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Investigation of the Cytotoxic Effect of A New N-Phenyl Benzimidazole Derivative on Cell Viability in A549 and HepG2 Cell Lines

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Abstract

Introduction: One of the important heterocyclic compounds that exhibit versatile biological activity properties such as anti-inflammatory, antioxidant, and anti-proliferative/anticancer activities are benzimidazole-based compounds. In this study, a new benzimidazolium salt was synthesized. The cytotoxicity of this newly synthesized compound on HepG2 and A549 cell lines was investigated.

Methods: In this study, a new benzimidazole salt was synthesized. Then, the cytotoxic effects of this compound **4** on the human liver cancer cell line (HepG2) and human lung cancer cell line (A549) were investigated by the MTT method.

Result: According to the results obtained, it was concluded that compound **4**, when applied to the human liver cancer cell line and the human lung cancer cell line under *in vitro* conditions, showed a significant cytotoxic effect on these cells. Cellular proliferation was measured for both lung A549 and liver HepG2 cells. Lung and liver cells exposed to various concentrations of compound **4** and cisplatin were observed at the 72-hour time point. Significant differences were detected in A549 cells treated with different concentrations of compound **4** and cisplatin ($p=0.001<0.05$). The doses that created the difference were found to be between 200 µM and 25 µM for compound **4** ($p = 0.017$) and between 200 µM and 50 µM concentrations ($p = 0.001$). There were also significant differences between doses for cisplatin ($p=0.001<0.05$). The concentrations that created the difference were found to be between 100 µM and 25 µM ($p = 0.042$), between 200 µM and 25 µM ($p = 0.001$), and between 200 µM and 50 µM ($p = 0.049$). As seen in Figure 1, cell viability in the high-dose compound **4** and cisplatin-treated groups (200 µM, 100 µM) is lower than the viability of cells in the low-dose compound **4** groups (50 µM, 25 µM). There is a difference between compound **4** and cisplatin ($p<0.05$). In terms of cell viability, the average of cisplatin was lower than compound **4**. In the treatment of HepG2 cells with different doses of compound **4** and cisplatin, significant differences were detected between compound **4** and cisplatin ($p=0.035<0.05$). In terms of cell viability, the average of cisplatin was higher than that of compound **4**.

Discussion and Conclusion: Benzimidazole derivatives have various biological activities, including antitumor activity. Several investigations have exhibited the bioactivities of benzimidazole derivatives as possible therapies against cancer by focusing on certain molecules or employing non-gene-specific approaches. We tested this compound against two different human cancer cell lines including lung and liver. The results show that compound **4** had cytotoxic effects on both A549 and HepG2.

Keywords: Cytotoxic activity; N-phenyl benzimidazole; HepG2, A549; cisplatin.

Introduction

Critical obstacles to cancer treatment are drug insensitivity and resistance. As a result, creating novel treatment alternatives that specifically target cancerous cells is seen as a top priority (1). Precision medicine's cornerstone is targeted therapy (2). The rapid increase in cancer cases and increasing death rates in recent years have made cancer a global burden (3). Hepatocellular carcinoma is the most common primary malignant tumor of the liver. It is the fifth most common cancer in men and the seventh in women worldwide, with more than half a million new cases diagnosed. Hepatoma cells are the most common cells used to understand both liver cancer phenotypes and the phenotype of hepatocyte cells in healty and diseased states (4). Among human hepatoma cell lines, HepG2 cells are the best characterized. The versatility and function of these cells make them very useful as a model system for liver function (5). Lung cancer, hepatocellular carcinoma, and colorectal cancer have become the most common types in the world (4). Tumor stem cell self-renewal or proliferation, aberrant drug metabolism pathways, DNA damage repair dysfunction, autophagy regulation, inhibition of apoptotic pathways, and decreasing drug concentration in tumor cells are all part of the complicated process of drug resistance in tumors (5, 6). The primary cause of tumor chemotherapy's inefficiency is the growing issue of tumor drug resistance brought on by the prolonged use of necessary chemotherapy

