Virtual Screening on Molecules Targeting the Interaction Between Estrogen Receptor Beta and Murine Double Minute 2

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Abstract

Estrogen receptor beta (ERβ) is an isoform of estrogen receptor that plays a role in breast cancer. An E3 ubiquitin ligase, murine double minute 2 (MDM2), can bind to ERβ and degrade it. Virtual screening and protein-protein docking studies are one of the approaches that can be performed to discover FDA-approved drugs targeting the interaction of the ERβ-MDM2 complex. This study aimed to conduct virtual screening of 1615 compounds targeting the interaction between ERβ-MDM2 as an initial study to discover potential breast cancer drugs. Biovia Discovery Studio 2021, ClusPro 2.0, PyRx 8.0, and PyMOL software were utilized in this study. ERβ (PDB ID: 3OLS) and MDM2 (PDB ID: 1T4E) receptors were docked to obtain the ERβ-MDM2 protein complex. The ligands used in the virtual screening were FDA-approved drugs downloaded from the ZINC database. PIC and PLIP web tools were also utilized to analyze the amino acid residues involved in the interaction. The virtual screening results showed that ergotamine was the drug with the lowest energy score (-12.0 kcal/mol) among 1057 drugs and was able to establish the strongest interaction between ERβ-MDM2. In conclusion, based on this computational study, ergotamine strengthens the interaction between ERβ-MDM2 and thus could be used as a candidate for breast cancer drug. Thorough validation of *in vitro*, biochemical, and *in vivo* studies are needed to confirm this finding.

Keywords: *Estrogen receptor beta, breast cancer, protein-protein interaction, MDM2.*

INTRODUCTION

Breast cancer remains the most dominantly occurs in women. Almost 30% of cancer cases in women are breast cancers (Loibl, *et al*., 2021). Out of 9.6 million cancer deaths, 12% were caused by breast cancer (Bray, *et al*., 2018). Recently, estrogen receptors (ER) have been found to play a role in breast cancer and have become the target of breast cancer treatment (Zhou & Liu, 2020). Estrogen receptor beta is an ER isoform which is found in 20-30% of breast cancer cases (Elebro, *et al*., 2017; Marotti, *et al*., 2010).

In phosphatidylinositol 3-kinase (PI3K/Akt) signaling pathway, ERβ plays a role in breast cancer cell proliferation. Activated ERβ will recruit the E3 ubiquitin ligase murine double minute 2 (MDM2) protein from the cytoplasm to the nucleus and degrade them (Sanchez, *et al*., 2013). On the other hand, MDM2 acts as ubiquitin ligase for

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a tumor suppressor p53. The interaction between MDM2 and p53 promotes protein degradation, leading to cancer cell proliferation. Furthermore, highly expressed 'free' MDM2 also increases cancer cell growth (Sanchez, *et al*., 2013). Thus, the interaction between ERβ and MDM2 becomes a promising target for breast cancer drug discovery. A molecule that can stabilize ERβ and MDM2 interaction will stimulate more 'free' MDM2 to bind to ERβ. Thus, it will decrease its expression and interaction with tumor suppressor p53, inhibiting cancer cell growth.

This study reported the virtual screening of FDA-approved drugs retrieved from ZINC database to find the compound which can stabilize ERβ and MDM2 interaction (Cavasotto, 2011). The first step, protein-protein docking was performed to build ERβ and MDM2 complex which then be used as macromolecule in the screening virtual step. Analysis of protein-protein docking model results was substantial to provide evidence that the interaction between ERβ and MDM2 was established virtually. After protein-protein docking analysis to confirm ERβ and MDM2 interaction, the virtual screening was performed utilizing PyRx 0.8 tool. To confirm the binding of the protein complex and the ligand, we performed protein hotspot analysis using FTMap (Kozakov, *et al*., 2015; Petta, *et al*., 2016), and PLIP and PIC web tools to analyze the amino acid residues involved in the interaction.

METHODS

Proteins and Ligands Preparation

The 3D structure proteins used in this study ERβ (PDB ID: 3OLS) and MDM2 (PDB ID: 1T4E) were retrieved from Protein Data Bank (https://www.rcsb.org/). Water molecules, native ligands, and any other molecules associated with both of 3D structure were removed by Biovia Discovery Studio 2021 software. The structures were then saved as PDB files and used for further protein-protein docking analysis on ClusPro 2.0 (Kozakov, *et al.*, 2017).

