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Anticancer Potential of a Novel Benzothiazole-Derived Heterocyclic Compound: In Vitro, In Vivo, and Molecular Docking Studies

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Abstract

Background: Benzothiazole (BTA) is a potent organic compound with numerous biological and medicinal effects, making it a valuable tool for drug development. The purpose of the study: This study aims to produce a heterocyclic compound belonging to benzothiazoles, which are known for their great medical effectiveness, and to test their biological effectiveness against cancer cell growth. Methods: The exposure to A2 compound (1-(benzo[d]thiazol-2-yl) pyrazolidine-3,5-dione) was 72 hours, followed by adding 200 µL of crystal violet stain solution to each well and incubating at 37°C for 30 minutes. A molecular docking experiment was conducted using the crystallographic framework of the epidermal growth factor receptor (EGFR) to evaluate the binding strength of the compound to this receptor. The lethal dose (LD50) was determined using the Miller and Tainter method. Results: Even at a lower concentration of 6 µg/ml A2 compound appears to have greater cytotoxicity against SKG-T4 cells and AMGM5, while lower cytotoxicity to inhibition of rat embryo fibroblasts (REF) in exposure time at 72 h and has good affinity at the active site of EGFR due to the formation of hydrogen bonds with critical amino acids, in acute toxicity experiments, the LD50 of compound A2 was determined to be 52.31 mg/kg.

Conclusion: The A2 compound, although present in a reduced amount, shows elevated cytotoxic effects on SKG-T4 cells and AMGM5, but shows decreased cytotoxicity toward REF cells after 72 hours. Furthermore, it shows a robust attraction to the active site of the EGFR.

Keywords: *Benzothiazole, Anticancer activity, Molecular Docking, Lethal Dose*

Introduction

Heterocyclic compounds are substances that comprise an important family of molecules of organic compounds that possess strong biology and medicinal effects. Due to their enormous pharmacological description, heterocyclic molecules, either alone or in fusion form, are continually utilized by scientists to create novel medications. Among oxygen, nitrogen, and sulfur (1). Many naturally occurring products contain benzothiazole (BTA), and the natural compounds in question have medicinal and pharmaceutical applications because of a fused benzo-heterocycle (2). The BTA is made up of a benzene ring and a thiazole ring. (3). A heterocyclic molecule of the aromatic class is benzothiazole (BTA). BTA derivatives are used for biological functions including, but not limited to, anticancer (4). anti-inflammatory (5), antiviral (6), antibacterial (7), antiproliferative (8), anti-diabetic (9), anticonvulsant (10), anti-tubercular (11), antimalarial (12), antileishmanial (13), antifungal (14), BTA derivatives reveal amazing and ubiquitous biological and pharmacological activity against various kinds of tumors and cancer cell lines (15); such as HeLa (human cervical carcinoma cell line) (16), SW480 (human colon carcinoma cell line) (17), HepG2 (human hepatic cancer cells) (18), mammary and ovary cell carcinoma lines (19), and the intestines, excluding small-cell pulmonary cell types (20). Cancer is the leading cause of death worldwide. Cancer is assumed to be a process of several phases of tumorigenesis, including numerous physiological cell processes such as cell signaling and death (21). These malignant tumor cells are varied, different cells with fast-proliferating characteristics (22). Malignant tumors (cancer) can infiltrate or spread to other sections of the body via the circulation and lymphatic system Risk indicators for the growth of squamous-cell carcinoma include poor income level (23), intake of alcohol, cigarettes, and hot drinks (24)

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The aim of this study is to investigate the anticancer potential of a novel benzothiazole-derived compound through a comprehensive set of studies, including in vitro, in vivo, and molecular docking analyses. The research focuses on evaluating the compound's efficacy against various cancer cell lines and exploring its effects at the molecular level. These combined approaches aim to provide a deeper understanding of the compound's biological activity and its potential as a therapeutic agent for cancer treatment.

