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DESIGN AND SYNTHESIS OF NEW EGFR- TYROSINE KINASE INHIBITORS CONTAINING PYRAZOLO[3,4-*d*]PYRIMIDINE CORES AS ANTICANCER AGENTS

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تضمن البحث تحضير بعض مضدات لعامل النمو المصممة وذلك من المركب الوسطى 14 ث. هذة المضدات تحتوى على وحدات مختلفة موجودة فى الوضع 4 علي جزئية الفنيل امينو وهي الاميديك والكربونيل الحلقية وشالكون والبيرازولين المشتقة والالكيل امينو بجانب مجموعة الفينول وذلك فى مركبات 7 **11**. المركبات التى تم تحضيرها تم اثبات الشكل البنائي لها بواسطة التحليل الدقيق للعناصر والتحليل الطيفى. ايضا تم عمل النمذجة الجزئية ضد عامل النمو EGFR باستخدام الاريلوتنيب كمركب رائد ليقارن به ، وايضا تم فحص المركبات كمضادات للخلايا السرطانية وقد جاءت نتائج النمذجة الجزئية متوافقة مع اختبار هذة المركبات كمضادات للخلايا السرطانية وبعض المركبات التى تم تحضيرها اضهرت فاعلية مثبطة لخلايا سرطان الثدى البشرى مثل مركب **17** الذى لة IC_{50} (14.86µM)=

*New designed EGFR inhibitors (**7-11**) were prepared from the pyrazolo[3,4-d]pyrimidine intermediates **4a-d** including different moieties. All newly synthesized compounds were confirmed by elemental analyses and spectral data. The molecular simulation docking to protein tyrosine kinase (EGFR), using erlotinib (Tarceva ™) as a lead compound was also studied. Some of the prepared compounds were screened for in-vitro cytotoxic activity. The docking results were in coincidence with the biological results that indicated compound **7a** showed an inhibitory activity against human breast carcinoma cell line (MCF-7) [IC_{50} (14.86µM)].*

INTRODUCTION

Protein kinases have become one of the most intensively pursued classes of drug target with approximately 30 distinct kinase targets being developed to the level of a phase I clinical trial[1]. Receptor protein tyrosine kinases play a role in signal transduction pathways that regulate cell division and differentiation. Among the growth factors receptor kinases that have been identified as being important in cancer is epidermal growth factor (EGFR) kinase (also known as erb-1 or Her-1 and the related human epidermal growth factor receptor (also known as erb-2 or HER-2)[2]. EGFR-dependent aberrant signaling is associated with cancer cell proliferation, apoptosis, angiogenesis and metastasis[3]. A number of small molecule EGFR kinase inhibitors have been evaluated in cancer clinical trials. Anilinoquinazoline-containing compounds, erlotinib **I** (Tarceva™)[3-5], gefitinib **II** (Iressa™)[6] (Fig. 1) and lapatinib (Tykerb™)[7-9] have been approved for the chemotherapeutic treatment of patients with advanced non small lung cancer. They are considered as 4-substituted amino pyrimidine pharmacophoric core derivatives that bind to the hinge region of the kinase enzyme. On the basis of the bioisosterism between benzene and pyrazole which is well known and widely documented in the biologically active drugs, pyrazolo-pyrimidine cores were evaluated as isosteres for quinazoline cores. Several analogues

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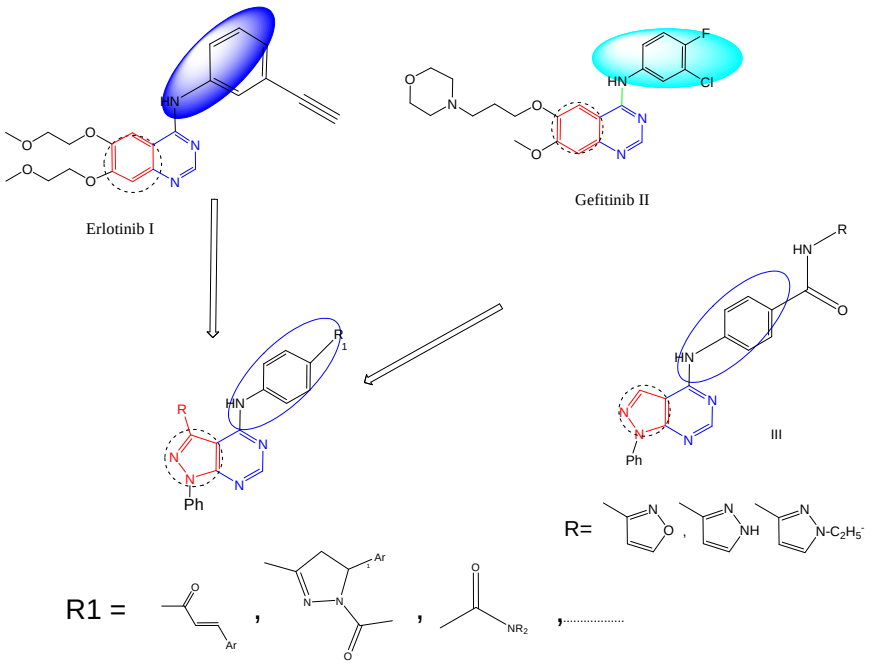


Fig. 1: Planned modification of certain kinase inhibitors.

containing 4-substituted phenylaminopyrazolo [3,4-*d*]pyrimidine III core have shown good affinity for enzyme binding site[10-16] and led to new models of kinase inhibitors.

In the present work, several pyrazolo-pyrimidines were designed and prepared that included different moieties which reported to be present in all active kinase inhibitors. These moieties are represented by amidic, fused quinazoline, chalcone, substituted pyrazoline, *o*-alkylamino to hydroxyl moiety as in compounds **(7-11)** respectively. Estimating the essentiality of phenylamino moiety, compounds **5** and **8** were prepared where this phenylamino moiety was omitted and replaced by C=O as in compound **5** and by a bicyclic structure fused with pyrazolopyrimidines as in **8**.

