

Novel 1,2,5-Trisubstituted Benzimidazoles Potentiate Apoptosis by Mitochondrial Dysfunction in Panel of Cancer Cells

Jagadeesha Gullahalli Swathantraiah,[#] Sudhanva Muddenahalli Srinivasa,[#] Anil Kumar Belagal Motatis, Akshay Uttarkar, Shwetha Bettaswamygowda, Sridhar Bilgumba Thimmaiah, Vidya Niranjana, Shobith Rangappa,* Rangappa Kanchugarakoppal Subbegowda, and Thimmegowda Naraganahalli Ramegowda*



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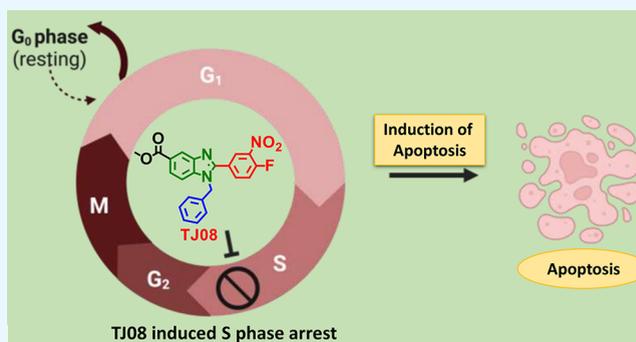


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ABSTRACT: Synthetic small molecules have been very effective in decimating cancer cells by targeting various aberrantly overexpressed oncogenic proteins. These small molecules target proteins involved in cell cycle regulation, cell division, migration, invasion, angiogenesis, and other regulatory proteins to induce apoptosis in cancer cells. In this study, we have synthesized a novel 1,2,5-trisubstituted benzimidazole chemical library of small molecules and unveiled their anticancer potential against a panel of cancer cell lines such as Jurkat, K-562, MOLT-4, HeLa, HCT116, and MIA PaCa-2 cancer cells. The MTT assay and Trypan blue dye exclusion assay clearly unveiled the cytotoxic effect of methyl 1-benzyl-2-(4-fluoro-3-nitrophenyl)-1*H*-benzo[*d*]-imidazole-5-carboxylate (TJ08) and its potential to induce apoptosis with effective IC₅₀ of 1.88 ± 0.51, 1.89 ± 0.55, 2.05 ± 0.72, 2.11 ± 0.62, 3.04 ± 0.8, and 3.82 ± 0.25 μM against Jurkat, K562, MOLT-4, HeLa, HCT116, and MIA PaCa-2 cancer cell lines, respectively. Altered mitochondrial membrane potential was observed in HeLa, HCT116, and Jurkat cells due to TJ08 treatment, which was unveiled by JC10 staining. Induction of early and late apoptosis by TJ08 treatment was also unveiled by apoptotic analysis and immunofluorescence imaging. Cell cycle analysis distribution confirms the accumulation of cells in the S-phase in a dose-dependent manner.



1. INTRODUCTION

Public health issues pose major challenges across the globe, especially cancer which increases every year and stands second in the causes of deaths across the globe. Hence, cancer is a major public health problem worldwide. Though many anticancer agents have been proven to be successful in the clinics, they have encountered various issues like low bioavailability and solubility. Cancer cells develop drug resistance to available drugs by various mechanisms, increasing drug efflux pumps (MDR1, P-gp) and drug transporters. Drug efflux by ATP binding cassette transporters is of major concern because it effectively eliminates the drug and inhibits the effective accumulation of drug inside the cells. Several cancer cells exhibit overexpression of these drug efflux transporters which results in developing drug resistance against various available drugs in the market. Hence multidrug resistance (MDR) and intolerable toxicities are a major concern for effective chemotherapeutic interventions against human malignancies. Hence, there is a constant demand to identify and develop structurally novel potent anticancer drugs to combat various cancers.

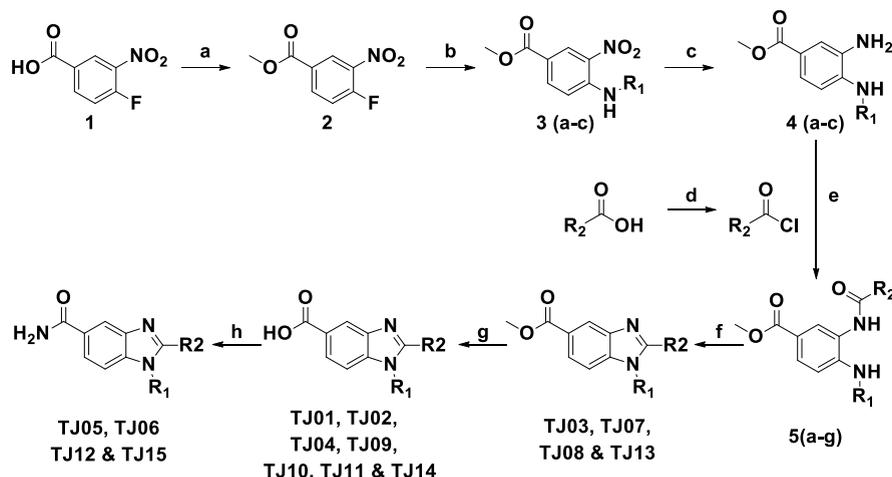
Benzimidazole is one of the most important heterocyclic compounds having structural similarity to nucleotides found in the human body, and it is an important pharmacophore in medicinal chemistry.¹ The benzimidazole nucleus is a structural isostere of purine nucleosides. The structural resemblance of the benzimidazole with the purine nucleus in nucleotides makes benzimidazole derivatives, which are attractive ligands for interaction with biopolymers. The benzene ring, which is present in 1*H*-benzimidazole, functions as an electron-attracting group on imidazole and thereby decreases imidazole's electronic density, which plays an important role in chemical interactions that have varied biological activities and thus are of great scientific interest

