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# Investigation of Cytotoxic Effect of a Benzimidazole Derivative in DLD-1 Cell Line Using MTT Cell Viability Test and Histopathological Parameter Methods

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**ABSTRACT**

A class of traditional chemotherapy medications with a wide range of pharmacological activity is benzimidazole. Imidazole compounds show anticancer activity both *in vitro* and *in vivo*, according to recent literature publications. Making a benzimidazole derivative and testing its anticancer properties in DLD-1 colon cancer cells was the study's goal.
Hematoxylin eosin staining, caspase-3 activity, and the MTT assay were among the biological investigations.
A microplate reader was used to measure the impact of SL-9 on cell density in light of the MTT analysis data, and a logarithmic slope line was produced. The $IC_{50}$ value of SL-9 was found 57.68 µM based on the logarithmic slope line. Over the course of treatment with 20 µM of SL-9, a notable decline in cell viability was noted. Typical apoptotic alterations, particularly nuclear condensation and nuclear fragmentation, were noted in the cells with morphological parameters 24 hours after the start of treatment. These results imply that SL-9 caused cell death by apoptosis. When measured in terms of mitotic activity, the control group showed higher levels of mitotic activity than the treatment group.
Therefore, it was discovered that the substance SL-9, a benzimidazole derivative, had a strong lethal impact when administered to colon cancer cells at varying concentrations. More thorough research should examine these beneficial effects of SL-9 treatment on colon cancer cells from a variety of angles

**Keywords:** Anti-cancer treatment, colon cancer, benzimidazole, DLD-1, Cas-3

## Inroduction

Colon cancer is one of the leading causes of cancer-related deaths in affluent countries (1,2). Colon cancer is the third most common malignant tumor and its incidence has increased over the last decade (3). According to Cusimano *et al.* (3), local invasion of colon cancer cells leads to metastases, which increases the mortality rate. Surgical resection, radiation, chemotherapy, and immunotherapy are used to treat colon cancer (4,5). More than 10% of cancer-related deaths worldwide are due to colorectal cancer, a common carcinoma (6). Drug repositioning is one of the strategies being investigated in light of the urgent need for effective treatments for colorectal cancer. Drug repositioning is the process of evaluating existing drugs for different therapeutic uses. A biological pharmacophore is the benzimidazole nucleus of the heterocyclic molecule that exhibits a wide range of biological activities (7) Benzimidazoles have attracted significant interest and research attention due to their potential therapeutic value. The benzimidazole skeleton has an important place in medicinal chemistry due to its broad spectrum of pharmacological activity (23). Several drugs containing the benzimidazole nucleus have been approved by the FDA for various clinical applications (24). Benzimidazole, as a structural isostere of nucleobases, can easily interact with various biomolecular targets and lead to various pharmacological effects including anticancer, anthelmintic, antihypertensive, antiulcer and anti-inflammatory activities (25). The nucleus of many different drugs contains benzimidazoles. Benzimidazole derivatives are frequently used in the creation of new medical agents to treat various diseases. Drug development greatly benefits from the diverse pharmacological properties of benzimidazole and its derivatives, which are the subject of numerous researches in the pharmaceutical industry. Anti-

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cancer, anti-tuberculosis, anti-acetylcholinesterase, antiviral, antiprotozoal, analgesic, antimalarial, anti-inflammatory, antifungal and antihistamine are just some of the numerous pharmacological properties reported for benzimidazole derivatives (8,9). Most anti-cancer molecules activate apoptosis, a regular process for transforming damaged cells from patients during development or homeostasis (26,27). Characteristic changes in apoptotic changes include shrinkage, condensation of chromatin, properties of mitochondrial membrane potential and fragmentation of genetic materials (26,27). In addition, the extrinsic pathway for apoptosis caspases is activated by receptors on membranes and intrinsically in life, different stress stimuli activate apoptosis (28,29). Usually, tumor cells are eliminated from the body by synthetic therapeutic agents and natural products that exploit apoptotic pathways (30,31). The cytotoxic effects of compound SL-9 on DLD-1 cell line were studied at varying doses (25, 50 and 100 μM) for 24 hours. Caspase-3 activity of the compound and hematoxylin eosin staining were evaluated. In this study, a benzimidazole molecule with possible anticancer properties was constructed and produced.

