

Published in final edited form as:

*Arch Pharm (Weinheim)*. 2011 February ; 344(2): 102–110. doi:10.1002/ardp.201000207.

## ***In vitro* Antiproliferative Activity of Benzopyranone Derivatives in Comparison with Standard Chemotherapeutic Drugs**

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### **SUMMARY**

The cytotoxic activities of five new benzopyranone derivatives containing basic amino side chain are described. Their cytotoxicities against ER (+) MCF-7 and ER (–) MDA-MB-231 human breast cancer cell lines, and Ishikawa human endometrial cell line were determined after 72 h drug exposure employing CellTiter-Glo assay at concentrations ranging from 0.01 – 1.0 × 10<sup>5</sup> nM. The antiproliferative activities of these compounds were compared to tamoxifen (TAM), 4-hydroxytamoxifen (4-OHT, active metabolite of tamoxifen) and raloxifene (RAL). *In vitro* results indicated that compounds **9**, **10**, **12** and **13** were more potent than TAM against the human breast cancer cell lines with IC<sub>50</sub> < 20 μM. The *in silico* structure-activity relationships of these compounds and their binding mode within the estrogen receptor (ER) binding site using AutoDock vina are discussed.

### **Keywords**

Coumarin; estrogen receptors; anticancer activity; antiproliferative agents; cytotoxic activity

### **INTRODUCTION**

Breast cancer is the second leading cause of cancer death in American women behind lung cancer [1]. Postmenopausal women, whose production of ovarian estrogen has ceased with remaining estrogens originating in extra-glandular tissues, accounted for approximately 80% of breast cancer cases [2]. It has been reported that about one-third of postmenopausal breast cancer patients have hormone-dependent tumors, which involve the stimulation of cancer cell proliferation by estrogens [3]. Therapeutic agents, which prevent the biosynthesis (e.g. aromatase inhibitors) and physiological action of estrogen on tumor cells (e.g. Selective Estrogen Receptor Modulators, SERMs), represent a new and very successful approach for the treatment of postmenopausal women with hormone-dependent breast tumors [4]. Currently, one way of blocking the estrogen action on tumor cells is preventing the binding

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**Conflict of Interest:** There is no conflict of interest associated with this publication.

of estrogen to estrogen receptor (ER) by using an antiestrogen compound capable of blocking the effects of 17 $\beta$ -estradiol (E2) [5–7].

Antiestrogens exhibit antitumor effect by displaying an antagonist action at the ER, and are widely used in the treatment of hormone-dependent ER (+) breast cancer [6, 7]. SERMs are antiestrogenic compounds possessing high affinity for ER. They are classified as ER agonists or antagonists based on conformational changes of the receptors, particularly at the helix 12 (H-12) [8, 9]. The nature of the amine-bearing side chain (dialkylaminoalkoxy group) and its orientation relative to the ligand backbone play an important role in the determination of SERMs' tissue-selective activity by preventing the proper positioning of H-12 for agonistic activity [10–13]. In this antagonist conformation, the basic amino side chain forces H-12 to move into a position occluding a hydrophobic pocket before coactivators can bind and produce a transcription complex, an interaction that is important in mediating agonist activity [14, 15]. Clinically, SERMs belong to two chemical families of non-steroidal compounds, triphenylethylenes e.g. TAM **1** and benzothiophenes e.g. RAL **2** (Fig. 1) with basic amino side chains [16, 17]. TAM is an antiestrogen drug currently used as adjuvant chemotherapy for the treatment of ER (+) breast cancer, particularly in the post-menopausal women [18–20]. TAM behaves as ER antagonist in the breast tissue and as ER agonist in bone, and has prophylactic use in breast cancer [21]. Although TAM has been very successful in breast cancer treatment, its agonistic effect on the uterus is said to be associated with increased risk of developing endometrial cancer [22]. Thus, alternative and similar effective drugs for the treatment of breast cancer are being sought.

Most recently, our group has been interested in the coumarins (2*H*-1-benzopyran-2-one) ring system **3** (Fig. 1) as a core pharmacophore of potential therapeutic agents for the treatment of hormone-dependent breast cancer [23]. Coumarin derivatives have been found to be useful in photochemotherapy, antitumor, anti-HIV therapy [24, 25] central nervous system (CNS) stimulants [26], antibacterial [27, 28], anti-inflammatory [29], anti-coagulants [30] and dyes [31]. In breast cancer cells, in particular, numerous coumarins have shown interesting pharmacological activities including binding affinities for ERs, antiproliferative activities, and inhibitory activities against sulfatase and aromatase enzymes. For example, SP500263 **4** (Fig. 1), a new category of SERM called a benzopyranone molecule or coumarin-based SERM, produced similar effects as the ideal SERMs like TAM [32, 33]. It binds with high affinity to ER $\alpha$ , functions as potent antiestrogen in *in vivo* models of breast cancer, and potently inhibits estrogen-dependent MCF-7 proliferation with similar IC<sub>50</sub> value to TAM [33, 34]. Compound **4** possessing an amine-bearing side chain attached to coumarin has a different core structural feature with respect to other SERMs currently on the market or in clinical development [34, 35]. Furthermore, recent investigation involving another type of benzopyranone compounds as ER ligands revealed that compound **5** and analogs possess both estrogen agonistic and antagonistic activities, and demonstrate significant *in vitro* antiproliferative activity with moderate *in vivo* anti-implantation activity [36].

