Synthesis, docking study, and structure activity relationship of novel anti-tumor 1, 2, 4 triazole derivatives incorporating 2-(2, 3- dimethyl aminobenzoic acid) moiety

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Abstract
A series of 1,2,4 triazole derivatives (H7-12) have been synthesized by reacting an excess of hydrazine hydrate with carbothioamide derivatives (H1-6). The final compounds (HB1-HB6) were synthesized by reacting the triazole derivatives with mefenamic acid using DCC as a coupling agent. The chemical structures were confirmed by FT-IR, 1H, and 13C-NMR spectra, and some physicochemical properties were determined. The cytotoxicity of the different compounds (HB1-HB6) was evaluated by the MTT assay against two human epithelial cancer cell lines, A549 lung carcinoma and Hep G2 hepatocyte carcinoma, and one normal human cell line WI-38 lung fibroblasts. The mode of cell killing (apoptosis versus necrosis), as well as the effect on cell cycle phases were evaluated via flow cytometry. Additionally, EGFR tyrosine kinase inhibition assay was performed. The results presented in the current study indicate that the six tested compounds exhibited cytotoxicity against both cancer cell lines, and the lowest IC50 was achieved with compound HB5 against Hep G2 cancer cells which was found to be highly selective against cancer cells. HB5-treated Hep G2 cells were arrested at the S and G2/M cell cycle phases. Compound HB5 caused cell killing via apoptosis rather than necrosis, and this was achieved by inhibiting EGFR tyrosine kinase activity needed for cell proliferation, and cell cycle progression. In silico pre-ADMET studies confirmed all final compounds don't cause CNS side effects, with little liver dysfunction effect.

Keywords
1,2,4 triazole, MTT assay, apoptosis, EGFR tyrosine kinase activity, pre-ADMET

Introduction
Cancer is one of the major global health burdens representing the second major cause of death worldwide (WHO 2020). Cancer is an intricate series of disease states that involves losing control of regular cell growth. For decades, the known protocols for cancer treatment have been either the use of single or combination therapeutics
which include chemotherapy, surgery, and radiation therapy (Debela et al. 2021). Today, several studies have been focusing on developing new therapies with limited side effects as compared to conventional medications (Iacopetta et al. 2020).

Epidermal growth factor has a role in cell growth stimulation and differentiation through binding to its receptor, the epidermal growth factor receptor (EGFR). EGFR is a transmembrane protein belonging to the ErbB family of receptors. The ErbB is a subfamily of tyrosine kinase receptors including EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). EGFR is often upregulated in several cancers such as breast cancer, non-small-cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), and glioblastoma (Sigsmund, Avanzato and Lanzetti 2018a; Arienti et al. 2019; Maenning et al. 2019; Liu et al. 2021). The binding of EGF to the extracellular domain of EGFR causes homodimerization or heterodimerization of ErbB family receptors, which results in phosphorylation of intracellular Tyr1173 and Tyr1068 residues of EGFR. This in turn initiates several signaling pathways such as, phosphoinositide-3-kinase (PI3K)/serine/threonine-specific protein kinase (Akt), RAS/mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and stimulation of transcription (Jak/Stat) pathways. The activation of these pathways mediates cellular proliferation and transformation, which in turn may cause invasion, growth, and cancer metastasis (Gurdal et al. 2019; Belli et al. 2020).

Heterocyclic compounds are interesting structures for medicinal chemists due to their important chemical and biological properties. Although many heterocyclic compounds have been developed, efforts are still ongoing to produce a new heterocyclic ring system with important biological activity (Al-Bayati et al. 2021a). One interesting heterocyclic compound is triazole, which consists of a five-member ring with 3N atoms and the chemical formula C₃H₆N₃. 1,2,4 triazole nucleus has the greater benefit for the p-member ring with 3N atoms and the chemical formula (biological activity (Al-Bayati et al. 2021a). One interesting produce a new heterocyclic ring system with important pounds have been developed, efforts are still ongoing to medicinal chemists due to their important chemical and biological properties. The nitrogen atoms in the triazole moiety play an important role in binding the receptor by forming bonds via H-bond donors or acceptors (Chopra et al. 2018; Kerru et al. 2020; Al-Bayati et al. 2021b). Significant improvement in both pharmacokinetics and pharmacodynamics of ligand-containing triazole rings is expected due to a rise in water solubility owing to the polar characteristics (Al-Bayati et al. 2021b). Many studies have shown that this important pharmacophore nucleus has important role in different types of anti-cancer compounds such as kinase inhibitors, tankyrase inhibitors, methionine aminopeptidase inhibitors, tubulin modulators, aromatase, nucleoside-based anticancer agents, steroid sulfatase inhibitors, and metal complex based anti-cancer agents (Zhang et al. 2020).

The ability of 1,2,4 triazole derivatives to form different types of non-covalent interactions such as hydrogen bonds, van der Waals forces, hydrophobic interactions, and dipole-dipole bonds with various biological targets, is responsible for diverse therapeutic properties including their antibacterial (Grytsai et al. 2020), antimalarial (Chu et al. 2019), antifungal (Peyton et al. 2015), antiviral (El-Sebaey 2020), antitubercular (Shaikh et al. 2019), and anticaner activities (Lakkakula et al. 2019; Krishna et al. 2020). Furthermore, compounds that have triazole moiety could display antioxidant activity either through inhibition of lipid peroxidation or scavenging activity for ROS (reactive oxygen species) (Das et al. 2019).

