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Anticancer Activity and Molecular Docking Studies of Selected Benzoxazole Derivatives as Apoptosis Inducers in Non-Small Cell Lung Cancer

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ABSTRACT

Objective: Non-small cell lung cancer (NSCLC), the most prevalent type of lung cancer, remains the leading cause of cancer-related deaths worldwide. Late-stage diagnosis and resistance to conventional treatments highlight the need for further research into its molecular mechanisms. This study aimed to evaluate the anticancer effects of several benzoxazole derivatives (2-(4-tert-butylphenyl)-5-nitrobenzoxazole (1a), 2-(4-tert-butylphenyl)-6-nitrobenzoxazole (1b), 2-(2,3-dimethylphenyl)-5-nitrobenzoxazole (2a), and 2-(2,3-dimethylphenyl)-6-nitrobenzoxazole (2b)) on the viability of A549 cells.

Materials and Methods: Cell viability was assessed using the MTT assay. We also performed a molecular docking study to investigate the interactions between the benzoxazole derivatives and caspase-3, a key executioner caspase involved in apoptosis.

Results: The benzoxazole derivatives coded 1a, 1b, 2a, and 2b exhibited anticancer activity against A549 cells, with half-maximal inhibitory concentration (IC_{50}) values of 17.41±0.16, 20.50±0.08, 32.17±0.08, and 31.13±0.07 μM, respectively. Among the tested benzoxazoles, 1a and 1b showed activity comparable to cisplatin (IC_{50}=19.65±0.09 μM). According to the docking results, all compounds demonstrated satisfactory docking scores ranging from -4.339 to -5.202 kcal/mol.

Conclusion: Our results demonstrate that the benzoxazole derivatives 1a and 1b exhibit significant anticancer effects by inhibiting lung cancer cell proliferation at low concentrations, similar to cisplatin. The structure-activity relationship suggests that substitution of a phenyl group at the 2-position of the benzoxazole ring with a tert-butyl group at the para position enhances anticancer activity against A549 cells. This preliminary study indicates that these benzoxazole derivatives have promising potential as cytotoxic agents for the treatment of NSCLC.

Keywords: Apoptosis, benzoxazole, cancer, molecular docking, non-small cell lung cancer.

INTRODUCTION

Lung cancer remains a major global health issue and the leading cause of cancer-related deaths, despite advances in prevention and treatment strategies. Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancer cases, with adenocarcinoma being the most prevalent subtype.[1,2] The five-year relative survival rate varies depending on the stage at diagnosis. However, a considerable proportion of NSCLC cases are diagnosed at an advanced stage, with 30%–40% identified at stage IV. Given its poor prognosis and limited response to conventional treatments such as radiation therapy and chemotherapy, further investigation into the molecular mechanisms underlying NSCLC is essential for the development of more effective therapeutic strategies.[2]

Cancer arises from the dysregulation of cell cycle progression and apoptotic mechanisms. Apoptosis is mediated by a series of cysteine-dependent aspartate-specific proteases known as caspases. Caspases are attractive therapeutic targets in several diseases, and the induction of caspase activity may offer new treatment strategies for cancer, which is characterized by uncontrolled cell proliferation.[3] Caspase-3, a key executioner caspase, plays an essential role in apoptosis; however, its specific involvement in the pathogenesis of lung cancer remains inadequately understood.[4]

A hallmark of cancer is its ability to evade apoptosis through modulation of anti-apoptotic and pro-apoptotic gene expression. Although chemotherapy aims to induce apoptosis and eliminate cancer cells, it has not achieved complete clinical success.[4] The identification of novel anticancer agents with high efficacy and low toxicity is a major priority in cancer drug development. Given that drug resistance is a significant challenge in cancer treatment, the development of alternative chemotherapeutic agents is essential.[5]

Heterocyclic compounds play a fundamental role in drug discovery and development. Numerous approved drugs and promising therapeutic agents are based on heterocyclic scaffolds.[6] Benzoxazole is one of the most extensively studied heterocycles in medicinal chemistry, with both naturally occurring and synthetic derivatives exhibiting diverse biological activities.[7] A broad spectrum of pharmacological effects, including anti-inflammatory,[8] antimicrobial,[9] antifungal,[10] and antitumor[5,11,12] activities, has been attributed to benzoxazole derivatives.

In 2004, a series of benzoxazole derivatives and their analogs were evaluated for inhibitory activity against eukaryotic DNA topoisomerase II (Topo II). Among these compounds, 2-(2-methoxyphenyl)-6-nitrobenzoxazole and 6-methyl-2-(2-nitrophenyl)benzoxazole demonstrated greater potency than etoposide, a clinically used reference drug.[13] Subsequent three-dimensional quantitative structure–activity relationship (3D QSAR) studies, employing comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA), were conducted on the same compound series in 2005[14] and 2006[15] to elucidate the structural features contributing to Topo II inhibitory activity. These investigations revealed that hydrophilic substitution at positions 5 or 6 of the heterocyclic core significantly enhanced enzyme inhibition compared to hydrophobic groups. Additionally, the presence of hydrophobic substituents at the ortho and/or para positions of the phenyl ring attached at the 2-position of the heterocyclic core was identified as a critical factor contributing to increased activity. As a continuation of these investigations, we reported the syntheses and hTopo I and hTopo IIα enzyme inhibition activities of a series of 2-(substituted-phenyl)benzoxazole derivatives bearing a nitro group at the 5- or 6-position in 2021.[5]

