Synthesis and Anti-proliferative Activity of Substituted-Anilinoquinazolines and Its Relation to EGFR Inhibition

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Abstract

4-Anilinoquinazoline is a privileged scaffold in developing small molecule inhibitors of tyrosine kinases (TK) especially epidermal growth factor receptor (EGFR). 2 series belonging to 3'-substituted-4-anilinoquinazoline scaffold were synthesized and screened in vitro on isolated and a breast cancer cell line. The research aims at exploring the activity of compounds having diverse substituents at 3' position of the aniline moiety. Generally, the meta-substituted-anilinoquinazolines exhibited significant inhibitory activity against isolated enzyme as well as MCF-7 cancer cell line. For instance, compound **10b** inhibited >99% of EGFR activities at 10µM concentration. 6 of the tested compounds exhibited range of anti-proliferative activity below 10µM potency. In particular, compounds **6e** and **10b** displayed the highest activity among the tested compounds with IC_{50} values equal to 8.6 and 4.84µM, respectively. Structure-based tools were utilized to rationalize EGFR-TK binding of compound **10b** since it is the most active compound in the enzyme inhibition test.

Introduction

Inappropriate or uncontrolled activation of protein tyrosine kinases (PTKs), either by overexpression, constitutive activation, or mutation has been shown to cause uncontrolled cell growth [1]. The EGFR (erb-B_1) PTK has been identified as a major target for medicinal chemistry programs especially in cancer therapy such as breast, ovarian, colon, and prostate [2,3] as it is often associated with vascularity and poor prognosis of patients [4]. Drug discovery efforts targeting this aberrant kinase activity resulted in introduction of several FDA approved small molecule anticancer agents such as gefitinib, erlotinib and lapatinib (**Fig. 1**) which all belong to 4-anilinoquinazoline scaffold [5]. Thus, among the great number of reported structural classes of tyrosine kinase inhibitors, 4-anilinoquinazilines along with their congeners 4-anilinoquinolines and pyrrolopyrimidines represent the most researched classes of EGFR-TK inhibitors.

Gefitinib and erlotinib, the first generation quinazoline EGFR-TK inhibitors, have similar therapeutic profile since they target the activated form of the enzyme [6]. Resistance to EGFR-TK inhibitors have been attributed to specific mutations which usually make receptors less sensitive to their inhibitors. Common point mutations include T790M (also reported as T766M), G719S, L747S, and L858R [7,8]. The latter, also called L834R in another sequence numbering convention, is the most discovered resistance source as it represents 40% of detected mutations in some tumors such as Non-Small Cell Lung Cancer (NSCLC) [9,10]. Fortunately, both drugs seem to be effective in single L858R patients as they possess higher affinity to this particular mutant enzyme [11,12]. Lapatinib, a dual EGFR/erb-B2 inhibitor, seems to overcome the resistance emerged due to the somatic mutations of T790M in the tyrosine kinase domain of the EGFR gene that occur in patients with breast cancer who showed a poor response to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib [13]. Chemically, all EGFR inhibitors currently in clinical use are functionalized on the meta position of the aniline moiety with small hydrophobic halo or hydrocarbon substituents. Nonetheless, we found no enough research addressing the SAR of this position with variety of bulkier groups [14,15]. Therefore, we realized that this point of

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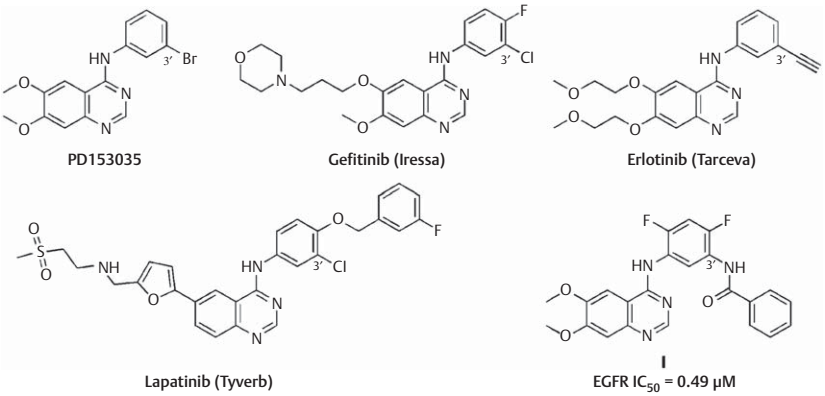

Fig. 1 Quinazoline derivatives as EGFR TK inhibitors.

diversity is an intriguing opportunity to develop novel antiproliferative agents which may act through EGFR-TK inhibition and/or another mechanism.

Design and Rationale

Most of the precedent SAR studies of 4-anilinoquinazolines were dedicated to substitutions on 4' position of the aniline moiety while halo group or small alkyl groups were recruited to satisfy a presumed small pocket around the 3' position [16, 17]. In these early works on quinazoline inhibitors of EGFR-TK, screening results discouraged researchers of pursuing SAR studies that cover a diverse molecular space around this position since methoxy and methyl analogues exhibited lower potency than halo compounds. Nevertheless, the success of erlotinib and availability of several crystal structures of EGFR-TK bound to quinazoline inhibitors brought us a renewed interest in this less explored position and we decided to investigate it with more challenging bulkier functionalities. The SAR around the 3' position was studied through the synthesis of 2 sets of compounds that have substituents linked through ether or amide linker. Moreover, the 2 compound series contained chloro group on the C2 of the core quinazoline nucleus because it was reported in recent studies to enhance the anticancer activity and blood brain barrier penetration [18]. We kept 2 methoxy groups at positions 6- and 7- of the quinazoline as they have been established as compatible groups to good EGFR-TK inhibition [19]. Accordingly, the proposed compounds were synthesized and evaluated for their in vitro enzyme inhibitory activity as well as antiproliferative potency on MCF-7 breast carcinoma cell line in which EGFR-TK is over expressed.

