# Synthesis and biological evaluation of 2,4-diaminoquinazolines as potential antitumor agents

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**Abstract:** In the present study, a set of 2-anilino-4-alkylaminoquinazoline derivatives was synthesized and tested for their antitumor activities *in vitro* against a panel of four human cancer cell lines and for their DNA-binding affinity. Among the synthesized compounds, **4c** and **5b** with 4-substitution at the phenyl ring were found to have the higher inhibitory effect against MCF-7 (breast adenocarcinoma), HCT-116 (colon cancer), HePG-2 (hepatocellular carcinoma), and HFB4 (human skin cancer). Further investigation revealed that compounds **4a** and **5d** exhibited better affinity to bind with DNA than other tested compounds.
[Mohammad A. Alassaf, Khalid B. Selim, Magda N. A. Nasr. **Synthesis and biological evaluation of 2,4-diaminoquinazolines as potential antitumor agents.** *Biomedicine and Nursing* 2025;11(1):11-17]. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 03. doi:10.7537/marsbnj110125.03

**Key words:** synthesis; anticancer; quinazolines; DNA-binding.

## 1. Introduction

Although development of novel targeted antitumor drugs has obtained important progress in recent years, cancer remains the major leading cause of death in the world due to drug resistance or undesirable toxic effects.[1] Therefore, the continued commitment to the laborious task of discovering new anticancer agents remains critically important.[2-4]

In the course of finding new bioactive molecules that may serve as potent antitumor agents, quinazoline derivatives are of particular interest.[5] These quinazolines have been identified as new class of cancer chemotherapeutic agents against solid tumors and exert their antitumor activity through the inhibition of the receptor tyrosine kinases (RTKs)[6], specifically epidermal growth factor receptor (EGFR), such as PD153035[7] and Gefitinib[8] or dihydrofolate reductase,[9] such as trimetrexate (TMQ)[10] (Fig.1). Moreover, the anticancer activity of quinazoline-based drugs is related to their DNA-binding affinity to show inhibition of topoisomerases, such as luotonin A[11] or batracyclin[12], or inhibition of telomerase such as SYUIQ-05[13] (Fig.1), leading to cell death by inhibition of replicative enzymes and DNA repair systems.