In the present study, as a continuation of this research, we aimed to investigate the anticancer effects of selected benzoxazole derivatives (2-(4-tert-butylphenyl)-5-nitrobenzoxazole (1a), 2-(4-tert-butylphenyl)-6-nitrobenzoxazole (1b), 2-(2,3-dimethylphenyl)-5-nitrobenzoxazole (2a), and 2-(2,3-dimethylphenyl)-6-nitrobenzoxazole (2b)) whose syntheses and hTopo I and hTopo IIα enzyme inhibition activities were previously reported by our group in 2021, on the viability of A549 human NSCLC cells. Additionally, due to the insufficient hTopo I and hTopo IIα inhibitory activities of these compounds,[5] we performed a molecular docking study to examine the interactions between these benzoxazole derivatives and caspase-3, an executioner caspase involved in apoptosis. Moreover, the ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) properties of these derivatives were evaluated using Schrödinger software suite to identify the most promising candidates as antitumor agents.

KEY MESSAGES

- Several 2-(substituted phenyl)-5(6)-nitrobenzoxazole derivatives were evaluated for cytotoxicity against A549 cells.
- Compounds 2-(4-tert-butylphenyl)-5-nitrobenzoxazole (1a) and 2-(4-tert-butylphenyl)-6-nitrobenzoxazole (1b) showed cytotoxic activity comparable to cisplatin.
- Molecular docking studies suggested possible interactions between the compounds and caspase-3 (PDB ID: 3GJQ).

MATERIALS AND METHODS

Chemicals

This study was conducted between August 2024 and February 2025. The in silico and *in vitro* investigations of molecules 1a, 1b, 2a, and 2b, whose syntheses were previously published by our group, were carried out.[5] As this study consisted solely of *in vitro* experiments and molecular docking analyses, ethical approval was not required.

In Vitro Studies

Cell Culture

Human NSCLC A549 cells (CCL-185™, ATCC, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM-HG) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell Viability Assay

The viability of A549 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded at a density of 7×10^3 cells/mL and cultured until 80% confluence was reached. The cells were then treated with various concentrations (0–100 µM) of compounds 1a, 1b, 2a, and 2b, as well as cisplatin (2.5, 5, 10, 20, and 50 µM), for 48 hours. Following treatment, 10 µL of MTT reagent (5 mg/mL) was added to each well and incubated for four hours. After removal of the medium, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using an Epoch 2 BioTek ELISA (enzyme-linked immunosorbent assay) reader. The half maximal inhibitory concentration (IC_{50}) values, defined as the concentration required to inhibit 50% of cell growth, were calculated by nonlinear regression analysis using a variable slope model in GraphPad Prism 9.5.1 (GraphPad Software, USA). Each experiment was performed in triplicate (n=3), based on previously established optimization studies and similar *in vitro* experiments.[12]

Statistical Analysis

Data were analyzed using GraphPad Prism 9.5.1 (GraphPad Software, USA). Data normality was assessed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was performed to assess overall group differences, followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was defined as follows: ***$p<0.001$, **$p<0.01$, *$p<0.05$. Data are presented as mean±standard error of the mean (SEM).

Molecular Docking Studies

Molecular docking studies were performed using the Schrödinger Maestro 2022-4 Glide package[16] to calculate the binding energies of protein-ligand complexes and to visualize their interactions.

Protein Preparation

The protein preparation process aims to optimize the protein structure prior to molecular docking.[17] The X-ray crystal structure of caspase-3 enzyme complexed with a peptide inhibitor was retrieved from the RCSB Protein Data Bank (PDB ID: 3GJQ) at a resolution of 2.60 Å, and the peptide inhibitor was used as the reference ligand.[18] The Protein Preparation Wizard module in Schrödinger Maestro was used to prepare the protein for optimization, hydrogen bond addition, elimination of atomic clashes, and removal of water molecules from protein crystal structures prior to docking. The protein was cleaned, bond orders and charges were corrected, missing hydrogens were added, and the protein structure was optimized.[19]

Ligand Preparation

All ligands were drawn using the 2D Sketcher module in Schrödinger Maestro. Further minimization of all ligands was performed using the Schrödinger Maestro "Minimize Selected Atoms" tool. Subsequently, all ligands were prepared using the "LigPrep" module by converting each ligand into 3D conformers, neutralizing charged structures, and ionizing the entire structure at neutral pH 7±2.0.[20] We set 32 stereoisomers as the maximum limit per ligand.

