Molecular Modeling Based, Design Synthesis and Cytotoxic Activity of Substituted Arylidene Piperazinoquinoline, a Hybrid Pharmacophore, Targeting Epidermal Growth Factor Receptor (EGFR), Tyrosine Kinase

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Abstract: A series of 1-(2-(4-substitutedarylidene) hydrazinyl-4-(7-chloroquinolin-4-yl) piperazine-2, 5-dione **VI** was designed by molecular hybridization approach and synthesized for biological evaluation. Virtual screening was carried out through docking the designed compounds into the ATP binding site of epidermal growth factor receptor (EGFR) to predict if these compounds have similar binding mode as the EGFR inhibitors. Subsequently, the compounds were examined for their cytotoxic effect on human breast cell-line (MCF-7) in which the EGFR is highly expressed. Although most of the compounds were quite effective on the breast cancer cell line examined, the compounds II, III, IV a, IVc, VIa, VIc emerged as the most active among the prepared series. Thus 7-chloro-4- (2, 5-dioxo 4-substitutedarylidene piperazinoquinoline can serve as the prototype molecule for further development of a new class of EGFR Tyrosine Kinase inhibitors and anti-breast cancer agents.
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1. Introduction:

The growing incidence of drug resistance to cancer chemotherapeutic agents represents a serious medical problem[1]. Therefore there is an urgent need to develop new classes of chemotherapeutic agents with different mechanism of action to treat cancer[2].It has been reported that designing a single molecule with more than one pharmacophore each with different mode of action could be beneficial for the treatment of cancer[3-6] ,as well as to reduce unwanted side effects [3,4,6,7] .

Many quinolines were found to possess antineoplastic activity. Although the antineoplastic activity of these quinolines was attributed to intercalating binding to DNA, there were additional advantages in quinolines that interact with DNA, with low association constant.The corresponding significant increase in the amount of free drug at equilirium may have an important effect upon distribution and hence the spectrum of activity and accessibility of these molecules to solid tumor[8,9]. In addition a number of quinoline derivatives were reported to reverse tumor cell multidrug resistance[10,11]

Recently it has been demonstrated that 10 uM chloroquine significantly increases cancer cell killing effects[3,4,12]. Several CQ analogues (Fig 1, 1-3) have been synthesized and examined as cytotoxic agents against (MCF-7) breast cancer cell line. Some of these compounds were very effective[13].It has been reported that CQ and its analogs has a unique property in being accumulated in the lysosomes, raising intra-lysosomal pH, and results in enhancement of cell killing by cancer therapeutic agents in a variety of different tumors[14]. Published data showed that the piperazinoquinoline pharmacophore, is one of the most effective newly emerging class of heterocyclic molecules that possessed antitumor activity[15,16] (Fig 2, 4&5). In addition many researches proved the importance of azomethine link or Schiff's base in anticancer drug[17,18].

Moreover there are accumulating line of evidence that hybridization of two or more bioactive molecules with complementary pharmacophoric functions or with different mechanisms of action often render synergestic effects[19-24]. Encouraged by these previous reports and in an effort towards developing effective anticancer agents by a hybrid pharmacophore approach, herein (7-chloroquinolines, azomethine, piperazinedione) we designed different set of compounds (scheme 1) in the aim of prospecting their anticancer potentiality.

2. Chemistry

Compounds (II-VI) described in this study were synthesized as outlined in scheme 1. 4,7-Dichloroquinoline I was reacted with glycine ethyl ester to afford compound II which upon reaction with hydrazine hydrate produced the key compound III. The target compounds VIa-e were obtained through two different pathways. The first includes the reaction of

compound III with different aromatic aldehydes to yield shiff's base IV a-f which upon cyclization with chloroacetylchloride afforded the desired compounds VIa-e. Alternatively the second pathway involves primarily cyclization of the key compound III via reaction with chloroacetylchloride to produce compound V which through interaction with different aromatic aldehydes ends into our target compounds.

2.2. In vitro cytotoxicity

Since breast cells are known to over express EGFR, which leads to continuous activation of the EGFR pathway involved in cell proliferation, therefore all the compounds synthesized were evaluated for their cytotoxicity on breast cancer cell line MCF-7 using the Sulfo-Rhodamine-B stain (SRB) assay using the method of skehan[25].

Cells were plated in 96 – multiwall microtiter plate (10^4 cells / well) for 24h before treatment with the compound (s) to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volumes. Different concentration of the compound under test (0.1, 2.5, 5 and 10 µg / ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48h at 37°C and in atmosphere of 5% CO_2. After 48h, cells were fixed, washed and stained for 30 minutes with 0.4% (wt / vol) with SRB dissolved in 1% acetic acid unbound dye was removed by four washes with 1% acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve. Most of the tested compounds exhibited potent inhibitory activity against MCF-7 cell line (Table 1, Fig. 3-6).