We herein report the *in vitro* antiproliferative activity of new benzopyranone derivatives containing basic amino side chain **9–13** (Table 1) against ER (+) MCF-7 and ER (–) MDA-MB-231 human breast cancer cell lines, and Ishikawa human endometrial adenocarcinoma cell line. These cell lines are widely accepted models for assessing potent antiproliferative and antiestrogenic agents including SERMs. An *in silico* analysis of these antiproliferative agents for their relative binding affinities (RBAs) to the ERs using molecular modeling studies has also been reported here. TAM, 4-OHT and RAL were used as standards for comparison purposes in these studies.

## RESULTS AND DISCUSSION

### Cytotoxicity

Compounds **9–13** were evaluated for *in vitro* antiproliferative activity against ER (+) MCF-7 and ER (–) MDA-MB-231 human breast cancer cell lines and Ishikawa human endometrial cell line at concentration range 0.01 to  $1.0 \times 10^5$  nM in the presence of 10 nM estradiol (E2) using CellTiter-Glo assay. Cell proliferation involved measuring the light emitted during the bioluminescence reaction of luciferine in the presence of ATP and luciferase. As shown in Table 1 and Fig. 2, compounds **9, 12** and **13** ( $IC_{50} = 10.9, 10.1$  and  $10.3 \mu\text{M}$ , respectively) demonstrated significant antiproliferative activity against ER (+) MCF-7 human breast cancer cell line in comparison to TAM ( $IC_{50} = 26.2 \mu\text{M}$ ), but were less potent than 4-OHT and RAL ( $IC_{50} = 1.2$  and  $0.66 \mu\text{M}$ , respectively) (Note:  $IC_{50}$  is the concentration of test drug where a 50% reduction is observed in cell growth compared to the untreated control after a 72 h period of exposure to test drug). E2 at physiological concentration (10 nM) was used to do competitive growth inhibitory studies. The results showed that compounds **9, 12** and **13** may act as ER antagonists in the context of human breast cancer cell and compound **12** possessing piperidinylethoxy side chain exhibits the most potent antiproliferative activity in MCF-7 cell line in comparison to TAM (Fig. 2a).

The antiproliferative mechanism of TAM in ER (+) MCF-7 breast cancer cells is mainly related to the inhibition of E2 binding to the ER, however ER-independent mechanisms have also been suggested [37, 38]. The ER (–) MDA-MB-231 breast cancer cell line constitutes an original model for identifying the ER-independent mechanisms of TAM antiproliferative effects [39, 40]. Thus, in the present study the antiproliferative activity of compounds **9–13** against ER (–) MDA-MB-231 human breast cancer cell lines were also investigated to shed some light on their mechanism of action. The results showed that compounds **9, 10, 12** and **13** ( $IC_{50} = 13.9, 12.3, 17.0$  and  $12.4 \mu\text{M}$ , respectively; Table 1) were more potent in this cell line than TAM ( $IC_{50} = 18.7 \mu\text{M}$ ) and RAL ( $IC_{50} = 32.8 \mu\text{M}$ ), but less potent than 4-OHT ( $IC_{50} = 7.8 \mu\text{M}$ ). Furthermore, compound **10** possessing dimethylaminoethoxyl side chain exhibits the most potent antiproliferative activity in comparison to TAM and RAL, suggesting an additional ER-independent mechanism (Fig. 2b).

In the development of antiestrogenic SERM drugs, it is critical that the candidate drug does not cause estrogenic stimulation of the uterus, which could lead to both increase in uterine bleeding and an increased risk of developing uterine cancer. The Ishikawa cell line is an endometrial adenocarcinoma cell line that expresses functional estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ) isofomers [41]. In the present investigation, evaluation of the antiproliferative activity of these compounds on the uterus using the Ishikawa human endometrial cell line revealed that compounds **10** and **13** ( $IC_{50} = 18.2$  and  $15.6 \mu\text{M}$ , respectively; Table 1) were more potent than TAM ( $IC_{50} = 32.9 \mu\text{M}$ ) and RAL ( $IC_{50} = 26.1 \mu\text{M}$ ), but less potent than 4-OHT ( $IC_{50} = 8.3 \mu\text{M}$ ). Compound **13** possessing pyrrolidinoethoxyl side chain exhibits the most potent antiproliferative activity in the presence of E2 (Fig. 2c).

### Molecular Modeling Studies

The X-ray co-crystal structure of the ER-ligand binding domain (LBD) has provided a significant understanding of the ER binding site [42]. In the present study, we examined various binding modes of compounds **9–13** in the receptor of antagonist  $ER\alpha$ -4-OHT complex (PDB code 3ERT) and  $ER\beta$ -RAL complex (PDB code 1QKN) using AutoDock Vina. The best poses (Fig. 3) retrieved through re-docking with AutoDock Vina were almost identical with the original poses of the cognate ligands with the root mean square deviation (rmsd) values between the two poses for both 4-OHT and RAL being  $<2\text{\AA}$ , a criterion often

used for the correct bound structure prediction [43]. This led us to conclude that the predicted binding modes from AutoDock Vina can be used reliably as a docking tool in our modeling studies. Then, the optimized structures of compounds **9–13** were docked in the ligand's binding pocket followed by the determination of the binding affinity.