Additionally, one of the important medications based on triazole nucleus which possess anti-proliferative activity against different types of cancer are the non-steroidal anti-inflammatory drugs (NSAIDs) (Kuntala et al. 2021). Their ability to inhibit cyclooxygenase-2 (COX-2) was proposed to be a mediator for their anti-tumor activity, but higher doses were required. Moreover, studies have indicated that NSAIDs induce apoptosis in many types of cancer cell lines such as colon, stomach, and prostate (Woo et al. 2004; Kazberuk et al. 2020).

In this regard, the objective of this study was to design and synthesize some novel 1, 2, 4-triazole derivatives incorporating 2-(2, 3- dimethyl aminobenzoic acid) moiety. Different electron withdrawal and donating groups at the p-position of benzene moieties were chosen to improve the physicochemical properties and cytotoxicity of the new synthesized compounds.

**Experimental part**

**Material and methods**

The chemical reagents and solvents were used without further purification from Sigma-Aldrich, Milano, Italy and Merck, Taufrichen, Germany. Mefenamic acid was purchased from Pioneer Company (Erbil, Iraq). Infrared spectra were measured using a Shimadzu model 8400s (Nakagyo, Japan) spectrophotometer on disk of KBr, (ν = cm⁻¹). Elemental microanalysis (CHNS) was done using a Euro EA300 elemental analyzer (Carlo Erba, Emmendingen, Germany). The proton (¹H) and carbon (¹³C) NMR spectra were measured using the Inova model Ultra shield at 500, and 125 MHz respectively, δ= ppm was used to express the chemical shift. The solvent used was DMSO_d6.

**Chemical synthesis**

The intermediates and final compounds were synthesized according to Scheme 1.

**Synthesis of methyl ester derivatives: compounds (3 and 4)**

Few drops of conc. H₂SO₄ were added to 10 mmol of benzoic acid (1) or p-nitro benzoic acid (2) in 30 mL MeOH, the mixture was then refluxed for 3h. The product was
formed after neutralization of the reaction mixture with Na₂CO₃. Compound (3) was obtained by extracting the aqueous layer with chloroform, and dried using anhyd. MgSO₄. While compound (4) was filtrated after precipitation and recrystallized with EtOH. The physical properties are mentioned in Table 1.

Methyl benzoate (3): FT-IR(v = cm⁻¹): 1724 (C=O) of ester.

Methyl 4-nitrobenzoate (4): FT-IR: 1731 (ν = cm⁻¹): 3233.16 br. (NH) str. of (CONHNHCSNH); 3171.65 str. of (Ar-CH).; 1690.76 (C=O) str. of amide; 1511.24 and 1453.61 Ar(C=C) str.; 1185.35 br. (Ar-C=S) str. of (C=S); 1029 sym str. (C-O).

2-benzoyl-N-phenylhydrazine-1-carbothioamide (H1): FT-IR(v=cm⁻¹): 1688.34 (C=N) str. of imine; 1384.76 (NH) str. of NO₂, 1376 (C=O) str. of (C=O) of ester.

2-benzoyl-N-(4-chlorophenyl)hydrazine-1-carbothioamide (H2): FT-IR(v=cm⁻¹): 3262.91 br. (NH) str. of (CONHNHCSNH); 1683.56 str. of (C=O) amide; 1181.14 str. of (C=S); 1088.46 str. of (C-Cl).

2-benzoyl-N-(4-methoxyphenyl)hydrazine-1-carbothioamide (H3): FT-IR(v=cm⁻¹): 3263.81 br. str. (NH) of (CONHNHCSNH); 3144.91 str. of (Ar-C=H); 1244 asym str. of (C-O); 1182.55 str. of (C=S); 1027 sym str. of (C-O).

2-(4-nitrobenzoyl)-N-phenylhydrazine-1-carbothioamide (H4): FT-IR(v=cm⁻¹): 3273.2 br. str. (NH) of (CONHNHCSNH); 1866.31 (C-O) str. of amide; 1541.22, 1511.24 and 1453.61 str. of (Ar-C=S); 1182.3 str. of (C=S).

2-(4-nitrobenzoyl)-N-(4-methoxyphenyl)hydrazine-1-carbothioamide (H5): FT-IR(v=cm⁻¹): 3271.55 br. str. (NH) of (CONHNHCSNH); 1690.76 (C=O) str. of amide; 1177.42 str. of (C=S); 1087.56 str. of (C-Cl).

2-(4-nitrobenzoyl)-N-(4-chlorophenyl)hydrazine-1-carbothioamide (H6): FT-IR (v=cm⁻¹): 3271.55 br. str. (NH) of (CONHNHCSNH); 1684.85 (C=O) str. of amide; 1566.35; 1511.64 and 1455.17 Ar(C=C) str.; 1245.22 asym str. of (C-O); 1185.55 str. of (C=S); 1029 sym str. of (C-O).

Synthesis of 1,2,4 triazole derivatives, compounds (H 7-12)

To 5 mmol of compounds (H 1-6) was suspended in 10 mL of MeOH, and 10 mmol of hydrazine hydrate was added. A red mixture was formed and refluxed for 24h at a cold place. The precipitate was washed repeatedly with diethyl ether, and dried overnight at 25 °C (Fattah 2014). The physical properties are mentioned in Table 1.

