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Synthesis and antiproliferative activities against breast cancer of N-(benzimidazol-2-yl)-substituted benzamide derivatives

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Abstract: The strategic design of benzimidazole structures, akin to nucleotides, facilitates intricate interactions with amino acids within protein active sites. This structural scaffold, endowed with favorable pharmacokinetic profiles and lipophilic attributes, serves as a cornerstone in crafting potent pharmaceutical entities. In this study, a series of N-(1*H*-benzo[d]imidazol-2-yl)-substituted benzamides was meticulously synthesized and characterized through comprehensive spectroscopic analyses. Leveraging the established pharmacophoric role of substituted benzimidazoles, renowned for their documented anti-proliferative properties, this study embarked on the synthesis of benzamides employing methoxy and substituted phenyl rings as key pharmacophores, known for their anticancer efficacy. Subsequent cytotoxicity evaluations using the MTT assay against breast cancer (MCF7) and normal mouse fibroblasts (L929) revealed compound 9 as the leading candidate, inducing significant cytotoxicity. This suggests its potential as a potent anticancer agent through apoptotic pathways. These findings highlight compound 9 as a promising molecular scaffold that requires careful optimization for the development of effective anticancer therapies.

Keywords: Benzimidazole; benzamide; synthesis, antiproliferative activity; MTT assay; structure-activity relationship. ©2024 ACG Publication. All right reserved.

1. Introduction

Cancer encompasses a group of diseases characterized by the uncontrolled proliferation of certain cells in the body, which can metastasize to other areas. Among these, breast cancer stands out as the most significant type affecting women's health globally. Breast cancer ranks first in terms of both incidence and mortality rates among women worldwide.[1,2] According to the World Health Organization (WHO), breast cancer affects approximately 2.3 million women annually.[3] The escalating number of breast cancer diagnoses underscores the urgent need for research into novel drugs capable of inhibiting cancer cell proliferation, owing to factors such as resistance to conventional therapies and severe side effects.[4-9] Consequently, the emergence of resistance to chemotherapy agents and the inadequate ability to discriminate between healthy and cancerous cells represent fundamental challenges. Conversely, drugs targeting specific pathways tend to accelerate the onset of resistance. It is well-established that drugs with multiple targets exert a stronger therapeutic effect compared to single-target agents.[10,11] Thus, newly developed drug candidates are anticipated to target multiple pathways while selectively exerting their effects on cancer cells.

Both benzimidazole structures and the carboxamide functional group have been extensively explored in anticancer drug development endeavors documented in the literature.[12-15] Substituted

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benzimidazole compounds are known for their high potency as anticancer agents. Notably, several anticancer drugs currently in clinical trials, including binimetinib and veliparib, feature carboxamide and/or benzimidazole structures (Figure 1).[16, 17.]

Nocodazole Veliparib Binimetinib

Figure 1. Compounds containing carboxamide and benzimidazole structures used in cancer treatment

On the other hand, the benzamide functional group has garnered attention in drug development studies due to its cytotoxic activity.[18-20] The strong bond polarity of the amide linkage, along with its inherent advantages such as good stability and conformational diversity, has led to significant interest in drug development efforts.[21] Approved anticancer drugs containing the benzamide functional group, such as cabozantinib, are depicted in Figure 2.

Cabozantinib

Figure 2. Structures of some anticancer drugs containing the carboxamide group

In recent years, particular attention has been paid to new drug units that stop cancer growth and proliferation. It is seen that among the groups with antiproliferative groups that bring together the literature studies, there are also associations with benzimidazole ring system. These separations aim to demonstrate that the substituted benzimidazole derivatives at the 1st, 2nd, and 5th positions exhibit significant activity compared to those at other positions, with particular emphasis on the high activity of derivatives in the second position.[16-19, 22-26]

In this study, we describe the synthesis of a compound where benzimidazole is linked to a benzamide structure, with a phenylcarboxamide substitution at the para position. We investigate the in vitro effects of these compounds and their cytotoxic activities against MCF7 cancer cell lines. Additionally, we propose a potential mechanism.

