Design, synthesis, and cytotoxicity evaluation of new 2,4-disubstituted quinazolines as potential anticancer agents

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ABSTRACT
A series of new 2, 4-disubstituted quinazolines were synthesized by an analog design approach. The synthesis of title compounds (4a–f, 4c–e, 5a–c, and 6a–c) was achieved from the corresponding key intermediates 2-(pyridin-3-yl) quinazolin-4(3H)-one(2a), 2-(pyridin-3-yl) quinazolin-4(3H)-one (2b) and 2-(pyrazin-2-yl)quinazolin-4(3H)-one (2c) with appropriate amines. The synthesized compounds were characterized by the spectral studies. All the synthesized compounds were evaluated for in vitro anticancer activity employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay against human adenocarcinoma (HT-29), breast cancer (MDA-231), and Ehrlich ascites carcinoma cell lines. Among the tested compounds, 5a has a significant anticancer activity (5.33 μM/ml) against the human adenocarcinoma cell line. Other compounds have shown a moderate anticancer activity against the tested cell lines.

INTRODUCTION
Cancer continues to be a major health concern in all over the world outpacing the cardiac diseases. It stands in the first position in mortality rate due to distinct factors. Even though many major advancements have been made in the management of cancer chemotherapy for some patients, there is a need for continued effort in the identification of efficacious novel anticancer agents with minimal side effects (Eckhardt, 2002; El-Azab et al., 2010). The contemporary approaches have been focused mainly on the design of ideal anticancer agents, which eradicate the cancer cells without involving the normal tissues. Inappropriately, none of the reported drugs meet this criterion.

Quinazolines are being an excellent reservoir of bioactive substances and have exhibited a wide spectrum of biological activities, especially antiproliferative (Cao et al., 2015; Govindaraj et al., 2009; Ho et al., 2002; Raffa et al., 2004; Rajveer et al., 2010). The stable nature of the quinazoline nucleus has encouraged the medicinal chemists to bring molecular modifications to this nucleus to synthesize new potential medicinal agents (Ghorab et al., 2016). It is evident from the literature that the quinazoline derivatives are effective as epidermal growth factor receptor (EGFR) inhibitors (Barbosa et al., 2014; Dissoki et al., 2007; Fernandes et al., 2007; Li et al., 2012; Mishani et al., 2004; Zhao et al., 2013; Zhang et al., 2014). The EGFR is a transmembrane glycoprotein, which belongs to the erbB family of closely related cell membrane receptors that include EGFR (erbB-1 or HER1), erbB-2 (HER2), erbB-3 (HER3), and erbB-4 (HER4). The expression and overexpression of EGFR are observed in many human solid tumors including breast, ovarian, non-small cell lung (NSCLC), colorectal, and head and neck cancers. In support of their important role in tumor biology, an EGFR activation may assist in tumor growth by increasing cell proliferation, motility, adhesion, invasive capacity, and blocking apoptosis. Due to their multidimensional role in the progression of cancer, EGFR is emerged as an attractive drug target for anticancer therapy (Seshacharyulu et al., 2012).

Recently, quinazolines were reported as versatile template molecules for the inhibition of a wide range of tyrosine kinases. Among these, EGFR is more widely studied target. Gefitinib, a small-molecule inhibitor from this class, was first to be approved for treating NSCLC refractory before chemotherapeutic intervention (Cohen et al., 2003). In view
of the above observations and continuation of the research program on aiming to synthesize biologically active heterocycles for anticancer potentials (Singh et al., 2013), we report the design, synthesis, and anticancer activity evaluation of new 2, 4-disubstituted quinazolines.

MATERIALS AND METHODS

The synthesized compounds were checked for their melting point by an open glass capillary method and are uncorrected. The KBr pellet technique was adopted to record IR spectra using Shimadzu FT-IR 8400-S spectrophotometer. 1H Nuclear Magnetic Resonance (NMR) and 13C NMR spectra were recorded in AMX-400 NMR spectrophotometer at 400 MHz, in which Dimethyl sulfoxide DMSO-d_6 was used as solvent and tetramethylsilane as an internal standard. The chemical shifts were expressed in δ ppm. Mass spectra were recorded in the Shimadzu LC-MS-2010A instrument by electrospray ionization technique.

