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RESEARCH ARTICLE

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Design, synthesis, characterization, and in vitro cytotoxic activity evaluation of 1,2-disubstituted benzimidazole compounds

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Abstract

A series of 2-*p*-tolyl-1*H*-benzo[*d*]imidazole derivatives were synthesized and characterized. For finding an effective anticancer drug, which could be used in future generations, the developed heterocyclic compounds were screened in the human epithelial breast adenocarcinoma cell line (MCF-7) and human liver epithelial hepatocellular carcinoma cell line (HepG2) using the MTT assay method. Two positive control drugs were used for comparison with the compounds. The substituents on the 1- and 2-positions of the benzimidazole core had an important effect on the antiproliferation of cancerous cells. According to the results obtained, a compound, namely, 1-(4-methylbenzyl)-2-*p*-tolyl-1*H*-benzo[*d*]imidazole, which has electron donating groups (CH_3) in the para position of a phenyl ring, showed higher cytotoxic activities compared to other compounds towards liver and breast cell lines. The compounds were found to have more cytotoxicity in HepG2 rather than MCF-7.

KEYWORDS

benzimidazole, cytotoxic activity, heterocyclic compound

1 | INTRODUCTION

Cancer is caused by the uncontrolled growth of abnormal cells anywhere in the body. It affects not only humans but also animals and other living organisms. Lung, prostate, colorectal, stomach, and liver cancers are the most common types of cancer in men while breast, colorectal, lung, cervix, and thyroid cancers are the most widespread in women. Every year, millions of people get these cancers, and half of them loose their lives due to it. In 2018 alone, 9.6 million people died due to cancer. As this is predicted to continue increasing in the following years, it is seen clearly how dreadful this malady can be. There is an increasing need for the development of new drug candidates due to high cancer-related human mortality rates.

The 1,2-disubstituted benzimidazole compounds exhibit significant activities towards various viruses such as HIV, herpes (HSV-1), influenza, and human cytomegalovirus (HCMV).[1] In addition, benzimidazole derivatives are used as topoisomerase inhibitors, selective neuropeptide YY1 receptor antagonists, smooth muscle cell proliferation inhibitors, and in the treatment of interstitial cystitis.[1] Furthermore, compounds containing benzimidazole nucleus have many other biological activities such as antimicrobial, anthelmintic, analgesic, antiulcer, antiviral, anticancer, antioxidant, antihypertensive, anti-inflammatory, antifungal, and diuretic activities.[2–15]

There are a lot of commercial drugs containing benzimidazole core, for example, bendamustine is a chemotherapy drug used to treat chronic lymphocytic leukemia,