2. Experimental

2.1. Chemistry

Melting points were measured with an Electrothermal-9200 digital melting points apparatus. Melting points of the compounds were recorded on an apparatus and are uncorrected. The Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) NMR spectra were recorded by a Bruker 400 NMR (for ^{1}H NMR) and 100 NMR spectrometer (for ^{13}CNMR). ^{1}H NMR spectra and ^{13}C NMR spectra were run in deuterated dimethyl sulfoxide (DMSO–d_6), deuterated chloroform ($CDCl_3$) or deuterated methanol (MeOD-D_4). Chemical shifts (δH) are reported in parts per million (ppm, δ) relative to TMS as an internal standard. Elemental analysis results were obtained on a Leco CHNS-932 instrument. Reaction courses and product mixtures were routinely monitored by thin layer chromatography (TLC) on silica

gel precoated F254 Merck plates. All chemicals used were of analytical grade and were used as received without any further purification and were obtained from Sigma-Aldrich.

*2.2. General Procedure for The Synthesis of N-(benzimidazol-2-yl)-2-substituted Benzamide (**6-9**)*

The 2-aminobenzimidazole (**1**) (1 mmol) were dissolved in tetrahydrofuran (THF) (15 mL), benzoyl chloride or its derivatives (1.1 mmol) (**2-5**) were added drop-wise over a 10-min period while maintaining the temperature at 0 °C, and the reaction was stirred for two hours at the same temperature. Afterward, 5 drops of trimethylamine (TEA) were added at room temperature, and the reaction mixture was refluxed for 24 h. According to the TLC analysis of the reaction's progression, the reaction mixture was evaporated and 25 mL DCM was added. The organic phase was washed with 1 N HCl (3×20 mL), a saturated solution of $NaHCO_3$ (2×15 mL), and distilled water (15 mL). The resulting organic layer was concentrated under vacuum to obtain a crude product, which was then subjected to column chromatography (silica gel 60–120 mesh) with ethyl acetate/hexane (1:6) (silica gel, 60–120 mesh, eluent; hexane/EtOAc gradient), and the white crystals obtained were recrystallized using chloroform.

N-(1H-benzo[d]imidazol-2-yl)benzamide (**6**): Compound **6** known in literature.[19-21, 27]

N-(1H-benzo[d]imidazol-2-yl)-4-chlorobenzamide (**7**): Compound **7** known in literature.[21, 27]

N-(1H-benzo[d]imidazol-2-yl)-4-methylbenzamide (**8**): Compound **8** known in literature.[19,21]

N-(1H-benzo[d]imidazol-2-yl)-4-methoxybenzamide (**9**) Compound **9** known in literature.[27]

2.3. Biological Assay

2.3.1 Cell Culture Conditions

Cell lines were procured from the American Type Culture Collection (ATCC). The study employed human breast cancer cell lines (ER-) (MCF7, HTB-22TM) and mouse fibroblast cell lines (L 929, CCL-1TM). MCF7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin under a 5% CO2 atmosphere at 37°C. L-929 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum and 1% penicillin. Both cell lines were maintained in a humidified incubator at 37°C with 5% CO_2. The experimental groups as detailed in Table 4 were subjected to cell culture assays.

Table 4. Study groups of cell cultures

Study groups	**Properties**
Negative Control Group 1	Untreated MCF7 cell line
Negative Control Group 2	Untreated L-929 cell line
Positive Control Group 1	MCF7 cell line treated with DOX
Positive Control Group 2	Cell line treated with DOX
Working Group 1	MCF7 cell line treated with various concentrations of the compounds
Working Group 2	L-929 cell line treated with various concentrations of the compounds

2.3.2. MTT Assay for In Vitro Cytotoxicity Assessment

Cytotoxicity of the compounds was assessed using the MTT assay method.[28-30] Compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted to appropriate concentrations in Dulbecco's modified Eagle's medium (DMEM), ensuring that the DMSO concentration in the culture medium was less than 1%. Cells were seeded at a density of 10^4 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 µg/mL streptomycin in 96-well microculture plates. Following incubation at 37°C for 24 hours in a 5% CO_2 incubator, cells were treated with the test compounds at various concentrations for 24 hours. Subsequently, 100 µL of MTT solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 hours. Afterward, the medium was aspirated from each well, and the formazan crystals formed by viable cells were dissolved in DMSO. Absorbance was measured at 570 nm using a microplate reader (Bioteck). Cell viability was

calculated as the percentage of viable cells in each sample relative to control wells. Each experiment was conducted in triplicate, and IC_{50} values were determined from concentration-response curves using SPSS software (SPSS Inc., Chicago).

