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Original article

Design, synthesis and anticancer screening of 3-(3-(substituted phenyl) acryloyl)-2H-chromen-2ones as selective anti-breast cancer agent

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ABSTRACT

By utilizing concept of molecular hybridization, involving combination of various Pharmacophore, novel substituted coumarin-chalcone hybrids was synthesized and evaluated for anti-proliferative activity against estrogen receptor-positive MCF-7 and negative MDA-MB-435 breast cancer cell lines. *In-vivo* study was carried out by N-methyl nitrosourea (MNU) induced mammary carcinoma in virgin female Spraque Dawly (SD) rats. The compound **5b** has highest potential than standard drug Adriamycin, comparable against Tamoxifen against ER-positive MCF-7 breast cancer cell lines. Docking study was performed to study the binding orientation and affinity of synthesized compounds on the ER-α enzyme.

1. Introduction

Despite immense advances in the field of basic and clinical research of new aggressive therapeutics, breast cancer, still remains a leading killer among women worldwide. Estrogens are well known to play crucial role in breast cancer development. ER-α is well characterized as a mediator of cell proliferation in breast cancer cells [1]. Tamoxifen (TAM) is the first selective estrogen receptor modulator (SERM) approved for the prevention of breast cancer [2].

Due to the development of drug resistance, undesired side effects, relapses and recurrences of cancer, there is a need to develop safe, potent and tissue selective anti-breast cancer agents with novel mode of action [3,4]. For many years important research studies have been devoted to natural phytoestrogens and to their synthetic analogues. These derivatives can bind to the ERs and lead to agonist or antagonist activities.

Coumarins are heterocyclic organic compounds known as benzo-2-pyrone derivatives. It is an important group of natural occurring products having varied activities [5]. The biological activities include anticoagulation, antibiotic, antifungal, antipsoriasis, antitumor, anti-HIV, anti-inflammatory [6].

Among diverse biological activities of coumarin the most intriguing bioactivity is effective against breast cancer. The literature reveals that coumarin containing compound neotanshin lactone which is 10 fold more potent and 20 fold more selective than TAM against estrogen receptor positive human breast cancer [7]. Several coumarin derivatives have been identified as nuclear hormone receptor modulator [8]. Chromane derivatives were found to possess Raloxifene like activity on serum cholesterol and bone mineral density, which explains beneficial estrogenic effect of coumarin containing compound [9].

Chalcones (1,3-diphenyl-2-propen-1-ones) considered as the precursor of flavonoids and isoflavonoids are widely distributed in nature from ferns to higher plants [10]. Chalcone derivatives are associated with some important biological activity such as antitubercular, anthelmintic, fungicidal, antioxidant, antimalarial, anti-inflammatory, anti-cancer agents and anti-tumouragent [11–14].

Both coumarin and chalcone are chemopreventive agents. Their chemopreventive efficacy includes their ability to interrupt or reverse the carcinogenesis process by acting on intracellular signalling network molecules involved in the initiation and or promotion of cancer or their potential to arrest or reverse the progression stage of cancer. They may also trigger apoptosis in cancerous cells through modulation of a number of key elements in cellular signal transduction pathways linked to apoptosis [15].

In recent years, combination chemotherapy was adopted to treat cancer. A single molecule containing more than one

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pharmacophore, each with different mode of action could be beneficial for the treatment of cancer [16].

In view of these observations, it was thought of interest to combine different pharmacophore to produce novel compounds with effective breast cancer treatment. This strategy has resulted in a combination of pharmacophoric moieties of different bioactive substances such as coumarin, chalcone and amine side chain [17] led us to discover a novel class of substituted coumarin-chalcone hybrids (Fig. 1) which could be with an improved affinity, efficacy and modified selectivity profile with different mode of action and reduce undesirable side effects.

2. Experimental section

2.1. Chemistry

Melting points were recorded in open capillaries with electrical melting point apparatus. IR spectra of all synthesized compounds in KBr were recorded using a (JASCO FT-IR 4000) spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on the Bruker Advance (400 MHz) Spectrometer in $CDCl_3$ solutions, with TMS as an internal reference. Mass spectra were recorded on a Varian Inc, 410 Prostar Binary LC with 500 MS IT PDA Detectors. All the reagents and solvents used were of analytical grade.

