6,7-Disubstituted-4-anilinoquinazoline: Design, Synthesis and Anticancer Activity as a Novel Series of Potent Anticancer Agents

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Abstract

Background: Epidermal Growth Factor Receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) are responsible for several pathological conditions such as the development of different kinds of tumors. The combined inhibition of both signal transduction pathways seems to be a promising novel approach for cancer treatment.

Methods: In this study, novel 4-anilinoquinazoline derivatives with various substituents on C-7 position of quinazoline moiety were designed, synthesized, and evaluated for their anti-proliferative activity against A431 and HU02 cell lines.

Results: Compounds 8a, 8d, and 8f displayed the most potent anticancer activities against A431 (IC50 = 1.78 µM, 8.25 µM, and 7.18 µM, respectively) in comparison with reference standards (erlotinib IC50=8.31 µM and vandetanib IC50=10.62 µM). Molecular docking studies proved that 8a as the most potent compound could be efficiently accommodated in the ATP binding site of EGFR and VEGFR-2 through the formation of essential hydrogen bonds between quinazoline N1 atom and the Met796 backbone of EGFR as well as the Cys919 backbone of VEGFR-2 with a distance of 1.94 Å and 1.398 Å, respectively.

Conclusion: Compound 8a as the most potent compound with morpholine and 3-bromoaniline at the 7 and 4 positions of quinazoline scaffold, respectively, deserves more study and structural optimization as an anticancer agent.

Introduction

Most of the cellular functions including proliferation, angiogenesis, differentiation, and migration are regulated by protein kinases and their overexpression plays a critical role in formation of cancer cells.1-3 Abnormal protein kinases signaling, particularly receptor tyrosine kinases (RTKs), which mediates numerous signal transduction pathways may cause both the proliferation of cancer cells and angiogenesis as well as tumor development.4,5 The ErbB family of RTK consists of four distinct members, including the epidermal growth factor receptor (EGFR, ErbB1; HER1 in humans), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4).6,7 Activation of the cytoplasmic tyrosine kinase domains and downstream signaling pathways happen by binding ligands such as growth factors to the extracellular domain of EGFR. It consequently induces dimerization and autophosphorylation.8 The activation of various signal transduction pathways (Jak/STAT, Ras/MAPK, PI3K/Akt) and intracellular processes due to EGFR dysregulation by upactivation, overexpression or mutation can cause tumorigenesis and is usually associated with a poor prognosis in cancerous disorders.1,2 Thus, EGFR can be considered as a useful therapeutic target for developing novel anticancer agents.9 Angiogenesis, through which new blood and lymphatic vessels form from pre-existing vasculature, is a vital process in both normal physiological development as well as continued tumor growth and metastasis-facilitating effect.10 The activation of vascular endothelial growth factor receptor (VEGFR-2) also known as kinase insert domain receptor (KDR), a member VEGFRs family, by vascular endothelial growth factor (VEGF) induces downstream signaling transduction pathways. It can lead to angiogenesis, high vascular permeability, and tumor growth and progression.11-13 Thus, by inhibition of VEGFR-2, the process of angiogenesis and tumor growth can be blocked.14 EGFR and VEGFR-2 are involved in various pathological conditions and the development of several types of cancers. Also, it has been proven that blockade of VEGFR-2 can boost the anticancer effect of EGFR inhibitors. In contrast, activation of VEGFR-2 without any impact on EGFR signaling

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pathway may contribute to EGFR inhibitors-resistance. So, concurrent inhibition of EGFR and VEGFR signaling pathways seems to be a great method of cancer treatment. Among different kinds of synthesized derivatives, quinazoline nucleus, in special 4-anilinoquinazolines, have drawn a lot of interest over the last decade because of their diverse biological activities, particularly as EGFR and VEGFR-2 inhibitors. There are several drugs as EGFR and VEGFR-2 blocking agents that reveal this structure, including erlotinib (Tarceva), gefitinib (Iressa), vandetanib (Caprelsa), lapatinib (Tykerb), icotinib (Commana), and afatinib (Tovok). Some potent drugs inhibiting the EGFR kinase have been designed with alkoxy, especially ethoxy at the C-7 position such as erlotinib, peltinib, and gefitinib. Vandetanib, a dual tyrosine kinase inhibitor, targets both EGFR and VEGFR-2 (Figure 1). In this work, some novel compounds designed and synthesized with various substituted anilines and basic side chains at C-4 and C-7 positions of the quinazoline core, respectively, based on the structures of erlotinib and vandetanib (Figure 2). The cytotoxicity of synthesized compounds was also evaluated against A431 human skin cancer (Epidermoid carcinoma) cells as an EGFR overexpressed cancer cell line as well as Hu02 (Foreskin fibroblast) as a normal cell line using MTT assay. Besides, docking studies were performed by AutoDock software on the crystal structure of EGFR (PDB ID: 1M17) and VEGFR (PDB ID: 2RL5) tyrosine kinase domains.