Table 1. Physical properties of the intermediates and final compounds.

| Compound | Appearance       | Yield (%) | M.p. b.p. °C | R₁  |
|----------|-----------------|-----------|--------------|-----|
| 3        | Oily liquid     | 90        | 199          | 0.8 |
| 4        | Pale-yellow     | 92        | 94-95        | 0.83|
| 5        | White crystal   | 85        | 120-121      | 0.49|
| 6        | Yellow crystal  | 88        | 142-143      | 0.41|
| H1       | White powder    | 78        | 148-149      | 0.35|
| H2       | White powder    | 79        | 166-168      | 0.36|
| H3       | White powder    | 84        | 160-161      | 0.33|
| H4       | Yellow powder   | 72        | 168-169      | 0.33|
| H5       | Yellow powder   | 80        | 180-181      | 0.32|
| H6       | Yellow powder   | 75        | 175-177      | 0.34|
| H7       | Red powder      | 62        | 160-161      | 0.54|
| H8       | Brown powder    | 50        | 178-179      | 0.56|
| H9       | Orange powder   | 70        | 185-186      | 0.53|
| H10      | Brown powder    | 63        | 190-191      | 0.6 |
| H11      | Brown powder    | 64        | 202-203      | 0.62|
| H12      | Orange powder   | 67        | 193-194      | 0.61|
| HB1      | Dark-orange powder | 47    | 206-207      | 0.83|
| HB2      | Dark-brown powder | 52    | 211-212      | 0.85|
| HB3      | Brown powder    | 48        | 218-219      | 0.83|
| HB4      | Brown powder    | 56        | 220-221      | 0.92|
| HB5      | Dark-red powder | 50        | 230-231      | 0.95|
| HB6      | Dark-brown powder | 43     | 225-226      | 0.94|

* Eluent: n-hexane: ethyl acetate (1:1).
str. (NH) of 1° amine; 3304.15 str. (NH) of sec. amine; 1690.21 (C=N) str. of imine; 1542.61 (N-O) asym str. of NO₂, 1379 (N-O) sym str. of (NO₂).

\( \text{N}^3 \) (4-methoxyphenyl)-5-(4-nitrophenyl)-4H-1,2,4-triazole-3,4-diamine (H12): FT-IR(v=cm⁻¹): 3411 and 3362 str. (NH) of 1° amine; 3332.65 str. (NH) of sec. amine; 1683.71 str. (C=N) of imine; 1543.11 (N-O) sym str. of NO₂, 1380 (N-O) asym str. of NO₂, 1380 (N-O) asym str. of NO₂.

### Synthesis of amide derivatives (compounds HB 1-6)

10 mmol of mefenamic acid was dissolved in (5mL dioxan and 10 mL THF) on ice bath, to this solution, 10 mmol of DMAP, and 10 mmol of the corresponding compounds (H7-12) were added, respectively. 10 mmol of N, N-dicyclohexylcarbodiimide (DCC) in 5 mL of dichloromethane CH₂Cl₂ was added. The mixture was stirred at 0 °C for 3 days. The product was filtered to remove DCU, and the solvent was reduced using a rotary evaporator. EtOAc (5mL) was added to the residual, and the mixture was washed with 10% HCl, 5%NaHCO₃, and H₂O, respectively. The organic layer was dried with anhyd. MgSO₄, and left to dry for 24h (Mahdi and Alsaad 2012). The physical properties are mentioned in Table 1.

2-((2,3-dimethylphenyl)amino)-N-(3-phenyl-5-(phenylamino)-4H-1,2,4-triazol-4-y1)benzamide (HB1): FT-IR(v=cm⁻¹): 3303.3 (NH) str; 1680.4 (C=O) str, 1665 (C=N). 1 HNMR(500MHz,DMSO d₆,δ=ppm): 2.1 (s, 3H, CH₃), 2.3 (s, 3H, CH₃), 3.7 (s, 2H, aliph. NH), 6.0–8.2 (m, 17H, Ar.CH), 8.5 (s, 1H, NH-C=O). 13C-NMR(125MHz,DMSO d₆,δ=ppm): 18.9 and 20.2 C (methyl); 115.4, 118.1, 118.8, 121.3, 125.5, 126.1, 126.9, 127.3, 127.7, 128.5, 129.0, 129.4, 130.0, 131.4, 132.5, 133.5, 138.2, 138.8, 139.3, 145.2 C (Aromatic), 155.0 and 157.9 C (Triazole), 180 C (carbonyl). CHN analysis: Calcd. for (C₂₉H₂₆N₆O): MW: 473.22, C: 73.40; H: 5.52; N: 17.71. Observed: C: 73.37; H: 5.53; N: 17.70.