2.2. Synthesis of 3-acetyl-2H-chromen-2-one [18] (1)

To a cold mixture of salicylaldehyde (2.1 ml, 20.051 mmol), ethyl acetoacetate (3.16 ml, 25.064 mmol) in 2.5 ml absolute ethanol, 1.0 ml piperidine was added drop wise. It was stirred vigorously on a magnetic stirrer. By continuing the stirring mixture started thickening, to it was added 2.5 ml absolute ethanol in order to facilitate stirring. After 30 min, added 5 ml absolute ethanol and stirred vigorously for further 30 min. Then 50 ml of ethanol was added and warmed just to dissolve fine particles. It was then allowed to crystallize into needle by cooling in freezer for 10–15 min. Then washed with cold ethanol and recrystallized with 100 ml distilled water to get cream colour needles crystals m. p. = 124–126 °C, yield = 2.07 g.

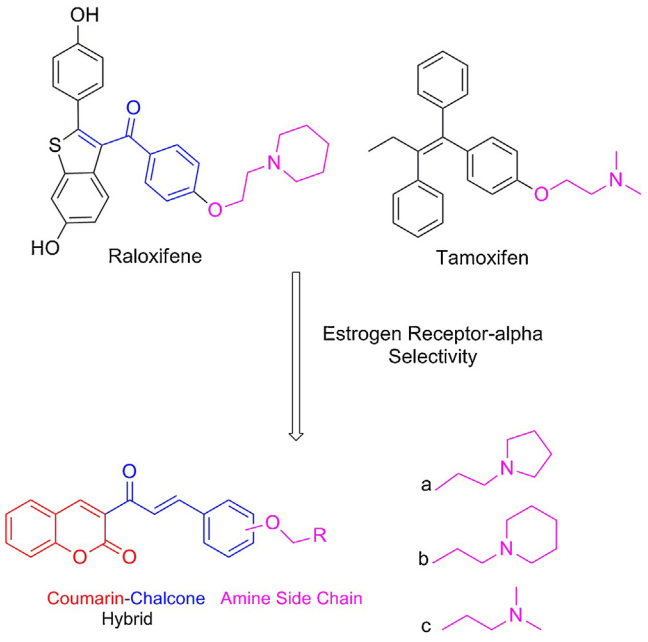


Fig. 1. Designed pharmacophor of substituted coumarin–chalcone hybrids.

2.3. General procedure for synthesis of substituted p-hydroxy chalcone [19] (2–4)

An equimolar mixture of 3-acetyl-2H-chromen-2-one, substituted benzaldehydes and KOH (2 mmol) was stirred in PEG-400 (15 ml) at 40 °C for 2–3 h. After the completion of the reaction (monitored by TLC), the crude mixture was worked up with ice-cold water (100 ml). The resultant product was separated out and recrystallized from absolute ethanol.

2.4. General procedure for the synthesis of 3-(3-(substituted phenyl) acryloyl)-2H-chromen-2ones [20] (**5a–7c**)

A mixture of substituted chalcone (0.625 mmol), anhydrous K_2CO_3 (3.12 mmol), amino hydrochloride chain (0.93 mmol), and dry acetone (10 ml) was refluxed for 24 h. K_2CO_3 was filtered off and acetone was distilled out. The residue was diluted with water and extracted with ethyl acetate. The organic layer was washed with water, brine, and dried over anhydrous Na_2SO_4. The precipitate was recrystallized from absolute ethanol.

2.5. In-vitro *anticancer screening*

In vitro testing done using SRB assay protocols [21], each drug is tested at 4 dose levels (1×10^{-7} M, 1×10^{-6}M, 1×10^{-5} M, 1×10^{-4} M, or 10, 20, 40, 80 μg/ml). Appropriate positive controls are run in each experiment and the experiment is repeated thrice. Results are given in terms of GI_{50}, TGI and LC_{50} values.

2.6. In-vivo *anticancer screening*

Female virgin SD rats were obtained from Wockhardt Pvt. Ltd. (Aurangabad) at 35 days of age. Rats were housed at 3 per cage and maintained at (25 ± 2) °C under 12 h dark/light cycle with access to standard diet and water *ad libitum*.