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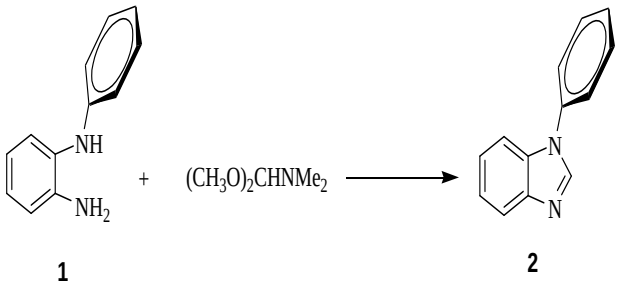
medications in clinical practice (7). To increase clinical efficacy, new medication development or the discovery of sensitizing agents is therefore important. While radiation therapy and surgery are quite effective in treating certain types of cancer, they still have limited success in curing aggressive tumors. The substantial toxicities of several anticancer drugs make chemotherapy difficult to administer and severely impair patients' quality of life (8). As a result, medicinal chemistry has to design anticancer medications with excellent selectivity and low toxicity (9). One of the first metal-based chemotherapy medications, cisplatin (cis-diamminedichloroplatinum) is still often used, even in our nation, to treat patients with a variety of cancers (10). Among the anticancer medications identified in recent years are several benzimidazole derivatives. Their diverse biological activity and clinical uses draw attention to the need for creating novel anticancer drugs. Because of their many biological properties, including their anti-inflammatory, antioxidant, and anti-proliferative properties, benzimidazole derivatives are among the most significant heterocyclic compounds (11). When it comes to anticancer medications, benzimidazole shows promise due to its ability to target specific or non-oncogene specific targets. In our study, a new N-phenylbenzimidazole derivative was prepared, and characterized and its cytotoxic potential was evaluated especially in hepatic and lung cancer cell lines.



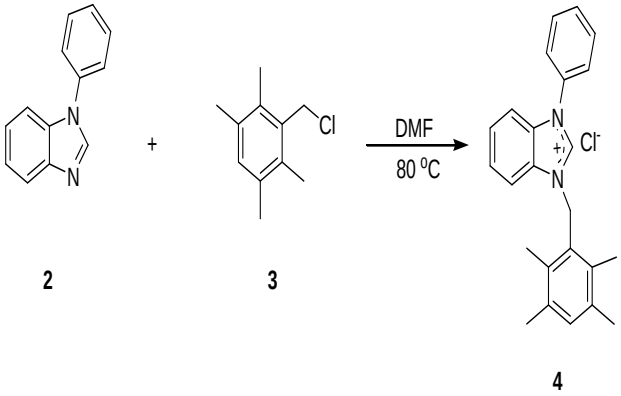
Scheme 1. Synthesis of a N-phenylbenzimidazole.

Material and Methods

Synthesis of *N*-phenylbenzimidazole

20% excess of N,N-dimethylformamide dimethylacetal (3.89 g) was added to N-phenyl-o-phenylenediamine (5 g). It was heated at water bath temperature for 3 hours to allow MeOH and $HNMe_2$ to be removed (Scheme 1). The remaining yellow oily part was distilled in a vacuum. The product was obtained with a 93% yield (28).

Synthesis of 1 – phenyl – 3 - (2,3,5,6-tetramethylbenzyl) benzimidazolium chloride: 2,3,5,6-Tetramethylbenzyl chloride (1.23 g) was added to the solution of N-phenylbenzimidazole (1.31 g) in 5 mL DMF and stirred at 60 °C for 18 hours (12) (Scheme 2).



Scheme 2. Synthesis of a benzimidazolium salt.

After the reaction mixture was cooled, diethyl ether (15 mL) was added and the resulting salt was precipitated, filtered, and dried in a vacuum. The product **4** was purified by crystallization in a mixture of ethyl alcohol-diethyl ether (1:2).