Materials and Methods

Chemistry

All commercially available solvents and reagents were prepared from Merck and Sigma Aldrich and used without further purification. The A431 human skin cancer (Epidermoid carcinoma) cells and the Hu02 (Foreskin fibroblast) as a normal cell line were purchased from the Iranian Biological Resource Center (IBRC). The melting points were measured using an Electrothermal-9100 melting point apparatus and are uncorrected. The infrared (IR) spectra were obtained on Perkin-Elmer Spectrum Two FT-IR spectrophotometer equipped with the universal attenuated total reflectance (UATR) with a ZnSe–Diamond composite crystal. 1HNMR and 13CNMR spectra were determined in DMSO-d6 on Bruker FT-500 and 400 MHz spectrometers and chemical shifts (δ) are given in parts per million (ppm) by tetramethylsilane (TMS) as an internal standard. Elemental analysis was done by a Perkin Elmer 2400 (automatic elemental analyzer) and results were within ±0.5 of the calculated values. The mass spectra were recorded on an Agilent 5973 mass spectrometer (70 eV). Merck silica gel 60 F254 plates were used for analytical TLC and monitoring the reactions. Column chromatography was performed on silica gel 60 (Merck, particle size 0.06–0.20 mm).

Methyl 4-hydroxy-3-methoxybenzoate (1)

To a solution of vanillic acid (1.68 g, 10 mmol) in dry methanol (15 mL), thionyl chloride (1.09 mL, 15 mmol) was added dropwise at 0 °C, over 10 minutes. After the mixture stirring at room temperature overnight, the solvent and excess thionyl chloride were evaporated under reduced pressure. Then, the residual was eluted by H2O (10 mL) before the mixture was extracted by EtOAc (3×10 mL). The EtOAc layer was dried over anhydrous sodium sulfate (Na2SO4) and concentrated to give I as a white precipitate. Yield: 69%; mp=125-127 °C; IR (KBr, cm−1) νmax: 3537(OH), 1698(C=O), 3025(C-H benzene). 1HNMR (DMSO-d6, 500 MHz): δppm 7.45 (d, J=8.5, 1H, H-C6 phenyl), 7.42(d, J=2 Hz, 1H, H-C2 phenyl), 6.8 (d, J=8 Hz, 1H, H-C5 phenyl), 3.80(s, 3H, OCH3), 3.78 (s, 3H, COOCH3). 13CNMR (DMSO-d6, 125 MHz) δppm 166.5, 152.1, 147.8, 123.8, 120.7, 115.6, 112.8, 56.0, 52.1. MS (ESI): m/z 182.2 [M+H]+.

Methyl 4-(2-chloroethoxy)-3-methoxybenzoate (2)

The mixture of 1 (1.52 g, 10 mmol), potassium

Figure 1. Structures of EGFR tyrosine kinase inhibitors
carbonate (2.76 g, 20 mmol), and a catalytic amount of tetrabutylammonium bromide (TBAB) was refluxed in methanol (20 ml) for 20 min. Then, 1-bromo-2-chloroethane (2.86 g, 20 mmol) was added to the mixture. The obtained mixture was refluxed for 4 hours and cooled to room temperature. The precipitate was filtered, washed with acetonitrile (3×5 ml), and the solvent was evaporated under reduced pressure and dried to give 2.

Yield: 82%; mp=115-117.3 °C; IR (KBr, cm⁻¹) νmax: 1705 (C=O), 1512 (CH₃O).

¹HNMR (DMSO-d₆, 500 MHz): δppm 7.56 (d, J=8.5 Hz, 1H, H-C₆ phenyl), 7.46 (s, 1H, H-C₂ phenyl), 7.09 (d, J=8.5 Hz, 1H, H-C₅ phenyl), 4.31 (t, J=5 Hz, 2H, OCH₂), 3.97 (t, J=5 Hz, 2H, ClCH₂), 3.82 (s, 3H, OCH₃), 3.81 (s, 3H, COOCH₃).

¹³CNMR (DMSO-d₆, 125 MHz) δppm 166.3, 152.0, 148.9, 123.4, 122.8, 112.9, 112.5, 69.1, 56.0, 52.3, 43.2. MS (ESI): m/z 244.2 [M+H]+.

Methyl 4-(2-chloroethoxy)-5-methoxy-2-nitrobenzoate (3)

A mixture of HNO₃ (5 mL, 65%) and H₂SO₄ (5 mL, 98%) was added to a solution of compound 2 (0.61 g, 2.5 mmol) in dry acetonitrile (5 ml) dropwise at 0-5 °C. After this mixture was stirred below 10 °C for about 6 h, it was slowly poured into ice water (5 mL). The organic layer was washed using saturated sodium bicarbonate (2 × 5 mL) and brine (2 × 5 mL) and dried over anhydrous Na₂SO₄. The acetonitrile was concentrated under reduced pressure, and the residue was dried to give 3 as a yellow solid.

Yield: 86%; mp=119-120 °C; IR (KBr, cm⁻¹) νmax: 3473, 3360 (NH₂), 1514 (CH₃O), 1203 (CH₂O). ¹HNMR (DMSO-d₆, 500 MHz): δppm 7.15 (s, 1H, H-C₆ phenyl), 6.37 (s, 1H, H-C₃ phenyl), 4.19 (t, J=5 Hz, 2H, OCH₂), 3.96 (t, J=5 Hz, 2H, ClCH₂), 3.74 (s, 3H, OCH₃), 3.66 (s, 3H, COOCH₃).

¹³CNMR (DMSO-d₆, 125 MHz) δppm 165.8, 152.8, 149.0, 121.4, 111.9, 109.2, 69.8, 57.0, 53.5, 43.0. MS (ESI): m/z 289.1 [M+H]+.