RESULTS AND DISCUSSION

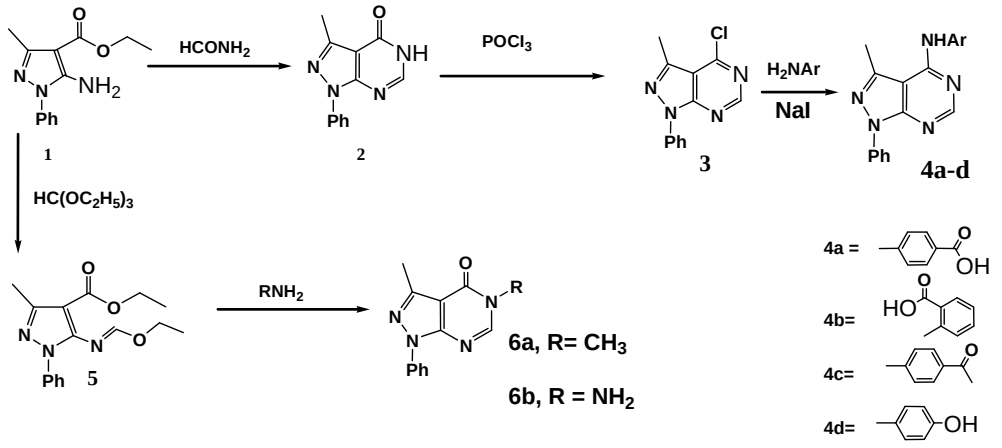
Chemistry

Reacting ethyl 5-amino-1-phenyl-3-methyl-1*H*-pyrazole-4-carboxylate **1**[17] with formamide resulted in formation of pyrazolopyrimidinone **2**[18] via a ring closure. The latter was chlorinated with phosphorous oxychloride to give 4-chloro-1-phenyl-3-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidine **3**[19] which was considered as the key intermediate used for alkylation of different aromatic amines giving **4a-d** (Scheme**1)**. The structures of compounds **4a-d** were elucidated by their elemental analyses and their spectral data. Compound **4a** showed IR absorption bands at 3600-2787 cm^{-1} indicated for (OH & NH) and 1684 cm^{-1} for (C=O). Also its ^{1}HNMR showed a characteristic peaks at δ 2.81ppm (s, 3H, CH_3); 7.33 ppm (dd J_1= 9 Hz, J_2= 3 Hz, 1H, ArH(H4 of 1-phenyl)); 7.54 ppm (t, 2H, ArH (H3&H5 of 1-phenyl)); 7.91 ppm (d, *J*= 9 Hz, 2H, ArH (H2&H6 of phenyl amino)); 7.97 ppm (d, *J*= 9 Hz, 2H, ArH (H3&H5 of phenyl amino)); 8.18 ppm (d, *J*= 9 Hz, 2H, ArH (H2,H6 of 1-phenyl)); 8.53 ppm (s, 1H, C6H); 8.99 (s,1H, NH, D_2O exchangeable. Also compound **4b** showed IR absorption bands at 3600-3063 cm^{-1} for (OH & NH) and at 1691 cm^{-1} for (C=O). While its ^{1}HNMR showed peaks at δ 2.80 ppm (s, 3H, CH_3); 7.14 ppm (t, *J*= 6 Hz, 1H, ArH (H4 of phenylamino)); 7.32 ppm (t, *J*= 9 Hz, 1H, ArH (H4 of 1-phenyl)); 7.53 ppm (t, *J*= 6 Hz, 2H, ArH(H3&H5 of 1-phenyl)); 7.64 ppm (t, *J*= 9 Hz, 1H, ArH (H5 of phenyl amino)); 8.03 ppm (d, *J*= 6 Hz, 1H, ArH (H6 of phenyl amino)); 8.15 ppm (d, *J*= 4.5 Hz, 2H, ArH (H2&H6 of 1-phenyl)); 8.57 ppm (s, 1H, C6H); 9.12 ppm (d, *J*= 9 Hz, 1H, ArH (H3 of phenyl amino)); 9.28 ppm (brs, 1H, NH, D_2O exchangeable); 11.56 ppm (s, 1H, OH, D_2O exchangeable). In addition compound 4c its IR showed absorption bands at 3409 cm^{-1} for (NH) and at 1671 cm^{-1} for (C=O). ^{1}HNMR of

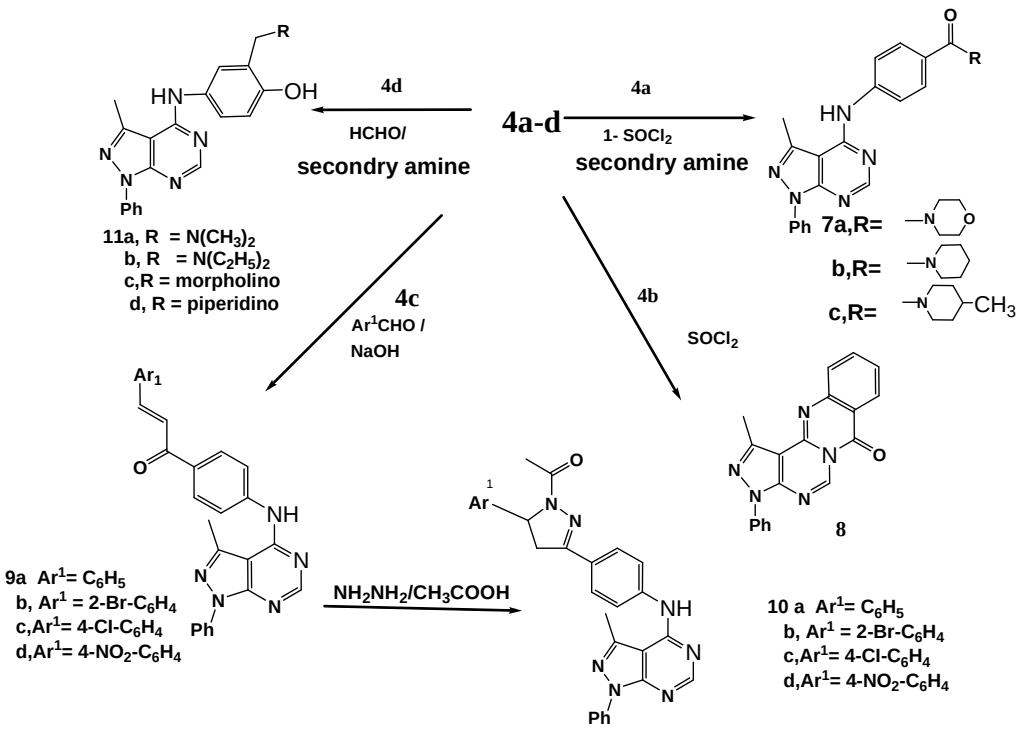
4c showed peaks at δ 2.60 ppm (s, 3H, O=C-CH_3); 2.82 ppm (s, 3H, CH_3); 7.33 ppm (t, *J*= 9, 1H, ArH (H4 of 1-phenyl)); 7.55 ppm (t, *J*= 6.7, 2H, ArH (H3&H5 of 1-phenyl)); 7.77 ppm (d, *J*= 7.8, 2H, ArH (H2 &H6 of phenyl amino)); 8.03 ppm (d, *J*= 7.8, 2H, ArH(H3, H5 of phenyl amino)); 8.18 (d, *J*= 9, 2H, ArH (H2&H6 of 1- phenyl)); 8.47 (s, 1H, C6H); 8.97 (s, 1H, NH, D_2O exchangeable). More over, **4d** its IR spectrum showed bands at 3435-2680 cm^{-1} for (OH & NH) and its ^{1}HNMR showed peaks at δ 2.73 ppm (s, 3H**,** CH_3); 6.80 ppm (d, *J*= 8.6, 2H, ArH (H2&H6 of phenylamino)); 7.24-7.62 ppm (m, 5H, ArH (H4 of 1-phenyl, (H3,H5) of phenyl amino and (H3,H5) of 1-phenyl)); 8.18 ppm (d, *J*= 9, 2H, ArH (H2,H6 of 1-phenyl)); 8.33 ppm (s, 1H, C6H); 8.67 ppm (s, 1H, NH, D_2O exchangeable); 9.41 (s, 1H, OH, D_2O exchangeable).

On the other hand, the aminoester **1** was reacted with triethyl orthoformate giving ethyl (5-ethoxymethyleneimino-3-methyl-1-phenyl-1*H*-pyrazole)-4-carboxylate **5.** Formation of compound **5** was confirmed via its ^{1}HNMR that revealed the presence of two triplet peaks at δ 1.32 ppm and 1.38 ppm and two quartet peaks at δ 4.24 ppm and 4.32 ppm corresponding to two ester moieties. Subsequentlly cyclization of compound **5** by treating with methylamine or hydrazine hydrate gave **6a** and **6b** respectively. ^{1}HNMR spectra of **6a** and **6b** revealed the disappearance of the two ester moieties and appearance of new singlet peaks at δ 3.48 ppm corresponding to N-C<u>H</u>$_3$ (**6a)** and at δ 5.74 ppm which is exchangeable with D_2O corresponding to NH_2 (**6b)** (Scheme1). The intermediate **4a** was heated with thionyl chloride followed by secondary amines in-situ giving a series of pyrazolopyrimidines **7a-c** that carry an amidic function group para to amino moiety (Scheme 2). The spectral data of **7a** confirmed its structure that IR showed a charachteristic band at 3392 cm^{-1} related to NH and at 1681 cm^{-1} which indicate amidic C=O. Its ^{1}HNMR spectrum showed two triplet peaks at δ 2.75 ppm and 3.30 ppm corresponding to its morpholino moiety. Additionally, upon heating **4b** with thionyl chloride, 1-methyl-3-phenylpyrazolo [3, 4:4, 5] pyrimido[6, 1-*b*]quinazolin-7(3*H*)-one (**8**) was obtained (Scheme 2). Its ^{1}HNMR spectrum revealed the disappearance of two exchangeable bands of NH and OH. Moreover, **4c** was reacted with appropriate aromatic aldehyde giving chalcone derivatives **9a-d**. The chalcones were cyclized through treatment with hydrazine hydrate in acetic acid affording the pyrazoline derivatives **10a-d** (Scheme 2). The ^{1}HNMR spectrum of **10a** displayed a singlet signal at δ 2.44 ppm attributed to (O=C-CH_3), a pair of doublet at δ3.15 ppm, 3.21 ppm corresponding to 1H of C4H of pyrazoline and another pair of doublet at δ 3.78-3.84 ppm belongs to another H of C4H,while C5H of pyrazoline appeared at δ 5.57, 5.61ppm as a pair of doublet.