Materials and Methods

Chemicals

All reagents and solvents utilized in this study were of reagent-grade quality and were sourced from Sigma-Aldrich and Fluka. The melting points were measured using open capillary tubes with a Stuarts SMP30 melting point apparatus (Germany), and the values were reported without correction. Infrared spectra (FT-IR) were recorded on a SHIMADZU FT-IR8400S spectrophotometer at the Department of Chemistry, College of Science, Mustansiriyah University.

Synthesis of 2-hydrazino-1,3-benzothiazole (A1)

A mixture of 0.01 moles (0.5 g) of 2-mercaptobenzothiazole and 0.01 moles (1.67 g) of hydrazine was refluxed in 15 ml of ethanol for three hours. After the mixture was cooled to produce a precipitate, the excess hydrazine was removed by filtering and rinsing it with cold water filtering and recrystallizing the precipitate from ethanol after the solvent was evaporated (25).

Synthesis of 1-(1,3-benzothiazol-2-yl) pyrazolidine-3,5-dione (A2)

In a round-bottom flask, 0.006 moles (1 g) of compound A1 was combined with 0.006 moles (0.76 g) of diethyl malonate, 0.015 moles of acetic acid, and 30 ml of absolute ethanol. It refluxed for around 11 to 12 hours. Compound (A2) was obtained by filtering and recrystallizing the precipitate from ethanol after the solvent was evaporated (26).

Cell culture and maintenance

Three cell lines were used in this study: SKG-T4 (esophageal cancer cells), AMGM5 (glioblastoma cell line), and REF (rat embryo fibroblasts). These cells were maintained locally at the Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University. The cell cultures were supplemented with 2 mM GlutaMAX (Invitrogen Products) and 10% fetal calf serum 100 mg/ml streptomycin sulfate and 100 U/ml penicillin (Sigma).). Each cell line was cultivated in an incubator with 5% CO2 and a dryer environment at 37°C. The cells were routinely subcultured twice a week in RPMI-1640 medium supplemented with 3.0 mM L-glutamine and 10% fetal bovine serum to maintain exponential growth.

Compound preparation

Chemicals' solubility was assessed independently, while their cytotoxicity was evaluated through in vitro assays The solubility of the manufactured substance was approved by the specified procedure. (28). The A2 compound was dissolved in (DMSO) and diluted with medium RPMI-1640 to preparation solutions, prepared at concentrations of 1, 4, 5, 6, 8, 10, 15, 25, 50, 85 µM.

Cytotoxicity assay

When the cells were monolayers in the falcon tissue culture flask, they were detached using tripsin-versinTM ml for 5 min to collect a single cell. Add 15 ml of the culture medium to the falcon that collects single cells. Transfer about 200µL from cells to 96-well plates, then seal and incubate at 37°C. After 24 h, the medium was withdrawn and 200 µL of synthesized chemical compound preparation solutions from C1 to C10 were added to the 96-well plates. About 200µLof freshly prepared media was given as a control. The same amount of DMSO is used as a positive control. Each concentration was carried out in triplicate. The total exposure to the chemical compounds were 72 hours. After 72 hours, 200 µlof crystal violet stain solution was added to each well and incubated at 37°C for 30 minutes. After that, the dish became purple when it was cleaned with tap water. After that, the plates were tested using a microplate reader at 495 nm (Biochrom, UK). Thus, the inhibitory rate was calculated after that by applying

$$growth\ inhibition\ \% = \frac{ab\ C. - ab\ T.}{ab\ C.} \times 100\%$$

Were, ab C. indicate mean absorption of control and ab T. absorption of chemical compound (29).