Characterization of 1 – phenyl – 3 - (2,3,5,6-tetramethylbenzyl) benzimidazolium chloride: Yield: 95%, m.p.: 205-206 °C. IR $\nu_{(CN)}$= 1590.00 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6), δ: 2.22, and 2.24 (s, 12H, C*H*$_3$); 5.82 (s, 2H, NC*H*$_2$C$_6$H(CH$_3$)$_4$); 7.70-8.56 (m, 10H, Ar-*H*); 9.59 (s, 1H, 2-C*H*). ^{13}C NMR (75.47 MHz, DMSO-d_6), δ: 15.9, and 20.6 (*C*H$_3$); 46.8 (N*C*H$_2$C$_6$H(CH$_3$)$_4$); 114.0, 114.5, 126.5, 127.6, 128.2, 128.5, 130.5, 130.9, 131.9, 132.2, 133.4, 134.6, and 135.1 (Ar-*C*); 141.9 (2-*C*H).

The cytotoxic activity studies of compound 4: Cell viability was measured via (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. A549 and HepG2 cell lines, which were purchased from the American Type Culture Collection (ATCC, USA), were cultured in DMEM supplemented with 10% FBS and 1% glutamax. Cells seeded in 25 cm^2 flasks were incubated in a CO_2 incubator. Cells were checked every day. After reaching 90% cells density, the medium in the flask was removed. Cells were washed twice with PBS. Then, 2,5 mL tyripsin-EDTA was added and incubated in CO_2 incubator for another 4 min. After the cells were seen to be lifted under the microscope, the cells were removed with a clean pipette into a centrifuge and 2.5 mL of medium was added. Cells were centrifuged at 1450 rpm for 5 min. The supernatant was discarded. 5 mL of medium was added to the remaining pellet and cell counting

was performed using a toma slide. After cell counting, 5 x 10^3 cells were seed in each well of the 96-well plate using a multichannel pipette. The plates were incubated in a CO_2 incubator for 24 hours. After the time was completed, the medium in the wells was removed and fresh medium was added instead. Cells were exposed to a compound **4** and cisplatin for 72 h (13, 14). Cisplatin and the substance were dissolved in PBS and applied to the cells. The same amount of PBS was added to the negative control group so that the PBS ratio was the same in all wells. After the 72-hour incubation period was completed, the medium in the wells was removed and 50 µL of MTT (5mg/mL) dye was added in the dark and incubated in the incubator again for two hours. After the time was reached, the medium was carefully discarded from the wells one by one with a pipette. 200 µL dimethylsulfoxide (DMSO) was added instead. It was mixed on the mixer for half an hour. Absorbance values at 590 nm were measured on an Elisa reader. After the calculations were done, IC_{50} concentration was determined using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA). All experiments were performed independently twice in two replicates and standart deviation values were calculated.

Statistical analysis: SPSS (Version: 29.02) package program was used in the evaluation. Kruskal Wallis H test was used to evaluate the change in cell viability depending on concentrations, and Mann-Whitney U test was used to compare the concentrations of compound **4** and cisplatin groups. For all analyses, $p<0.05$ was considered statistically significant.

Results

Synthesis of a benzimidazolium salt:

A benzimidazolium salt **4** was synthesized in high yield and its structure was characterized by IR, ^{1}H NMR, and ^{13}C NMR. The characteristic C=N peak was observed at 1590.0 cm^{-1} in the IR spectrum. The methylene signal belonging to the 2,3,5,6-tetramethylbenzyl group was seen in the ^{1}H NMR spectrum at 5.82 ppm. The characteristic NCHN signal indicating that the salt was observed in the ^{1}H NMR spectrum at 9.59 ppm. In ^{13}C NMR, the characteristic signal of the carbene precursor belonging to NCHN was obtained at 141.90 ppm in the low field. Additionally, other signals also supported the accuracy of the structure. Cytotoxic activity studies of a benzimidazolium salt The benzimidazole compound **4** was evaluated against two common human cancer types, A549 and HepG2 cell lines. For comparative purposes, the cytotoxicity of cisplatin (a platinum-containing anticancer drug) was evaluated under the same conditions. The MTT test was employed in cell culture investigations to determine the link between cell viability under various treatments (15, 16, 17). Compounds are evaluated for their possible anti-cancer activity using the IC_{50} value, which is the dose of the molecule that results in a 50% reduction in survival value as determined by the MTT test (18, 19). Findings regarding the viability rates of A549 and HepG2 cells cultured with cisplatin and compound **4** are given in Figures 1 and 2.