Chemistry

General synthesis of target compounds are depicted in **Fig. 2, 3** from the central intermediate 2,4-quinazolinedione (**2**) [20]. Chlorination of the dione with $POCl_3$ in the presence *N,N*-dimethylaniline gave the key starting material, 2,4-dichloro-6,7-dimethoxyquinazoline **3** [21]. In the second figure, the synthesis of the final compounds 4-anilinoquinazolines (**6a–i**) was described where the C-3' bulky substituent was linked to the aniline ring via amide linker. 4-(3-Carboxyanilino)-2-chloro-6,7-dimethoxyquinazoline (**4**) was synthesized via the reaction of (**3**) with *m*-aminobenzoic acid under carefully controlled conditions to strictly direct the S_NAr substitution reaction towards the C-4 chloro group without affecting the chlorine atom at C-2 position. At the following stage, 4-(3-carboxyanilino)-2-chloro-6,7-dimethoxyquinazoline **4** was refluxed with thionyl chloride to produce the unstable acyl chloride hydrochloride derivative (**5**). Without isolation, the acid chloride **5** was reacted with amines at ambient temperature, gave the final compounds (**6a–i**). On the other hand, **Fig. 3** describes the synthetic pathway of the other series of the 4-anilinoquinazoline compounds (**10a–f**). The protected *m*-acetamidophenol (**7**) was reacted with the corresponding alkyl or alkyl halides to afford the corresponding alkoxy derivatives (**8a–e**). After removal of the protecting acetyl group using alkaline hydrolysis, the aminophenyl ethers (**9a–e**) were reacted with the 2,4-dichloro-6,7-dimethoxyquinazoline (**3**) in presence in basic conditions to furnish the final compounds (**10a–e**). The structures of all the newly synthesized compounds were elucidated with ^{1}H NMR, EI-Mass, FT-IR and elemental analyses.

In Vitro EGFR Inhibition and Antiproliferative Cell Assay

EGFR-TK inhibitory activities of the title compounds were determined using K-LISA (Kinase activity ELISA) technique. K-LISA enabled us to measure kinase activity semi-quantitatively using enzyme-linked immunosorbent assays (ELISA) with phospho-specific antibodies utilizing colorimetric detection [22, 23]. The test compounds that showed >50% inhibition, were evaluated for IC_{50} on the MCF-7 breast cancer cell lines where the EGFR-TK is over-expressed (**Table 1**) [24, 25].

Results and Discussion

Most of the tested compounds exhibited significant inhibitory activity against the isolated EGFR enzyme except compounds **6b, 6d, 6g, 6i** and **10d**. Generally the ether series (**10a–e**) showed higher EGFR inhibition and cellular activities than the amide series (**6a–i**). In particular, the allyloxy analogue **10b** was approximately as potent as the reference lapatinib. Also, the cellular assay results are consistent with enzyme inhibition, implicating that anticancer activities are partially or mostly related to EGFR-TK inhibition.

To rationalize the EGFR-TK binding potency of **10b**, a structure-based modelling study was performed by docking this molecule on the crystal structures of EGFR-TK (pdb codes: 1M17, 2ITY and

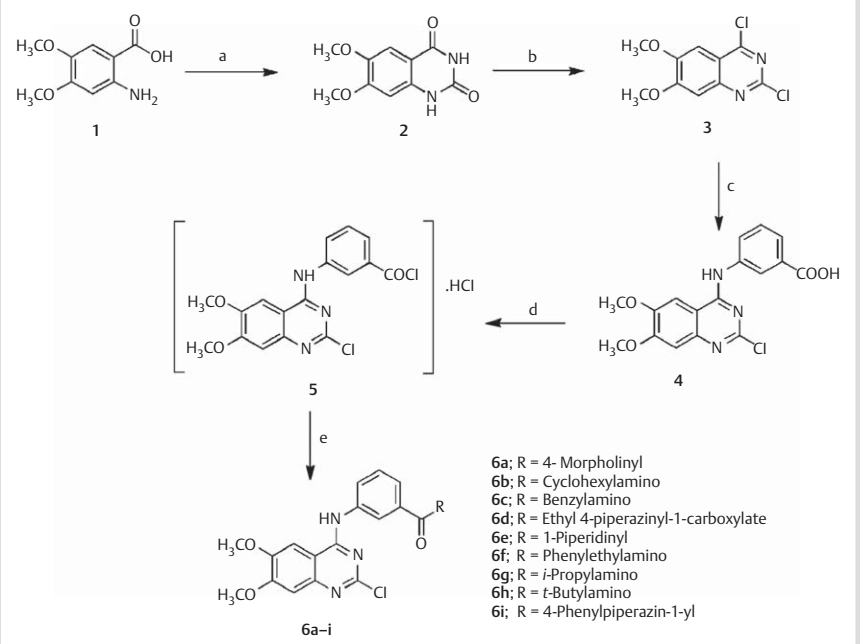

Fig. 2 Synthetic pathway of 4-anilinoquinazolines (**6a–i**). Reagents and conditions **a** KCNO, AcOH then NaOH; **b** $POCl_3$, *N,N*-dimethylaniline; **c** *m*-aminobenzoic acid, ethanol, rt, 24 h; **d** $SOCl_2$, reflux; **e** Amine compound, dioxane, K_2CO_3, rt.