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Scheme 1. Synthesis of Benzimidazole Derivatives TJ01–TJ15^a

^aReagents and reaction conditions: (a) SOCl_2 (1.5 equiv), MeOH (10 vol), 70 °C, 2 h, 98.0% yield; (b) K_2CO_3 (2 equiv), $\text{R}_1\text{-NH}_2$ (1.1 equiv), THF (10 vol), 0 °C to rt, 2 h, 95.0–98.6% yield; (c) iron powder (10% w/w), saturated aqueous NH_4Cl solution (5 vol), IPA (10 vol), 90 °C, 2–3 h, 83.3–86.5% yield; (d) SOCl_2 (1.5 equiv), toluene (20 vol), 50 °C, 2 h; (e) acyl chloride (1.1 equiv), THF (20 vol), DIPEA (2 equiv), 0 °C to rt, 3 h, 78.3–93.6% yield; (f) CH_3COOH (10 vol), 100 °C, 3–5 h, 73.7–93.9% yield; (g) $\text{LiOH}\cdot\text{H}_2\text{O}$ (1.5–3.1 equiv), $\text{CH}_3\text{OH}/\text{THF}/\text{H}_2\text{O}$ (1:1:1), 0 °C to rt to 60 °C, 2–8 h, 57.2–98.3%; (h) CDI (1.5 equiv), aqueous NH_3 (3 vol), THF (10 vol), 0 °C to rt, 6 h, 77.5%–90.2%.

Table 2. Structures of R_1 and R_2 of Intermediate Compounds 3a–c, 4a–c, and 5a–g

	<p>3a (98.1%)</p>	<p>3b (98.6%)</p>	<p>3c (95.0%)</p>		<p>4a (83.3%)</p>	<p>4b (84.0%)</p>	<p>4c (86.5%)</p>
	<p>5a–g</p>						
	<p>5a (92.9%)</p>	<p>5b (91.7%)</p>	<p>5c (93.6%)</p>	<p>5d (88.4%)</p>	<p>5e (78.3%)</p>	<p>5f (81.1%)</p>	<p>5g (90.3%)</p>
R_2							

the benzimidazole core. The cytotoxic potency of newly synthesized 1,2,5-trisubstituted benzimidazole derivatives against various cancer cells reveals that the TJ08 compound is a promising hit which efficiently induces apoptosis in cancer cells. Here we enumerated the efficiency of the synthesized library of compounds against panels of cancer cells to unveil their anticancer potential.

2. RESULTS AND DISCUSSION

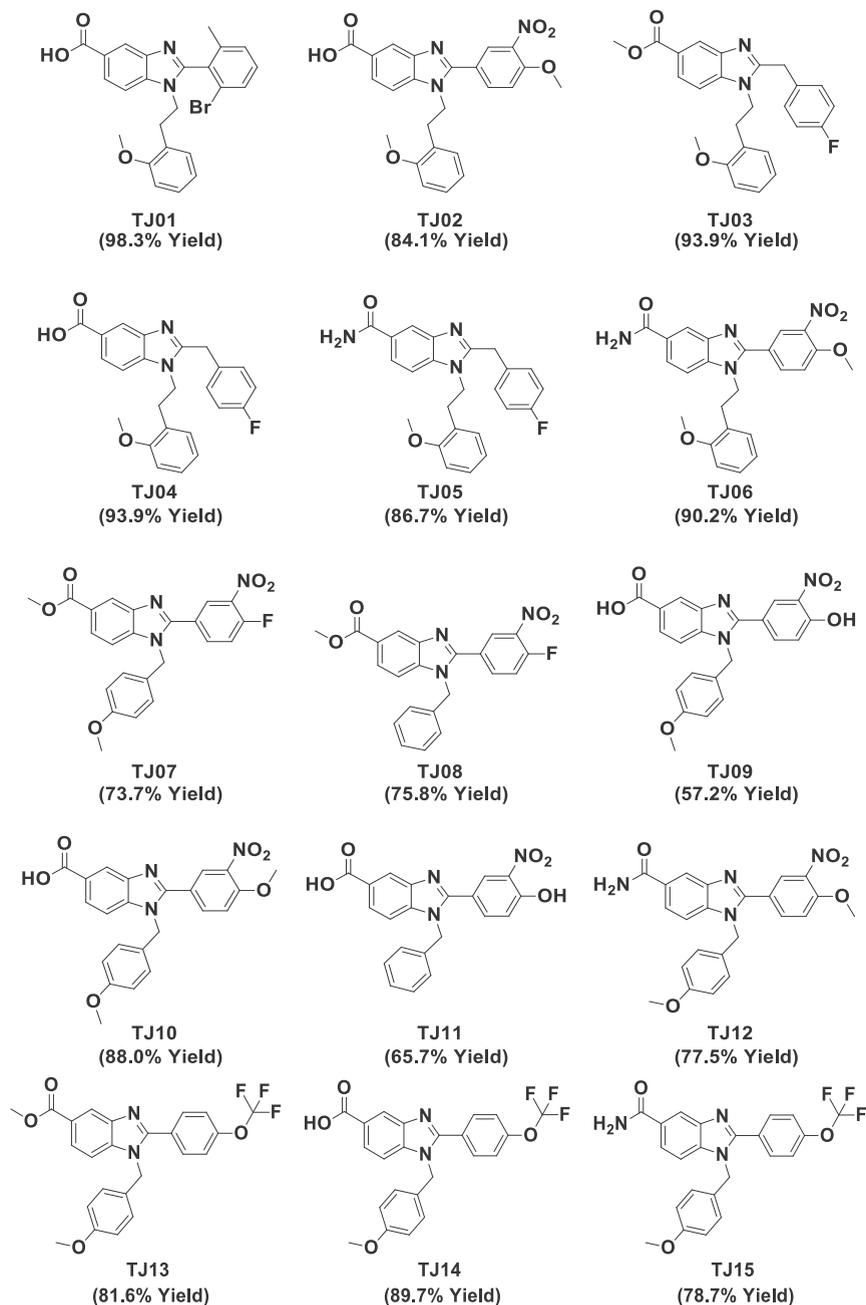
2.1. Chemistry. The synthetic procedures that we followed for the synthesis of designed benzimidazole derivatives TJ01–TJ15 are outlined in Schemes 1–4. The structures of the substituents R_1 and R_2 of synthesized intermediate compounds

3a–c, 4a–c, and 5a–g are shown in Table 2. The structures of synthesized final compounds TJ01–TJ15 are displayed in Table 3.

