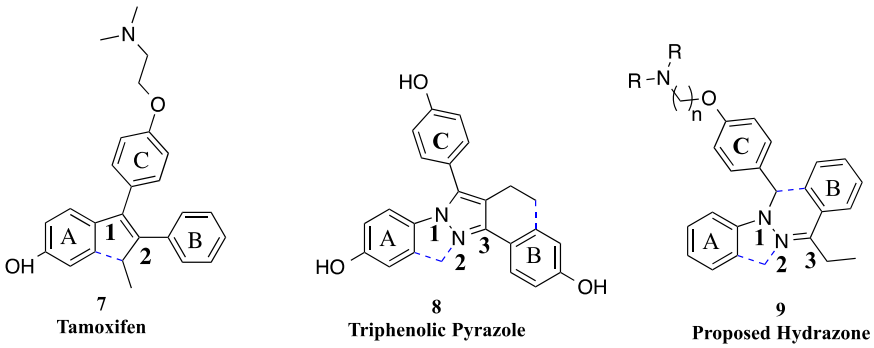


Fig. 2 Structures of tamoxifen, triphenolic pyrazole and proposed hydrazone

scaffold with specific alignment of three aryl substituents suitable for ER affinity and activity. Here, our motivating factor was to see the flexibility effect of the benzylic group, and also the effect of distance between A and B rings as in tamoxifen (**7**) at 2-position and in pyrazole (**8**) at 3-position on the biological activity against ER+ve and ER−ve breast cancer cell lines. The flexible alignment of an acyclic or pseudo ring (compound **9**) may help in the adoption of a favorable (antagonist) conformation by the molecules within the receptor cavity resulting in the enhanced affinity of the ligand to the receptor amino acids thereby potentiating the biological efficacy of hydrazones (Fig. 2).

Experimental section

Materials and methods

All the reagents were purchased commercially from Sigma-Aldrich and were used without further purification. The reactions were monitored by thin-layer chromatography on silica gel F_{254} plates. Melting points were taken in open capillaries on Complab melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer AC-1 spectrometer. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded using Bruker Supercon Magnet DRX-300 spectrometer (operating at 200, 300 MHz for ^{1}H and 50, 75 MHz for ^{13}C) using $CDCl_3$ and DMSO-d_6 as solvents and tetramethylsilane as internal standard. Chemical shifts are reported in parts per million. Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), multiplet (m), and broad singlet (brs). FAB mass spectra were recorded on JEOL SX 102/DA 6000 mass spectrometer using argon/xenon (6 Kv, 10 mA) as the FAB gas. Pure compounds were obtained by column chromatography using silica gel (60–120 and 100–200 mesh).

MTT assay in Breast cancer cell line

The MTT assay was used to determine the anti-proliferative activities of the compounds as described in our earlier

communication (Shankar et al. 2009). In short, 3000 cells/well were seeded in 100 µL Dulbecco's modified eagle medium media, supplemented with 10% fetal bovine serum in each well of 96-well cell culture plates and incubated for 24 h at 37 °C in a CO_2 incubator so that cells reaches about 70–80% confluency. Test compounds were dissolved in DMSO and diluted to the desired concentrations in culture medium so that final DMSO concentration is 0.001% or less. Cells were treated with 100 µL of each compound of desired concentration by adding to the wells along with untreated vehicle control and incubated for 48 h. Post-treatment period, the media were removed and 100 µL/well MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added and the plates were further bioincubated for 4 h. Then the supernatant from each well was carefully removed, formazon crystals were dissolved after adding 100 µL of DMSO with shaking under incubation for 10 min and absorbance at 540 nm wavelength were recorded with enzyme-linked immunosorbent assay reader.

Molecular modeling method

Additionally, the comparative docking study of computationally energy minimized conformation of active molecule **5e** and 4-hydroxy tamoxifen (OHT) was performed to rationalize the activity profile in ER (ERα). The molecular modeling study was carried out to obtain an insight of the molecular mechanism of compound **5e** by using the Schrodinger suite (Shiau et al. 1988). The crystal structure of ER ligand binding domain (LBD) in complex with 4-hydroxytamoxifen and reference protein coordinates used for protein preparation were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/; PDB ID: 3ERT) and further modified for Glide docking calculations. The builder module of Maestro has been used for the structure preparation of ER LBD in complex with 4-hydroxytamoxifen from the crystal structure (3ERT.pdb). Furthermore, the manually inspected and corrected structure of ER was subjected to a single run by multistep Schrödinger's Protein preparation wizard to optimize the structure and ensure its chemical correctness, and to determine the most energetically favorable conformation. Minimizations were performed until the average root mean square deviation of the non-hydrogen atoms reached 0.3 Å. The starting conformation for the most active compound **5e** and 4-hydroxy tamoxifen were obtained by 5000 steps of Monte Carlo conformational search under GB/SA continuum water solvation model and Polak-Ribiere Conjugate Gradient method used for the subsequent energy minimization (EM). After ensuring that the protein and ligands were in the correct form for docking, the receptor-grid files were generated using a grid-receptor generation program of glide. To soften the potential for non-polar parts of the receptor, we scaled van der Waal radii of receptor atoms by 1.00 Å with a partial atomic charge of 0.25. The ligands were docked with the active site using the 'xtra precision' (XP) Glide algorithm. After successful docking, enzyme-ligand complexes were subjected to EM to obtain the final binding mode. All the calculations were performed on Schrödinger Suite 2007 (Schrödinger Inc.) with GB/SA continuum water solvation model. OPLS_2001 force field was used for 'XP' docking and then the Merck molecular force fields force field was implemented in MACROMODEL 9.1 for EM.

*General procedure for the synthesis of N-benzylated hydrazones (**4a–g**)*

To a suspension of NaH (60% suspension in oil, 3 mM) in 5 mL of dry N,N-Dimethylformamide (DMF) was added hydrazone (**3a**, 20 mM, dissolved in 5 mL dry DMF) at 0 °C with stirring under nitrogen atmosphere. After 15 min, substituted benzyl bromide (30 mM, dissolved in 10 mL DMF) was added dropwise and stirring was continued at room temperature for 1.5 h. On completion, the reaction was quenched with cold water to decompose any unreacted sodium hydride and the solution was extracted with dichloromethane, washed with water and dried over anhydrous calcium chloride. The drying agent was filtered off and the solvent was removed by distillation. The residue was purified on a silica gel column using hexane/EtOAc as eluent to give the corresponding pure hydrazone derivatives.