Methyl 4-(2-chloroethoxy)-2-amino-5-methoxybenzoate (4)

A mixture of compound 3 (0.19 g, 0.667 mmol), powdered iron (0.13 g, 2.33 mmol), and NH₄Cl (0.177 g, 3.34 mmol) in MeOH:H₂O (5 mL: 2.5 mL) was refluxed for about 4.5 h. The obtained mixture was filtered while hot; the filter cake was washed with chloroform (2 × 5 mL). The filtrate was extracted with chloroform (3 × 15 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated and the column chromatography was used for purification of the residue using ethyl acetate/hexane as eluent (30:70, v:v) to give 4 as a yellowish-brown compound.

Yield: 68%; mp=108-107 ºC; IR (KBr, cm⁻¹) νmax: 3473, 3360 (NH₂), 1514 (CH₃O), 1203 (CH₂O). ¹HNMR (DMSO-d₆, 500 MHz): δppm 7.15 (s, 1H, H-C₆ phenyl), 6.37 (s, 1H, H-C₃ phenyl), 4.19 (t, J=5 Hz, 2H, OCH₂), 3.96 (t, J=5 Hz, 2H, ClCH₂), 3.74 (s, 3H, OCH₃), 3.66 (s, 3H, COOCH₃).

¹³CNMR (DMSO-d₆, 125 MHz) δppm 165.8, 152.8, 149.0, 121.4, 111.9, 109.1, 69.8, 57.0, 53.5, 43.0. MS (ESI): m/z 259.1 [M+H]+.

7-(2-chloroethoxy)-6-methoxyquinazolin-4(3H)-one (5)

A solution of 4 (0.1 g, 0.33 mmol) and formamidine acetate (57 mg, 0.55 mmol) was dissolved in absolute ethanol (10 ml) and refluxed for 6 h. Then, the mixture was cooled to 0°C, and the precipitated crystals were filtered, washed with cold ethanol, and dried to give 5 as a white solid.

Yield: 81%; mp=252-254 ºC; IR (KBr, cm⁻¹) νmax: 3473, 3360 (NH₂), 1514 (CH₃O), 1203 (CH₂O). ¹HNMR (DMSO-d₆, 500 MHz): δppm 7.15 (s, 1H, H-C₆ phenyl), 6.37 (s, 1H, H-C₃ phenyl), 4.19 (t, J=5 Hz, 2H, OCH₂), 3.96 (t, J=5 Hz, 2H, ClCH₂), 3.74 (s, 3H, OCH₃), 3.66 (s, 3H, COOCH₃).

¹³CNMR (DMSO-d₆, 125 MHz) δppm 165.8, 125.7, 152.0, 149.0, 121.4, 111.9, 109.1, 69.8, 57.0, 53.5, 43.0. MS (ESI): m/z 259.1 [M+H]+.
7-(2-chloroethoxy)-4-chloro-6-methoxyquinazoline (6)

To a solution of 4 (0.136 g, 0.527 mmol) in dichloromethane (2 mL), oxalyl chloride (0.33 g, 2.63 mmol) and dimethyl formamide (DMF) (0.2 mL) were added dropwise and stirred at room temperature for 2 days. Chloroform (10 mL) was added to the mixture and then, it was neutralized with saturated sodium bicarbonate, and dried over anhydrous Na2SO4. The chloroform was evaporated to give the product 6 as a yellow solid.

Yield: 92%; mp= 173.3-174.8 °C; IR (KBr, cm-1): 3229(NH-aniline), 1675(C=O), 1491(CH3C=O) νmax: 1624.8, 1564.8, 1556.8, 1491.7, 1418.5, 1259.7, 1290.2, 1276.7, 1238.4, 1217.2, 1167.5, 1092.8, 1043.7, 1017.3, 959.4, 826.2, 780.4, 747.8, 699.3, 641.2, 593.7, 545.4, 500.2, 451.0, 401.6, 352.0, 302.9, 253.7, 203.6, 154.5, 103.8, 87.7, 69.8, 58.1, 52.7, 43.3. MS (EI): m/z 327.76 [M+H]+.

7-(2-chloroethoxy)-N-(3-bromo-4-fluorophenyl)-6-hydroxyquinazolin-4-amine (7a)

Yield: 94%; mp= 243.4-245 °C; IR (KBr, cm-1) νmax: 3250(NH-aniline), 1491(CH3O), 1280(CH3O), 776(C-Cl). 1HNMR (DMSO-d6, 500 MHz): δ ppm 11.72 (s, 1H, H-N aniline), 8.74 (s, 1H, H-C, quinazoline), 8.49 (s, 1H, H-C, quinazoline), 7.64 (s, 1H, H-C, quinazoline), 7.52 (d, J=5.10 Hz, 1H, H-C, aniline), 7.51 (s, 1H, H-C, aniline), 7.32 (d, J=5.10 Hz, 1H, H-C, aniline), 4.49 (t, J=5.5 Hz, 2H, OCH3), 4.11 (t, J=6 Hz, 2H, CICH3), 4.05 (s, 3H, OCH3), 2.23 (s, 3H, CH3-C, aniline). 13CNMR (DMSO-d6, 125 MHz) δ ppm 158.6, 155.4, 150.6, 149.2, 139.1, 136.0, 131.0, 129.2, 127.6, 124.0, 121.6, 108.2, 104.9, 101.1, 69.8, 57.6, 42.8. MS (EI): m/z 423.1 [M+H]+.