The methyl 4-fluoro-3-nitrobenzoate (2) was obtained (98% yield) from 4-fluoro-3-nitrobenzoic acid (1) by an esterification reaction using thionyl chloride and DMF (catalytic amount) in methanol as a solvent under reflux conditions. Aromatic nucleophilic substitution reaction of compound 2 with different substituted aromatic amines in the presence of potassium carbonate as a base and tetrahydrofuran as solvent gives compounds 3a–c (95–98.6% yield).

Compounds 3a–c were converted to 4a–c (83.3–86.5% yield) by reduction of the nitro group to the corresponding

Table 3. Structures of the Final Compounds TJ01–TJ15



amines. These conversions were performed by treatment with iron powder, saturated aqueous ammonium chloride solution, and isopropyl alcohol as a solvent under reflux conditions. The intermediate compounds **5a–g** (78.3–93.6% yield) were obtained by reacting the compounds **4a–c** with different aromatic acyl chlorides (acyl chlorides were prepared by reacting the corresponding carboxylic acids with thionyl chloride, a catalytic amount of DMF, and toluene as a solvent) in the presence of *N,N*-diisopropylethylamine as a base and tetrahydrofuran as a solvent.

The 1,2-disubstituted benzimidazole-5-carboxylates **TJ03**, **TJ07**, **TJ08**, and **TJ13** were synthesized (73.7–93.9% yield) as described in **Scheme 1** by heating intermediate compounds **5c**, **5d**, **5g**, and **5f**, respectively, in acetic acid under reflux conditions. The 1,2-disubstituted benzimidazole-5-carboxylic

acids **TJ04**, **TJ09**, **TJ11**, and **TJ14** were prepared (57.2–93.9% yield) from the compounds **TJ03**, **TJ07**, **TJ08**, and **TJ13**, respectively, by hydrolysis of the ester group of these compounds to the corresponding acids (during hydrolysis of ester of **TJ07** and **TJ08**, in addition to hydrolysis of ester, the fluorine was also substituted by a hydroxyl group and produced compounds **TJ09** and **TJ11**, respectively) by reacting with lithium hydroxide monohydrate, tetrahydrofuran, methanol, and water mixture as solvent. The compounds **TJ01**, **TJ02**, and **TJ10** (84.1–98.3% yield) were obtained from intermediate compounds **5a**, **5b**, and **5e** by heating in acetic acid under reflux conditions followed by hydrolysis of the ester to the corresponding acid.

The 1,2-disubstituted benzimidazole-5-carboxamides **TJ05**, **TJ06**, **TJ12**, and **TJ15** were obtained (77.5–90.2% yield) from

Table 4. IC₅₀ Values of TJ08 against Various Cancer Cells and Normal Cells^a

compd	IC ₅₀ (μM)						
	Jurkat	K562	MOLT-4	HeLa	HCT116	MIA-PaCa-2	HEK
TJ08	1.88 ± 0.51	1.89 ± 0.55	2.05 ± 0.72	2.11 ± 0.62	3.04 ± 0.8	3.82 ± 0.25	>50
DOXO	4.89 ± 0.11	0.99 ± 0.21	>1	0.21 ± 0.13	0.26 ± 0.19	0.059 ± 0.63	>50

^aMTT assay, cell treated with TJ08 and incubated for 48 h. IC₅₀ (μM) (mean ± SD, n = 3).