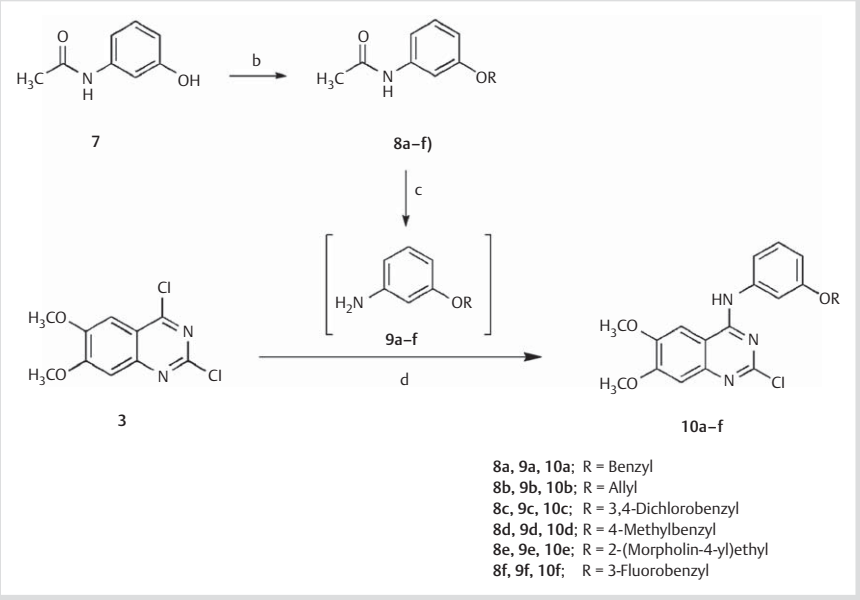

Fig. 3 Synthesis of 4- alkyloxyanilinoquinazolines (**10a–f**); Reagents and conditions **a** $(CH_3CO)_2O$, CH_3COOH, rt.; **b** Alkyl/Aralkyl halide, K_2CO_3, KI, Acetone, reflux.; **c** NaOH, H_2O, EtOH, reflux.; **d** CH_3COONa & EtOH, rt, 24h.

Table 1 EGFR-TK inhibition and MCF cell line inhibition activity of test compounds. Note that IC_{50} > 10 µM is considered to be out of range. IC_{50} of reference lead compound (Lapatinib) was 7.70 nM. ND means Not Determined.

Compound No.	inhibition % at 10 µM	Cytotoxicity (IC50) µM
6a	84	17.9
6b	0	ND
6c	69.7	11.9
6d	0	ND
6e	65.4	8.6
6f	33	ND
6g	0	ND
6h	48	ND
6i	2.4	ND
10a	0	ND
10b	99.5	4.8
10c	53	out of range
10d	22.2	ND
10e	70.5	14.3

2ITZ) after modifying each ligand to the required structure while keeping the rest of the structural features as given [26,27]. The new structure was then re-docked into the ATP site. Concerning the alkoxy side chain, the least energy conformer (all anti-conformation) was used. Also, 2 docking experiments were performed due to difference in orientation of the allyl with respect to quinazoline ring as it either attains *cis* or *trans* conformation (**Fig. 4**). In the process, it was worthy to compare the orientation and contacts of this allyloxy group with erlotinib's alkynyl group in its crystal structure (PDB Code: 1M17) which is proved to acquire *cis* conformation in its binding with the activated conformation of EGFR. The compound **10b** with extended conformation of allyloxy group (least energy conformer) rested comfortably with good VdW radii in this medium size pocket composed of hydrophobic side chains of Leu764, Thr766, Met742 and Lys721 (**Fig. 4b**). Additionally, the ether oxygen established a hydrogen bond with Thr766 terminal hydroxyl group. On the other hand, the chloro group approached and

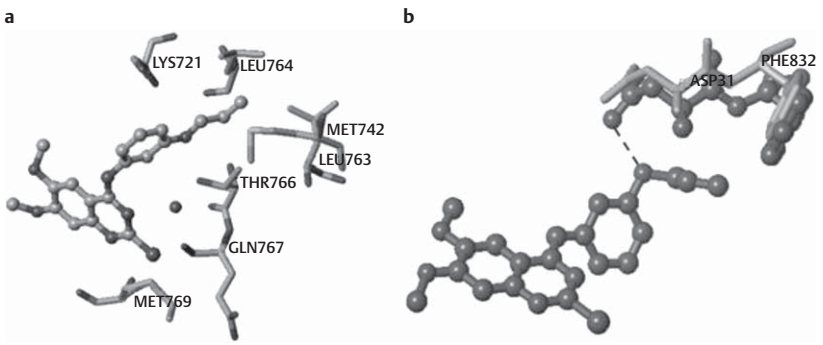

Fig. 4 **a** Docking of *cis*-conformation of **10b** on crystal structure of wild type EGFR-TK (PDB Code 1M17). **b** Docking of *trans*-conformation of **10b** on mutant L834R EGFR-TK (PDB Code: 2ITZ) shown in dark grey. Same amino acid residues from wild type are shown in light grey sticks.

backbone carbonyl of Gln767 in unsuitable short distance. Conformational search about the 2 bonds between the C$ atom of the quinazoline and the phenyl ring suggested that rotating of the 2 dihedral angles between the 2 rings resulted in relaxation of inter-atomic distances between the chloro group and atoms around without affecting other interactions such as the critical bridged H-bond between quinazoline N-3 and Thr766 (hinge region interaction). This way, the chloro group had contacts with a small pocket in the hinge loop composed of residues Gln767-Met769. Similar results were obtained from docking of **10b** *cis* conformation on the wild-type EGFR crystal structure complexed with gefitinib (PDB Code: 2ITY).