N-[4-(2-Chloroethoxy)-benzyl]-N-phenyl-N′-(1-phenyl-ethylidene)-hydrazone (**4a**) and N-[4-(3-Chloropropoxy)-benzyl]-N-phenyl-N′-(1-phenyl-ethylidene)-hydrazone (**4b**) **4a** and **4b** were very unstable at room temperature and were used as such in the next reaction.

N-[4-(2-Chloroethoxy)-benzyl]-N-phenyl-N′-(1-phenyl-propylidene)-hydrazone (**4c**) oil ^{1}H NMR ($CDCl_3$): δ 0.84 (t, 2H), 2.71 (q, 2H), 3.79 (t, 2H), 4.22 (t, 2H), 4.67 (s, 2H), 6.86 (d, 2H, $J = 8.4$ Hz), 6.92–6.99 (m, 3H, ArH), 7.23–7.28 (m, 2H), 7.32–7.34(m, 2H), 7.41–7.43 (m, 3H) 7.78–7.81 (m, 2H, ArH).

N-[4-(2-Chloroethoxy)-benzyl]-N-phenyl-N′-(1-phenyl-butylidene)-hydrazone (**4d**) oil, ^{1}H NMR($CDCl_3$): δ 0.94 (t, 3H), 1.29 (q, 2H), 2.64 (t, 2H), 3.80 (t, 2H), 4.22 (t, 2H), 4.67 (s, 2H), 6.87 (d, 2H, $J = 8.4$ Hz), 6.93–6.98 (m, 3H, ArH), 7.23–7.28 (m, 2H, ArH), 7.33 (d, 2H, $J = 8.5$ Hz), 7.41–7.43 (m, 3H), 7.75–7.78 (m, 2H); ^{13}C NMR($CDCl_3$): δ 12.97, 18.71, 30.31, 40.26, 60.75, 66.65, 113.12, 116.19, 119.66, 125.87, 127.04, 127.59,128.29, 128.53, 130.38, 136.15, 150.14, 155.92, 170.38; ESIMS: 407($M^+ + 1$).

N-[4-(2-Chloroethoxy)-benzyl]-N′-[1-(4-methoxy-phenyl)-ethylidene]-N-phenyl-hydrazone (**4e**) oil, ^{1}H NMR ($CDCl_3$): δ 2.15 (s, 3H, CH_3), 3.81 (t, 2H), 3.87 (s, 3H, OCH_3), 4.22 (t, 2H), 4.72 (s, 2H), 6.87 (d, 2H, $J = 8.6$ Hz), 6.92–6.96 (m, 5H, ArH), 7.24–7.35 (m, 4H, ArH), 7.88 (d, 2H, $J = 8.6$ Hz); ^{13}C NMR($CDCl_3$): δ 16.78, 41.99, 55.39, 61.51, 68.13, 113.69, 114.00, 117.08, 120.58, 128.22, 128.95, 129.50, 130.78, 131.94, 150.68, 157.16, 161.12, 166.13; ESIMS: 409($M^+ + 1$).

N-[4-(2-Chloroethoxy)-benzyl]-N-(4-methoxy-phenyl)-N′-(1-phenyl-ethylidene)-hydrazone (**4f**) oil, ^{1}H NMR ($CDCl_3$): δ 2.19 (s, 3H, CH_3), 3.89 (s, 3H, OCH_3), 3.90 (t, 2H, $ClCH_2$), 4.25 (t, 2H, OCH_2), 4.63 (s, 2H), 6.97–6.81 (m, 5H, ArH), 7.41–7.28 (m, 6H, ArH), 7.82–7.79 (m, 2H); ESIMS: 409($M^+ + 1$).

N-[4-(2-Chloroethoxy)-benzyl]-N′-(3,4-dihydro-2H-naphthalen-1-ylidene)-N-phenyl-hydrazone(**4g**) oil, ^{1}H NMR ($CDCl_3$): δ 1.62 (m, 2H), 2.17 (t, 2H),2.65 (t, 2H), 2.66 (t, 2H), 3.88 (t, 2H), 4.43 (s, 2H), 6.72–6.87 (m, 3H, ArH), 7.32–7.47 (m, 6H, ArH), 7.80–7.84 (m, 4H); ESIMS: 404 (M^+).

*General procedure for the synthesis of N-substituted hydrazones (**5a–m**)*

N-Phenyl-N′-(1-phenyl-ethylidene)-N-[4-(2-pyrrolidin-1-yl-ethoxy)-benzyl]-hydrazone (**5a**) A solution of **4a** (1.12 mM), pyrrolidine (5 mL) and tetrabutyl ammonium iodide (10 mg) in dry DMF (15 mL) was heated at 70–75 °C with stirring for 7 h. After completion, the solution was cooled and extracted with dichloromethane. The organic phase was washed with water and dried over anhydrous calcium chloride. It was filtered, concentrated and the residue purified on a silica gel column using hexane/EtOAc as eluent to give the pure compound **5a** as colorless oil; yield (69%); ^{1}H NMR ($CDCl_3$): δ 1.80–1.84 (m, 4H), 2.13 (s, 3H), 2.60–2.63 (m, 4H), 2.89 (t, 2H), 4.08 (t, 2H), 4.71 (s, 2H), 6.84 (d, 2H, $J = 8.5$ Hz), 6.90–6.95 (m, 3H, ArH), 7.20–7.29 (m, 4H, ArH), 7.38–7.40 (m, 3H), 7.82–7.85 (m, 2H, ArH); ^{13}C NMR($CDCl_3$): δ 15.73, 22.27, 53.41, 53.79, 61.08, 65.66, 112.98, 116.45, 119.67, 125.32, 126.93, 127.59, 128.10, 128.23, 129.75, 137.11, 149.54, 156.56, 163.56; MS(ESI) *m/z* 414($M^+ + 1$). Anal. calcd for $C_{27}H_{31}N_3O$: C 78.42, H 7.26, N 10.16%. Found: C 78.55%, H 7.30, N10.02%.