General procedure for the synthesis of 7a-7d

A solution of 6 (0.1 g, 0.36 mmol) and appropriate aniline derivatives (0.73 mmol), including 3-bromoaniline (7a), 4-bromo-2-methylaniline (7b), 3-bromo-4-fluorooaniline (7c), and 3-aminobenzonitrile (7d) in isopropanol (5 mL) was heated to reflux for 3 h. The reaction mixture was cooled to 0°C, and the resulting precipitate was filtered, washed with cold isopropanol (5 mL), and dried to afford 7a-7d products.

7-(2-chloroethoxy)-N-(3-bromophenyl)-6-methoxyquinazolin-4-amine (7a)

Yield: 88%; mp= 243.4-245 °C; IR (KBr, cm-1) νmax: 3250(NH-aniline), 1491(CH3O), 1280(CH3O), 776(C-Cl). 1HNMR (DMSO-d6, 500 MHz): δ ppm 11.68 (s, 1H, H-N aniline), 8.90 (s, 1H, H-C, quinazoline), 8.48 (s, 1H, H-C, quinazoline), 7.06 (s, 1H, H-C, aniline), 7.81 (d, J=10 Hz, 1H, H-C, aniline), 7.52 (t, J=10 Hz, 1H, H-C, aniline), 7.47 (s, 1H, H-C, quinazoline), 7.43 (d, J=7 Hz, 1H, H-C, aniline), 4.48 (t, J=8.4 Hz, 2H, OCH3), 4.12(t, J=8.4 Hz, 2H, CICH3), 4.06 (s, 3H, OCH3). 13CNMR (DMSO-d6, 125 MHz) δ ppm 158.6, 155.4, 150.6, 149.2, 139.1, 136.0, 131.0, 129.2, 127.6, 124.0, 121.6, 108.2, 104.9, 101.1, 69.8, 57.6, 42.8. MS (EI): m/z 408.0 [M+H]+.

7-(2-chloroethoxy)-N-(4-bromo-2-methylphenyl)-6-methoxyquinazolin-4-amine (7b)

Yield: 92%; mp= 173.3-174.8 °C; IR (KBr, cm-1) νmax: 3250(NH-aniline), 1495(CH3O), 1290(CH3O). 1HNMR (DMSO-d6, 500 MHz): δ ppm 11.72 (s, 1H, H-N aniline), 8.74 (s, 1H, H-C, quinazoline), 8.49 (s, 1H, H-C, quinazoline), 7.64 (s, 1H, H-C, quinazoline), 7.52 (d, J=5.10 Hz, 1H, H-C, aniline), 7.51 (s, 1H, H-C, aniline), 7.32 (d, J=5.10 Hz, 1H, H-C, aniline), 4.49 (t, J=5.5 Hz, 2H, OCH3), 4.11 (t, J=6 Hz, 2H, CICH3), 4.05 (s, 3H, OCH3), 2.23 (s, 3H, CH3-C, aniline). 13CNMR (DMSO-d6, 125 MHz) δ ppm 158.6, 155.4, 150.6, 149.3, 138.4, 135.5, 133.6, 130.3, 129.8, 120.8, 107.6, 107.6, 105.1, 101.0, 69.8, 57.5, 42.8, 18.1. MS (EI): m/z 423.1 [M+H]+.
Novel 4-Anilinoquinazoline Derivatives as Potent Anticancer Agents

(1H, H-C\textsubscript{2} aniline), 7.27 (d, J=8 Hz, 1H, H-C\textsubscript{4} aniline), 7.19 (s, 1H, H-C\textsubscript{1} quinazoline), 4.24 (t, J=3 Hz, 2H, OCH\textsubscript{2}), 3.92 (s, 3H, OCH\textsubscript{3}), 3.38 (brs, 4H, H-C\textsubscript{15} morpholine), 2.77 (brs, 2H, NCH\textsubscript{2}), 2.49 (brs, 4H, H-C\textsubscript{24} morpholine). 1\textsuperscript{13}CNMR (DMSO-d\textsubscript{6}, 125 MHz) \delta ppm 160.1, 157.4, 147.8, 141.4, 138.5, 132.1, 130.3, 123.0, 112.1, 109.1, 106.2, 80.9, 79.3, 66.8, 64.6, 54.2, 52.9, 50.5, 21.0. MS (ESI): m/z 472.2 [M+H]\textsuperscript{+}. Anal. Calcd for C\textsubscript{25}H\textsubscript{24}BrN\textsubscript{4}O\textsubscript{3}: C, 55.82; H, 5.32; N, 11.84. Found: C, 55.64; H, 5.33; N, 11.78.