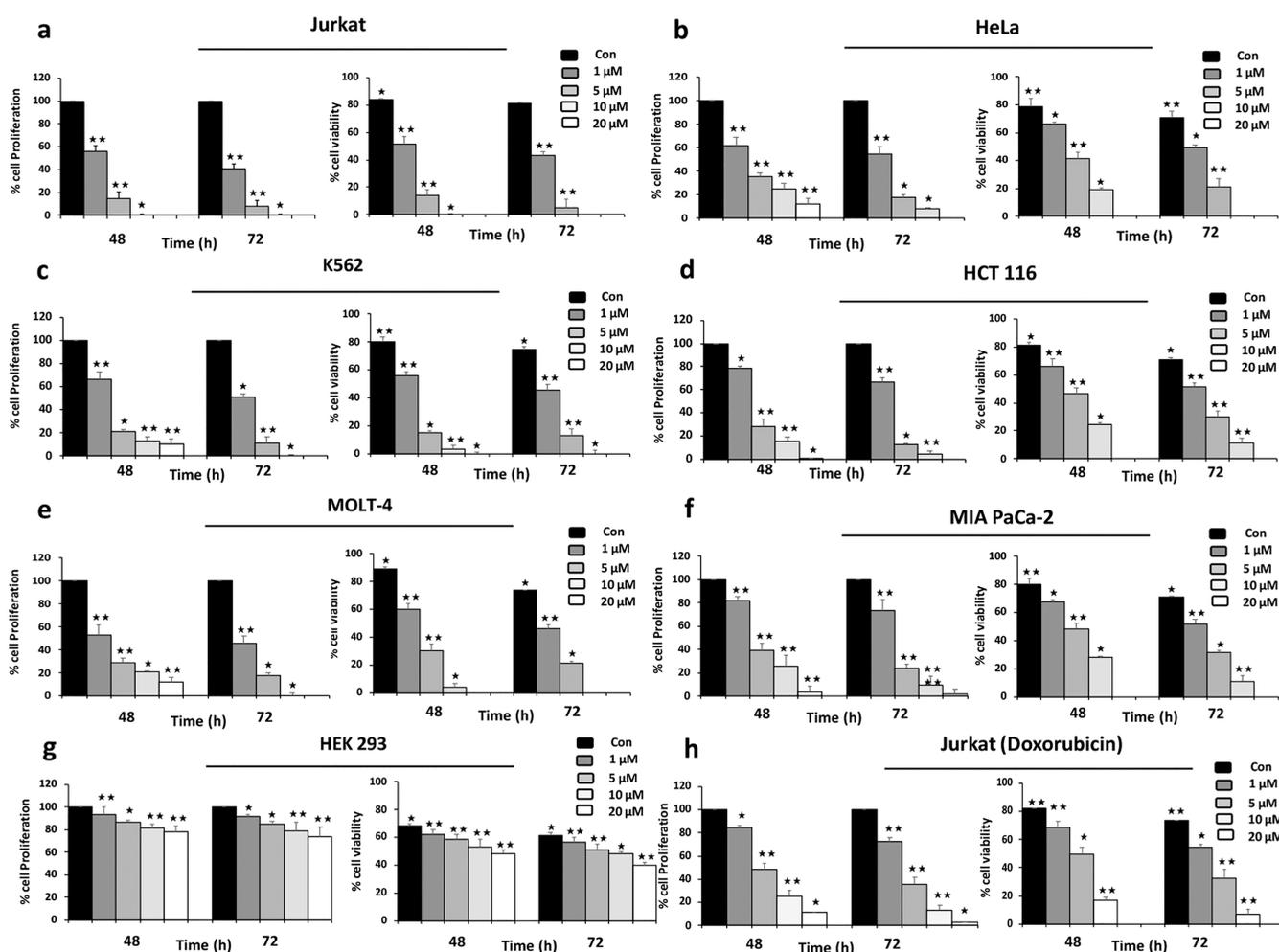


Figure 2. Effect of TJ08 on cell proliferation and cell viability on a panel of cancer cell lines: Cells were seeded in a 96-well plate, incubated for 12 h, treated with an increased concentration of TJ08, incubated for 48 and 72 h, and subjected to MTT and Trypan blue dye exclusion assay to assess the cytotoxic potential of TJ08. DMSO-treated wells served as vehicle control. (a) Jurkat, (b) K562, (c) MOLT4, (d) HeLa, (e) HCT 116, (f) MIA PaCa-2, (g) HEK, and (h) Jurkat (doxorubicin). Each experiment has been repeated a minimum of 3 times. Error bars indicate the SEM and P-values were calculated by comparing the mean of control group with the mean of treated group. *, $p < 0.05$; **, $p < 0.005$.

the compounds TJ04, TJ02, TJ10, and TJ14, respectively, by reacting with the carbonyldiimidazole (CDI) and aqueous ammonia (~25% solution) in DMF as a solvent.

2.2. Biological Evaluation. **2.2.1. Antiproliferative Effect of TJ08 on Various Cancer Cell Lines.** All the synthesized compounds were tested against various cancer cells, including human leukemic cancer cells (Jurkat, K562, and Molt4), human cervical cancer cells (HeLa), human colorectal carcinoma cells (HCT116), and human pancreatic ductal adenocarcinoma (MIAPaCa-2) (Table S1). The results revealed that the compound TJ08 was very effective against various cells with IC₅₀ ranging from 1.88 to 3.82 μM (Table 4), and doxorubicin displayed apoptotic activity against Jurkat cells at 4.89 μM. Jurkat cells were most sensitive, whereas MIA

PaCa-2 cells were least sensitive to TJ08, which was unveiled by MTT assay (Figure 2a–f). Trypan Blue dye exclusion assay also unveiled a drastic reduction in the survival percentage of cancer cells. Interestingly, TJ08 displayed the least cytotoxic effect against normal kidney cell lines (Hek cells) (Figure 2g).

2.2.2. TJ08 Treatment Diminishes Foci Formation and Mitigates Cell Migration and Invasion. Foci formation is the hallmark process of transformed cells; due to the loss of density-dependent growth they grow in multiple layers and form foci. The capability of cancer cells to form foci was examined with the TJ08 treatment. Interestingly, TJ08 treatment diminished the colony formation capability of HeLa, HCT116, and MIA PaCa-2 cells when compared to the DMSO-treated well in a concentration-dependent manner