The triad of Glu 353, Arg 394 and buried water as well as His 524 in the ERs forms the basis of the favorable binding interaction for ER binding pocket. This sort of interaction normally shifts H-12 into a position antiparallel to H-11 (Met 517-Met 528), thereby sealing the ligand in a hydrophobic core and exposing the LXXLL cofactor-binding motif on the receptor surface [44]. The docking results indicated that compounds **9–13** showed a favorable binding affinity toward antagonist ER $\alpha$  (3ERT) binding mode than antagonist ER $\beta$  (1QKN) binding mode (Table 2). Furthermore, in the ER $\alpha$  (3ERT) receptor active site compound **9** ( $K_i = 0.06 \mu\text{mol}$ ) exhibits higher binding affinity and compound **13** ( $K_i = 0.11 \mu\text{mol}$ ) exhibits comparable binding affinity; in comparison with compounds **10–12** and E2 (Table 2). However, these binding affinities are considerably lower than those of 4-OHT, TAM and RAL.

The best-docked position of compounds **9** and **13** in the receptor of 3ERT (Fig. 4a) revealed the expected classical ER binding mode. However, a closer inspection of the ER binding pocket of compound **9** revealed that this compound does not exhibit the classical ER binding mode associated with agonists and antagonist compounds i.e. there is no hydrogen bonding interaction between the methoxy group, water, Glu 353, Arg 394 and His 524 (Fig. 4a). This lack of such hydrogen bonding is perhaps largely compensated by hydrophobic interaction. For example, recent investigation of benzochromen-6-one analogs as selective ER $\beta$  agonists revealed that the presence of methyl group could allow steric clashes with residues in helix 3 (such as Ala 350), which could prevent hydrogen-bonding interaction with the usual Glu/Arg pair [45]. Based on this result, when we replaced the methoxy group with hydroxyl group in compound **9**, hydrogen bonding with Arg 394 and Glu 353 in the receptor of 3ERT (ER $\alpha$ ) was observed with the eventually predicted  $\Delta G_{\text{binding}}$  value of  $-10.2 \text{ kcal/mol}$  (equivalent to  $0.033 \mu\text{M}$ ). This finding proved that the presence of the methoxy group was responsible for the lack of the observed hydrogen-bonding interaction with water, Glu 353, Arg 394 and His 524. The aromatic rings and the coumarin moieties are largely accommodated by a hydrophobic cavity lined by residues notably Leu 391, Ala 350, Ile 424, Gly 521, Leu 524, Met 343 and Thr 347 (Fig. 4b). However, the protonated (at physiological pH 7.4) amino nitrogen atom of the basic side chain of compounds (e.g. **9**) seems to engage in hydrogen bonding interaction with a buried water molecule that is hydrogen bonded to Thr 347 (Fig. 4b). Kekenus-Huskey and colleagues using computational docking methods have suggested this alternative mode of ER-ligand binding through hydrogen bonding interaction involving the amino nitrogen atom of imidazoles, imidazolines and piperazines with Thr 347 (residue 399 for ER $\beta$ ) as opposed to His 524 [46]. Most recently, Shankar *et al.* have also shown such hydrogen bonding interaction involving Thr 347 [47], which was also observed in the present studies.

## Conclusions

The evaluation of a new group of benzopyranone compounds **9–13** with basic amino side chain as antiproliferative agents have been described. Our studies have shown that addition of basic amino side chains to the coumarin structure widely affect the activity of the molecules, as further demonstrated in this investigation. The *in vitro* results indicated that compounds **9**, **10**, **12** and **13** possessing amine groups: piperidine, dialkylamine and pyrrolidine moieties are among the best side chain for antagonist potency against ER (+) MCF-7, ER (-) MDA-MB-231 and Ishikawa human cancer cell lines. The antiproliferative activities of these compounds were compared with TAM, RAL and 4-OHT. Among the

synthesized compounds, compound **13** exhibited the highest antiproliferative activity against ER (+) MCF-7 (hormone-dependent) and ER (-) MDA-MB231 (hormone-independent) cancer cell lines as well as Ishikawa human endometrial cell line as evident by lowest IC<sub>50</sub> value, in comparison with TAM.

## Experimental Section

### The Southern Research Institute (SRI) screening procedures [48]

The cytotoxic activity of compounds **9–13** was evaluated at the Southern Research Institute (SRI, Birmingham, Alabama, USA). The compounds were screened against ER (+) MCF-7 and ER (-) MDA-MB-231 human breast cancer cell lines and Ishikawa human endometrial cell line in comparison to TAM, 4-OHT and RAL.

### Cytotoxicity Studies

MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the NCI. The Ishikawa human endometrial cancer cell line was purchased from Sigma. All three-cell lines were cultured in phenol red-free RPMI-1640 (Hyclone) (500 mL) supplemented with L-glutamine-dipeptide (Hyclone) (5 mL), and 10% fetal bovine serum (Atlanta Biologicals) (50 mL). All three cell lines were maintained in exponential growth phase by sub-culturing twice weekly in 150-cm<sup>2</sup> flasks at 37 °C, 95% air with 5% CO<sub>2</sub>. The media was removed from the flasks, the cells washed with phosphate buffer solution (PBS) (Hyclone), and cells were then detached using 5 mL of TrypLEExpress solution (Invitrogen) (incubation 5–10 minutes) followed by the addition of growth media. Cells were centrifuged (1,500 rpm) for 5 minutes, and resuspended in growth media at 10<sup>5</sup> cells per mL. To each well of 96-well microplate, 50 µL of cell suspension was added at final density of 5,000 cells per well. After the cells were allowed to attach and grow overnight, 25 µL of 40 nM estradiol (Sigma) was added to each well, followed by 25 µL of the different drugs treatments (compounds **9–13**, and TAM, 4-OHT and RAL) ranging from 400,000 nM to 0.04 nM, resulting in a final concentration of 10 nM estradiol in every well, and drug treatments ranging from 0.01 – 100,000 nM. Incubation lasted for 3 days at 37 °C air with 5% CO<sub>2</sub> followed by CellTiter-Glo assay (Promega). All of the compounds were tested in triplicate for each cell lines. The results expressed as IC<sub>50</sub> (Inhibitory concentration of 50%) were the averages of three data points for each concentration and were calculated using GraphPad Prism 4.0.