Finally, certain mannich bases **11a-d** were prepared by reaction of **4d** with formaldehyde and secondary amines. Actually, compounds of **11a-d** were obtained as mixtures as indicated by TLC which upon crystallization yielded the desired product **11a-d** (Scheme 2). Physical and spectral data of all the prepared compounds are illustrated in the experimental section.



Scheme 1: Synthesis of compounds **4a-d, 6a** and **6b.**



Scheme 2: Synthesis of compounds **7a-c, 8, 9a-d, 10a-d** and **11a-d.**

Molecular docking

Protein kinases (PKs) are essential enzymes in cellular signaling processes that regulate cell growth, differentiation, migration and metabolism[20]. They transfer phosphate from ATP to tyrosine, serine and threonine residues in protein substrates[21]. Aberrant catalytic activity of many PKs via mutation or overexpression plays an important role in numerous pathological conditions including cancer[21&22] Protein kinase inhibitors have been widely used to probe the role of protein phosphorylation in cellular signaling and constitute an important new class of potential therapeutic agents in the management of cancer[23-25]. Literature review[26&27] revealed that the kinase inhibitor binding site can be visualized as five main pharmacophoric regions: hydrophilic region formed of adenine binding site, sugar and phosphate regions in addition to two hydrophobic areas I and II. Most kinase inhibitors share common properties: low molecular weight (small molecules) hydrophobic heterocycles which act by competing with ATP for binding in kinase ATP binding site[26] Kinase inhibitors should contain the following attributes to gain selectivity and potency[28]. A portion that closely mimics ATP molecule and one to three hydrogen bonds to the amino acids located in the hinge region of the target kinases, as in erlotinib **I**[26], gefitinib **II**[29] and lapatinib[26]. An additional hydrophobic binding site which is directly adjacent to the ATP binding site (allosteric site), as in imatinib mesylate[30] and sorafenib[31]. However, other mechanism could be achieved through binding outside the ATP-binding site at an allosteric site[32] and by forming irreversible covalent bond to the kinase active site[33&34] EGFR tyrosine kinase is a target for a remarkable variety of antitumor drugs, such as erlotinib **I**[4]. The 3D structure of EGFR protein kinase complexed with erlotinib was obtained from Protein Data Bank (PDB entry: 1M17) at Research Collaboration for Structural Bioinformatics (RCSB) protein database[35] (Fig. 2).

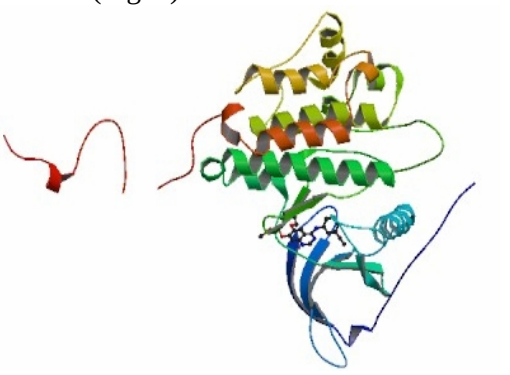


Fig. 2: 3D Structure of EGFR tyrosine kinase with erlotinib.

In order to qualify the molecular docking results in terms of accuracy of the predicted binding conformations in comparison with the experimental results, the internal ligand (erlotinib) was used as a testing molecule. The molecular docking result indicated that the binding conformation of the internal ligand, derived by MOE (Molecular Operating Environment, 2008, 10) superposed well with the crystallographic one. The vicinity where erlotinib bound was considered as the active site of EGFR tyrosine kinase and erlotinib as template. The interactions of erlotinib with EGFR (can be visualized using MOE site finder program), red dotted line representing H-bonding interaction between N3 and Thr 766 through a water bridge (red sphere), and violet dotted line representing H-bonding interaction between N1 and Met 769 as in figure 3.

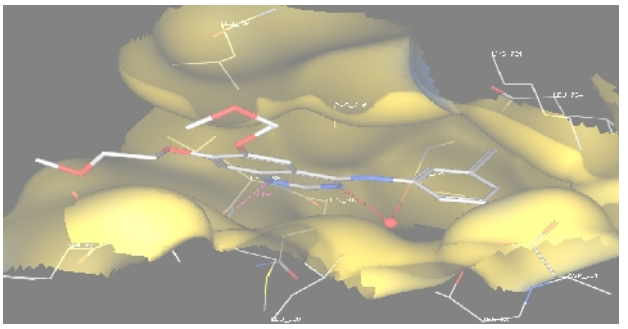


Fig. 3: Binding interaction of erlotinib with EGFR binding site[35].

The molecular docking study revealed that some of the designed compounds showed promising activity to inhibit EGFR tyrosine kinase active site. The data obtained for the prepared compounds from the docking study were explained in table 1.

Table 1: Results of molecular docking analyses, binding energy scores (Kcal/mol): energy of ligands and erlotinb in the active site of EGFR tyrosine kinase.

Compound number	Number of H-bonds	Atoms of compound forming H-bonds	Amino acid Residues forming-bonds (H-bond length in A^o)	Binding Energy Score Kcal/mol
Erlotinib	2	N1,N4	**Met**769 (2.70), **Thr**766 (2.78)	-21.35
6a	2	N7, C=O	**Thr**766 (2.78), **Lys**721 (2.43)	-11.80
6b	1	NH_2	**Met**769 (1.93)	-15.05
7a	2	N5,N7	**Met**769 (2.89), **Thr**766 (2.71)	-17.23
7b	1	NH	**Met**769 (1.64)	-18.05
7c	2	N5,N7	**Met**769 (2.896), **Thr**766 (2.94)	-16.75
8	0	-	-	-13.28
10a	1	N7	**Thr**766 (2.80)	-16.02
10b	4	N5, NH, N7, C=O	**Met**769 (1.92), **Met**769 (2.807), **Thr**766 (2.90), **Lys**692 (2.64)	-17.38
10c	3	N5, NH, N7	**Met**769 (2.15), **Met**769 (3.07), **Thr**766 (2.97)	-18.70
11a	2	OH	**Asp**831 (1.37) **Lys**721 (2.64)	-14.49

Attachment of a morpholino carbonyl moiety para to amino phenyl function as in compound **7a** resulted in the same mode of ligand erlotinib interaction with the amino acids of ATP active site in EGFR tyrosine kinase (Thr 766, and Met 769). It seems that compound **7a** has favorable binding to the kinase that led to high docking score (-17.23). Figures 4A & 4B show the 3D and 2D interaction of compound **7a** with the active site of EGFR tyrosine kinase respectively.

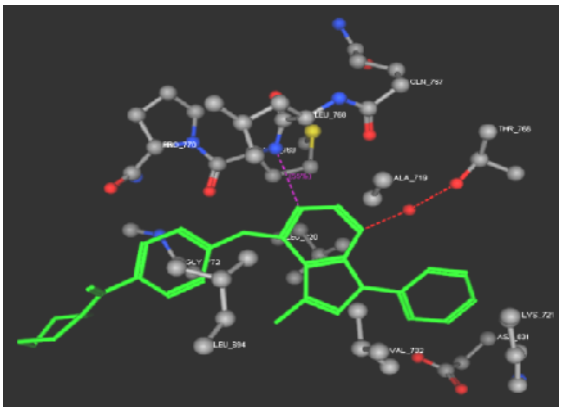


Fig. 4(A): 3D Interaction of **7a** with the binding site of EGFR tyrosine kinase.