The 1615 molecules of FDA-approved drugs were downloaded from ZINC Database (Sterling & Irwin, 2015). These compounds were minimized their energy and converted to pdqt format using the Open Babel built in PyRx 0.8. The energy minimization resulted in 1058 compounds, which were all applied as ligands in the virtual screening.

Protein-protein Docking of Estrogen Receptor Beta (ERβ) and Murine Double Minute 2 (MDM2)

Web server ClusPro 2.0 (https://cluspro.bu.edu/login.php?redir=/home.php) was applied to perform protein-protein docking between protein ERβ and MDM2 (Kozakov, *et al*., 2017). Web server ClusPro 2.0 have a three-step computation: 1) rigid-body docking using billions of global protein conformation database, 2) clustering 1000 structure with the lowest binding energy based on root-mean-square derivation (RMSD) to get a representation of model cluster, and 3) sorting the chosen structure based on the minimum energy.

The pdb files of ERβ and MDM2 were used as receptor and ligand in ClusPro 2.0 for protein-protein docking. All chains of ERβ were used and subsequentially docked with MDM2 chain A or B. The model structure of ERβ and MDM2 chain A or B with the lowest score was then used for further analysis. There are four algorithm scores from ClusPro 2.0: balanced, electrostatic-favored, hydrophobic-favored, and Van der Waals + electrostatic. The lowest energy on cluster model "balanced" was then visualized using PyMOL 2.0 software. The best cluster model from ClusPro 2.0 was validated in Ramachandran Plot Server (https://zlab.umassmed.edu/bu/rama/index.pl) followed by protein interface analysis using PIC web server (http://pic.mbu.iisc.ernet.in/). Web server online FTMap (https://ftmap.bu.edu/queue.php) was utilized to predict the hot spot of ERβ-MDM2 complex where

Table 1. Model score of the complex of ERβ and MDM2 protein chain A and B.

Cluster	Members	Representative	Weighted Score
Chain A			
1	81	Center	-627.8
		Lowest Energy	-761.0
Chain B			
1	50	Center	-593.0
		Lowest Energy	-705.0

the molecule with the lowest binding energy score can bind.

Virtual Screening for Compounds that Binds to Estrogen Receptor Beta (ERβ) and Murine Double Minute 2 (MDM2) Complex

Virtual screening of 1615 compounds of FDA-approved drugs from the ZINC database was performed toward the ERβ-MDM2 complex using PyRx 0.8 (Dallakyan & Olson, 2015; Sterling & Irwin, 2015). Energy optimization of the FDA-approved drugs molecule using default energy minimization parameters set by Open Babel built in PyRx 0.8 tools (uff force field and conjugate gradients for optimization algorithm with 'total number of steps'; 'number of steps for update'; and 'stop if energy difference is less than number' were set on 200; 1; and 0.1, respectively) (Dallakyan & Olson, 2015; Kozakov, *et al*., 2017). Among 1615 molecules, only 1057 could be minimized its energy and converted to pdbqt format. The center was set (X: 18.4920, Y: -23.0923, and Z: 4.7846), the grid box was set to maximum (X: 74.1007, Y: 60.8801, and Z: 73.9575), and equal 8 for exhaustiveness. Virtual screening resulted in the energy binding score and RMSD values of all molecules. The top five molecules with the lowest energy binding score were then reported and discussed. All visualization of the 3D structures were performed in PyMOL software.