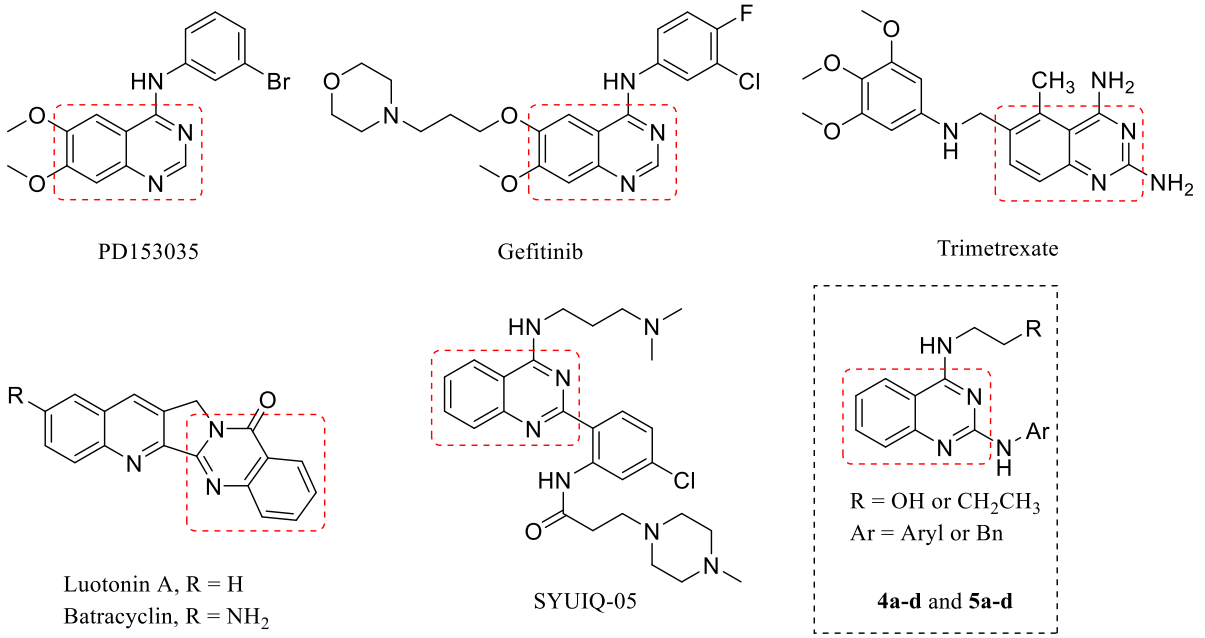


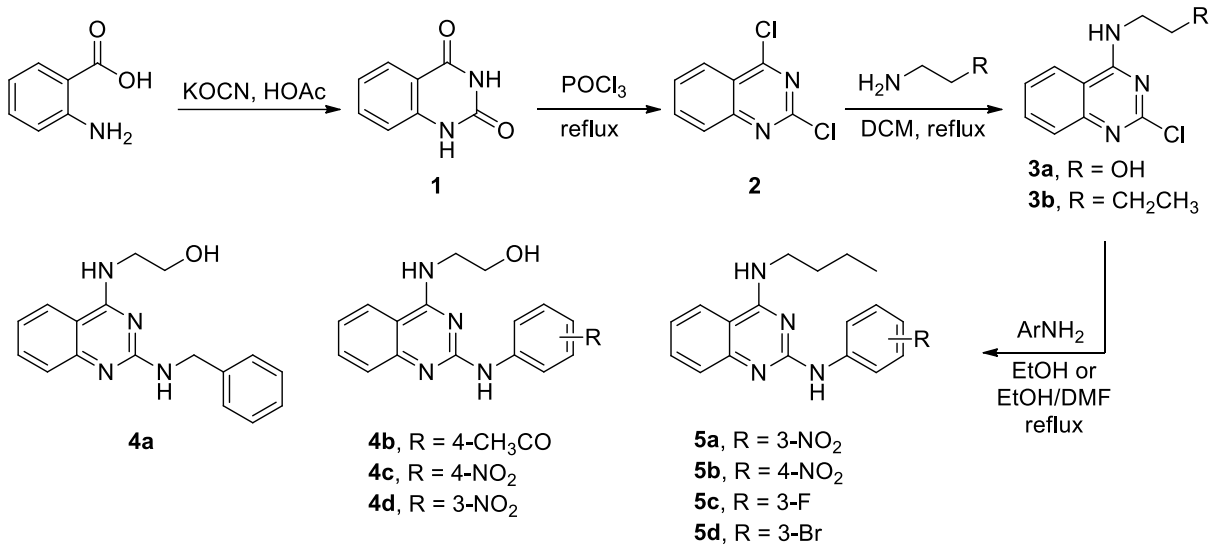

**Fig. 1.** Recently reported anticancer agents with quinazoline moiety and target compounds.

In the current study, we have synthesized a series of 2,4-disubstituted quinazolines **4a-d** and **5a-d** (Fig. 1) contains a simple alkyl amino side chain at position 4 instead of the diaminopropyl moiety of SYUIQ-05.[13] In addition, an aryl amino moiety was introduced at position 2 of the quinazoline ring instead of the substituted aryl fragment. This can significantly increase the binding ability of these compounds with the relevant desired receptors such as EGFR tyrosine kinase or DNA through formation of hydrogen bonding. An evaluation of their antiproliferative activity was carried out using four human tumor cell lines, in addition to their DNA-binding affinity.

## 2. Results and discussion

### 2.1. Chemistry

2-Arylamino-4-alkylaminoquinazoline derivatives **4a-d** and **5a-d** were prepared as shown in Scheme 1. The starting material 2-aminobenzoic acid conveniently cyclized to quinazolidine-2,4-dione (**1**) by condensation with potassium cyanate as described in the literature.[14] Compound **1** yielded the corresponding 2,4-dichloroquinazoline (**2**) upon refluxing with phosphorous oxychloride.[15] Selective substitution of the chlorine at the 4-position with ethanolamine and *n*-butylamine in dichloromethane under reflux gave the corresponding 4-alkylamino-2-chloro-quinazoline **3a** and **3b**,[16] respectively. Finally, compounds **3a** and **3b** were treated with the appropriate amines in ethanol (for **4a-d**) or in a mixture of ethanol and dimethylformamide (for **5a-d**) to give the target quinazolines.




