

Molecular Docking study, and In vitro Evaluation of Antitumor Activity of Some New Isoxazoline and Pyrazoline Derivatives of Nabumetone against breast cancer cell line (MCF-7)

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Abstract:

A variety of new pyrazolines, isoxazolines, and amide derivatives were designed, synthesized, and tested in vitro for their cytotoxic potential against the breast cancer cell line MCF-7. Nabumetone is a prodrug that is used as non-steroidal anti-inflammatory drug

(NSAID). Before synthesis, the Molecular docking program (GOLD suite v. 5.7.1) was used to evaluate the selectivity for ER- α receptor, which demonstrated good agreement with the in vitro findings. Specifically, compounds 1e and 2e that target the ER- α receptor had the greatest PLP fitness values of (75.61 and 73.36), respectively, when compared to the tamoxifen reference medication, which had a PLP fitness of (92.78). The IC₅₀ values for the synthesized compounds revealed that compound (1e) has a high IC₅₀ value of 19 μ M against MCF-7, compared to tamoxifen, which has an IC₅₀ value of (18.02) μ M.

Key words: Nabumetone, Pyrazoline, Isoxazoline, Docking Study, Breast Cancer.

الارساء الجزئي والتقييم المختبري للفعالية المضادة للسرطان لبعض مشتقات الايزوكسازولين والبيرازولين الجديدة للنايوميتون ضد خلايا سرطان الثدي (MCF-7)

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الخلاصة:

تم تصميم مجموعة جديدة من البيرازولين والاييزوكسازولين ومشتقات الأמיד التي تحتوي على جزء النابوميتون ، وتوليفها واختبارها في المختبر من حيث قدرتها على تسمم الخلايا ضد خط خلايا سرطان الثدي MCF-7 نابوميتون هو دواء أولي يستخدم كعقار التهابي غير ستيرويدي (مضادات الالتهاب غير الستيروئيدية) . قبل تحضير المركبات، تم استخدام برنامج الالتحام الجزئي) مجموعة GOLD الإصدار 5.7.1 (لتقييم انتقائية مستقبلات ER- α ، والتي أظهرت توافقاً جيداً مع النتائج المختبرية. كان للمركبين 1 e و 2 e على وجه التحديد اللذان يستهدفان مستقبل ER- α أعلى قيم لياقة PLP (75.61 و 73.36) ، على التوالي ، عند مقارنته بالدواء القياسي تاموكسيفين ، الذي كان له لياقة PLP (92.78) . كشفت قيم IC₅₀ للمركبات المحضرة أن المركب (e1) له قيمة IC₅₀ عالية تصل إلى (19) ميكرومولار ضد خلايا MCF-7 ، مقارنة مع عقار تاموكسيفين الذي يحتوي على قيمة IC₅₀ تبلغ (18.02) ميكرومولار.

الكلمات المفتاحية: نابيوميتون, بايرازولين, ايزوكسازولين, دراسة الارساء الجزيئي, سرطان الثدي.

Introduction

A tumor is a pathological cell growth disturbance, identified by abnormal cell proliferation. Tumors are irregular tissue masses that are either rigid or fluid-filled [1]. Tumors are divided into two types: benign and malignant. Benign tumors are masses of cells which are localized with uncontrolled cell growth that do not undergo metastasis [2]. They are often housed in a fibrous capsule and are normally simple to be removed surgically. Malignant tumors are uncontrolled cell growth masses that are locally invasive and do undergo metastasis. They are difficult to be surgically resected because of their irregular border and its ability to invade tissues [2]. Cancer remains the most complicated illness to treat and the main worldwide cause of mortality. Mutation occurs due to various causes, including exposure to chemicals, smoking, and other risk factors [3]. Because of one or more mutations in its DNA, a human cell becomes a cancer cell. These may be inherited or acquired, usually due to viruses or carcinogenic agents exposition (e.g. tobacco smoking products, asbestos) [4]. Breast cancer may be treated with surgery, which is regarded a first-line treatment, radiation also can be used with surgery, and cytotoxic chemotherapy, which comprises a variety of chemical classes such as DNA-alkylating agents, antimetabolites, and others or hormonal therapy such as SERMS Tamoxifen [5]. Recently, new kinds of medicines have emerged that target cancer cells with a high degree of selectivity, resulting in fewer adverse effects than traditional cytotoxic treatments [6]. At the moment, scientists are focusing their efforts on creating new anticancer medicines and heterocyclic chemicals that have been widely researched for this purpose [7]. Molecular docking is a type of bioinformatic modeling that predicts a ligand's preferred orientation in relation to

a receptor (Protein) in order to create a stable compound. Using scoring methods, the preferred orientation may be used to estimate the strength of the contact or binding affinity between ligand and protein [8].