In this modelling study, the possibility of the allyloxy group to attain *trans* conformation with respect to the quinazoline moiety was investigated according to findings of Yun et al. about binding mode of gefitinib with mutated EGFR (L834R) [27].The authors reported an inverted (*trans*) positioning of the chloro group as they formed a halogen bond with Asp831. Docking of *trans* conformation of compound **10b** on crystal structure L834R EGFR crystal structure (PDB Code: 2ITZ) resulted in a convincing short hydrogen bond with Asp831. In addition, the allyl group attained a bent conformation make suitable hydrophobic contact with the aromatic ring of Phe832 (▶ **Fig. 4b**).

Conclusion

In this work, we present compounds with low micromolar level of cell growth inhibition on human breast carcinoma cell line (MCF-7). The exact mechanism of its cytotoxic activity of our compounds especially those belonging to amide series could not be confirmed because the enzyme inhibition is not as high as the cell line inhibition.

The SAR also indicates that the 3′ position is less tolerant to bulkier groups than 4' position because the allyloxy group gave better results in the isolated EGFR-TK inhibition tests. Nonetheless, the results reveal that EGFR inhibition is not highly sensitive to group size at 3' position as previously implicated. Therefore, this diversity point can be utilized to introduce novel TK inhibitors with versatile biological and/or pharmacokinetic properties.

Experimental

Melting points are uncorrected and were determined on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK). Elemental analyses (C, H, N) were performed on Perkin-Elmer 2400 analyser (Perkin-Elmer, Norwalk, CT, USA) at the microanalytical laboratories of the Faculty of Science, Cairo University. All analyses of the new compounds were within ±0.4% of the theoretical values. The IR spectra (KBr) were measured on Shimadzu IR 110 spectrophotometer (Shimadzu, Koyoto, Japan), ^{1}H-NMR spectra were obtained on a Bruker proton NMR-Avance 300 (300 MHz) (Bruker, Munich, Germany), in DMSO-d_6 as a solvent, using tetramethylsilane (TMS) as internal standard. The electron impact (EI) mass spectra were recorded on Finnigan Mat SSQ 7000 (70 ev) mass spectrometer. All reactions were monitored by thin layer chromatograph (TLC) using precoated Aluminium sheets Silica gel Merck 60 F254 and were visualized by UV lamp (Merck, Darmstadt, Germany). All reagents and solvents were purified and dried using standard techniques. Intermediates **8a**, **8b**, **8c** were synthesized according to reported methods [28–30].

4-(3-Carboxyanilino)-2-chloro-6,7-dimethoxyquinazoline (4)

2,4-Dichloro-6,7-dimethoxyquinazoline (**3**) (0.5 g, 1.93 mmol) was added portion-wise to a mixture of 3-aminobenzoic acid (0.265 g, 1.93 mmol) and sodium acetate (0.2 g, 2 mmol) in absolute ethanol (20 mL). The mixture was stirred at room temperature overnight. The resulting faint green precipitate was collected and dissolved in saturated solution of sodium carbonate. The solution was filtered, acidified using glacial acetic acid. The produced precipitate was collected, washed with water and dried then crystallized from acetone. Yield: 77%; m.p.: >250 °C; IR (ν, cm^{-1}): 3500–3078 (NH, OH acidic), 1692 (C=O), 1642 (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.92 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 7.16 (s, 1H, C5-H of quinazoline), 7.51-8.29 (m, 4H, Ar-H), 7.92 (s, 1H, C8-H of quinazoline), 10.02 (s, 1H, NH, D_2O exchangeable), 10.71 (s, 1H, OH, D_2O exchangeable). EIMS, *m/z*: 359 (M^+, 100%), 361 (M+2, 32.2%).

2-Chloro-6,7-dimethoxy-4-{[3-(morpholin-4-yl) carbonyl]anilino}quinazoline (6a)

A mixture of **4** (3.59 g, 10 mmol) and thionyl chloride (15 mL, excess) was refluxed for 4 h. Excess thionyl chloride was distilled under vacuum. The residue obtained was washed with dry ether (Na_2SO_4) and dried. The freshly prepared 3-(2-chloro-6,7-dimethoxyquinazolin-4-ylamino)benzoyl chloride hydrochloride (**5**) (0.2 g, 0.53 mmol) was mixed with morpholine (0.2 g, excess) and anhydrous K_2CO_3 (0.2 g) in dioxane (1.5 mL) and cooled in ice bath for 30 min then stirred at room temperature overnight. The mixture was poured on warm freshly prepared concentrated solution of K_2CO_3 (10–15 mL) then stirred for 30 min. The formed precipitate was filtered, washed with water, dried and crystallized from the ethanol/methanol mixture. Yield: 77%; m.p.: 270–272 °C; IR (ν, cm^{-1}): 3317 (NH), 1619