N-Phenyl-N′-(1-phenyl-ethylidene)-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5b**) Oil; yield 71%; ^{1}H NMR ($CDCl_3$): δ 1.47–1.49 (m, 2H), 1.61–1.68 (m, 4H), 2.13 (s, 3H), 2.52–2.54 (m, 4H), 2.78 (t, 2H), 4.10 (t, 2H), 4.71 (s, 2H), 6.84 (d, 2H, $J = 8.5$ Hz), 6.90–6.95 (m, 3H, ArH), 7.20–7.29 (m, 4H, ArH), 7.38–7.40 (m, 3H, ArH), 7.82–7.85 (m, 2H); ^{13}C NMR($CDCl_3$): δ 15.73, 22.93, 24.57, 53.75, 56.66, 61.08, 64.46, 113.00, 116.46, 119.68, 125.32, 126.93, 127.59, 128.11, 128.23, 129.23, 129.80, 137.10, 149.59, 156.48, 163.55; MS(ESI) *m/z* 428($M^+ + 1$). Anal. calcd for $C_{28}H_{33}N_3O$: C 78.65, H 7.78, N 9.83%. Found: C 78.96, H 7.89, N 9.67%.

N-Phenyl-N′-(1-phenyl-ethylidene)-N-[4-(3-pyrrolidin-1-yl-propoxy)-benzyl]-hydrazone (**5c**) Oil; yield 65%; ^{1}H NMR ($CDCl_3$): δ 1.74–1.77 (m, 4H), 1.94–1.98 (m, 2H), 2.09 (s, 3H), 2.45–2.49 (m, 4H), 2.61 (t, 2H), 3.99 (t, 2H), 4.67 (s, 2H), 6.78 (d, 2H, $J = 8.4$ Hz), 6.83–6.90 (m, 3H, ArH), 7.16–7.24 (m, 4H, ArH), 7.32–7.36 (m, 3H), 7.76–7.79 (m, 2H); MS(ESI) *m/z* 428($M^+ + 1$). Anal. calcd for $C_{28}H_{33}N_3O$: C 78.65, H 7.78, N 9.83%. Found: C 78.44, H 7.82, N 9.71%.

N-Phenyl-N′-(1-phenyl-ethylidene)-N-[4-(3-piperidin-1-yl-propoxy)-benzyl]-hydrazone (**5d**) Oil; yield 76%; ^{1}H NMR ($CDCl_3$): δ 1.24–1.29 (m, 2H), 1.59–1.65 (m, 4H), 2.51–2.52 (m, 4H), 2.76 (t, 2H, NCH_2), 4.09 (t, 2H, OCH_2), 6.84 (d, 2H, $J = 8.4$ Hz), 6.89–6.96 (m, 3H), 7.20–7.30 (m, 5H), 7.39–7.41(m, 3H), 7.78–7.79(m, 2H); ^{13}C NMR ($CDCl_3$): δ 17.03, 24.54, 26.01, 26.91, 54.68, 56.02, 62.35, 66.27, 96.17, 114.19, 117.70, 120.93, 126.60, 128.22, 128.87, 129.34, 129.51, 130.82, 138.38, 150.80, 158.03, 164.84; MS(ESI) *m/z* 442($M^+ + 1$). Anal. calcd for $C_{29}H_{35}N_3O$: C 78.87, H 7.99, N 9.52%. Found: C 78.97, H 7.68, N 9.76%.

N-Phenyl-N′-(1-phenyl-propylidene)-N-[4-(2-pyrrolidin-1-yl-ethoxy)-benzyl]-hydrazone (**5e**) Oil; yield 68%; ^{1}H NMR ($CDCl_3$): δ 0.82 (t, 3H), 182–1.86 (m, 4H), 2.66–2.90 (m, 4H), 2.92 (t, 2H), 4.13 (t, 2H), 4.68 (s, 2H), 6.90 (d, 2H, $J = 8.4$ Hz), 6.93–7.01 (m, 3H, ArH), 7.25–7.35 (m, 4H, ArH), 7.42–7.44 (m, 3H, ArH), 7.77 (d, 2H, $J = 8.5$ Hz); ^{13}C NMR($CDCl_3$): δ 11.09, 22.83, 23.53, 54.77, 55.16, 61.58, 67.05, 114.36, 117.04, 120.66, 127.26, 128.44, 128.92, 129.72, 130.93, 137.03, 151.28, 157.28, 173.71; MS(ESI) *m/z* 428($M^+ + 1$). Anal. calcd for $C_{28}H_{33}N_3O$: C 78.65, H 7.78, N 9.83%. Found: C 78.46, H 7.92, N 9.68%.

N-Phenyl-N′-(1-phenyl-propylidene)-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5f**) Oil; yield (69%); ^{1}H NMR ($CDCl_3$): δ 0.80 (t, 3H), 1.42–1.46 (m, 2H), 1.59–1.67 (m, 4H), 2.51–2.53 (m, 4H), 2.67 (q, 2H), 2.78 (t, 2H), 4.11 (t, 2H), 4.66 (s, 2H), 6.87 (d, 2H, $J = 8.4$ Hz), 6.89–6.99 (m, 3H, ArH), 7.23–7.33 (m, 4H, ArH), 7.42–7.44 (m, 3H, ArH), 7.75–7.78 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 11.05, 22.82, 24.20, 25.93, 55.08, 57.99, 61.55, 65.91, 114.10, 115.77, 117.01, 120.62, 127.23,

128.41, 128.89, 129.68, 130.91, 137.01, 151.25, 157.84, 173.71; MS(ESI) *m/z* 442 ($M^+ + 1$). Anal. calcd for $C_{29}H_{35}N_3O$: C 78.87, H 7.99, N 9.52%. Found: C 78.76, H 7.82, N 9.44%.