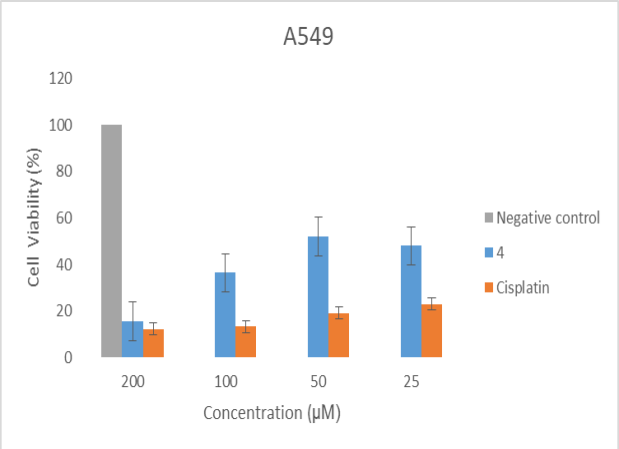


Figure 1. Cell viability assay. Graph of MTT assay showing the rate of viability A549 cells for 72 h exposure of different compound **4** and cisplatin concentrations (25, 50, 100, 200 µM).

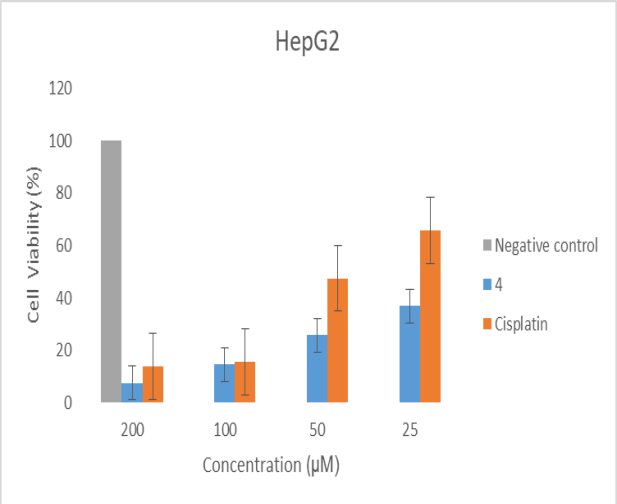


Figure 2. Cell viability assay. Graph of MTT assay showing the rate of viability HepG2 cells for 72h exposure of different compound **4** and cisplatin concentrations (25, 50, 100, 200 µM).

Table 1: IC_{50} results for compound **4** and cisplatin against human cell lines.

Compouns	IC_{50} (μM)	
	A549	HepG2
4	40.32 ± 3.90	15.85 ± 2.21
Cisplatin	9.87 ± 0.74	37.32 ± 4.53

Accordingly, when different concentrations of cisplatin (25, 50, 100, 200 μM) and compound **4** (25, 50, 100, 200 μM) were applied to A549 and HepG2 cells for 72 hours, the reached IC_{50} dose corresponded to the dose required to kill half of the cells was determined (Table 1). Compound **4** exhibited an IC_{50} value of 40.32 ± 3.90 μM against the A549 cell line. This result indicates that compound **4** shows a moderate anticancer activity in A549 cells. Cisplatin showed an IC_{50} value of 9.87 ± 0.74 μM against the A549 cell line. This result revealed that cisplatin showed a higher anticancer effect in A549 cells compared to compound **4**. Compound 4 demonstrated an IC_{50} value of 15.85 ± 2.21 μM against HepG2 cell line. Compound **4** exhibited a more effective anticancer profile compared to cisplatin, showing a lower IC_{50} value in HepG2 cell line. This lower value suggest that compound **4** is effective in HepG2 cells and has the potential to inhibit liver cancer cells. Cellular proliferation was measured for both lung A549 and liver HepG2 cells. Lung and liver cells exposed to various concentrations of compound **4** and cisplatin were observed at the 72-hour time point. Significant differences were detected in A549 cells treated with different concentrations of compound **4** and cisplatin ($p=0.001<0.05$). The doses that created the difference were found to be between 200 μM and 25 μM for compound **4** ($p = 0.017$) and between 200 μM and 50 μM concentrations ($p = 0.001$). There were also significant differences between doses for cisplatin ($p=0.001<0.05$). The concentrations that created the difference were found to be between 100 μM and 25 μM ($p = 0.042$), between 200 μM and 25 μM ($p = 0.001$), and between 200 μM and 50 μM ($p = 0.049$). As seen in Figure 1, cell viability in the high-dose compound **4** and cisplatin-treated groups (200 μM, 100 μM) is lower than the viability of cells in the low-dose compound **4** groups (50 μM, 25 μM). There is a difference between compound **4** and cisplatin ($p<0.05$). In terms of cell viability, the average of cisplatin was lower than compound **4**. In the treatment of HepG2 cells with different doses of compound **4** and cisplatin, significant differences were detected between compound **4** and cisplatin ($p=0.035<0.05$). In terms of cell viability, the average of cisplatin was higher than that of compound **4** (Figure 2).