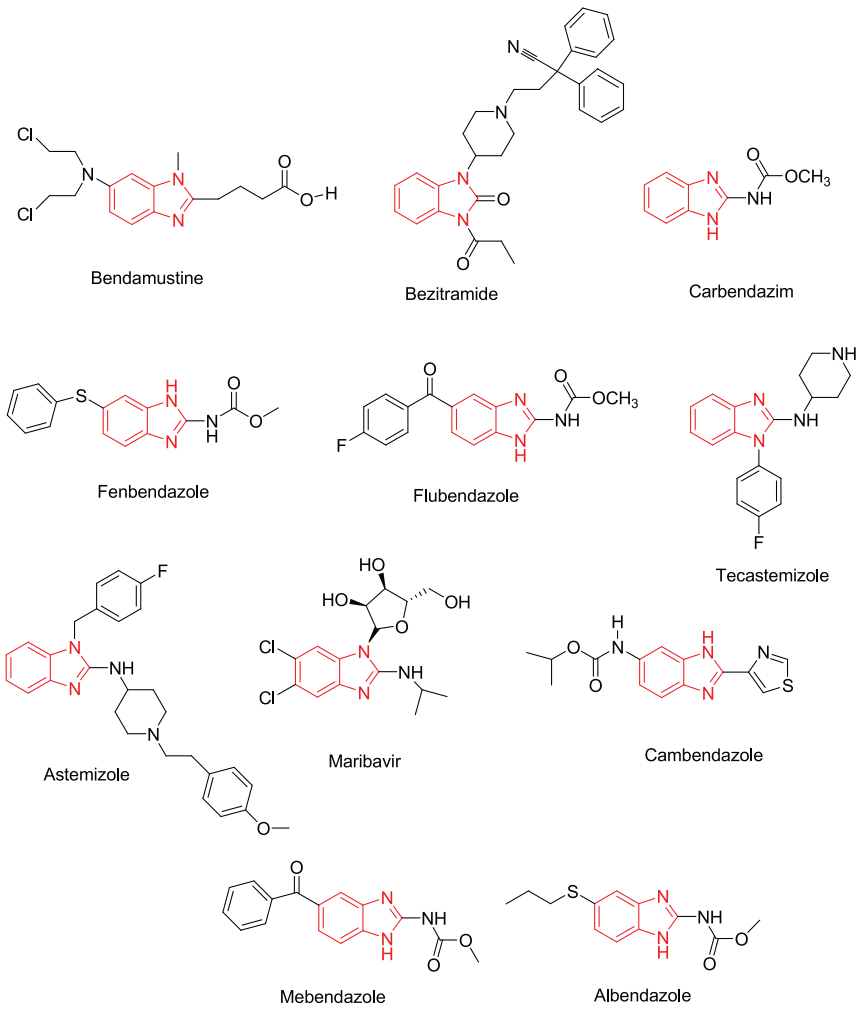
multiple myeloma, and non-Hodgkin's lymphoma. Bezitramide is an opioid analgesic. Carbendazim is a commonly used broad-spectrum benzimidazole fungicide. Fenbendazole is an anthelmintic drug used against gastrointestinal parasites. Flubendazole is an anthelmintic. Candesartan is an angiotensin receptor blocker used in the treatment of hypertension and congestive heart failure. Tecastemizole is used as an antiparasitic drug. Astemizole is a second-generation antihistamine drug. Maribavir is an antiviral drug used for the prevention and the treatment of human cytomegalo virus disease in hematopoietic stem cell/bone marrow transplant patients. Cambendazole is used in the treatment of animals. Mebendazole is used in the treatment of the intestine parasites. Albendazole is used to treat many parasitic infections (Scheme 1). On the other hand, there is also benzimidazole in the structure of vitamin B12.[16] Samples can be increased further.

In this study, a few potential anticancer drug candidates were synthesized in two steps. In the first step, 2-*p*-tolyl-1*H*-benzo[*d*]imidazole was prepared from *o*-phenylenediamine and 4-methylbenzaldehyde. In the second step, a series of 1,2-disubstituted benzimidazole compounds were synthesized using various aryl halides. Their structures were characterized by IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Theoretical calculations of the molecules were done using Spartan 10 in ethyl alcohol (in the Supporting Information). The antiproliferative activity studies of the compounds were performed against the MCF-7 and HepG2 cell lines for 72 h.

2 | MATERIALS AND METHODS

2.1 | Used reagents, solvents, and materials

The necessary chemical materials, such as *o*-phenylenediamine, benzyl chloride, 4-methylbenzylchloride, 2,3,4,5,6-pentamethylbenzylchloride, 3-methoxybenzylchloride, 1-(2-bromoethyl)-4-nitrobenzene, nickel (II) chloride



SCHEME 1 Commercially available drugs containing benzimidazole nucleus

hexahydrate ($NiCl_2.6H_2O$), chloroform ($CHCl_3$), potassium hydroxide (KOH), ethyl alcohol, dimethyl sulfoxide (DMSO), and *N*,*N*-dimethylformamide (DMF), were bought from chemical companies such as Sigma-Aldrich (Interlab A.S., USA), Merck (Darmstadt, Germany), or Isolab (Isolab Laborgaräte GmbH, Germany).

MCF-7 (ATCC® HTB-22™) and HepG2 (ATCC® HB-8065™) cell lines were bought from ATCC (American Type Culture Collection, USA). Trypsin-EDTA, phosphate buffered saline (PBS), fetal bovine serum (FBS), dulbecco's modified eagle's medium (DMEM), and other necessary laboratory materials such as tissue culture flasks, 96-well plates, centrifuge tubes, serological pipettes, PCR tubes, and tips were purchased from Sigma-Aldrich Chemie GmbH, Thermo Fisher (Germany), BioFrox (Germany), PAN Biotech (South America), Gibco by Life Technologies, Wisent Inc., Toronto Research Chemicals Inc. (Toronto/Canada), VWR life science, Costar Corning Incorporated (USA), Nest biotechnology Co. LTD. (China), and LP Italiana SPA (Italy).