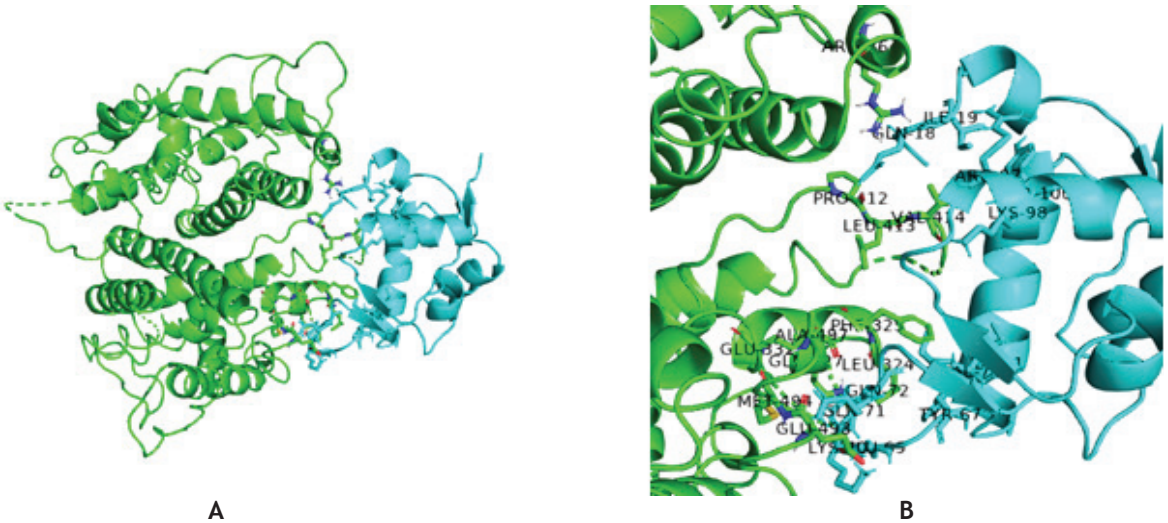

Figure 1. Visualization of 3D structure of ERβ and MDM2 protein complex. (A) The structure of ERβ is displayed in green and MDM2 is in tosca; (B) zoomed in with amino acid residues label.

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Table 2. Amino acid residues in the interaction between ERβ and MDM2 model structure.

Types	ERβ		with	MDM2	
	Position	Residues		Position	Residue
Hydrophobic interactions (within 5Å)					
	324	LEU		62	MET
	324	LEU		67	TYR
	325	PHE		61	ILE
	325	PHE		62	MET
	325	PHE		67	TYR
	325	PHE		75	VAL
	325	PHE		93	VAL
	413	LEU		93	VAL
	414	VAL		100	TYR
	414	VAL		19	ILE
Hydrogen bonds					
Main chain-side chain					
	324	LEU		62	MET
	324	LEU		72	GLN
	324	LEU		72	GLN
	412	PRO		18	GLN
	412	PRO		18	GLN
	414	VAL		96	HIS
	497	ALA		73	HIS
Side chain-side chain					
	314	LYS		69	GLU
	332	GLU		94	LYS
	332	GLU		94	LYS
	327	GLN		72	GLN
	327	GLN		72	GLN
	327	GLN		72	GLN
	327	GLN		72	GLN
	364	ARG		18	GLN
	364	ARG		18	GLN
	493	GLU		71	GLN
	493	GLU		71	GLN
	493	GLU		70	LYS
	493	GLU		70	LYS
	493	GLU		71	GLN
	493	GLU		71	GLN
	494	MET		71	GLN
	494	MET		71	GLN
	494	MET		71	GLN
	494	MET		71	GLN
Ionic interactions					
	314	LYS		69	GLU
	332	GLU		73	HIS
	332	GLU		94	LYS
	493	GLU		70	LYS
	365	ASP		97	ARG
Atomic-atomic interactions (within 4.5Å and 7Å)					
	325	PHE		67	TYR
Aromatic-sulfur interactions (within 5.3 Å)					
	325	PHE		62	MET

Table 3. Top five lowest energy binding affinity between FDA-approved drugs and ERβ and MDM2 complex.

Rank	Name	ZINC ID	Energy binding affinity (kcal/mol)
1	Ergotamine	ZINC000052955754	-12
2	Dihydroergotamine	ZINC000003978005	- 11.6
3	Bromocriptine	ZINC000053683151	-11
4	Telmisartan	ZINC000001530886	-10.8
5	Alectinib	ZINC000066166864	-10.8
Native ligand	Estradiol	ZINC000002563085	- 8.1

RESULTS

Estrogen Receptor Beta (ERβ) and Murine Double Minute 2 (MDM2) Established Protein-protein Interaction

The result from ClusPro 2.0 simulation exhibited the thirty best models of ERβ-MDM2 protein-protein interaction. Cluster size with the number of neighbors within radius 9Å C-α RMSD was applied as the best model score category selection parameter. Both ERβ chains (chain A and B) were inputted as receptors. Based on protein-protein docking analysis, B chain of MDM2 showed better model score and the most neighbors than the A chain MDM2 (Table 1). The B chain of MDM2 was then applied for further analysis. Visualization of the best model was performed using PyMOL (Figure 1).