N-(3-((4-chlorophenyl)amino)-5-phenyl-4H-1,2,4-triazol-4-yl)-2-((2,3-dimethylphenyl)amino)benzamide (HB2): FT-IR=v(cm⁻¹): 3320.81 (N-H) str; 1681.61 (C=O) str; 1660.23 (C=N). 1 HNMR(500MHz,DMSO d₆,δ=ppm): 2.4 (s, 3H, CH₃), 2.7 (s, 3H, CH₃), 4.2 (s, 2H, aliph. NH), 6.4–7.9 (m, 16H, Ar.CH), 8.8 (s, 1H, NH-C=O). 13C-NMR(125MHz,DMSO d₆,δ=ppm): 19.2 and 20.3 C (methyl), 115.4, 118.1, 118.8, 121.3, 125.5, 126.3, 126.8, 127.3, 127.7, 128.1, 128.7, 129.0, 130.0, 131.4, 132.5, 133.5, 138.2, 138.8, 139.3, 145.2 C (Aromatic), 155.0 and 157.9 C (Triazole), 180 C (carbonyl). CHN analysis: Calcd. for (C₂₉H₂₅ClN₆O): MW: 509.01, C: 68.43; H: 4.95; Cl: 6.96; N: 16.51. Observed: C: 68.44; H: 4.93; Cl: 6.98; N: 16.52.
2-((2,3-dimethylphenyl)amino)-N-(3-((4-methoxyphenyl)amino)-5-phenyl-4H-1,2,4-triazol-4-yl)benzamide (HB3): FT-IR (v=cm⁻¹): 3326.62 (NH), 2874.99, and 2825.51 (C-H) aliph. str, 1688.22 (C=O); 1564.65 (C=N). HNMR(500MHz, DMSO_d6, δ=ppm): 2.3 (s, 3H, CH3), 2.7 (s, 3H, CH3), 3.5 (s, 3H, O-CH3), 4.1 (s, 2H, aliph. NH), 6.5–8.2 (m, 16H, Ar, CH), 8.4 (s, 1H, NH-C=O). ¹CNMR(125MHz, DMSO_d6, δ=ppm): 19.2 and 20.3 C_(aliph); 62.9 C_(methoxy); 115.1, 115.8, 118.1, 118.7, 120.3, 121.3, 126.1, 126.9, 127.3, 128.5, 129.0, 129.6, 130.1, 131.1, 132.6, 133.7, 138.1, 138.8, 139.3, 145.2 C_(Aromatic); 152.5 C_(Triazole); 120.3, 121.3, 126.1, 126.9, 127.3, 128.5, 129.1, 130.1, and 157.1 C_(Triazole); 181.2 C_(carbonyl). Elemental analysis: Calcd. for (C29H25N7O3): MW: 519.57, C: 145.7, 149.3 C_(Aromatic); 152.0 and 157.0 C_(Triazole), 182.0 C_(carbonyl).

128.5, 129.0, 129.7, 131.4, 132.5, 133.6, 138.2, 138.8, 139.5, 114.2, 118.2, 118.7, 121.9, 124.8, 126.1, 126.7, 127.4, 127.6, 128.5, 129.0, 132.4, 133.6, 138.3, 138.8, 139.3, 145.2 C_(Aromatic); 152.5 and 157.1 C_(Triazole); 181.2 C_(carbonyl). Elementary analysis: Calcd. for (C29H24ClN7O3): MW: 554.01, C: 62.87; H: 4.37; Cl: 6.40; N: 17.70. Observed: C: 62.87; H: 4.34; Cl: 6.42; N: 17.68.

In vitro biological study

Biological study

Method of the docking study

Molecular Operating Environment 19.0901 software was used to predict the binding modes of the designed compounds inside target sites of EGFR tyrosine kinase. The ligand-binding sites were created from a co-crystallized structure within PDB (ID: 1M17) (https://www.rcsb.org).

Initially, water molecules were reduced from the complex. The clean protein options and protein report and utility were used to correct unfilled valence atoms and crystallographic disorders. The protein energy was reduced using MMFF94 force fields, while fixed atom constraint was applied to obtain the protein rigid binding site. 2D structures were generated using ChemBioDraw Ultra16.0 and saved in MDL-SD form. The 3D structures were protonated after opening in MOE, 0.05 RMSD kcal/mol was applied to minimize the energy, and the docking protocol was used for docking the minimized structures. CDOCKER protocol was used to accomplish the process of molecular docking. The ligands were permitted to be flexible while the receptor was held rigid. Thirty conformation poses were used in the placement process, scored by London ΔG, and the best 10 docking scores (ΔG) of fitted poses with the active site at EGFR tyrosine kinase (scored by GBVI/WSA) were used and 3D perspective was done by Discovery Studio 2019 Client software.

These methods were also used to anticipate the binding profile, ideal orientation of each docking pose, affinity, and binding free energy (ΔG) of the prepared compounds with EGFR tyrosine kinase.

In silico ADMET and drug-likeness prediction

In-silico study ADMET along with drug-likeness prediction assist the drug discovery and development process. Here, Pre-ADME online software was utilized for estimating absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiling of the tested compounds. These methods were also used to anticipate the binding profile, ideal orientation of each docking pose, affinity, and binding free energy (ΔG) of the prepared compounds with EGFR tyrosine kinase.

Docking study

The chemical structure of the designed compounds was compared with crystal ligands to choose the molecular targets. A protein data bank (PDB) (http://www.rcsb.org/) was used to select the target site, additionally, the main requirements for binding with essential amino acids in the target site were determined.

Human A549 lung cancer cell line was grown in Ham's F-12K medium (Thermo Scientific, USA) supplemented with fetal bovine serum (10%) (FBS; Gibco, USA), human Hep G2 hepatocyte carcinoma cell line and human WI-38 cell line (lung fibroblasts) were grown in Eagle's minimum
essential medium (LONZA, Switzerland), to which 10% FBS was added. Trypsin-EDTA (Millipore-Merck, USA) was used during subcultures. Growth of cells was established at 37 °C in 5% CO₂ and 95% air.

**In vitro MTT cell proliferation assay**

The MTT assay was used to evaluate the proliferation of control and treated cells (Mosmann 1983). 96-well plates were used throughout the experiment. 30,000 cells were added to each well containing the appropriate media and grown for 24 h. The stock solutions of each of the tested compounds were prepared in DMSO. Eight concentrations (100, 30, 10, 3, 1, 0.3, 0.1, and 0.03 µM) were prepared in the growth media for each compound and added to the cells for 48 h. MTT salt (Freshly prepared; 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (5 mg/ml; Sigma) was then added to each well to obtain 0.5 µg/µL as a final concentration. 200 µL of DMSO and isopropanol mixture 1:1 was added to each well and incubated for 30–45 min. Cell proliferation was detected by measuring the absorbance of each well at 590 nm using Multiskan EX (Thermo Scientific, USA).