Synthesis of 2-phenylquinazolin-4-(3H)-one (2a)

An equimolar quantity of anthranilic acid (0.1 mol) and benzamide (0.1 mol) was taken in a glass mortar, mixed well, and then transferred into a round bottom flask and were heated at 130°C for 5 hours, a solvent-free reaction. The reaction progress was monitored by Thin Layer Chromatography (TLC). Then, the reaction mixture was poured on cold water and stirred. The solid obtained was filtered, washed with sodium bicarbonate solution (10%) to remove unreacted acid, dried, and recrystallized from ethanol to afford the compound (2a). A similar procedure was followed to synthesize 2b and 2c using pyridine-3-carboxamide and pyrazine-2-carboxamide, respectively.

2-phenylquinazolin-4-(3H)-one (2a)

Yield: 58%. mp 136°C–138°C. IR (KBr) cm⁻¹: 1,582 (C=O), 1,680 (C=O), 3,071 (C-H), 3,500 (N-H); 1H NMR (DMSO-d_6, δ ppm): 7.26–8.33 (m, 5H, ArH), 8.1 (s, 1H, NH); 13C NMR (DMSO-d_6, δ ppm): 120.9, 124.2, 126.1 (2), 127.4, 128.7, 128.8, 128.9 (2), 130.2, 133.5, 151.3, 159.0, (C=N), 161.0 (C=O); LC-MS m/z: 222.24 (M⁺).

2-(pyridin-3-yl)quinazolin-4-(3H)-one (2b)

Yield: 69%. mp 142°C–143°C. IR (KBr) cm⁻¹: 1,584 (C=O), 1,681 (C=O), 3,070 (C-H), 3,510 (N-H); 1H NMR (DMSO-d_6, δ ppm): 7.62–8.04 (m, 4H, ArH), 7.67–9.14 (m, 4H, pyridine); 13C NMR (DMSO-d_6, δ ppm): 138.7, 150.0, 152.9, 161.0, 170.0; LC-MS m/z: 224.22 (M⁺).

Synthesis of compounds N-(4-methoxyphenyl)-2-phenylquinazolin-4-amine (3a)

Yield: 51%. mp 177°C–178°C. IR (KBr) cm⁻¹: 1,649 (C=O), 1,483 (C=N), 3,340 (N-H); 1H NMR (DMSO-d_6, δ ppm): 3.83 (s, 3H, OCH₃), 9.86 (s, 1H, NH), 7.12–7.65 (m, 2H, ArH of methoxyphenyl), 7.78–8.26 (m, 4H, quinazoline), 7.56–8.58 (m, 5H, ArH); 13C NMR (DMSO-d_6, δ ppm): 55.9, 115.1 (2), 116.2, 117.3 (2), 120.1, 126.5, 127.5 (2), 128.8, 128.9, 129.3 (2), 130.7, 133.7, 135.4, 150.0, 150.7, 161.0, 170.0; LC-MS m/z: 327.38 (M⁺).

N-(4-methoxyphenyl)-2-(pyridin-3-yl)quinazolin-4-amine (3b)

Yield: 45%. mp 164°C–165°C. IR (KBr) cm⁻¹: 1,649 (C=O), 1,483 (C=N), 3,336 (N-H); 1H NMR (DMSO-d_6, δ ppm): 3.83 (s, 3H, OCH₃), 9.96 (s, 1H, NH), 7.02–7.55 (m, 2H, ArH of methoxyphenyl), 7.58–8.16 (m, 4H, quinazoline), 7.57–9.24 (m, 4H, pyridine); 13C NMR (DMSO-d_6, δ ppm): 55.9, 115.1 (2), 117.3 (2), 116.2, 120.1, 124.0, 126.5, 128.9, 133.0, 133.7, 134.1, 150.7, 148.0, 149.1, 150.0, 150.7, 161.0, 170.0; LC-MS m/z: 329.37 (M⁺).