(C=O), 1606 (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.93 (s, 3H, O**CH$_3$**), 3.95 (s, 3H, O**CH$_3$**), 4.33 (t, 4H, **CH$_2$**-O-**CH$_2$** of morpholine), 4.07 (t, 4H, **CH$_2$**-N-**CH$_2$** of morpholine), 7.16 (s,1H, C5-H of quinazoline), 7.21–7.79 (m, 4H, Ar-H), 7.86 (s, 1H, C8-H of quinazoline), 9.931 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 428 (M^+, 63.2%), 430 (M+2, 17.2%). Anal. Calcd. for $C_{21}H_{21}ClN_4O_4.0.5H_2O$: C, 57.56; H, 5.02; N, 12.78. Found: C, 57.60; H, 5.34; N, 12.50.

2-Chloro-4-[3-(cyclohexylcarbamoyl)anilino]-6,7-dimethoxyquinazoline (6b)

Compound **6b** was prepared as described for **6a** from **4** and cyclohexylamine then the product was recrystallized from acetonitrile. Yield: 63%; m.p.: 248–250 °C; IR (ν, cm^{-1}): 3378 (NH amidic), 3354 (NH), 1628 (C=O), 1574 cm^{-1} (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.03–1.83 (m,10H, cyclohexyl), 3.93 (s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 3.34–3.49 (m, 1H, NH-**CH** of cyclohexyl), 7.18 (s, 1H, C5-H of quinazoline), 8.09 (s, 1H, C8-H of quinazoline), 7.47–8.04 (m, 4H, Ar-H), 8.23 (d, 1H, **NH** amidic, D_2O exchangeable), 9.94 (s, 1H, **NH**, D_2O exchangeable). MS, *m/z*: 440 (M^+, 100%), 442 (M+2, 30.1%). Anal. Calcd. for $C_{23}H_{25}ClN_4O_3.0.5H_2O$: C, 62.65; H, 5.71; N, 12.71. Found: C, 62.76; H, 5.75; N, 12.79.

4-[3-(*N*-Benzylcarbamoyl)anilino]-2-chloro-6,7-dimethoxyquinazoline (6c)

Compound **6c** was prepared as described for **6a** from **4** and benzylamine. The product was recrystallized from methanol. Yield: 76%; m.p.: 170–173 °C; IR (ν, cm^{-1}): 3346 (NH amidic), 3289 (NH), 1626 (C=O), 1578 cm^{-1} (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.93 (s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 4.50 (s, 2H, NH-**CH$_2$**-Ph), 7.18 (s, 1H, C5-H of quinazoline), 7.33–8.18 (m, 9H, 2 Ar-**H**), 7.90 (s, 1H, C8-H of quinazoline), 9.06 (t, 1H, **NH** amidic, D_2O exchangeable), 9.96 (s, 1H, **NH**, D_2O exchangeable). MS, *m/z*: 448 (M^+, 41.9%), 450 (M+2, 14.4%). Anal. Calcd. for $C_{24}H_{21}ClN_4O_3$: C, 64.21; H, 4.72; N, 12.28. Found: C, 63.88; H, 5.11; N, 11.98.

4-[3-[(4-Carbethoxypiperazinyl)carbonyl]anilino]-2-chloro-6,7-dimethoxyquinazoline (6d)

Compound **6d** was prepared as described for **6a** from **4** and ethyl piperazinyl-1-carboxylate. The product was recrystallized from ethanol. Yield: 80%; m.p. 255–257 °C; IR (ν, cm^{-1}): 3501 (NH), 1686 (C=O ester), 1623 (C=O amidic), 1579 (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.12 (t, 3H, COOCH$_2$**CH$_3$**), 3.46 (t, 4H, 2-**CH$_2$** of piperazine), 3.56 (t, 4H, 2-**CH$_2$** of piperazine), 3.94(s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 4.07 (q, 2H, COO**CH$_2$**CH$_3$), 7.18 (s, 1H, C5-H of quinazoline), 7.80 (s, 1H, C8-H of quinazoline), 7.16–7.84 (m, 4H, Ar-**H**), 9.94 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 499 (M^+, 100%), 501 (M+2, 38.1%). Anal. Calcd. for $C_{24}H_{26}ClN_5O_5.0.65H_2O$: C, 56.33; H, 5.32; N, 13.65. Found: C, 56.07; H, 4.94; N, 13.42.

2-Chloro-6,7-dimethoxy-4-[3-[(piperidin-1-yl)carbonyl]anilino]quinazoline (6e)

Compound **6e** was prepared as described for **6a** from **4** and piperidine then the product was recrystallized from ethanol. Yield: 75%; m.p.: 240–241 °C; IR (ν, cm^{-1}): 3246 (NH), 1676 (C=O), 1579 (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.53–1.64 (m, 6H, piperidine- H), 3.50 (m, 4H, **CH$_2$**-N-**CH$_2$** of piperidine), 3.93 (s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 7.17 (s, 1H, C5-H of quinazoline), 7.15–7.86 (m, 4H, Ar-H), 7.77 (s, 1H, C8-H of quinazoline), 9.83 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 426 (M^+, 46.2%), 428 (M+2, 14.1%). Anal. Calcd. for $C_{22}H_{23}ClN_4O_3$: C, 61.90; H, 5.43; N, 13.12. Found: C, 62.24; H, 5.52; N, 13.21.

