

Fig. 3 (a) — $^1\text{H-NMR}$ and (b) $^{13}\text{C-NMR}$ spectra of chalcone 1 (CDCl_3)

Fig. 4 — FT-IR spectrum of compound 1Aii

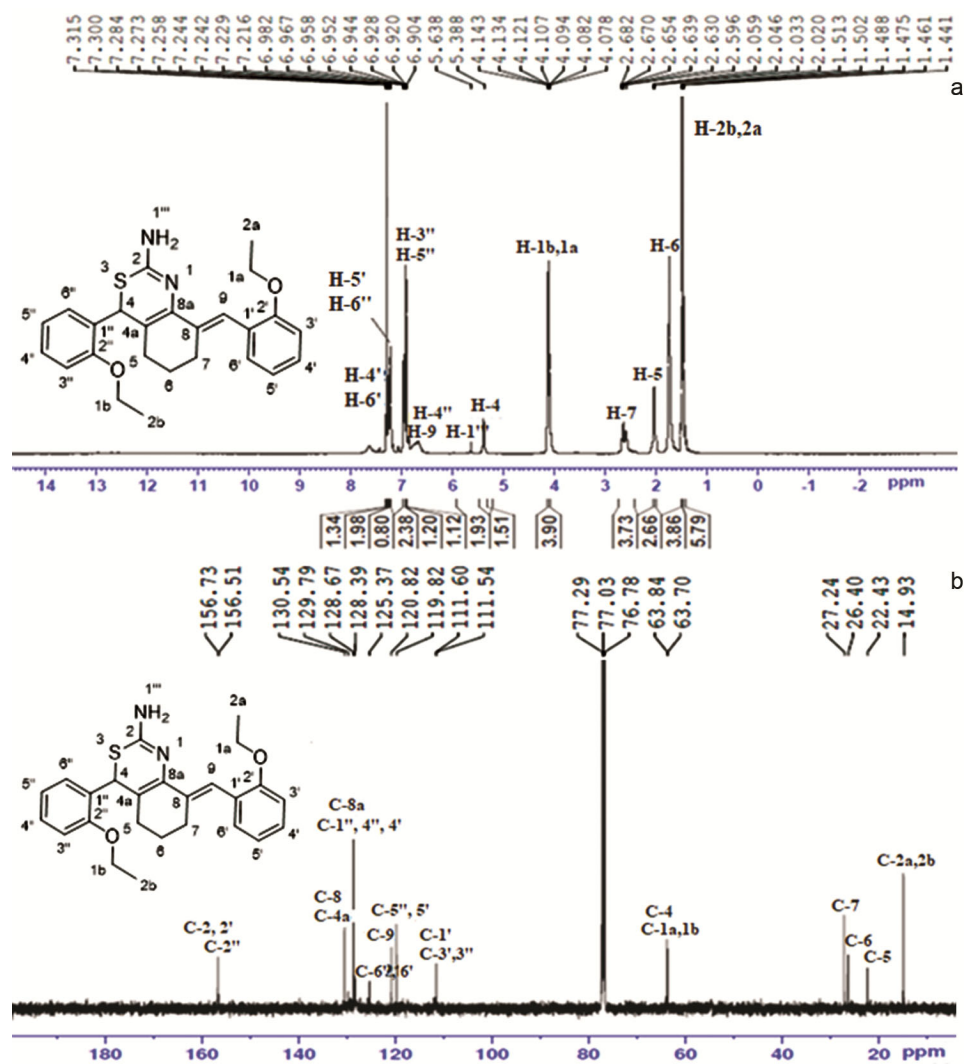


Fig. 5 (a) — ¹H-NMR and (b) ¹³C-NMR spectra of pyrimidine 1Aii (CDCl₃)

Molecular docking study of the synthesized compounds

Various molecules have been investigated to find whether they bind well with ER α , a crucial receptor for breast cancer that plays an important role in cell proliferation²⁸. The docking study was carried out between the human estrogen receptor alpha (ER α) with all the designed compounds (ligands) to obtain the type of interaction and binding energy of the ligands. MCF-7 (high ER/ER ratio), T47D (low ER/ER ratio), and MDA-MB-231 are three human breast cancer cell lines with different oestrogen receptors (ER-negative). Cancer cells that are ER-negative do not need estrogen to grow. Around 75% of breast cancers are estrogen receptor alpha (ER α)-positive and are treatable²⁹. Thus, continuous research on drug discovery led to the design of molecules with natural product scaffolds.