Molecular docking has become a popular technique of computer-aided drug design (CADD) because it can anticipate the binding mechanism and energy of receptor-ligand complexes [9].

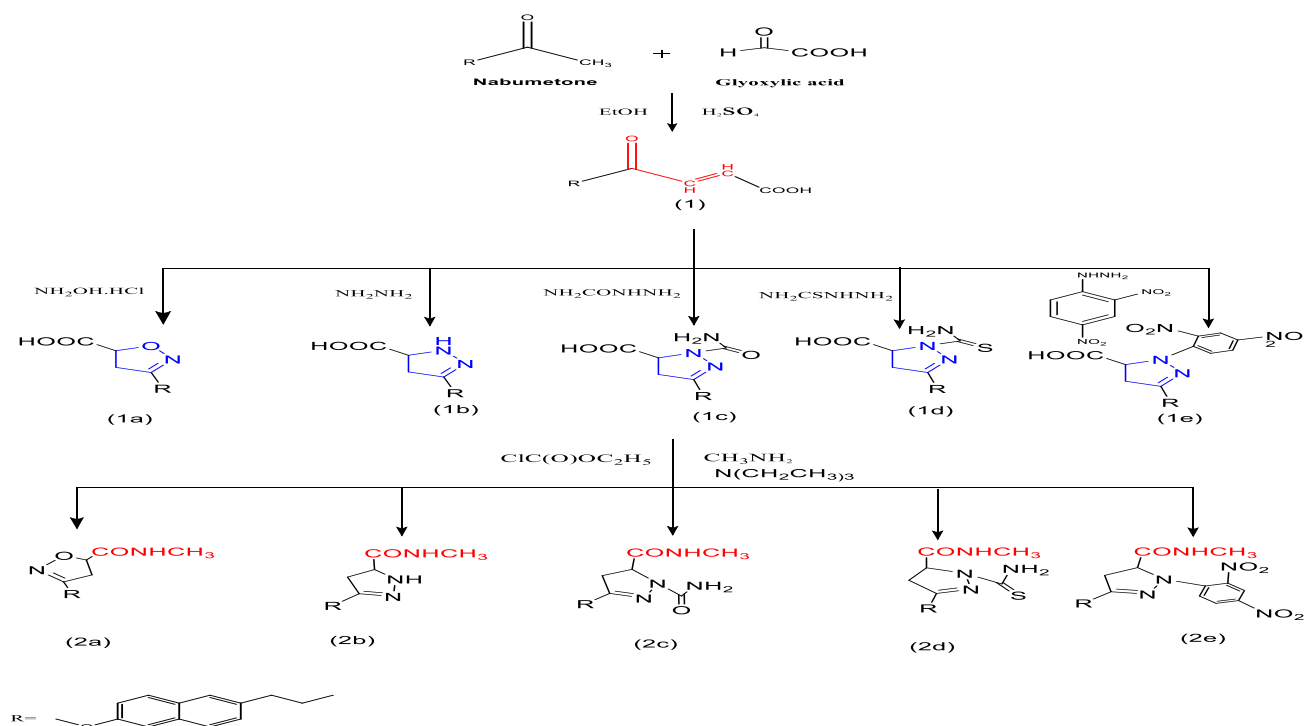
The docking software determines the binding energies and specificity of the designed compounds to protein (ER- α) by studying the molecular interaction between the protein's active binding sites and the chemical compounds [9]. The ER- α inhibitory activity of designed compounds and tamoxifen, were performed depending on their PLP fitness associated in the complex formation at the active sites. GOLD software also shows the distance of hydrogen bonding between our designed compounds and a protein along with all bonds length was $\leq 3\text{\AA}$ [9].