2-Chloro-6,7-dimethoxy-4-[3-(2-phenylethylcarbamoyl)anilino]quinazoline (6f)

Compound **6f** was prepared as described for **6a** from **4** and 2-phenylethylamine. The product was recrystallized from methanol. Yield: 72%; m.p.: 122–125 °C; IR (ν, cm^{-1}): 3301–3500 (NH, NH amidic), 1629 (C=O), 1581 (C=N). ^{1}H NMR (DMSO-d_6) δ: 2.86 (t, 2H, Ph-**CH$_2$**- CH$_2$-NH), 3.50 (t, 2H, Ph-CH$_2$- **CH$_2$**-NH), 3.93(s, 3H, O**CH$_3$**), 3.95 (s, 3H, O**CH$_3$**), 7.16 (s, 1H, C5-H of quinazoline), 7.91 (s, 1H, C8-H of quinazoline), 7.12–8.07 (m, 9H, Ar-H), 8.59 (t, 1H, NH amidic, D_2O exchangeable), 10.02 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 462 (M^+, 26.4%), 464 (M+2, 7.1%). Anal. Calcd. For $C_{25}H_{23}ClN_4O_3$: C, 64.86; H, 5.01; N, 12.10. Found: C, 65.03; H, 4.98; N, 12.43.

2-Chloro-4-[3-(isopropylcarbamoyl)anilino]-6,7-dimethoxyquinazoline (6g)

Compound **6g** was prepared as described for **6a** from **4** and *i*-propylamine then the product was recrystallized from methanol. Yield: 55%; m.p.: 170–171 °C; IR (ν, cm^{-1}): 3312–3700 (NH, NH amidic), 1628 (C=O), 1575 (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.18 (d, 6H, NHCH(**CH$_3$**) **CH$_3$**), 3.936 (s, 3H, O**CH$_3$**), 3.965 (s, 3H, O**CH$_3$**), 4.140 (septet, 1H, NH**CH**(CH$_3$) CH$_3$), 7.187 (s, 1H, C5-H of quinazoline), 7.92 (s, 1H, C8-H of quinazoline), 7.48–8.10 (m, 4H, Ar-H), 8.24 (d, 1H, **NH** amidic, D_2O exchangeable), 9.96 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 400 (M^+, 100%), 402 (M+2, 35.5%). Anal. Calcd. For $C_{20}H_{21}ClN_4O_3$: C, 59.92; H, 5.28; N, 13.98. Found: C, 60.01; H, 5.63; N, 13.71.

4-[3-(*t*-Butylcarbamoyl)anilino]-2-chloro-6,7-dimethoxyquinazoline (6h)

Compound **6h** was prepared as described for **6a** from **4** and *t*-butylamine then the product was recrystallized from methanol. Yield, 63%; m.p. 200–202 °C; IR (ν, cm^{-1}): 3374–3600 (NH, NH amidic), 1630 (C=O), 1575 (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.39 (s, 9H, NHC(**CH$_3$**)$_3$), 3.93 (s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 7.19 (s, 1H, C5-H of quinazoline), 7.91 (s, 1H, C8-H of quinazoline), 7.41(m, 4H, Ar-H), 8.03 (s, 1H, **NH** amidic, D_2O exchangeable), 9.945 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 414 (M^+, 73.6%), 416 (M+2, 32.4%). Anal. Calcd. for $C_{21}H_{23}ClN_4O_3$: C, 60.79; H, 5.59; N, 13.50. Found: C, 61.10; H, 5.93; N, 13.17.

2-Chloro-6,7-dimethoxy-4-[3-(4-phenylpiperazin-1-yl)anilino]quinazoline (6i)

Compound **6i** was prepared as described for **6a** from **4** and 1-phenylpiperazine then the product was recrystallized from methanol. Yield: 75%; m.p.: 198–200 °C; IR (ν, cm^{-1}): 3322 (NH), 1630 (C=O), 1594 (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.592–3.801 (m, 8H, piperazine-H), 3.93 (s, 3H, O**CH$_3$**), 3.96 (s, 3H, O**CH$_3$**), 6.78–7.86 (m, 9H, Ar-H), 7.17 (s, 1H, C5-H of quinazoline), 7.83 (s, 1H, C8-H of quinazoline), 9.87 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 504 (M^+, 82.0%), 506 (M+2, 20.8%). Anal. Calcd. For $C_{27}H_{26}ClN_5O_3.H_2O$: C, 62.12; H, 5.36; N, 13.42. Found: C, 61.54; H, 5.61; N, 12.98.

3-(3-Methylbenzyloxy)acetanilide (8d)

A mixture of *m*-hydroxyacetanilide (**7**) (1g, 6mmol), 3-methylbenzyl chloride (0.84g, 6mmol), anhydrous K_2CO_3 (0.2 g, 1.4mmol) and KI (0.2 g, 1.2mmol) in dry acetone (15mL) was heat to reflux for 11h. The mixture was cooled to room temperature, solvent was evaporated and the residue was partitioned between water and ether (3×30mL). The organic layer was washed with 10% aqueous sodium hydroxide, brine and dried

(anhydrous potassium carbonate) and evaporated. The residue was washed with dry *n*-hexane then crystallized from chloroform. Yield, 76%; m.p. 92–95 °C; IR (ν, cm^{-1}): 3310 (NH amidic) and 1668 (C=O). ^{1}H NMR (DMSO-d_6) δ: 2.03 (s, 3H, **CH_3**CONHPh), 2.32 (s, 3H, PhOCH$_2$Ph**CH_3**), 5.01 (s, 2H, PhO**CH_2**PhCH_3), 6.66–7.36 (m, 8 H, Ar-H), 9.89 (s, 1H, **NH** amidic, D_2O exchangeable). MS, *m/z*: 255 (M^+, 5.7%).

