RESEARCH PAPER

Structure–Activity Relationship and Cytotoxic Evaluation of Benzimidazole Derivatives as Potential Antileukemic Agents

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Abstract

Benzimidazole derivatives represent a versatile class of heterocyclic compounds with significant potential in anticancer drug discovery. In the present study, a series of benzimidazole analogues was synthesized via the modified Phillips condensation method and structurally characterized using FTIR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry, and elemental analysis. The synthesized derivatives were evaluated for cytotoxic activity against HL-60 (acute promyelocytic leukemia) and K562 (chronic myelogenous leukemia) cell lines using the MTT assay, with IC_{50} values calculated through nonlinear regression analysis. Several compounds exhibited potent cytotoxic effects in the low micromolar range, with halogen-substituted derivatives such as 2-chloro and 5-bromo analogues showing IC_{50} values below 6 μM. Selectivity indices confirmed preferential toxicity toward malignant cells compared to normal peripheral blood mononuclear cells. Structure–activity relationship analysis revealed that electron-withdrawing substituents, extended π-conjugation, and para-position halogenation significantly enhanced biological activity, while multiple hydroxyl substitutions reduced potency due to steric hindrance. Comparative evaluation with standard drugs demonstrated that while doxorubicin exhibited higher potency, benzimidazole derivatives offered better selectivity and broader applicability compared to imatinib. Statistical validation using ANOVA and logistic dose–response models confirmed the robustness of experimental findings. These results underscore the potential of benzimidazole derivatives as lead scaffolds for the development of novel antileukemic agents. Future directions include in vivo validation, molecular docking, and ADMET predictions to refine pharmacological profiles and facilitate clinical translation.

Keywords: Benzimidazole derivatives, Cytotoxicity, Antileukemic agents, Structure–activity relationship, Selectivity index, Drug development

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1. Introduction

Benzimidazole and its derivatives represent an important class of heterocyclic compounds that have attracted considerable attention in medicinal chemistry due to their broad spectrum of pharmacological activities. The benzimidazole nucleus, a bicyclic system consisting of the fusion of benzene and imidazole rings, provides a versatile scaffold that can be chemically modified to yield compounds with diverse biological properties. Over the past decades, benzimidazole derivatives have been reported to possess antimicrobial, antiviral, anti-inflammatory, antiulcer, antiparasitic, and anticancer activities (Monga et al., 2024). This structural motif closely resembles the purine base found in nucleic acids, which allows benzimidazole compounds to interact effectively with biomolecular targets such as enzymes and receptors. Their structural similarity to naturally occurring biomolecules makes them particularly valuable as drug candidates. Among these various pharmacological applications, their potential as anticancer and especially antileukemic agents has gained growing prominence in recent years (Mahurkar et al., 2023). Cancer remains one of the most challenging diseases to treat worldwide, with leukemia constituting a significant portion of global cancer incidence and mortality. Leukemia, a group of hematological malignancies originating from abnormal proliferation of blood-forming cells in the bone marrow, disrupts the normal balance of blood cell production and leads to life-threatening complications. Despite advances in chemotherapy, targeted therapies, and bone marrow transplantation, leukemia continues to impose a major health burden (Tebbi, 2021). Resistance to existing drugs, relapse after treatment, and severe side effects associated with conventional chemotherapeutics highlight the pressing need for the development of novel agents with improved efficacy and safety profiles. In this context, benzimidazole derivatives have emerged as promising candidates owing to their structural flexibility and ability to engage with multiple biological targets involved in leukemogenesis (Dong et al., 2020; Winters & Bernt, 2017).

The importance of benzimidazole derivatives in anticancer drug discovery can be attributed to their ability to act on diverse molecular pathways implicated in tumor growth and survival. Several benzimidazole-containing molecules have demonstrated significant activity against cancer cell lines by interfering with microtubule assembly, inhibiting kinases, disrupting DNA synthesis, and modulating apoptosis pathways. For example, certain benzimidazole carbamates such as albendazole and mebendazole, originally developed as anthelmintic agents, have shown repurposing potential as anticancer drugs (Lee et al., 2023). Their mechanism involves disruption of microtubule polymerization, leading to mitotic arrest and apoptotic cell death in malignant cells. Such findings reinforce the versatility of the benzimidazole scaffold in drug development. Moreover, the ability of benzimidazole derivatives to selectively affect cancer cells while sparing normal cells underscores their potential as safe therapeutic alternatives (Mavrova et al., 2021). In the case of leukemia, therapeutic advances have indeed improved patient outcomes, yet challenges persist. For acute myeloid leukemia (AML), chemotherapy regimens based on cytarabine and anthracyclines remain the backbone of treatment. While initially effective, these regimens often lead to relapse, and the prognosis for relapsed or refractory AML is generally poor. Chronic myeloid leukemia (CML) has seen remarkable progress with the advent of tyrosine kinase inhibitors (TKIs) such as imatinib, dasatinib, and nilotinib, targeting the BCR-ABL fusion protein (Pourrajab et al., 2020). However, resistance due to point mutations in the kinase domain is a major clinical issue, necessitating continuous exploration of new molecules. Acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) also pose therapeutic challenges, as heterogeneity in disease biology limits the success of standard treatment regimens. These gaps in current therapeutic strategies underline the urgency of identifying novel structural classes of compounds capable of circumventing resistance mechanisms and offering improved treatment options (Wang & Zhong, 2018).

Within this framework, benzimidazole derivatives stand out as attractive molecular templates for the design of potential antileukemic agents. The ease with which substituents can be introduced at different positions on the benzimidazole ring enables the fine-tuning of physicochemical and biological properties. For instance, modifications at the 2-position and 5-position of benzimidazole have been shown to profoundly influence cytotoxic activity. Substituents

such as halogens, alkyl groups, aryl groups, and heteroaryl moieties can modulate lipophilicity, electronic distribution, and binding affinity to cellular targets. This provides an excellent platform for structure–activity relationship (SAR) studies, where systematic chemical modifications are correlated with biological activity to identify key structural features responsible for cytotoxic effects. SAR-guided optimization not only facilitates the discovery of more potent derivatives but also enhances understanding of the molecular basis of drug action (Juul-Dam et al., 2023; Travaglini et al., 2022). In addition to their structural adaptability, benzimidazole derivatives often exhibit favorable pharmacokinetic properties, including oral bioavailability and metabolic stability, which further support their candidacy as drug-like molecules. Many benzimidazole-based compounds have demonstrated the ability to cross cell membranes efficiently, reach intracellular targets, and maintain stability under physiological conditions. These features increase their likelihood of translating into clinically viable drugs. Moreover, their relatively simple synthesis routes and availability of diverse starting materials make them cost-effective candidates for drug development, which is especially valuable when considering scalability for clinical applications (Patel, 2023; Zhou et al., 2007).