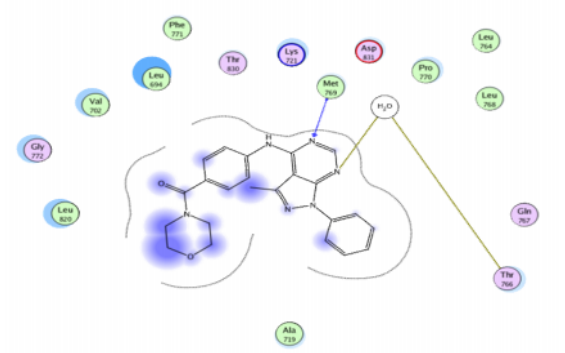


Fig. (4B): 2D Interaction of **7a** the binding site of EGFR tyrosine kinase.

Biological study

Five compounds **6b**,**7a**, **8, 10c**, **11a** were tested for their anticancer activity using human breast carcinoma cell line (MCF-7)[36]: The survival fraction ratio was calculated according to the following equation:Survival fraction= optical density (O.D.) (treated cells)/O.D. (control cells).

IC_{50} values (the concentration required to produce 50% inhibition of cell growth) were calculated using sigmodial dose response curve fitting models (GraphPad, Prizm software incorporated). Human breast cancer cell line (MCF7) was challenged to the antiproliferative effect of the tested compounds at four concentrations and doxorubicin was used as a reference in the biology experiment. From the synthesized compounds, those compounds having the moderate binding energy score (Kcal/mol) were chosen for the *in-vitro* cytotoxic activity and the results are shown in (Table 2).

Table 2: Biological screening results of Doxorubicin, **6b,7a, 8, 10c** and **11a** against MCF7.

Compound	Survival fraction ratio				IC_{50} (µM)
	Concentration (µM)				
	5	12.5	25	50	
Doxorubicin	0.19	0.17	0.18	0.20	5.47
6b	0.73	0.64	0.44	0.53	89.21
7a	0.53	0.32	0.18	0.30	14.86
8	0.82	0.69	0.72	0.90	-ve
10c	0.51	0.46	0.34	0.38	14.94
11a	0.90	0.72	0.69	0.80	-ve

Each concentration was repeated 3 times.

The results of biological screening indicated that MCF-7 cells appeared to be sensitive to inhibitory activity of three of the target compounds. (**6b, 7a, and 10c**) Table 2. They showed more than 50% inhibition activity towards cells, while the other tested compounds (**8 and 11a**) were devoid of activity.

It was observed that the substituted pyrazoline **10c** resulted in an additional hydrogen bonding with Met 769 besides the two interactions reported for the lead erlotinib and which may rationalize the cytotoxic activity of **10c** (IC_{50} = 14.94) which is less potant than doxorubicin.

In an attempts to rationalize the relationship between the cytotoxic activity and the presence of a phenylamino moiety, compound **6** was prepared in which the phenylamino was replaced by oxo, as in compound **6b,** that showed lower activity against MCF-7 less potent than doxorubicin. Docking results showed that in **6b** only one hydrogen bond with EGFR tyrosine kinase site was detected and could explain its lower potency. On the other hand compound **8** and **11a** were prepared, such modification were not

successful hence giving two compounds with abolished inhibitory activity against MCF-7 cells. Exploring the docking result of compound **8** no any hydrogen bonding interaction was seen. Also **11a** interact with receptor active sire with different amino acids Asp 831 and Lys 721 that seems not efficient for activity.

Experimental

Chemistry

Melting points were determined on Griffin apparatus (U.K) and are uncorrected. IR spectra were recorded on Shimadzu 435 Spectrometer (Japan), using KBr discs and values were represented in cm^{-1}. ^{1}H-NMR spectra were carried out on Varian Gemini 200 or 300 MHz Spectrometer (Germany) at the microanalytical center, Cairo University, Egypt using TMS as an internal standard and chemical shifts were recorded in ppm on δ scales. The electron Impact (EI)mass spectra recorded on Hewlett Packard 5988 Spectrometer (U.S.A), at the microanalytical center, Cairo University, Egypt and National Research Center, Cairo, Egypt. Elemental analyses were carried out at the microanalytical center, Cairo, Egypt. Progress of all reactions was monitored by TLC using TLC sheets precoated with UV fluorescent silica gel MERCK 60 F 254 that was visualized by UV lamp, using $CHCl_3$/ CH_3OH (9.5/ 0.5 or 9/1) as eluent. Compounds 1, **2** and **3** were prepared according to reported[17-19] procedures.

3-Methyl-1-phenyl-4-substituted amino-1*H*-pyrazolo[3,4-*d*]pyrimidines (4a-d)

A mixture of 4-chloro-3-methyl-1-phenyl-1*H*-pyrazolo [3, 4-*d*] pyrimidine **(3)** (2.44g,0.01 mol), the appropriate aromatic amine (0.01 mol) and sodium iodide (0.2 gm) in isopropyl alcohol (20 mL) was heated under reflux for 2 h as indicated by TLC. After cooling, the reaction mixture was neutralized to litmus paper with sodium carbonate solution (20%). The formed precipitate was collected by filtration, washed with water and crystallized from ethanol to afford **4a-d**, their physical and spectral data are given in table 3.

Ethyl (5-ethoxymethyleneimino-3-methyl-1-phenyl-1*H*-pyrazole)-4-carboxylate (5)

A mixture of ethyl (5-amino-3-methyl-1-phenyl-1*H*-pyrazole)-4-carboxylate (**1**) (2.45 gm, 0.01 mol) and triethyl orthoformate (2.96 gm, 0.02 mol) in acetic anhydride (25 mL) was heated under reflux for 5 h as indicated by TLC. After cooling, the solution was poured on ice-cold water and the precipitate formed was filtered, dried and crystallized from ethanol to give white crystals of **5**.

Yield: (66%), m.p.: 52-4°C, IR cm^{-1}: 1701 (C=O); 1640 (C=N).

^{1}H-NMR($CDCl_3$): δ 1.32 (t, *J*=7.5Hz, 3H, $OCH_2C\underline{H}_3$); 1.38 (t, *J*=7.5Hz, 3H,$COOCH_2C\underline{H}_3$); 2.49 (s, 3H, CH_3); 4.24 (q, *J*=7.5 Hz, 2H, $OC\underline{H}_2CH_3$); 4.32 (q, *J*=7.5 Hz, 2H, $COOC\underline{H}_2CH_3$); 7.27-7.64 (m, 5H, ArH of 1-phenyl); 8.05 (s, 1H, N=CH).

Analysis for $C_{16}H_{19}N_3O_3$ (301.35): Calcd. C%63.77, H%6.36, N%13.94 Found C%63.38, H%5.90, N%13.70

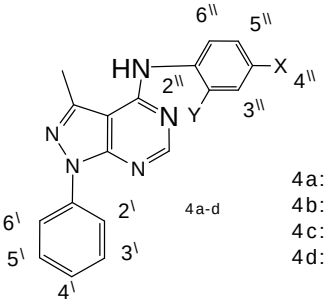
3-Methyl-1-phenyl-5-substituted-1*H*-pyrazolo[3,4-*d*] pyrimidin-4(5*H*)-ones (6a,b)

A mixture of **5** (3 gm, 0.01 mol) and methyl amine or hydrazine hydrate (99.9%) (0.01 mol) in benzene (25 mL) was heated under reflux for 1h as indicated by TLC. After cooling, the precipitate formed was filtered, dried and crystallized from benzene to give **6a, b** their physical and spectral data are given in Table 4.

3-Methyl-1-phenyl-4-[4-(substituted aminocarbonyl)anilino]-1*H*-pyrazolo[3,4-*d*]pyrimidines (7a-c)

A mixture of **4a** (3.4 gm, 0.01 mol) and excess of thionyl chloride (15 mL) was heated under reflux for 4h as indicated by TLC. Excess thionyl chloride was distilled under vacuum; the obtained residue was washed with diethyl ether and dried in vacuum oven. The appropriate secondary amine (0.01 mol) was added to the resulting acid chloride, followed by dioxane (15 mL) and triethylamine (2.8 mL, 0.02 mol) and the reaction mixture was stirred at room temperature for an overnight. Water (15 mL) was added and the stirring was continued for further 0.5 h. The separated product was filtered, dried and crystallized from ethanol to give **7a-c** (Table 5).