## Materials and Methods

### Compound synthesis protocol

**2-p-tolyl-1H-benzo[d]imidazole:** In this study, the following synthesis protocol was applied to obtain compound SL-9. In here, benzene-1,2-diamine (0.50 g, 1 mmol), 4-methylbenzaldehyde (0.56 g, 1 mmol) and $NiCl_2.6H_2O$ (0.11 g, 0.1 mmol) were dissolved in chloroform ($CHCl_3$) and stirred at 25 °C for 3 h on a magnetic stirrer. At the end of the time, cyclohexane was added to the reaction mixture followed by filtration using a 50 mL glass Gooch crucible. The resulting compound 2-*p*-tolyl-1*H*-benzo[*d*]imidazole (SL-9) was washed several times with cyclohexane for purification and dried under vacuum (10).

**The Cytotoxic Activity Studies:** Cytotoxic activity studies of compounds were performed according to the procedure described in the literature (11). The human colon cancer cell line (DLD-1) was purchased from the American Type Culture Collection (ATCC, USA). DLD-1 cells were cultured in Dulbecco's modified Eagle's Medium-High-Glucose (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% glutamax. The cell seeding was done at a density of $5\times10^3$ cells/well into sterile 96-well plates. The cells were exposed to the compounds at 100, 50 and 25μM concentration for 24 h. The MTT stock solution (50 μL, 5 mg/mL) was added to the plate wells and incubated for an additional 2 h. Absorbance values were measured in the Epoch 2 Elisa plate reader device at 590 nm. $IC_{50}$ values were calculated by GraphPad Prism Software 5.

**Hematoxylin-eosin Staining:** Prior to cell staining, the cells were incubated in PBS at 37°C for 15 minutes after the top medium in the culture medium was removed. Following the removal of the PBS, the cells were fixed for 30 minutes at 4 °C in 70% ethanol. Following the fixation procedure, the alcohol was discarded, the cells were stained for one minute with hematoxylin, and any extra dye was washed off. Following an ethanol treatment, the cells were stained for two minutes with eosin. They were run through a succession of escalating alcohol concentrations (50, 80, and 100%) after the excess eosin was eliminated, and they were then allowed to dry. At last, the preparations were prepared for analysis by mounting them on the slide using entellan.

**Immunohistochemical Staining:** Prior to cell staining, the cells were incubated in PBS at 37 °C for 15 minutes after the top medium in the culture medium was removed. Following the removal and disposal of the PBS, the cells were fixed for 30 minutes at 4°C in 70% ethanol. Following 10 minutes in PBS, the coverslips were incubated for 10 minutes in a 0.5% $H_2O_2$ solution made in methanol before being rinsed three times for five minutes each with distilled water. Following a 30-minute incubation period in 4N HCl, the samples underwent three 5-minute PBS washes. After that, a 20-minute protein block was applied. A 1:100 dilution of Cleaved Caspase-3 (Asp175) (Cell Signaling, 9661) primary antibody and HRP-conjugated goat anti-rabbit antibody (Abcam, ab236466) were utilized for caspase-3 detection. Following washing, coverslips were counterstained with Mayer hematoxylin and treated for equal amounts of time with DAB chromogen. After passing through a succession of escalating alcohol concentrations (50, 80, and 100%), they were allowed to dry. At last, the preparations were ready for analysis and they were placed on the slide using entellan. Cells were manually counted according to immunopositivity in ten randomly chosen fields at 20× magnification (bar 50 μm) in order to assess the staining. The Chi-square test was used in the Jamovi program to statistically compare the two groups.

## Results

**Determination of anti-proliferative effect of SL-9 by MTT method:** Variations in cell survival rates were assessed by MTT assay during a 72-hour incubation period of DLD-1 cells with varying doses of SL-9 (25, 50, and 100 μM). A microplate reader was used to measure the impact of SL-9 on cell density in light of the MTT analysis data, and a logarithmic slope line was produced. The $IC_{50}$ value of SL-9 was found 57.68 μM based on the logarithmic slope line (Table 1).