Nabumetone (nab ue' me tone) is a naphthyl alkanone and orally available anti-inflammatory agent. Like other NSAIDs, nabumetone is a potent cyclo-oxygenase (Cox-1 and -2) inhibitor, which blocks the formation of prostaglandins that are important mediators in pain and inflammatory pathways. Nabumetone is a pro-drug and exerts anti-cyclo-oxygenase activity only after absorption and activation in the liver. Like other NSAIDs, it has analgesic, antipyretic and anti-inflammatory activities. Some New Isoxazoline and Pyrazoline Derivatives of Nabumetone were synthesized [10].

Materials and Methods

Chemical synthesis

Scheme of synthesis of new compounds (1, 1a-e & 2a-e) is illustrated below (Scheme 1).



Scheme (1): Synthesis of intermediate and final compounds ^[10].

Computational Methods

This study has many steps as mentioned in the following sections.

Molecular docking studies for the synthesized compounds

Docking studies for the synthesized compounds were conducted using the CCDC (Cambridge Crystallographic Data Center) GOLD (Genetic Optimization for Ligand Docking) (v5.7.1.) program.

Ligands and protein receptor preparation

ChemDraw expert (v.16.0) software was used to draw the chemical structures of the newly designed and synthesized compounds. The energy of the novel compounds was decreased using Chem3D (v.16.0) and the MM2 force field. After that, the newly developed ligands were docked utilizing the active target's three-dimensional structure: the crystal structure of ER- α protein complexes with Tamoxifen (PDB code: 3ERT). The receptor then was loaded into GOLD's Hermes module from the protein data bank (PDB) ^[11]. The ER- α receptor was cleaned

up by deleting all water molecules that were not in the active site (except for water molecules (HOH31, 58, 68)). To achieve correct ionization and tautomeric states, hydrogen atoms were added to the amino acid residues ^[11].

Docking Methodology

Hermes visualizer tool from the CCDC GOLD suite was used to prepare the receptor for docking. The protein binding site was found within the (10 Å) of the reference ligand for the docking process. The number of created poses was retained at ten, the default solution was preserved, and the early termination option was deactivated. As a setup guide, Chemscore kinase was employed. The piecewise linear potential (ChemPLP) is used as a scoring function. The results were then saved as mol.2 files. These results were examined in order to determine the best interaction between our newly developed ligands and the receptor's amino acids (ER- α receptor) ^[12].

Cell Line Studies

The anticancer activity of the newly developed and synthesized compounds [1,1a-e & 2a-e] on the MCF-7 human breast cancer cell line is determined using the MTT colorimetric assay [6].

Cell Culture

ATCC provided the human MCF-7 breast cancer cell line. It was preserved in the cell bank of Mustansiriyah university/college of pharmacy's tissue culture research laboratory [6].

Cell Line Storage and Resuscitation

Under liquid hydrogen, cells were held at (-80 °C) for 24 hours. The cells were thawed at 37 °C, and then 10 ml fresh media were added. Centrifugation was used to gather the cells. After that, 25 ml of new media was added to re-suspend the cells, and they were moved to a flask with a surface area of 75 cm² and allowed to grow there [6].

Cell Maintenance

Dulbecco's modified Eagle's medium (DMEM) was used to keep human MCF-7 breast cancer cells alive (500 ml). Then complete media was added (50 mL of 10% FBS, 5 ml of 1% L-Glutamine, and 5 ml of Penicillin-Streptomycin-Amphotericin B100X as an antiseptic). These cells were propagated in 75 cm² flasks. The cells were incubated in a 5 percent CO₂ environment with 95 percent humidified air (37 °C). When the MCF-7 cells had reached 90% confluence, the flasks were put through sterile conditions [7]. Following that, these cells were washed with a PBS 5ml solution. The cells were then maintained in trypsin solution at 37°C for 2 minutes to ensure that they were detached from the flask bottom. After that, equivalent amounts of complete growth medium were added, and the resulting cell suspension was transferred to a (50 ml) conical tube. After that, the cells were centrifuged for 3 minutes (1200 rpm). Finally, the supernatant was discarded, and

the cell pellet was resuspended in growth medium (fresh media). As a result, cells were counted using a Haemocytometer (under a microscope) and used as required [7].