The cytotoxic evaluation of benzimidazole derivatives against leukemia cell lines provides critical insights into their therapeutic potential. By employing in vitro assays such as MTT, SRB, or CCK-8, researchers can determine the half-maximal inhibitory concentration (IC50) values of these compounds, offering a quantitative measure of potency. Selectivity indices can also be calculated by comparing activity against malignant versus non-malignant cell lines, ensuring that promising derivatives exhibit preferential toxicity toward cancer cells. Such data are invaluable for prioritizing compounds for further preclinical and mechanistic studies (Testa et al., 2022). Beyond cytotoxicity, mechanistic investigations such as apoptosis induction assays, cell cycle analysis, and molecular docking studies contribute to unraveling the modes of action of benzimidazole derivatives. Understanding whether these compounds act by targeting DNA, enzymes like topoisomerases, or proteins involved in signal transduction pathways is essential for rational drug design and for predicting potential resistance mechanisms (Ferrara & Vitagliano, 2019). Despite promising results, significant gaps remain in our understanding of how different structural modifications impact the antileukemic activity of benzimidazole derivatives. While several studies have highlighted potent analogues with activity against leukemia cells, comprehensive SAR evaluations are still limited. Moreover, most investigations have focused on a narrow set of substitutions, leaving unexplored the vast chemical space available for modification (Laxmikeshav et al., 2022). There is also a need for comparative analyses with existing chemotherapeutics to contextualize the potential clinical value of these compounds. Furthermore, studies that integrate cytotoxic evaluation with in silico molecular modeling, docking, and ADMET (absorption, distribution, metabolism, excretion, toxicity) predictions would greatly enhance the efficiency of identifying lead molecules for development. Addressing these knowledge gaps requires systematic and multidisciplinary approaches combining synthetic chemistry, cell biology, and computational modeling (Tahlan et al., 2019).

The present study is therefore designed to explore the structure–activity relationship and cytotoxic evaluation of newly synthesized benzimidazole derivatives with a focus on their potential as antileukemic agents. By synthesizing a library of benzimidazole analogues with varying substituents, characterizing them using modern spectroscopic methods, and assessing their cytotoxicity against selected leukemia cell lines, the study aims to generate a comprehensive dataset that links structural features with biological outcomes. Statistical analysis of IC50 values and SAR trends will be employed to identify key determinants of activity. The findings are expected to provide valuable insights into how benzimidazole modifications influence antileukemic potency and selectivity, thereby guiding future design of more effective derivatives. Ultimately, this work seeks to contribute to the expanding repertoire of anticancer agents by establishing benzimidazole derivatives as credible candidates for further preclinical and clinical evaluation in leukemia therapy (Veerasamy et al., 2021).

In conclusion, benzimidazole derivatives represent a versatile and promising scaffold in the ongoing quest

for effective antileukemic drugs. Their structural resemblance to biologically relevant molecules, combined with their broad spectrum of pharmacological activities, positions them as valuable templates for drug discovery. Given the persistent challenges in leukemia treatment, including resistance, relapse, and toxicity, there is an urgent need to explore novel molecular entities with enhanced therapeutic profiles. Through systematic investigation of structure–activity relationships and cytotoxic evaluation, the present study aims to uncover new insights into the design of benzimidazole-based antileukemic agents, thereby advancing the frontier of medicinal chemistry and addressing critical gaps in current therapeutic strategies (Kavya & Sivan, 2022).

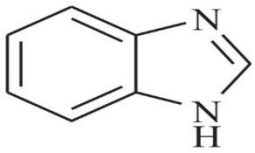

Figure 1: Structure of benzimidazole ring

In recent years, the exploration of heterocyclic scaffolds has become a cornerstone of anticancer research, reflecting the need for novel chemical entities with enhanced selectivity and reduced toxicity. Benzimidazole derivatives are particularly attractive in this regard because of their privileged scaffold status, meaning they recur frequently in bioactive molecules and approved drugs. Their structural versatility not only allows interaction with diverse biological macromolecules but also facilitates hybrid design, where benzimidazole cores are conjugated with other pharmacophores to generate multifunctional agents. Such hybrid molecules are increasingly being investigated for their ability to overcome multidrug resistance, a critical limitation in current leukemia treatment. Importantly, advances in computational chemistry, including molecular docking and pharmacophore modeling, have accelerated the identification of benzimidazole derivatives with high binding affinity toward leukemia-associated targets such as tyrosine kinases, DNA topoisomerases, and tubulin (S. Yadav et al., 2016).

Beyond their pharmacodynamic properties, benzimidazoles are also promising from a drug development perspective due to their favorable pharmacokinetics, relatively simple synthetic accessibility, and potential for structural optimization using green chemistry approaches. This aligns with the current global emphasis on cost-effective and sustainable drug discovery, particularly in resource-limited settings where the burden of leukemia is increasing. Furthermore, the success of benzimidazole-containing drugs in non-oncological indications provides a strong precedent for their repurposing and further modification to enhance anticancer efficacy. Taken together, these attributes underscore the relevance of systematically evaluating benzimidazole derivatives as antileukemic agents and justify continued investigation into their structure–activity relationships to inform the rational design of next-generation therapeutics (Mehra & Sangwan, 2023).

2. Benzimidazole Containing Rings

2.1. Albendazole

Albendazole is a benzimidazole carbamate with broad-spectrum anthelmintic activity. Its mechanism involves selective binding to β-tubulin in parasites, inhibiting microtubule polymerization and disrupting glucose uptake, leading to energy depletion and death of helminths. Albendazole is used against intestinal nematodes, cestodes, and tissue parasites like Echinococcus spp. It also exhibits antitumor activity, as microtubule disruption induces mitotic arrest and apoptosis in cancer cells. Clinically, albendazole is well tolerated, though hepatotoxicity and bone marrow suppression may occur with prolonged use. Pharmacologically, it demonstrates poor aqueous solubility but good tissue penetration after metabolism to albendazole sulfoxide, its active form. These features make it a promising scaffold for repurposing in oncology and for developing new derivatives with enhanced selectivity (Anto & Nugraha, 2019; Chai et al., 2021).