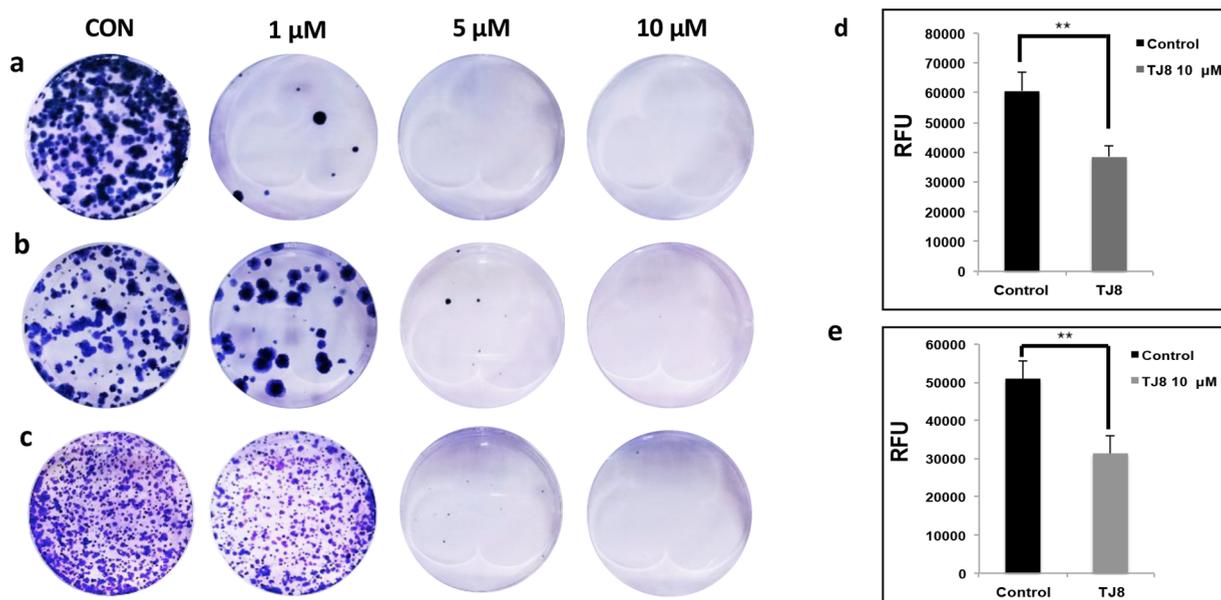


Figure 3. Effect of TJ08 on foci formation, migration, and invasion: Cells were seeded into a 6-well plate and treated with increased concentrations of TJ08, allowed for 2 weeks to form colonies, and stained with crystal violet. (a) HeLa cells, (b) HCT116, and (c) MIA PaCa-2 cells were used for the study. DMSO-treated wells served as the vehicle control; each experiment was repeated 3 times. (d, e) Effect of TJ08 on migration and invasion, respectively. HeLa cells were seeded and treated with TJ08, incubated, and subjected for assay as described in the procedure, and DMSO-treated wells served as the vehicle control; each experiment was repeated 3 times. Error bars indicate the SEM and *P*-values were calculated by comparing the mean of control group with the mean of TJ08-treated group. **, *p* < 0.05.

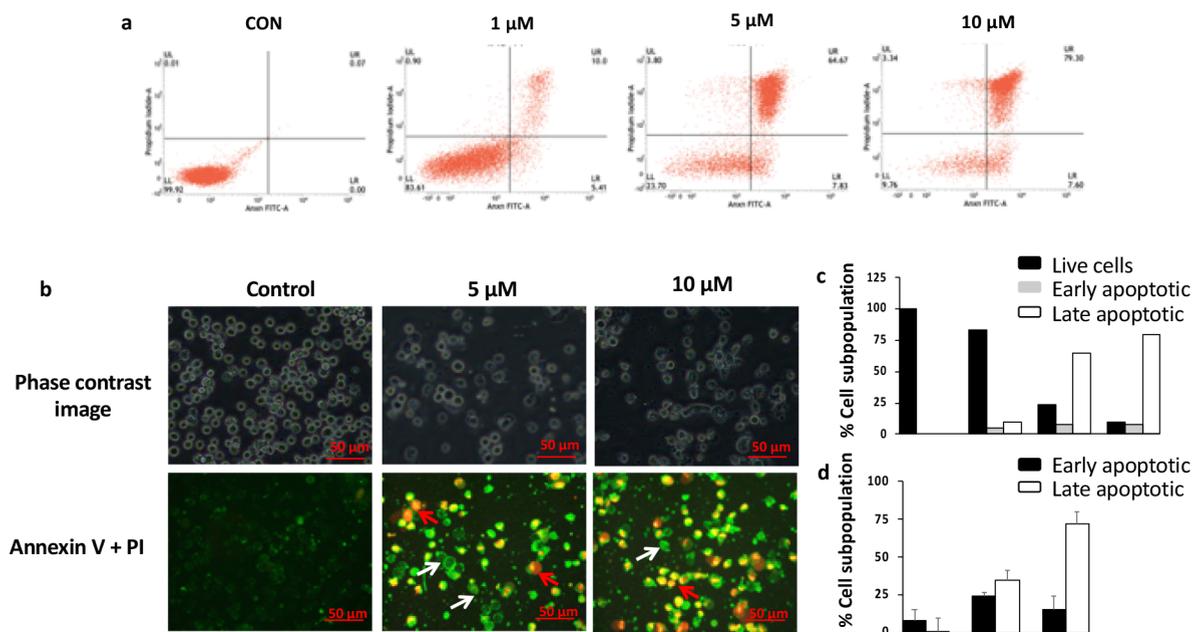


Figure 4. Effect of TJ08 on apoptosis. Jurkat cells were seeded in 6-well dishes, treated with increased concentrations of TJ08, and incubated for 24 h, and cells were harvested and incubated with Annexin V/PI. (a) Cytometric analysis of apoptotic cells. (b) Fluorescence microscopic imaging of apoptotic cells. (c, d) Histogram of percentage of apoptotic cells cytometric and fluorescence analysis. Cells marked with white arrow represents early apoptotic cells, and cells marked with red arrow represent late apoptotic cells.

(Figure 3a–c). Metastasis plays a pivotal role in the development of secondary tumors, which was facilitated by the degradation of the extracellular matrix and promotes migration and invasion of cancer cells. TJ08 treatment diminishes migration and invasion of HeLa cells, which was unveiled by Roche's cell migration and invasion kits (Figure 3d,e).