### Molecular Modeling Studies

**Preparation of proteins**—Crystal structures of ligand binding domains of human estrogen receptor  $\alpha$  (PDB code: 3ERT complexed with 4-hydroxytamoxifen) and  $\beta$  (PDB code: 1QKN, complexed with RAL) were obtained from the protein databank. The water molecules except those within 4.5 Å of the SERM (4-OHT or RAL) binding site were removed and missing hydrogens were added to the protein structures using the Reduce algorithm implemented in the MolProbity server (<http://molprobity.biochem.duke.edu/>). The latter allows optimization of local H-bonding environment avoiding steric clashes and also necessary correction for misorientation of Asn, Gln and His residues [45]. For further structural refinement, the protein structures together with the associated SERMs were then energy minimized using GROMOS96 force field implemented in Swiss-PdB viewer (Deep View). The associated SERM molecules were later removed from the corresponding energy-minimized protein structures to which the Gasteiger partial atomic charges [49] were added using the AutoDockTool (ADT) [50]. The prepared structures were eventually used as input files for the docking.

**Preparation of ligands**—The 3D structures of the benzopyranone derivatives containing basic side chain were drawn in ChemBioDraw Ultra 11.0 module of the ChemBioOffice



2008 package. All the structures were then energy-minimized in ChemBio3D ultra 11.0 using MM2 force field. Later, using ADT, the Gasteiger partial atomic charges were added and all flexible torsions were defined for the ligands. The prepared structures were used as input files for the docking.

**Docking**—Flexible-ligand docking was carried out using AutoDock Vina [51]. Vina uses a sophisticated gradient optimization method in its local optimization procedure and a modified X-score-based scoring function [51]. For this, a grid box of  $60 \times 60 \times 60$  number of points (grid spacing of  $0.375 \text{ \AA}$ ) was built around the ligand-binding region of the protein chains. Several key residues known to be involved in SERM binding (e.g. Asp 351, Glu 353, Arg 394, Thr 347 and His 524) were held flexible during the docking. From the estimated free energy of ligand binding ( $\Delta G_{\text{binding}}$ , kcal/mol), the inhibition constants ( $K_i$ ) was calculated taking into consideration of the dissociation of the enzyme inhibitor complex using basic thermodynamics formula (Arrhenius equation) of  $\Delta G = RT \ln K_i$ . Only the best pose (the one with the lowest  $\Delta G_{\text{binding}}$ ) is considered. The docking was run on windows operating environment implemented on a PC with an Intel dual core processor and the docked structures shown in PyMol.

## Acknowledgments

Faculty Research Development Funds (NIH/NCRR grant G12 RR0 3020, NIH/NCRR grant 1 C06 RR12512-01 and NIH/DHHS grant 1 S11 ES011182 01) is gratefully acknowledged for financial support. The authors also gratefully acknowledge Dr. David H. Powell of the Mass Spectrometry Services, Department of Chemistry at University of Florida for assistance with Mass Spectra analysis.