2.2 | Synthesis of heterocyclic compounds

2.2.1 | 1-Benzyl-2-p-tolyl-1*H*-benzo[d] imidazole, 2

Xie et al[17] synthesized 1-benzyl-2-*p*-tolylbenzimidazole by the presence of CuI as catalyst, K_3PO_4 as base, and DMF as solvent. They conducted reaction under N_2 at 120°C for 20 h. Bera et al[18] synthesized a series of N-heteroaromatics with nickel-catalyzed dehydrogenative coupling of alcohols with aromatic diamines. They used a Ni-based catalyst.

In this study, the following method for the synthesis of compound **2** was used. For the first step of the reaction, *o*-phenylenediamine (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 the reaction mixture was stirred for 3 h at 25°C. After 3 h, cyclohexane was added to the reaction medium, and it was filtered using a glass Gooch crucible (50 ml). The product, namely, 2-*p*-tolyl-1*H*-benzo[*d*] imidazole (**1**), was washed several times with cyclohexane and was dried using a vacuum pump. For the second step of the reaction, compound **1** (0.18 g, 1 mmol) was dissolved in ethyl alcohol (3 ml), and KOH (0.048 g, 1 mmol) was added to this solution. It was mixed 1 h at 25°C. Then, benzyl chloride (0.109 g, 1 mmol) was slowly added to this solution, and the reaction mixture was stirred for 7 h at 80°C. The aimed final product was washed a few times with diethyl ether and dried under vacuum. By with this method, compound **2** was synthesized in a moderate yield like 43%.

2.2.2 | 1-(4-Methylbenzyl)-2-p-tolyl-1*H*-benzo[d]imidazole, 3

Xu et al[19] prepared 1-(4-methylbenzyl)-2-*p*-tolylben zimidazole with a different synthetic method. In this study, *o*-phenylenediamine (0.50 g, 1 mmol), 4-methyl benzaldehyde (0.56 g, 1 mmol), and $NiCl_2.6H_2O$ (0.11 g, 0.1 mmol) were dissolved in chloroform ($CHCl_3$), and the reaction mixture was stirred for 3 h at 25°C. After 3 h, cyclohexane was added to the reaction medium, and it was filtered using a glass Gooch crucible (50 ml). The product, namely, 2-*p*-tolyl-1*H*-benzo[*d*]imidazole (**1**), was washed several times with cyclohexane and was dried using a vacuum pump. Compound **3** was synthesized from 4-methylbenzyl chloride (0.202 g, 1 mmol) and **1** (0.30 g, 1 mmol) in ethyl alcohol. The product was purified by crystallization in ethyl acetate. Compound **3** was obtained in 51% yield.

2.2.3 | 1-(3-Methoxybenzyl)-2-p-tolyl-1*H*-benzo[d]imidazole, 4

o-Phenylenediamine (0.50 g, 1 mmol), 4-methylbenzal dehyde (0.56 g, 1 mmol), and $NiCl_2.6H_2O$ (0.11 g, 0.1 mmol) were dissolved in chloroform ($CHCl_3$), and the reaction mixture was stirred for 3 h at 25°C. After 3 h, cyclohexane was added to the reaction medium, and it was filtered using a glass Gooch crucible (50 ml). The product, namely, 2-*p*-tolyl-1*H*-benzo[*d*]imidazole (**1**), was washed several times with cyclohexane and was dried using a vacuum pump. Compound **4** was synthesized from 3-methoxybenzyl chloride (279.4 µl, 1 mmol) and **1** (0.4 g, 1 mmol) in ethyl alcohol. This new product was purified by crystallization in isopropyl alcohol. Yield: 53%, m.p.: 98–99°C, color:cream. IR: 1249.4 (C–O); 1447.0 (C═N); 2912.3 and 2960.0 cm^{-1} (C–H). ^1H NMR (400 MHz, DMSO-d_6, 298 K), δ: 2.36 (s, 3*H*, $C_6H_4CH_3$); 3.42 [s, 3*H*, $C_6H_4(OCH_3)$-3]; 5.53 [s, 2*H*, $NCH_2C_6H_4(OCH_3)$-3]; 6.51–8.06 (m, 12*H*, Ar-*H*). ^{13}C NMR (100 MHz, DMSO-d_6, 298 K), δ: 21.38 ($C_6H_4CH_3$); 47.75 [$C_6H_4(OCH_3)$-3]; 55.40 [$NCH_2C_6H_4(OCH_3)$-3]; 111.48, 112.53, 113.05, 118.53, 119.59, 122.64, 123.05, 126.83, 127.70, 129.39, 129.86, 129.98, 130.44, 136.32, 139.03, 140.05, 143.07, 153.81 and 159.91 (Ar-*C* and N*C*N). Elemental analysis for $C_{22}H_{20}N_2O$ (328.41 g/mol) %: Found C: 80.35; H: 6.28; N: 8.48. Anal. Calc. C: 80.46; H: 6.14; N: 8.53.