2.3.3 Flow Cytometry Analysis

Flow cytometric analysis was conducted using Annexin-V/PI commercial kits.[31] MCF7 and L-929 cells were treated with the IC_{50} values of compound **4** and DOX for 24 hours. After incubation, the plate contents were aspirated, cells were washed with PBS, and trypsin-EDTA treatment was applied, followed by centrifugation at 800 rpm for 8 minutes. The resulting cells were counted using trypan blue, and then 5 µL of Annexin V and 5 µL of PI were added. The mixture was incubated in the dark at room temperature for 15 minutes. Afterward, 400 µL of binding buffer was added on ice, and flow cytometry measurements were conducted (emission: 530 nm, excitation: 488 nm).

2.3.4 Cell Cycle Analysis

Flow cytometry was utilized to perform cell cycle analysis in MCF7 cells.[32-36] A total of $1x10^5$ cells were treated with 5 µg/mL of compound 4 for 24 hours. Subsequently, all cells were collected and centrifuged for 5 minutes at 300g and 25 °C. To each tube, 250 µL of trypsin buffer was added and incubated at 25 °C for 10 minutes. Following this, 200 µL of trypsin was added to the mixture, which underwent an additional 10-minute incubation. Finally, 200 µL of cold propidium iodide (PI) stain solution was added to each tube, and data from 3×10^4 cells were measured. All data were then compared with untreated control cells for analysis.

2.3.5 Statistical Analysis

Cytotoxicity results were expressed as mean ± standard error of mean (SEM).

3. Results and Discussion

3.1. Chemistry

We carried out the reaction of substituted benzoyl chloride (**2-5**) (1.1 mmol) with 2-aminobenzimidazole (**1**) (1 mmol) in the THF and TEA of under conventional reaction (Scheme 1).The efficient synthesis of N-(benzimidazol-2-yl)-2-substituted benzamide structures (**6-9**) in a single step. The compounds were obtained in good yield. All compounds were characterized using spectroscopic analyses (Tablo 1).

H N N NH_2 + O Cl R
1) THF 0 °C
2) TEA, rt, 24h
H N N NH O R

1

2 H
3 Cl
4 CH_3
5 OCH_3

6-9

Scheme 1. Synthesis of N-(1H-benzo[d]imidazol-2-yl)benzamide derivatives (6-9)

3.2. Biological Assay

3.2.1 In Vitro Cytotoxic Activity

The synthesized compounds **6-9** were evaluated for their anti-proliferative activity against the MCF7 cell line using the MTT assay method. The IC_{50} values of the compounds ranged from 3.84 ±

0.62 to 13.10 ± 0.57 µM. Doxorubicin (DOX), an established antitumor drug, exhibited IC_{50} values ranging from 5.93 ± 0.33 µM in the same cell line. Comparative analysis revealed that our compounds demonstrated sensitivity towards MCF7, with most exhibiting similar or higher IC_{50} values compared to DOX. Notably, compound **9** (IC_{50} = 3.84 ± 0.62 µM) displayed the most potent anti-proliferative activity against MCF7 cancer cells (Table 2).

Evaluation of compound **9** against MCF7 cells indicated lower activity compared to the standard drug DOX, with a specificity index (SI) of 15.01 compared to DOX on normal mouse fibroblast cells. This suggests that while compound **9** may be less potent than the standard drug, it possesses a broader therapeutic index than other compounds.

O
N
NH
R
N
H

Table 1. Chemical structures, m.p., IR and NMR spectral data of synthesized compounds

Compound	R	Yield (%)	m.p. (˚C)	IR (cm^{-1})	^{1}H-NMR (ppm)
6[19-21, 27]	H	60	200	3311, 3061, 2833, 1660, 560, 1427, 1265, 1017	12.27 (s, 2H-), 8.17 (q, *J* = 1.7 Hz, 1H), 8.15 (t, *J* = 1.7 Hz, 1H), 7.65 – 7.60 (m, 1H), 7.58 – 7.53 (m, 2H), 7.51 – 7.46 (m, 2H), 7.19 – 7.13 (m, 2H)
7[21, 27]	Cl	45	195	3308, 2759, 1622, 1568, 1468, 1270	12.38 (s, 2H), 8.18 (d, J = 1.8 Hz, 1H), 8.16 (d, J = 2.4 Hz, 1H), 7.61 (d, J = 2.4 Hz, 1H), 7.59 (d, J = 1.8 Hz, 1H), 7.49 – 7.44 (m, 2H), 7.20 – 7.15 (m, 2H)
8[19, 21]	CH_3	50	180	3325, 2756, 1659, 1571, 1552, 1519, 1473, 1270	12.19 (s, 2H), 8.06 (d, J = 8.1 Hz), 7.50 – 7.45 (m, 2H), 7.36 (d, J = 8.1 Hz, 2H), 7.17 – 7.12 (m, 2H), 2.41 ppm (s, 3H).
9[27]	OCH_3	60	230	3460, 3015, 2969, 1739, 1581, 1216	12.14 (s, 2H), 8.23 – 8.06 (m, 2H), 7.53 – 7.43 (m, 2H), 7.18 – 7.12 (m, 2H), 7.11 – 7.05 (m, 2H), 3.87ppm (s, 3H)