In this study, tamoxifen was used as the reference standard. Tamoxifen is the most widely used hormonal therapy drug for breast cancer which inhibits the expression of estrogen-dependent response genes. In receptor alpha (ER α)+ breast cancer cells, it works as an antagonist to ER α and suppresses its signalling pathway. The tamoxifen-bound ER complex inhibits the genes from being switched on by estrogen, preventing the estrogenic actions that cause cancer cell proliferation. Tamoxifen therapy dramatically reduces the risk of breast cancer recurrence. However, tamoxifen has adverse effects, and its efficacy is limited by the presence of potential resistance³⁰.

An ideal drug molecule usually complies with Lipinski's Rule of Five, which predicts the solubility and permeability of a chemical compound, indicating whether the compound can be taken orally or not³¹.

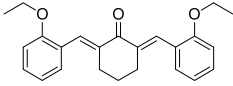
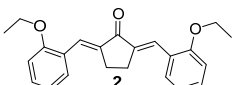
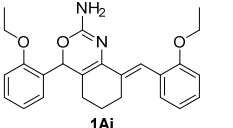
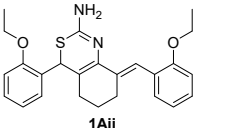
The rule states that a compound's molecular weight should be less than 500 g/mol, that the log P value should be less than 5, that there should be no more than 5 hydrogen-bond donors, and that there should be no more than 10 hydrogen-bond acceptors³². Table 1 shows the theoretical evaluation of the designed compounds (ligands) based on Lipinski's

Rule. The study suggested that most of the designed compound showed the properties which necessary for a drug candidate. Docked conformation of estrogen receptor alpha with all the designed compounds and Tamoxifen showed possible interaction which led to their free binding energy (ΔG) and inhibition constant (Table 2).

Table 1 — Computed data of the designed compounds based on Lipinski's Rule

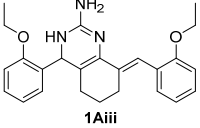
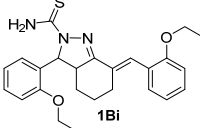
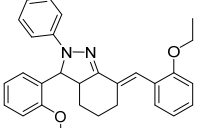
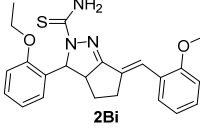
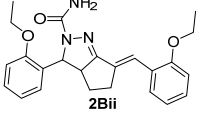
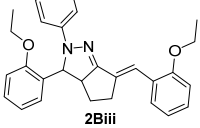
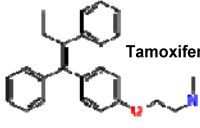
Compound	Mol. Wt.	Binding energy (kcal/mol)	K _i (nM)	Log P	H-bond donor	H-bond acceptor	Interactions with amino acids	
							H-bond	van der Waals (Hydrophobic)
1	362.47	-8.92	287.95	5.06	0	0	-	LEU391, LEU349, LEU346, ALA350, LEU387, TRP383, LEU354, ASP351, MET343, MET388
2	348.44	-9.28	157.74	4.64	0	0	-	LEU391, LEU349, LEU346, LEU525, TRP383, ASP351, LEU354, ALA350, MET343, LEU387
1Ai	404.51	-7.83	1820	4.21	1	0	CYS530	LYS529, VAL533, LEU525, ASP351, TRP383, ALA350
1Aii	420.57	-7.76	2050	4.77	1	0	CYS530	LEU536, TRP383, LEU525, MET528, LYS529, VAL533, TYR526, MET522
1Aiii	403.53	-8.44	651.31	3.97	1	1	ASP351, CYS530	THR347, ALA350, LEU346, MET343, LEU525, VAL533, LYS529, TRP383
1Bi	435.59	-7.77	2030	4.77	0	0	-	LEU536, LEU525, LEU34, ALA350, LEU346, TRP383
1Biii	452.60	-8.72	406.80	6.49	0	0	-	MET522, LEU525, ASP351, LEU354, ALA350, TRP383, LEU536
2Bi	421.56	-8.53	557.72	4.35	1	1	TRP384, ASP351	MET522, LEU536, LEU539, LEU354, ALA350
2Bii	405.50	-8.96	269.92	3.79	0	1	ASP351	LEU346, MET343, MET421, MET528, LEU525, ALA350
2Biii	438.57	-9.57	96.53	6.07	0	0	-	LEU346, MET421, MET343, LEU525, ASP351, LEU354, ALA350
Tamoxifen	371.51	-10.46	21.55	6.07	0	0	-	PHE404, LEU391, LEU387, LEU428, LEU346, MET421, LEU525, THR347, ASP351, ALA350

