Supplementary data

Design and synthesis of novel quinazolinone-based derivatives as EGFR inhibitors with potential antitumor activity

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This PDF file includes the biological evaluation of anticancer activity by NCI against a panel of 60 cell lines, molecular docking into the active sites of EGFR enzyme, and spectral data (¹H NMR and ¹³C NMR) of the newly synthesized quinazolinone derivatives.

Supplementary data

(Biological evaluation - Molecular docking validation - Chemistry)

<u>1-</u> Experimental

1.1 Biological evaluation

1.1.1. Measurement of anticancer activity against a panel of 60 cell lines.

Anticancer activity screening of the newly synthesized compounds was measured *in vitro* utilizing 60 different human cancer cell lines provided by US NCI according to previously reported standard procedure as follows:

- Cells were seeded into 96-well microtiter plates in a density of 5,000-1,000 cells per 100 μL/well. Cells were then incubated at 37°C, 5% CO₂, 95% air, and 100% relative humidity for 24 h before addition of experimental compounds. After 24 hr, two plates of each cell line were fixed in situ with trichloroacetic acid (TCA), to present a measurement of the cell population for each cell line at the time of compound exposure (Tz).
- Experimental compounds were solubilized in DMSO at 400-fold, the desired final maximum test concentration, and stored frozen before use. At the time of compound addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 µg/mL gentamicin.
- Additional four, 10-fold or $\frac{1}{2}$ log serial dilutions were made to provide a total of five compound concentrations plus control. Aliquots of 100 μ L of these different compound dilutions were added to the appropriate microtiter wells containing 100 μ L of the medium, resulting in the required final compound concentrations.
- Following compound addition, the plates were incubated for an additional 48 hr at 37°C, 5% CO₂, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried.
- Sulforhodamine B (SRB) solution (100 µL) at 0.4% (w/v) in 1% acetic acid was added to each well and plates were incubated for 10 min at room temperature. After staining, the unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. The bound stain was subsequently solubilized with 10 mM Trizma base, and the absorbance was obtained using an automated plate reader at a wave-length of 515 nm. For suspension cells, we used the same methodology except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 µL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of a compound at the five concentration levels (Ti)], the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition is calculated as follows:

 $[(Ti - Tz)/(C - Tz)] \times 100$ for concentrations for which $Ti \ge Tz$,

 $[(Ti - Tz)/Tz] \times 100$ for concentrations for which Ti > Tz

For each experimental agent. Growth inhibition of 50% (GI₅₀) is calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$, which is the compound concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the compound incubation; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

In vitro growth inhibition % (GI %) of the synthesized compounds **3a-g** against a panel of 60 tumor cell lines at 10 µM.

	3 a	3b	3c	3d	3e	3f	3g
Subpanel							
Leukemia							
K-562			24.8				
SR			16.0				
NSC lung cancer							
HOP-92							16.01
NCI-H226			15.23				
Colon cancer							
No inhibition on all colon cell lines							
CNS cancer							
No inhibition on all CNS cancer cell lines							
Melanoma							
No inhibition on all melanoma cell lines							
Ovarian cancer							
No inhibition on all ovarian cancer cell lines							
Renal cancer							
RXF 393				NT			25.11
UO-31			21.64				20.99
Prostate cancer							
No inhibition on all prostate cancer cell lines							
Breast cancer							
No inhibition on all breast cancer cell lines							
Mean inhibition	<0	<0	1.6	<0	<0	<0	<0

-- Growth inhibition % produced by the compound is below 15%.

1.1.2. In vitro cell based EGFR inhibitory assay

In vitro EGFR inhibitory activities of compounds **6a**, **6b**, **6d**, **6f**, **6j**, doxorubicin and erlotinib were evaluated using serial dilutions (1.0, 0.1, 0.01, 0.001 μ M) against EGFR (KDR) Kinase Assay Kit Catalog # 40325 according to manufacturer's instructions. In brief, the master mixture was produced (25 μ L per well) and poured into each well. Each well received 5 μ L of inhibitor solution designated as "Test Inhibitor". The "Positive Control" and "Blank" groups received 5 μ L of the same solution without the inhibitor (Inhibitor buffer). In order to prepare 3 mL of kinase buffer, 600 μ L of kinase buffer were combined with 2400 μ L of water. The blank wells received 20 μ L of kinase buffer. The amount of EGFR required for the test was measured and the enzyme was diluted to 1 ng/ μ L with kinase buffer. 20 μ L of diluted EGFR enzyme was added to the wells designated as "Test Inhibitor Control" and "Positive Control" to start the reaction and the mixtures were incubated at 30 °C for 45 minutes. After the 45 minutes, each well received 50 μ L of Kinase-Glo Max reagent and the plate was incubated at room temperature for 15 minutes. The luminescence was measured with a microplate reader.