Grid Box Preparation

After opening the "Receptor Grid Generation" module, the grid box was generated by selecting the reference ligand complexed with the protein. The grid box was defined within a 20 Å region around the reference ligand.[21]

Docking Studies

Molecular docking of compounds 1a, 1b, 2a, 2b, and the original ligand was performed to evaluate their binding to the target caspase-3 enzyme using the Schrödinger Maestro "Ligand Docking" module.[22] Following the docking procedure, the Schrödinger Maestro Grid-Based Ligand Docking with Energetics (GLIDE) tool calculated the docking score, Glide score, Glide Emodel, and Glide energy to assess protein-ligand interaction energies. The Glide scores of compounds 1a, 1b, 2a, and 2b were compared with that of the peptide inhibitor used as the reference ligand.[23]

RESULTS

In Vitro Anticancer Activities of Benzoxazole Derivatives Against A549 Cells

The anticancer effects of several previously synthesized benzoxazole derivatives (1a, 1b, 2a, and 2b) were evaluated by treating A549 lung cancer cells with varying concentrations

of these compounds. All derivatives exhibited anticancer activity against A549 cells (Fig. 1, Appendix 1). The IC_{50} values for compounds 1a, 1b, 2a, and 2b were 17.41±0.16, 20.50±0.08, 32.17±0.08, and 31.13±0.07 μM, respectively (Table 1). The IC_{50} value of cisplatin was determined to be 19.65±0.09 μM. Comparison of IC_{50} values revealed that compounds 1a and 1b exhibited activity similar to cisplatin, whereas compounds 2a and 2b showed higher IC_{50} values than both cisplatin and compounds 1a and 1b ($p<0.01$). In addition to differences in IC_{50} values, treatment with compounds 2a and 2b at concentrations of 40, 60, 80, and 100 μM resulted in a significant reduction in cell viability compared with 20 μM (Appendix 1).

Results of Docking Studies

3D molecular docking of all benzoxazole derivatives (1a, 1b, 2a, and 2b), along with the original ligand, into the crystal structure of the caspase-3 enzyme (PDB ID: 3GJQ) (Figs. 2-6) was conducted for the first time using Schrödinger Release 2022-4 molecular modeling software (Schrödinger Release 2022–4, Glide, LLC, New York, NY, USA).[22,24,25] The study aimed to evaluate binding energies and investigate the interaction modes within the enzyme's active site. To estimate binding affinities and optimal alignment of the benzoxazole derivatives within the active site, different types of noncovalent interactions with surrounding amino acids, along with Glide scores, were analyzed. Based on the docking analysis, all compounds exhibited satisfactory docking scores, ranging from -4.339 to -5.202 kcal/mol (Table 2). The interaction diagrams of the original ligand and compounds 1a, 1b, 2a, and 2b are shown in Figures 2-6, respectively. As illustrated in Figures 3-6, the benzoxazole derivatives interacted with amino acid residues such as ASN208, ALA162, CYS163, GLN161, SER209, TRP206,