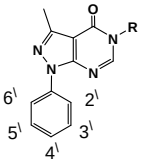
Table 3: Physical and spectral data of compounds **4a-d.**



4a: X=COOH, Y=H
4b: X=H, Y= COOH
4c: X= $COCH_3$, Y= H
4d: X=OH, ,Y=H

4	m.p.(°C) Yield%,	Mol. formula (M.Wt.)	Elemental Analyses %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ(ppm), J(HZ)
				Calcd.	Found		
a	> 300 (88)	$C_{19}H_{15}N_5O_2$ (345.36)	C H N	66.08 4.38 20.28	65.90 4.48 20.19	3600-2787 (OH & NH); 1684 (C=O) and 1608 (C=N).	2.81 (s, 3H, CH_3); 7.33 (t, *J*= 5.6, 1H, ArH(H4'); 7.54 (t, *J*= 5.8 , 2H, ArH(H3'&H5'); 7.91 (d, *J*= 9 , 2H ,ArH(H2''&H6''); 7.97 (d, *J*= 9, 2H, ArH (H3''&H5''); 8.18 (d, *J*= 6, 2H, ArH(H2',H6'); 8.53 (s, 1H, C6H); 8.99 (s,1H, NH, D_2O exchangeable).
b	235-7 (72)	$C_{19}H_{15}N_5O_2$ (345.36)	C H N	66.08 4.38 20.28	65.90 4.40 20.38	3600-3063 (OH & NH); 1691 (C=O) and 1578 (C=N).	2.80 (s, 3H, CH_3); 7.14 (t, *J*= 6 , 1H, ArH (H4''); 7.32 (t, *J*= 9 , 1H, ArH (H4); 7.53 (t, *J*= 6 , 2H, ArH(H3'&H5'); 7.64 (t, *J*= 9, 1H, ArH (H5''); 8.03 (d, *J*= 6, 1H, ArH (H6''); 8.15 (d, *J*= 4.5, 2H, ArH (H2'&H6'); 8.57 (s, 1H, C6H); 9.12 (d, *J*= 9, 1H, ArH (H3''); 9.28 (brs, 1H, NH, D_2O exchangeable); 11.56 (s, 1H, OH, D_2O exchangeable).
c	170-1 (76)	$C_{20}H_{17}N_5O$ (343.39)	C H N	69.96 4.99 20.39	70.18 5.14 20.51	3409 (NH); 1671 (C=O) and 1614 (C=N).	2.60 (s, 3H, O=C-CH_3); 2.82 (s, 3H, CH_3); 7.33 (t, *J*= 9 , 1H, ArH (H4'); 7.55 (t, *J*= 6.7 , 2H, ArH (H3'&H5'); 7.77 (d, *J*= 7.8 , 2H, ArH (H2'' &H6''); 8.03 (d, *J*= 7.8, 2H, ArH(H3'', H5''); 8.18 (d, *J*= 9 , 2H, ArH (H2'&H6'); 8.47 (s, 1H, C6H); 8.97 (s, 1H, NH, D_2O exchangeable).
d	274-5 (62)	$C_{18}H_{15}N_5O$ (317.35)	C H N	68.13 4.76 22.07	68.09 4.75 21.91	3435-2680 (OH & NH) and 1613 (C=N).	2.73 (s, 3H**,** CH_3); 6.80 (d, *J*= 8.6 , 2H, ArH (H2''&H6''); 7.24-7.62 (m, 5H, ArH (H4, (H3'',H5'') and (H3',H5')); 8.18 (d, *J*= 9 , 2H, ArH (H2',H6'); 8.33 (s, 1H, C6H); 8.67 (s, 1H, NH, D_2O exchangeable); 9.41 (s, 1H, OH, D_2O exchangeable).

Table 4: Physical and spectral data of compounds **6a,b**.

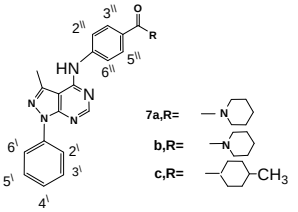


6a, R= CH_3

6b, R = NH_2

6	%, m.p. (^oC)Yield	Mol.Formul (M.Wt.)	Elemental Analyses %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ(ppm), *J*(HZ)
				Calcd.	Found		
a	150-2 (60)	$C_{13}H_{12}N_4O$ (240.27)	C H N	64.99 5.03 23.32	64.92 4.60 23.60	1681 (C=O) and 1587 (C=N).	2.54 (s, 3H, CH_3); 3.48 (s, 3H, N-$C\underline{H}_3$); 7.37 (t, *J*= 6 , 1H, ArH (H4$^\backslash$); 7.53 (t, *J*= 6 , 2H, ArH (H3$^\backslash$&H5$^\backslash$); 8.02 (d, *J*= 9 , 2H, ArH (H2$^\backslash$&H6$^\backslash$); 8.43 (s, 1H, C6H).
b	214-5 (65)	$C_{12}H_{11}N_5O$ (241.25)	C H N	59.74 4.60 29.03	59.81 4.91 29.36	3319, 3272 (NH_2); 1676 (C=O) and 1576 (C=N).	2.91 (s, 3H, CH_3); 5.74 (s, 2H, NH_2, D_2O exchangeable); 7.31-7.33 (m, 1H, ArH (H4$^\backslash$); 7.45-7.48 (m, 2H, ArH (H3$^\backslash$&H5$^\backslash$); 7.96 (d, *J*= 9, 2H, ArH (H2$^\backslash$&H6$^\backslash$) ; 8.40 (s, 1H, C6H).

Table 5: Physical and spectral data of compounds **7a-c**.



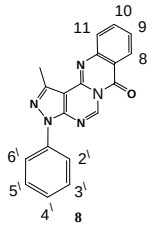
7a,R=

b,R=

c,R=

7	m.p. (^oC) Yield %	Mol.Formula (M.Wt)	Elemental Analysis %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ(ppm), J(HZ)
				Calcd	Found		
a	205-7 (60)	$C_{23}H_{22}N_6O_2$ (414.47)	C H N	66.65 5.35 20.28	66.52 5.20 19.88	3392 (NH); 1681 (C=O); and 1604 (C=N).	2.46 (s, 3H, CH_3); 2.75 (t, *J*= 6, 4H, CH(H3,H5 of morpholinyl)); 3.30 (t, *J*= 6, 4H, CH(H2&H6 of morpholinyl)); 7.32-7.57 (m, 3H, ArH(H4$^\backslash$,H3$^\backslash$&H5$^\backslash$); 7.75 (d, *J*= 8.5 , 2H, ArH (H2$^{\backslash\backslash}$&H6$^{\backslash\backslash}$); 7.79 (d, *J*= 8.5, 2H, ArH (H3$^{\backslash\backslash}$&H5$^{\backslash\backslash}$); 8.14 (d, *J*= 9 , 2H, ArH(H2$^\backslash$&H6 $^\backslash$)); 8.46 (s, 1H, C6H).
b	235-7 (56)	$C_{24}H_{24}N_6O$ (412.50)	C H N	69.88 5.86 20.37	69.88 5.50 20.83	3412 (NH); 1686 (C=O) and 1594 (C=N).	1.42-1.65 (m, 6H, CH (H3,H4&H5 of piperidinyl)); 2.75 (s, 3H, CH_3); 3.25-3.45 (m, 4H, CH (H2&H6 of piperidinyl)); 7.26-7.56 (m, 5H, ArH(H3,H5,H4,H2$^{\backslash\backslash}$&H6$^{\backslash\backslash}$); 8.09 (d, *J*= 9, 2H, ArH(H3$^{\backslash\backslash}$&H5$^{\backslash\backslash}$); 8.19 (d, *J*= 6 , 2H, ArH(H2$^\backslash$&H6$^\backslash$); 8.59 (s, 1H, C6H); 8.82 (s, 1H, NH, D_2O exchangeable).
c	229-31 (49)	$C_{25}H_{26}N_6O$ (426.53)	C H N	70.40 6.14 19.70	70.33 5.88 19.24	3335 (NH); 1693 (C=O); and 1611 (C=N).	0.92 (s, 3H, CH_3,(4-methyl piperdinyl)); 1.45-1.62 (m, 5H, CH,H3,H4&H5 of 4-methyl piperidinyl)); 2.80 (s, 3H, CH_3); 4.25-4.35 (m, 4H, CH(H2,H6 of 4-methyl piperidinyl)); 7.33-7.42 (m, 3H, ArH(H4$^\backslash$ and H2$^{\backslash\backslash}$,H6$^{\backslash\backslash}$); 7.50-7.55 (m, 2H, ArH(H3$^\backslash$,H5$^\backslash$); 7.79 (d, *J*= 9 , 2H, ArH(H3$^{\backslash\backslash}$&H5$^{\backslash\backslash}$); 8.19 (d, *J*= 9 , 2H, ArH(H2$^\backslash$&H6$^\backslash$); 8.49 (s, 1H, C6H); 8.90 (s,1H, NH, D_2O exchangeable).