3-[2-(Morpholin-4-yl)ethoxy]acetanilide (8e)

Compound **8e** was prepared as described for **8d** from **7** and 2-(4-morpholinyl)ethyl chloride (0.89 g, 6 mmol) and then crystallized from chloroform. Yield: 73.5%; m.p. 67–70 °C; IR (ν, cm^{-1}): 3296.7 (NH amidic) and 1654.6 (C=O). ^{1}H NMR (DMSO-d_6) δ: 2.03 (s, 3H, **CH_3**CONHPh), 2.47 (t, 4H, **CH_2**-N-**CH_2** of morpholine), 2.68 (t, 2H, PhOCH$_2$**CH_2**-morpholine), 3.57 (t, 4H, **CH_2**-O-**CH_2-** of morpholine), 4.03 (t, 2H, PhO**CH_2**CH$_2$morpholine), 6.59-7.31 (m, 4H, Ar-H), 9.92 (s,1H, **NH** amidic, D_2O exchangeable). MS, *m/z*: 266 (M^+, 7%).

4-(3-Benzyloxyanilino)-2-chloro-6,7-dimethoxyquinazoline (10a)

A mixture of **8a** (0.75 gm, 3.12 mmol), NaOH (6.25 g, 156 mmol) in ethanol (125 mL) and water (30 mL) was heated to reflux with stirring for 7 h. The mixture was cooled and the solvent was removed under vacuum. The residue was partitioned between chloroform (100 mL) and water (100 mL). The organic phase was washed with water (2×100 mL), dried ($MgSO_4$), and evaporated under vacuum to give **9a** as oily residue that was mixed with sodium acetate (0.2 g, 2 mmol) in absolute ethanol (20 mL) was warmed gently till complete dissolution. The 2,4-dichloro-6,7-dimethoxyquinazoline (**3**) (0.5 g, 1.93 mmol) was added portionwise and stirred at room temperature overnight. Precipitate formed was collected, washed with ethanol, dried and crystallized from ethanol. Yield: 77%; m.p.: 165–167 °C; IR (ν, cm^{-1}): 3371 (NH), 1573 (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.92 (s, 3H, O**CH_3**), 3.96 (s, 3H, O**CH_3**), 5.14 (s, 2H, Ph**CH_2**O), 6.85-7.93 (m, 9H, Ar-H), 7.16 (s, 1H, C5-H of quinazoline), 7.93 (s, 1H, C8-H of quinazoline), 9.87 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 421 (M^+, 20.5%), 423 (M+2, 9.0%). Anal. Calcd. for $C_{23}H_{20}ClN_3O_3$: C, 65.48; H, 4.78; N, 9.96. Found: C, 65.55; H, 4.92; N, 10.05.

4-(3-Allyloxyanilino)-2-chloro-6,7-dimethoxyquinazoline (10b)

Compound **10b** was prepared as described for **10a** from **8b** (0.595 gm, 3.12 mmol) and **3** then the product was recrystallized from ethanol. Yield, 60%; m.p. 190–193 °C; IR (ν, cm^{-1}): 3207 (NH), 1594 (C=N), 1548 (C=C). ^{1}H NMR (DMSO-d_6) δ: 3.93(s, 3H, O**CH_3**), 3.96 (s, 3H, O**CH_3**), 4.45 (d, 2H, O**CH_2**CH=CH_2), 5.25 (dd, 1H, OCH$_2$CH=(CH)**H**), 5.36 (dd,1H,OCH$_2$CH=(**CH**)H), 6.02 (m,1H, OCH$_2$**CH**=CH_2), 6.73–7.91 (m, 4H, Ar-H), 7.14 (s, 1H, C5-H of quinazoline),8.05 (s, 1H, C8-H of quinazoline), 10.25 (s,1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 371 (M^+, 16.7%). Anal. Calcd. for $C_{19}H_{18}ClN_3O_3$: C, 61.38; H, 4.88; N, 11.30. Found: C, 61.39; H, 4.91; N, 11.33.

2-Chloro-4-[3-(3,4-dichlorobenzyloxy)anilino]-6,7-dimethoxyquinazoline (10c)

Compound **6c** was prepared as described for **10a** from **8c** (0.964 gm, 3.12 mmol) and **3** then the product was recrystallized from ethanol. Yield: 80.3%; m.p.: 200–202 °C; IR (ν, cm^{-1}): 3421 (NH) and 1580 (C=N). ^{1}H NMR (DMSO-d_6) δ: 3.92 (s, 3H, O**CH_3**), 3.96 (s, 3H, O**CH_3**), 5.16 (s, 2H, Ph**CH_2**O), 6.83–8.07 (m, 7H, 2 Ar-H), 7.16 (s, 1H, C5-H of quinazoline), 7.91 (s, 1H, C8-H of quinazoline), 9.87 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 489 (M^+, 45.5%), 491 (M+2, 14.2%). Anal. Calcd. For $C_{23}H_{18}Cl_3N_3O_3$: C, 56.29; H, 3.70; N, 8.56. Found: C, 56.38; H, 4.00; N, 8.65.