The NMU was purchased from Sigma (USA). An aqueous solution at a concentration of 10 mg/ml was made by wetting the NMU powder with 3% acetic acid and then dissolving it in 0.9% NaCl solution; a fresh solution was prepared for each injection. Rats were given intra peritoneal (i.p.) 50 mg/kg of NMU on the 50th and 57th day. Animals were divided into different groups with six animals in each group. Group I (intact control) received 0.9% NaCl solution Groups II–V was introduced with MNU. Two weeks after MNU treatment, animals were treated with synthesized compounds (5 mg/kg) and TAM (10 mg/kg in 1% Tween 80) by gavage once per day for six weeks. Animals in intact control group and untreated MNU group were given vehicle (Tween 80) according to experimental protocol. After MNU treatment the animals were palpated weekly to detect the presence of mammary tumors. MNU yields a high incidence of estrogen receptor positive mammary tumors. The time of appearance of first tumor (latency period), the number of tumors/rat (tumor burden) and relative size of every tumor was recorded. The tumor diameter was measured by micrometer caliper, and volume was calculated using the formula:

$$V = 4/3\ \pi\ r^3$$

Where, r is half of the average diameter.

After completion of treatment, blood was collected from retro orbital puncture and analyzed for estrogen level measurement.

2.7. Histopathological studies

Tumors larger than 0.5 cm in diameter were removed from the mammary gland and washed with ice cold buffered saline solution (pH 7.4) and preserved in 4% formaldehyde in 0.1 mol/l phosphate

buffer solution (PBS, pH 7.4). They were fixed overnight in 10% phosphate buffered formalin, dehydrated in alcohol, cleared of fat with toluene, rehydrated and tumor sections (5 μm) stained with H&E.

2.8. Docking study

In order to explore the main interactions with the target ER-α receptor of the chalcone derivatives was subjected to molecular docking studies using Glide v5.8 (Schrödinger, LLC). The coordinates for ER-α receptor was taken from RCSB Protein Data Bank and prepared for docking using protein preparation wizard. Water molecules in the structures were removed and termini were capped by adding ACE and NMA residue. The bond orders and formal charges were added for hetero groups and hydrogens were added to all atoms in the structure. Side chains that were not close to the binding cavity and do not participate in salt bridges were neutralized. After preparation, the structures were refined to optimize the hydrogen bond network using OPLS_2005 force field. The minimization was terminated when the energy converged or the RMSD reached a maximum cutoff of 0.30 Å. Grids were then defined around refined structure by centering on ligand using default box size. The extra precision [22] (XP) docking mode for compounds, optimized by Ligprep, was performed on generating grid of protein structure. The results of molecular docking studies indicated an acceptable reliability of the parameters specified in Maestro-Glide in reproducing the binding mode for these compounds.