**Cell cycle analysis**

Hep G2 cells were treated with the IC₅₀ concentration of drug HB5 (2.87 µM) for 48 h. Apoptosis (early and late)/necrosis induction was detected in control and treated cells by phosphatidylserine translocation to the cell surface using annexin V-FITC apoptosis detection kit (Elabscience Biotechnology, USA).

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

The quantity of BAX, Bcl-2, p53, and caspase 3 mRNA in control and HB5 (at the IC₅₀ concentration)-treated Hep G2 cells were assessed using the manufacturer’s instructions (RNeasy mini kit, Qiagen, Germany). After RNA extraction, cDNA was prepared using the Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, USA). Amplification of target cDNA for apoptosis markers and GAPDH [as a normalization (housekeeping) gene] was done using one-step RT-PCR SYBR Green kit Master Mix (Bio-Rad Laboratories, USA) on Rotor-Gene Q real-time PCR thermal cycler instrument. cDNA (2 µL aliquots) was mixed with forward primer (1 µL), reverse primer (1 µL) (Table 2), master mixture (10 µL) and the reaction volume was completed to (20 µL) with nuclease-free water. All experiments were performed in triplicate.

**Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitory assay**

The in-vitro inhibitory activity of compound HB5 and erlotinib [standard receptor-tyrosine kinase inhibitor (TKI)] against EGFR tyrosine kinase was done using EGFR Kinase Assay Kit (BPS Bioscience, USA). Briefly, EGFR and its substrate were incubated either with HB5 or erlotinib (1000, 300, 100, 30, 10, 3, 1, and 0.3 nM) in the enzymatic buffer at 30 °C for 40 min to initiate the enzymatic reaction. Detection reagent (Kinase-Glo MAX; Promega, USA) was added to terminate the reaction, followed by incubation for 15 min at 25 °C. The

Table 2. The sequence of qRT-PCR primers, forward (F) and reverse (R), used in the current study.

| Gene    | Primer sequence          | Reference                   |
|---------|--------------------------|-----------------------------|
| p53     | F: 5’- GCCCAACAAACACGAGTCTCT -3’ (Zhang et al. 2019) |                          |
| R: 5’- CCTGGGATCTGTTAACGTTCC -3’      |                             |
| BAX     | F: 5’- CCGAGAGGCTTTTTGCCGAG -3’ (Yang et al. 2020) |                          |
| R: 5’- CCAGCCCAACATGATTTGCTGAT -3’    |                             |
| Bcl-2   | F: 5’- TGTGCGCTTCTTGAAGTGGTG -3’ (Khodapasand et al. 2015) |                          |
| R: 5’- GGTGCCGGTTCAGGTACTCAGTCA -3’   |                             |
| Caspase-3| F: 5’- ACAATGGAGGGAATCAATGGACTC -3’ (Peluffo, Young and Stouffer 2005) |                          |
| GAPDH   | F: 5’- GCCACCTTCATGACCTCAAC -3’                      |                             |
| R: 5’- CTCTTCTCACTGTGTGGTAA -3’       |                             |

Table 3. The energy of binding (ΔG) kcal/mol of the candidate compounds (HB1-HB6) against EGFR tyrosine kinase target site PDB ID: 1M17.

| Ligand | RMSD value (Å) | Docking score(ΔG) (kcal/mol) | Interactions | H-bond | pi |
|-------|----------------|-----------------------------|--------------|---------|----|
| HB1   | 1.85           | -7.08                       |              | -       | 7  |
| HB2   | 1.90           | -7.47                       |              | 1       | 8  |
| HB3   | 1.98           | -7.50                       |              | 1       | 6  |
| HB4   | 1.45           | -7.82                       |              | 1       | 6  |
| HB5   | 1.74           | -8.32                       |              | 1       |    |
| HB6   | 1.99           | -7.15                       |              | 1       | 9  |
| Erlotinib | 1.90          | -6.55                       |              | 1       | 6  |
remaining activity of EGFR tyrosine kinase was observed by measuring chemiluminescence using BioTek Synergy2 Microplate Reader (BioTek, USA). The concentration-percent remaining EGFR tyrosine kinase activity curve was used to calculate the concentration that caused 50% kinase activity inhibition (the effective concentration that inhibits 50% of EGFR kinase activity; EC\textsubscript{50}). All samples and controls were verified in triplicate.

**Statistical analysis**

Results are recorded as mean ± SEM. GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The student's t-test was used to determine significance between means, p-value < 0.05 was considered significant.

**Results and discussion**

**Docking study**

The binding form of the reference (Erlotinib) displayed an energy binding of -6.55 kcal/mol against EGFR tyrosine kinase. The quinazoline ring formed pi-Alkyl interactions with amino acids Val702, Leu694, Leu820, Ala719, and one H-bonding with Met769 with a distance of 2.66 Å. The 3-ethynyl phenyl moiety interacted with Lys721 and Leu764 by pi-Alkyl interactions as shown in Fig. 1.

The energy of binding (∆G) of the tested compounds (HB1-6), the number of H-bonds, and pi interactions are displayed in Table 3. The 2D docking of compounds (HB1-6) against EGFR tyrosine kinase is shown in Fig. 2, where H-bonds are represented in green lines and the pi interactions are represented in purple, orange, and pink lines. Additionally, each of the compounds (HB1-6) were superimposed with erlotinib and both were docked inside the target site of EGFR tyrosine kinase (Fig. 2). The superimposition shows that the fittest compounds were compound HB4 and HB5.