1-Methyl-3-phenylpyrazolo[3,4:4,5] pyrimido[6,1-*b*]quinazolin-7(3*H*)-one (8)

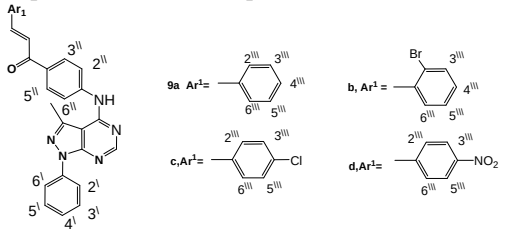


A mixture of **4b** (0.34 gm, 0001 mol) and excess thionyl chloride (4 mL) was heated under reflux for 4 h as indicated by TLC. Excess thionyl chloride was distilled off and the residue was crystallized from ethanol to give 8.
Yield: (62.5%), m.p.: 170-171°C, IR cm^{-1}: 1708 (C=O); 1617 (C=N), ^{1}H-NMR ($CDCl_3$): δ 2.66 (s, 3H, CH_3);.738-7.51 (m, 2H, ArH(H4\ and H11); 7.54-7.57 (m, 4H,ArH(H3\,H5\ and H9, H10); 7.73 (d *J*=8.1Hz, 2H, ArH(H2\,H6\); 8.23 (d, *J*= 6.3 Hz,1H, ArH(H8); 8.26 (s, 1H, C5H of pyrimidinyl)). Analysis for $C_{19}H_{13}N_5O$ (327.35): Calcd. C% 69.52, H% 4.00, N% 21.39 Found C% 69.52, H% 4.21, N% 20.89.

(E) 3-Methyl-1-phenyl-4-[4-(2-arylvinyl-carbonyl)anilino]-1*H*-pyrazolo[3,4-*d*] pyrimidines (9a-d)

To a stirred solution of compound **4c** (0.34 gm, 0.001 mol) in ethanol (10 mL), was added an aqueous solution of sodium hydroxide (1 mL, 10%). After cooling in an ice bath, the appropriate aromatic aldehyde (0.001 mol) was added while stirring at a temperature not exceeding 20°C. The reaction mixture was stirred at room temperature for 12 h as indicated by TLC. The solid obtained was filtered, washed with water and crystallized from the appropriate solvent to give **9a-d**, (Table 6).

Table 6: Physical, analytical and spectral data of compounds **9a-d**.



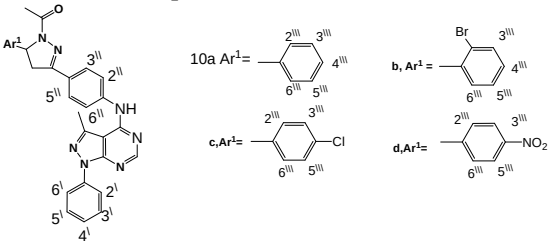
9	m.p.(°C),Yield%, Solvent of Crystallization	Mol. Formula (M.Wt)	Elemental Analyses %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ(ppm), J(HZ)
				Calcd	Found		
a	> 300 (90) (a)	$C_{27}H_{21}N_5O$ (431.50)	C H N	75.16 4.91 16.23	74.91 4.69 16.34	3424 (NH); 1665 (C=O) and 1591 (C=N).	2.83 (s, 3H, CH_3); 7.27-7.37 (m, 4H, ArH(H4\ and H3\\\,H4\\\,H5\\\ ); 7.42 (d, *J*=6, 2H, ArH(H3\,H5\); 7.47-7.55(m,3H,=CH-Ar & ArH(H2\\& H6\\); 7.57-7.83 (m, 5H, O=C-CH=& ArH (H3\\,H5\\,H2\\\&H6\\\ ); 8.14 (d, *J*= 9, 2H, ArH (H2\&H6\); 8.58 (s, 1H, C6H).
b	205-7 (70) (b)	$C_{27}H_{20}BrN_5O$ (510.40)	C H N	63.54 3.95 13.72	63.89 4.34 13.92	3437 (NH); 1659 (C=O) and 1604 (C=N).	2.83 (s, 3H, CH_3); 7.26-7.41 (m, 4H, ArH(H4\ and H4\\\,H5\\\&H6\\\); 7.51-7.64 (m, 3H, =CH-Ar& ArH, H3\\,H5\\ ); 7.83-7.88 (m, 4H, O=C-CH=& ArH(H2\\,H6\\ and H3\\\); 8.03-8.17 (m, 4H, ArH(H2\,H6\ and H3\,H5\ ); 8.58 (s, 1H, C6H).
c	212-4 (81) (b)	$C_{27}H_{20}ClN_5O$ (465.95)	C H N	69.60 4.33 15.03	68.91 4.60 14.80	3428 (NH); 1656 (C=O) and 1608 (C=N).	2.83 (s, 3H, CH_3); 7.34 (t, *J*= 6 , 1H, ArH(H4\); 7.51-7.63 (m, 3H, ArH(H3\,H5\ ) & =CH-Ar); 7.85 (d,J= 6 .1H, O=C-CH=); 8.02 (d, *J*= 6 , 2H, ArH(H2\\&H6\\ ); 8.08-8.18 (m, 6H, ArH(H3\,H5\ and H2\\\,H3\\\,H5\\\,H6\\\); 8.29 (d, *J*= 6, 2H, ArH (H2\&H6\); 8.47 (s, 1H, C6H); 9.03 (s, 1H, NH, D_2O exchangeable).
d	220-1 (80) (b)	$C_{27}H_{20}N_6O_3$ (476.50)	C H N	68.06 4.23 17.64	68.45 4.35 17.32	3440 (NH), 1661 (C=O) and 1609 (C=N).	2.82 (s, 3H, CH_3); 7.35 (t, *J*= 6 , 1H, ArH,H4\); 7.53 (t, *J*= 6 , 2H, ArH(H3\&H5\); 7.62 (d, *J*= 6 , 1H, =CH-Ar); 7.86 (d, *J*= 6 , 1H, O=C-CH=); 8.07-8.20 (m, 8H,ArH (H3\\,H5\\,H2\\,H6\\&H3\\\,H5\\\,H2\\\,H6\\\); 8.29 (d, *J*= 7.5 , 2H, ArH(H2\,H6\); 8.47 (s, 1H, C6H); 9.02 (s, 1H, NH, D_2O exchangeable).

(a); acetic acid (b); isopropyl alcohol

3-Methyl-1-phenyl-4-[4-(1-acetyl-5-aryl-4,5-dihydro-1*H*-pyrazol-3-yl)anilino]-1*H*-pyazolo[3,4-*d*]pyrimidines (10a-d)

To a solution of hydrazine hydrate (99.9%, 0.1 mL, 0.002 mol) in glacial acetic acid (5 mL), the appropriate chalcone **9a-d** (0.001 mol) was added. The reaction mixture was heated under reflux for 5 h as indicated by TLC. After cooling, the solution was poured on an ice-cold water. The obtained solid was collected by filtration, washed with water and crystallized from ethanol to yield **10a-d**, (Table 7).

Table 7: Physical and spectral data of compounds **10a-d**.