Table 2 — Binding energies of all the ligands and their IC₅₀ value

Compound	Time (h)	IC ₅₀ (μM)			*Selective index	
		MCF-7	MDA-MB-231	MCF-10A	MCF-7	MDA-MB-231
	24	100 ± 0	100 ± 0.01	3.66 ± 0.05	0.04	0.03
	48	89.83 ± 2.37	100 ± 0.01	3.26 ± 0.12	0.04	0.03
	72	87.11 ± 2.01	100 ± 0.01	3.23 ± 0.07	0.04	0.03
	24	100 ± 0.01	14.52 ± 4.08	100 ± 0.01	1	6.89
	48	100 ± 0.01	33.14 ± 6.95	100 ± 0.01	1	3.18
	72	100 ± 0.01	40.8 ± 2.82	100 ± 0.01	1	2.45
	24	100 ± 0	100 ± 0.01	100 ± 0.01	1	1
	48	100 ± 0	100 ± 0.01	100 ± 0.01	1	1
	72	97.07 ± 5.08	100 ± 0.01	100 ± 0.01	1.03	1
	24	56.73 ± 3.3	16.1 ± 0.43	3.24 ± 0.01	0.06	0.2
	48	18.64 ± 1.12	14.58 ± 0.69	3.19 ± 0.04	0.17	0.22
	72	15.03 ± 2.39	9.86 ± 0.69	3.09 ± 0.01	0.21	0.31

(Contd.)

Table 2 — Binding energies of all the ligands and their IC₅₀ value

Compound	Time (h)	IC ₅₀ (μM)			*Selective index	
		MCF-7	MDA-MB-231	MCF-10A	MCF-7	MDA-MB-231
 1Aiii	24	37.74 ± 1.32	9.85 ± 0.26	3.16 ± 0.04	0.08	0.32
	48	6.98 ± 1.12	9.45 ± 1.08	3.09 ± 0.01	0.44	0.33
	72	5.68 ± 0.54	7.59 ± 1.21	3.16 ± 0.01	0.56	0.42
 1Bi	24	100 ± 0.01	21.4 ± 1.32	72.22 ± 8.33	0.72	3.37
	48	100 ± 0.01	21.41 ± 1.28	39.52 ± 1.38	0.39	1.85
	72	100 ± 0.01	18.2 ± 0.05	32.65 ± 1.9	0.32	1.79
 1Biii	24	100 ± 0.01	100 ± 0.01	100 ± 0.01	1	1
	48	100 ± 0.01	100 ± 0.01	100 ± 0.01	1	1
	72	100 ± 0.01	100 ± 0.01	100 ± 0.01	1	1
 2Bi	24	100 ± 0.01	25.96 ± 5.84	100 ± 0.01	1	3.85
	48	100 ± 0.01	26.73 ± 1.4	100 ± 0.01	1	3.74
	72	100 ± 0.01	48.4 ± 8.88	100 ± 0.01	1	2.07
 2Bii	24	100 ± 0.01	100 ± 0.01	87.52 ± 10.81	0.88	0.87
	48	83.19 ± 1.92	100 ± 0.01	89.14 ± 2.05	1.07	0.89
	72	70.57 ± 8.26	100 ± 0.01	90.51 ± 1.2	1.28	0.91
 2Biii	24	100 ± 0.01	100 ± 0.01	100 ± 0.01	1	1
	48	56.9 ± 6.51	100 ± 0.01	89.83 ± 2.37	1.58	0.9
	72	53.83 ± 4.55	100 ± 0.01	87.93 ± 6.63	1.63	0.88
 Tamoxifen	24	42.66 ± 2.19	43.03 ± 1.60	12.52 ± 2.46	0.29	0.29
	48	35.01 ± 3.28	34.19 ± 3.04	7.11 ± 1.32	0.2	0.21
	72	26.95 ± 3.01	23.36 ± 3.84	7.04 ± 1.48	0.26	0.3