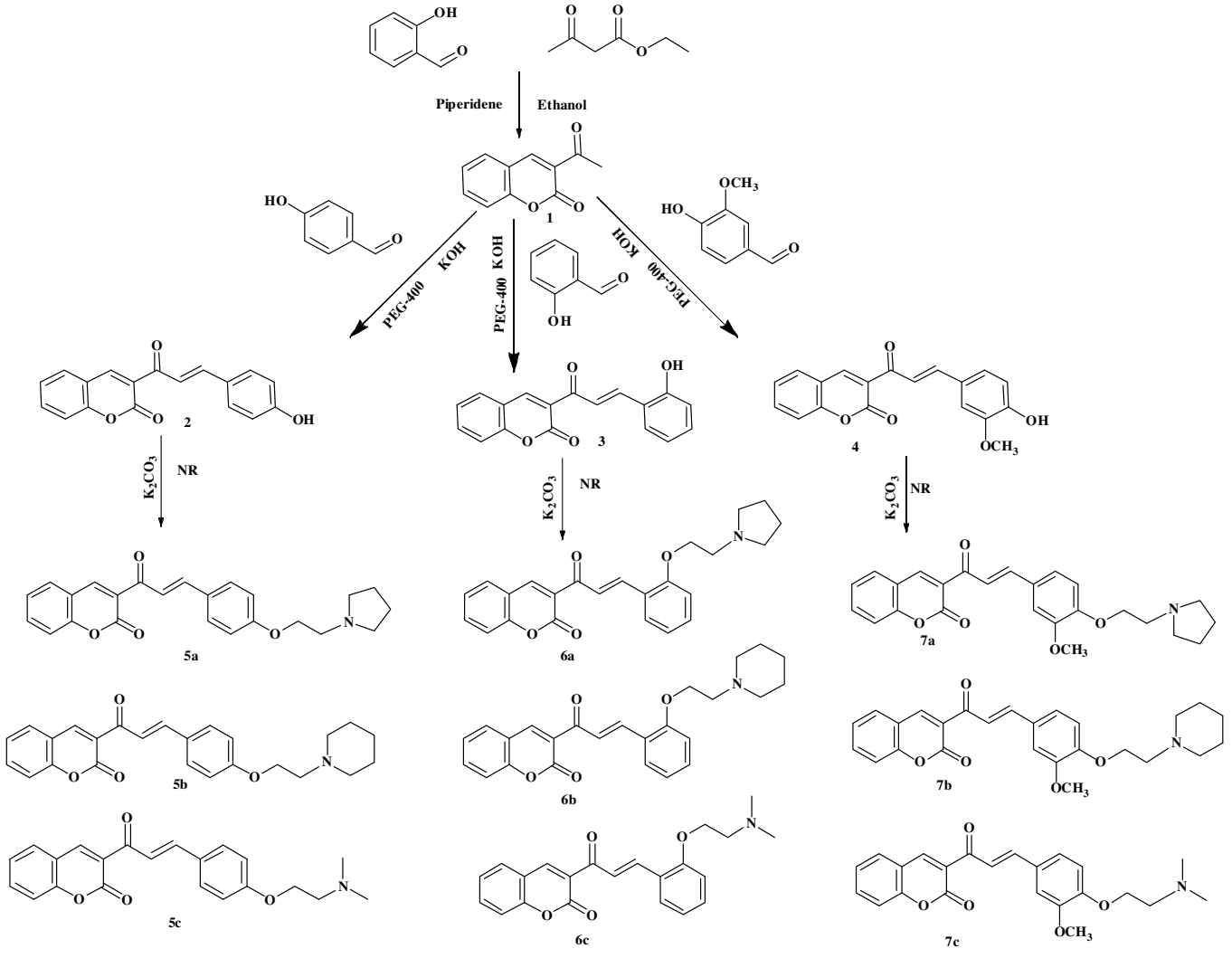
In order to better describe the possible binding modes of chalcone compounds with hER-α, hER-α ligand-binding domain (LBD) with non-steroidal antagonists, *i.e.* raloxifene (RAL) was used. The crystal structure of hER-α LBD (PDB entry: 1ERR) was obtained from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/). To identify the binding modes for the chalcone compounds, the docked states (ligand conformation and orientation relative to the estrogenic receptor; hereafter referred to as poses) of each compound were compared.

3. Result and discussion

3.1. Chemistry

We describe herein a convenient approach to the synthesis of 3-acetylcoumarin (**1**), intermediate hydroxychalcones(**2, 3, 4**) and the target compound 3-(3-(3–4-subtitutedphenyl) acryloyl)-2H-chromen-2one (**5, 6, 7**) [as shown in Scheme 1].

The key reactions involved in the formation of compound **1** are Knoevenagel condensation reaction, the intermediate formation of



Scheme 1. Synthesis of designed compounds.

Chalcone is based on Claisen-Schmidt condensation on 3-acetylcoumarin with three different aldehydes (4-hydroxybenzaldehyde,2-hydroxy benzaldehyde and 4-hydroxy-3-methoxy benzaldehydes). The synthesis of target compounds involves alkylation of chalcones.

3.2. Biological evaluation

The target compounds were evaluated for their anti-breast cancer activity against the MCF-7 and MDA-MB-435 cell line and Adriamycin and Tamoxifen was taken as the standard. LC_{50}, TGI and GI_{50} value of *in-vitro* anticancer activity profile was shown in Table 1.

3.3. Structural activity relationship

The results from *in-vitro* screening suggest that, anti-proliferative activity increases with newly synthesized compounds substituted with amine side chain containing piperidine ring. The compounds **5b, 6b, 7b** were found to be more active against the MCF-7 than MDA-MB-435 cell line. The highest anti-proliferative activity with GI_{50} value below 10 μg/ml obtained with compound **5b**.