N-Phenyl-N′-(1-phenyl-butylidene)-N-[4-(2-pyrrolidin-1-yl-ethoxy)-benzyl]-hydrazone (**5g**) Oil; yield 62%; ^{1}H NMR ($CDCl_3$): δ 0.73 (t, 3H), 1.21–1.28 (m, 2H), 1.82–1.86 (m, 4H), 2.61–2.66 (m, 6H), 2.92 (t, 2H), 4.13 (t, 2H), 4.67 (s, 2H), 6.89 (d, 2H, $J = 8.4$ Hz), 6.93–6.99 (m, 3H, ArH), 7.27–7.34 (m, 4H, ArH), 7.41–7.43 (m, 3H), 7.74 (d, 2H, $J = 9.4$ Hz); ^{13}C NMR($CDCl_3$): δ 14.18, 20.02, 23.51, 31.71, 54.76, 55.13, 61.59, 67.02, 114.35, 117.06, 120.62, 127.20, 128.40, 128.73, 128.88, 129.66, 130.90, 137.49, 151.25, 157.88, 172.69; MS(ESI) *m/z* 442($M^+ + 1$). Anal. calcd for $C_{29}H_{35}N_3O$: C 78.87, H 7.99, N 9.52%. Found: C 79.11, H 8.05, N 9.34%.

N-Phenyl-N′-(1-phenyl-butylidene)-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5h**) Oil; yield 69%; ^{1}H NMR ($CDCl_3$): δ 0.74 (t, 3H), 0.90–0.92 (m, 2H), 1.21–1.26 (m, 2H), 1.59–1.64 (m, 4H), 2.50–2.52 (m, 4H), 2.62 (t, 2H), 2.76 (t, 2H), 4.08 (t, 2H), 4.63 (s, 2H), 6.84 (d, 2H, $J = 8.5$ Hz), 6.90–6.96 (m, 3H, ArH), 7.20–7.23 (m, 2H, ArH), 7.26–7.30 (m, 2H, ArH), 7.38–7.41 (m, 3H, ArH), 7.73–7.76 (m, 2H, ArH); ^{13}C NMR($CDCl_3$): δ 14.24, 19.99, 24.29, 25.97, 31.56, 55.11, 58.01, 61.99, 65.86, 114.24, 117.32, 120.77, 127.14, 128.29, 128.82, 129.50, 129.62, 130.80, 137.46, 151.42, 157.84, 171.73; MS(ESI) *m/z* 456 ($M^+ + 1$). Anal. calcd for $C_{30}H_{37}N_3O$: C 79.08, H 8.19, N 9.22%. Found: C 79.46, H 8.42, N 9.01%.

N′-[1-(4-Methoxy-phenyl)-ethylidene]-N-phenyl-N-[4-(2-pyrrolidin-1-yl-ethoxy)-benzyl]-hydrazone (**5i**) Oil; yield 69%; ^{1}H NMR ($CDCl_3$): δ 1.80–1.85 (m, 4H), 2.12 (s, 3H), 2.61–2.64 (m, 4H), 2.91 (t, 2H), 3.86 (s, 3H, OCH_3), 4.11 (t, 2H), 4.70 (s, 2H), 6.87 (d, 2H, $J = 8.6$ Hz), 6.90–6.95 (m, 5H, ArH), 7.22–7.31 (m, 4H, ArH), 7.81 (d, 2H, $J = 8.6$ Hz); ^{13}C NMR($CDCl_3$): δ 16.76, 23.50, 54.72, 55.11, 55.37, 61.51, 66.92, 113.65, 114.32, 116.97, 120.43, 128.17, 128.88, 129.41, 130.88, 131.19, 150.72, 157.77, 161.03, 166.10; MS(ESI) *m/z* 456($M^+ + 1$). Anal. calcd for $C_{28}H_{33}N_3O$: C 75.81, H 7.50, N 9.47%. Found: C 75.99, H 7.76, N 9.32%.

N′-[1-(4-Methoxy-phenyl)-ethylidene]-N-phenyl-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5j**) Oil; yield (68%); ^{1}H NMR ($CDCl_3$): δ 1.43–1.48 (m, 2H), 1.59–1.64 (m, 4H), 2.12 (s, 3H), 2.49–2.51 (m, 4H), 2.78 (t, 2H), 3.86 (s, 3H, OCH_3), 4.10 (t, 2H), 4.70 (s, 2H), 6.86 (d, 2H, $J = 8.5$ Hz), 6.90–6.95 (m, 5H, ArH), 7.22–7.31 (m, 4H, ArH), 7.81 (d, 2H, $J = 8.5$ Hz); ^{13}C NMR($CDCl_3$): δ 16.76, 24.19, 25.92, 55.05, 55.37, 57.98, 61.52, 65.88, 113.65, 114.32, 114.47, 116.96, 120.42, 128.17, 128.89, 129.40, 130.88, 131.17, 150.72, 157.76, 161.03, 166.08; MS(ESI) *m/z* 470 ($M^+ + 1$). Anal. calcd for $C_{29}H_{35}N_3O$: C 76.12, H 7.71, N 9.18%. Found: C 76.30, H 7.76, N 9.32%.

N′-(3,4-Dihydro-2H-naphthalen-1-ylidene)-N-phenyl-N-[4-(2-pyrrolidin-1-yl-ethoxy)-benzyl]-hydrazone (**5k**) Oil; yield (69%); ^{1}H NMR ($CDCl_3$): δ 1.74–1.82 (m, 2H), 1.83–1.85 (m, 4H), 2.53 (t, 2H), 2.61–2.66 (m, 4H), 2.77 (t, 2H), 2.92 (t, 2H), 4.11 (t, 2H), 4.71 (s, 2H), 6.86 (d, 2H, $J = 8.5$ Hz), 6.90–6.95 (m, 3H, ArH), 7.15 (d, 1H, $J = 7.5$ Hz), 7.21–7.32 (m, 6H, ArH), 8.37 (d, 1H, $J = 7.5$ Hz); ^{13}C NMR($CDCl_3$): δ 22.24, 23.47, 28.87, 29.77, 54.68, 55.07, 61.83, 66.86, 114.28, 117.35, 120.64, 125.47, 126.26, 128.67, 128.85, 129.32, 129.32, 129.76, 131.19, 140.26, 150.61, 157.73, 164.89; MS(ESI) *m/z* 440($M^+ + 1$). Anal. calcd for $C_{29}H_{33}N_3O$: C 76.73, H 7.51, N 8.95%. Found: C 76.88, H 7.72, N8.52%.