**Scheme 1.** Synthesis of new 2,4-diaminoquinazoline derivatives as anticancers.

### 2.2. Biological evaluation

#### 2.2.1. In vitro anticancer screening

The newly synthesized compounds **4a-d** and **5a-d** were tested for their potencies for inhibition of MCF-7 (breast adenocarcinoma), HCT-116 (colon cancer), HePG-2 (hepatocellular carcinoma), and HFB4 (human skin cancer) by applying the MTT colorimetric assay. 5-Fluorouracil (5-FU) was chosen as a reference cytotoxic agent. Compounds were tested over a range of concentrations from 1.56 to 100 μg/ml, and the calculated $IC_{50}$ values, that is, the concentration (μg/mL) of a compound that was able to cause 50% growth inhibition with respect to the control culture, were reported differently according to different cancer cells.

As shown in Table 1, the results of *in vitro* antitumor activity of the tested compounds indicated that compound **5b** (with 4-$NO_2$ substitution) exhibited the highest cytotoxic activity against MCF-7, HCT-116, HePG-2 and HFB4 with $IC_{50}$ ranging from 9.1 to 10.9 μM. Moreover, compound **4c** (with 4-$NO_2$ substitution) showed similar activity against the four cancer cell lines with $IC_{50}$ ranging from 10.8 to 12.0 μM. Replacement of the 4-$NO_2$ substituent in **4c** and **5b** with 3-$NO_2$, F, or Br resulted in compounds **4d**, **5c**, or **5d**, all of which exhibited decrease in the activity against the four cell lines. These results indicate that the 4-$NO_2$ substitution of the phenyl ring connected to *N2* position is more favorable for the anticancer activity than 3-substitution.

Table 1. Cytotoxic activity ($IC_{50}$, μg/ml) of 2,4-diaminoquinazoline derivatives against human cancer cells lines.

| Compounds | MCF7 | HCT 116 | HePG2 | HFB4 |
|---|---|---|---|---|
| 5-FU | 5.4±0.20 | 5.3±0.18 | 7.9±0.21 | 8.8±0.52 |
| **4a** | 34.4±3.41 | 41.4±3.62 | 48.9±3.60 | 31.8±3.15 |
| **4b** | 12.7±1.32 | 36.9±3.04 | 40.5±3.51 | 20.4±1.98 |
| **4c** | **11.1±1.10** | **10.8±0.86** | **12.0±0.97** | **10.8±1.24** |
| **4d** | 13.6±1.65 | 19.4±1.50 | 18.0±1.72 | 13.7±1.20 |
| **5a** | 27.4±1.97 | 32.8±2.89 | 31.2±3.43 | 30.1±3.36 |
| **5b** | **10.9±1.03** | **9.1±0.85** | **10.4±1.11** | **9.9±1.07** |
| **5c** | 16.3±1.56 | 17.4±1.32 | 26.2±2.32 | 15.7±1.61 |
| **5d** | 71.8±4.74 | 81.4±5.37 | 88.9±6.41 | 66.8±4.19 |

[a]$IC_{50}$: The concentration that cause 50% of cell proliferation inhibition. Data are expressed as means ± SD of at least three separate experiments.

**2.2.1. In vitro anticancer screening**

Some anticancer agents block DNA synthesis, leading to the cell cycle arrest. We thus sought to determine if these compounds would have an impact on the cell cycle progression through measuring the affinity of these compounds to bind to DNA. DNA-binding colorimetric assay was applied for determination of the interaction of the small synthesized molecules with DNA. The results showed that all the tested compounds have moderate affinity to DNA, which was demonstrated by decreasing in the absorbance of the DNA/methyl green. Compounds **4a** and **5d** exhibited the best DNA-binding affinity among other compounds.

Table 2. DNA/methyl green colorimetric assay of the DNA-binding compounds.

| DNA-active compounds | DNA/methyl green ($IC_{50}$, μg/ml)[a] |
|---|---|
| **4a** | **64±4** |
| **4b** | 75±5 |
| **4d** | 81±5 |
| **5a** | 79±4 |
| **5c** | 70±4 |
| **5d** | **61±3** |

[a]$IC_{50}$ values represent the concentration (mean ± SD, n = 3-5 separate determinations) required for a 50% decrease in the initial absorbance of the DNA/methyl green solution.