7-(2-(4-methylpiperazin-1-yl)ethoxy)-N-(4-bromo-2-methylphenyl)-6-methoxyquinazolin-4-amine (8e)

Yield: 38%; mp=219-221 °C; IR (KBr, cm\textsuperscript{-1}) vmax 3209 (NH aniline), 1493 (CH\textsubscript{2}), 1205 (CH=O). 1HNR (DMSO-d\textsubscript{6}, 500 MHz): \delta ppm 9.41 (s, 1H, H-N aniline), 8.27 (s, 1H, H-C\textsubscript{1} quinazoline), 8.20 (s, 1H, H-C\textsubscript{1} quinazoline), 7.84 (s, 1H, H-C\textsubscript{1} aniline), 7.53 (s, 1H, H-C\textsubscript{4} aniline), 7.42 (d, J=10 Hz, 1H, H-C\textsubscript{8} aniline), 7.27 (d, J=9.5 Hz, 1H, H-C\textsubscript{2} aniline), 4.23 (brs, 2H, OCH\textsubscript{2}), 3.93 (s, 3H, OCH\textsubscript{3}), 2.77 (brs, 2H, NCH\textsubscript{2}), 2.55 (brs, 4H, H-C\textsubscript{15} piperazine), 2.42 (brs, 4H, H-C\textsubscript{24} piperazine), 2.21 (s, 3H, CH\textsubscript{3} aniline), 2.17 (s, 3H, NCH\textsubscript{2}). 1\textsuperscript{13}CNMR (DMSO-d\textsubscript{6}, 125 MHz) \delta ppm 159.7, 152.7, 151.7, 146.8, 135.6, 128.2, 126.3, 122.8, 119.4, 119.3, 116.0, 110.2, 94.7, 66.9, 54.6, 54.1, 52.1, 52.0, 51.8, 18.4. MS (ESI): m/z 385.0 [M+H]\textsuperscript{+}. Anal. Calcd for C\textsubscript{25}H\textsubscript{24}BrN\textsubscript{4}O\textsubscript{3}: C, 56.79; H, 5.80; N, 14.40. Found: C, 56.60; H, 5.82; N, 14.43.

7-(2-(diethylamino)ethoxy)-N-(3-bromophenyl)-6-methoxyquinazolin-4-amine (8f)

Yield: 38%; mp=219-221 °C; IR (KBr, cm\textsuperscript{-1}) vmax 3415 (NH aniline), 1439 (CH\textsubscript{2}), 1205 (CH=O). 1HNR (DMSO-d\textsubscript{6}, 500 MHz): \delta ppm 9.48 (s, 1H, H-N aniline), 8.26 (s, 1H, H-C\textsubscript{1} quinazoline), 8.24 (s, 1H, H-C\textsubscript{1} quinazoline), 7.88 (s, 1H, H-C\textsubscript{4} aniline), 7.53 (s, 1H, H-C\textsubscript{4} aniline), 7.42 (d, J=11 Hz, 1H, H-C\textsubscript{8} aniline), 7.27 (d, J=9.5 Hz, 1H, H-C\textsubscript{2} aniline), 4.20 (brs, 2H, OCH\textsubscript{2}), 3.93 (s, 2H, OCH\textsubscript{3}), 2.90 (brs, 2H, NCH\textsubscript{2}), 2.63 (d, J=6.5 Hz, 4H, H-CH\textsubscript{2} dieetyleylamine), 2.17 (s, 3H, H-CH\textsubscript{3} C\textsubscript{15} aniline), 1.01 (t, J=7 Hz, 6H, H-CH\textsubscript{2} dieetyleylamine). 1\textsuperscript{13}CNMR (DMSO-d\textsubscript{6}, 125 MHz) \delta ppm 158.0, 153.7, 148.4, 147.4, 143.8, 133.4, 130.2, 129.5, 118.9, 108.4, 104.5, 103.0, 67.6, 56.8, 51.5, 47.6, 18.5, 12.2. MS (ESI): m/z 458.4 [M+H]\textsuperscript{+}. Anal. Calcd for C\textsubscript{25}H\textsubscript{24}BrN\textsubscript{4}O\textsubscript{3}: C, 57.52; H, 5.92; N, 12.20. Found: C, 57.54; H, 5.94; N, 12.16.

7-(2-morphinoethoxy)-N-(3-bromo-4-fluorophenyl)-6-methoxyquinazolin-4-amine (8g)

Yield: 34%; mp=217-220 °C; IR (KBr, cm\textsuperscript{-1}) vmax 3543 (NH aniline), 1499 (CH\textsubscript{2}), 1289 (CH=O). 1HNR (DMSO-d\textsubscript{6}, 500 MHz): \delta ppm 9.77 (s, 1H, H-N aniline), 8.47 (s, 1H, H-C\textsubscript{1} quinazoline), 8.27 (d, J=6 Hz, 1H, H-C\textsubscript{4} quinazoline), 7.94 (s, 1H, H-C\textsubscript{4} aniline), 7.93 (brs, 1H, H-C\textsubscript{4} aniline), 7.39 (t, J=9 Hz, 1H, H-C\textsubscript{2} aniline), 7.23 (s, 1H, H-C\textsubscript{4} quinazoline), 4.24(t, J=5.5 Hz, 2H, OCH\textsubscript{2}), 3.96(s, 3H, OCH\textsubscript{3}), 3.57(brs, 4H, H-C\textsubscript{15} morpholine), 2.88(s, 3H, OCH\textsubscript{3}), 2.61(d, J=8 Hz, 1H, H-C\textsubscript{2} aniline), 1.01(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline), 1.01(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline), 0.94(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline), 0.94(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline), 0.94(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline), 0.94(t, J=8 Hz, 3H, H-C\textsubscript{15} aniline).
Yield: 45%; mp = 219.3-222.1 °C; IR (KBr, cm⁻¹): vmax = 3410 (NH aniline), 1424 (CH₃O), 1282 (CH₂O). ¹HNMR (DMSO-d₆, 500 MHz): δppm 9.60 (s, 1H, H-N aniline), 8.49 (s, 1H, H-C₅ quinazoline), 7.82 (s, 1H, H-C₆ aniline), 7.80 (brs, 1H, H-C₆ aniline), 7.44 (brs, 1H, H-C₄ aniline), 7.22 (s, 1H, H-C₅ quinazoline), 4.23 (brs, 2H, OCH₂morpholine), 2.75 (brs, 2H, NCH₂piperazine), 2.50 (brs, 4H, H-C₄piperazine). ²CNMR (DMSO-d₆, 125 MHz) δppm 156.6, 154.7, 153.98, 149.9, 147.6 (d, J CF₂ = 207.3 Hz), 137.4, 124.0, 122.8 (d, J CF₂ = 23.8 Hz), 119.2, 117.1 (d, J CF₂ = 12.1 Hz), 117.0, 109.4, 108.8, 102.6, 56.2, 56.9, 55.3, 53.6, 46.3. MS (ESI): m/z 491.0 [M+H]+. Anal. Calcld for C₂₃H₂₄BrFNO₅: C, 53.89; H, 5.14; N, 14.28. Found: C, 54.13; H, 5.15; N, 14.21.