2-Chloro-6,7-dimethoxy-4-[3-(3-methylbenzyloxy) anilino]quinazoline (10d)

Compound **10d** was prepared as described for **10a** from **8d** (0.795 gm, 3.12 mmol) and **3** then the product was recrystallized from ethanol. Yield, 71.2%; m.p. 185–188 °C; IR (ν, cm^{-1}): 3111 (NH) and 1590 (C=N). ^{1}H NMR (DMSO-d_6) δ: 2.30 (s, 3H, **CH_3**Ph-CH_2O), 3.91(s, 3H, O**CH_3**), 3.94 (s, 3H, O**CH_3**), 4.99 (s, 2H, Ph**CH_2**O), 7.02–7.42 (m, 10H, Ar-H and quinazoline – H), 7.87 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 435 (M^+, 7.4%), 437 (M+2, 3.9%). Anal. Calcd. For $C_{24}H_{22}ClN_3O_3$: C, 66.13; H, 5.09; N, 9.64. Found: C, 66.52; H, 5.15; N, 9.67.

2-Chloro-6,7-dimethoxy-4-[3-[2-(morpholin-4-yl) ethoxy]anilino]quinazoline (10e)

Compound **10e** was prepared as described for **10a** from **8e** (0.823 gm, 3.12 mmol) and **3** then the product was recrystallized from ethanol. Yield: 85.7%; m.p. 245–247 °C; IR (ν, cm^{-1}): 3283 (NH) and 1572 (C=N). ^{1}H NMR (DMSO-d_6) δ: 1.91 (t, 4H, **CH_2**-N-**CH_2** of morpholine), 2.73 (t, 2H, PhOCH$_2$**CH_2**N of morpholine), 3.75 (t, 4H, **CH_2**-O-**CH_2** of morpholine), 3.93(s, 3H, O**CH_3**), 3.96 (s, 3H, O**CH_3**), 4.32 (t, 2H, PhO**CH_2**CH_2N of morpholine), 6.81–7.51 (m, 4H, Ar-H), 7.18 (s, 1H, C5-H of quinazoline), 7.93 (s, 1H, C8-H of quinazoline), 9.90 (s, 1H, **NH**, D_2O exchangeable). EIMS, *m/z*: 444 (M^+, 1.2%) 446 (M+2, 1.0%). Anal. Calcd. for $C_{22}H_{25}ClN_4O_4$: C, 59.39; H, 5.66; N, 12.59. Found: C, 59.43; H, 5.75; N, 12.77.

Biological Testing

The human tumor cell lines (MCF-7) were obtained as a gift from NCI, MD, USA. All chemicals and solvents were purchased from Sigma-Aldrich.

Assay for EGFR-TK activity inhibition using K-LISA technique

EGFR TK inhibitory activity was assayed according to reported methods [22,23] using EGFR enzyme kit (Merck KGaA, Darmstadt, Germany). The assay was performed in 96-well plates precoated with a substrate. In each well, 85 µL of 8 µM ATP solution and 100 µL of the standards were added. Then 100 µL of each test compound solution were added at 10 µM concentrations. A positive control for EGFR kinase was used. 100 µl of sample diluents were added to the blank well. After incubation for 1 h at 37 °C on a rotator set at 100 rpm, the plate was washed 3 times with 250–300 µL PBS containing 1% Tween 20 (T-PBS) per well. After the last wash, microwell strips were tapped on absorbent paper towel to remove excess Wash Buffer. The microwell strips were used immediately after washing or placing upside down on a wet absorbent paper for not longer than 15 min. Next, 100 µL HRP-conjugate anti-phosphotyrosine monoclonal antibody was added to all wells. After 1 h of incubation at 37 °C, the plate was washed 3 times as previously. TMB substrate solution (100 µL) diluted in T-PBS containing 5 mg/mL BSA was added to all wells including the blank well. The plate was re-incubated at room temperature (18–25 °C) for 15 min on a rotator set at 100 rpm. Direct exposure to intense light was avoided. The point at which

the substrate reaction should be stopped may be determined by the ELISA reader being used. Finally, the reaction was terminated by the addition of 100 µL of 1 M H_2SO_4 as stop solution, and A_{492} was measured using an ELISA reader. Results should be read immediately after addition of the Stop solution or within 1 h if the microwell strips are stored at 4 °C in the dark. Absorbance of each microwell was measured on a spectrophotometer at 450 nm (620 nm served as the reference wave length; 610 nm to 650 nm is acceptable). The plate reader was blanked using the blank well. The absorbance of the samples and the EGFR Standards were determined. The inhibition rate (%) was calculated using the equation stated that the inhibition % = [1−(A_{492}/A_{492} control)] ×100%.

In vitro anti-proliferative activity

Potential cytotoxic activity on human breast carcinoma cell line (MCF-7) was performed using the Sulfo-Rhodamine-B stain (SRB) assay according to the method of Skehan et al. [24]. Cells were plated in 96-multiwell microtiter plate (104 cells/well) for 24 h before treatment with the compound(s) to allow the attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compound under test (0.1, 2.5, 5, and 10 µM/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the test compounds for 48 h at 37 °C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed, and stained for 30 min with 0.4% (wt/vol) with SRB dissolved in 1% acetic acid. Unbound dye was removed by 4 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 for breast tumor cell line after the specified time.

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Conflict of Interest

The authors report no conflict of interest.

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