2.2.4 | 1-(4-Nitrophenethyl)-2-p-tolyl-1*H*-benzo[d]imidazole, 5

o-Phenylenediamine (0.50 g, 1 mmol), 4-methylbenzal dehyde (0.56 g, 1 mmol), and $NiCl_2.6H_2O$ (0.11 g, 0.1 mmol) were dissolved in chloroform ($CHCl_3$), and the reaction mixture was stirred for 3 h at 25°C. After 3 h, cyclohexane was added to the reaction medium, and it was filtered using a glass Gooch crucible (50 ml). The product, namely, 2-*p*-tolyl-1*H*-benzo[*d*]imidazole (**1**), was washed several times with cyclohexane and was dried using a vacuum pump. Compound **5** was synthesized from 2-(4-nitrophenyl)-1-bromoethane (0.36 g, 1 mmol) and **1** (0.33 g, 1 mmol) in ethyl alcohol. This new product was crystallized in butyl alcohol. Yield: 45%, IR: 1441.5 (C═N); 2903.7 and 2968.6 cm^{-1} (C–H). ^{1}H NMR (400 MHz, DMSO-d_6, 298 K), δ: 2.23 (s, 3*H*, $C_6H_4CH_3$); 2.50 [t, *J*: 8.0 Hz, 2*H*, $NCH_2CH_2C_6H_4(NO_2)$-4]; 5.51 [t, *J*: 8.0 Hz, 2*H*, $NCH_2CH_2C_6H_4(NO_2)$-4]; 6.88-7.82 (m, 12*H*, Ar-*H*). ^{13}C NMR (100 MHz, DMSO-d_6, 298 K), δ: 21.04 ($C_6H_4CH_3$); 21.39 [$NCH_2CH_2C_6H_4(NO_2)$-4]; 47.68 [$NCH_2CH_2C_6H_4(NO_2)$-4]; 111.50, 119.58, 122.55, 122.96, 126.46, 126.83, 127.77, 129.38, 129.79, 129.81, 134.40, 136.32, 137.10, 139.98, 143.14 and 153.79 (Ar-*C* and N*C*N). Elemental analysis for $C_{22}H_{19}N_3O_2$ (357.41 g/mol) %: Found C: 73.81; H: 5.43; N: 11.71. Anal. Calc. C: 73.93; H: 5.36; N: 11.76.

2.2.5 | 1-(2,3,4,5,6-Pentamethylbenzyl)-2-p-tolyl-1*H*-benzo[d]imidazole, 6

o-Phenylenediamine (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 the reaction mixture was stirred for 3 h at 25°C. After 3 h, cyclohexane was added to the reaction medium, and it was filtered using a glass Gooch crucible (50 ml). The product, namely, 2-*p*-tolyl-1*H*-benzo[*d*]imidazole (**1**), was washed several times with cyclohexane and was dried using a vacuum pump. Compound **6** was synthesized from 2,3,4,5,6-pentamethylbenzyl chloride (0.35 g, 1 mmol) and **1** (0.37 g, 1 mmol) in ethyl alcohol. Yield: 48%, m.p.: 174-176°C. ^{1}H NMR (400 MHz, DMSO-d_6, 298 K), δ: 1.96, 2.15, 2.33 and 2.50 [s, 18*H*, $C_6H_4CH_3$ and $NCH_2C_6(CH_3)_5$]; 5.55 [s, 2*H*, $NCH_2C_6(CH_3)_5$]; 7.08–7.71 (m, 8*H*, Ar-*H*). ^{13}C NMR (100 MHz, DMSO-d_6, 298 K), δ: 16.79, 17.00, 21.45 and 58.47 [$C_6H_4CH_3$ and $NCH_2C_6(CH_3)_5$]; 67.44 [$NCH_2C_6(CH_3)_5$]; 111.57, 115.87, 119.82, 121.97, 129.52, 130.02, 132.72, and 149.95 (Ar-*C* and N*C*N). Elemental analysis for $C_{26}H_{28}N_2$ (368.51 g/mol) %: Found C: 84.92; H: 7.58; N: 7.52. Anal. Calc. C: 84.74; H: 7.66; N: 7.60.