The 4H-1,2,4-triazole ring of compound HB1 generated pi interactions with the essential amino acids Leu820, Ala719, and Val702 of EGFR tyrosine kinase, while the phenyl moiety formed other pi interactions with Ala719, Lys721, and Val702, and the phenylamino moiety formed pi interaction with leu694. The binding features of compound HB2 to EGFR tyrosine kinase was through 4H-1,2,4-triazole ring which showed pi interactions with the essential amino acids Leu820, Ala719, and Val702 of EGFR tyrosine kinase, while the phenyl moiety formed another sulfur-pi interaction with Met742, and pi-Alkyl interaction with Lys721. The 4-chloro phenylamino moiety formed pi interaction with Leu694, and the (2,3-dimethyl phenyl) amino moiety formed one H-bond with Asp831 (2.45 Å), and pi-pi interaction with Phe699, while the benzamide moiety interacted with Cys773 by sulfur-pi interaction.

Compound HB4 interacted with different essential amino acids of EGFR tyrosine kinase: the 4H-1,2,4-triazole ring created pi interactions with Leu820, Ala719, and

![Figure 1. Erlotinib docked on EGFR tyrosine kinase. H-bonds (green) and pi interactions (purple lines) with the mapping surface showing erlotinib occupying the active pocket of EGFR tyrosine kinase.](image-url)
Val702, while the phenylamino moiety formed $\pi$ interaction with Lys721. The 4-nitrophenyl group interacted with Leu694 by $\pi$ interactions, and the (2, 3-dimethyl phenyl) amino moiety formed one H-bond with Cys773 by sulfur-$\pi$ interaction.

The 4H-1, 2, 4-triazole ring of compound HB5 created $\pi$ interaction with EGFR tyrosine kinase amino acid Leu694, while the (2,3-dimethyl phenyl) amino moiety formed $\pi$ interactions with Cys773 and Arg817, and the 4-nitrophenyl group interacted with Leu820, Ala719, Val702, and Lys721 by $\pi$ interactions, and one H-bond with Thr766 (2.33 °A).

Finally, the binding features of compound HB6 to essential amino acids of EGFR tyrosine kinase were mediated by 4H-1,2,4-triazole ring which demonstrated $\pi$ interactions with Leu820, Ala719, Leu694, and one H-bond with Met769 (2.44 °A), while the 4-methoxy phenylamino moiety formed $\pi$-alkyl and $\pi$-sulfur interactions with Ala719 and Cys751, respectively. The 4-nitrophenyl group formed $\pi$-sigma interaction with Leu694, the (2, 3-dimethyl phenyl) amino moiety interacted with Cys773 by sulfur-$\pi$ interaction, and the benzamide moiety interacted with Asp831 and Val702 by $\pi$-anion and $\pi$-alkyl interactions, respectively.

**In silico ADMET and Drug likeness prediction**

The ADMET properties of drugs are usually predicted by intestinal absorption, activity against colon cancer cell line (Caco-2), P-glycoprotein binding, and skin permeability levels (Han et al. 2019). The data displayed in Table 4 showed that among the tested compounds, HB1 had the best ADMET properties. Additionally, the results indicated the inability of all compounds to penetrate the blood-brain barrier (BBB), this in turn indicate that central nervous system (CNS) side effects are not expected.

**Table 4.** ADMET properties for compounds HB1-HB6.

| Properties                  | HB1 | HB2 | HB3 | HB4 | HB5 | HB6 | Erlotinib |
|-----------------------------|-----|-----|-----|-----|-----|-----|-----------|
| Human gastrointestinal absorption | High | Low | Low | Low | Low | Low | High     |
| Caco2                       | 66.8 | 36.76 | 41.8 | 45.8 | 56.8 | 30.8 | 45.8     |
| Bioavailability score       | 0.55 | 0.17 | 0.17 | 0.55 | 0.17 | 0.17 | 0.55     |
| BBB penetration             | NO  | NO  | NO  | NO  | NO  | NO  | YES      |
| PBPP                        | 95.35 | 80.46 | 81.30 | 91.69 | 84.36 | 89.39 | 75.69    |
| CYP1A2 inhibitor            | NO  | NO  | NO  | NO  | NO  | NO  | NO       |
| CYP2C19 inhibitor           | YES | YES | YES | YES | YES | YES | YES       |
| CYP2C9 inhibitor            | NO  | NO  | NO  | NO  | NO  | NO  | NO       |
| CYP2D6 inhibitor            | NO  | NO  | NO  | NO  | NO  | NO  | NO       |
| CYP3A4 inhibitor            | YES | YES | YES | YES | YES | YES | YES       |
| Carcinogenicity             | NO  | NO  | NO  | NO  | NO  | NO  | mutant   |
| Ames Test                   | NO  | NO  | NO  | NO  | NO  | NO  | mutant   |

![Figure 2. The 2D docking of compounds (HB1-6) against EGFR tyrosine kinase focusing on the interacting amino acids. H-bonds are shown in green dashed lines, and the $\pi$ interactions are shown in purple, orange, and pink dashed lines. Superimposition of each compound with erlotinib inside EGFR tyrosine kinase target site is shown.](image-url)
upon administration. The metabolism profile was predicted based on the CYP models, in particular (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4), the results showed no inhibitory effect against most of the tested liver enzymes. Moreover, the toxicity of the tested compounds was predicted based on the AMES test, and the data displayed in Table 4 showed carcinogenicity profile of most derivatives close to zero.