*Selective index = (IC₅₀ in normal cell/IC₅₀ in cancer cells)

Fig. 6(a-b) shows the 3D and 2D molecular interaction of compound **1Aii** in the binding site of estrogen ER α which shows both the hydrogen and noncovalent interactions. A conventional covalent bond was observed with CYS530 amino acid. The alkyl interaction was observed with VAL533 and LYS529 while π -alkyl interaction occurs between LEU536, LEU525, and MET528. There was also π -sulfur interaction observed with TYR526 and MET522 amino acids. On the other hand, compound **1Aiii** shows both hydrogen and noncovalent interactions in Fig. 6(c-d) of the 3D and 2D molecular interactions. A conventional hydrogen bond was observed with CYS530 and ASP351 amino acids.

Other hydrophobic interaction includes alkyl interaction with LYS529, π -alkyl interaction with ALA350 and TRP383 together with π - σ interaction with LEU525 and VAL 533.

Fig. 7 shows the molecular interaction of (a) 3D and (b) 2D between Tamoxifen and ER α which displayed only the hydrophobic interaction without hydrogen bond. Tamoxifen released the binding energy (ΔG) of -10.46 kcal/mol with an inhibition constant of 21.55 nM. The π -alkyl interaction was observed with LEU525, LEU387, LEU346, and LEU391 as well as alkyl interaction with MET421, LEU428, and PHE404. The π - σ interaction has also been observed with ALA350.

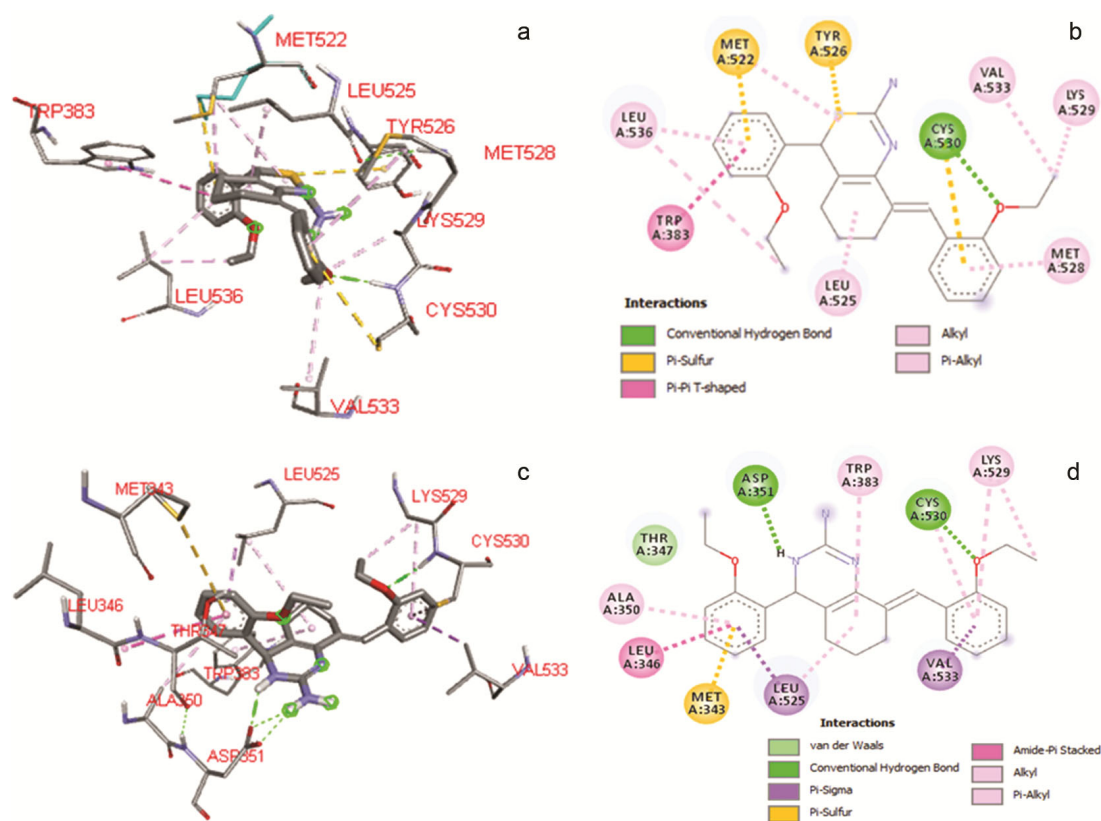


Fig. 6 — The docked pose of ligand in the binding site of estrogen receptor alpha ($ER\alpha$): (a) 3D and (b) 2D interactions of compound **1Aii**; (c) 3D and (d) 2D interactions of compound **1Aiii**