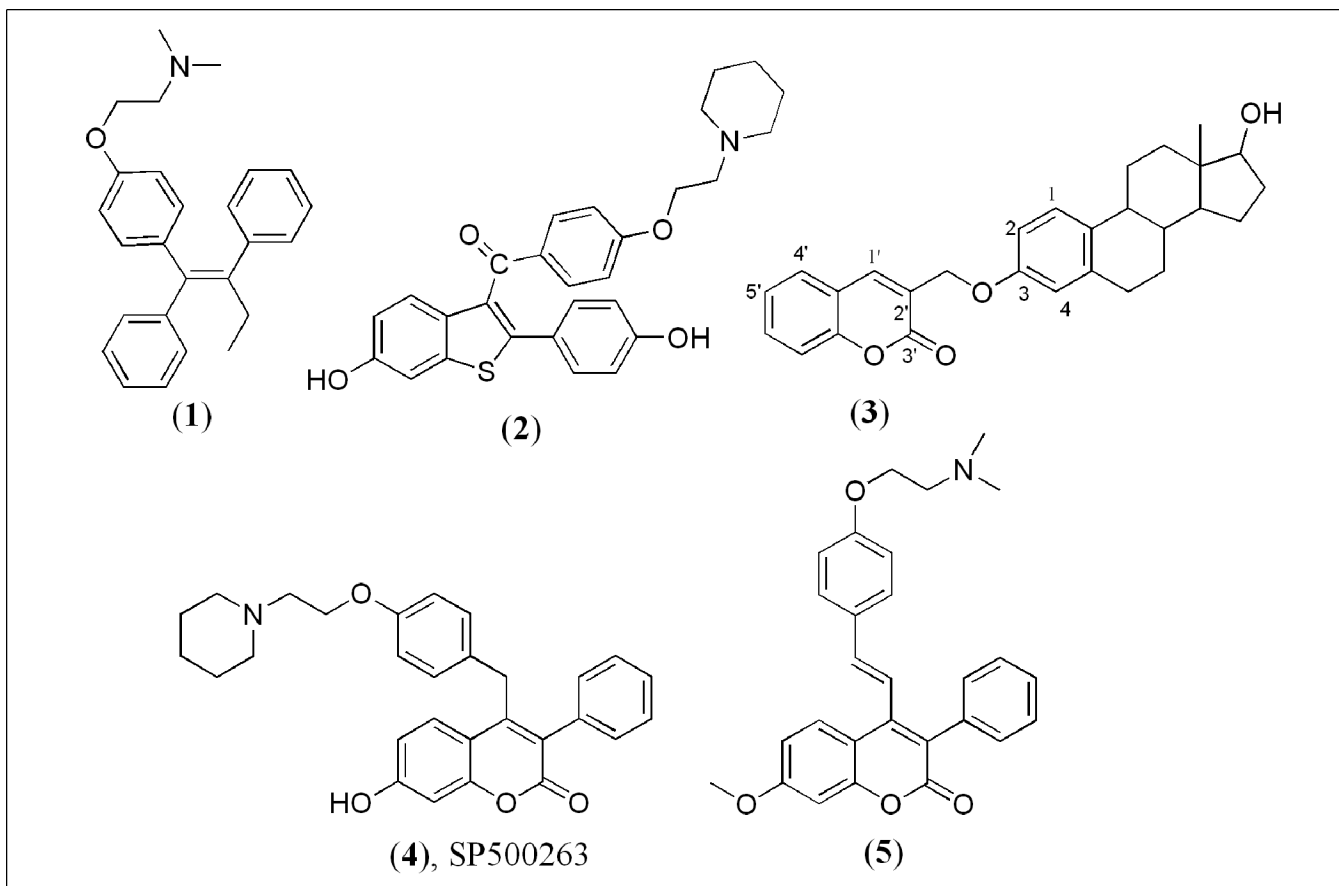
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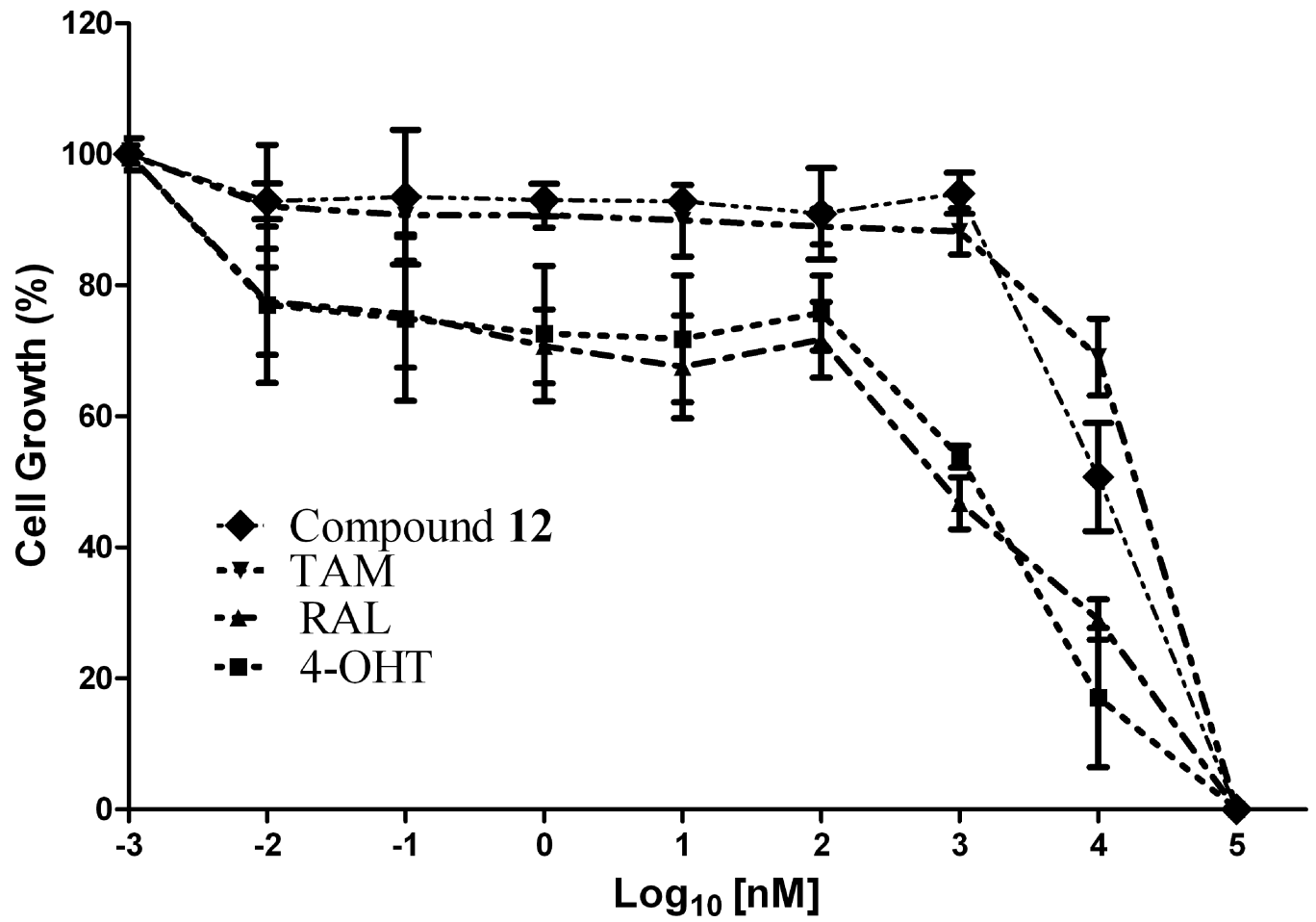
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