Furthermore, protein-protein interaction was validated by Ramachandran Plot server, and its protein-protein interface between ERβ and MDM2 was analyzed in PIC web server. PIC analysis showed the presence of interaction in amino acid residues (Table 2). Model validation of interaction between ERβ and MDM2 in Ramachandran plot server obtained a highly preferred area for the interaction reached 91.14%. In addition to hydrophobic and ionic interaction, as well as hydrogen

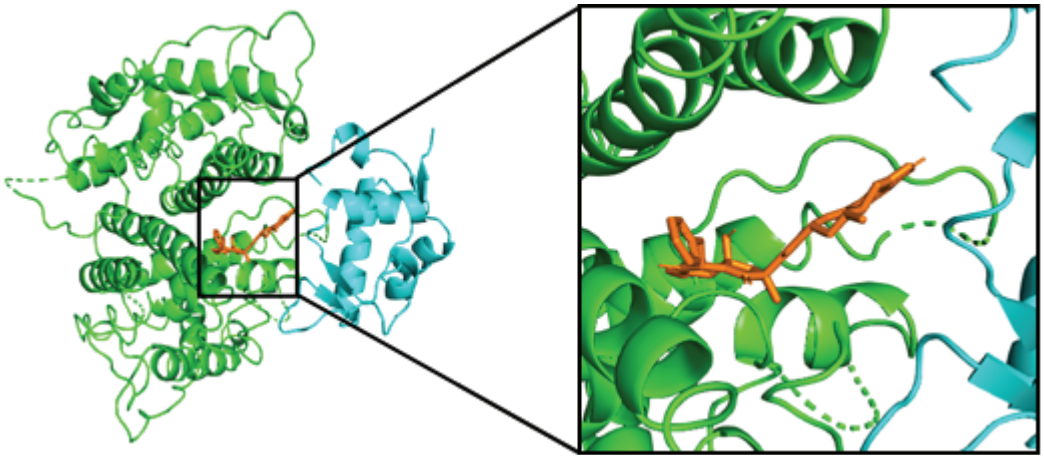

Figure 2. Visualization of ergotamine binding to ERβ-MDM2 complex. Ergotamine (orange) was docked toward ERβ (green)-MDM2 (tosca) complex protein.

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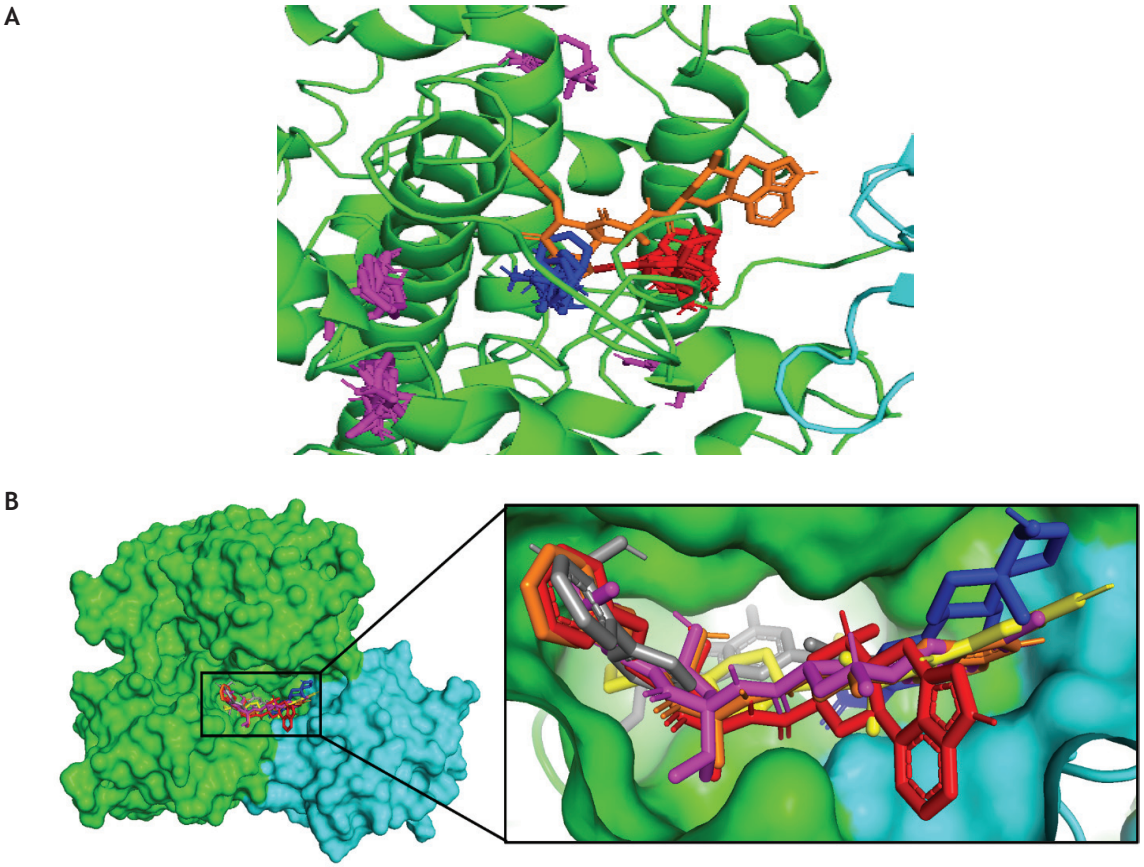