Molecular modeling

Using the binding affinity scoring approach, molecular docking analyzes protein-protein or ligand-protein interactions and scores the results, highlighting the bonds in the interaction. (30). The crystallographic structure of the epidermal growth factor receptor (EGFR) was obtained from Protein Data Bank (code: 8JFQ, Resolution: 1.60) (31) and was considered a target for the simulation of the binding. The docking study was done using the MOE 2014 program to examine the free energy and docking mode of the proposed compound to EGFR. At first, the protein's structure remained protonated, and hydrogen atoms were removed. Next, the protein's binding pockets were identified, and the energy was reduced to a minimum, using the default protocol. The 2D structures of the synthesized molecule and the reference compound, 2-morpholinoethane-1-sulfonate, were generated using the ChemDraw 20.0 application, then copying the mdb file allowed the database to be constructed. This molecule was supplemented with polar and nonpolar hydrogens, created a three-dimensional structure, and saved as a mdb file.

Animals

After a seven-day acclimation period to the laboratory environment, laboratory mice weighing 25–30 g and aged 10–12 weeks were used. Food and tap water were provided to the animals according to the ICCMGR protocol. The light and dark cycles were shorter than the standard 12:12 LD period. The temperature was maintained at 65-75°F (18-23°C) with 40-60% humidity.

Examination of Acute Toxicology

Miller and Tainter's standard procedure were followed for the acute toxicity tests (32). Using the probit analysis technique, male albino mice were divided into six groups of six animals each for LD50 determination. The A2 compound was administered intraperitoneally at doses of 65, 60, 55, 50, 45, and 40 mg/kg using an injection syringe. Toxicity signs and mortality were monitored over 24 hours, and the lethal dose for fifty percent (50%) of the animals (LD50) was determined. The animals were continuously monitored for the first two hours, followed by checks every four hours. Observations included instances of animals succumbing to toxicity, while others survived for the full 24-hour period. The LD50 was determined through both quantitative scientific methods and visual assessment First, the percentage of deaths was determined. by the ratio was adjusted to 0 % and 100 % (33) (34).

to 0% death ¼ 100 × (0.25/n)

to 100% death ¼ 100 × [(n- 0.25)/n]

where n is the overall number of animals in each group.

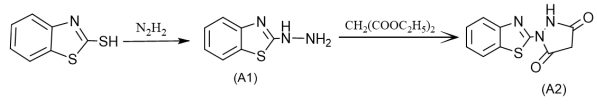
Statistical analysis

In this investigation, we employed Student's t test and Analysis of Variance (ANOVA) to determine variations in concentrations between SKG-4, AMGM5, and RFE cell lines. The probability p was established. p is less than 0.05. Using Graph Pad Prism V9.5.1, this statistical test was performed.

Results

preparation of 1-(benzo[d]thiazol-2-yl) pyrazolidine-3,5-dione

The compound 2-hydrazino-1,3-benzothiazole (A1) was prepared from a reaction of 2- mercaptobenzothiazole with hydrazine (35). The 1-(benzo[d]thiazol-2-yl) pyrazolidine-3,5-dione (A2) was synthesized from cyclization of hydrazine compound (A1) with diethyl malonate in previous study (m.p. =170 °C) (26) Scheme (1).



Scheme (1): Synthesis of compounds A (1 and 2)

Cytotoxic activity

The cytotoxicity of compound A2 was evaluated in vitro against three cell lines: SKG-T4, AMGM5, and REF (Figure 1). Our findings demonstrate that A2 exhibits significant cytotoxicity towards SKG-T4 cells, with 97% inhibition observed at a low concentration of 6 µg/ml after 72 hours of exposure. In AMGM5 cells, the inhibition reached 94%, while for REF cells, it was 45%. Cytotoxic effects at varying concentrations (1, 4, 5, 6, 8, 10, 15, 25, 50, 85 µg/ml) over a 72-hour period were as follows: for SKG-T4 cells (51%, 50%, 82%, 97%, 93%, 81%, 93%, 91%, 90%, 93%), for AMGM5 cells (47%, 52%, 79%, 94%, 91%, 81%, 83%, 85%, 86%, 84%), and for REF cells (30%, 32%, 34%, 45%, 41%, 36%, 36%, 43%, 39%, 37%).