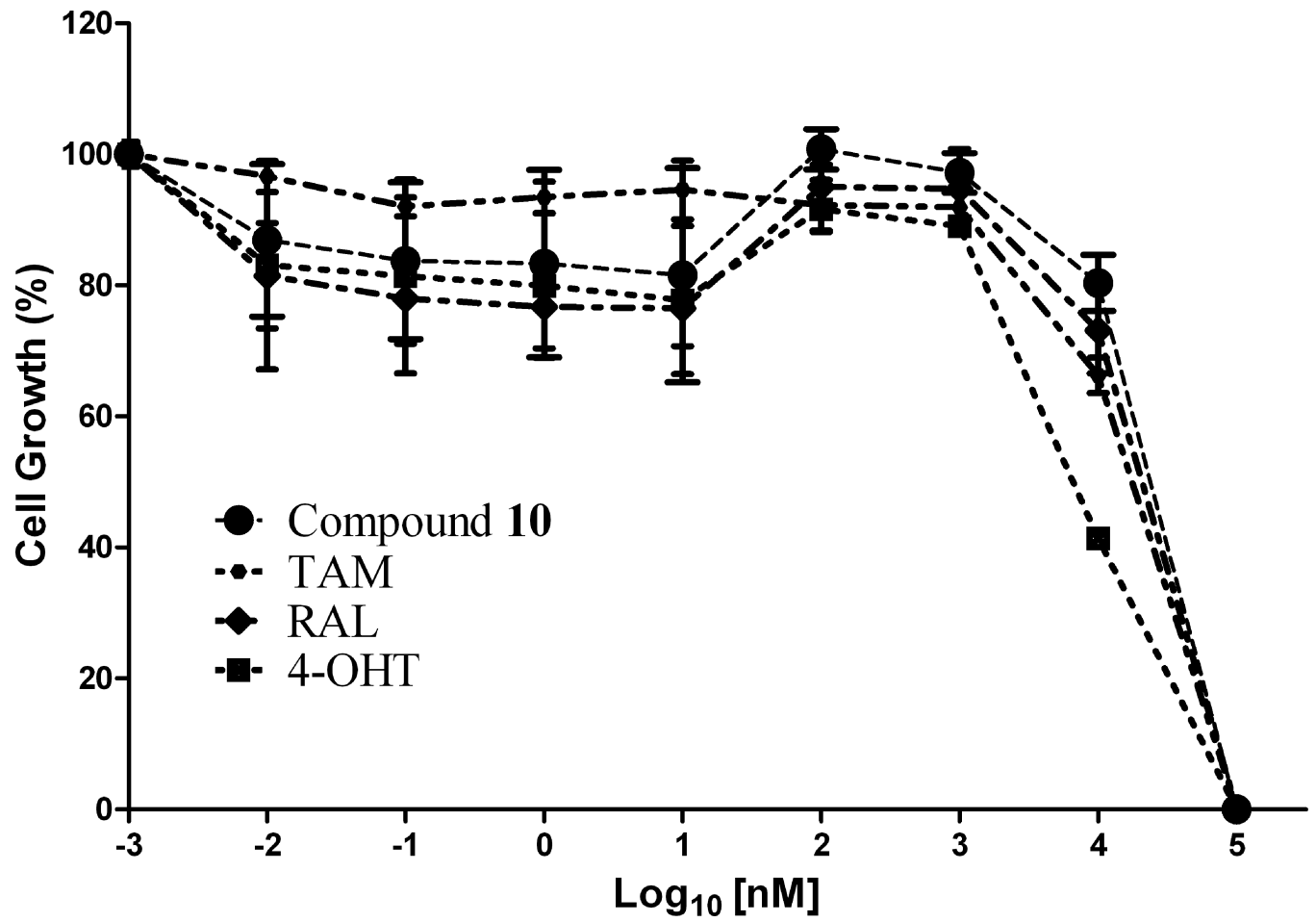
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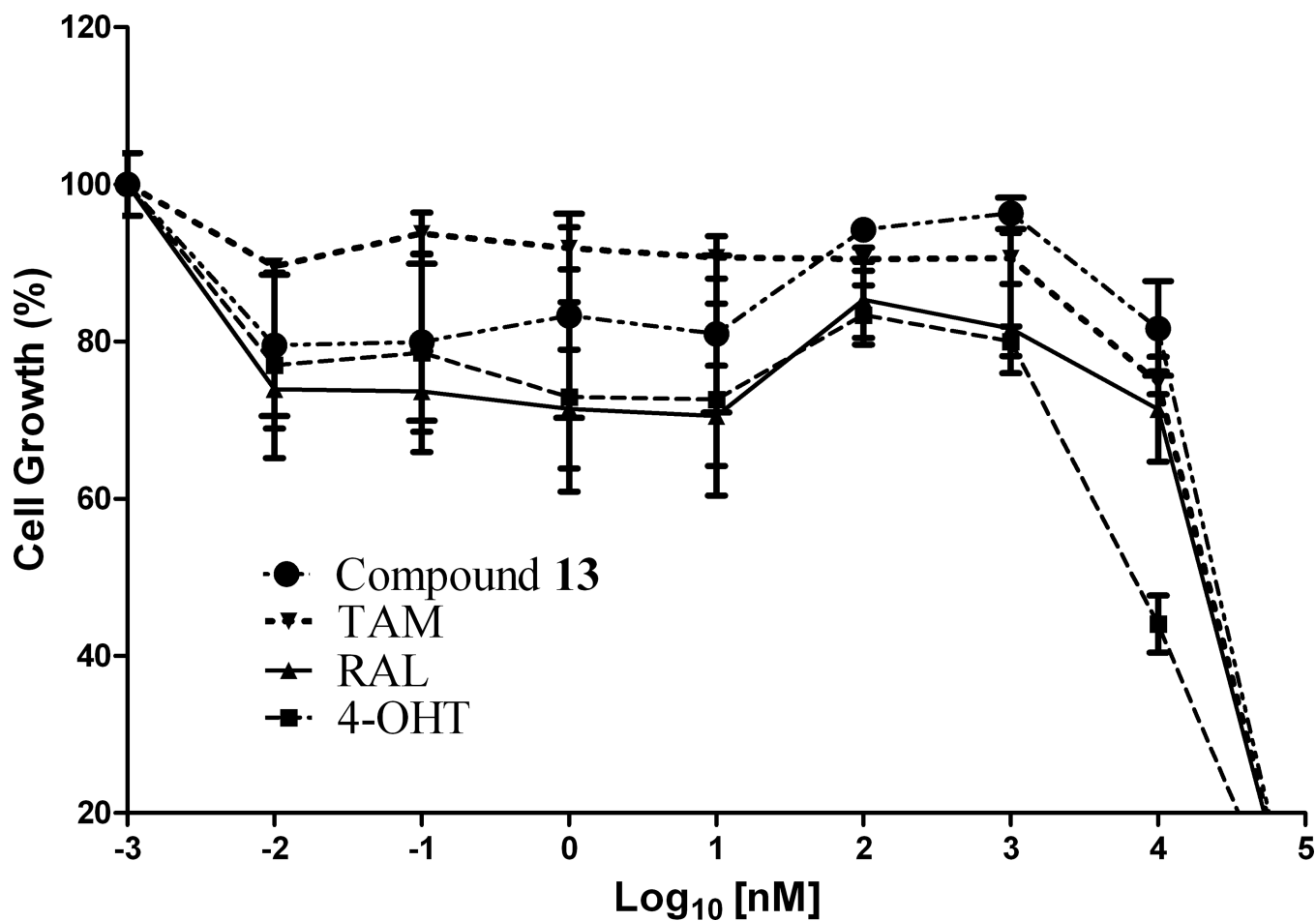