Cell Viability by MTT Colorimetric Assay

The cytotoxic effects of recently designed and synthesized compounds [1, 1a-e and 2a-e] on breast cancer cells were determined using the MTT colorimetric assay. The cell suspension (100 µL) was poured onto 96-well flat-bottom tissue culture plates at a concentration of (5 x 10³ cells per well), and then incubated under normal conditions for 24 hrs., (4 x 10³ cells per well) for 48 hrs., and (3 x 10³ cells per well) for 72 hrs. After the 24-hour period has passed, treat the cells with 50 µM of each of the newly synthesized compounds. The culture media of the cells was removed after the recovery period (24hr, 48hr, and 72hr) was completed, and the cultures were incubated at 37°C for 4 hours with a medium containing 30 µL of MTT solution (3mg/ml of MTT powder in PBS). When the four hours were over, the medium was removed by flipping the paper over and turning it over. The control wells were then filled with growth media (100 µL). After that, 100 µL of DMSO was put into each well, which was then left at room temperature (25 °C) for 15-20 minutes in a dark environment [13]. The MTT experiment was done in triplicate, and the optical density of each plate was evaluated at a transmission wavelength using a Multiscan Reader (520-600 nm). The following equation may be used to compute the rate of cell growth inhibition (cytotoxicity percentage):

[Inhibition Rate percentage= (A-B/A)*100] Where [A, and B] represent the optical density of control & tested compounds [14].

IC₅₀ (half-maximal inhibitory concentration) determination

The IC₅₀ of the studied compounds may be calculated using a dose-response curve. The IC₅₀ in the MTT experiment (*in vitro*) may be defined as the concentration of newly produced compounds (1, 1a-e and 2a-e) required to achieve 50% cell inhibition. The concentration ranges of compounds (1, 1a, 1b, 1c, 1d, 1e, 2a, 2b, 2c, 2d, 2e) used to calculate IC₅₀ values were (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, and 0.195 μM) ⁽¹⁵⁾.

Statistical Analysis

The MTT test and IC₅₀ data were analyzed using prism pad software, which is a nonlinear curve fitting program. To compare all of the groups that were included in the same MTT plate, one-way ANOVA with Tukey (prism pad software) was used (p > 0.05 was regarded statistically significant) ⁽¹⁶⁾.

Results and Discussion

Molecular Docking Studies

The results of the docking studies of the newly designed compounds were shown in table (1) and figures (1, 2, 3 and 4). Docking results demonstrated that all of the designed ligands (1, 1a-e, and 2a-e)

had good binding energies in the receptor's active pocket, indicating a potential interaction with the estrogen receptor alpha (ER-α) protein, as it binds to the amino acid (AAs) residues of the active site on the protein receptor through H-bonds along with other short contacts that enhance the binding.

The interaction with estrogen receptor alpha (ER-α) protein docking results showed that the compounds (1e, and 2e) give the best values of (75.61, and 73.36) respectively. The AAs and short contact that support the binding is shown in table (1) and figures (2 and 3) respectively. The binding of compound (1e) is via oxygen of nitro group that form two H-bond with Arg394. Whereas, compound (2e) form H-bond between nitro group with water molecule (HOH-AM1) ⁽¹²⁾.

Other amide-pyrazoline derivative compound (2c) give (66.29) PLP fitness value. The binding of (2c) is through two H-bond with water molecule via the nitrogen of pyrazoline moiety as shown in figure (4). These compounds still have lower binding energy than the standard drug tamoxifen that give (92.78) PLP fitness value and bind through H-bond with Thr347 through HOH-AM1 Bridge as represented in figure (1).

Table (1) The binding energies of nabumetone derivatives and tamoxifen, a conventional medication, docked with ER- α .

Compound	ER- α binding energy (PLP fitness)	Amino acids includes in H-bonding	Amino acids includes in short contact
1	65	Thr347, Glu353, Arg394	Thr347(2), Met388
1a	68.07	Thr347, Glu353, Arg394	Arg394, Thr347
1b	69.05	Asn519	Met522(4), Cys530, Lys529(3), Tyr526(4)
1c	64.37	Glu353,	Glu353, Met343, Ala350(2), Leu387
1d	68.67	Asn519	Glu523, Met522(5), Tyr526(2), Lys529(5), Cys530
1e	75.61	Arg394(2)	Leu525(6), Thr347, Tyr526, Ala350(3)
2a	62.09	Cys530,	Cys530, Phe404, Lys529, Met528(2), Leu525, Leu346(2)
2b	61.56	HOH-AM2	HOH-AM2, Leu525(2), Trp383, Phe404, Leu346(3)
2c	66.29	HOH-AM2(2)	HOH-AM2(2), Leu525(4), Phe404, Leu346(6)
2d	67.17	----	Leu354, Ala350(2), Leu346(4), Phe404, Leu349
2e	73.36	HOH-AM2	HOH-AM2, Leu525, Thr347, Leu346(2), Met343
Tamoxifen	92.78	Thr347 through HOH-AM1 bridge	Ala350, Met343(3), Leu525(2), Met528, Thr347, HOH-AM1(2)