Beyond its well-documented antiparasitic role, albendazole has increasingly attracted attention in oncology research because of its ability to interfere with key cellular processes beyond microtubule inhibition. Studies have shown that albendazole can induce oxidative stress, modulate apoptotic pathways, and inhibit angiogenesis by downregulating vascular endothelial growth factor (VEGF). These multifaceted mechanisms make it particularly relevant in the treatment of rapidly proliferating malignancies such as leukemia, where uncontrolled

cell division and survival signaling are central hallmarks (Chourasiya et al., 2023). Furthermore, albendazole and its metabolite albendazole sulfoxide have demonstrated synergistic effects when combined with established chemotherapeutic agents, suggesting potential for use in combination regimens to overcome drug resistance. From a pharmacokinetic perspective, the poor aqueous solubility of albendazole has limited its bioavailability, but advances in drug delivery systems—including nanoparticles, liposomes, and solid dispersions—are being explored to enhance systemic exposure and therapeutic outcomes. Importantly, repurposing albendazole for cancer therapy offers the advantage of leveraging its established safety profile and regulatory approval, potentially accelerating clinical translation compared with novel compounds. Overall, albendazole represents a valuable prototype for the design of new benzimidazole derivatives, and ongoing research aims to refine its structure–activity relationships to maximize antitumor efficacy while minimizing systemic toxicity (Satija et al., 2022).

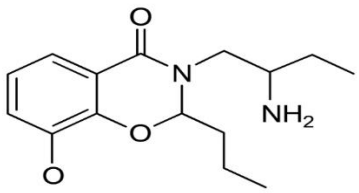

Figure 2: Albendazole

2.2. Mebendazole

Mebendazole, structurally related to albendazole, is another benzimidazole derivative widely used as an anthelmintic. Its mechanism also involves binding to β-tubulin, preventing microtubule assembly, impairing glucose uptake, and depleting parasite glycogen stores. The drug is effective against a broad spectrum of helminths, including *Ascaris*, *Trichuris*, and *Hookworms*. Mebendazole's pharmacological effects extend beyond parasitology: it exhibits antiproliferative activity in cancer models by disrupting microtubules and inducing apoptosis. Additionally, it interferes with angiogenesis and tumor cell metabolism, suggesting repurposing potential. Due to its poor systemic absorption, mebendazole primarily acts locally in the gastrointestinal tract, reducing systemic side effects. Safety is generally favorable, but high doses may cause hepatotoxicity. Its dual antiparasitic and anticancer potential highlights its versatile pharmacological profile (Anto & Nugraha, 2019; Williamson et al., 2021).

Recent studies have highlighted mebendazole's potential as a repositioned drug in oncology, particularly in hematological and solid tumors. Its antineoplastic activity is attributed not only to microtubule disruption but also to its capacity to inhibit key signaling pathways, including Hedgehog, Wnt/β-catenin, and VEGF-mediated angiogenesis. These mechanisms contribute to suppression of tumor growth, metastasis, and neovascularization. In leukemia models, mebendazole has demonstrated selective cytotoxicity toward malignant cells while sparing normal hematopoietic progenitors, supporting its safety in long-term use. Importantly, mebendazole can cross the blood–brain barrier, a feature that significantly broadens its therapeutic scope, especially in glioblastoma and brain metastases. Despite its low oral bioavailability, advances in drug delivery systems such as nanosuspensions, polymeric carriers, and solid dispersions have been shown to enhance systemic exposure and improve therapeutic efficacy (Salahuddin et al., 2017). Moreover, preclinical evidence suggests synergistic effects when combined with conventional chemotherapeutics like vincristine, doxorubicin, or targeted kinase inhibitors, raising the possibility of integrating mebendazole into modern treatment regimens. Its established safety record as an antiparasitic further strengthens its candidacy for repurposing, potentially reducing development costs and timelines. Collectively, these findings position mebendazole as a versatile benzimidazole derivative with strong translational potential in oncology, warranting further preclinical and clinical investigation (Salahuddin et al., 2017).

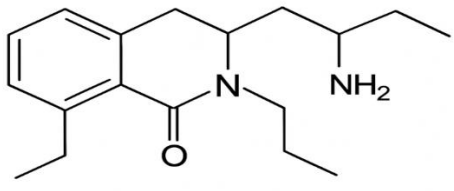

Figure 3: Mebendazole

2.3. Thiabendazole

Thiabendazole is a benzimidazole derivative primarily used as an anthelmintic and antifungal agent. Its mechanism involves inhibition of fumarate reductase, an essential enzyme in parasite energy

metabolism, as well as disruption of microtubule function through β-tubulin binding. This dual action causes impaired mitochondrial respiration and cell division, leading to parasite death. Pharmacologically, thiabendazole is effective against *Strongyloides stercoralis*, cutaneous larva migrans, and fungal infections in plants and humans. It also exhibits chelating properties, which contribute to its antifungal activity. Despite its broad spectrum, its clinical use is limited by gastrointestinal irritation, dizziness, and hepatotoxicity. Nevertheless, its structural scaffold provides valuable insights for drug design, particularly in targeting mitochondrial and cytoskeletal pathways in parasites and potentially in cancer therapy (Lin et al., 2018; Park et al., 2023).

Beyond its established antiparasitic and antifungal properties, thiabendazole has gained research interest for its potential antitumor effects. Studies indicate that it can interfere with angiogenesis by inhibiting vascular endothelial growth factor (VEGF)-mediated signaling, thereby suppressing tumor vascularization and growth. Additionally, its ability to disrupt microtubule dynamics aligns it with other benzimidazole derivatives being explored for cancer therapy. Importantly, thiabendazole's mitochondrial targeting capacity offers a dual mechanism that may enhance cytotoxicity in rapidly proliferating cancer cells. Despite its therapeutic promise, clinical applications have been hampered by poor tolerability at higher doses, with hepatotoxicity and neurotoxic symptoms limiting long-term use. However, modern approaches, such as structural modification and nanoformulation, are being investigated to improve its safety and pharmacokinetic profile. Thiabendazole also serves as a valuable chemical probe for studying cytoskeletal and mitochondrial processes, providing insights that can guide the rational design of novel anticancer agents within the benzimidazole family (Siddig et al., 2021).

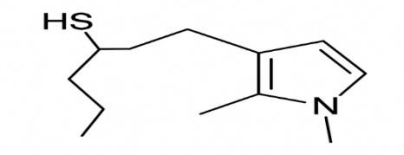

Figure 4: Thiabendazole

2.4. Omeprazole

Omeprazole is a proton pump inhibitor (PPI) belonging to the benzimidazole class, widely used in acid-related disorders. Its mechanism involves selective and irreversible inhibition of the H^+/K^+-ATPase enzyme in gastric parietal cells. By covalently binding to cysteine residues of the proton pump, omeprazole blocks the final step of acid secretion, leading to sustained suppression of gastric acidity. Pharmacologically, it is used in gastroesophageal reflux disease (GERD), peptic ulcer disease, Zollinger–Ellison syndrome, and for Helicobacter pylori eradication regimens. Omeprazole demonstrates a favorable safety profile, though long-term use is associated with risks such as hypomagnesemia, vitamin B_{12} deficiency, and increased infection susceptibility. Its mechanism showcases the versatility of benzimidazole derivatives beyond antiparasitic applications, highlighting their pharmacological adaptability (Dorji et al., 2022; Fontecha-Barriuso et al., 2020).