The drug-likeness study indicated that all of the synthetic compounds (HB1-6) have most of Lipinski’s Rule criteria for oral administration. In addition, it is known that for good bioavailability after oral administration the value of TPSA must not exceed 140 Å² (Abbas et al. 2022), thus all tested compounds have good bioavailability, as shown in Table 5.

### Biological study

**The newly synthesized 1,2,4 triazole derivatives (HB1-6) are cytotoxic to cancer cells**

The cytotoxic effects of the 6 compounds (HB1-HB6) were evaluated against Hep G2 and A549 human cancer cell lines using MTT assay (Suppl. material 1: Fig. S1). The IC₅₀ values are shown in Table 6. The results revealed that all tested compounds exhibited cytotoxicity against Hep G2 hepatocyte carcinoma, and A549 lung cancer cell lines, 4 compounds (HB2, HB4, HB5, and HB6) were more cytotoxic to Hep G2 than to A459 cancer cells (lower IC₅₀; Table 6), while compounds HB1 and HB3 were more cytotoxic against A549 lung cancer cells. The lowest IC₅₀ value achieved was with compound HB5 against Hep G2 hepatocyte carcinoma (2.87 µM; Table 6); thus, this compound was selected for further investigation at the IC₅₀ concentration. The results of the MTT assay confirmed the superimposition results shown in Fig. 2, where the most cytotoxic compounds (HB5 and HB4) were the best superimposed (fittest) with erlotinib.

To study the selectivity of compound HB5 against cancer cells, the cytotoxicity of this compound was evaluated against the normal lung fibroblast cell line WI-38. The IC₅₀ concentration of compound HB5 against WI-38 cells was 23.67 µM (Suppl. material 1: Fig. S1), resulting in a selectivity index (SI) of 8.25 (SI = IC₅₀ normal cells/IC₅₀ cancer cells (Indrayanto et al. 2021)). These data indicate that compound HB5 (molecular weight = 554.01 g/mol) exhibits strong cytotoxicity indicated by IC₅₀ between 10 and 100 µg/mL (HB5 IC₅₀= 2.87 µM= 13.1 µg/mL), and high selectivity against cancer cells (SI ≥ 3) (Weerapreeyakul et al. 2012).

### Table 5. Physicochemical properties of compounds (HB1–HB6).

| Properties     | HB1   | HB2   | HB3   | HB4   | HB5   | HB6   | Erlotinib |
|----------------|-------|-------|-------|-------|-------|-------|-----------|
| clog P         | 3.43  | 3.58  | 4.07  | 1.95  | 2.03  | 2.60  | 4.06      |
| clog S         | 10.83 | 10.93 | 11.41 | 10.16 | 10.25 | 10.07 | 7.26      |
| H-bond acceptor | 3     | 4     | 3     | 5     | 6     | 5     | 6         |
| H-bond donor   | 3     | 3     | 3     | 3     | 3     | 1     |           |
| Skin permeation |       |       |       |       |       |       |           |
| (log Kp) cm/s  | 83.87 | 93.10 | 83.87 | 129.35| 138.92| 129.69| 129.69    |

### Table 6. In-vitro anti-proliferative activities of the tested compounds against Hep G2 and A549 cell lines. Data are presented as the mean of the IC₅₀ values (µM) from three different experiments.

| Compound | HepG2/IC₅₀ | A549/IC₅₀ |
|----------|------------|-----------|
| HB1      | 34.81      | 12.40     |
| HB2      | 12.96      | 18.62     |
| HB3      | 16.36      | 10.82     |
| HB4      | 8.29       | 9.94      |
| HB5      | 2.87       | 9.69      |
| HB6      | 17.87      | 18.74     |

![Figure 3. Flow cytometric analysis of Hep G2 cell cycle phases 48h-post compound HB5 treatment. Representative histograms showing the distribution of cell cycle phases of control and HB5-treated cells. Hep G2 hepatocyte carcinoma cells were treated with 2.87 µM (IC₅₀ value) of compound HB5 for 48 h.](image-url)
Compound HB5-treated Hep G2 hepatocyte carcinoma cells are arrested at S and G2/M cell cycle phases

The effect of 48 h treatment of compound HB5 on Hep G2 cell cycle progression was evaluated by staining cells with PI followed by flow cytometry (Fig. 3). Data shown in Table 7 indicate that treating Hep G2 cells with the IC$_{50}$ concentration of compound HB5 resulted in cell cycle arrest in the S and G2/M phases. The percentage of cells arrested in these phases increased by 50% when compared to control Hep G2 cells (Table 7).

Cytotoxicity of compound HB5 is attributed to apoptosis not necrosis

To investigate the mode of cell death (apoptosis versus necrosis) caused by 48h of compound HB5 treatment, Hep G2 cells were treated, stained with PI and annexin V-FITC, and analyzed by flow cytometry (Fig. 4A). As shown in Fig. 4A, Table 8, compound HB5 induced early apoptosis in treated cells as compared to control untreated cells.

Additionally, the level (fold change) of mRNA of apoptosis markers such as, p53, BAX (pro-apoptosis), Bcl-2 (anti-apoptosis), and caspase-3 was measured by qRT-PCR.
The results shown in Fig. 4B indicate that the fold change of p53, BAX, and caspase-3 mRNA (normalized over GAPDH) increased, while the that of Bcl-2 mRNA decreased in HB5-treated Hep G2 cell line as compared to vehicle-treated control cells (set as 1 arbitrary unit) confirming induction of apoptosis in HB5-treated Hep G2 cells.