**Cytopathological findings:** Over the course of treatment with 20 μM SL-9, a notable decline in cell viability was noted. Typical apoptotic alterations, particularly nuclear condensation and nuclear fragmentation, were noted in the cells with morphological parameters 24 hours after the start of treatment. These results imply that SL-9 caused cell death by apoptosis. When measured in terms of mitotic activity, the control group showed higher levels of mitotic activity than the treatment group (Figure 1).

**Immunohistochemical findings:** Cells were treated with SL-9 for 24 hours in order to investigate the apoptotic effects of SL-9 on DLD-1 cells. Using immunocytochemical staining, changes in the activities of the caspase-3 enzyme were evaluated. When DLD-1 cells were incubated with 20 μM SL-9 for 24 hours, their caspase-3 activity increased statistically significantly ($p<0.001$) in comparison to untreated cells (Figure 2). These results imply that caspase-3 activity may be connected to SL-9-induced apoptosis.

**Table 1:** $IC_{50}$ Value Obtained After 24 Hours of Incubation of SL-9 Applied to DLD-1 Cell Culture

| Compounds | $IC_{50}$ (μM) |
|---|---|
| | DLD-1 |
| SL-9 | 57.68 |

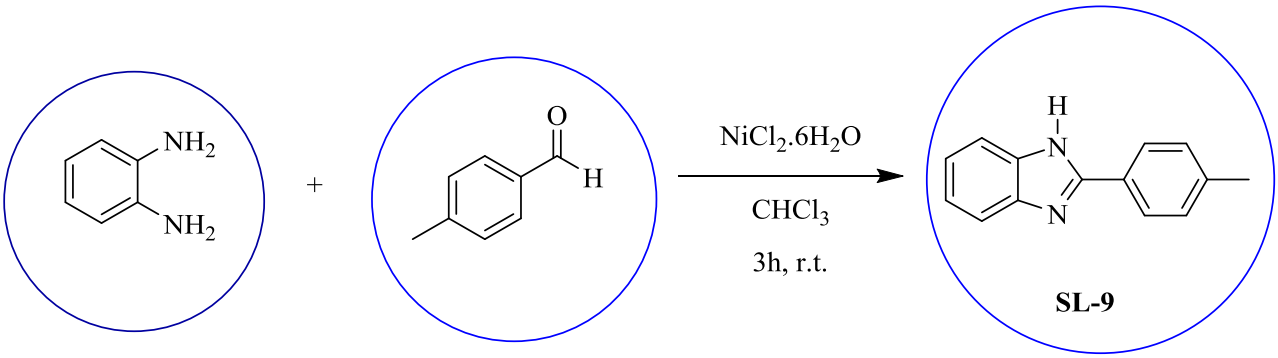
## Discussion

Identification of abnormalities in cell death signaling has become an important reason for the search for new treatment strategies. Targeting cell death in cancer covers an important area of treatment (12). One of the major challenges in the treatment of colorectal cancer is the high incidence of recurrence. 20% of patients experience recurrence after surgery and adjuvant therapy is required to reduce the chance of recurrence (13). Most drugs used to treat carcinomas are associated with the activation of apoptosis via mitochondrial pathways (32,33). The change in mitochondrial membrane potential is believed to be an important indicator of the onset of apoptosis (34,35). Activation of apoptosis by anticancer compounds has been investigated mainly to treat various types of cancer (27,28). In the present study, benzimidazole exposure showed a concentration-dependent inhibitory effect on DLD-1 cell proliferation. Apoptotic properties were observed in DLD-1 cells when exposed to benzimidazole for 24 h. Exposure of DLD-1 cells to benzimidazole resulted in an increase in the proportion of apoptotic cells compared to control cultures. The aim of this study was investigate the effects of a benzimidazole analog on *in vitro* colorectal cancer cell growth and apoptosis. Human DLD-1 colorectal cancer cells were treated with increasing concentrations of a structural analog of benzimidazole. The results showed that SL-9 induced cell death and suppressed *in vitro* colorectal cancer cell proliferation.

A key benefit of this strategy is the significantly lower time and risk associated with bringing a drug to market compared to creating new drugs from scratch. The pharmacokinetics, pharmacodynamics, and side effects of repurposed drugs have been described in detail (14). In particular, a number of repurposed drugs, including sirolimus, aspirin, and metformin, have shown promise in the treatment of colorectal cancer (15,16,17).