3.4. In-vivo *anticancer screening*

It is observed that administration of synthesized compounds shows the protective effect on mammary tumorigenesis. The animals treated with **5b, 6b, 7b** prolonged the latency, reduced tumor burden and volume compared to that of MNU treated rats. The effect of treatment on tumor latency, burden and volume is given in Table 2. Histomorphological examination of rat mammary gland revealed that the normal architecture was disturbed by carcinogen. Rat mammary gland adenocarcinoma and tumor response to treatment is given in Fig. 3.

Table 1
In-vitro anticancer activity (μg/ml) of synthesized compounds.

Comp No.	MCF-7			MDA-MB-435		
	LC_{50}	TGI	GI_{50}	LC_{50}	TGI	GI_{50}
5a	>80	>80	65.2	>80	>80	>80
5b	74.5	40.2	<10	>80	78.2	75.3
5c	>80	>80	>80	>80	>80	>80
6a	>80	>80	>80	>80	>80	>80
6b	>80	62.9	18.9	>80	77.1	>80
6c	>80	>80	38.8	>80	>80	>80
7a	>80	70.6	35.1	>80	>80	>80
7b	>80	>80	32.5	>80	>80	54.2
7c	>80	>80	>80	>80	>80	>80
ADR	>80	54.5	<10	>80	61.8	<10
TAX	29.4	11.2	<10	54.2	21.5	<10

3.5. Detection of estrogen level

Hormone concentrations measured in rat serum on days 30 and 45 of intoxication were compared with the control and MNU group values which shows prominent selectivity on estrogen receptor. Mean of the concentrations of all groups is depicted in Table 3. The result shows that the compound **5b** has selectivity as standard TAM (tamoxifen) towards estrogen receptor. All the synthesized compounds show marked antagonistic activity.

3.6. Docking study

The level of ER-α inhibition and anti-proliferative activities of synthesized compounds prompted us to perform molecular docking studies to understand the ligand–protein interactions in detail. Docking of compounds was done with ER-α and the results not only validated our docking protocol but also established the following findings. The docking studies showed that the compounds **5–7(a–c)** were binding to the binding site of Raloxifen in the co-activator groove. Amongst the analogues synthesized significant interactions were seen in a few cases. Foremost amongst them was compound **5b**. The comparison of compound **5b** docked with ER-α shows that it has a binding site similar to RAL in the co-activator groove (Fig. 2**a–c**).

In general, it is observed that the coumarin fragment of the compounds fitted into the cavity formed by Phe404, Glu353, Leu387, Leu428, Met388 and Met421 while the other amine side chain fitted into the other cavity formed by Thr347, Asp351, Trp383, Met543, Leu536 and Met528. Compound **5b** produces a deep, moving into the hydrophilic pocket of ER-α with which the coumarin and 4-ethoxy piperidine side chain fragments are able to reach the hydrophilic pocket and was involved in strong hydrogen bonding with Asp351 (C=O···NH; 2.74 Å) as similar to that of RAL and TAM. In addition to this compound **5b** shows π-π stacking interaction with Phe404 which was also observed in the TAM interaction (Fig. 2**a andc**). Substitution/removal of the N-substituted piperidine/pyrrolidine ring by an alkyl group in the side chain resulted in weaker binding affinity and no significant interactions with the receptor were observed. The compound **5b** shows the same binding pattern as that of standards which fails in the other compounds. This difference in the binding modes may lead to distinct activities.

4. Conclusion

The synthesis and characterization of new series of 3-(3-(substituted phenyl)acryloyl)-2H-chromen-2one derivatives were carried out and all the compounds were evaluated for their anti-breast cancer activity against the MCF-7 and MDA-MB-435 cell line. The majority of the tested compound possessed antiproliferetive activity against the tested cancer cell line. Compound **5b** was fond to be similar effect compared to TAM and ADR against the MCF-7 cell line, having GI_{50} value less than 10 μg/ml. NMU-induced mammary carcinomas express functional ERα and are

Table 2
Effect of treatment on mammary tumorigenesis.

Group	Treatment	Tumor Latency (week)	Tumor burden	Tumor volume (mm^3)
I	MNU	4.5 ± 0.6	4.45 ± 0.1	4.8 ± 0.14
II	TAM	6.2 ± 1.0[c]	2.8 ± 0.21[c]	2.5 ± 0.16[c]
III	**5b**	5.5 ± 0.7[c]	3.1 ± 0.22[c]	3.3 ± 0.27[c]
IV	**6b**	4.7 ± 0.2[c]	3.6 ± 0.12[c]	3.9 ± 0.14[b]
V	**7b**	3.8 ± 0.4[a]	4.25 ± 0.13[c]	4.5 ± 0.16[a]

The results are expressed as Mean ± SEM. The data is analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey's test. (n = 6). [a]P < 0.001, [b]P < 0.01, [c]P < 0.05.