N′-(3,4-Dihydro-2H-naphthalen-1-ylidene)-N-phenyl-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5l**) Oil; yield (69%); ^{1}H NMR ($CDCl_3$): δ 1.45–1.47 (m, 2H), 1.58–1.66 (m, 4H), 1.69–1.78 (m, 2H), 2.51–2.55 (m. 6H), 2.75–2.79 (m, 4H), 4.09 (t, 2H), 4.70 (s, 2H), 6.83 (d, 2H, $J = 8.5$ Hz), 6.87–6.95 (m, 3H, ArH), 7.14 (d, 1H, $J = 7.5$ Hz), 7.29–7.32 (m, 3H, ArH), 8.35 (d, 1H, $J = 7.5$ Hz); MS (ESI) *m/z* 454($M^+ + 1$). Anal. calcd for $C_{30}H_{35}N_3O$: C 76.96, H 7.71, N 8.69%. Found: C 76.74, H 7.58, N8.42%.

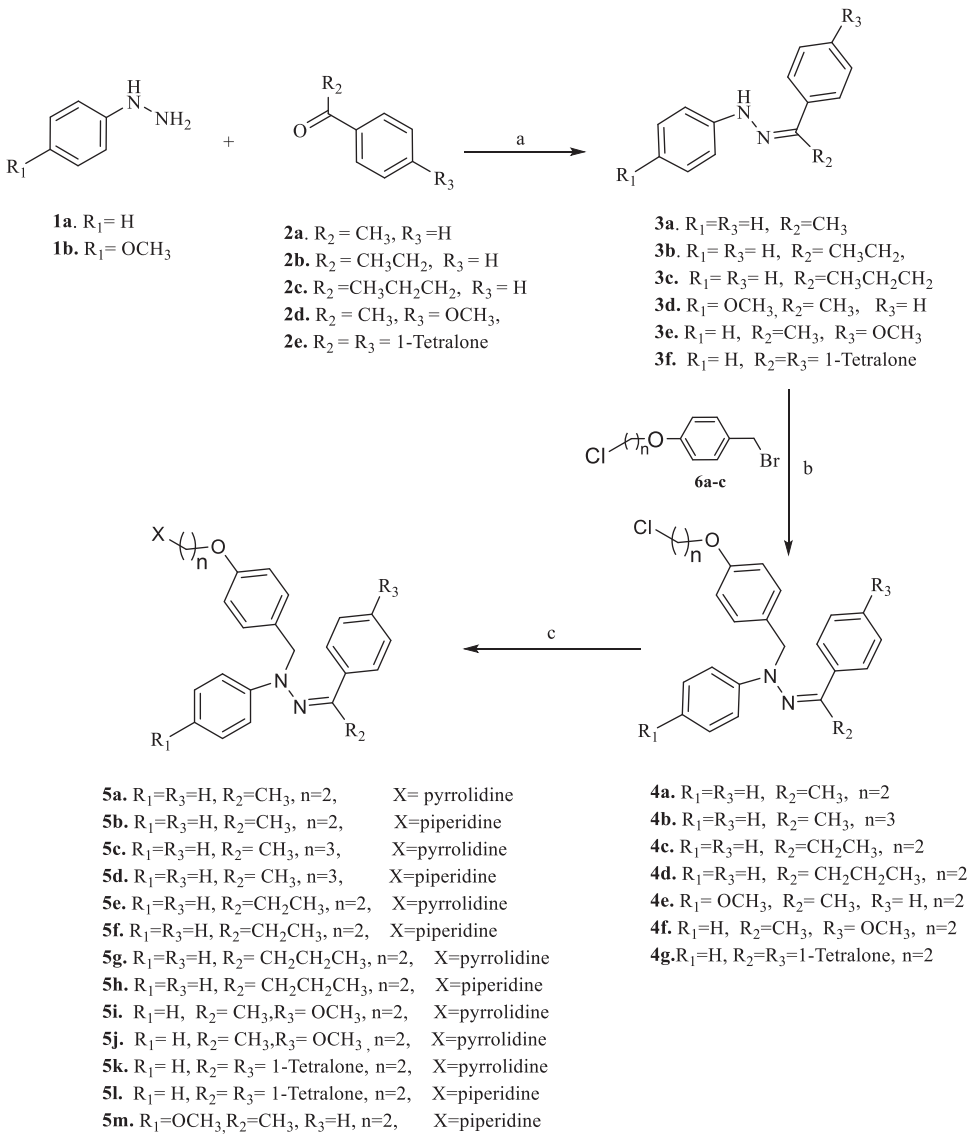
N-(4-Methoxy-phenyl)-N′-(1-phenyl-ethylidene)-N-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-hydrazone (**5m**) Oil; yield 64%; ^{1}H NMR ($CDCl_3$): δ 1.47–1.51 (m, 6H), 2.42–2.45 (m, 4H), 2.76 (t, 2H), 3.88 (s, 3H, OCH_3), 4.08 (t, 2H, OCH_2), 4.68 (s, 2H), 6.84 (d, 2H, $J = 8.5$ Hz), 6.90–6.93 (m, 5H, ArH), 7.25–7.29 (m, 4H, ArH), 7.79 (d, 2H, $J = 8.5$ Hz); MS(ESI) *m/z* 458($M^+ + 1$). Anal. calcd for $C_{29}H_{35}N_3O_2$: C 76.12, H 7.71, N 9.18%. Found: C 76.45, H 7.98, N 9.06%.

Result and Discussion

Chemistry

The proposed compounds were synthesized as outlined in the Scheme 1. Phenyl hydrazones (**3a–f**) were prepared by heating a mixture of phenyl hydrazines (**1a** and **b**) with acyclic or cyclic ketones (**2a–e**) under acid catalyzed conditions in excellent yields. Since the hydrazones were unstable and decomposed rapidly, they were used in situ for N-alkylation with 4-(ω-chloroalkyl) benzyl bromides in the presence of NaH/DMF at 0 °C under nitrogen atmosphere to

Scheme 1 Reagents and conditions: **a** $CH_3COOH/EtOH$, **b** NaH/DMF, **c** TBAI, DMF pyrrolidine/piperidine



1a. R_1= H
1b. R_1= OCH_3

2a. R_2 = CH_3, R_3 =H
2b. R_2 = CH_3CH_2, R_3 = H
2c. R_2 =$CH_3CH_2CH_2$, R_3 = H
2d. R_2 = CH_3, R_3 = OCH_3,
2e. R_2 = R_3 = 1-Tetralone