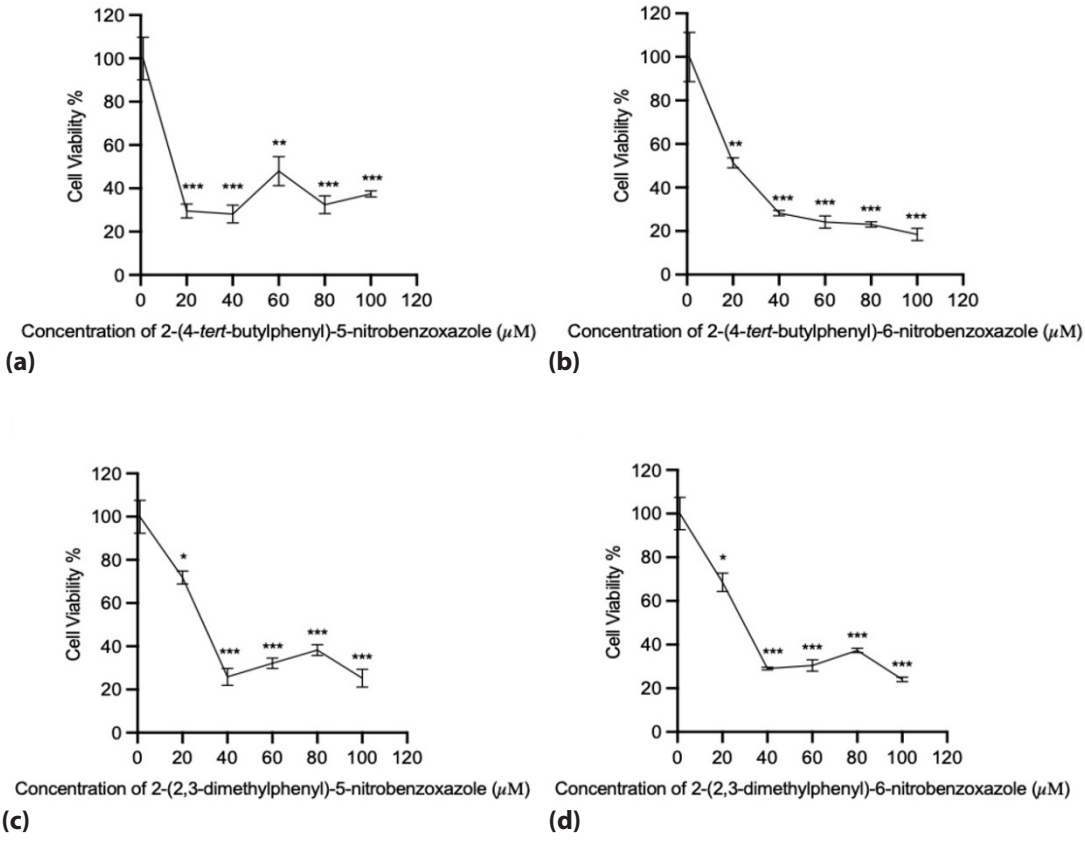

Figure 1. Effects of benzoxazole derivatives at different concentrations on the cell viability of A549 cells after 48 hours. The graphs demonstrate the percentage of cell viability after treatment with **(a)** 2-(4-tert-butylphenyl)-5-nitrobenzoxazole (1a), **(b)** 2-(4-tert-butylphenyl)-6-nitrobenzoxazole (1b), **(c)** 2-(2,3-dimethylphenyl)-5-nitrobenzoxazole (2a), and **(d)** 2-(2,3-dimethylphenyl)-6-nitrobenzoxazole (2b). Data are presented as mean±standard error of the mean (SEM) from three independent experiments (n=3). Statistical analyses were performed using one-way analysis of variance (ANOVA) for multiple-group comparisons and unpaired t-tests for comparisons between two groups. Significance levels are indicated as follows: ***p<0.001, **p<0.01, *p<0.05 versus the untreated group.

Table 1. IC_{50} values of benzoxazole derivatives and reference compound against A549 cells

Compound	IC_{50} (μM)±SEM
1a	17.41±0.16
1b	20.50±0.08
2a	32.17±0.08
2b	31.13±0.07
Cisplatin	19.65±0.09

IC_{50}: Concentration required to cause 50% inhibition of cell growth; μM: Micromolar; SEM: Standard error of the mean; 1a: 2-(4-tert-butylphenyl)-5-nitrobenzoxazole; 1b: 2-(4-tert-butylphenyl)-6-nitrobenzoxazole; 2a: 2-(2,3-dimethylphenyl)-5-nitrobenzoxazole; 2b: 2-(2,3-dimethylphenyl)-6-nitrobenzoxazole. Data represent the mean±standard error of the mean (SEM) of three independent experiments (n=3).

TRP214, HIS121, ARG64, ARG207, TRP206, PHE252, PHE256, and SER249 through hydrogen bonds, π-π stacking, π-cation interactions, salt bridges, and hydrophobic interactions.

DISCUSSION

Lung cancer has the highest incidence and mortality rates among all malignant neoplasms, and 80%–85% of cases are classified as NSCLC. Although treatment modalities such as radiotherapy, targeted therapy, immunotherapy, and chemotherapy are available, they are often associated with severe side effects and the development of drug resistance, which significantly affect patients' quality of life. As a result, the overall cure and survival rates for NSCLC remain low.[26]

Benzoxazole is a combination of a benzene ring and an oxazole ring. This heterocyclic compound is widely utilized as a core scaffold structure in drug research and development, significantly contributing to the discovery of new therapeutic agents.[27] Several benzoxazoles are already used in the treatment of various diseases, and some are currently in clinical trials. Additionally, an increasing number of benzoxazole derivatives are being explored in the early stages of drug discovery as potential hit or lead compounds.[7] Benzoxazole is an important heterocyclic compound due to its diverse pharmacological properties, including anticancer activity. Both synthetic and naturally occurring benzoxazole derivatives have demonstrated strong anticancer effects against various human cancer cell lines.[28] The anticancer activities of benzothiazoles and their bioisosteres (benzimidazoles and benzoxazoles), have been extensively documented.[29-31] In particular, a series of benzothiazole and benzoxazole derivatives were evaluated for antitumor activity against human breast cancer cells (MCF-7 and MDA-MB-231), with N-methylpiperazinyl