Yield: 33%; mp = 232-234 °C; IR (KBr, cm⁻¹): vmax = 3508 (NH aniline), 1500 (CH(O)), 1203 (CH(O)). ¹HNMR (DMSO-d₆, 500 MHz): δppm 9.58 (s, 1H, H-N aniline), 8.50 (s, 1H, H-C₅ quinazoline), 8.21 (s, 1H, H-C₆ quinazoline), 7.87 (brs, 1H, H-C₄ aniline), 7.84 (s, 1H, H-C₆ aniline), 7.42 (t, J = 9 Hz, 1H, H-C₅ aniline), 7.24 (s, 1H, H-C₅ quinazoline), 4.27 (brs, 2H, OCH₂morpholine), 3.96 (s, 3H, OCH₃morpholine), 3.04 (brs, 2H, NCH₂piperazine), 2.75 (brs, 4H, H-C₄piperazine). ²CNMR (DMSO-d₆, 125 MHz) δppm 163.9, 157.9, 156.5, 152.4, 151.3 (d, J CF₂ = 25.7 Hz), 140.0, 137.7, 126.7, 123.5, 122.5, 117.0 (d, J CF₂ = 19.6 Hz), 110.9, 109.9, 91.3, 67.9, 66.9, 56.9, 47.8, 12.30. MS (ESI): m/z 462 [M+H]+. Anal. Calcld for C₂₃H₂₃BrFNO₅: C, 54.44; H, 5.22; N, 12.09. Found: C, 54.66; H, 5.23; N, 12.05.

Yield: 30%; mp = 210-213 °C; IR (KBr, cm⁻¹): vmax = 3459 (NH aniline), 1442 (CH(O)), 1228 (CH(O)), 2228 (CN). ¹HNMR (DMSO-d₆, 500 MHz): δppm 9.90 (s, 1H, H-N aniline), 8.53(s, 1H, H-C₅ quinazoline), 8.42 (s, 1H, H-C₆ quinazoline), 8.21(d, J = 7 Hz, 1H, H-C₆ aniline), 7.95 (s, 1H, H-C₅ aniline), 7.58 (t, J = 7.5 Hz, 1H, H-C₆ aniline), 7.53 (d, J = 7.5 Hz, 1H, H-C₆ aniline), 7.24 (s, 1H, H-C₅ quinazoline), 4.26(t, J = 5 Hz, 2H, OCH₃), 3.98 (s, 3H, OCH₃), 3.58(brs, 4H, H-C₆piperazine), 2.77 (t, J = 5 Hz, 2H, NCH₂piperazine), 2.50 (brs, 4H, H-C₆morpholine). ²CNMR (DMSO-d₆, 125 MHz) δppm 155.9, 153.6, 152.4, 149.3, 147.1, 140.6, 129.4, 126.2, 124.4, 118.8, 111.1, 109.0, 108.0, 108.01, 102.2, 66.3, 61.1, 56.7, 56.4, 53.6. MS (ESI): m/z 405.3 [M+H]+. Anal. Calcld for C₂₅H₂₅N₇O₇C₂: 65.17; H, 5.72; N, 17.27. Found: C, 65.05; H, 5.74; N, 17.32.

Antiproliferation assay
The antiproliferative activities of the synthesized 4-anilinoquinazoline derivatives on A431 (Human carcinoma cell) and HU02 (Foreskin fibroblast) cell lines were evaluated using MTT assay. Briefly, cells were plated in 96-well plates (cell density of 5×10⁴ cells/well) and incubated at 37 °C under a humidified atmosphere with 5% CO₂ for 24h. The cells were treated with etrolitbin and vandetanib as reference standards and test compounds at different concentrations (1, 2, 5, 10, 20, 50, 100, and 200 µM). MTT solution (Sigma; 5mg/ml of PBS) was added to each well, and the cells were incubated for 3h at 37 °C, after 72h of treatment. Then, the supernatant was removed, 150 µl DMSO was added into each well for solubilization. Absorbance was recorded using Biotek Epoch™ microplate reader at 570 nm. All experiments were performed in 5% CO₂ atmosphere.
triplicate. The IC₅₀ value [the concentration needed for 50% inhibition of cell viability] for reference compounds, vandetanib and erlotinib, and each synthesized compound was determined in µM by GraphPad Prism (GraphPad Software, Inc. San Diego, CA).