3a. R_1=R_3=H, R_2=CH_3
3b. R_1= R_3= H, R_2= CH_3CH_2,
3c. R_1= R_3= H, R_2=$CH_3CH_2CH_2$
3d. R_1= OCH_3, R_2= CH_3, R_3= H
3e. R_1= H, R_2=CH_3, R_3= OCH_3
3f. R_1= H, R_2=R_3= 1-Tetralone

4a. R_1=R_3=H, R_2=CH_3, n=2
4b. R_1=R_3=H, R_2= CH_3, n=3
4c. R_1=R_3=H, R_2=CH_2CH_3, n=2
4d. R_1=R_3=H, R_2= $CH_2CH_2CH_3$, n=2
4e. R_1= OCH_3, R_2= CH_3, R_3= H, n=2
4f. R_1=H, R_2=CH_3, R_3= OCH_3, n=2
4g.R_1=H, R_2=R_3=1-Tetralone, n=2

5a. R_1=R_3=H, R_2=CH_3, n=2, X= pyrrolidine
5b. R_1=R_3=H, R_2=CH_3, n=2, X=piperidine
5c. R_1=R_3=H, R_2= CH_3, n=3, X=pyrrolidine
5d. R_1=R_3=H, R_2= CH_3, n=3, X=piperidine
5e. R_1=R_3=H, R_2=CH_2CH_3, n=2, X=pyrrolidine
5f. R_1=R_3=H, R_2=CH_2CH_3, n=2, X=piperidine
5g. R_1=R_3=H, R_2= $CH_2CH_2CH_3$, n=2, X=pyrrolidine
5h. R_1=R_3=H, R_2= $CH_2CH_2CH_3$, n=2, X=piperidine
5i. R_1=H, R_2= CH_3,R_3= OCH_3, n=2, X=pyrrolidine
5j. R_1= H, R_2= CH_3,R_3= OCH_3, n=2, X=pyrrolidine
5k. R_1= H, R_2= R_3= 1-Tetralone, n=2, X=pyrrolidine
5l. R_1= H, R_2= R_3= 1-Tetralone, n=2, X=piperidine
5m. R_1=OCH_3,R_2=CH_3, R_3=H, n=2, X=piperidine

give the desired triaryl substituted hydrazone framework (**4a–g**). To convert these hydrazones into potential estrogen antagonists or ER modulators, tert.amino groups, the most essential determinant known to impart mixed agonist/antagonist activity were introduced by substituting the chloride group with secondary amines such as piperidine or pyrrolidine in the presence of the catalytic quantity of tetrabutylammonium iodide (TBAI) forming the target molecules in good yields (**5a–m**).

In-vitro anti-proliferative activity

Antagonistic action on ER in breast cancer cells results in the inhibition of its estrogen-induced proliferation. Therefore, in order to evaluate our hypothesis for synthesizing novel hydrazones as ER-targeting anti-cancer agents, the molecules were screened against functional ER bearing breast and uterine cancer cells. Triaryl substituted hydrazones (**4a–i)** without the basic amino functionality were found to be inactive against both the cancer cell lines, however, subsequent replacement of terminal halogen with basic amino groups like piperidine or pyrrolidine rendered these hydrazones (**5a–m**) significantly active, showing anti-proliferative activity against the cancer cell lines. Of all the hydrazones tested, anti-proliferative activity against ER-positive breast cancer cell line michigan cancer foundation-7 (MCF-7) was highest for **5e** (IC_{50}: 7.8 μM) followed by **5i** (IC_{50}: 8.4 μM) > **5d** (IC_{50}: 9 μM) > **5f** (IC_{50}: 11 μM) > **5m** (IC_{50}: 11.8 μM) > **5a** (IC_{50}: 16 μM), rest of the compounds were either inactive or moderately active (Table 1).

When screened against ER−ve MD Anderson metastatic breast-231 (MDA-MB-231) cancer cell line, compound **5i** was found to be the most active (IC_{50}: 4.7 μM). Other compounds showing significant activity were

Table 1 Physical characteristics and anti-proliferative activity of N-substituted hydrazones **5a–m**

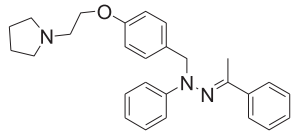
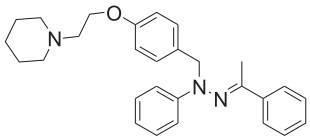
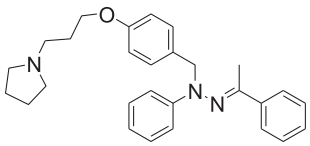
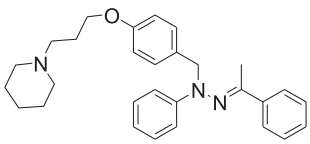
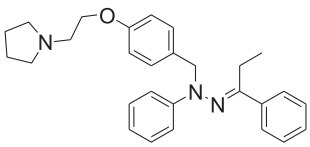
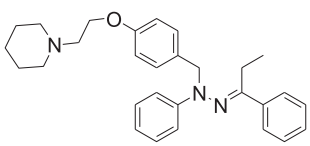
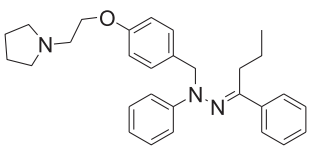
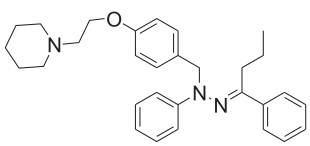
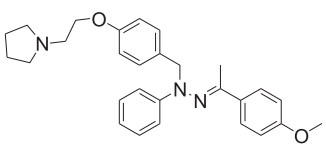
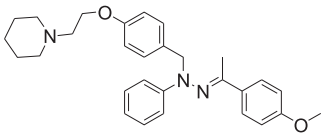
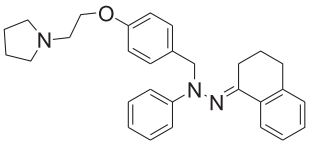
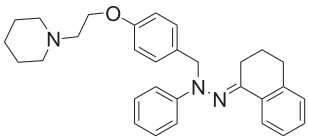
Compd. No	Molecular formula	Molecular Mass	Structure	IC_{50} (μM)			
				MCF-7	MDA-MB-231	Ishikawa	HEK-231
5a	$C_{27}H_{31}N_3O$	413.25		16	16	NA	16
5b	$C_{28}H_{33}N_3O$	427.26		NA	NA	NA	NA
5c	$C_{28}H_{33}N_3O$	427.26		NA	NA	NA	NA
5d	$C_{29}H_{35}N_3O$	441.28		9	8	14.7	28.9
5e	$C_{28}H_{33}N_3O$	427.26		7.8	6.2	7.3	23.3
5f	$C_{29}H_{35}N_3O$	441.28		11	13	18	24.7
5g	$C_{29}H_{35}N_3O$	441.28		NA	NA	NA	14.7
5h	$C_{30}H_{37}N_3O$	455.29		NA	NA	NA	NA
5i	$C_{28}H_{33}N_3O_2$	443.26		8.4	4.7	6	17
5j	$C_{29}H_{35}N_3O_2$	457.27		NA	8.8	19	NA