Figure 3. (A) Prediction of hot spot in the ERβ-MDM2 complex by FTMap. Ergotamine (orange) was docked in the ERβ-MDM2 complex on region CS0 (red). Blue probes were located in CS2, whereas probes in purple showed located in another CS. (B) The same hotspot was favored by estradiol (blue) and four other ZINC compounds; dihydroergotamine (red), bromocriptine (purple), telmisartan (grey), and alectinib (yellow) to bind to ERβ-MDM2 complex .

bonds, it revealed that atomic-atomic interactions and aromatic-sulfur interaction also occurred. The detailed interaction between amino acid residues in ERβ and MDM2 are displayed in Table 2. These interaction was in line with the fact that domain 26-109 of MDM2 (SWIB/MDM2 domains) participate in protein-protein interaction (https://prosite.expasy.org/rule/PRU01273) with the functional role is poorly characterized.

Ergotamine Binds to Estrogen Receptor Beta (ERβ) and Murine Double Minute 2 (MDM2) Complex with the Lowest Binding Energy Score and the Presence of Ergotamine Strengthen its Binding Affinity

PyRx 0.8 was then applied to virtually screened FDA-approved drugs. The best ERβ and MDM2 complex model was used as macromolecules to screen 1615 FDA-approved drugs from

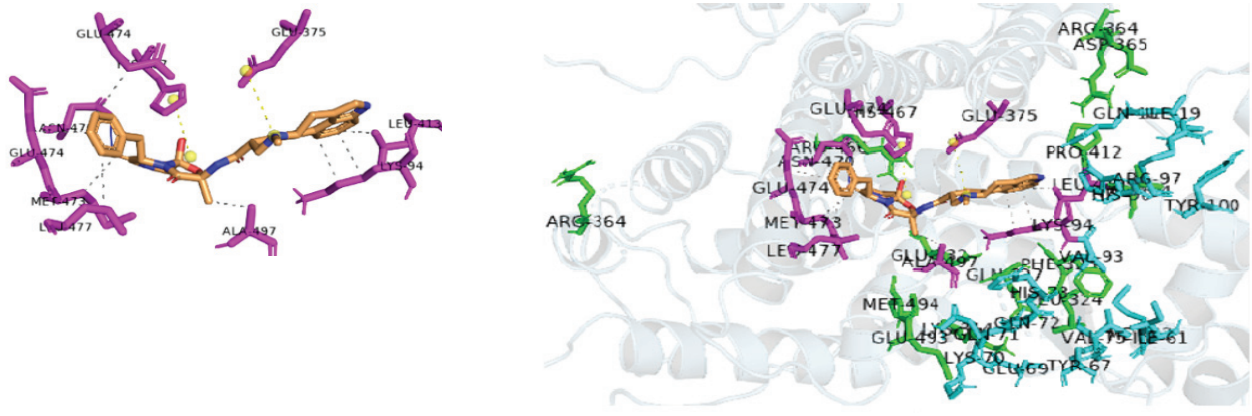
Table 4. Amino acid residues in the interaction ERβ-MDM2 complex with ergotamine.