Table 2. IC_{50} values of compounds 6-9 against breast cancer (MCF7) and normal mouse fibroblasts (L929) after 24 h

Compounds	IC_{50} values ± SD (µM) *		Selective index (SI)**
	MCF7	L-929	
6	13.10 ±0.57	48.18±2.04	3.67
7	5.52 ± 2.10	56.60±3.52	10.25
8	4.34± 0.31	50.40±4.40	11.61
9	3.84 ± 0.62	57.64±3.20	15.01
DOX	5.93 ± 0.33	93.99±2.68	15.85

*IC_{50} values for all test compounds and all cell lines used in the study, obtained by the MTT test after 24 h treatment. The results are presented as mean ± standard deviation (SD) of three separate experiments, with three wells each. ** SI (L-929/ MCF7)

Furthermore, antiproliferative activity studies were conducted using non-toxic concentrations of compounds determined on the L-929 cell line. Compound **9**, identified as the most specific, along with DOX, was administered to MCF7 cells for 0–72 hours, and their cytotoxic responses were assessed using an MTT assay (Fig. 3). Treatment with the compound at non-toxic and lower concentrations resulted in a time-dependent inhibition of cancer cell proliferation. While compound **9** exhibited lower efficacy than DOX after 24, 48, and 72 hours of treatment, with a higher number of live cells observed, its antiproliferative activity increased with prolonged exposure. After 72 hours, the tested compounds demonstrated activity equivalent to that of DOX.