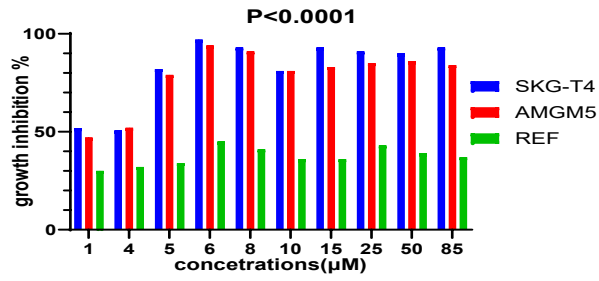


Figure (1): The cytotoxic effects of compound (2) on cancer cell lines SKG-T4, AMGM5 and REF were observed when varying doses were synthesized and exposed for 72 hours.

Docking Study

Molecular docking studies were conducted to evaluate the interaction between the reference ligand and the synthesized compound (A2) with the active site of EGFR. The docking score for the reference ligand, as shown in Table 1, was -8.139 kcal/mol. The sulfite group of 2-morpholinoethane-1-sulfonate formed hydrogen bonds with three amino acids: GLN 1020, LEU 703, and SER 768, along with an ionic bond with ARG 831. Furthermore, the morpholine ring established a hydrogen bond with ARG 776 (Figure 2).

Table 1: The interactions occurring between the reference ligand and the active site of the EGFR receptor.

Ligand	Receptor	Interaction	Distance (Å)	E (kcal/mol)
O 14	NE ARG 776 (A)	H-acceptor	-2.0	3.20
O 22	CB GLN 1020 (A)	H-acceptor	-1.5	3.39
O 23	N LEU 703 (A)	H-acceptor	-4.6	2.95
O 24	CA SER 768 (A)	H-acceptor	-0.8	3.22
O 24	NH2 ARG 831 (A)	Ionic	-1.6	3.57

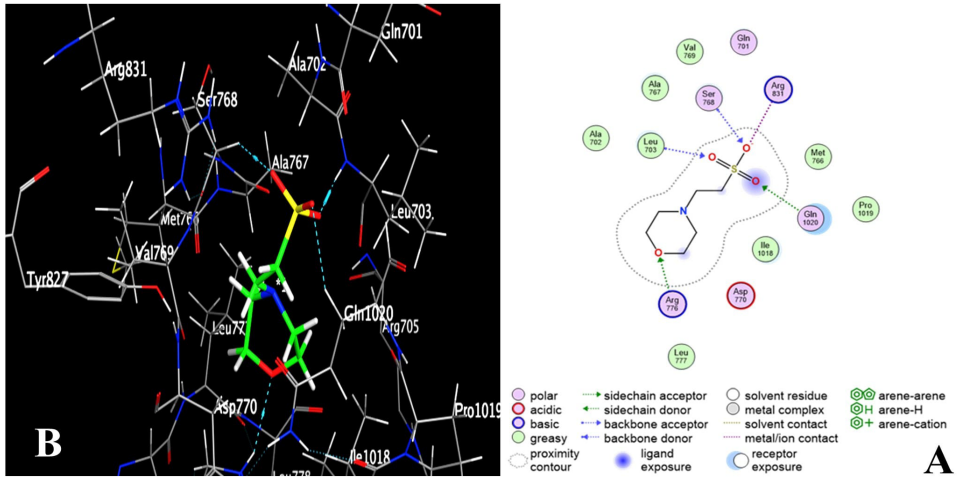
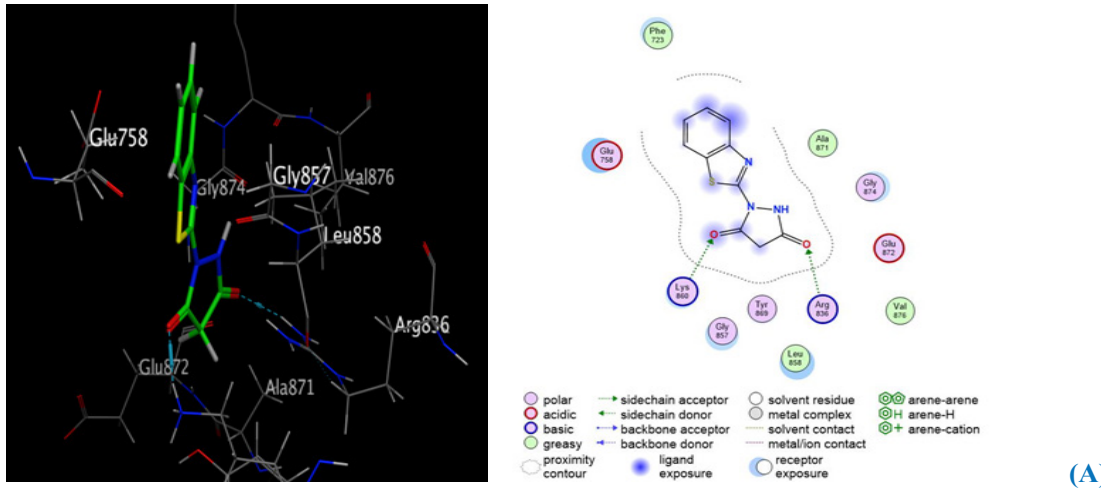