2.3 | The cytotoxic activity studies of benzimidazole-based compounds

The cytotoxic activity studies were conducted according to literature procedures.[20,21] MCF-7 and HepG2 cells were cultured in DMEM including high glucose supplemented with 1% penicillin streptomycin and 10% FBS. The cell seeding was done in a density of 4×10^3 cells/well into sterile 96-well plates and maintained at 37°C for 24 h. MCF-7 and HepG2 cells were exposed to synthesized compounds (**2**–**4**) at nine different concentrations (200–0.5 μM) and six dissimilar concentrations ranging from 200 to 5 μM, respectively. Cells were exposed to drug candidates for 72 h. The plates were incubated for 4 h after adding MTT stock solution to each well. After this period, the MTT stock solution was aspirated and 200 μl of DMSO was added to each well. The plates were then shaken for 30 min on a plate rocker. Absorbance values were read using an ELISA micro plate reader (Biorad 6800) device at 560 nm. The GraphPad Prism software was used for calculating IC_{50} values.

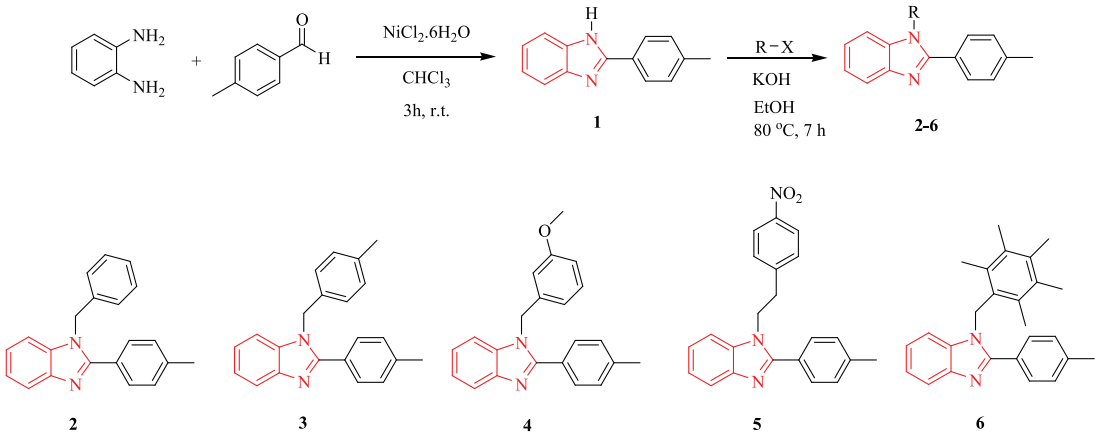
3 | RESULTS AND DISCUSSION

3.1 | Synthesis and spectral characterization of 1,2-disubstituted benzimidazole derivatives

A series of heterocyclic compounds including 2-*p*-tolyl-1*H*-benzo[*d*]imidazole structure were synthesized in two steps in moderate yields (Scheme 2). The structures of these heterocyclic compounds were verified with elemental analysis, ^{1}H NMR, ^{13}C NMR, and IR.

The ^{1}H NMR spectra of the molecules were run in $CDCl_3$ for **3** and DMSO-d_6 for **2**, **4**-**6** at 400 MHz. In ^{1}H NMR, the signal belonged to an aryl-linked methyl group –$C_6H_4CH_3$ resonated upfield at δ 2.38, 2.35, 2.36, 2.23, and 2.33 ppm as 3H singlets for **2**–**6**, respectively. The most upfield signals were found to belong to CH_3 in all compounds (**2**–**6**). The methylene protons, which were aryl-linked CH_2, were seen downfield as 2*H* singlet signals at δ 5.57, 5.44, 5.53, 5.51, and 5.55 ppm for **2**–**6**, respectively. The aryl-linked methoxy group (OCH_3) of compound **4** resonated more downfield than the CH_3 group at δ 3.42 ppm as a singlet. All the aromatic proton signals of these compounds were seen downfield of tetramethyl silane, around δ 6.51–8.08 ppm.

