

New pyrimidine-5-carbonitrile derivatives as EGFR inhibitors with anticancer and apoptotic activity: Design, molecular modeling and synthesis

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Biological testing

1. *In vitro* anti-proliferative activity

MTT assay protocol was applied as described previously to assess the anti-proliferative activity of the synthesized compounds. Two human cancer cell lines (HepG2 and A549) were used in this test. At first, the cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) were added at 37°C in a 5% CO₂ incubator. The cell lines were seeded in a 96-well plate at a density of 1.0 x 10⁴ cells / well at 37 °C for 48 h under 5% CO₂. After incubation, the cells were treated with different concentration of the synthesized compounds and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl was added into each well to dissolve the purple formazan formed. The colorimetric assay was 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. The half maximal inhibitory concentration (IC₅₀) values are presented using non-linear regression analysis of the data set from three experiments with three plate wells were used for an individual concentration.

2. *In vitro* EGFR kinase assay

The most active derivatives (**10a, 10b, 13a, 13b, 15a, 15e and 15j**) that exhibited promising antiproliferative activities against HepG2 and A549 cell lines were tested for its inhibitory activity against EGFR. Human EGFR ELISA kit (Enzyme-Linked Immunosorbent Assay) was utilized in this test. At first, specific antibody for EGFR was seeded on a 96-well plate and 100 µL of the standard solution or the tested compound was added, all were incubated at room temperature for 2.5 h. Then washed, 100 µL of the prepared biotin antibody was added, then incubated at room temperature for additional 1 h. and washed. Then, 100 µL of streptavidin solution was added and incubated for 45 min. at room temperature. Washed again, 100 µL of

TMB Substrate reagent was added and incubated for 30 min. at room temperature. 50 μL of the stop solution was added, then read at 450 nm immediately. The standard curve was drawn, concentrations on the X-axis and the absorbance on the Y-axis.

3. Flow cytometry analysis for cell cycle

To determine the role of the synthesized compounds in cell cycle distribution, cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry analysis for compound **10b**. Flow Cytometry Kit for Cell Cycle Analysis (ab139418_Propidium Iodide Flow Cytometry Kit/BD) was used in this test. HepG2 cells were treated with compound **10b** (3.56 μM) for 24 h. Then, the cells were fixed in 70% ethanol at 4 $^{\circ}\text{C}$ for 12 h. After that, the cells were washed with cold PBS, incubated with 100 μl RNase A at 37 $^{\circ}\text{C}$ for 30 min, and stained with 400 μl PI in the dark at room temperature for further 30 min. The stained cells were measured using Epics XL-MCL™ Flow Cytometer (Beckman Coulter), and the data were analyzed using Flowing software (version 2.5.1, Turku Centre for Biotechnology, Turku, Finland).

4. Flow cytometry analysis for apoptosis

Flow cytometry cell apoptosis analysis was used to investigate the apoptotic effect of the synthesized compounds. HepG2 cells were treated with compound **10b** (3.56 μM) for 24 h, collected by trypsin, centrifuged, washed two successive times with PBS, suspended in 500 μl binding buffer, and double stained with 5 μl Annexin V-FITC and 5 μl PI in the dark at room temperature for 15 min. The stained cells were measured using Epics XL-MCL™ Flow Cytometer and analyzed using Flowing software.

5. Statistical analysis

Graph Pad Prism 6 software was used for statistical evaluation of the grouped data. Values are expressed as the mean \pm SEM of the triplicates of each experiment. p value of less than 0.05 was accepted as statistically significant.

Molecular docking studies

MOE.14 software was used in the docking studies, where the binding affinities of target compounds against the EGFR were evaluated. The 3D crystal structure of EGFR was obtained from the Protein Data Bank, <http://www.rcsb.org/pdb> (EGFR, PDB ID: 1M17: 2.05 \AA). At first, water molecules were deleted from the downloaded protein molecule. Energy minimization was performed by applying CHARMM and MMFF94 force fields. The active binding site was

identified and prepared for docking protocol. The 2D structures of erlotinib and the target compounds were drawn using ChemBioDraw Ultra 14.0 and saved in MDL-SD file format. After opening of the SD file and protonation of the 3D structures, energy was minimized. Preparation of the target structures was done through optimization of the parameters. The docking studies were carried out using the protocol in the interface of MOE. A maximum of 10 conformers was considered for each molecule in the docking analysis. Then, the docking scores of the most ideal pose for each of the docked molecules were recorded. The final figures obtained from MOE were visualized using Discovery studio 4.0 software.

Spectral data of final target compounds 10a-h, 14, and 15a-l