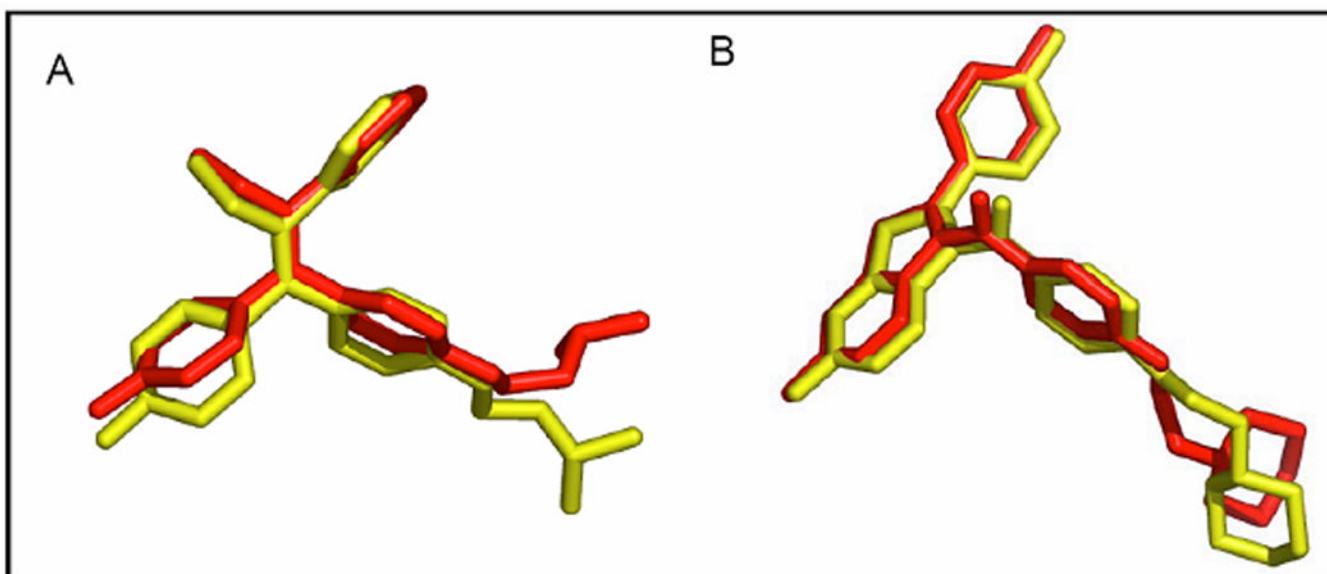
**Figure 1.** Structures of Tamoxifen **1**, Raloxifene **2**, Coumarin-E2 conjugate **3**, Coumarin-based benzopyranone, SP500263 **4** and 7-methoxy-3-phenyl-4-phenylvinyl benzopyranone derivative **5**.