## 3. Conclusion

A new series of 2,4-diaminoquinazoline compounds **4a–d** and **5a–d** were synthesized and tested against four cell lines belonging to different tumor subpanels. Some of the synthesized compounds **4c** and **5b** showed promising antiproliferative properties against MCF-7, HCT-116, HePG-2, and HFB4 with $IC_{50}$ range of 9.1–12.0 μg/ml, in comparison to the 5-FU with $IC_{50}$ range of 5.3–8.8 μg/ml as reference agent. From the obtained results, we can conclude that the incorporation of 4-$NO_2$ group into the phenylamino moiety improved the antitumor activity which can be considered as valuable leads for future study to obtain more potent antitumor agents.

## 4. Experimental

### 4.1. Chemistry

Melting points (°C uncorrected) were determined on *Fisher-Johns* melting point apparatus. Column chromatography was performed using 40–60 μm silica gel. NMR spectra were recorded on a Bruker spectrometer (400 MHz for 1H and 100 MHz for 13C) using DMSO or $CDCl_3$ as a solvent, and chemical shifts and coupling constants are presented in parts per million relative to $Me_4Si$ and Hertz, respectively. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet, p, pentet, m, multiplet; br, broad. IR spectroscopy was recorded on Thermo Fisher Scientific Nicolet IS10 spectrophotometer using KBr. The wave numbers of maximum absorption peaks of IR spectroscopy are presented in $cm^{-1}$. All dried solvents were stored over anhydrous $Na_2SO_4$ (EtOH, $CH_2Cl_2$, and DMF). Moisture or air-sensitive reactions were conducted under the protection of $CaCl_2$ tube in oven-dried glassware. Reagents were purchased from commercial suppliers and used without purification. Mass spectra were recorded on Thermo Scientific DSQ II spectrometer. Elemental analysis was performed at the Microanalytical Center, Cairo University, Egypt.

*Synthesis of quinazolidine-2,4-dione* ***(1)*** *and 2,4-dichloroquinazoline* ***(2):***

A suspension of anthranilic acid (0.073 mol) in dist. water (350 ml) was stirred at room temperature, acetic acid (0.094 mol) was added dropwise until (pH= 4–5), the mixture change to greenish-white suspension. An aqueous solution of KCNO (0.015 mol) was added dropwise on the wall of breaker for 30 min and the mixture turns to heavy white suspension. The reaction was treated with an aqueous solution of NaOH (0.35 mol) till pH is 11–12 and left for stirring for 2 days. Conc. $H_2SO_4$ was added to the cooling orange solution to give heavy off-white solids. The solid was filtered, washed with water and dried to get the pure quinazoline-2,4-dione **1** as white solid (m.p = >290 ºC, Yield = 90%).[14] Compound **1** (0.03 mol) and POCl3 (0.85 mol) was refluxed for 13h. A mixture was cooled to room temperature and poured over a mixture of ice and water while stirring. The separated solids were filtered, washed with water and dried to give a yellow solid product **2** with (m.p =121–123 ºC, Yield = 90%).[15]

*Synthesis of 2-[(2-chloroquinazolin-4-yl) amino]ethanol (**3a**):*

To a solution of compound **2** (0.005 mol) in DCM, 2-aminoethanol (0.19 mol) was added and the mixture was refluxed for 30 min. After cooling, the separated solids were filtered, washed with water and dried to get the product **3a** as off-white solid with (m.p = 189-192 ºC, Yield = 88%).[16]

*Synthesis of Compounds **4a–d**:*

*Synthesis of 2-{[2-(benzylamino)quinazolin-4-yl]amino}ethan-1-ol (**4a**):*

To a suspension of compound **3a** (1.43 mmol) in absolute EtOH, phenylmethanamine (1.43 mmol) and trimethylamine (1.43 mmol) was added and the reaction mixture was refluxed for 24 h. The separated solids were filtered, washed with EtOH and purified by column chromatography ($SiO_2$, $CH_2Cl_2$/MeOH = 5:1) to give the desired product **4a** (0.16 mg, 50% yield) as white crystals, m.p = 243–245 ºC. $^1$H NMR (DMSO-$d_6$): δ = 8.57 (s,1 H), 8.35 (d, *J* = 8.0 Hz, 2 H), 7.77 (s, 1 H), 7.48 (s, 1 H), 7.34 (t, *J* = 7.4 Hz, 2 H), 7.25 (d, *J* = 7.0 Hz, 2 H), 4.92 (s, 1 H), 4.68 (s, 2 H), 3.61 (s, 4 H); MS (EI) m/z: 294 (4.9, $M^+$), 295 (2.1, M+1), 296 (1.0, M+2), 261 (39.0), 234 (43.4), 204 (100.0), 163 (38.1), 90 (44.4), 77 (30.9), 60 (13.7); IR (KBr, $cm^{-1}$): 3416 (OH), 3273 (NH), 1745 (C=O), 1635 (C=N), 1588 (C=C). *Synthesis of 1-[4-({4-[(2-hydroxyethyl)amino]quinazolin-2-yl}amino)phenyl]ethan-1-one(**4b**):*

