4. Experimental*4.1. Chemistry*

All melting points are uncorrected and were taken in open capillary tubes using Electrothermal apparatus 9100. Elemental microanalyses were carried out at Microanalytical Unit, Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt, using Vario Elementar and were found within ±0.4% of the theoretical values. Infrared spectra were recorded using KBr pellets using Perkin Elmer FT-IR 1650 spectrophotometer. 1H- and 13C NMR spectra were recorded at 400 (100) MHz using Jeol ECA 500 MHz Spectrometer and performed at Micro Analytical Laboratory Center, Faculty of Pharmacy, Cairo University, Cairo, Egypt. DMSO‑*d*6 was used as a solvent and chemical shifts were given in parts per million (ppm) relative to TMS as internal standard. Coupling constants were reported in Hertz (Hz). Mass spectra were measured (JEOL JMS-AX 500 Mass Spectrometer) at 70 eV.Elemental analysis, IR and mass spectra were performed at the Central Services laboratory, National Research Centre, Cairo, Egypt.

Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-precoated aluminum sheets (Type 60, F 254, Merck, Darmstadt, Germany) using chloroform/methanol (10:0.8, v/v) and the spots were detected by exposure to UV lamp at λ254 nanometer for few seconds and by iodine vapor. The chemical names given for the prepared compounds are according to the IUPAC system.

*4.2.1*. In vitro *anticancer activity*

MTT assay was used to evaluate the in vitro cytotoxicity of the new compounds against cervix cancer cell lines Hela (Thavamani et al., 2013; Prasetyaningrum et al., 2018). Furthermore, the biologically active benzimidazoles were evaluated for their cytotoxicity against normal cell line WI-38. HeLa (human cervical cancer) cell line and non-tumour WI-38 cell line was obtained from the American Type Culture Collection. MTT assay depends on the reduction of the soluble 3-(4,5- methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a blue purple formazan product, mainly by mitochondrial reductase activity inside the living cells. The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2×104 cells/mL) were plated in 96-well culture plates and incubated at 37 °C in a 5% CO2 incubator. After 12 h, the test sample (2 μL) was added to the cells (2×104) in 96-well plates and cultured at 37◦C for 3 days. The cultured cells were mixed with 20μL of MTT solution and incubated for 4 h at 37 °C. The supernatant was carefully removed from each well and 100 μL of DMSO were added to each well to dissolve the formazan crystals which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by a microplate reader using a test wavelength of 570 nm. The results were expressed as the IC50 (μM), which inducing a 50% inhibition of cell growth of the treated cells when compared to the growth of control cells. Each experiment was performed at least 3 times. There was a good reproducibility between the replicate wells with standard errors.

*4.2.2. Kinase assays*

The activities of the examined compounds against EGFR, HER2, PDGFR-β and VEGFR2 were *in vitro* testedusing abcam’s EGFR Human In cell ELISA Kit (ab 126419), ADP-Glo™ Kinase Assay for Her2, PDGFR-β, Active, Recombinant protein expressed in Sf9 cells for VEGFR2 (KDR) Kinase Assay Kit Catalog # 40325, respectively. The procedure of the used kits was done according to the manufacturer, s instructions.

*4.2.3.* *Cell cycle analysis and apoptosis detection*

Cell cycle analysis and apoptosis investigation were carried out by flow cytometry (He et al., 2009; Cao et al., 2020; Yang et al., 2020). Hela cells were seeded at 8×104 and incubated at 37 °C, 5% CO2 overnight. After treatment with the tested compound, for 24 h, cell pellets were collected and centrifuged (300g, 5 min). For cell cycle analysis, cell pellets were fixed with 70% ethanol on ice for 15 min and collected again. The collected pellets were incubated with propidium iodide (PI) staining solution (50 mg/mL PI, 0.1 mg/Ml RNaseA, 0.05% Triton X-100) at room temperature for 1 hand analyzed by Gallios flow cytometer (Beckman Coulter, Brea, CA, USA). Apoptosis detection was performed by FITC Annexin V/PI commercial kit (Becton Dickenson, Franklin Lakes, NJ, USA) following the manufacture protocol. The samples were analysed by fluorescence-activated cell sorting (FACS) with a Gallios flow cytometer (Beckman Coulter, Brea, CA,USA) within 1 h after staining. Data were analyzed using Kaluzav 1.2 (Beckman Coulter).



**Fig. 1** Analysis of cell cycle and induction of apoptosis of compounds **9** and **13** on Hela cells.

*4.2.4. Molecular docking study*

The HER2 kinase domain complexed with a ligand was downloaded from the protein databank (PDB ID: 3RCD, <http://www.rcsb.org>). Protein preparation wizard of Schrödinger Suite (Sastry et al., 2013, Schrödinger, 2018) was used to correct the protein structural file by correcting bond orders, adding any missing hydrogen atoms, and completing unresolved side chains or loops. Water molecules which are beyond 5 Å of bound ligand and are not involved in at least two hydrogen bonds with non-water residues were removed from the protein complex. The structure was then energetically minimized to remove atomic clashes. The ligand coordinates were designated as the center for docking. Glide receptor grid preparation (Friesner et al., 2006; Halgren et al., 2004; Friesner et al., 2004; Schrödinger, 2018) was used to define and construct the receptor for the docking step. To decrease penalties for close contacts between ligands and protein, 0.85 was used to scale van der Waals radii of nonpolar atoms. The hydroxyl groups of Ser, Thr, or Tyr residues in the active site were identified as rotatable groups to account for all possible hydrogen bonds with incoming ligands. Ligand structures were prepared by LigPrep (Schrödinger, 2018). Standard-precision (SP) docking option was chosen for the docking experiment, and Glide was directed to generate ligand conformations through the docking process.

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**(A) (B)**

**Fig. 2** 2D diagram **(A)** and 3D representation **(B)** of compound **2c** showing its interaction with the HER2 kinase active site.

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**(A) (B)**

**Fig. 3** 2D diagram **(A)** and 3D representation **(B)** of compound **Erlotinib** showing its interaction with the HER2 kinase active site.

**Abbreviations**

|  |  |
| --- | --- |
| **RTK** | receptor tyrosine kinase |
| **EGFR** | epidermal growth factor receptor |
| **HER** | human epidermal growth factor receptor |
| **VEGFR  PDGFR** | vascular endothelial growth factor receptor platelet-derived growth factor receptors |