2.2.3. Induction of Apoptosis by TJ08 Treatment. Evasion of apoptosis is a characteristic feature of cancer cells to ensure rapid proliferation, but chemotherapeutic drugs forcibly induce apoptosis in cancer cells. TJ08-induced early and late apoptotic events were confirmed by Annexin V/PI and also by Hoechst and PI double staining. Accumulation of propidium iodide inside the cells confirms the induction of apoptosis upon TJ08

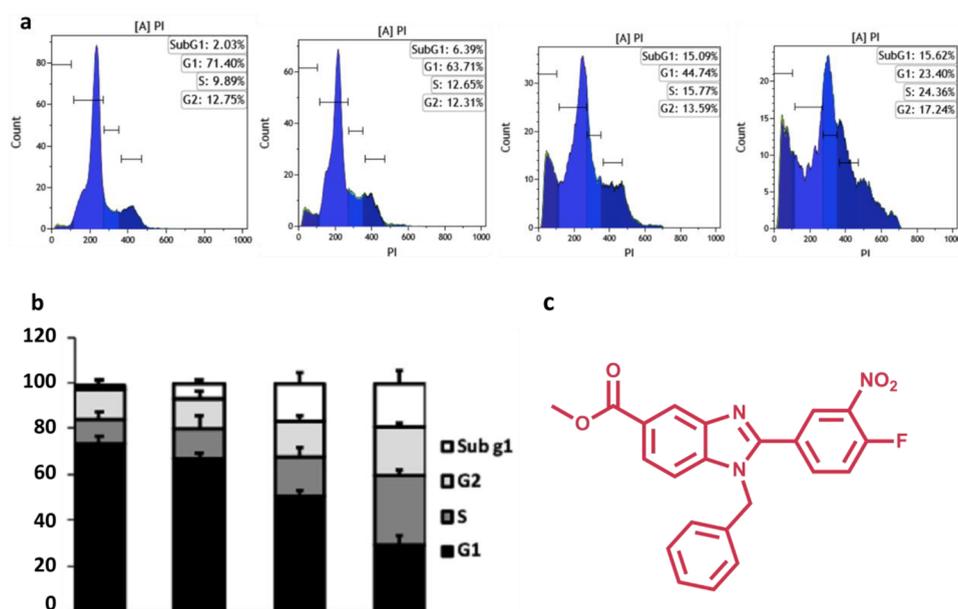


Figure 5. Impact of TJ08 on cell cycle distribution. Jurkat cells were seeded and treated with different concentrations of TJ08 (1, 5, and 10 μM) and incubated for 24 h. Cells were harvested and fixed with ethanol, stained with propidium iodide, and analyzed using flow cytometer. (a) Effect of TJ08 on cell cycle. (b) Percentage of cells distribution in different phases of cell the cycle. (c) Structure of TJ08. DMSO-treated cells served as the vehicle control.

treatment, and the same was further confirmed by staining the cells with Annexin V/PI double stain. Early apoptotic cells were stained with green alone, and late apoptotic cells were stained with both green and red (Figure 4). Interestingly, we did not find any PI-stained cells, which confirms that TJ08 treatment induces cell death by apoptosis, not by necrosis, in a concentration-dependent manner.

2.2.4. TJ08 Instigates S Phase Arrest and Abrogates Cancer Cell Progression. Interference in any protein pathway obstructs the progression of cell cycle and triggers cell cycle arrest. Competency to instigating cell cycle arrest by TJ08 is unveiled by cell cycle analysis which clearly unveils the accumulation of cells at the S phase in a concentration-dependent manner followed by increased accumulation of a sub-G1 population of cells (Figure 5). The results clearly demonstrate that the compound TJ08 induces cell cycle arrest upon treatment and promotes apoptosis of cancer cells.

2.2.5. TJ08 Treatment Permutes Mitochondrial Membrane Potential and Triggers Apoptosis. Membrane potential is a critical component to maintain the integrity of mitochondria. Alterations in the membrane potential result in release of cytochromes into the cytosol of cells, which actuates the apoptotic cascade events inside the cells by activating initiator caspases. Alteration of membrane potential by TJ08 treatment was analyzed using JC10 dye. The inherent negative potential of the intact mitochondrial membrane allows the positively charged JC10 dye to enter the mitochondria. TJ08 treatments alter the membrane potential and results in accumulation of JC10 monomers in the cytosol and exhibit intense green fluorescence in a dose-dependent manner (Figure 6a–c). The obtained result clearly confirms the alteration of mitochondrial membrane potential due to TJ08 treatment.

2.2.6. TJ08 Alters the Expression of Pro-apoptotic and Anti-apoptotic Proteins. Pro-apoptotic and anti-apoptotic proteins play important roles in cell survival and cell death,

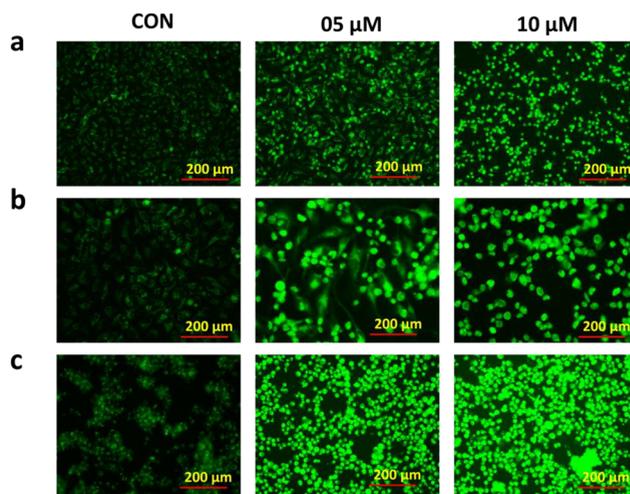


Figure 6. Effect of TJ08 on mitochondrial membrane potential. Cells were seeded and treated with an increased concentration of TJ08, incubated for 24 h, harvested, and subjected for MMP Assay according to manufacturer's protocol. (a) HCT 116, (b) HeLa, and (c) Jurkat cells were used for the study. DMSO-treated cells served as the vehicle control.

specifically BCL2, a major antiapoptotic protein involved in cell survival. TJ08 treatment specifically alters the expression of these proteins and induces apoptosis in cancer cells. Western blot results clearly unveil the upregulation of cleaved caspase and downregulation of antiapoptotic BCL2 proteins. Cleavage of PARP proteins also indicates clear induction of cell death due to TJ08 treatment (Figure 7).