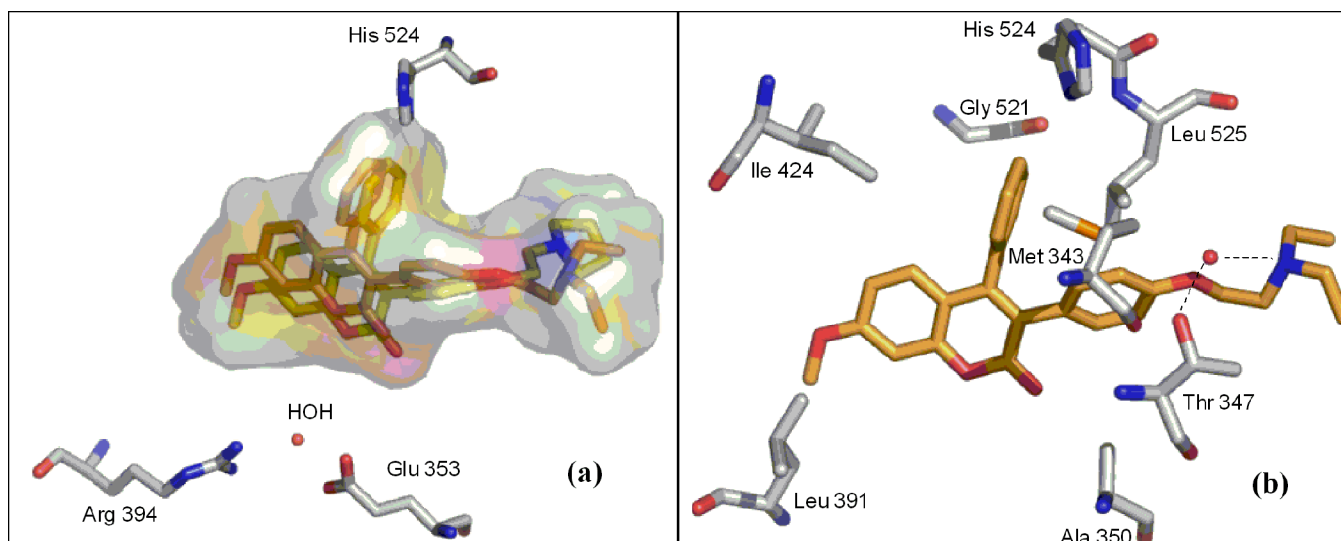




**Figure 2.**  
*In vitro* antiproliferative activity of (a) compound 12 against MCF-7 (ER+) cell line, (b) compound 10 against MDA-MB-231 (ER-) cell line and (c) compound 13 against Ishikawa human endometrial cell line; in comparison to TAM, 4-OHT and RAL



**Figure 3.**  
Validation of AutoDock Vina against: A) ER $\alpha$ -4-OHT and B) ER $\beta$ -RAL complex.

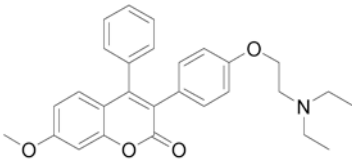
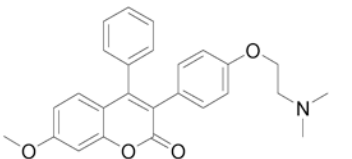
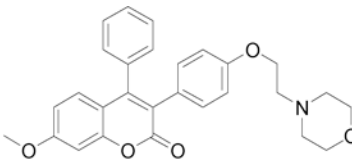
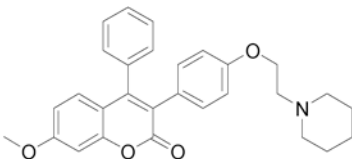
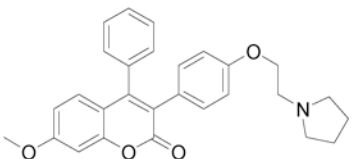


**Figure 4.** Best docked position of; a) compounds **9** and **13**, and b) compound **9** in the ligand-binding pocket of ER $\alpha$  (PDB 3ERT).



**Table 1**

IC<sub>50</sub> values (μM) for compounds **9–13** tested against MCF-7 (ER+) and MDA-MB231 (ER-) human breast cancer cell lines, and Ishikawa human endometrial cells.

Compounds	IC <sub>50</sub> values (μM)		
	MCF-7	MDA-MB231	Ishikawa
 <p><b>9</b></p>	10.9	13.9	40.6
 <p><b>10</b></p>	16.5	12.3	18.3
 <p><b>11</b></p>	48.3	33.5	41.8
 <p><b>12</b></p>	10.1	17.0	26.2
 <p><b>13</b></p>	10.3	12.4	15.6
RAL-HCl	0.66	32.8	28.0
4-OHT	1.2	7.8	8.3
TAM	26.2	18.7	33.0

<sup>1</sup>The data represent the average of triplicate determinations at various concentrations.

<sup>2</sup>The IC<sub>50</sub> values were determined from the graphs (GraphPad Prism) using mean values of data points at various concentrations.

**Table 2**Binding affinities of the compounds with ER $\alpha$  and ER $\beta$ 

Compound	ER $\alpha$ (3ERT)		ER $\beta$ (1QKN)	
	$\Delta G_{\text{binding}}$ (kcal/mol)	$K_i$ ( $\mu\text{mol}$ )	$\Delta G_{\text{binding}}$ (kcal/mol)	$K_i$ ( $\mu\text{mol}$ )
<b>9</b>	-9.85	0.06	-7.7	2.27
<b>10</b>	-9.0	0.252	-7.5	3.18
<b>11</b>	-8.7	0.419	-7.8	1.91
<b>12</b>	-9	0.252	-7.2	5.27
<b>13</b>	-9.5	0.11	-7.0	7.39
<b>4-OHT</b>	-11.5	0.004	-9.06	0.228
<b>E2</b>	-9.5	0.11	-8.5	0.587
<b>TAM</b>	-10.0	0.047	-8.1	1.15
<b>RAL</b>	-11.5	0.004	-10.38	0.025