2.2.1. Results and Discussion

The biological screening results (table 1) revealed that cytotoxic activities increase following the conversion of the ester II to the corresponding acid hydrazide III with IC_{50} values equal to 3.09 and 2.84 M respectively. Converting the acid hydrazide III to the corresponding arylidene derivatives reduces the cytotoxic activities as seen from compounds IVa and IVc where IC_{50} were 5.37 and 8.83 µg respectively. Unfortunately cyclization of compound III to produce compound V abolishes the activity, which was restored again as seen from compound VIa and VIc with IC_{50} 1.41 and 2.75 µg respectively; a result that supports our hypothesis of designing a hybrid compound bearing three cytotoxic pharmacophore; herein: 7-chloroquinoline, a piperazine moiety and an azomethine group. It is also clear from the obtained data that electronic effect of the substituent in the para position of the arylidene group in the series comprising compounds VI(a-e), seems to be an important determinant of activity since the bromo derivative VIa was more active than its methoxy congenere VIc (IC_{50} equal to 1.41 and 2.75 µg respectively).

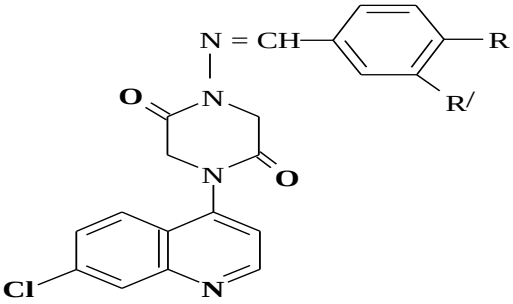
3. Molecular modeling study:

Docking study was carried out for the target compounds into EGFR using SYBYL version 7.3. Tripos Inc with malegro virtual docker program version 2007. The crystal structure of the enzyme and lapatinib (1XKK) was obtained from protein data bank PDB[26] since it was found that lapatinib mimic ATP and bind to the ATP binding region of the kinase active site. Therefore our compounds were modeled by positioning them in the lapainib binding site in accordance with the published crystal structures of quinazoline derivatives bound to kinase[27]. The entire complex was then subjected to alternate cycles of minimization and dynamics the intent was to get a satisfactory structure for the complex that was consistent with the published crystal structure[28,29].

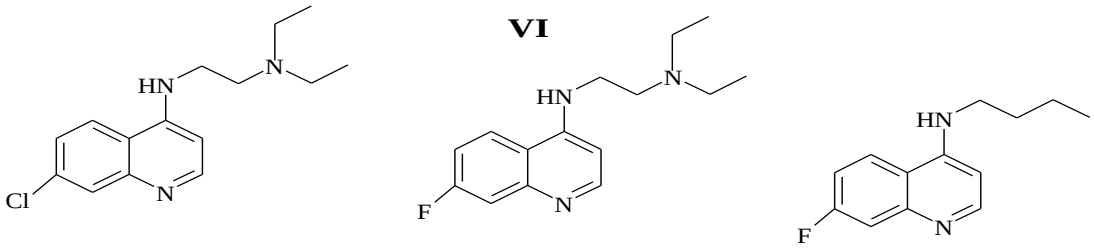
From the comparative docking study of our compounds with many structurally related lead compounds, such as lapatinib and gefitinib we could observe how our compounds might bind to the kinase binding site. Based on a knowledge of the structure of similar active sites, we docked Lapatinib into the active site of the enzyme (Fig. 7). Docking studies have revealed that Lapatinib ring binds to a narrow hydrophobic pocket in the EGFR TK domain with three hydrogen bond interaction with amino acids in vicinity while the aniline moiety lies in a deep and hydrophobic pocket. The bulky sulfamoyl group at C-4 of aniline moiety lies at the same position of the 3' chloro-4'-(3-fluorobenzyloxy) moiety of Lapatinib with total interaction energy equal to – 71 k cal/mol and RMSD equal 0.004 indicating that the ligand chosen interact with the enzyme at the same sites as do the main ligand. Compound IVc was then docked in the ATP binding site of EGFR TK with total interaction energy equal to -65.3 kcal/mol and showing hydrogen bond with D 855 (Fig. 8). Compound VIc was also docked in the ATP binding site of EGFR TK with total interaction energy equal to – 66.3 k cal/mol and showing two hydrogen bonds with R 841&C 797 (Fig. 9). We can observe that the quinoline ring lies in a deep and hydrophobic pocket in the EGFR as in case of the choosen lead compounds with total interaction energy which nearly approaches that of the lead compound Lapatinib.