3. CONCLUSION

In this study, we have synthesized 1,2,5-trisubstituted benzimidazole derivatives TJ01–TJ15 and evaluated their anticancer properties, and the compound TJ08 is identified as

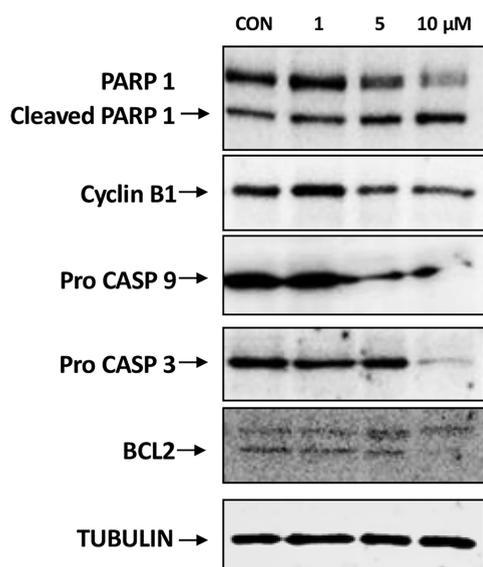


Figure 7. Effect of TJ08 on regulatory proteins. Jurkat cells were seeded in a 100 mm dish, treated with increased concentrations of TJ08, and incubated for 48 h. Cells were harvested and lysed by using RIPA buffer, electrophoresed, electroblotted, and incubated with respective antibodies, cyclin B1, PARP1, CASP 9, CASP3, BCL2, and tubulin.

a structurally novel, potential anticancer agent among the synthesized compounds. Our results revealed that the compound TJ08 efficiently decreases the proliferation capability of various screened cancer cells which was unveiled by both cell proliferation and cell viability assays. The compound TJ08 also diminished the colony formation capabilities of the cancer cells and mitigates invasion and migratory capabilities. Efficient induction of apoptosis will be achieved by triggering cell cycle arrest in the cancer cells. The compound TJ08 efficiently induces G1/S phase arrest and promotes apoptosis in various cancer cells. The compound TJ08 instigates apoptosis by permuting mitochondrial membrane potential, which alters the mitochondrial charges and results in leakage of cytochromes into the cell cytosol which preliminarily activates the initiator caspases and executionary caspases to initiate the apoptotic cascade events. Compound TJ08 efficiently alters the pro- and anti-apoptotic proteins to induce cell death in cancer cells, cleavage of PARP proteins which was the critical evidence for the activation of caspase protein.

The structure–activity relationship results indicates that the key structural features, the 4-fluoro-3-nitrophenyl group at the R₂ position and the benzyl group at the R₁ position of the benzimidazole core, are crucial for the potential anticancer activity of TJ08. Replacement of these groups with other substituents abolished the anticancer potency, which clearly indicates the importance of these groups at the terminal ends. All consolidated results from *in vitro* assays unveil the potency of TJ08 as a promising structurally novel anticancer agent, and hence, we have selected this molecule to develop further to enhance its cytotoxic potential. The synthesis of designed TJ08 analogues and evaluation of their anticancer effect is in progress and will be published in the future.

4. MATERIALS AND METHODS

4.1. Chemistry. Starting materials, reagents, and solvents were purchased from commercial sources and used as received unless stated otherwise. Moisture or air-sensitive reactions were conducted under nitrogen atmosphere in an oven-dried glass apparatus. Reaction progress was monitored by thin-layer chromatography (TLC) on precoated silica gel plates (silica gel 60 F₂₅₄; Merck grade), and visualization was accomplished with UV light and potassium permanganate stains followed by heating. The solvents were removed under reduced pressure using rotary evaporator. Purification of intermediates and final compounds were carried out by silica gel 230–400 mesh (particle size 40–63 μm) flash column chromatography. The NMR spectra were recorded on Bruker 400 and 300 MHz and Agilent 100 MHz spectrometers, and chemical shifts are reported in ppm using the solvents (DMSO-*d*₆). Chemical shifts are reported in ppm using TMS as the internal standard. Data are reported as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constant(s) in hertz. LC–MS analysis were performed on Agilent Technologies, and HRMS was performed on a Waters SYNAPT G2. FTIR spectra were recorded on a Cary 630 spectrometer and Bruker Alpha 2 spectrometer.

4.1.1. Synthesis of Methyl 4-Fluoro-3-nitrobenzoate (2). To a stirred solution of 4-fluoro-3-nitrobenzoic acid (**1**) (5.0 g, 27.01 mmol, 1.0 equiv) in methanol (50 mL) was added thionyl chloride dropwise (3.0 mL, 41.51 mmol, 1.5 equiv) at 0 °C under nitrogen atmosphere, and then the reaction mixture was refluxed at 70 °C for 2 h. The reaction progress was monitored by TLC. Upon completion, the solvent was removed *in vacuo* to obtain the crude product. The crude product was further purified by recrystallization using a mixture of solvents diethyl ether and hexane (30 mL, 1:2 V/V) to obtain pure methyl 4-fluoro-3-nitrobenzoate (**2**) as an off-white solid with 98% yield (5.3 g). ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.56–8.59 (m, 1H, Ar-H), 8.31–8.36 (m, 1H, Ar-H), 7.72–7.78 (m, 1H, Ar-H), 3.91 (s, 3H, -OCH₃). ESI/MS (*m/z*): Calculated for [C₈H₆FNO₄] [M + H]⁺: 200.03. Found *m/z*: 199.9 [M + H]⁺; LCMS purity = 99.361%.