Figure 2: (A)2D diagram & (B) 3D views of the reference ligand docked at the active site of EGFR.

The docking analysis of the novel compound A2 with the EGFR receptor revealed two favorable binding poses with minimal binding energy. The first pose showed a docking score of -7.214 kcal/mol, characterized by hydrogen bond interactions between the carbonyl group of A2 and the amino acids ARG 836 and LYS 860 (Figure 3, Table 2). The second pose exhibited a docking score of -6.784 kcal/mol, where hydrogen bond interactions were observed between the carbonyl group of A2 and the amino acid GLY 874, in addition to interactions between the thiazole ring and ARG 836, as well as the carbonyl group and GLY 874.

Table 2: The interactions occurring between the compound A2 and the active site of the EGFR receptor.

Ligand-receptor interaction distance E (kcal/mol)						
Pose 1						
O	22	NH2	ARG 836 (A)	H-acceptor	-1.2	3.19
O	23	NZ	LYS 860 (A)	H-acceptor	- 6.8	2.99
Pose2						
C	16	O	GLY 874 (A)	H-donor	- 0.5	3.30
N	13	NH1	ARG 836 (A)	H-acceptor	- 6.3	3.01
O	23	N	GLY 874 (A)	H-acceptor	-2.5	3.03



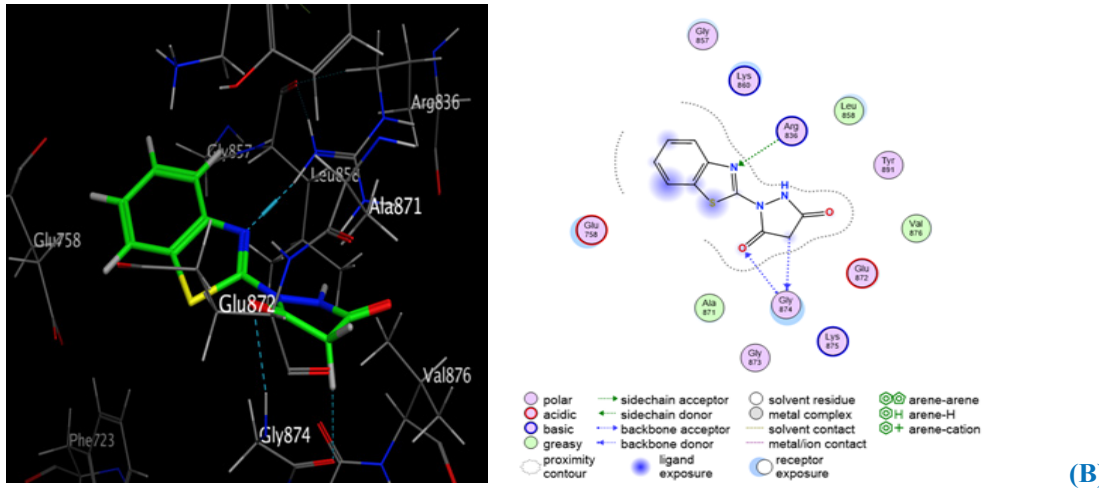


Figure 3: (A)2D diagram & 3D Views of the pose1(B) 2D diagram & 3D views of pose1 (B) 2D diagram & 3D views of pose2 of the A2 compound ligand attached to the active site of EGFR.