Akkoc et al (18), after synthesizing three benzimidazole derivatives, found that a compound had the most promising anti-breast cancer property compared to regular cisplatin ($IC_{50}$: 1.26 ± 0.85 vs. 5.77 ± 0.40). In a different study, Atmaca et al (19) found that the chemical was significantly more cytotoxic than 5-fluorouracil, with $IC_{50}$ values of 17.8 ± 0.24, 10.2 ± 1.4, and 49.9 ± 0.22 μg/ml against lung cancer (H69AR), prostate cancer (DU-145), and breast cancer (MCF-7), respectively. Compared to cisplatin ($IC_{50}$: 30.38 and 60.79 μM), the compounds showed higher anticancer properties against HepG2 ($IC_{50}$: 26.62 and 20.29 μM, respectively) and DLD-1 cells ($IC_{50}$: 21.29 and 19.23 μM, respectively) (20).

In the present study, the mechanism of *in vitro* activity of benzimidazole analog (SL-9) against cancer cells was investigated using caspase-3 activity. The findings showed that treatment with SL-9 increased the apoptotic cell rate in DLD-1




**Scheme 1.** Synthesis of a compound including benzimidazole core

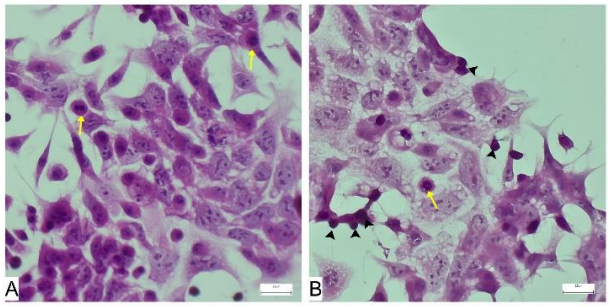



**Fig. 1.** Image of DLD-1 cells stained with hematoxylin-eosin. A. Mitotic activity is indicated by yellow arrows (control). B. Black arrowheads indicate apoptotic cells, while yellow indicates mitotic activity (SL-9). 20 µm is the bar

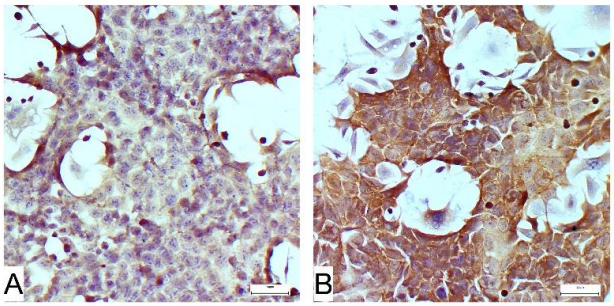



**Fig. 2.** Immunoreaction of DLD-1 cells with cleaved-caspase3 antibody. A. Control. B. SL-9. Bar: 50 µm

cells in a dose-dependent manner (Figure 2). In a separate study, α-mangostin, a xanthones derived from Garcinia mangostana, was shown to similarly reduce cell viability and induce apoptosis when applied at concentrations higher than 20 µM (21). In another study, Bortezomib, an anticancer drug used in cancer treatment, was shown to significantly increase the rate of necrotic and apoptotic cells in DLD-1 colon cancer cells. According to some theories, bortezomib treatment caused higher apoptosis and necrosis, especially in hypoxic conditions than in normoxic conditions (22). In this study, benzimidazole injection was found to increase caspase-3 activity in human colon cancer DLD-1 cells.

Therefore, it was discovered that the substance SL-9, a benzimidazole derivative, had a strong lethal impact when administered to colon cancer cells at varying concentrations. The DLD-1 cell line's cell survival and proliferation were considerably inhibited by this chemical, according to the analyses. Although the MTT results indicated a dose-dependent reduction in the cells' metabolic activity, cytopathological analyses and immunocytochemical data verified that this effect resulted in notable alterations to the morphology of the cells. According to these results, SL-9 may have antiproliferative and maybe anticancer effects on colon cancer cells. More thorough research should examine these beneficial effects of SL-9 treatment on colon cancer cells from a variety of angles.

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**Ethics Committee Approval:** Our study did not involve invasive procedures on animals, so ethics committee approval was not necessary.

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