Graphical Structure:

IV

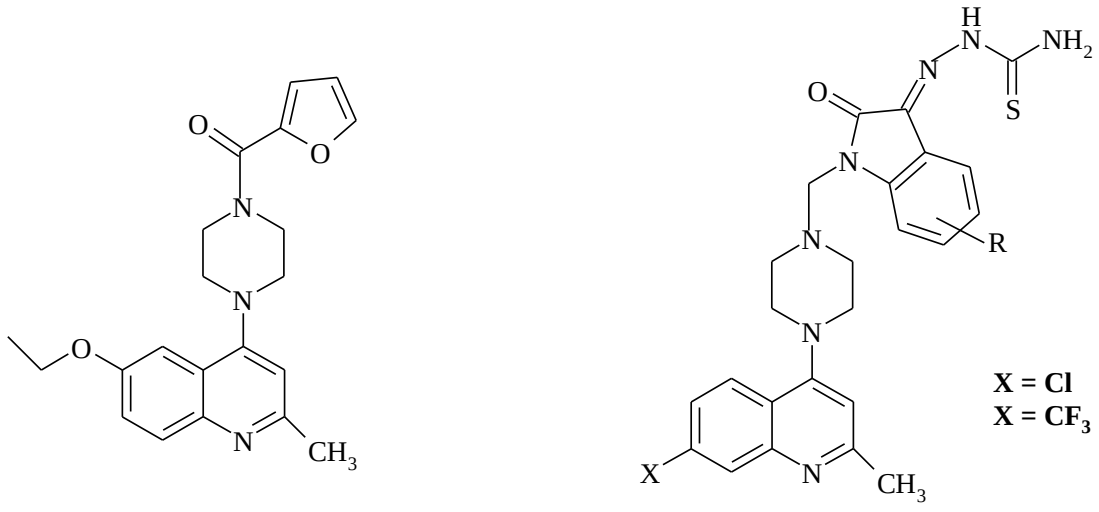


VI



1 **2** **3**

Fig. 1. Chemical structure of 4-aminoquinolines reported for anticancer activity



4 **5**

Fig. 2. The design of hybrid compounds

Scheme

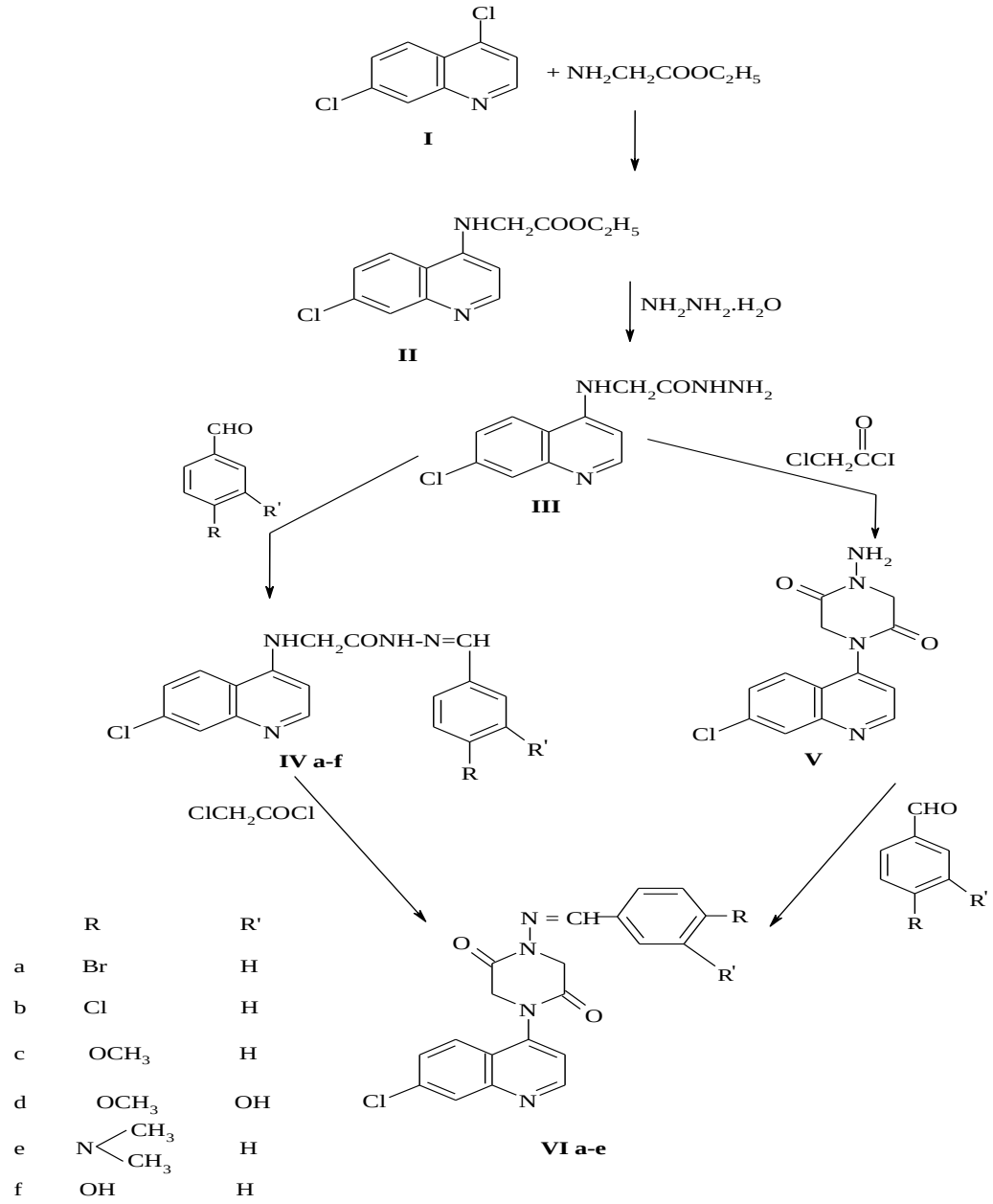


Table1:In vitro cytotoxic activities of some synthesized compounds against human breast cancer cell (MCF-7)

Compounds	Cytotoxicity (IC_{50})[a,b]
II	3.097
III	2.84
IVa	5.37
IVc	8.83
V	n.a.
VIa	1.41
VIb	1.5
VIc	2.75

[a]IC_{50}, Compounds concentration required to inhibit tumor cell proliferation by 50%.
[b]Values are averages of three experiments n.a. = No activity