Molecular docking
Compound 8a was selected as the most potent agent for molecular docking with EGFR and VEGFR2. AutoDock 4.2 and AutoDock Tools 1.5.4 (ADT) was used for docking study. The X-ray crystal structures of the EGFR tyrosine kinase domain in complex with 4-anilinoquinazoline inhibitor erlotinib (PDB ID: 1M17) and the VEGFR2 kinase domain with a 2,3-dihydro-1,4-benzoxazine inhibitor (PDB ID: 2RL5) were downloaded from RCSB Protein Data Bank (http://www.rcsb.org). Ligand and water molecules, except for ones that were necessary for binding with the active site, were removed from crystal structures of receptors followed by adding hydrogens and Kollman charges and merging non-polar hydrogens. Structures of test compounds were sketched, and optimization of their molecular geometries was done by molecular mechanics MM+ and then semi-empirical AM1 methods by HyperChem 8.0 software. Grid box dimensions were set to x=90, y=90, z=90, and grid spacing of 0.375 Å. The Lamarckian genetic search algorithm was utilized for conformational search with 100 GA runs. Co-crystallized ligands were used for validation of the docking procedure based on the method as mentioned above.

Results and Discussion
The synthetic pathway was depicted in Figure 3. Vanillic acid was converted to its methyl ester (intermediate 1) using thionyl chloride and methanol. The reaction of 1 with 1-bromo-2-chloroethane in the presence of potassium carbonate and TBAB gave 2. Sulfuric acid and nitric acid were used for the nitrification of 2 to afford compound 3. The reduction of intermediate 3 to 4 was performed using powdered iron and ammonium chloride and followed by the ring-closure reaction in the presence of formamidine acetate and ethanol to achieve quinazoline skeleton (Compound 5).19,20 Intermediate 5 was chlorinated using oxalyl chloride to yield 6 which was then coupled with appropriate aniline groups to afford the desired intermediate 7a-7d.20,21 Finally, the desired compounds (8a-8l) were generated through the reaction of 7a-7d and various secondary amines using anhydrous potassium iodide.19

Cytotoxic activities of compounds were tested against A431 (human carcinoma cell) as an EGFR overexpressed cancer cell line as well as HU02 (Foreskin fibroblast) as a normal cell line by MTT assay.22-24 As is presented in Table 1, most of the final compounds showed significant anti-proliferative activities on A431 (1.78 µM - >100 µM).

Figure 3. Synthetic route for the synthesis of the target compounds 8a–8l. Reagents and conditions: (a) SOCl₂, MeOH, Reflux; (b) 1-Bromo-2-chloroethane, TBAB, K₂CO₃, Reflux; (c) H₂SO₄, HNO₃, 0–5 °C; (d) Fe, NH₄Cl, MeOH/H₂O, Reflux; (e) formamidine acetate, Ethanol, Reflux; (f) Oxalyl chloride, DMF, Dichloromethane, rt; (g) Aniline Derivatives, i-PrOH, Reflux; (h) KI, DMF, Secondary amine, Reflux.
Table 1. Cytotoxic activity (IC\textsubscript{50}, µM) of synthesized compounds \textit{8a-l} on A431 and HU02 cell lines.

| Compounds | R\textsuperscript{1} | R\textsuperscript{2} | A431  | HU02  |
|------------|---------------------|----------------------|-------|-------|
| 8a         | ![Structure](structure8a.png) | ![Structure](structure8a.png) | 1.78±0.47 | >100  |
| 8b         | ![Structure](structure8b.png) | ![Structure](structure8b.png) | 14.3±3.43 | >100  |
| 8c         | ![Structure](structure8c.png) | ![Structure](structure8c.png) | >100  | >100  |
| 8d         | ![Structure](structure8d.png) | ![Structure](structure8d.png) | 8.25±3.56 | >100  |
| 8e         | ![Structure](structure8e.png) | ![Structure](structure8e.png) | 21.7±4.62 | >100  |
| 8f         | ![Structure](structure8f.png) | ![Structure](structure8f.png) | 7.18±2.15 | >100  |
| 8g         | ![Structure](structure8g.png) | ![Structure](structure8g.png) | 61.97±8.92 | >100  |
| 8h         | ![Structure](structure8h.png) | ![Structure](structure8h.png) | 10.3±3.22 | >100  |
| 8i         | ![Structure](structure8i.png) | ![Structure](structure8i.png) | 12.94±3.21 | >100  |
| 8j         | ![Structure](structure8j.png) | ![Structure](structure8j.png) | 46.70±5.32 | >100  |
| 8k         | ![Structure](structure8k.png) | ![Structure](structure8k.png) | 36.69±4.73 | >100  |
| 8l         | ![Structure](structure8l.png) | ![Structure](structure8l.png) | >100  | >100  |