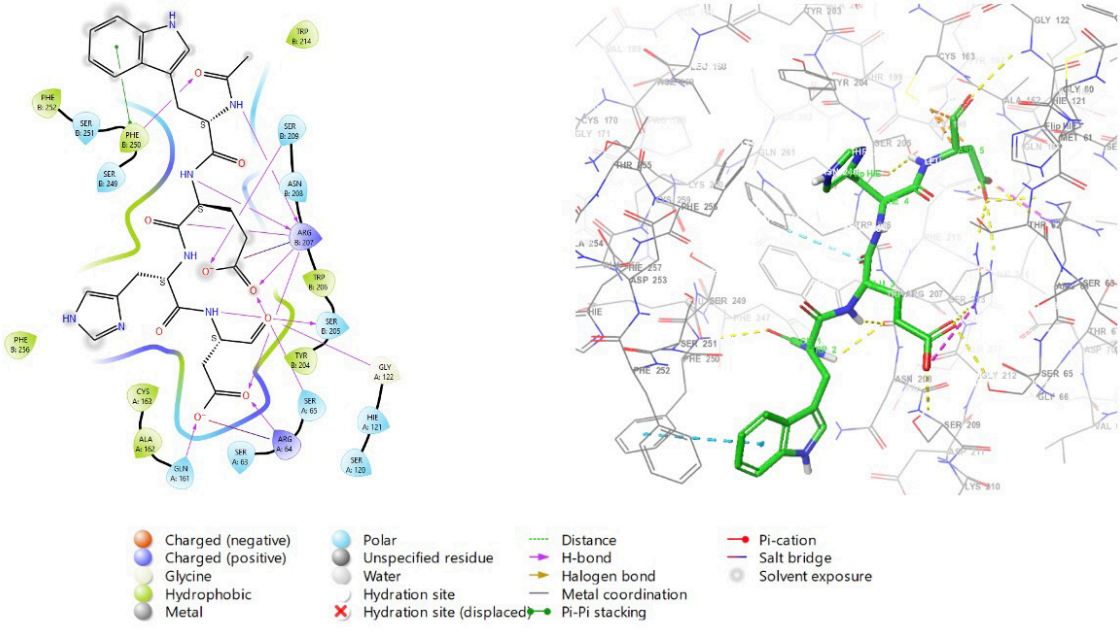

Figure 2. 2D and 3D interaction diagrams of the original ligand with the caspase-3 enzyme (PDB ID: 3GJQ). The 2D diagram shows the ligand's interactions with active-site amino acids: hydrogen bonds (purple arrows), π–π stacking (green arrows), and salt bridge interactions (two-colored lines).

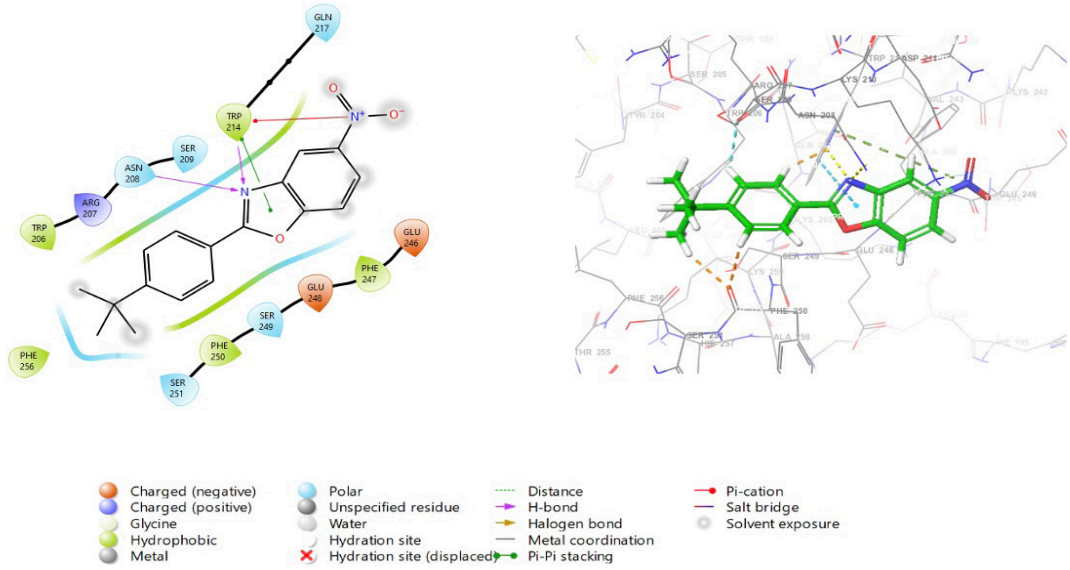

Figure 3. 2D and 3D interaction diagrams of compound 1a with the caspase-3 enzyme (PDB ID: 3GJQ). The 2D diagram shows the interactions of compound 1a with active-site amino acids: hydrogen bonds (purple arrows), π–π stacking (green arrows), and π-cation interactions (red line).

derivatives demonstrating exhibiting notably strong inhibitory effects. Docking studies have revealed potential interactions with epidermal growth factor receptors (EGFR), indicating therapeutic potential in cancer treatment.[12] Moreno-Rodríguez et al.[32] reported that 2-[(5-chlorobenzoxazol-2-yl)thio]-N-(3-fluorophenyl)-2-phenylacetamide, a benzoxazole-based amide/sulfonamide derivative, was the most cytotoxic compound in their series, demonstrating antiproliferative activity and caspase activation in colorectal cancer cells HT-29 and HCT116. Three benzoxazole derivatives were shown to exhibit strong cell growth inhibition in the HCT-116 cell line, accompanied by increased caspase-3 levels.[33] Tricyclic decylbenzoxazole