Table 1 continued

Compd. No	Molecular formula	Molecular Mass	Structure	IC_{50} (μM)			
				MCF-7	MDA-MB-231	Ishikawa	HEK-231
5k	$C_{29}H_{33}N_3O$	439.26		11.8	9.3	NA	14
5l	$C_{30}H_{35}N_3O$	453.28		NA	7	15	15
5m	$C_{29}H_{35}N_3O_2$	457.27		NA	NA	NA	NA
Tamoxifen				6	11	27	13

NA not active up to 100 μM

5e (IC_{50}: 6.2 μM) > **5k** (IC_{50}:7 μM) > **5d** (IC_{50}: 8 μM) > **5j** (IC_{50}: 8.8 μM) > **5m** (IC_{50}: 9.3 μM) > **5f** (IC_{50}:13 μM) and **5a** (IC_{50}: 16 μM). Since MDA-MB-231 is an ER-negative cell line, significant activity of **5i** and **e** indicated the possibility of these compounds exerting the inhibitory effect through some target other than ER. Further, screening of compounds in uterine cancer Ishikawa cell line, **5i** again showed considerable activity (IC_{50}: 6 μM) followed by compounds, **5e** (IC_{50}: 7.3 μM) > **5d** (IC_{50}: 14.7) > **5k** (IC_{50}: 15) > **5f** (IC_{50}: 18) > **5j** (IC_{50}:19). In order to confirm if the cytotoxic effects of the compounds were specific to cancer cells only, they were also tested for their cytotoxicity in normal non-cancer specific human kidney epithelial cell line HEK-293, wherein, compounds, **5d** (IC_{50}: 18.9 μM), **5k** (IC_{50}: 14 μM), **5g** (IC_{50}: 14.7 μM), **5l** (IC_{50}: 15 μM) and **5a** (IC_{50}: 16 μM) were found to be cytotoxic to HEK-293 cells, whereas, **5e** (IC_{50}: 23.3 μM) and **5i** (IC_{50}: 27 μM) did not exhibit any significant cytotoxicity towards normal cell line. Further, it is also interesting to note that of the two active compounds, **5e** is antagonistic on both the cell types, MCF-7 cells (IC_{50}: 7.8 μM) and Ishikawa cells (IC_{50}: 7.3 μM) whereas, compound **5i** was selectively most active against ER-negative MDA-MB 231 cells (IC_{50}: 4.7 μM). Following the similarity of triaryl substituted hydrazone pharmacophore as structural acyclic proptotype of triaryl pyrazole and tamoxifen, we decided to focus our initial structure–activity relationship exploration efforts around the triaryl hydrazone moiety with differently substituted phenyl rings. A change in the structure of the alkyl group at position-1 from phenyl-ethylidene to phenyl-propylidene to phenyl-butylidene and to 3,4-dihydro-2H-napthalen-1-ylidene resulted in minor change in the anti-proliferative activity. Increasing the length of carbon chain from methyl to ethyl or propyl caused an increase in the activity of ER +ve MCF cells (IC_{50}: 7.8 μM) and Ishikawa cells (IC_{50}: 7.3 μM), however, a decrease in the activity was observed for ER-negative MDA-MB (231) cells (**5i**, IC_{50}: 4.7 μM). Replacing the 5-membered pyrrolidine ring to 6-membered piperidine ring in the basic side chain (**5e** to **5f**) led to a decrease in the anti-proliferative activity against both the cell lines. An exploration of the structure activity relationship study indicated that oxyethyl-pyrrolidine substituted hydrazones (**5e** and **i**) showed better inhibitory effect as compared to the oxyethyl-piperidine group (**5d** and **f**, Table 1).