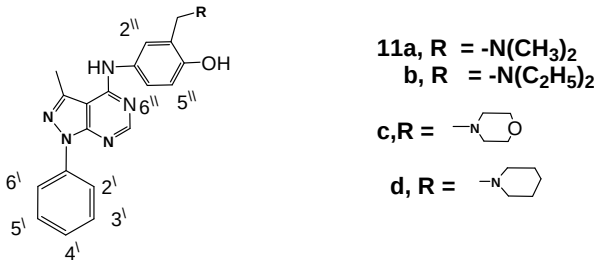


10	m.p. °C Yield %	Mol. Formula (M.Wt)	Elemental Analyses %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ (ppm), *J* (Hz)
				Calcd	Found		
a	> 300 (60)	$C_{29}H_{25}N_7O$ (487.57)	C H N	71.44 5.17 20.11	71.20 5.58 20.16	3315 (NH) 1652 C=O) 1587 C=N).	2.44 (s, 3H, O=C-CH_3); 2.60 (s, 3H, CH_3); 3.15, 3.21 (dd, J_1= 4.8 , J_2= 4.8 ,1H, CH_2 of pyrazoline); 3.78, 3.84 (dd, J_1= 12 , J_2= 12, 1H, CH_2 of pyrazoline); 5.57, 5.61 (dd, J_1= 4.5 , J_2= 12 , 1H, CH of pyrazoline); 7.17 (d, *J*= 7.5 , 2H, ArH(H2$^{\prime\prime\prime}$,H6$^{\prime\prime\prime}$); 7.26-7.38 (m, 4H, ArH(H4$^{\prime}$,H3$^{\prime\prime\prime}$,H4$^{\prime\prime\prime}$,H5$^{\prime\prime\prime}$); 7.55-7.61 (m, 4H, ArH(H3$^{\prime}$,H5$^{\prime}$,H2$^{\prime\prime}$,H6$^{\prime\prime}$); 7.76 (d, *J*= 9, 2H, ArH(H3$^{\prime\prime}$,H5$^{\prime\prime}$); 8.08 (s, 1H, NH, D_2O exchangeable); 8.12 (d, *J*= 9, 2H, ArH(H2$^{\prime}$,H6$^{\prime}$); 8.53 (s, 1H, C6H).
b	270-1 (50)	$C_{29}H_{24}BrN_7O$ (566.46)	C H N	61.49 4.27 17.31	61.39 4.28 17.00	3438 (NH); 1663 C=O); and 1602 (C=N).	2.51 (s, 3H, O=C-CH_3); 2.70 (s, 3H, CH_3); 3.06, 3.11 (dd, J_1= 4.5 , J_2= 4.5 , 1H, CH_2 of pyrazoline); 3.88, 3.93 (dd, J_1= 12.3 , J_2= 4.5 , 1H, CH_2 of pyrazoline); 5.91, 5.94 (dd, J_1= 4.2, J_2= 4.5 , 1H, CH of pyrazoline); 7.06 (d, *J*= 6 , 1H,ArH(H6$^{\prime\prime\prime}$); 7.11-7.18 (m, 1H, ArH(H5$^{\prime\prime\prime}$); 7.26-7.36 (m, 2H, ArH(H4$^{\prime}$ &H4$^{\prime\prime\prime}$); 7.46-7.55 (m, 3H, ArH(H3$^{\prime}$,H5$^{\prime}$) & NH(D_2O exchangeable)); 7.60 (d, *J*= 8.1 , 2H, ArH(H2$^{\prime\prime}$&H6$^{\prime\prime}$); 7.81 (d, *J*= 8.1, 2H, ArH(H3$^{\prime\prime}$&H5$^{\prime\prime}$); 7.91 (s, 1H, ArH(H2$^{\prime\prime\prime}$); 8.14 (d, *J*= 9 , 2H, ArH(H2$^{\prime}$&H6$^{\prime}$); 8.55 (s, 1H, C6H).
c	230-1 (45)	$C_{29}H_{24}ClN_7O$ (522.01)	C H N	66.73 4.63 18.78	66.71 5.02 18.68	3320 (NH); 1705 (C=O); and 1619 (C=N).	2.37 (s, 3H, O=C-CH_3); 2.81 (s, 3H, CH_3); 3.00,3.06 (dd, J_1= 4.8 , J_2= 4.8 , 1H, CH_2 of pyrazoline); 3.96, 4.06 (dd, J_1=12 Hz, J_2= 12 , 1H, CH_2 of pyrazoline); 5.72, 5.74 (dd, J_1= 4.5 , J_2= 12 , 1H, CH of pyrazoline); 7.04 (d, *J*= 7.8 , 2H, ArH (H2$^{\prime\prime\prime}$,H6$^{\prime\prime\prime}$); 7.24 (d, *J*= 7.8 , 2H, ArH(H3$^{\prime\prime\prime}$,H5$^{\prime\prime\prime}$); 7.33-7.35 (m, 1H, ArH(H4$^{\prime}$); 7.45-7.50 (m, 2H, ArH(H3$^{\prime}$&H5$^{\prime}$); 7.67 (d, *J*= 7.8, 2H, ArH(H2$^{\prime\prime}$&H6$^{\prime\prime}$)); 7.89 (d, *J*= 7.8 , 2H, ArH(H3$^{\prime\prime}$&H5$^{\prime\prime}$); 8.18 (d, *J*= 8 , 2H, ArH(H2$^{\prime}$&H6$^{\prime}$); 8.44 (s, 1H, C6H); 8.91 (s, 1H, NH, D_2O exchangeable).
d	252-4 (66),	$C_{29}H_{24}N_8O_3$ (532.57)	C H N	65.40 4.54 21.04	65.44 4.77 21.14	3426 (NH); 1655 (C=O); and 1588 (C=N).	2.44 (s, 3H, O=C-CH_3); 2.81 (s, 3H, CH_3); 3.15, 3.21 (dd, J_1= 4.8 , J_2= 4.8 , 1H, CH_2 of pyrazoline); 3.76, 3.81 (dd, J_1= 12 , J_2= 12, 1H, CH_2 of pyrazoline); 5.57, 5.60 (dd, J_1= 4.2, J_2= 4.5 , 1H, CH of pyrazoline); 7.19 (d, *J*= 9 , 2H, ArH(H2$^{\prime\prime\prime}$,H6$^{\prime\prime\prime}$); 7.27-7.32 (m, 3H, ArH(H4$^{\prime}$ & H3$^{\prime\prime\prime}$,H5$^{\prime\prime\prime}$); 7.49-7.55 (m, 4H, ArH(H3$^{\prime}$,H5$^{\prime}$ &H2$^{\prime\prime}$,H6$^{\prime\prime}$); 7.83 (d, *J*= 9 , 2H, ArH(H3$^{\prime\prime}$,H5$^{\prime\prime}$); 8.15 (d, *J*= 9,2H, ArH(H2$^{\prime}$,H6$^{\prime}$); 8.17 (s, 1H, NH, D_2O exchangeable); 8.57 (s, 1H, C6H).

3-Methyl-1-phenyl-4-[3-(substituted aminomethyl)-4-hydroxyanilino]-1*H*-pyrazolo[3,4-*d*]pyra- midines (11a-d)

To a solution of **4d** (3.17gm, 0.01mol) in absolute ethanol (10 mL), a mixture of secondary amine (0.012 mol) and paraformaldehyde (0.45gm, 0.015 mol) was added. The mixture was heated under reflux for 5 h as indicated by TLC on a steam bath and left overnight. The solvent was distilled off under reduced pressure. The solid residue was dissolved in ethanol, precipitated with water, filtered, dried and crystallized from ethanol to give **11a-d**, (Table 8).

Table 8: Physical and spectral data of compounds **11a-d**.