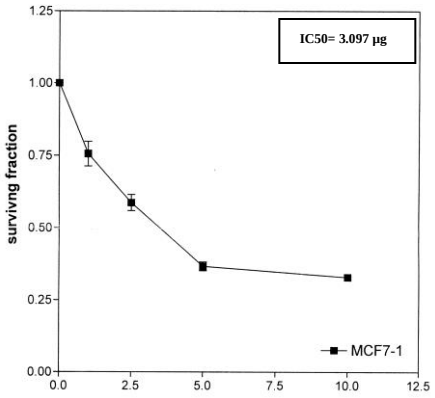


Fig. 3. Concentration of II in µg /ml

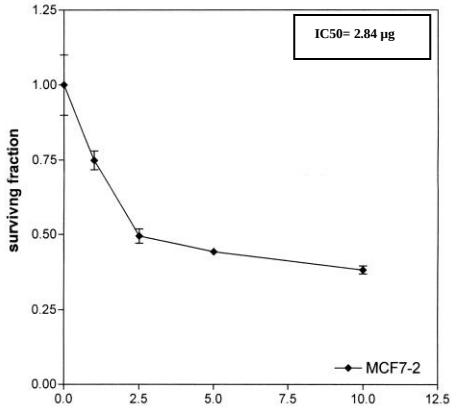


Fig. 4. Concentration of III in µg /ml

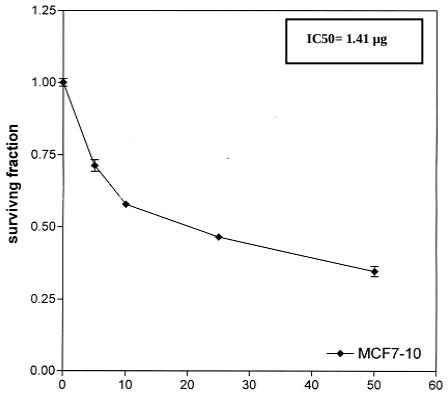


Fig.5.Concentration of VIa in µg /ml

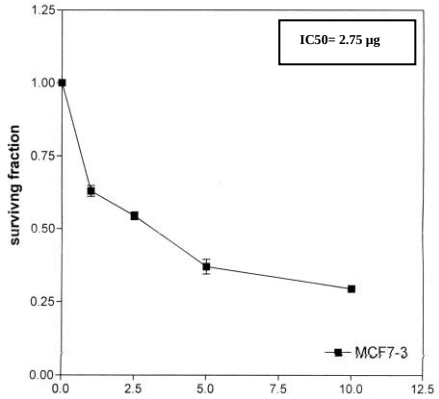


Fig. 6. Concentration of VIc in µg /ml

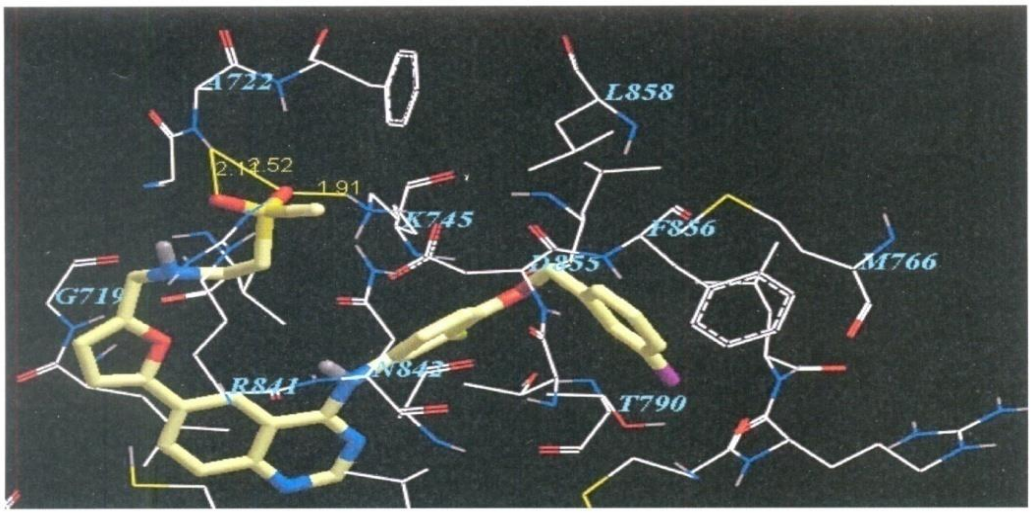