The ^{13}C NMR spectra were run in $CDCl_3$ or DMSO-d_6 at 100 MHz. The aryl-linked methyl carbon signals resonated at δ 21.38 for **2**, 21.11 and 21.47 for **3**, 21.38 for **4**, 21.04 for **5**, 16.79, 17.00, 21.45, and 58.47 ppm for **6**. The formation of compounds was validated by the presence of



SCHEME 2 Synthesis of 1,2-disubstituted benzimidazole

methylene (CH_2) carbon signal at δ 47.86, 48.25, 55.40, 47.68, and 67.44 ppm for **2–6**, respectively. The presence of a CH_2 signal in ^{13}C NMR verified the structures of the aimed molecules **2–6**, because there was not this CH_2 signal in the precursor compound **1**.

The IR spectra data of the compounds revealed the stretching frequencies of the characteristic C═N peak at 1445.3, 1455.6, 1447.0, and 1441.5 cm^{-1} for **2–5**, respectively. The stretching frequency bands of C–H were observed at 2960.7 cm^{-1} for **2**, 2914.9 and 3009.1 cm^{-1} for **3**, 2912.3 and 2960.0 cm^{-1} for **4**, and 2903.7 and 2968.6 cm^{-1} for **5**. An additional band was noticed in molecule **4** at 1249.4 cm^{-1}, which represents the C-O group.

Electronegativity (χ), chemical hardness (η), the energy of the lowest unoccupied molecular orbital (E_{LUMO}), and the energy of the highest occupied molecular orbital (E_{HOMO}) of molecules **1–6** were calculated using the Spartan 10 program. The results are given in the Supporting Information.

3.2 | Antiproliferative activity studies of 1,2-disubstituted benzimidazoles

Using the MTT assay method, the developed compounds **2–4** were evaluated for their cytotoxicity at 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 μM concentrations in the MCF-7 cell line and 5, 10, 20, 50, 100, and 200 μM concentrations in the HepG2 cell line. In this study, under the same experimental conditions, two positive control drugs (irinotecan hydrochloride trihydrate and 5-fluorouracil) were used as reference compounds for comparison. The cancerous cells were treated with compounds for 72 h. The results are given in Table 1.

Although compounds **2** and **4** were found to be inactive in MCF-7, molecule **3** was much less active compared to the reference drugs against the same cell line.

TABLE 1 IC_{50} results for compounds **2–4** and positive control drugs against human cancer cell lines

Compounds	IC_{50}, μM	
	MCF-7	HepG2
2	>200	63.88
3	130	48.17
4	>200	66.60
Irinotecan hydrochloride trihydrate	27.84	N.T.
5-Fluorouracil	26.31	55.48

Abbreviation: N.T., not tested.

Compound **2** was the least potent drug candidate and was seen more than 70% cell viability ratio even it was used at the high concentration of 200 μM. Therefore, this compound has no toxic effect on MCF-7. Compound **3**, having 4-methylbenzyl group, was the most potent compound and induced important cytotoxicity at 100 and 200 μM. Cell viability ratios in these concentrations were obtained under 50% (cell viability: <50%). However, this molecule (**3**) did not show noteworthy cytotoxicity in concentrations such as 0.5, 1, 2, 5, 10, 20, and 50 μM towards MCF-7 (Figure 1). Compound **4** demonstrated cytotoxicity only at a 200 μM concentration and was found to be ineffective in other eight concentrations ranging from 100 to 0.5 μM against MCF-7 (Figure 1).

As seen in the Figure 2, the prepared drug concentrations affected cell viability in different rates. That is, cell viability changes depending on the concentration. When the drug concentration was increased, the cell viability mostly dropped.