1.1.3. Cell cycle analysis

Flow cytometry was used to analyse the cell cycle using ab139418 propidium iodide flow cytometry kit/BD (Abcam, Cambridge, UK), as directed by the manufacturer guidelines. HS 578T cells were treated with compound **6d** at its IC₅₀ concentrations (2.17 μ M) for 24 h. The cells were washed twice with ice-cold phosphate buffer saline (PBS) and collected by centrifugation. The cells were then fixed using ice-cold 66% (v/v) ethanol, washed with PBS, and re-suspended with 0.1 mg/mL RNase to digest cellular RNA and thus minimize stained RNA in the background. The cells were next stained with PI, a fluorescent molecule that may bind to nucleic acid, at a concentration of 40 mg/mL. In cells, PI attaches to DNA in proportion to its amount. Because the DNA content of cells at different stages of the cell cycle differs, the fluorescence intensity can be used to assess the stage of cell growth. FacsCalibur (BD Biosciences, USA) was used to estimate cell fluorescence, which was then examined using Cell-Quest software (Becton Dickinson). Cell cycle analysis of HS 578T cells without any treatment was used as control.

1.1.4. Apoptosis assay

According to the manufacturer's instructions, flow cytometry was used to analyze apoptosis using Annexin V-FITC and propidium iodide double-staining apoptosis detection kit (Biovision, USA). Annexin V is a phosphatidylserine (PS) binding protein with a high affinity. After beginning apoptosis, the latter is a cell membrane component that translocate from the inner face of the plasma membrane to the cell surface. PS can be detected on the cell surface using a fluorescent Annexin V conjugate. HS 578T cells ($5x10^5$) were exposed to compound **6d** at its IC₅₀ concentrations, and then subsequently incubated for 24 hours. After that, the cells were centrifuged and resuspended in 500 mL of binding buffer. Annexin V-FITC and PI double staining was accomplished by mixing 5 µL of Annexin V-FITC with 5 µL of PI. The cells were then incubated for 15 minutes in the dark at room temperature. FacsCalibur was used to assess cell fluorescence after incubation (BD Biosciences, USA). The results were represented using dot-plot graphs.

1.1.5. Caspase-3 enzyme assay

The level of the apoptotic marker caspase-3 was measured using Invitrogen ELISA Kit Catalog # KHO1091. The procedure of the used kit was performed according to the manufacturer's instructions. Let all components to reach room temperature before use. Gently combine all liquid reagents prior to use. Add 100 μ L of the standard diluent buffer to the zero standard wells. Add 100 μ L of standards and controls or diluted samples to the appropriate microtiter wells. Incubate for 2 h at room temperature. Pipette 100 μ L of Caspase-3 (Active) detection antibody solution into each well. Incubate for 1 h at room temperature. Add 100 μ L anti-rabbit IgG HRP working solution to each well. Prepare the working dilution and incubate for 30 minutes at room temperature. Add 100 μ L of stop solution to each well. The liquid in the wells will begin to turn blue. Incubate for 30 minutes at room temperature and in the dark. Add 100 μ L of stop solution to each well. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm. Read the plate within 2 h after adding the stop solution. Use a curve fitting software to generate the standard curve. Read the concentrations for unknown samples and controls from the standard curve.



Figure 1. 3D representation of molecular docking of compounds A) **6a**; B) **6b**; C) **6f**; and D) **6j** in the binding site of EGFR (PDB: 1M17).

3- ¹H NMR and ¹³ C NMR spectra of the new derivatives.

Compound 3a



Compound 3b



Compound 3c



Compound 3d

Amr Motawie_H_ABCCl



Compound 3e





Compound 3f



Compound 3g



Compound 6a



Compound 6b



Compound 6c



110 100 f1 (ppm) -10

Compound 6d



Compound 6e



Compound 6f



Compound 6g



Compound 6h



Compound 6i

Amr Motawie_H_ABphH-NMP



Compound 6j



Compound 6k



Compound 61