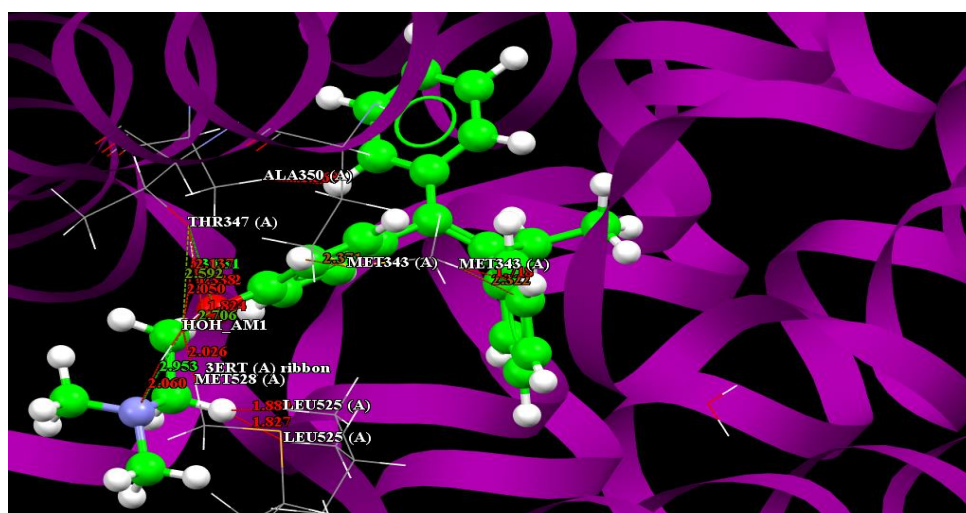


Figure (1):Binding interaction and orientation of Tamoxifen compound inside ER- α receptor.

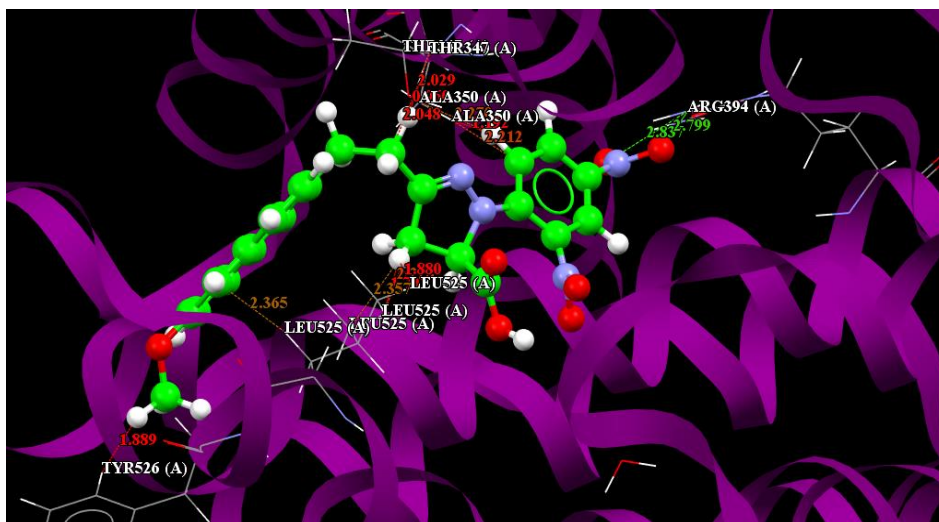


Figure (2): Binding interaction and orientation of (1e) compound inside ER- α receptor.