The heterocyclic compounds (**2–4**) inhibited the proliferation of rapidly dividing liver cancerous cells rather than breast cancerous cells. It is worth noting that all compounds tested had more antiproliferative activity at 100 and 200 μM than reference drug 5-fluorouracil

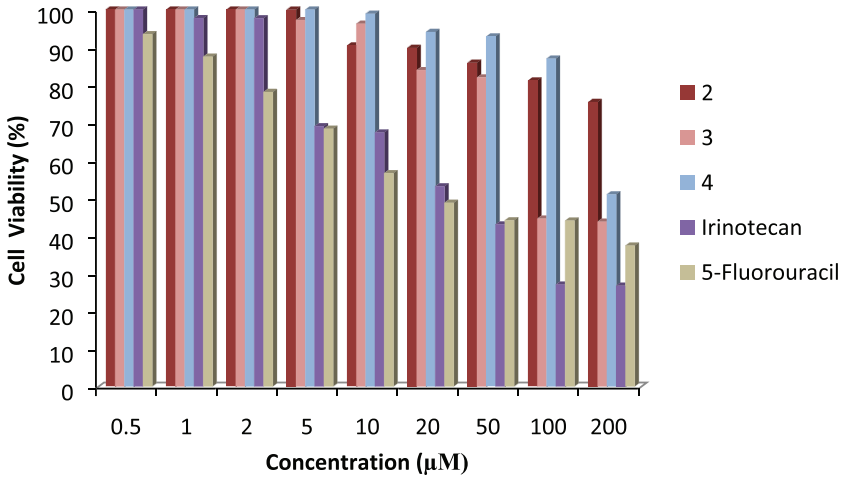


FIGURE 1 Cell viability ratio of MCF-7 cells

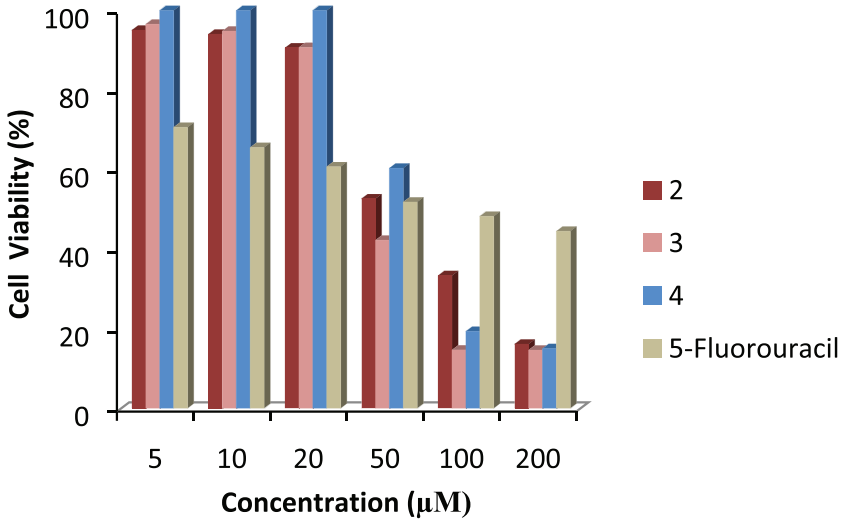


FIGURE 2 Cell viability ratio of HepG2 cells

towards HepG2. Compound **4** demonstrated cytotoxicity at 50, 100, and 200 µM, but not at 5, 10, and 20 µM concentrations (Figure 2).

4 | CONCLUSIONS

Five heterocyclic compounds were synthesized and the confirmations of the chemical structure of these benzimidazole scaffold-based molecules (**2**-**6**) were done with ^{1}H NMR, ^{13}C NMR, IR, and elemental analysis. Synthesized compounds, together with positive control drugs, namely, 5-fluorouacil and irinotecan hydrochloride trihydrate, were evaluated for in vitro cytotoxic activity against the MCF-7 and HepG2 cell lines. Compounds **2** and **4** did not demonstrate measurable cytotoxic activity in MCF-7 with IC_{50} values of >200, although compound **4** indicated cytotoxic activity at a concentration of 200 µM. Molecules **2** and **4** were found highly active in the liver cell line. The electron donating CH_3 group on the benzene rings in the para position played a crucial role in activity and increased the antiproliferative effect of compound **3** on the liver and breast cancerous cell lines. Based on the in vitro cytotoxic activity results, compound **3** was the most effective drug candidate among the other tested compounds **2** and **4**.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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