Discussion

Benzimidazole derivatives have various biological activities, including antitumor activity (20). Several investigations have exhibited the bioactivities of benzimidazole derivatives as possible therapies against cancer by focusing on certain molecules or employing non-gene-specific approaches (2). In the conducted study by Mamedov et al. (21), cytotoxicity in cell lines administered benzimidazole derivatives was examined in the concentration range of 1-100 μM. Cell viability decreased as concentration increased in A549 and cervical carcinoma (HeLa) cell lines. In parallel with our study, Refaat (22) found excellent anticancer activity potential of benzimidazole derivatives against HepG2 cell line. In a different study, Taherian and colleagues (23) used the MTT colorimetric test to assess the cytotoxic effects of chemicals, including benzimidazole core, against MCF-7 and HeLa cell lines at 100, 200, 300, 400, and 500 μM. They found that almost all compounds had good cytotoxic activity against both cell lines. In another study, benzimidazole derivatives were found to have anti-proliferative activity not only against cancer cells but also against most of the tumor cells and were reported as strong antitumor agents (24). Argirova and colleagues evaluated the anticancer activity of benzimidazole compounds against human malignant cell lines MCF-7 and AR-230 and normal fibroblast cell lines 3T3 and CCL-1 (25). They reported that compounds exhibit significant antitumor activity. In the study by Hsieh et al. (26), against every tumor cell line tested, the majority of the different benzimidazole compounds demonstrated possible anticancer activity. IC_{50} values were measured in the concentration range of 9.73–12.47 μM in the A549 cell line and the concentration range of 10.16–10.93 μM in the HepG2 cell line. In our study, the IC_{50} value of the benzimidazole derivative **4** applied to A549 cells was measured as 40.32 ± 3.90 μM, and the IC_{50} value in HepG2 cells was measured as 15.85 ± 2.21 μM. In light of these results, compound **4** was a more potent inhibitor than cisplatin in the HepG2 cell line and therefore could be investigated as a therapeutic candidate against liver cancer. In the A549 cells line, cisplatin showed a higher cytotoxic effect than compound **4**. Taherian et al. looked into the cytotoxic effects of benzimidazole compounds on HeLa cells at various doses (μM) of the

compounds. Cell survival was assessed by means of the MTT technique. A positive control that was employed was doxorubicin. The results were reported as significant $p<0.001$ for all doses (23). All benzimidazole-based compounds were evaluated *in vitro* for potential cytotoxic effects against HepG2, and Khalifa et al. (27) discovered that the compounds' anticancer efficacy varied. It was administered at concentrations of 100 µg/mL to 500 µg/mL. Increasing concentration has been shown to lead to lower percent vitality.

Conclusion

Cancer is very common nowadays and is expected to be among the leading causes of death in the future. Currently, pharmaceutical companies are investing huge amounts of money in the innovation and development of potential and safe anticancer drugs with fewer side effects. It is well known that certain benzimidazole medications target several biomarkers, which may enhance therapy response and result in synergistic reduction of tumor growth. Therefore, in our study, we synthesized a new benzimidazolium salt and characterized its structure using IR and NMR. We tested this compound against two different human cancer cell lines including lung and liver. The results show that compound **4** had cytotoxic effects on both A549 and HepG2 cell lines.

Ethical approval: This study is not subject to the ethics committee.

Conflict of interest: The authors declare no competing interests.

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Author contributions: The authors contributed equally to the article.

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