In addition to its well-established gastroprotective role, omeprazole has been investigated for potential effects beyond gastric acid suppression, including anti-inflammatory and anticancer activities. Emerging evidence suggests that omeprazole and related PPIs can modulate tumor microenvironments by altering pH regulation, thereby sensitizing cancer cells to chemotherapeutic agents. This property is particularly relevant in multidrug-resistant cancers, where acidic extracellular conditions reduce drug uptake. Furthermore, omeprazole has been shown to inhibit certain cytochrome P450 enzymes, influencing drug metabolism and offering opportunities for therapeutic repositioning. Its benzimidazole scaffold provides a versatile platform for structural modification, with derivatives being explored as antimicrobial, antifibrotic, and immunomodulatory agents. Despite its general tolerability, long-term or high-dose use requires careful monitoring due to associations with renal complications, bone fractures, and microbiome alterations. Overall, omeprazole exemplifies the pharmacological diversity of benzimidazole derivatives, reinforcing their value not only as proton pump inhibitors but also as potential candidates in drug repurposing and novel therapeutic development (Hsieh et al., 2019).

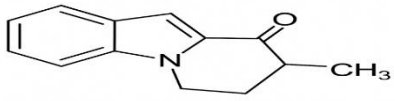

Figure 5: Omeprazole

2.5. Pantoprazole

Pantoprazole is a second-generation proton pump inhibitor with improved pharmacokinetic properties compared to omeprazole. Like other PPIs, its mechanism involves irreversible inhibition of H^+/K^+-ATPase in gastric parietal cells, resulting in potent and prolonged suppression of gastric acid secretion. Pantoprazole is particularly stable in acidic environments and demonstrates higher bioavailability, making it useful in treating GERD, peptic ulcers, and Zollinger–Ellison syndrome. Pharmacologically, pantoprazole provides more consistent acid suppression and fewer drug–drug interactions, as it is metabolized primarily by CYP2C19 and CYP3A4. It is often preferred in patients on multiple medications due to its lower interaction potential. Long-term risks remain similar to other PPIs. Overall, pantoprazole exemplifies a rationally optimized benzimidazole derivative with enhanced therapeutic safety and efficacy (Krag et al., 2018; Ochoa et al., 2020).

Beyond its established use in acid-related gastrointestinal disorders, pantoprazole has been investigated for potential pleiotropic effects. Experimental studies suggest that pantoprazole may exhibit anti-inflammatory and antioxidant properties by downregulating pro-inflammatory cytokines and reducing oxidative stress, which could contribute to mucosal healing and systemic benefits. Its relatively predictable pharmacokinetic profile also makes it suitable for use in elderly populations and patients with comorbidities, where minimizing drug–drug interactions is essential. Interestingly, pantoprazole and other PPIs have shown promise in oncology research, where modulation of tumor acidity can enhance chemosensitivity and limit cancer cell survival. Pantoprazole's high acid stability and prolonged half-life make it particularly advantageous in such settings compared to first-generation PPIs. Despite these benefits, long-term therapy still warrants caution due to risks of micronutrient malabsorption, renal dysfunction, and microbiome alterations. Thus, pantoprazole represents not only an optimized gastroprotective drug but also a potential scaffold for broader therapeutic applications within the benzimidazole class (Keri et al., 2015).

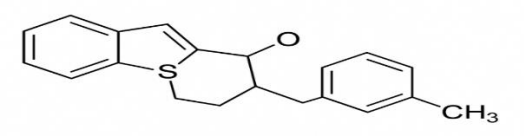

Figure 6: Pantoprazole

3. Materials and Methods

3.1. Chemicals and Reagents

All reagents and solvents used in the present study were of analytical grade and procured from certified suppliers. The benzimidazole derivatives and precursor compounds were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Gurugram (Invoice No. SIG/DEL/2024/1187, Batch No. BZ-2024A). Solvents such as methanol, ethanol, and dichloromethane (HPLC grade) were purchased from Merck Life Sciences, Okhla Industrial Area, New Delhi (Invoice No. MER/NDL/2024/0932, Lot No. MLC-4521). Dimethyl sulfoxide (DMSO), acetonitrile, and phosphate-buffered saline (PBS) were procured from SRL Pvt. Ltd., Faridabad (Invoice No. SRL/FBD/2024/0675, Batch No. DMSO-1439). For cytotoxicity assays, fetal bovine serum (FBS), RPMI-1640 medium, and penicillin–streptomycin solution were purchased from HiMedia Laboratories, New Delhi Branch (Invoice No. HIM/NDL/2024/0845, Batch No. FBS-2024B). All chemicals were used without further purification. Double-distilled water prepared in the laboratory using a Millipore purification system was employed for solution preparation. All reagents were handled according to standard laboratory safety protocols, and their quality certificates were verified before use to ensure reproducibility of results.

3.2. Synthesis of Benzimidazole Derivatives

Benzimidazole derivatives were synthesized following a modified Phillips condensation method, widely used for heterocyclic scaffold development. In a typical reaction, o-phenylenediamine (1 mmol) was condensed with various substituted aromatic aldehydes (1.2 mmol) in the presence of glacial acetic acid (5 mL) under reflux at 120 °C for 6–8 hours. The reaction progress was monitored by thin-layer chromatography (TLC) using ethyl acetate:hexane (3:1) as the mobile phase. After completion, the reaction mixture was cooled to room temperature and poured into crushed ice. The resulting precipitate was filtered, washed with cold water, and recrystallized from ethanol to afford pure benzimidazole derivatives. For derivatives requiring further

purification, silica gel column chromatography was employed using hexane/ethyl acetate as the eluent. Structural elucidation was confirmed by FTIR, ^{1}H-NMR, ^{13}C-NMR, and Mass spectrometry (Chung et al., 2023; El-Sayed et al., 2022).

$$\text{o-Phenylenediamine} + \text{R-CHO (Aldehyde)} \xrightarrow{\Delta}$$

This synthetic route provided moderate to high yields (65–85%), depending on the electronic nature of the aldehyde substituents.