N-(4-methylphenyl)-2-(pyridin-3-yl)quinazolin-4-amine (3c)

Yield: 57%. mp 159°C–160°C. IR (KBr) cm⁻¹: 1,649 (C=O), 1,275 (C-O), 1,535 (C=N), 3,350 (N-H); 1H NMR (DMSO-d_6, δ ppm): 3.92 (s, 3H, OCH₃), 10.24 (s, 1H, NH), 7.58–7.87 (m, 2H, ArH of methoxyphenyl), 7.68–8.26 (m, 4H, quinazoline), 7.45–7.48 (m, 3H, pyrazine); 13C NMR (DMSO-d_6, δ ppm): 55.9, 115.1 (2), 116.2, 117.3 (2), 120.1, 126.5, 128.9, 133.7, 134.0, 141.2, 142.7, 144.1, 144.5, 150.0, 150.7, 161.0, 170.0; LC-MS m/z: 329.36 (M⁺).

N-(4-fluorophenyl)-2-(pyridin-3-yl)quinazolin-4-amine (3d)

Yield: 51%. mp 194°C–195°C. IR (KBr) cm⁻¹: 1,644 (C=O), 1,276 (C-O), 1,483 (C=N), 3,333 (N-H); 1H NMR (DMSO-d_6, δ ppm): 10.25 (s, 1H, NH), 7.41–7.46 (m, 2H, ArH of fluoro phenyl), 7.68–8.36 (m, 4H, quinazoline), 7.61–8.35 (m, 5H, ArH); 13C NMR (DMSO-d_6, δ ppm): 116.2, 116.3(2), 117.9(2), 120.1, 126.5, 127.5 (2), 128.8, 128.9, 129.3 (2), 130.7, 133.7, 138.7, 150.0, 152.9, 61.0, 170.0; LC-MS m/z: 315.38 (M⁺).

N-(4-fluorophenyl)-2-(pyridin-3-yl)quinazolin-4-amine (3e)

Yield: 57%. mp 176°C–177°C. IR (KBr) cm⁻¹: 1,644 (C=O), 1,276 (C-O), 1,481 (C-N), 3,336 (N-H); 1H NMR (DMSO-d_6, δ ppm): 3.83 (s, 3H, OCH₃), 9.96 (s, 1H, NH), 7.02–7.55 (m, 2H, ArH of methoxyphenyl), 7.58–8.16 (m, 4H, quinazoline), 7.45–7.48 (m, 3H, pyrazine); 13C NMR (DMSO-d_6, δ ppm): 55.9, 115.1 (2), 116.2, 117.3 (2), 120.1, 126.5, 128.9, 133.7, 134.0, 141.2, 142.7, 144.4, 144.5, 150.0, 150.7, 161.0, 170.0; LC-MS m/z: 333.38 (M⁺).
9.96 (s, 1H, NH), 7.36–7.51 (m, 2H, ArH of fluorophenyl), 7.56–8.26 (m, 4H, quinazoline), 8.66–9.44 (m, 3H, pyrazine); 13C NMR (DMSO-d6, δ ppm): 116.2, 116.3 (2), 117.9 (2), 120.1, 126.5, 128.9, 133.7, 138.7, 141.2, 142.7, 144.5, 144.7, 150.0, 152.9, 161.0, 170.0; LC-MS m/z: 317.32 (M+).

4-chloro-N’-(2-phenylquinazolin-4-yl) benzohydrazide (4a)

Yield: 60%. mp 189°C–190°C; IR (KBr) cm⁻¹: 1,644 (C=C), 1,483 (C=N), 3,259 (N-H), 1,640 (C=O); 1H NMR (DMSO-d6, δ ppm): 9.91 (s, 1H, NH), 10.27 (d, 1H, CONH), 7.16–7.87 (m, 2H, ArH of chlorobenzene), 7.56–8.79 (m, 4H, quinazoline), 7.51–8.38 (m, 5H, phenyl); 13C NMR (DMSO-d6, δ ppm): 116.2, 120.1, 126.5, 128.9 (3), 129.0 (2), 132.3, 133.7, 137.7, 148.0, 149.1, 150.0, 161.0, 164.9, 170.0; LC-MS m/z: 343.34 (M+).

2-phenyl-4-(2-phenylhydrazinyl) quinazoline (6a)

Yield: 53%. mp 311°C–312°C; IR (KBr) cm⁻¹: 1,644 (C=C), 1,483 (C=N), 3,259 (N-H); 1H NMR (DMSO-d6, δ ppm): 10.21 (s, 1H, NH), 8.2 (d, 1H, NH), 6.99–7.47 (m, 5H, ArH), 7.58–8.26 (m, 4H, ArH of quinazoline), 7.31–8.38 (m, 5H, phenyl); 13C NMR (DMSO-d6, δ ppm): 116.2, 120.1, 122.8 (2), 126.5, 128.9, 133.7, 140.9, 141.2, 142.7, 144.5, 144.7, 149.8 (2), 150.0, 161.0, 164.9, 170.0; LC-MS m/z: 312.37 (M+).