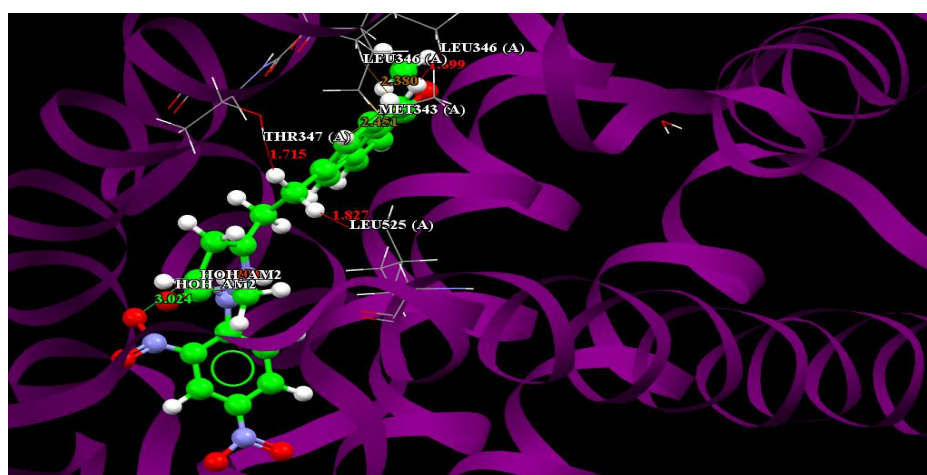


Figure (3): Binding interaction and orientation of (2e) compound inside ER- α receptor.

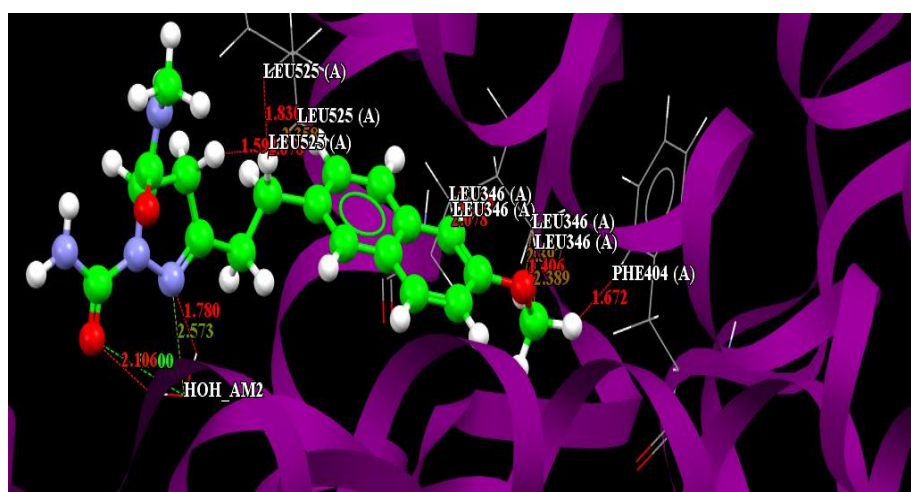


Figure (4): Binding interaction and orientation of (2c) compound inside ER- α receptor.

Cytotoxicity Assay Results of Compounds Against Breast Cancer Cell Line (MCF-7)

The IC₅₀ values of the synthesized compounds were determined using the MTT assay. The assay was done in 96-well flat plates to a range of concentrations (100 – 0.195 μM) of the synthesized compounds (1,1a-e & 2a-e) and the IC₅₀ values were obtained after treating the cells with the

compounds for 72 hrs. The resulted IC₅₀ values of these compounds were compared with reference antitumor drug tamoxifen. The IC₅₀ values were demonstrated in table (2). The dose-response curves were generated by Prism Pad 8.1 using nonlinear regression analysis for the synthesized compounds in MCF-7 cells are shown in below figures (5 to 8) [17].

Table (2): The IC₅₀ values of the effective compounds (1e, 2c, and 2e) and tamoxifen as standard against breast cancer cell line (MCF-7).

Compound	IC ₅₀ values μM
1e	19
2e	23.51
2c	34.09
Tamoxifen	18.02

According to the above results, the newly synthesized compounds [1e, 2e, and 2c] showed potential anticancer activity. The most significant cytotoxic effect was for compound [1e], which had an IC₅₀ value of 19 μM, making it

nearly as active as the reference drug tamoxifen, which had an IC₅₀ value of 18.02 μM. While compounds [2e and 2c] have higher IC₅₀ values (23.51 and 34.09 μM) than tamoxifen, which means, they show lower anticancer activity than the standard.