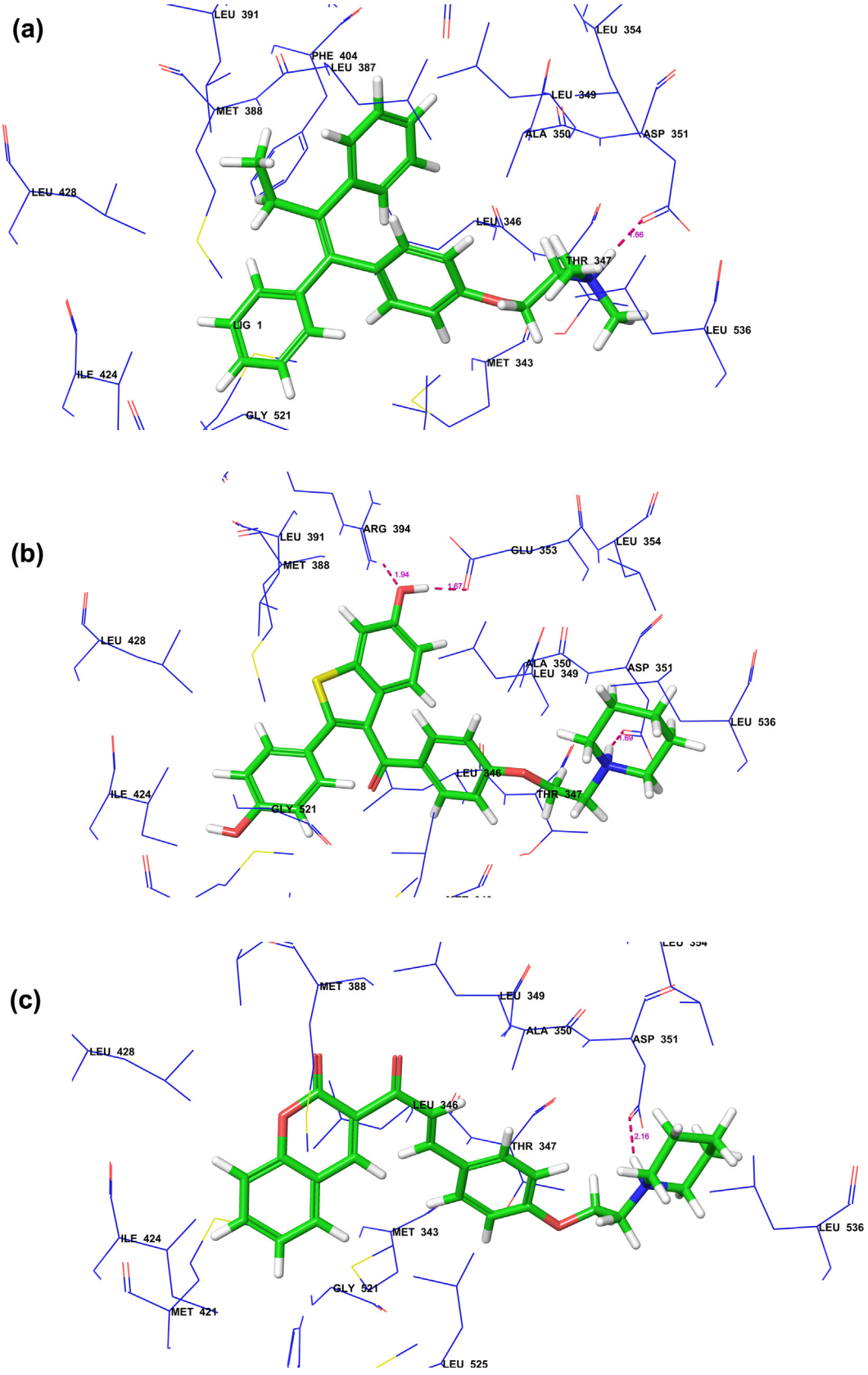


Fig. 2. Ligand interaction diagrams of – **a)** Tamoxifen, **b)** Raloxifene & **c)** Compound 5b respectively.