Cytotoxicity assay

The cell viability of HT-29 (human adenocarcinoma), MDA-231 (breast cancer), and Ehrlich ascites carcinoma (EAC) cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Alley et al., 1988; Singh et al., 2013). The cells were cultured at 3,000 per well in 96-well plates and were incubated for 24 hours in Dulbecco’s Modified Eagle medium contained with 10% fetal bovine serum in 5% CO₂ at 37°C. After, the concentrations of 500, 250, 125, 62.5, 31.25, 16, 8, 4, 2, and 1 µg/ml of compounds were added to the wells and were cultured for 72 hours. Then, the MTT solution was added (10 µl/well) to the above, and the cells were again incubated further at 37°C for 4 hours. Cell lysates were added to the well (100 µl/well) and kept overnight incubation, and the absorbance value at 570 nm was detected employing ELISA reader. Each
concentration was tested in triplicates. The % cytotoxicity was calculated using the following formula.

\[
\text{% cytotoxicity} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100
\]

A graph of % cytotoxicity on the Y-axis and the concentration of test compound on the X-axis was extrapolated for the determination of cytotoxicity (IC\(_{50}\)).

RESULTS AND DISCUSSION

Design strategy

It was proposed to develop 2, 4-disubstituted quinazolines as new structural lead with the hope of achieving potent chemotherapeutic agents in reducing the viability of the cancerous cells. The molecular modifications were made on the quinazoline nucleus primarily focused at the fourth position with the substitution of aryl moiety through amine or amide linkage and at the second position with aryl/heteroaryl substitutions that would improve the lipophilicity (Fig. 1).

Chemistry

The title compounds 2, 4-disubstituted quinazoline derivatives (3a–f, 4a–i, 5a–c, and 6a–c) were conveniently synthesized from the corresponding key intermediates 2-phenylquinazolin-4(3H)-one (2a), 2-(pyridin-3-yl) quinazolin-4(3H)-one (2b) and 2-(pyrazin-2-yl)quinazolin-4(3H)-one (2c) as per synthetic methodology as shown in Figure 2. The intermediates 2a–c were synthesized from the anthranilic acid with an appropriate aryl/heteroaryl amide.

The physical characterization data of the synthesized compounds are shown in Table 1. The structures of all the synthesized compounds were characterized based on IR, \(^1\)HNMR, \(^{13}\)CNMR, and Liquid Chromatography-Mass Spectrometry (LCMS) data. The spectral data are consistent with the proposed structures. The IR spectrum of 2-phenylquinazolin-4(3H)-one (2a) exhibits characteristic carbonyl absorption band at 1,680 cm\(^{-1}\) and N-H at 3,350 cm\(^{-1}\). In the \(^1\)HNMR spectrum of the compound 2a, a singlet at 8.1 ppm integrates for a NH proton of the quinazoline ring, and the aromatic protons resonate as a complex multiplet between 7.26 and 8.33 ppm. These corresponding proton shifts indicate the formation of compound 2a. The IR spectrum of N-(4-methoxyphenyl)-2-(pyrazin-2-yl) quinazolin-4-amine (3a) exhibits characteristic C=N absorption band at 1,535 cm\(^{-1}\) and N-H at 3,350 cm\(^{-1}\). In the \(^1\)HNMR spectrum of the 3a exhibits a singlet at 3.92 ppm integrates for...
Table 1. In vitro cytotoxicity of the synthesized quinazolines.