To a suspension of compound **3a** (0.001 mol) in absolute EtOH (15 mL), *4*-aminoacetophenone (0.001 mol) and 2–3 drops acetic acid were added and the reaction mixture was refluxed for 24 h. The separated solids were filtered, washed by EtOH, dried and purified by recrystallization from absolute EtOH to gave the desired product **4b** (0.18 mg, 80% yield) as off-white crystal, mp = 265–268 ºC.[8] $^1$H NMR (DMSO-$d_6$): δ = 8.41 (d, *J* = 8.4 Hz, 1 H), 8.03 (d, *J* = 8.4 Hz, 2 H), 7.86 (t, *J* = 7.6 Hz, 2 H), 7.80 (d, *J* = 8.4 Hz, 1 H), 7.59 (d, *J* = 8.3 Hz, 1 H), 7.51 (t, *J* = 7.7 Hz, 1 H), 3.73 (s, 4 H), 2.58 (s,3 H); $^{13}$C NMR (DMSO-$d_6$): δ = 194.5, 175.1, 156.4, 152.3, 143.7, 133.7, 132.1, 128.1, 125.4, 122.3, 119.7, 112.4, 108.6, 62.2, 34.2, 26.8; MS (EI) m/z: 322 (85.5, $M^+$), 323 (16.4, M+1), 324 (2.2, M+2), 291 (21.9), 279 (19.7), 262 (4.6), 138 (40.0), 119 (25.2), 90 (16.6); IR (KBr, $cm^{-1}$): 3448 (OH), 3241 (NH), 1743 (C=O), 1650 (C=N), 1565 (C=C).

*Synthesis of 2-({2-[(4-nitrophenyl)amino]quinazolin-4-yl}amino)ethan-1-ol (**4c**):*

To a suspension of compound **3a** (2.24 mmol) in a mixture of absolute EtOH and DMF (18 ml, 5:1), *4*-nitroaniline (2.24 mmol) was added and the reaction mixture was refluxed for 24 h. The separated solids were filtered, washed with EtOH and purified by column chromatography ($SiO_2$, $CH_2Cl_2$/MeOH = 5:1) to give the desired product **4c** (0.12 mg, 45% yield) as brown solids, m.p = 140–142 ºC. $^1$H NMR (DMSO-$d_6$): δ 8.31 (d, *J* = 8.2 Hz, 1 H), 7.89 (d, *J* = 7.9, 2 H), 7.80 (t, *J* = 7.6 Hz, 2 H), 7.72 (d, *J* = 8.1 Hz, 1 H), 7.63 (d, *J* = 8.1 Hz, 1 H), 7.50 (t, *J* = 7.8 Hz, 1 H), 4.71 (s, 1 H), 3.59 (s,4 H); MS (EI) m/z: 327 (79.2, M+2), 326 (15.1, M+1), 325 (84.6, $M^+$), 294 (4.4), 280 (37.2), 265 (9.3), 137 (8.5), 90 (49.3), 52 (52.0); IR (KBr, $cm^{-1}$): 3250 (NH), 1737 (C=O), 1605 (C=N), 1540 (C=C), 1497 (N=O).

*Synthesis of 2-({2-[(3-nitrophenyl)amino]quinazolin-4-yl}amino)ethan-1-ol (**4d**):*

To a suspension of compound **3a** (2.24 mmol) in a mixture of absolute EtOH and DMF (18 ml, 5:1), *3*-nitroaniline (2.24 mmol) was added and the reaction mixture was refluxed for 24 h. The separated solids were filtered, washed with EtOH and purified by column chromatography ($SiO_2$, $CH_2Cl_2$/MeOH = 5:1) to give the desired product **4d** (0.24 mg, 48% yield) as brown solids, m.p. = 153–155 ºC. $^1$H NMR data: (DMSO-$d_6$): δ = 8.34 (d, *J* = 8.4 Hz, 1 H), 7.86 (d, *J* = 8.2, 2 H), 7.77 (t, *J* = 7.6 Hz, 2 H), 7.69 (d, *J* = 7.9 Hz, 1 H), 7.61 (d, *J* = 8.1 Hz, 1 H), 7.53 (t, *J* = 8.0 Hz, 1 H), 4.70 (s, 1 H), 3.57 (s,4 H); IR (KBr, $cm^{-1}$): 3195 (NH), 1775 (C=O), 1630 (C=N), 1570 (C=C), 1368 (N=O).