Acute toxicity of 1-(benzo[d]thiazol-2-yl) pyrazolidine-3,5-dione

The results of the LD50 estimate for the acute toxicity studies were tabulated. As shown (Table 3, Figure 4), use the Miller and Tainter equation to calculate the fatal dose (36). The toxicity measurements were carried out and at doses equivalent to (40, 45, 50, 55, 60, 65) mg/kg, respectively, the percentage mortality recorded was (0, 10, 20, 40, 50, 100) %. A slope that at probit 5 extended to a value of 1.719 on the x-axis was obtained when a plot of log dose (on the X-axis) vs. probability units (on the Y-axis) was created. Consequently, it was found that the LD 50 of the A2 compound was 52.31 mg/kg.

Table 3: Conversion table for the dosage to probits.

Groupe	(n)	(dose) (mg/kg)	Logarithmic dose	(Md)	(dead%)	(%corrected)	(probits)
1	6	40	1.602	0	0	4.16	3.25
2	6	45	1.653	1	16	16	4.01
3	6	50	1.699	2	33	33	4.56
4	6	55	1.740	4	66	66	5.41
5	6	60	1.778	5	83	83	5.95
6	6	65	1.813	6	100	95.8	6.75

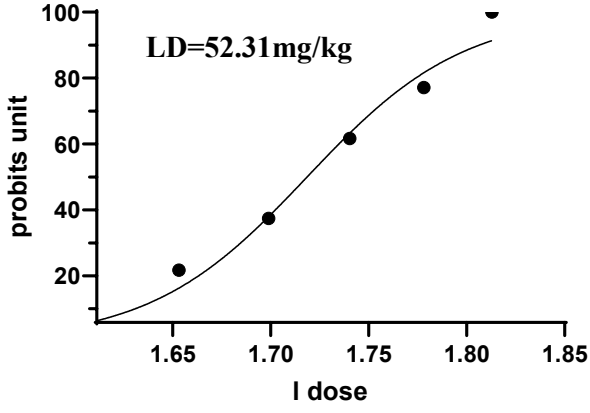


Figure 4: The graph representing dose-response in measures of probability units.

Discussion

The in vitro experiments of the current study indicated that the chemical compound (A2) had potential anticancer properties, at a lower concentration 6 µg/ml appears and appeared to have higher cytotoxicity against the exposure time of SKG-T4, AMGM5, REF cell lines at 72 h with a 97%, 94%, and 45% inhibition rate, respectively. AMGM5 human glioblastoma cancer cell lines are considered a type of cancer characterized by resistance to chemotherapy, especially a subset of cancer patients with the methylated MGMT gene. and this is associated with switching from methylated to an undesired nonmethylated MGMT state (37) Therefore, this compound(A2) can be used as a chemotherapy treatment for this type of cancer. In the study by Kumbhare et al. (2012), test in vitro cytotoxicity of their synthesized compound compared to etoposide, the benzothiazole compound 3 showed the strongest antiproliferative efficacy against the human monocytic cell line U-937 cell line, and its IC50 values were greater than the standard compound etoposide's IC50 values (17.94 ± 0.89), (18.69 ± 0.94), and (2.16 ± 0.11 mM)) against human monocytic cell lines (U-937and THP-1) and mouse melanoma cell line(B16-F10) cell lines, respectively (16.23 ± 0.81 mM), (4847.73 ± 2.39 mM), and (34.58 ± 1.73 mM) respectively. (38). In another study by Gabr et al. (2014), The creation of new benzothiazole compounds targeting 60 types of tumor cells at a single dose of 10 mM, they found the two most effective derivatives, 32 and 33, showed micromolar and submolar doses against each of the 60 types of tumor cells with GI50 values in the low micromolar to submicromolar range. The moiety enhanced both compounds' antitumor properties. (39).