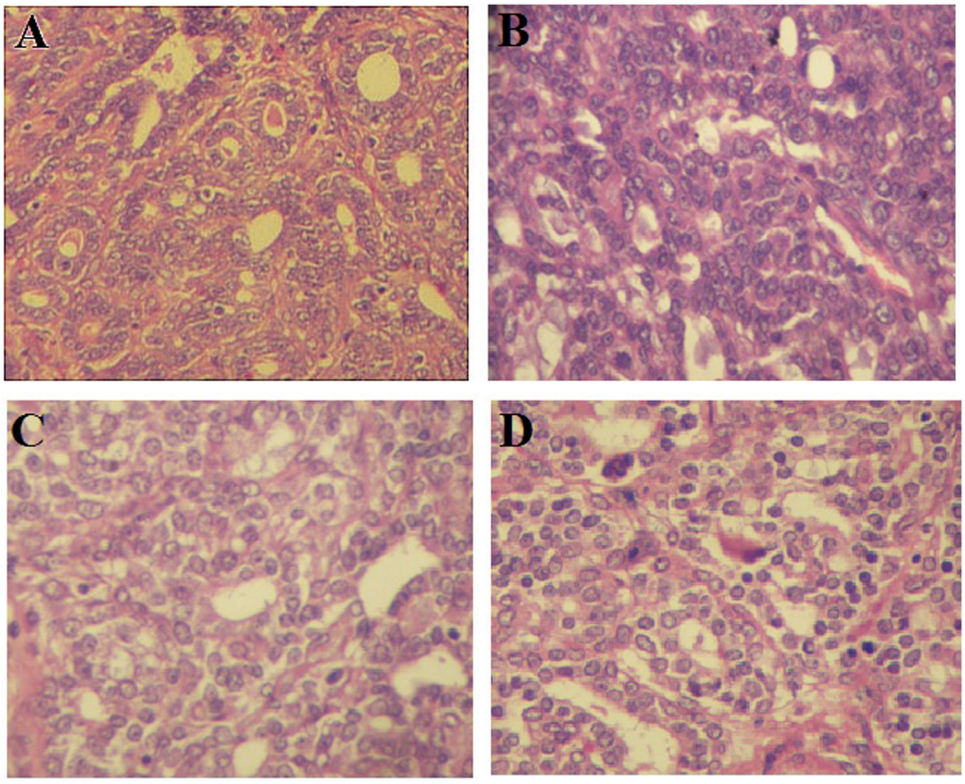


Fig. 3. Histomorphology of mammary gland adenocarcinoma in rats treated with NMU **(A)** Histology of the normal mammary gland. ×100. **(B)** Cribiform carcinoma in a NMU control animal. Masses of proliferating epithelial cells surround acinar spaces of varying size and shape, a few of which contain secretion. ×200. **(C)** Benign tubular adenoma in a tumor responded to **5b** treatment. The tumor is composed of simple tubular structures separated by only a small amount of stroma. ×200. **(D)** Benign pericanalicular fibroadenoma in TAM treated tumor. Bands of fibrous tissue surround islets of secretory epithelium and myoepithelium with loss of lobular structure. ×200.

Table 3
Estrogen levels (pg/ml) of animals on day 30 and 45.

Group	Compound No.	Estrogen level on	
		30th Day (mean ± SE)	45th Day (mean ± SE)
I	Control	9.9 ± 1.22	9.7 ± 1.36
II	MNU	18.96 ± 2.09	20.77 ± 1.98
III	**5b**	11.08 ± 1.36	11.14 ± 1.88
IV	**6b**	12.84 ± 2.58	13.36 ± 2.12
V	**7b**	14.22 ± 1.03	14.12 ± 1.46
VI	TAM	11.06 ± 1.18	11.28 ± 1.69

hormone responsive; probably the observed *in vivo* antitumor activity of **5b** compound in this model may similarly be mediated by inhibition of ER functional activity. Molecular dockingsuggested that multiple hydrophobic and hydrogen bond interactions are two predominant factors that affect the binding process. The decomposition of binding free energy to each residue revealed that the most favorable contributions came from Asp351 similar to that of RAL and TAM. These preliminary results are beneficial for further lead optimization and rational design and synthesis of novel compound with potent activity against breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.02.089.

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