Fig. 7. Lapatinib in the ATP binding site of EGFR –TK. With ΣE equal to -71, RMSD = 0.004 with 3 HB. This picture was created with SYBYL version 7.3.

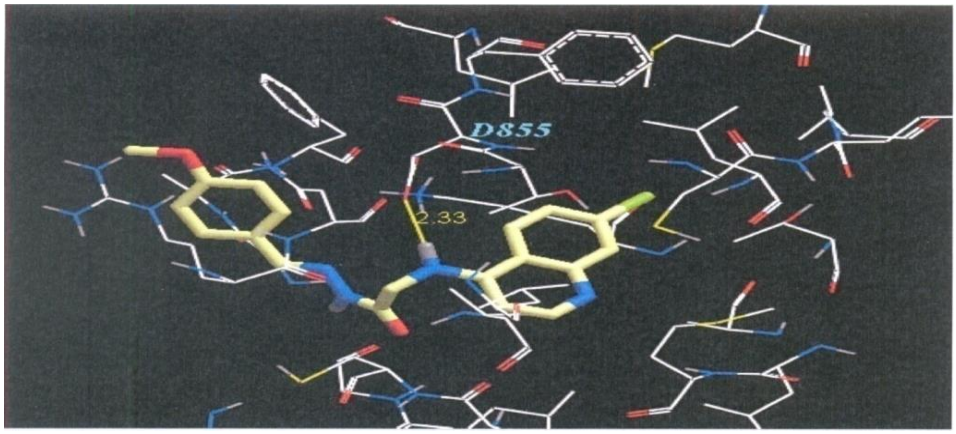


Fig. 8. Compound IVc docked at the ATP binding of EGFR – TK with ΣE equal to–65.3 the fig shows HB with D 855.

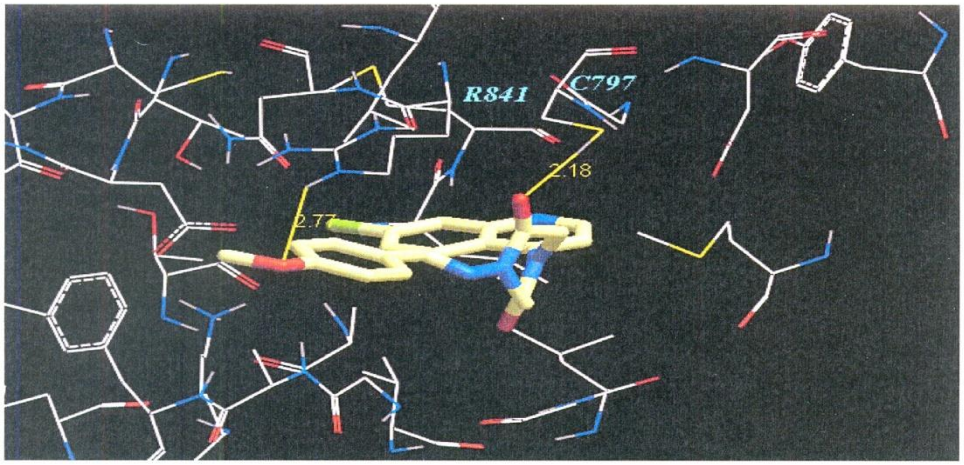


Fig.9. Binding mode of compound VIc in the ATP binding site of EGFR–TK with ΣE equal to–66.3 and showing 2HB with R841 & C 797.