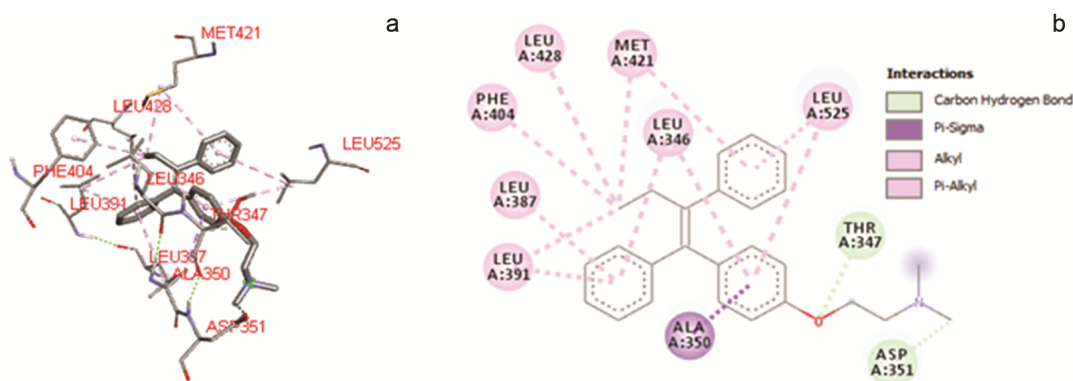


Fig. 7 — The docked pose of Tamoxifen in the binding site of estrogen receptor alpha ($ER\alpha$): (a) 3D and (b) 2D interactions

As from the result, the binding affinity of compounds **1Aii** (ΔG : -7.76 kcal/mol) and **1Aiii** (ΔG : -8.44 kcal/mol) can be considered comparable to the control, Tamoxifen (ΔG : -10.46 Kcal/mol). However, the inhibition constant of compound **1Aii** (2050 nM) and **1Aiii** (651.31 nM) were higher than Tamoxifen (21.55 nM). However, compounds **1Aii** and **1Aiii** showed better interactions compared to Tamoxifen, which might be due to the presence of a hydrogen bond compared to no hydrogen bond observed in Tamoxifen.

Cytotoxicity study

Two chalcones, **1** and **2**, three heterocyclic ring compounds **1A(i-iii)**, and five new pyrazoline compounds, **1Bi**, **1Biii**, **2B(i-iii)** were evaluated against two types of breast cancer cell lines, MCF-7 (with a receptor) and MDA-MB-231 (without receptor), and also the normal breast cell lines, MCF-10A (as control). The $ER\alpha$ was docked with all the synthesized compounds (ligands) and Tamoxifen (positive control). The IC_{50} and the selective index values are presented in

Table 2. Compounds **1Aii** and **1Aiii** were found to show moderate IC₅₀ values when exposed to the MCF-7 cell line for 24 h. Hence these compounds will be discussed.

The effects of all ligands against breast cancer cell lines MCF-7 were measured using the MTT assay. Following 24 h of exposure to pyrimidine compound **1Aiii**, significant inhibition of cancer cell proliferation in the treated cells was observed with the IC₅₀ values of 37.74 ± 1.32 µM, as compared to the Tamoxifen (control). The thiazine compound **1Aii** also showed the IC₅₀ values of 56.73 ± 3.3 µM which are comparable to the IC₅₀ value of Tamoxifen, 42.66 ± 2.19 µM. The presence of the amine group (NH₂) which is an electron-donating group, attached to the pyrimidine ring in compound **1Aiii** showed better inhibition activity against the MCF-7 breast cancer cell, with better IC₅₀ values compared to the thiazine ring in compound **1Aii**.