**Compound HB5 inhibited Hep G2 cell proliferation and induced apoptosis by inhibiting EGFR tyrosine kinase**

EGFR is one of the most frequently mutated genes in solid epithelial cancers, either through overexpression of the EGFR protein or a kinase-activating mutation (Sigismund et al. 2018b). In addition, the results of the molecular docking studies of compounds HB1-6 indicated that these compounds might be able to target EGFR tyrosine kinase. After analyzing the results of the EGFR kinase inhibition assay, it was found that compound HB5 exhibited inhibitory activity against EGFR tyrosine kinase (EC₅₀ = 38.3 nM), which was comparable to the EC₅₀ concentration of the standard chemotherapeutic drug erlotinib (EC₅₀ = 9.6 nM) (Suppl. material 2: Fig. S2). All tested compounds have the same pattern of pharmacophoric queries of EGFR tyrosine kinase inhibitors, however, compound HB5 has the highest fitting with these queries. Compound HB5 showed

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**Table 7.** Effect of compound HB5 on cell cycle progression in Hep G2 cells after 48 h treatment. Values are given as mean ± SEM of three independent experiments.

| Cell cycle distribution (%) | Sub-G1 | G1     | S      | G2/M   |
|-----------------------------|--------|--------|--------|--------|
| Control Hep G2              | 0.63 ± 0.18 | 57.77 ± 2.68 | 25.35 ± 1.69 | 16.25 ± 1.34 |
| HB5-treated Hep G2          | 1.16 ± 0.17 | 35.56 ± 5.08* | 37.87 ± 3.87* | 25.41 ± 2.18* |

*p < 0.05 as compared to control cells.

**Table 8.** Effect of compound HB5 on the mode of cell death in Hep G2 cells after 48h treatment. Values are given as mean ± SEM of three independent experiments.

|                  | % Viable   | % Apoptosis  | % Necrosis |
|------------------|------------|--------------|------------|
| Control Hep G2   | 93.55 ± 0.29 | 5.69 ± 0.30  | 0.62 ± 0.05 |
| HB5-treated Hep G2 | 28.21 ± 1.80**** | 70.44 ± 1.80**** | 1.21 ± 0.20  |

****p < 0.0001 as compared to control cells.

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**Figure 5.** The synthesized compounds predicted feature as EGFR tyrosine kinase inhibitors.
the lowest ΔG score (-8.32 Kcal/mol; Table 3) in the interaction with EGFR tyrosine kinase, which indicates the stability of interaction between HB5 and the “Hit” amino acids in the target site of EGFR tyrosine kinase. This in turn indicates high selectivity and activity against the EGFR tyrosine kinase target site, confirmed by docking studies and superimposition as well as the EGFR inhibitory assay, which explains the ability of compound HB5 to cause the strongest cytotoxicity, i.e., lowest IC50 value (Table 6).

**Structure-Activity Relationship (SAR) of compounds HB1-HB6**

The SAR of the compounds (HB1-6) revealed several common findings (Fig. 5). For anti-cancer activity, the fragment binding to the hydrophilic linker is essential to be an aryl or heteroaryl (Gaber et al. 2021). Accordingly, the compounds which have 4-nitrophenyl moiety (compounds HB4, HB5, and HB6) showed good activity nearly equal with those compounds containing 4-phenyl moiety (compounds HB1, HB2, and HB3). Additionally, compounds that contain chloro (Cl) groups in hydrophobic tail, showed the best activity against targeted cells according to the MTT assay (compounds HB2 and HB5), moreover, the 4-nitrophenyl ring attached to central aromatic is more preferable than non-derivatization. Thus, for the tested compounds (HB1- HB6), chloro derivative in the hydrophobic tail (HB5) is better than methoxy(HB6), and phenyl derivative (HB4).

**Conclusion**

A series of 1,2,4 triazole derivatives (HB1-6) have been successfully synthesized, and their chemical structures were confirmed using FT-IR, 1H, and 13CNMR spectra and elemental microanalysis. After performing molecular docking studies and MTT assay for the tested compounds, the results showed a high correlation between the expected results from the molecular modeling and the wet-lab biological evaluations, which indicated high selectivity against the EGFR tyrosine kinase target site. Additionally, in silico pre-ADMET study showed that all tested compounds have no CNS side effects, and carcinogenicity score close to zero. Finally, compound HB5 showed the strongest cytotoxicity and highest selectivity against cancer cells through its stable interaction with EGFR tyrosine kinase leading to inhibition of its activating, and thus inducing cancer cell apoptosis.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

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Supplementary material 1

Figure S1
Authors: Hiba Alsaad, Ammar Kubba, Lubna H. Tahtamouni, Ali H. Hamzah
Data type: JPG file
Explanation note: HB5-treated Hep G2 hepatocyte carcinoma and A549 lung cancer cells show reduced cell proliferation. Representative line chart of the MTT assay results. Cells were treated for 48h and the growth inhibition percentage (Inhibition %) relative to the vehicle-treated control cells was measured. Percent inhibition was calculated according to the following formula: optical density (OD) treated/OD vehicle-treated control×100%. The experiment was performed three times in triplicates.

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Link: https://doi.org/10.3897/pharmacia.69.e83158.suppl1

Supplementary material 2

Figure S2
Authors: Hiba Alsaad, Ammar Kubba, Lubna H. Tahtamouni, Ali H. Hamzah
Data type: JPG file
Explanation note: The EGFR tyrosine kinase inhibitory activity of compound HB5 was comparable to that of erlotinib. The inhibitory activities of compound HB5 and erlotinib (a standard TKI) against EGFR kinase were evaluated by chemiluminescence and the EC50 values were determined in nM.

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Link: https://doi.org/10.3897/pharmacia.69.e83158.suppl2