4. Material and methods

4.1. Experimental

Melting points were determined with a Gallenkamp (London, U.K.) melting point apparatus and are uncorrected.IR spectra (KBr,cm^{-1}) were recoreded on Bruker Vector, 22 FT-IR (Fourier Transform Infrared (FTIR)) (Germany) spectrometer. ^{1}HNMR spectra were recorded on a Varian Gemini-200 (200-MHz, Foster City Calif., USA) and Varian Mercury-300 (300-MHz, City: Palo Alto, State: Calif.,USA spectrometers using dimethylsulphoxide (DMSO)-d_6 as a solvent and tetramethylsilane *(TMS)* as an internal standard (Chemical shift in δ, ppm). Mass spectra were determined using Mass spectrometers GC/MS Shimadzu QP 1000 EX (Shimadzu Corporation, Tokyo, Japan) with ionization energy 70 ev. Elemental analyses were determined using Automatic Elemental Analyzer CHN Model 2400 Perkin Elmer (USA) at Microanalytical Center, Faculty of Science, Cairo University, Egypt. All the results of elemental analyses corresponded to the calculated values within experimental error. Progress of the reaction was monitored by thin-layer chromatography (TLC) using precoated TLC sheets with Ultraviolet (UV) fluorescent silica gel (Merck 60F254) and spots were visualized by iodine vapors or irradiation with UV ligh (254 nm). All the chemicals were purchased from *Sigma-Aldrich*.

4.1.1. Ethyl-2-(7-chloroquinolin-4-ylamino) acetate II

A mixture of glycine ethyl ester (0.01 mole) and 4,7-dichloro-quinoline I (0.01 mol) in absolute ethanol (10 ml) containing few drops of HCl was refluxed for 2 h. to give a creamy white precipitate which was filtered, washed with cold aqueous alcohol and recrystallized from absolute alcohol yield 80%; MP: 185-87^{o}C; IR (KBr, cm^{-1}) 3300,1610; ^{1}H NMR: 1.2 (t, 4H, CH_3) 2.4 (s, 2H, CH_2) 4.2 (q, 2H, CH_2) 6.8-8.5 (m, 5H, aromatic) 9.5 (s, 1H, NH) MS m/z 265. Anal. Calcd for $C_{13}H_{13}N_2ClO_2$: C, 58.9; H, 4.9; N, 10.5. Found: C, 58.72; H. 4.62; N, 10.5.

4.1.2. 7-Chloro-N-(2-hydrazinyloxy)-2-oxoethyl) quinoline-4-amine III

A mixture of (0.01 mol) of II and hydrazine hydrate (0.03 mol) was refluxed in absolute ethanol (10 ml) for 2h. The solvent was evaporated under vacuum; the resulting oil was triturated with ice. The buff precipitate formed, was filtered, washed with alcohol/water and crystallized from ethanol. Yield 85% MP: 195-97^{o}C; IR (KBr, cm^{-1}) 3150-3300, 1650; ^{1}H NMR: 2.4(s, 2H, CH_2); 6.8-7.8 (m, 5H, aromatic) 8 (s, 2H, NH_2) 9-9.4 (2s, 2H, 2 NH) exchangeable with D_2O MS m/z 250. Anal. Calcd. for $C_{11}H_{11}N_4ClO$: C, 52.8; H, 4.3; N, 22.11. Found: C, 52.6; H, 4.58; N, 22.32.

4.2. General procedure for the preparation of N-(2-(2-(4-substituted arylidene) hydrazinyloxy)-2-oxoethyl)-7-chloroquinoline-4-amine IVa-f

A mixture of III (0.01 mol) and the appropriate aldehyde (0.01 mol) in absolute ethanol and drops of glacial acetic acid was refluxed for 3 h. The precipitate formed was filtered off, washed with alcohol dried and recrystallized from DMF/water.

4.2.1 N-(2-(2-(4-Bromobenzylidene) hydrazinyloxy)-2-oxoethyl)-7-chloroquinoline-4-amine IVa

Yield: 70%; mp: 198-200^{o}C, IR (KBr, cm^{-1}) 3300-3400, 1650; ^{1}H NMR 2.4 (s, 2H, CH_2), 7.2-8.6 (m, 9H aromatic) 9.4 (s, 1H, ald), 11.6-11.8 (2s, 2H, 2 NH) exchangable with D_2O, MSm/z 416. Anal. Calcd. for $C_{18}H_{14}N_4$ Br ClO: C, 51.7; H, 3.3; N, 13.4. Found: C, 51.8; H, 3.16; N, 13.4.