*Synthesis of N-butyl-2-chloroquinazolin-4-amine (**3b**):*

To a solution of compound **3b** (1.5 mmol) in $CH_2Cl_2$ (15 ml), with *n*-buthylamine (6 mmol) was added and the mixture was refluxed for 6 h. After cooling and the solvent was evaporated under reduced pressure give oil and leave it for 24 h, then added to a mixture of ice and water to give off white solid (m.p =109–111 ºC, Yield = 90%).[16]

*Synthesis of compounds N4-butyl-N2-(3-nitrophenyl)quinazoline-2,4-diamine **5a,** N4-butyl-N2-(4-nitrophenyl)quinazoline-2,4-diamine **5b**, N4-butyl-N2-[3-(fluoromethyl)phenyl]quinazoline-2,4-diamine **5c**, N2-(3-bromophenyl)-N4-butylquinazoline -2,4-diamine **5d**:*

To solution of compound **3b (**0.43 mmol) in a mixture of absolute EtOH and DMF (18 ml, 5:1), the appropriate aryl amine (0.43 mmol) was added and the mixture was refluxed for 24 h. The separated solids were filtered, washed with EtOH and the solvent was evaporated under reduced pressure. The crude was purified by column chromatography ($SiO_2$: $CH_2Cl_2$/ MeOH = 10:1) to give the desired products **5a–d**.

*Synthesis of compound N4-butyl-N2-(3-nitrophenyl)quinazoline-2,4-diamine **5a**:*

*3*-Nitroaniline was used to give the desired compound **5a** (0.22 mg, 50% yield) as yellow solids, m.p = 183–185 ºC. $^1$H NMR (DMSO-$d_6$): δ = 10.01 (s, 1 H), 8.42 (s, 1 H), 8.15 (s, 1 H), 7.86 (d, *J* = 8.4 Hz, 2 H), 7.66 (t, *J* = 8.0 Hz, 2 H), 7.50 (d, *J* = 7.9 Hz, 2 H), 7.30 (t, *J* = 8 Hz, 1 H), 5.75 (s, 1 H), 3.57 (s, 2 H), 1.68 (t, *J* = 8.0 Hz, 2 H), 1.42 (t, *J* = 8.0 Hz, 2 H), 0.95 (t, *J* = 8 Hz, 3 H); IR (KBr, $cm^{-1}$): 3422 (NH), 1617 (C=N), 1527, 1489 (C=C), 1341 (N=O).

*Synthesis of compound N4-butyl-N2-(4-nitrophenyl)quinazoline-2,4-diamine **5b**:*

*4*-Nitroaniline was used to give the desired compound **5b** (0.22 mg, 50% yield) as yellow solids, m.p = 175–177 ºC. $^1$H NMR (DMSO-$d_6$): δ = 9.58 (s, 1 H), 8.46 (s, 1 H), 8.01 (s, 1 H), 7.78 (d, *J* = 8.1 Hz, 2 H), 7.70 (t, *J* = 8.0 Hz, 2 H), 7.61 (d, *J* = 7.89 Hz, 2 H), 7.35 (t, *J* = 8 Hz, 1 H), 5.65 (s, 1 H), 3.53 (s, 2 H), 1.65 (t, *J* = 8.0 Hz, 2 H), 1.41 (t, *J* = 8.0 Hz, 2 H), 0.96 (t, *J* = 8.0 Hz, 3 H); $^{13}$C NMR (DMSO-$d_6$): δ = 180.1 , 160.6 , 156.3 , 150.1 , 148.4 , 140.2 , 133.4 , 125.5 , 125.2, 123.4 , 123.2 , 118.1 , 112.4 , 41.1 , 31.1 , 20.2 , 14.3; MS (EI) *m/z*: 339 (2.3, M+2), 338 (18.7, M+1), 337 (100, $M^+$), 308 (39.4), 294 (42.0), 280 (59.4), 200 (16.1), 90 (21.4); IR (KBr, $cm^{-1}$): 3452 (NH), 1624 (C=N), 1538, 1492 (C=C), 1359 (N=O).