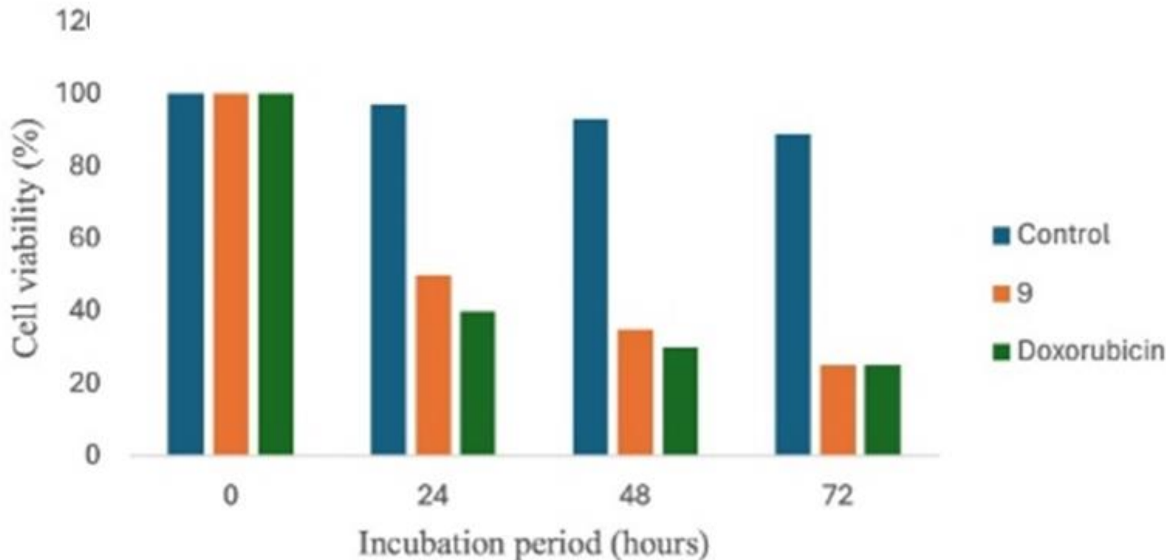

Figure 3. MTT assay on MCF7 cell line after 72 h with DOX and compound **9**. The absorbance values were selected as 570 nm for the MTT method. Control cells not containing compounds and DOX were incubated same conditions. Cell viability was calculated as the ratio of absorbance of treated cells with compound or DOX to untreated cells. Given values show the mean standard deviations from three independent experiments carried out in triplicate. Note: p values: 24 h: control—**9**; $p < .000$; control—DOX; $p < .000$; control—DOX $p < .05$. 48 h: control—**9**; $p < .000$; control—DOX; $p < .000$; control—DOX $p < .05$. 72 h: control—**9**; $p < .000$; control—DOX; $p < .000$; control—DOX $p < .05$

3.2.2. Flow Cytometric Analysis

Based on the cytotoxicity and selectivity results, compound **9** exhibited a decrease in cell viability. Subsequently, flow cytometry analysis was conducted to assess the apoptotic and necrotic effects of these compounds using the Annexin V (FITC)/PI kit. The percentages of live, early apoptotic, late apoptotic, and necrotic cells for both cell lines are presented in Table 3. In these flow cytometry experiments, DOX served as a positive control to evaluate their apoptotic effects. The results showed an increased apoptosis rate after 24 hours of treatment compared to the control group. Anticancer drugs and drug candidates typically induce apoptosis/necrosis.[37] A higher apoptosis/necrosis ratio indicates greater drug efficacy. Comparing our synthesized molecules with DOX, compound **9** exhibited the highest ratio in cancer cells. Compound 9 demonstrated a low apoptosis-necrosis rate (10.50) in the MCF7 cancer cell line compared to doxorubicin, our positive control. Moreover, compound **9** showed

high viability in a healthy cell line. These findings indicate that the compound selectively targeted breast cancer cells while sparing healthy cells.

Table 3. Apoptosis and necrosis rates of compound **9** and DOX exposure on MCF7 and L929 cell lines

Cell Lines	Compound	Live (%)	Early Apoptosis (%)	Late Apotosis (%)	Necrosis (%)	Apoptosis / Necrosis
MCF7	**13**	43.05±2.18	22.50±1.90	29.50±1.25	4.95±1.10	10.50
	DOX	45.08±1.20	38.52±2.38	12.25±2.24	4.15±0.20	12.23
L-929	**13**	97.92±2.30	1.48±0.10	0.60±0	0.00±0	2.08
	DOX	95.19±2.20	0.67±0.75	1.20±0.50	2.94±0.80	0.63

4. Conclusion

In this study, N-(benzimidazol-2-yl)-2-substituted benzamide analogs (**6–9**) were synthesized, characterized, and evaluated for their antiproliferative activities against MCF7 cancer cells. Specifically, benzimidazole derivatives substituted with benzamide, including N-(1*H*-benzo[d]imidazol-2-yl)-4-methylbenzamide (**6**), N-(1*H*-benzo[d]imidazol-2-yl)-4-chlorobenzamide (**7**), N-(1*H*-benzo[d] imidazol-2-yl)benzamide (**8**), and N-(1*H*-benzo[d]imidazol-2-yl)-4-methoxybenzamide (**9**), were synthesized, and their structures were elucidated using spectroscopic methods. The antiproliferative effects of these compounds on MCF7 cancer cells were assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method.

Overall, most compounds in the series demonstrated significant antiproliferative activity against MCF7 cancer cells. A structure-activity relationship analysis revealed that para-substituted phenyl rings with methoxy substituents exhibited increased compound potency compared to non-substituted compounds. Additionally, electron-donating groups significantly enhanced activities against cancer cell lines. Compound **9**, in particular, emerged as the most active agent against MCF7 cancer cells, with IC_{50} values ranging from 3.84 to 13.10 μM.

Considering the clinical application of anticancer drugs and their potential side effects, the study emphasizes the importance of selectivity towards cancer cells. A pharmacophore hypothesis was developed to analyze the structure–activity relationships between the molecular structures of the synthesized compounds and the observed biological activity in MCF7 cell lines.

In conclusion, our findings suggest that N-(benzimidazol-2-yl)-2-substituted benzamide scaffolds possess potent antiproliferative effects on cancer cell lines. Substitutions on the phenyl ring, particularly at the para position, significantly contribute to the observed activity.

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