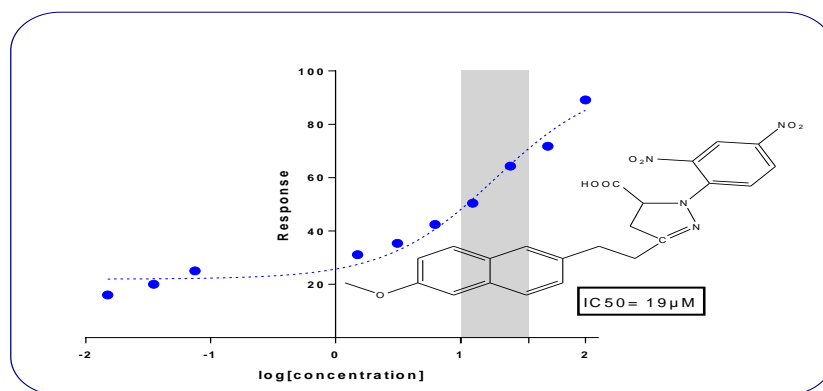


Figure (5): IC₅₀ dose-response curves for (1e). IC₅₀ of compound (1e) in Breast Cancer Cell Line (MCF-7) = 19μM Vs Tamoxifen (control) in the same cell line =18.02μM

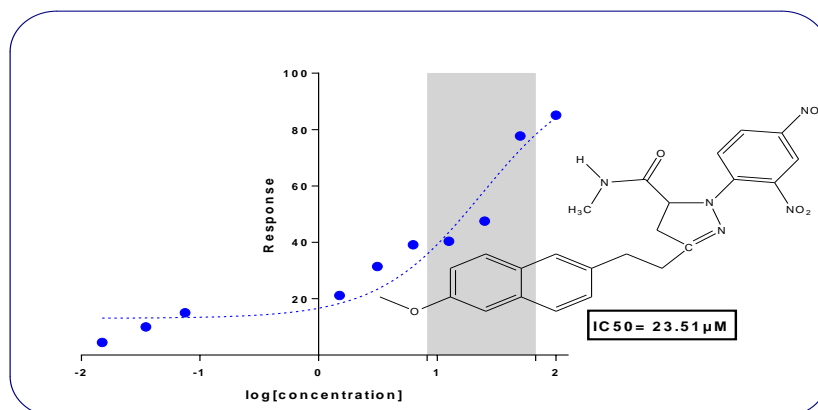


Figure (6): IC₅₀ dose-response curves for (2e). IC₅₀ of compound (2e) in Breast Cancer Cell Line (MCF-7) = 23.51μM Vs Tamoxifen (control) in the same cell line =18.02μM

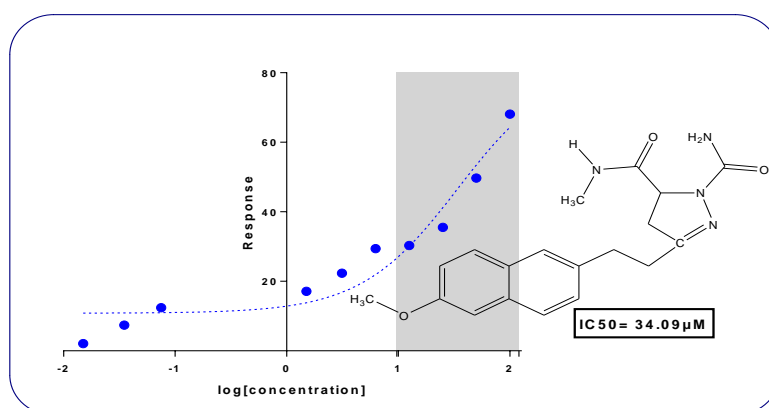


Figure (7) :IC₅₀ dose-response curves for (2c). IC₅₀ of compound (2c) in Breast Cancer Cell Line (MCF-7) = 34.09μM Vs Tamoxifen (control) in the same cell line =18.02μM