11	m.p. (°C) Yield %	Mol. Formula (M.Wt)	Elemental Analyses %			IR (cm^{-1})	^{1}H-NMR(DMSO), δ (ppm), J (HZ)
				Calcd.	Found		
a	115-8 (56)	$C_{21}H_{22}N_6O$ (374.45)	C H N	67.36 5.92 22.44	67.55 5.82 22.44	3500-3250 (OH& NH) and 1588 (C=N).	2.69 (s, 3H, CH_3); 2.75 (s, 6H, $N(CH_3)_2$); 3.41 (s, 1H, OH, D_2O exchangeable); 4.23 (s, 2H, CH_2-N); 7.31-7.59 (m, 5H, ArH(H4\',H3\',H5\',& H2\\H5\\); 7.63 (d, *J*= 6 Hz, 1H, ArH(H6\\); 8.17 (d, *J*= 9 Hz, 2H, ArH(H2\',H6\'); 8.37 (s, 1H, C6H); 8.80 (s, 1H, NH, D_2O exchangeable).
b	101-3 (60),	$C_{23}H_{26}N_6O$ (402.50)	C H N	68.63 6.51 20.88	68.39 6.41 20.90	3414-3102 (OH& NH) and 1591 (C=N).	1.21 (t, *J*=7.8 Hz, 6H, $2CH_2$-$C\underline{H}_3$); 2.72 (s, 3H, CH_3); 3.10 (q, *J*=7.8 , 4H, $2C\underline{H}_2CH_3$); 4.21 (s, 2H, CH_2-N); 6.81 (d,1H, ArH(H2\\); 7.08-7.51 (m, 5H, ArH(H4\',H3\',H5\'& H5\\,H6\\); 8.17 (d, *J*= 8.1, 2H, ArH(H2\',H6\'); 8.32 (s, 1H, C6H); 8.70 (s, 1H, NH, D_2O exchangeable); 9.51 (1H, OH, D_2O exchangeable).
c	120-2 (42)	$C_{23}H_{24}N_6O_2$ (416.49)	C H N	66.33 5.81 20.18	66.69 5.54 20.31	3393-3250 (OH& NH) and 1589 (C=N).	1.49 (t, 4H, CH morpholino); 2.73 (s, 3H, CH_3); 2.98 (t, 4H, CH morpholino); 3.62 (1H, OH, D_2O exchangeable); 3.91 (s, 2H, CH_2-N); 7.30-7.51 (m, 5H, ArH(H4\',H3\',H5\' &H2\\ ,H6\\); 8.01 (d, *J*= 9 , 1H, ArH(H5\\); 8.16 (d, *J*= 9, 2H, ArH(H2\',H6\'); 8.32 (s, 1H, C6H); 8.74 (s, 1H, NH, D_2O exchangeable).
d	190-2 (66)	$C_{24}H_{26}N_6O$ (414.51)	C H N	69.54 6.32 20.27	69.63 6.18 19.91	3416-3138 (OH& NH) and 1610 (C=N).	1.59-1.70 (m, 6H, CH piperidino); 2.64-2.75 (m, 4H, CH piperidino); 2.83 (s, 3H, CH_3); 4.00 (s, 2H, CH_2-N); 5.40 (s, 1H, NH, D_2O exchangeable); 6.90 (d, *J*= 7.8 , 1H, ArH(H2\\); 7.26-7.50 (m, 4H, ArH(H4\',H3\',H5\'&H6\\ amino)); 7.98 (d, *J*= 9 , 1H, ArH(H5\\); 8.10 (d, *J*= 4.5 , 2H, ArH(H2\',H6\'); 8.46 (s, 1H, C6H).

Molecular docking: The docking was performed using Molecular Operating Environment (MOE) 2008, 10 software according to the following procedures.

- Downloading step: This step includes downloading of EGFR from the protein data bank (PDB code: 1M17) and hydrogen was clarified hence the downloaded proteins from PDB do not show any hydrogen.
- The charging step: the protein needs to be charged to calculate the total charge over it and this allows the free energy calculation step.
- The Surface conversion step: the appearance of the protein is changed by converting its surface to molcad or slab surface, which helps to see the active site in details, extract the ligand and modify it.
- Then extraction step: through it the a ligand could be separated from protienand modified to a form that enhances determination of its binding affinity.
- Modifying step that includes adding or removing any atom or group of atoms from the ligand.
- The minimization step: that include the geometrical optimization process that enables the ligand before docking step to give us the lowest conformational energy for a ligand.
- The molecular docking step that includes fitting the ligand after extraction, modifying and minimization into the active site.

  Finally Minimization of ligand inside the active site: this step includes selection of the active site with the ligand to minimize their conformational energy resulted after ligand binding process.
- Energy calculation step: for every change to the ligand, the free energy (ΔE) is used to determine the binding affinity of each ligand with the receptor site.

Biology screening

General methodology: Study was operated in National Cancer institute, Cairo University,Egypt.

All reagents and authentic samples used during the biology experiment were obtained through (sigma & Aldrichcompany)and are of analytical grades.

MCF was grown as monolayer culture in RPM 11640 medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. The cell line was incubated at 37°C 5% CO_2 95% air and high humidity atmosphere in the water jacketed incubator (Revco, GS laboratory equipment, RCO 3000 TVBB, U.S.A). The cell line was regularly subcultured to be maintained in the exponential growth phase. The sterile conditions were strictly attained by working under the equipped laminar flow (Microflow Laminar flow cabinet, Hamsphire SP 105aa, U.K.).

A- Maintenance of the human tumor cell line

1. A cryotube containing frozen cells was taken out of the nitrogen container and then thawed in a water bath at 37°C.
2. The cryotube was opened under strict aseptic conditions and its contents were transferred into sterile 50 ml disposable falcon tube supplemented by 5 ml medium drop by drop.
3. The tube was incubated for 2h then its contents were centrifuged at 1200 rpm for 10 min.
4. The supernatant was discarded and the cell pellet was suspended and seeded in 5 ml supplemented medium in T25 Nunclon sterile tissue culture flasks.
5. The cell suspension was incubated and followed up daily with replacing the supplemented medium every 2-3 days.
6. Incubation was continued until a confluent growth was achieved and the cells were freshly subcultured before each experiment to be in the exponential phase of growth.

B- Collection of cells by trypsinization

1. The medium was discarded.
2. The cell monolayer was washed with 10 ml phosphate-buffered saline (PBS).
3. All the adherent cells were dispersed from their monolayer by the addition of I ml trypsin solution (0.025% trypsin w/v).
4. The flask was left in incubator till complete detachment of the cells and checked with the inverted microscope (Olympus 1×70, Tokyo, Japan).
5. Trypsin was inactivated by the addition of 5 ml of the supplemented medium containing fetal calf serum (FCS). The trypsin content was discarded by centrifugation (Boaco Germany) at 1200 rpm for 10 min. Cells

were separated in a single suspension by gentle dispersion several times.

C- Determination and counting of viable cells

1. 100 µl of 0.05% trypan blue solution were added to 100 µl of the single cell suspension.
2. The cells were examined under the inverted microscope using the haemocytometer.
3. Non stained (viable) cells were counted and the following equation was used to calculate the cell count /ml of cell suspension.
4. Viable cells/ml= [Number of cells in 4 quarters x 2 (dilution factor) $\times 10^4$]/4.
5. The cells were then diluted to give the required concentration of single cell suspension.

D- Cytotoxicity of the test compounds using Sulphordhodamine-B (SRB) assay

The principle

The cytotoxicity of the prepared compounds were tested on the MCF cell line determined using SRB assay. SRB is a bright pink aminoxanthene dye with two sulfonic groups. It is a protein stain that binds to the amino groups of intracellular protein under mild acidic conditions to provide a sensitive index of cellular protein content.

Procedure[36]

The breast cancer cells were seeded in 96-well microlitre plates and left to attach for 24 h.Cells were incubated with the tested compounds at concentration range from 0, 5, 12.5, 25 and 50 µg/ml)as well as doxorubicin and incubation was continued for 48 h. After 48h treatment, the cells were fixed with 50 µl cold 50% trichloroacetic acid (TCA) for 1h at 4°C.Wells were then washed 5 times with water and stained for 30 min at room temperature with 50 µl of 0.4% SRB dissolved in 1% acetic acid. The wells were then washed 4 times with 1% acetic acid. The plates were air dried and the dye was solubilized with 100 µl/well of 10Mm tris base (pH 10.5) for 5 min on a shaker (Orbital Shaker OS, Boaco, Germany) at 1600 rpm. The optical density (O.D) of each well was measured spectrophotometrically at 564 nm with enzyme linked immunosorbent assay (ELISA) micraplate reader (Tecan Sunrise, Austria). The mean values for each drug concentration was calculated**.**

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