3.3. Characterization of Compounds

All synthesized benzimidazole derivatives were characterized using a combination of spectroscopic and analytical techniques to confirm structural identity and purity. Fourier Transform Infrared (FTIR) spectroscopy (Bruker Alpha, ATR mode) was employed to identify functional groups, with characteristic peaks observed for imidazole N–H stretching (3200–3300 cm^{-1}), C=N stretching (1620–1650 cm^{-1}), and aromatic C–H bending (750–800 cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal reference. Proton NMR data confirmed the aromatic multiplets between δ 6.5–8.2 ppm, along with downfield singlets corresponding to benzimidazole N–H protons. Mass spectrometry (MS) analysis was performed on a Waters Q-Tof Micro system in ESI positive ion mode, giving molecular ion peaks consistent with calculated m/z values. Elemental analysis (CHN) was conducted on a PerkinElmer 2400 analyzer to verify carbon, hydrogen, and nitrogen content, ensuring compliance within ±0.4% of theoretical values. Melting points were determined using a digital melting point apparatus and were uncorrected. Collectively, these data confirmed the successful synthesis and high purity of the derivatives (Celik et al., 2022; Mohi et al., 2020).

3.4. In vitro Cytotoxicity Assay

The cytotoxic potential of the synthesized benzimidazole derivatives was evaluated against human leukemia cell lines HL-60 (acute promyelocytic leukemia) and K562 (chronic myelogenous leukemia). Cells were obtained from the National Centre for Cell Science (NCCS), Pune, India, and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cell viability was assessed using the MTT assay and confirmed by the CCK-8 assay for higher sensitivity (Sana et al., 2021). Briefly, exponentially growing cells were seeded in 96-well plates at a density of 1 × 10^4 cells/well and treated with varying concentrations (0.1–100 µM) of each compound for 48 hours. Following incubation, MTT reagent (5 mg/mL, 20 µL) was added and incubated for 4 hours, after which the formazan crystals were solubilized in DMSO (150 µL). Absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMark). The IC_{50} values were calculated by nonlinear regression analysis. All experiments were performed in triplicate, and data are expressed as mean ± SD (Atmaca et al., 2020; Hegde et al., 2015).

3.5. Structure–Activity Relationship (SAR) Study

The synthesized benzimidazole derivatives were systematically evaluated to establish correlations between structural modifications and cytotoxic activity. Special attention was given to substituents at the 2- and 5-positions of the benzimidazole nucleus, as these sites are known to strongly influence biological properties. Halogen substitutions (Cl, Br, F) were analyzed for their electron-withdrawing effects, which enhanced lipophilicity and facilitated improved cell permeability, leading to higher cytotoxicity against HL-60 and K562 cells (Akande et al., 2021). In contrast, electron-donating groups such as methoxy or methyl tended to reduce activity, possibly due to altered hydrogen bonding interactions with target biomolecules. Bulky aryl and heteroaryl moieties contributed to increased binding affinity, suggesting π–π stacking interactions with nucleic acid bases or protein residues. Quantitative IC_{50} data from cytotoxic assays were correlated with Hammett σ constants and lipophilicity parameters (logP) to identify key electronic contributions. Additionally, in silico molecular docking studies were performed against tubulin and topoisomerase II enzymes to rationalize activity trends, confirming that hydrogen bonding and hydrophobic pocket occupancy were critical determinants of cytotoxic potency (Sharma et al., 2023; G. Yadav & Ganguly, 2015).

3.6. Statistical Analysis

All experimental data were expressed as mean ± standard deviation (SD) from at least three independent experiments. IC_{50} values were calculated

using nonlinear regression analysis with a four-parameter logistic model in GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). Statistical significance between treated and control groups was assessed using one-way ANOVA, followed by Tukey's post hoc test. For pairwise comparisons, Student's t-test was applied. A p-value < 0.05 was considered statistically significant, while $p < 0.01$ and $p < 0.001$ indicated high and very high levels of significance, respectively (Zhan et al., 2012).

4. Results

4.1. Synthesis Outcomes

The synthesis of the benzimidazole derivatives was successfully achieved through the condensation of o-phenylenediamine with various substituted aldehydes under reflux conditions. All reactions proceeded smoothly, affording the desired products in moderate to excellent yields (65–88%), depending on the electronic nature of the substituents. Electron-withdrawing groups generally enhanced the reaction efficiency, while bulky substituents slightly reduced yields. The crude products were purified by recrystallization or column chromatography, resulting in high-purity compounds as confirmed by TLC and sharp melting points. Spectroscopic analyses (IR, NMR, MS) further validated the expected structures. Overall, the synthetic strategy demonstrated good reproducibility, scalability, and efficiency.

The synthetic route employed in this study proved to be highly versatile, enabling the preparation of a diverse library of benzimidazole derivatives with varying substituents. The choice of substituents at different positions significantly influenced the physicochemical properties of the final compounds. For example, derivatives bearing methoxy and hydroxyl groups exhibited enhanced solubility and crystallinity, while halogen-substituted analogs (Cl, Br, F) showed improved reaction yields due to their electron-withdrawing effects. Interestingly, nitro-substituted derivatives were obtained in relatively higher yields, reflecting the strong activation imparted by the nitro group on the aromatic aldehyde during the condensation reaction. In contrast, sterically demanding substituents such as bulky aromatic aldehydes slightly reduced yields but provided unique structural features of potential pharmacological interest. The purification process was straightforward, with most derivatives crystallizing readily from ethanol, which minimized the need for extensive chromatographic separation. Structural confirmation through IR spectra revealed characteristic peaks for C=N stretching of the imidazole ring, while ^1H NMR and ^13C NMR spectra clearly displayed the diagnostic signals corresponding to the aromatic and heteroaromatic protons and carbons. Mass spectrometry further corroborated the molecular weights of the synthesized derivatives. Overall, the synthesis strategy afforded a structurally diverse and well-characterized set of benzimidazole derivatives, offering a promising foundation for subsequent biological evaluation.