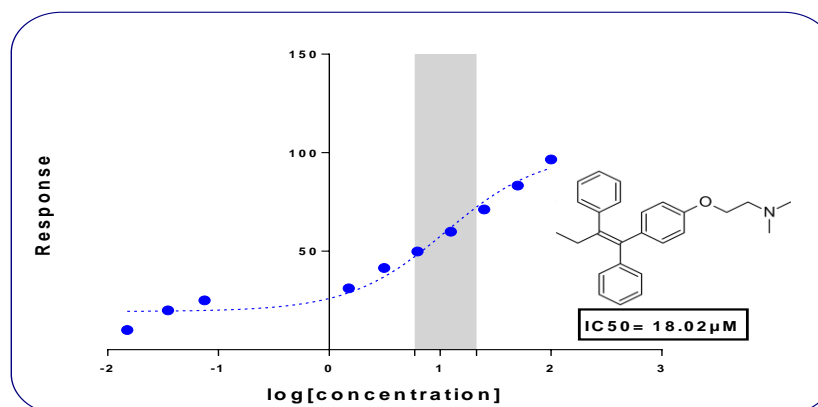


Figure (8): IC₅₀ dose-response curves for Tamoxifen (Control). IC₅₀ value of tamoxifen in breast Cancer Cell Line (MCF-7) =18.02 μM

Estimation of Percentage (%) Of Breast Cancer (MCF-7) Cell Death

The percentage (%) of cell death of the investigated compounds [1,1a-e and 2a-

e] was found using the MTT assay on human breast cancer cell line (MCF-7) cells at (35μM). The conventional anticancer medication tamoxifen was

used as the standard reference at the same concentration, at varied contact durations of 24, 48, and 72 hours. Results represent the mean absorbance \pm SEM of 3 independent experiments using graph pad prism 8.1 software to draw the dose time response versus percent (%) of cell death graph line which is shown in figure (9) [18].

The findings demonstrated that the percentage of cancer cells that die is

associated with time increasing from 24 to 72 hours. When compared to standard, all of the tested compounds reveal significant variation in cell death response. With breast cancer cells compound (1e) give (89.13%) of cell death. While compound (2e) give (85.10%) and compound (2c) (68.10%) of cell death, in comparison to standard drug tamoxifen that give (96.61%) still have lower percentage of cell death [19].

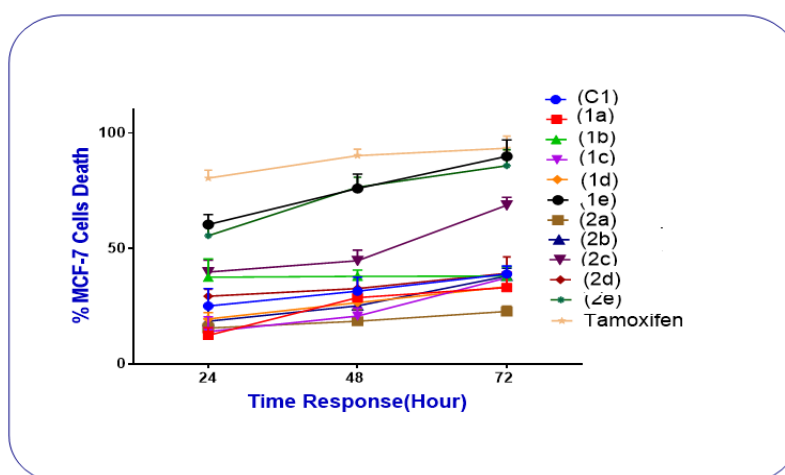


Figure (9): *In vitro* % cell death of the breast cancer (MCF-7) cells.

Conclusion

A new series of nabumetone derivatives were successfully synthesized. Anticancer activity evaluation of the synthesized compounds against the MCF-7 (breast) cancer cell line was done. Among the tested compounds the most promising compounds were (1e, 2e and 2c) with an IC_{50} values of 19 μ M, 23.51 μ M and 34.09 μ M respectively. Compound (1e) is nearly as active as the reference drug tamoxifen, which had an IC_{50} value of 18.02 μ M. While compounds (2e and 2c) have higher IC_{50} values (23.51 and 34.09 μ M) than tamoxifen, which means that, they show lower anticancer activity than the standard. Docking studies for ligands interaction with ER- α protein were done and the results were consistent with *In vitro* results.

Conflict of interest

The authors declared no conflict of interest

Acknowledgments

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