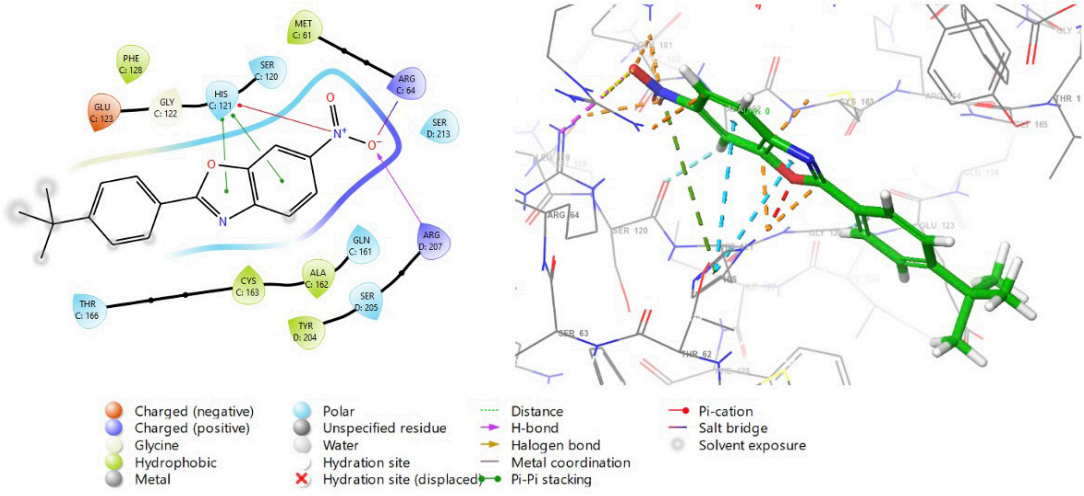

Figure 4. 2D and 3D interaction diagrams of compound 1b with the caspase-3 enzyme (PDB ID: 3GJQ). The 2D diagram shows the interactions of compound 1b with active-site amino acids: hydrogen bonds (purple arrows), π–π stacking (green arrows), π-cation interactions (red line), and salt bridge interactions (two-colored lines).

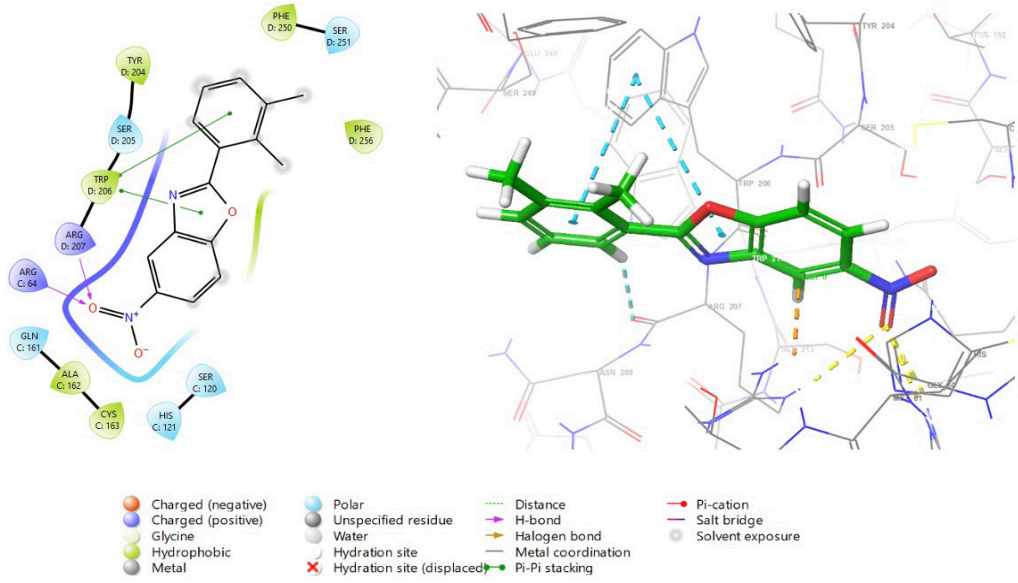

Figure 5. 2D and 3D interaction diagrams of compound 2a with the caspase-3 enzyme (PDB ID: 3GJQ). The 2D diagram shows the interactions of compound 2a with active-site amino acids: hydrogen bonds (purple arrows) and π–π stacking (green arrows).

has also been reported to inhibit proliferation and induce apoptosis in liver cancer cells (SMMC-7721).[34] In another study, several benzoxazole derivatives were synthesized as potential anticancer agents targeting sirtuin 1 (SIRT1) in NSCLC. Two of these compounds emerged as promising orally bioavailable SIRT1 modulators for targeted NSCLC therapy.[35] In a previous investigation, a 2-aminobenzothiazole derivative demonstrated the strongest activity against NSCLC HOP-92 cells. Molecular docking studies supported that the synthesized compounds bind in a manner similar to EGFR inhibitors, highlighting key interactions that may guide the design of more potent inhibitors.[30] Furthermore, novel benzoxazole-hydrazone and