In the docking model, as demonstrated in compound 17, the results of the coupling energy research indicated that concurrent EGFR/HER2 inhibitors should require at least one hydrogen-bonded interaction with the active site of the EGFR receptor to reduce the expression of TS (40).). In another study Abdellatif et al. (2020), compounds 39 and 40 demonstrated a binding pattern like lapatinib as the reference ligand, according to the active site of EGFR, as they satisfied crucial contacts where the hydrogen bond with Lys745 was created, compounds 39 and 40 (41).). In the study of Mokhtar et al. (2020), molecular modeling studies were also conducted for the synthesized compounds, including docking into the active site of EGFR and surface mapping. The results showed that the superior binding of the hydrazone derivatives 39 and 40 with EGFR suggested that they are good candidates for targeted antitumor therapy through inhibition of EGFR kinase, which is important, was entirely contained within the cavity of the active enzyme site and deeply buried within it. This contributed to their increased inhibitory action against the EFGR enzyme (42). In another study of Samia et al (2023), M they found that the compound 2a inside the binding pocket of the EGFR receptor (S = -6.20 kcal mol-1 and RMSD = 1.44 Å) was found to form two H-bonds with MET793 and LYS745 amino acids at 3.16 and 3.74 Å, respectively (43). The study of Aziz et al. (2022), Compound 5g which had an affinity value of -81.06 kcal mol−1 and four H bonds. Threonine854 (2.78 Å), the main amino acid, and the carbonyl group of the acetamide linker produced one H-bond. Methionine 793 (2.02 Å) and the carbonyl group at position-4 of thiazolidine-2,4-dione created one H-bond (44). Abd El-Meguid et al. (2021), Their studies showed that negative energy scores for benzo[d]thiazole targets 5, 6, and 7 were 13.55, 13.25 and -12.83 kcal/mol, indicating that their benzo[d]thiazole scaffold fits in the active site of EGFR by hydrophobic arene-arene and hydrogen bonding (45).). The results of the study conducted by Tarfah et al. (2023) showed that the thiazolyl pyrazolines that were tested demonstrated good activity due to their ability to interact with key amino acids at the binding site, as demonstrated by their binding pattern and docking score. These values ranged from -11.14 to -10.64 kcal/mol, which is like erlotinib's (S = -10.86 kcal/mol) (46). In the study by Raghu et al. (2023), the derivatives of thiazole 4i and 4j have docking scores that are higher than the docking score of 8.21 kcal/mol of the commonly prescribed the drug Osimertinib, according to docking investigations. Three hydrogen bonding contacts were observed between the most active molecule, 4i, and important amino acids, including Cys-781, Gly-857, and Thr-903(47). All docking activities, from the best contact to those with modest binding, showed an underlying mechanism of action; current docking study results suggested that the integration of a carbonyl group with a thiazole ring enhances binding affinity at the active site of EGFR due to the formation of hydrogen bonds with critical amino acids such as ARG 836 and GLY 874.

Acute toxicity was observed with the A2 molecule, resulting in 100% of the animals dying at 65 mg/kg.

Conclusions

Compound A2 demonstrated promising anticancer activity against the SKG-4, AMGM5, and REF cell lines in vitro, showing the highest activity against SKJ-T4 and AMGM5 cells at a low concentration of 6 µg/ml, compared to REF cells. The presence of a pyrazole ring in compound A2 allows it to bind to multiple sites on the EGFR receptor. This study, the first of its kind in Iraq, highlights the potential of compound A2 as a target for cancer diagnosis and treatment. The authors recommend further investigation into the molecular pathways through which this compound affects cancer cell growth.

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Authors' Contribution

All authors have made equal contributions to this work.

Conflict of interest

The authors disclosed that they had no conflicts of interest.

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