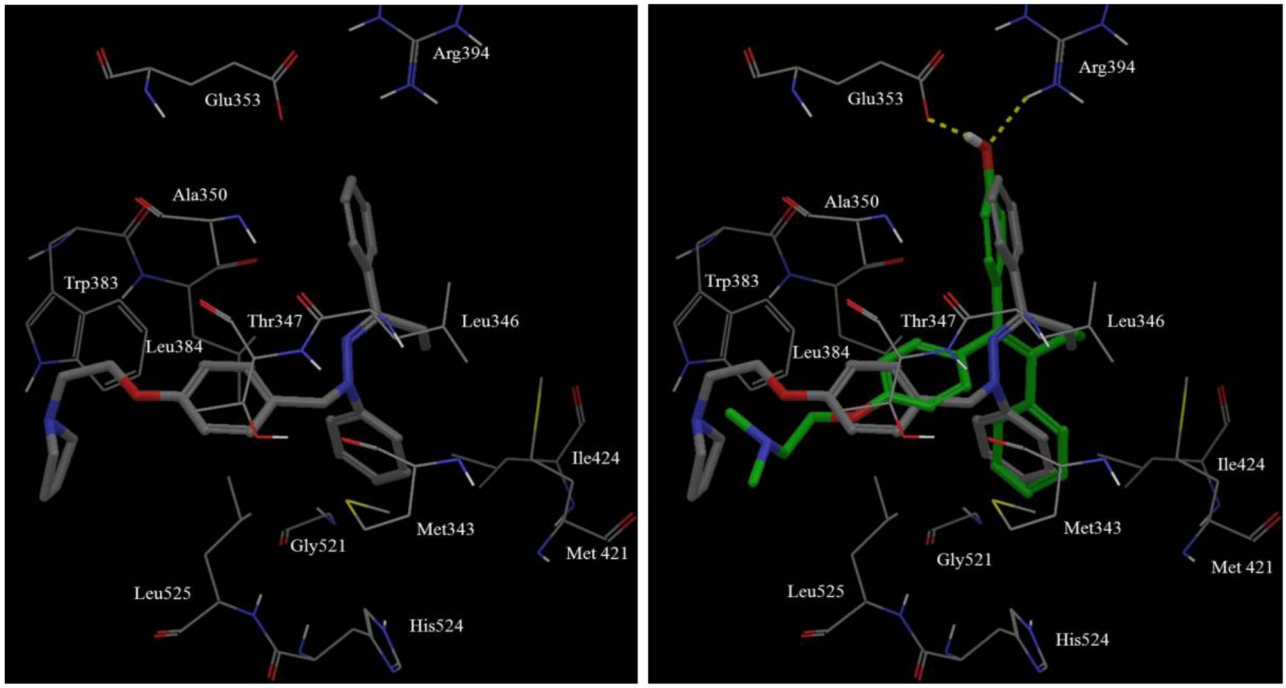


Fig. 3 Docking pose of most active compound **5e** (**a**) and comparative docking orientation and receptor interactions of **5e** (*gray*), 4-hydroxytamoxifen (OHT; *green*) (**b**) and ERα (PDB Code: 3ERT). *Yellow dotted lines* are hydrogen bonding interactions (<2.5 Å)

Molecular docking studies

To rationalize the activity profile in terms of interaction with ER (ERα), the molecular modeling study was carried out to obtain an insight into the molecular mechanism of compound **5e** by comparing docking of OHT and computationally energy minimized conformation of active molecule **5e** using the Schrodinger suite. The crystal structure of ERLBD in complex with 4-hydroxytamoxifen and reference protein coordinates used for protein preparation were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/; PDB ID: 3ERT) and further modified for Glide docking calculations. Figure. 3a, b, shows the orientation and interaction of the active molecule **5e** within the ER cavity in comparison to 4-hydroxy tamoxifen.

As is evident from Fig. 3a, the compound **5e** with its three aryl rings A, B and C (Fig. 2) occupies the same pocket that recognizes OHT, also the orientation of the aryl ring A of **5e** is in the same location as the ring A of OHT with its phenolic hydroxyl forming hydrogen bond with the side chains of Glu-353 and Arg-394. However, absence of any hydroxyl group or a hydrogen bond donor/acceptor group at the 4th position of the aryl ring A of **5e** results in the loss of this crucial H-bond interaction with Glu-353 and Arg-394, which is reflected in weak binding interaction with ERα as seen in (Fig.3b). Further, it was also observed that the binding orientation of pyrrolidino ethyl side chain of **5e** is comparable to that of the bulky dimethylaminoethyl side chain of OHT and occupies the nearly the same space within the cavity of ERα, though it is slightly bent outwards due to an additional benzylic (CH_2) group causing an increase in the length. Outward deviation of the C-aryl ring and unfavorable orientation of amino chain of **5e** from that of tamoxifen (Fig. 3b) may be responsible for weak binding interaction of the ligand with the salient amino acids essential for receptor binding affinity. The ring B of compound **5e** makes contacts with the residues (Met-343, Leu-346, Met-421, Ile-424, Gly-521, His-524 and Leu-525), most of which also interact with the ring B of OHT. These favorable interactions and the similarity in orientation of aryl rings A, B and C and the basic amino chains of 4-OHT over that of **5e** at the aryl ring C, conform for the antagonistic profile shown in MCF-7, MDA-MB-231 and Ishikawa cell lines.

Conclusion

The biological activity results show, that compound **5e** is comparable in its antagonistic activity to tamoxifen against MCF-7, MDA-MB-231 and Ishikawa cell lines, which is reflected in the docking studies also, but a slight increase in length due to an additional methylene group causes

unfavorable twisting and weak binding interactions with salient amino acids. Structural modification of the active compounds with insertion of groups and optimization of chain length and its orientation for specific binding interactions within the receptor cavity is in progress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Agatonovic-Kustrin S, Turner JV (2008) Molecular structural characteristics of estrogen receptor modulators as determinants of estrogen receptor selectivity. Mini Rev Med Chem 8:943–951

Kumar P, Narasimhan B (2013) Hydrazides/hydrazones as antimicrobial and anticancer agents in the new millennium. Mini Rev Med Chem 13:971–987

LaFrate AL, Gunther JR, Carlson KE, Katzenellenbogen JA (2008) Synthesis and biological evaluation of guanylhydrazone coactivator binding inhibitors for the estrogen receptor. Bioorg Med Chem 16:10075–10084

Morgan LR, Thangaraj K, LeBlanc B, Rodgers A, Wolford LT, Hooper CL, Fan D, Jursic BS (2003) Design, synthesis, and anticancer properties of 4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone and analogues. J Med Chem 46:4552–4563

Pandey J, Pal R, Dwivedi A, Hajela K (2002) Synthesis of some new diaryl and triaryl hydrazone derivatives as possible estrogen receptor modulators. Arzneimittelforschung 52:39–44

Shankar R, Chakravarti B, Singh US, Ansari MI, Deshpande S, Dwivedi SKD, Bid HK, Konwar R, Kharkwal G, Chandra V, Dwivedi A, Hajela K (2009) Synthesis and biological evaluation of 3,4,6-triaryl-2-pyranones as a potential new class of anti-breast cancer agents. Bioorg Med Chem 17:3847–3856

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL (1988) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937

Stauffer SR, Coletta CJ, Tedesco R, Sun J, Katzenellenbogen JA (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. J Med Chem 43:4934–4947

Stauffer SR, Huang YR, Aron ZD, Coletta CJ, Sun J, Katzenellenbogen BS, Katzenellenbogen JA (2001) Triarylpyrazoles with basic side chains: development of pyrazole-based estrogen receptor antagonists. Bioorg Med Chem 9:151–161

Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA, Katzenellenbogen BS (1999) Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-alpha or estrogen receptor-beta. Endocrinology 140:800–804

Singh US, Shankar R, Yadav GP, Kharkwal G, Dwivedi A, Keshri G, Singh MM, Moulik PR, Hajela K (2008) Synthesis and structure guided evaluation of estrogen agonist and antagonist activities of some new tetrazolyl indole derivatives. Eur J Med Chem 43:2149–2158