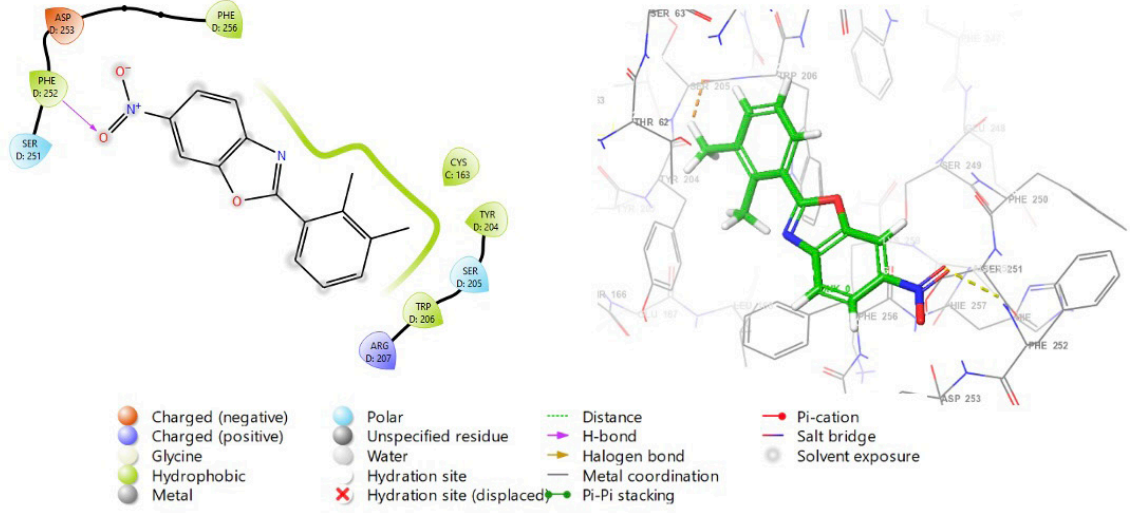

Figure 6. 2D and 3D interaction diagrams of compound 2b with the caspase-3 enzyme (PDB ID: 3GJQ). The 2D diagram shows the interaction of compound 2b with an active-site amino acid: hydrogen bond (purple arrow).

Table 2. Docking interactions of compounds 1a, 1b, 2a, 2b, and the original ligand with the caspase-3 enzyme (PDB ID: 3GJQ) predicted by Glide docking simulations

Compound	Docking Score (kcal/mol)	Glide Score (kcal/mol)	Glide Energy (kcal/mol)	Glide Evdw (kcal/mol)	Glide Emodel (kcal/mol)
1a	-5.202	-5.202	-30.874	-27.424	-39.451
1b	-4.373	-4.373	-34.514	-32.767	-43.593
2a	-4.339	-4.339	-31.524	-28.572	-40.109
2b	-4.640	-4.640	-27.456	-24.857	-35.161
Original ligand	-12.490	-13.053	-77.411	-47.695	-175.847

Docking score, Glide score, Glide energy, Glide evdw, and Glide emodel are expressed in kcal/mol. More negative docking or Glide scores indicate stronger predicted binding affinity.

benzoxazole-1,3,4-oxadiazole derivatives have been reported to induce apoptosis in human A549 lung cancer cells, consistent with their cytotoxic activity against these cells.[36]

In the present study, previously synthesized benzoxazole derivatives (1a, 1b, 2a, and 2b) were evaluated for their anticancer activities against A549 cells using the MTT cell viability assay, with cisplatin serving as the reference drug. Compounds 1a, 1b, 2a, and 2b exhibited potent anticancer activity and produced significant cytotoxic effects on A549 lung cancer cells by suppressing cell proliferation in a dose-dependent manner. The IC_{50} values of the benzoxazole derivatives ranged from 17.41 µM to 32.17 µM. Compounds 1a and 1b demonstrated greater antiproliferative activity than compounds 2a and 2b, with IC_{50} values comparable to that of cisplatin. These findings suggest that compounds 1a and 1b possess potential anticancer activity similar to that of cisplatin.

To investigate the relationship between these benzoxazole derivatives and caspase-3 enzyme activity, molecular docking studies were performed. To our knowledge, this is the first study to evaluate the interactions of these benzoxazole derivatives with caspase-3 using molecular docking analysis. According to the docking results, the tested benzoxazoles (1a, 1b, 2a, and 2b) may interact favorably within the binding site of caspase-3 (PDB ID: 3GJQ). Our compounds were observed to interact with amino acid residues similar to those of the original ligand of caspase-3, including ARG64, ARG207, TRP214, TRP206, GLY122, ASN208, TRP206, GLN161, ALA162, CYS163, PHE256, and SER249. The nitro group appeared to play an important role in mediating interactions with caspase-3. The NO_2 group at the 5-position of the benzoxazole ring in compound 1a exhibited a π-cation interaction with TRP214. In contrast, the same group at the 6-position of heterocyclic structure in compound 1b formed a π-cation interaction with HIS121 and a hydrogen bond with ARG207. While the nitrogen atom of the benzoxazole ring in compound 1a formed H-bonds with ASN208 and TRP214, no such interaction was observed for compound 1b. In compound 1a, a π–π interaction was observed with TRP214 only at the oxazole section of the benzoxazole structure, whereas in compound 1b, π–π interactions were observed at both the oxazole and benzene sections of the bicyclic structure with the HIS121 residue. Furthermore, compound 1a demonstrated a relatively lower docking score and IC_{50} value compared with the other derivatives, suggesting a preliminary indication of cytotoxic potential that warrants further investigation. Evaluation of the structure-activity relationship suggests that substitution of the phenyl group at the 2-position of the benzoxazole ring with a para-tert-butyl group contributes to increased antiproliferative activity against A549 cells.