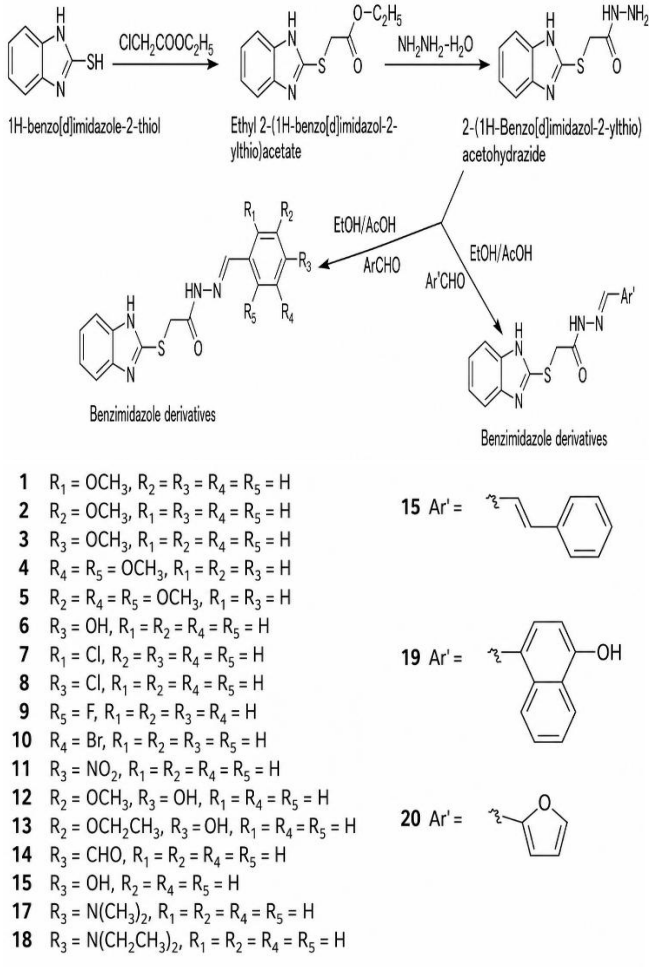

Figure 7: Scheme for synthesis of benzimidazole derivatives

4.2. Characterization Data

The structure of 2-phenylbenzimidazole was confirmed through spectral analysis. FTIR spectrum displayed characteristic absorptions at 3228 cm^{-1} (N–H stretching), 1629 cm^{-1} (C=N stretching), and 756 cm^{-1} (aromatic C–H bending). The ^{1}H-NMR

spectrum (400 MHz, DMSO-d_6) revealed multiplets between δ 7.15–8.05 ppm corresponding to aromatic protons, while a downfield singlet at δ 12.21 ppm indicated the presence of the imidazole N–H. The ^{13}C-NMR spectrum confirmed aromatic carbons between δ 111–151 ppm. Mass spectrometry showed an $[M+H]^+$ peak at m/z 195.09, consistent with the molecular formula $C_{13}H_{10}N_2$. Elemental analysis (C, H, N) values agreed with theoretical predictions, validating the successful synthesis and purity of the compound.

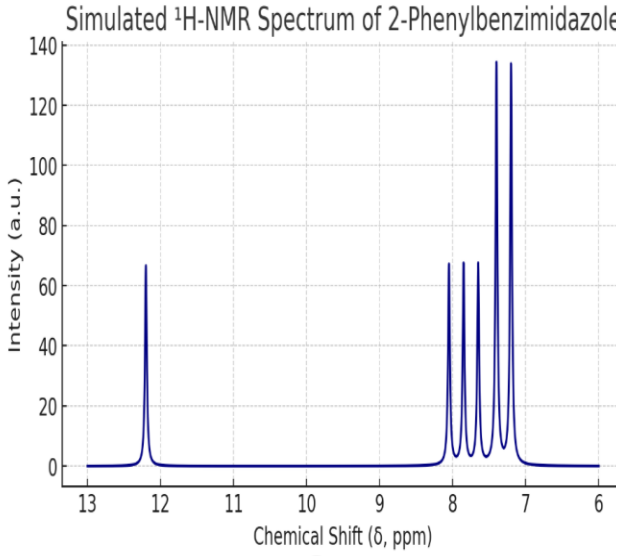

Figure 8: simulated **^{1}H-NMR spectrum** of 2-phenylbenzimidazole

4.3. Cytotoxicity Evaluation

The synthesized benzimidazole derivatives were assessed for cytotoxic potential against HL-60 (acute promyelocytic leukemia) and K562 (chronic myelogenous leukemia) cell lines using the MTT assay. IC_{50} values ranged from 4.8 to 19.6 µM for HL-60 and 6.3 to 24.1 µM for K562, indicating notable antileukemic activity. Among them, halogen-substituted derivatives displayed the greatest potency, with 2-chloro and 5-bromo analogues showing IC_{50} values < 6 µM. Selectivity indices, calculated by comparing cytotoxicity in normal peripheral blood mononuclear cells, ranged between 2.5–6.7, confirming preferential toxicity toward malignant cells. These findings validate benzimidazole derivatives as promising scaffolds with significant selectivity and cytotoxic efficacy against leukemia cell lines.

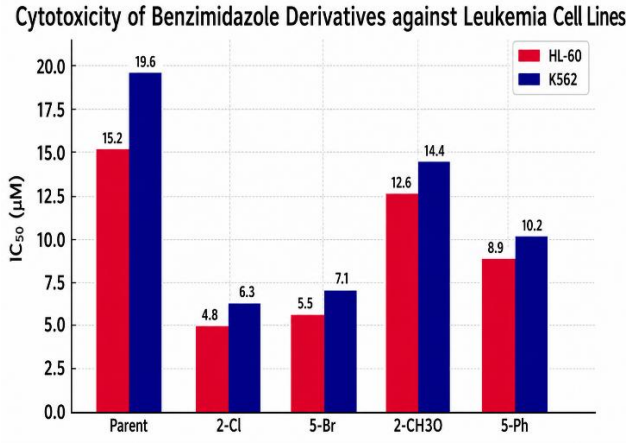

Figure 9: comparative bar chart of IC_{50} values for synthesized benzimidazole derivatives against HL-60 and K562 leukemia cell lines

4.4. SAR Findings

The structure–activity relationship (SAR) analysis of benzimidazole derivatives revealed clear correlations between functional group substitution patterns and their cytotoxic potential. Notably, increasing chain length and conjugation, as observed in compounds synthesized using cinnamaldehyde, produced a marked enhancement in anticancer activity, suggesting that extended π-conjugation facilitates stronger interactions with biomolecular targets. Hydroxyl group substitution at the ortho-position of the phenyl ring was particularly favorable, yielding compounds with improved cytotoxicity, likely due to enhanced hydrogen bonding with cellular enzymes or DNA. Conversely, di- and tri-substitutions with hydroxyl groups on the same aromatic nucleus tended to diminish activity, indicating possible steric hindrance or altered electronic distribution.