4.2.2. N-(2-(2-(4-Chlorobenzylidene) hydrazinyloxy)-2-oxoethyl)-7-chloroquinoline-4-amine IVb

Yield: 65% mp: 205-7^{o}C, IR (KBr, cm^{-1}) 3200-3400, 1680; ^{1}H NMR 2.4 (s, 2H, CH_2) 7.2-8.6 (m, 9H, aromatic) 9.4 (s, 1H, ald), 9.8, 10.2 (2s, 2H, 2 NH) exchangeable with D_2O Anal. Calcd. for $C_{18}H_{14}N_4Cl_2O$: C, 58; H, 3.7; N, 16.5. Found: C, 58.1; H, 3.8; N, 17.

4.2.3 N-(2-(2-(4-Methoxybenzylidene) hydrazinyloxy)-2-oxoethyl)-7-chloroquinoline-4-amine IV c

Yield: 68% mp: 118-20^{o}C; IR (KBr, cm^{-1}) 3200-3400, 1600; ^{1}H NMR 2.4 (s, 2H, CH_2) 4 (s, 3H, OCH_3) 7-8.4 (m, 9H, aromatic + 1H ald) 11.8 (broad, 2H, 2 NH) exchangeable with D_2O. Anal. Calcd. for $C_{19}H_{17}N_4ClO_2$: C, 61.8; H, 4.6; N, 15.1. Found: C, 61.83; H, 4.59; N, 14.91.

4.2.4. 5- (2-(2-(7-Chloro quinoline-4-ylamino) acetoxy) hydrazino) methyl) -2- methoxy phenol IVd

Yield. 80%; mp: 190-2^{o}C; IR (KBr, cm^{-1}) 3200-3400-3500, 1650; ^{1}H NMR 2.4 (s, 2H, CH_2), 4 (s, 3H, OCH_3), 6.8-8.2 (m, 9H, aromatic), 8.8 (s, 1H, ald), 9.9 (s, 1H, OH), 11.8 (br. 2H, 2NH) exchangeable with D_2O. Anal. Calcd. for $C_{19}H_{17}N_4ClO_3$: C, 59.2; H, 4.3; N, 14.5. Found: C, 59.12; H, 4.7; N, 14.9.

4.2.5.N-(2-(2-(4-Dimethylaminobenzylidene) hydrazinyloxy)-2-oxoethyl)-7-chloroquinoline-4-amineIVe

Yield 80%; mp. 245-7^{o}C; IR (KBr, cm^{-1}) 3200-3400, 1650; ^{1}H NMR 2.4 (s, 2H, CH_2), 3.4 (s, 6H, $2CH_3$) 6.8-8.6 (m, 9H, aromatic + 1H, ald) 9.8, 12 (2s, 2H, 2 NH) exchangeable with D_2O Anal. Calcd. for $C_{20}H_{20}N_5ClO$: C, 62.9; H, 5.2; N, 18.3. Found: C, 63; H, 5.2; N, 18.69.

4.2.6.N-(2-(2-(4-hydroxybenzylidene) hydrazinyloxy) -2-oxoethyl)-7-chloroquinoline-4-amine IV f

Yield 65%; mp. 173-5^{o}C; IR (KBr, cm^{-1}) 3300-3400, 1600; ^{1}H NMR. 2.4 (s, 2H, CH_2) 4 (s, 3H, OCH_3), 7-8.6 (m, 10H, aromatic + CH ald.) 10 (s, 1H, OH), 11.8 (broad, 2H, 2NH) exchangeable with D_2O. Anal. calcd for $C_{18}H_{15}N_4ClO_2$: C, 60.9; H, 4.2; N, 15.7. Found: C, 60.88; H, 4.3; N, 15.41.

4.3. 1 (Amino)-4- (7-chloroquinolin-4-yl)piperazine-2,5-dioneV

An equimolar amount of compound III and chloroacetyl chloride was refluxed in methylene chloride for 3h. The resulted buff precipitate was filtered, washed with alcohol/water and recrystallized from DMF/water, yield 60% MP: 295-97^{O}C; IR (KBr, cm^{-1}) 1610, 1700cm^{-1}, 3450cm^{-1}; ^{1}HNMR 4.2 (s,4H, 2CH $_2$) 7.9-8.7 (m,5H, aromatic) 11.4 (s, 2H, NH_2) exchangeable with D_2O. MS m/z 290.5 Anal. Calcd. for $C_{13}H_{11}N_4ClO_2$: C,53.7; H, 3.78; N, 19.2. Found: C, 54.01; H, 4.11; N, 17.44.