It was observed that the nitro group played an important role in the interaction with the caspase-3 enzyme in both compounds 2a and 2b, regardless of whether it was located at the 5- or 6-position. In compound 2a, the nitro group formed H-bonds with ARG207 and ARG64, whereas in compound 2b, it formed an H-bond only with PHE252. No π–π interactions with the enzyme were observed in compound 2b, either within the heterocyclic structure or the phenyl ring. In contrast, compound 2a exhibited interactions with the TRP206 residue involving both the oxazole part of the benzoxazole structure and the phenyl ring at the 2-position. It is also suggested that substitution at the para position of the phenyl ring attached to the 2-position of the benzoxazole ring is important for hydrophobic interactions. This is supported by the observation that the tert-butyl group of the phenyl at the para position of compound 1a forms a hydrophobic interaction with the PHE256 residue.

Considering both the *in vitro* activity and molecular docking results, these compounds demonstrate preliminary cytotoxic potential, which may be associated with caspase-3 activation. Although the compounds exhibited limited activity against the hTopo IIα enzyme,[5] they may exert their effects on A549 cells through caspase-3. A limitation of the present study is that the cytotoxic effects of the benzoxazole derivatives were

evaluated only in the A549 lung cancer cell line. Comparative analysis using normal lung epithelial cells would provide additional insight into the selectivity and potential safety profile of these compounds. Future studies are planned to include mechanistic assays, such as Western blotting and Annexin V/PI staining, as well as *in vivo* efficacy and toxicity studies in NSCLC models. These investigations will provide a clearer understanding of the cytotoxic potential and underlying mechanisms of benzoxazole derivatives and guide the design of new analogs based on these compounds.

CONCLUSION

Our findings indicate that the previously synthesized benzoxazole derivatives, 1a and 1b, exhibit preliminary cytotoxic potential by inhibiting lung cancer cell proliferation at low concentrations, similar to cisplatin. Based on the structure-activity relationship within this series, substitution of the phenyl at the 2-position of the benzoxazole ring with a para-tert-butyl group may enhance anticancer activity against A549 cells. Moreover, molecular docking studies demonstrate that all benzoxazole derivatives may interact with the active site of caspase-3, suggesting a possible involvement in caspase-3–mediated mechanisms. Therefore, the anticancer effects of these compounds in A549 cells may be associated with caspase-3 activation. This preliminary study suggests that these benzoxazole derivatives have potential as cytotoxic agents for the treatment of NSCLC. Future research will focus on optimizing the potency of these compounds, elucidating their mechanisms of action through mechanistic assays, and evaluating their *in vivo* efficacy and toxicity in murine NSCLC models.

Ethics Committee Approval: As this study consisted solely of *in vitro* experiments and molecular docking analyses, ethical approval was not required.

Informed Consent: This study reports the results of experimental investigations that did not involve human or animal subjects; therefore, informed consent is not required.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Appendix 1. Cell viability (%) of A549 cells treated with different concentrations of benzoxazole derivatives for 48 hours

Concentration (X±SEM)	0 µM	20 µM	40 µM	60 µM	80 µM	100 µM
2-(4-tert-butylphenyl)-5-nitrobenzoxazole	100±9.84	29.60±3.27 [a]*p*<0.001	28.15±4.15 [a]*p*<0.001	47.97±6.70 [a]*p*=0.002	32.44±4.13 [a]*p*<0.001	37.43±1.39 [a]*p*<0.001
2-(4-tert-butylphenyl)-6-nitrobenzoxazole	100±11.34	51.36±2.35 [a]*p*=0.007	28.21±1.19 [a]*p*<0.001	24.16±2.76 [a]*p*<0.001	23.06±1.24 [a]*p*<0.001	18.43±2.83 [a]*p*<0.001
2-(2,3-dimethylphenyl)-5-nitrobenzoxazole	100±7.63	71.81±2.99 [a]*p*=0.048	25.83±3.89 [a]*p*<0.001 [b]*p*=0.001	32.11±2.39 [a]*p*<0.001 [b]*p*=0.014	38.30±2.59 [a]*p*<0.001 [b]*p*=0.033	25.23±4.09 [a]*p*<0.001 [b]*p*=0.006
2-(2,3-dimethylphenyl)-6-nitrobenzoxazole	100±7.48	68.59±4.28 [a]*p*=0.019	29.03±0.57 [a]*p*<0.001 [b]*p*=0.009	30.43±2.63 [a]*p*<0.001 [b]*p*=0.011	37.34±0.99 [a]*p*<0.001 [b]*p*=0.032	24.01±0.97 [a]*p*<0.001 [b]*p*=0.005

Cell viability (%) of A549 cells after 48-hour treatment with benzoxazole derivatives is presented as mean±standard error of the mean (SEM) from three independent experiments (n=3). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Significant p-values are indicated in the table. [a]Significantly different from the untreated group (0 µM). [b]Significantly different from the 20 µM treatment group. µM: Micromolar; SEM: Standard error of the mean.