Interaction Type	Residue Number	Residue Type	Protein Unit	Distance (Å)
Hydrophobic interactions				
	94B*	LYS	MDM2	3.53
	94B*	LYS	MDM2	3.65
	413A*	LEU	ERβ	3.32
	473A	MET	ERβ	3.78
	474A	GLU	ERβ	3.78
	474B	GLU	ERβ	3.64
	477A	LEU	ERβ	3.57
	497A*	ALA	ERβ	3.68
Hydrogen bonds				
	470A	ASN	ERβ	2.88
Salt bridges				
	375B	GLU	ERβ	4.58
	467B	HIS	ERβ	4.82

Note: amino acid residues in star represented the amino acid residues that initially showed interaction in the ERβ-MDM2 protein complex.

the ZINC database. From 1615 compounds, 1057 compounds were obtained after energy minimization. These compounds were docked to the ERβ and MDM2 complex using the maximum grid box setting. The docking resulted in compounds' top five lowest energy binding affinity as shown in Table 3.

To confirm the "hot spot" of the ERβ-MDM2 complex, we performed an analysis on FTmap (https://ftmap.bu.edu). There were seven consensus sites (CS), considered the "hot spot" of ERβ-MDM2 complex. The hot spots are showed where the probes were displayed in different colors in (Figure 3). The probe cluster in red color



A B

Figure 4. The 3D visualization of amino acid residues involved the interaction between ERβ-MDM2 (purple) and ergotamine (orange) (A). Overlay visualization of amino acid residues of ERβ-MDM2 before (ERβ in green and MDM2 in tosca) and after (ERβ-MDM2 in purple) ergotamine was docked into the ERβ-MDM2 complex (B).

Table 5. Model score of the interaction ERβ and MDM2 in the presence of ergotamine.

Cluster	Members	Representative	Weighted Score
0	84	Center	-631.8
		Lowest Energy	-765.9

represented CS0, which was considered as the primary hotspot. This CS0 exhibited the most number of probes than another six CS found in the ERβ-MDM2 complex. Ergotamine indeed, binds to the ERβ-MDM2 complex in its main hot spot. This main hot spot was also favored by dihydroergotamine, bromocriptine, telmisartan, alectinib, and estradiol to bind to the ERβ-MDM2 complex. It also explained the slight difference in energy binding scores between five virtually screened ZINC compounds (Table 3).

PLIP tool was also applied to analyze amino acid interactions between ergotamine and ERβ-MDM2 protein complex. The result is displayed in Table 4 and Figure 4. Interestingly, amino acid residues initially interacted in the ERβ-MDM2 complex and were replaced by ergotamine after the docking. LEU413 and ALA497 residue ERβ, which initially interacted with VAL93 and HIS73 of MDM2, it established hydrophobic interactions with ergotamine. Similar observation on LYS94 residue MDM2, which initially interacted with GLU332 of ERβ, established hydrophobic interaction with ergotamine.

Protein-protein docking was also carried out to predict the binding affinity of ERβ-MDM2 complex in the presence of ergotamine using web server ClusPro 2.0 (Jena & Duttaroy, 2022; Rajendaran, *et al*., 2020). The binding affinity of the complex showed a lower score than of ERβ-MDM2 without ergotamine (see Table 1 and 4). Thus, the presence of ergotamine strengthens the interaction between ERβ and MDM2.

DISCUSSION

The uncontrolled expression of ERs can be used to predict breast cancer. More than 70% ERs are found in breast cancer cases as they are the master transcription factor in breast cancer phenotypes (Scabia, *et al*., 2022; Zheng, *et al*., 2016). One of ER isoform, ERβ, is highly expressed in normal breast epithelial cells and in 20-30% of invasive breast cancers (Elebro, *et al*., 2017; Hawse, *et al*., 2020). So, targeting ERβ for novel breast cancer drug discovery is promising.