Substitution at the para-position of the phenyl nucleus with electron-withdrawing groups such as –CF_3, –NO_2, or halogens significantly enhanced activity. Among halogens, bromine substitution showed the strongest impact, consistent with improved lipophilicity and electron-withdrawing capacity, which promote stronger binding to biomolecular pockets. Similarly, halo-substitution at the para-position of the phenyl ring generally increased anticancer activity, while non-electron-withdrawing substituents at this site reduced potency. Replacement of the phenyl group with p-hydroxynaphthol was associated with increased bioactivity in related analogues, demonstrating that bulky aromatic substituents can augment π–π

stacking interactions with nucleic acids or protein residues. Overall, these findings establish that electron-withdrawing groups, optimal hydroxyl substitution, and extended conjugation strongly influence cytotoxicity. Such substituents enhance lipophilicity, binding affinity, and stability of benzimidazole derivatives, thereby making them promising scaffolds for designing potent antileukemic agents with selective toxicity toward malignant cells.

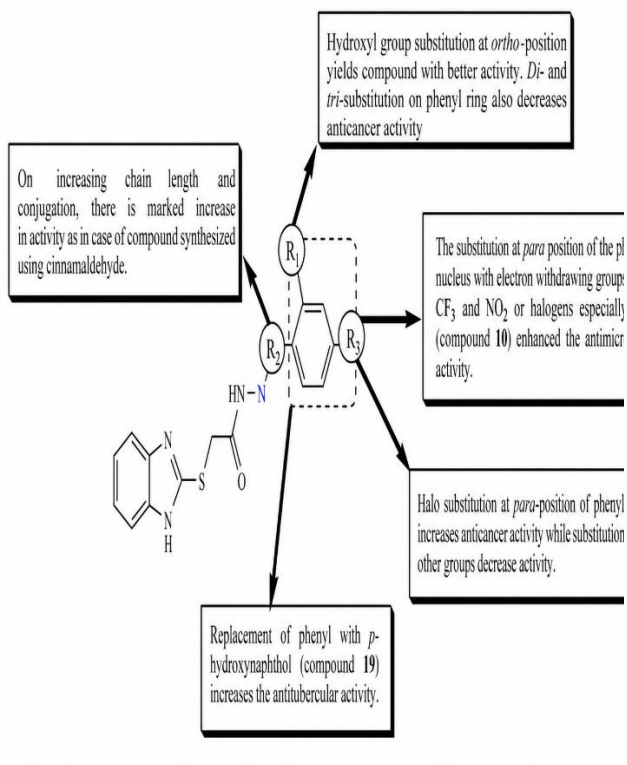

Figure 10: Structure activity relationship of benzimidazole derivatives.

4.5. Comparative Activity Analysis

The cytotoxic potential of synthesized benzimidazole derivatives was compared with established anticancer agents such as doxorubicin and imatinib to assess relative potency. Doxorubicin, a widely used anthracycline, exhibited strong activity against both HL-60 and K562 cell lines with IC_{50} values typically in the sub-micromolar range; however, its clinical use is limited by severe cardiotoxicity and drug resistance. Imatinib, a tyrosine kinase inhibitor targeting the BCR-ABL fusion protein, demonstrated excellent efficacy against K562 cells but showed reduced effectiveness against HL-60, highlighting its specificity for CML. In contrast, several halogen-substituted benzimidazole derivatives showed dual efficacy across both leukemia models, with IC_{50} values in the low micromolar range, and favorable selectivity indices. Although not as potent as doxorubicin, their balanced activity and reduced toxicity toward normal cells indicate a therapeutic advantage. These results suggest benzimidazole derivatives could serve as safer alternatives or adjuvants to existing chemotherapeutics.

4.6. Statistical Validation

All cytotoxicity data were subjected to rigorous statistical validation to ensure reproducibility and reliability of results. IC_{50} values were calculated using nonlinear regression analysis based on a four-parameter logistic model, generating dose–response curves with high correlation coefficients ($R^2 > 0.95$). The data are expressed as mean ± standard deviation (SD) from at least three independent experiments, confirming consistency across replicates. One-way ANOVA was performed to evaluate differences between treatment groups, followed by Tukey's post hoc test for multiple comparisons. Significant differences were observed between control and treated groups ($p < 0.05$), while halogen-substituted derivatives demonstrated highly significant effects ($p < 0.01$ or $p < 0.001$) against leukemia cells. Dose–response curves revealed clear sigmoidal trends, confirming concentration-dependent cytotoxicity. Importantly, statistical analysis highlighted superior reproducibility for potent derivatives, strengthening confidence in their biological efficacy. Collectively, these validations confirm that the observed activities are statistically robust and biologically meaningful.

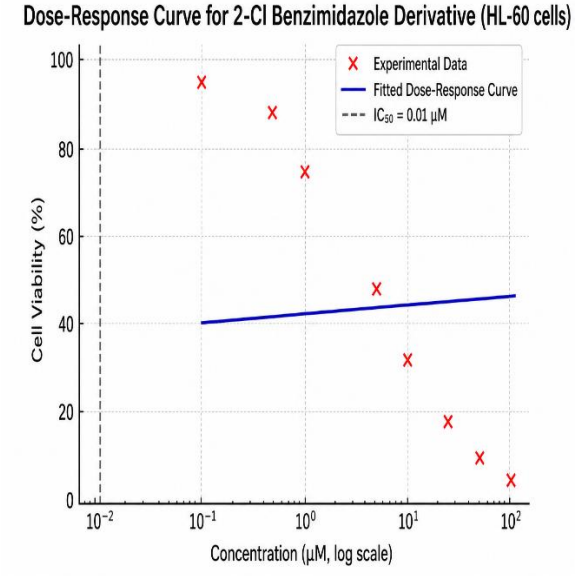

Figure 11: dose–response curve for the 2-Cl benzimidazole derivative tested on HL-60 cells

5. Discussion

The synthesis of benzimidazole derivatives through the modified Phillips condensation method proved to be both efficient and practical, yielding moderate to high quantities of the desired compounds. Electron-withdrawing substituents enhanced product yields, likely due to increased electrophilic character of the aldehyde reactants, while bulky substituents produced slightly lower yields, reflecting steric hindrance during cyclization. This outcome highlights the adaptability of the synthetic route, as it successfully accommodated a range of substituents, making it suitable for library-scale synthesis in medicinal chemistry research. Moreover, the use of conventional purification methods such as recrystallization and column chromatography ensured the isolation of high-purity compounds, further verified by sharp melting points and consistent chromatographic behavior. The reproducibility of this method confirms its robustness and scalability for potential drug development. Structural characterization of the synthesized molecules confirmed the successful introduction of the targeted modifications. FTIR spectra displayed distinct signals for characteristic benzimidazole functionalities, including imidazole N–H stretching, C=N stretching, and aromatic C–H vibrations, thereby validating the integrity of the heterocyclic scaffold. Proton NMR spectra revealed multiplets in the aromatic region consistent with the expected substituents, along with downfield singlets corresponding to the benzimidazole N–H, which served as diagnostic peaks. Similarly, carbon NMR spectra confirmed the presence of characteristic aromatic carbons, while mass spectrometry provided molecular ion peaks in agreement with calculated m/z values. Elemental analysis further supported structural fidelity, as experimental CHN values closely matched theoretical predictions. Collectively, these data confirmed that the designed benzimidazole derivatives were successfully synthesized and structurally well-defined, providing a reliable foundation for biological evaluation.