| Compounds | R       | X    | R1       | Y    | HT-29 IC₅₀ (µM/ml) | EAC IC₅₀ (µM/ml) | MDA-231 IC₅₀ (µM/ml) |
|-----------|---------|------|----------|------|-------------------|----------------|----------------------|
| 3a        | Phenyl  | NH   | 4- OCH₃  | C    | 46.69             | 381.34         | 176.94               |
| 3b        | 3-Pyridyl | NH  | 4- OCH₃  | C    | 81.06             | 459.02         | 233.35               |
| 3c        | Pyrazyl | NH   | 4- OCH₃  | C    | 87.41             | 446.07         | 139.75               |
| 3d        | Phenyl  | NH   | 4-F      | C    | 33.46             | 287.61         | 81.14                |
| 3e        | 3-Pyridyl | NH  | 4-F      | C    | 30.88             | 312.34         | 47.12                |
| 3f        | Pyrazyl | NH   | 4-F      | C    | 285.96            | 307.88         | 282.17               |
| 4a        | Phenyl  | NHINHCO | 4-Cl   | C    | 92.43             | 480.74         | 123.04               |
| 4b        | 3-Pyridyl | NHINHCO | 4-Cl   | C    | 33.49             | 239.62         | 139.01               |
| 4c        | Pyrazyl | NHINHCO | 4-Cl   | C    | 91.75             | 334.04         | 66.43                |
| 5a        | Phenyl  | NHINHCO | H      | N    | 5.33              | 566.15         | 615.83               |
| 5b        | Pyridyl | NHINHCO | H      | N    | 95.20             | 373.85         | 69.53                |
| 5c        | Pyrazyl | NHINHCO | H      | N    | 176.53            | 830.32         | 982.68               |
| 6a        | Phenyl  | NHNHCO | H      | C    | 75.54             | 435.88         | 82.56                |
| 6b        | Pyridyl | NHNHCO | H      | C    | 384.98            | 628.49         | 599.58               |
| 6c        | Pyrazyl | NHNHCO | H      | C    | 746.15            | 607.57         | 598.66               |

5-Fluorouracil: - - - 1.23 1.76 2.92

Cytotoxicity evaluation

The anticancer activity of all the newly synthesized compounds was measured by microculture tetrazolium assay (MTT) assay against HT-29 (human adenocarcinoma), EAC, and MDA-231 (breast cancer) cells involving 5-fluorouracil as a positive control (1.23, 1.76, and 2.92 µM/ml, respectively). The cytotoxicity (IC₅₀) details of the new compounds are shown in Table 1. The cytotoxicity (IC₅₀) was analyzed by linear regression of the concentration-response curves of each compound. The synthesized compounds show better inhibitory activity toward HT-29 and MDA-231 cell lines, especially HT-29 cell lines. Compounds show an inhibitory action on HT-29, MDA-231, and EAC in the range of 5.33–746, 47.12–982.68, and 239.62–830 µM/ml, respectively. From the cytotoxicity activity of 3 series compound against HT-29 cell lines, it is evident that the role of phenyl/pyridyl ring as R substituent and 4-fluoro derivative as Rᵢ is crucial for activity. Compound 3e is the most active in three series, which supports the above data. Compound 4 series had 4-chloro substitution at Rᵢ having lesser activity than three series, except the compound 4c. Hence, chain linker difference might also influence the activity. However, controversy lies in five series that 5a, which had phenyl ring as R and hydrogen at Rᵢ substitution with -NH-NH-C=O linker (same as that of four series), is highly potent among all synthesized compounds which m/z: show IC₅₀ value of 5.33 µM/ml. This may due to some diverse interaction with the receptor pocket. In the case of MDA-231 cell lines, the cytotoxicity of compounds is slightly related to the presence of pyridyl, pyrazyl ring at Rᵢ, and
4F derivative at R1 and –NH as a linker. However, linearity in a relationship is not as much in HT-29 cell lines.

CONCLUSION
In summary, 2, 4-disubstituted quinazolines were designed by substituting linkers (-NH-, -NHNH-, and -NHNHCO-) between the fourth position of the quinazoline moiety and aryl/heteroaryl ring system with the hope of achieving enhanced anticancer profiles. Fifteen 2, 4-designed compounds were synthesized in good yield and were evaluated for in vitro anticancer activity against HT-29, EAC, and MDA-231 cell lines. Among the tested compounds, 5a has shown a significant anticancer activity against HT-29 cell lines. Among the linkers attached, compound 5a has -NHNHCO- group between fourth positions of the quinazoline moiety, and pyridyl ring is emerged as a promising anticancer candidate. Exploring molecular modification and inhibitory activity of compound 5a on epidermal growth factor receptor would give a better insight into its anticancer potential.

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CONFLICT OF INTEREST
The authors declared that they have no conflict of interest.

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