Tamoxifen, as an antagonist, plays a role in ER beta mitochondria of breast cancer cells which increases reactive oxygen species (ROS) and is required for cytotoxicity (Razandi, *et al*., 2012). Tamoxifen regulation in ER is common and is thought to be via the PI3K-PTEN/AKT/mTOR signaling pathway (Hosford & Miller, 2014; Yin, *et al*., 2014). AKT controls the activity of the E3 ubiquitin ligase MDM2, which plays a role in the tumor suppressor p53 degradation, which promotes cancer cell growth. The increase of MDM2 expression also stimulated cancer cell growth. On the other hand, the interaction between ERβ and MDM2 leads to ERβ degradation, which is now believed to be a prognostic marker for cancer progression (Sanchez, *et al*., 2013). Thus, targeting the stabilization of the interaction between ERβ and MDM2 is promising to reduce breast cancer cell growth. MDM2 will interact more with ERβ, thus decreasing its expression and interaction with tumor suppressor p53 so that the cancer cell growth is inhibited.

Based on protein-protein docking analysis using ClusPro 2.0, this study successfully confirmed the interaction between ERβ and MDM2. This protein complex was then applied as the macromolecule on the virtual screen of 1.057 energy minimized molecules of FDA-approved drugs using PyRx 0.8. The virtual screen resulted in the top 5 compounds which showed the lowest

energy binding affinity score. These five compounds are ergotamine, dihydroergotamine, bromocriptine, telmisartan, and alectinib. Interestingly, all these compounds have no indications as breast cancer drugs.

Ergotamine is a member of the ergot alkaloids and is used for migraine, various migraines, or cluster headache medication (Ngo & Tadi, 2022). Dihydroergotamine is commonly used for migraines, and bromocriptine is known for treating galactorrhea due to prolactin-related disorders and adjunct treatment in surgery or radiotherapy for acromegaly (Dhiani, *et al*., 2022). Telmisartan is used for hypertension treatment. Alectinib is an orally available inhibitor of the receptor tyrosine kinase anaplastic lymphoma kinase (ALK) with antineoplastic activity (https://pubchem.ncbi.nlm.nih.gov/).

In the cytotoxicity test of ergotamine using human primary cells RPTEC and NHA, high viability cell values were obtained as high as 70% in RPTEC and 80% in NHA cells (Mulac & Humpf, 2011). A weak toxic effect was also observed from the induction of ergotamine to liver cancer cells HepG2 and colon cancer cells HT-29 (Mulac, *et al*., 2013). No studies are reported on the activity of ergotamine for inhibition of breast cancer cell growth.

A study on the interaction between ERβ and MDM2 by immunoprecipitation reported the inhibitory function of MDM2 on ERβ, which resulted in breast cancer cell proliferation. The increasing level of MDM2 down-regulated ERβ and the presence of mutation C462A of MDM2, which is important for its E3-ubiquitin ligase activity, restore the level of ERβ (Sanchez, *et al*., 2013). The quest for breast cancer drug discovery has been extensively carried out based on the discovery of mere MDM2 inhibitors and exploited the role of these inhibitors on the MDM2-p53 interaction (Li, *et al*., 2021; Tortorella, *et al*., 2016; Zhu, *et al*., 2022). None has reported the role of ligands on the protein complex ERβ-MDM2. Based on this virtual screen and protein-protein docking analysis, the presence of ergotamine in the ERβ and MDM2 complex strengthen the affinity between the two proteins. The higher affinity of MDM2 to ERβ decreased the 'free' MDM2 which can promote cancer cell proliferation and inhibit the binding of MDM2 to p53, thus ultimately inhibiting breast cancer cell growth.

However, this study is merely a computational approach that cannot be used to draw a strong, evidenced conclusion. Thorough investigation using *in vitro*, biochemical, and *in vivo* studies are needed to support the evidence of ergotamine's role in inhibiting breast cancer cell growth.

CONCLUSION

Virtual screening of FDA-approved drugs exhibited that ergotamine, dihydroergotamine, telmisartan, bromocriptine, and alectinib bind to the ERβ-MDM2 protein complex, in which ergotamine strengthens ERβ and MDM2 interaction, thus could be developed as breast cancer drug.

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