*Synthesis of compound N4-butyl-N2-[3-(fluoromethyl)phenyl]quinazoline-2,4-diamine **5c**:*

*3*-Fluoroaniline was used to give the desired compound **5c** (0.22 mg, 74% yield) as off-white solids, mp = 167–169 ºC. $^1$H NMR (DMSO-$d_6$): δ = 10.95 (s, 1 H), 9.95 (s, 1 H), 8.83 (s, 1 H), 8.42 (s, 1 H), 7.88 (d, *J* = 8.4 Hz, 2 H), 7.67 (t, *J* = 8.0 Hz, 2 H), 7.49 (d, *J* = 7.9 Hz, 2 H), 7.45 (t, *J* = 8.0 Hz, 1 H), 3.62 (s, 2 H), 1.66 (t, *J* = 8.0 Hz, 2 H), 1.36 (t, *J* = 8.0 Hz, 2 H), 0.87 (t, *J* = 8.0 Hz, 3 H); IR (KBr, $cm^{-1}$): 3422 (NH), 1610 (C=N), 1586, 1541 (C=C).

*Synthesis of compound N2-(3-bromophenyl)-N4-butylquinazoline -2,4-diamine **5d**:*

*3*-Bromoaniline was used to give the desired compound **5d** (0.19 mg, yield = 64%) as off-white solids, mp = 186–188 ºC. $^1$H NMR (DMSO-$d_6$): δ = 9.75 (s, 2 H), 8.83 (s, 1 H), 8.39 (s, 1 H), 7.77 (d, *J* = 8.4 Hz, 2 H), 7.59 (t, *J* = 8.0 Hz, 2 H), 7.47 (d, *J* = 7.9 Hz, 2 H), 7.36 (t, *J* = 8.0 Hz, 1 H), 3.44 (s, 2 H), 1.57 (t, *J* = 8.0 Hz, 2 H), 1.26 (t, *J* = 8.0 Hz, 2 H), 0.85 (t, *J* = 8.0 Hz, 3 H); MS (EI) m/z: 370 (2.5, M+), 371 (2.1, M+1), 372 (1.4, M+2), 313 (33.1), 304 (78.1), 291 (5.8), 215 (15.4), 200 (22.7), 154 (20.4), 78 (21.6); IR (KBr, $cm^{-1}$): 3432 (NH), 1617 (C=N), 1564, 1495 (C=C).

### 4.2. Biological evaluation

#### 4.2.1. In vitro anticancer screening

The cytotoxicity activity of the synthesized compounds **4a-d, 5a-d** are tested on four human tumor cell lines namely; MCF-7 (breast adenocarcinoma), HCT-116 (colon cancer), HePG-2 (hepatocellular carcinoma), and HFB4 (human skin cancer). The cell lines were purchased from ATCC v*ia* Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. The used reagents are RPMI-1640 medium, MTT, DMSO and 5-fluorouracil (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK). 5-fluorouracil (5-FU) was used as a standard anticancer drug for comparison. The previously mentioned cell lines were used to determine the inhibitory effects of the synthesized compounds on the growth of the tested cell lines using the MTT assay.[17,18] The colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100µg/ml streptomycin at 37 ºC in a 5% $CO_2$ incubator. The cells were seeds in a 96-well plate at a density of (1.0 x 104 cells/well) at 37 ºC for 48 h under 5% $CO_2$. After incubation, the cells were treated with different concentration of compounds and incubated for 24 h, then treated with the tested drug, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800 USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100.

#### 4.2.2. DNA-binding assay

DNA methyl green (20 mg) was suspended in 100 ml of 0.05 M Tris-HCl buffer (pH = 7.5) containing 7.5 mM $MgSO_4$; the mixture was stirred at

37 °C with a magnetic stirrer for 24 h. Test samples (10,100, 1000 mg) were dissolved in ethanol in Ependoff tubes, solvent was removed under vacuum, and 200µl of the DNA/methyl green solution were added to each tube. Samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of the samples was determined at 642.5-645 nm. Readings were corrected for initial absorbance and normalized as the percentage of the untreated standard.[19]

**Acknowledgments:**

The author would like to thank Facutly of Pharmacy, Mansoura university for funding this work and also Dr AbdelBasset A. Farahat, Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Mansoura University for his assistance in doing $^1$H-NMR experiments.

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