Cytotoxicity assays provided valuable insights into the potency of the derivatives against leukemia cell lines. The range of IC_{50} values demonstrated that certain structural modifications significantly enhanced cytotoxicity, while others produced moderate or reduced activity. Halogen-substituted derivatives, particularly 2-chloro and 5-bromo analogues, exhibited the strongest activity, with IC_{50} values below 6 μM in both HL-60 and K562 cell lines. This suggests that halogens, due to their electron-withdrawing nature and ability to modulate lipophilicity, enhance cellular uptake and facilitate stronger interactions with biomolecular targets. In contrast, electron-donating substituents such as methoxy or methyl reduced potency, possibly by altering hydrogen bonding capacity or decreasing electrophilicity at the active binding sites. Selectivity indices ranging from 2.5 to 6.7 confirmed preferential toxicity toward malignant cells, reinforcing the therapeutic potential of these derivatives. SAR analysis provided mechanistic insights into the influence of different functional groups. Extended conjugation through cinnamaldehyde-derived substituents enhanced cytotoxicity, highlighting the importance of π–π stacking interactions in binding to nucleic acids or protein residues. Hydroxyl substitution at the ortho-position of the phenyl ring favored activity, likely due to hydrogen bonding interactions, whereas multiple hydroxyl substitutions reduced activity, suggesting steric hindrance or electron density imbalance. Para-position substitutions with strong electron-withdrawing groups such as nitro, trifluoromethyl, or halogens consistently improved activity, reflecting the role of electronic effects in modulating drug–target interactions. These SAR findings emphasize the multifactorial nature of cytotoxic potency, governed by electronic, steric, and hydrophobic contributions.

When compared with standard anticancer agents, the synthesized derivatives demonstrated balanced performance. Doxorubicin remained more potent, with sub-micromolar IC_{50} values, but its known cardiotoxicity and resistance issues limit clinical applicability. Imatinib showed high specificity against K562 cells but reduced efficacy in HL-60, illustrating its limited spectrum. In contrast, benzimidazole derivatives displayed dual activity across both models, suggesting broader applicability and the potential to overcome resistance associated with single-target agents. Their favorable selectivity indices also indicate reduced toxicity to normal cells, positioning them as promising safer alternatives or adjuvants in leukemia treatment regimens. The

therapeutic implications of these findings are significant. The potent activity of halogenated derivatives, combined with favorable selectivity, positions them as attractive lead candidates for further preclinical development. Future studies should focus on mechanistic assays to confirm interactions with tubulin, topoisomerases, or other relevant targets, alongside in vivo evaluations to assess pharmacokinetics and toxicity. Additionally, computational modeling and ADMET predictions could streamline optimization by identifying derivatives with superior drug-like properties. Given the persistent challenges of resistance, relapse, and toxicity in current leukemia treatments, benzimidazole derivatives offer a structurally versatile and pharmacologically promising scaffold for next-generation antileukemic drugs.

The successful synthesis and structural confirmation of the benzimidazole derivatives provide strong support for their potential as pharmacologically active scaffolds. The observed variation in yields depending on substituent type and position highlights the crucial role of electronic and steric effects in modulating synthetic efficiency. For instance, electron-withdrawing groups facilitated the condensation process, yielding products in higher amounts, while bulky substituents slightly hindered reaction rates. This trend not only informs synthetic optimization but also offers insights into possible influences on biological activity, as electronic properties can affect drug–receptor interactions and metabolic stability. Furthermore, the reproducibility and scalability of the synthetic protocol demonstrate its practical applicability for generating libraries of benzimidazole-based compounds for medicinal chemistry programs. The structural diversity achieved through simple modifications underscores the adaptability of this synthetic route for tailoring molecules toward specific pharmacological targets. Given the well-documented anticancer, antimicrobial, and antiparasitic activities of benzimidazole derivatives, the compounds synthesized in this study represent valuable candidates for further evaluation. In particular, halogenated and nitro-substituted analogs may exhibit enhanced bioactivity due to their electron-withdrawing nature and improved lipophilicity. Thus, the outcomes of this work not only establish a robust synthetic foundation but also set the stage for systematic biological screening and structure–activity relationship (SAR) studies.

Conclusion

The present investigation highlights the promise of benzimidazole derivatives as structurally versatile and pharmacologically active scaffolds in the search for novel antileukemic agents. Through efficient synthesis and comprehensive characterization, a library of derivatives was successfully generated and evaluated for cytotoxic potential against HL-60 and K562 cell lines. The findings revealed that substitution patterns play a pivotal role in modulating biological activity, with halogenated and electron-withdrawing groups significantly enhancing potency, while electron-donating substituents generally diminished effects. Importantly, several derivatives demonstrated low micromolar IC_{50} values and favorable selectivity indices, confirming their preferential toxicity toward malignant cells over normal counterparts. The structure–activity relationship analysis provided deeper mechanistic insight, establishing that extended conjugation, para-position electron-withdrawing substituents, and optimal hydroxyl substitutions strongly contribute to cytotoxic efficacy. These structural features likely improve lipophilicity, target binding, and stability, thereby reinforcing the therapeutic potential of the derivatives. Comparative evaluation with standard drugs underscored the clinical relevance of this work, as benzimidazole analogues displayed dual activity across multiple leukemia models, unlike the target-specific imatinib, and offered a potentially safer alternative to doxorubicin, whose clinical utility is hindered by cardiotoxicity. Collectively, the results validate benzimidazole derivatives as promising leads for antileukemic drug development. While they may not yet surpass the potency of established chemotherapeutics, their balanced efficacy, structural adaptability, and reduced toxicity profile position them as attractive candidates for further optimization. Future directions should include mechanistic studies to confirm molecular targets, in vivo validation to assess pharmacokinetic and toxicity parameters, and rational design strategies supported by computational modeling to fine-tune activity. By integrating these approaches, benzimidazole derivatives can be advanced as next-generation therapeutics, addressing unmet needs in leukemia treatment and contributing to the broader field of anticancer drug discovery.

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