| Erlotinib hydrochloride | 8.31±1.96 | 72.61±4.75 |
| Vandetanib             | 10.62±2.54 | >100       |
Compound 8a bearing 3-bromoaniline at the 4-position and morpholine at the 7-position of quinazoline core demonstrated the best cytotoxicity on A431 (IC_{50}=1.78 \mu M) which was better than the reference drugs. Also, 8d and 8f were two other potent compounds which showed cytotoxicity more than reference standards (7.18 and 8.25 \mu M, respectively) with 4-bromo-2-methylaniline at C-4 position as well as morpholine and diethylamine at C-7 position of quinazoline scaffold, respectively. Likewise, compounds 8h (IC_{50}=10.3 \mu M) and 8i (IC_{50}=10.3 \mu M) bearing 3-bromo-4-flouroaniline at the 4-position along with N-methylpiperazine and dimethyl amine at the 7-position of quinazoline skeleton exhibited good potency against A431. Introduction of 3-bromoaniline and N-methylpiperazine at 4 and 7 positions of quinazoline skeleton in compound 8b led to significant antitumor activity (IC_{50}=14.3 \mu M). Compounds with 3-aminobenzonitrile at 4 position (8j-8l) resulted in loss of inhibitory activities on A431. Other compounds displayed moderate to weak cellular anti-proliferative activities against A431. In order to investigate the nonspecific cytotoxic activity, the same assay was performed on the HU02 (Foreskin fibroblast) as a normal cell line. Surprisingly, these compounds showed no significant effects on the proliferation of HU02 cells. The in vitro insensitivity of this cell line to synthesized agents was most closely related to the normal EGFR expression.

The structure-activity relationship (SAR) analysis showed that for morpholine (8a, 8d, 8g, and 8j) and N-methylpiperazine (8b, 8e, 8h, and 8k) substituted derivatives, the introduction of electron-donating group (–CH₃) at position-2 of the aniline ring in addition to moving bromo substituent from the meta site to the para position showed a moderate decrease in cytotoxicity. Also, the substitution of 3-bromine at the position-3 aniline ring with nitrile moiety as a strong electron-withdrawing substituent group (8j and 8k) led to a drastically reduced anticancer activity. In morpholine derivatives, the introduction of fluorine at the para position of the aniline ring (8g) led to a significant decrease in anticancer activity. In contrast, analysis of N-methylpiperazone derivatives proved that the presence of fluorine as H-bond acceptor group at the para position of aniline ring (8h) interestingly enhanced the anticancer activity. In compounds with diethylamine group, it seems that 2, 4-disubstituted (8f) and 3, 4-disubstituted (8i) aniline groups were more potent anticancer agents than 3-monomosubstituted ones (8c and 8i). Moreover, In order to study the interaction between the target compound 8a (As the most potent compound) and vandetanib, molecular docking studies were conducted using the AutoDock 4.2 and AutoDock Tools 1.5.4 (ADT). As is presented in Figure 4, N1 of the quinazoline formed one hydrogen bond with Met769 in the ATP binding site of EGFR (PDB: 1M17) for both compounds with the distances of 1.94 Å (8a) and 1.638 Å (vandetanib) and bond angle values of 162.5º (8a) and 176.9º (vandetanib). Besides, aniline rings of 8a and vandetanib coplanarly interacted with amino acids, including Lys-721, Val-702, and Ala-719 into EGFR's hydrophobic pocket. Also docking study was done for compound 8a and vandetanib into inactive DFG-out conformation of VEGFR-2 (PDB: 2RL5) (Figure 5). 8a and vandetanib could be fitted to the DFG-out conformation of VEGFR-2 impeccably. A hydrogen bond was formed between quinazoline N1 and the backbone amide NH of Cys919 in the hinge region (8a: 1.367 Å and 144.5º, vandetanib: 2.127 Å and 138.9º). The quinazoline core is located in a hydrophobic pocket composed of Leu840, Ala866, Phe918, and Leu1035. The oxygen of morpholine formed a hydrogen bond interaction with the Lys868 side chain.

Figure 4. Docking interaction of compound 8a and vandetanib with EGFR.
Conclusion
A series of 4-anilinoquinazoline derivatives containing diethylamine, morpholine, and N-methylpiperazine have been designed and synthesized potent anticancer agents. Most of the tested compounds exhibited good antiproliferative activities. The most potent ones were 8a, 8d, and 8f which showed better cytotoxic activity than both standards. Molecular docking proved the interaction of 8a with the ATP binding site of EGFR and inactive DFG-out conformation of VEGFR-2. All compounds did not show any significant activities on HU02 as the normal cell line. So, 8a, 8d, and 8f can be an appropriate ground for further structure optimization and evaluation of biological activities for developing novel potent cancer therapeutic agents.

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Ethical Issues
This study was approved by the Ethical Committee of Guilan University of Medical Sciences (ID: IR. GUMS. REC. 1398. 219, Date: 3.August.2019).

Author Contributions
FA: Synthesis of compounds, ME: MTT assay and cytotoxicity evaluation, HK: spectroscopic data interpretation, SG: design of the work, conducting experimental procedures, data interpretation, drafting the manuscript. All authors read and gave approval of the final manuscript.

Conflict of Interest
The authors confirm that this article content has no conflicts of interest.

Supplementary Data
Supporting information contains IR, NMR, and mass spectra which is available on the journal’s web site along with the published article.

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Figure 5. Docking interaction of compound 8a and vandetanib with VEGFR.
Novel 4-Anilinoquinazoline Derivatives as Potent Anticancer Agents

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