4.4. General procedure for the preparation of 1-(2-(4- substituted arylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dioneVI a-f

Method A

Compounds IV (a-f) (0.1 mole) were refluxed with chloracetyl chloride (10 ml) in absolute ethanol(10 ml) for 4 h. The refluxed mixture was poured into ice/ water to produce compounds VI a-f.

Method B

A mixture of compound V (0.1 mole) and the appropriate aromatic aldehyde (0.1 mole) in absolute ethanol (10 ml) containing few drops of glacial acetic acid was refluxed for 4h. The solid product was filtered off and recrystallized from DMF/water.

4.4.1. 1-(2-(4- Bromobenzylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dione VI a

Yield 75%; mp. 240-2^{o}C; IR (KBr, cm^{-1}) 1610-1620; ^{1}H NMR: 2.4 (s, 4H, $2CH_2$) 6.6-8.8 (m, 10H, aromatic + CH ald) Anal. Calcd for $C_{20}H_{14}N_4$ $BrClO_2$: C, 52.4; H, 3.0; N, 12.2; Found C, 52.5; H, 2.8; N, 12.25.

4.4.2. 1- (2-(4- Chlorobenzylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dione VI b

Yield 60%; mp: 295-7^{o}C; IR (KBr, cm^{-1}) 1608-1670; ^{1}H NMR 2.4 (s, 4H, $2CH_2$), 7.5-8.8 m (10H, arom + CH ald). Anal. Calcd. For

$C_{20}H_{14}N_4Cl_2O_2$: C, 58.25; H, 3.39; N, 13.59; Found: C, 58.4; H, 3.3; N, 13.93.

4.4.3. 1- (2-(4- Methoxybenzylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dione VIc

Yield 65%; mp: 275-7°C, IR (KBr, cm^{-1}) 1610-1620; ^1H NMR 2.4 (s, 4H, $2CH_2$), 3.8 (s, 3H, OCH_3) 7 – 8.8 (m, 10H, arom. + CH ald) MS/Z 409. Anal. Calcd for $C_{21}H_{17}N_4ClO_3$: C, 61.6; H, 4.16; N, 13.7; Found: C, 61.29, H, 3.79; N, 13.8.

4.4.4. 1- (2-(3- Hydroxy -4-methoxybenzylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dione VId

Yield: 65%; mp: 246-8°C; IR (KBr, cm^{-1}) 1600-1650; ^1H NMR. 24 (s, 4H, $2CH_2$) 3.8 (s, 3H, OCH_3) 6.9-8.7 (m, 10H, arom. + CH ald.) 9.8 (s, 1H, OH) exchangeable with D_2O. MS/mz 423.5. Anal. Calcd. for $C_{21}H_{17}N_4ClO_4$: C, 59.36; H, 4.0; N, 13.1. Found: C, 59.22; H, 4.11; N, 13.5.

4.4.5. 1- (2-(4- Dimethylaminobenzylidene) hydrazinyl-4-(7-chloroquinolin-4-yl)piperazine-2,5-dione VIe

Yield: 70%; mp: 228-30°C; IR (KBr, cm^{-1}) 1600-1610; ^1H NMR. 2.4 (s, 4H, $2CH_2$) 3.4 (s, 6H, $2CH_3$) 6.7-8.5 (m, 10H, arom. + CH ald.). Anal. Calcd. for $C_{22}H_{20}N_5ClO_2$: C, 62.6; H, 4.7; N, 16.6. Found: C, 62.33; H, 5.33; N, 17.

5. Conclusion

We have synthesized and tested a series of 1-(2-(4-substitutedarylidene) hydrazinyl-4-(7-chloroquinolin -4-yl) piperazine 2,5-dione VI variously substituted at the 3 and 4 phenyl moiety. Cytotoxic activity against human breast cell line (MCF-7) was evaluated as well as molecular modeling study was carried out through docking the designed compounds into the ATP binding site of EGFR. Biological screening results revealed that compounds VIa and VIc were the most active as shown in Table 1. It is crucial and advantageous that the electronic effect and the lipophilocity of the substituent in the para position of the arylidene moiety seems to be an important determinant of activity since the bromosubstitued derivative VIa was more active than the methoxy substituted derivative VIc. A parallel correlation was observed concerning molecular modeling study and cytotoxic activity. We can conclude that the designed hybrid pharmacophore VI might present good antitumor lead targeting EGFR-TK.

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