This result has also been supported by the molecular docking analysis which showed that the presence of the amine group at the pyrimidine ring of compound **1Aiii** enabled the formation of another hydrogen bond inside the active site of ER α . This resulted in better ΔG (-8.44 kcal/mol) than the thiazine ring of compound **1Aii** (ΔG = -7.76 kcal/mol). Despite compound **1Aii** and **1Aiii** showed good inhibition towards the breast cancer cell lines, it also gives serious toxic effect towards the control cell (MCF-10A) with the IC₅₀ values of 3.24 ± 0.01 µM and 3.16 ± 0.04 µM, respectively. Tamoxifen also was found to be cytotoxic to normal breast cell lines with the IC₅₀ values of 12.52 ± 2.46 µM after 24 h of subjection. This finding showed similar reported work by Petinari *et al.*³³ whereby Tamoxifen was found to be toxic towards cancerous and non-cancerous cells at µM concentration which might be due to the presence of estrogen receptors. In a related series, compounds **1Aii** and **1Aiii** also showed convincing prevention toward the growth of estrogen-negative human breast cancer cell line MDA-MB-231 with the IC₅₀ values of 16.1 ± 0.43 µM and 9.85 ± 0.26 µM, respectively. As the result, Tamoxifen also showed an impressive cytotoxic activity towards MDA-MB231 with IC₅₀ values of 43.03 ± 1.60 µM. This trend suggested that the cytotoxicity of tamoxifen probably involves more than one pathway, which included one pathway of the estrogen receptor-independent and another pathway of the estrogen receptor-dependent. The results also showed that compounds **1Aii** and **1Aiii** has a similar trend of cytotoxicity activity as tamoxifen but both

compounds were more sensitive towards the estrogen receptor-negative cell line compared to estrogen receptor-positive.

Pyrazoline compounds **1Bi** and **2Bi** showed only preferential inhibition towards MDA-MB-231 cell line with the IC₅₀ values of 21.4 ± 1.32 µM and 25.96 ± 5.84 µM, respectively, when exposed to the cell line for 24 h. It also showed good selectivity towards the breast cancer cell line (MDA-MB-231). They were found not interfering with the proliferation of the normal breast cell lines as they are specifically cytotoxic towards the estrogen-negative breast cancer cell line.

Conclusion

Chalcones **1** and **2** were synthesized via a Claisen-Smith condensation in basic media. The ring-closing reactions of these chalcones with urea, thiourea, and guanidine formed compounds with oxazine, thiazine and pyrimidine rings, **1A(i-iii)**, respectively. Reactions with thiosemicarbazide, semicarbazide and phenylhydrazine formed pyrazoline derivatives, **1Bi**, **1Biii**, **2B(i-iii)** accordingly. The molecular structures of these compounds were confirmed by the analysis using FT-IR, ¹H and ¹³C NMR, and elemental analysis which showed that the syntheses were successful. The molecular docking study of the synthesized compound determined their binding energies and inhibition constants of the compounds towards the estrogen receptor. Compound with the least binding energy was capable to be used as a breast cancer drug. Further modification of the substituents also can be done for better properties. All the synthesized compounds were evaluated against two types of breast cancer cell lines, MCF-7 (with a receptor) and MDA-MB-231 (without receptor), and the normal breast cell lines, MCF-10A as control. The results showed that pyrimidine ring compound **1Aiii** has a significant inhibition of cancer cell proliferation with the IC₅₀ values of 37.74 ± 1.32 µM when treated with MCF-7 cells as compared to the control, Tamoxifen of IC₅₀ values 42.66 ± 2.19. Thiazine ring compound **1Aii** also showed the IC₅₀ values of 56.73 ± 3.3 which are comparable to the IC₅₀ value of Tamoxifen. Both compounds **1Aii** and **1Aiii** also revealed the anticancer activity toward MDA-MB-231 with the IC₅₀ value of 16.1 ± 0.43 µM and 9.85 ± 0.26 µM, respectively. In addition, pyrazoline compounds **1Bi** and **2Bi** also showed preferential inhibition towards MDA-MB-231 cell line with the IC₅₀ values of 21.4 ± 1.32 µM, and 25.96 ± 5.84 µM, respectively when exposed to the cell line for 24 h.

Finally, compounds **1Bi** and **2Bi** showed good selectivity towards the MDA-MB-231 breast cancer cell line and were less sensitive toward the normal breast cell line (MCF-10A).

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