4.1.2. General Procedure A for the Synthesis of Compounds 3a–c. To a stirred solution of methyl 4-fluoro-3-nitrobenzoate (**2**) (1.0 equiv) in tetrahydrofuran (10 V) was added potassium carbonate (2.0 equiv) followed by slow addition of different substituted amines (1.1 equiv) at 0 °C under nitrogen atmosphere. The reaction mixture was gradually allowed to warm to room temperature and stirred for additional 2 h. The reaction progress was monitored by TLC. Upon completion, the reaction mixture was poured into ice cold water, the organic phase was extracted with ethyl acetate (150 mL × 3), and the combined organic phase was washed with water and saturated brine. The organic phase was dried over anhydrous sodium sulfate, and the solvent was filtered and concentrated *in vacuo* to obtain crude product. It was further triturated with *n*-pentane (50 mL), filtered, and dried *in vacuo* to obtain pure compounds 3a–c.

4.1.2.1. Synthesis of Methyl 4-((2-Methoxyphenethyl) amino)-3-nitrobenzoate (3a). Following the general procedure A, compound 3a was obtained from compound **2** (5.1 g, 25.61 mmol, 1.0 equiv) using 2-(2-methoxyphenyl)ethanamine (4.2 mL, 28.69 mmol, 1.1 equiv) as a yellow solid with 98.10% yield (8.3 g).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 8.87–8.88 (d, *J* = 2.1 Hz, 1H, –N-H), 8.879 (s, 1H, Ar-H), 8.02–8.06 (m, 1H, Ar-H), 7.19–7.30 (m, 2H, Ar-H), 6.90–6.97 (m, 3H, Ar-H), 3.91–3.92 (d, 6H, –(OCH₃)₂), 3.56–3.63 (m, 2H, –CH₂), 3.06–3.10 (t, *J* = 6.9 Hz, 2H, –CH₂). ESI/MS (*m/z*): Calculated for [C₁₇H₁₈N₂O₃][M + H]⁺: 331.12; Found *m/z*: 331.20 [M + H]⁺; LCMS purity = 99.614%.

4.1.2.2. Synthesis of Methyl 4-((4-Methoxybenzyl) amino)-3-nitrobenzoate (3b). Following the general procedure A, compound 3b was obtained from compound 2 (3.0 g, 15.06 mmol, 1.0 equiv) using (4-Methoxyphenyl) methanamine (2.2 mL, 16.83 mmol, 1.1equiv) as a yellow solid with 98.6% yield (4.7 g).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 9.00–9.01 (d, *J* = 7.6 Hz, 1H, Ar-H), 8.62–8.63 (d, *J* = 1H, –N-H), 7.88–7.91 (m, 1H, Ar-H), 7.30–7.33 (d, *J* = 11.2 Hz, 2H, Ar-H), 7.02–7.05 (d, *J* = 12.4 Hz, 1H, Ar-H), 6.89–6.92 (d, *J* = 11.6 Hz, 2H, Ar-H), 4.60–4.62 (d, *J* = 8.0 Hz, 2H, –CH₂), 3.72–3.81 (d, 6H, –(OCH₃)₂). ESI/MS (*m/z*): Calculated for [C₁₆H₁₆N₂O₅][M + H]⁺: 317.11. Found *m/z* = 317.2 [M + H]⁺; LCMS purity = 99.303%.

4.1.2.3. Synthesis of Methyl 4-(Benzylamino)-3-nitrobenzoate (3c). Following the general procedure A, compound 3c was obtained from compound 2 (3.0 g, 15.06 mmol, 1.0 equiv) using benzylamine (1.8 mL, 16.47 mmol, 1.1 equiv) as a yellow solid, with 95.0% yield (4.1 g).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 9.10 (s, 1H, Ar-H), 8.64–8.64 (d, *J* = 2.0 Hz, 1H, Ar-H), 7.78–7.79 (m, 1H, Ar-H), 7.33–7.36 (m, 4H, Ar-H), 7.26–7.28 (t, *J* = 4.2 Hz, 1H, Ar-H), 6.99–7.02 (d, *J* = 1.2 Hz, 1H, Ar-H), 4.70–4.71 (d, *J* = 6.0 Hz, 2H, –CH₂), 3.81 (s, 3H, –OCH₃). ESI/MS (*m/z*): Calculated for [C₁₅H₁₄N₂O₄][M + H]⁺: 287.10. Found *m/z* = 287.0 [M + H]⁺; LCMS purity = 97.288%.

4.1.3. General Procedure B for the Synthesis of Compounds 4a–c. The suspensions of nitro compounds 3a–c (1.0 equiv) in isopropanol (10 V) taken in separate round-bottomed flasks were heated to 70 °C (reaction mixture turns to clear solution), and then iron powder (20% w/w) was added to the reaction mixtures followed by saturated aqueous ammonium chloride solution (5 V). Then the reaction mixtures were heated to 90 °C and stirred for 2–3 h. The reaction progress was monitored by TLC. Upon completion, the reaction mixtures were filtered through a Celite pad and thoroughly washed with isopropanol. The collected clear filtrate was concentrated *in vacuo* to obtain the crude product, which was further purified by flash column chromatography (using 25–40% ethyl acetate in petroleum ether mobile phase) to obtain pure amine compounds 4a–c, respectively.

4.1.3.1. Synthesis of Methyl 3-Amino-4-((2-methoxyphenylethyl)amino)benzoate (4a). Following the general procedure B, compound 4a was obtained from compound 3a (8.05 g, 24.37 mmol, 1.0 equiv) using iron powder (1.6 g, 20% w/w) and saturated aqueous ammonium chloride solution (40 mL, 5 V) as a pale brown solid with 83.3% yield (6.1 g).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 7.18–7.25 (m, 4H, Ar-H), 6.98–7.00 (d, *J* = 8.8 Hz, 1H, Ar-H), 6.87–6.91 (t, *J* = 7.4 Hz, 1H, Ar-H), 6.53–6.56 (d, *J* = 8.4 Hz, 1H, Ar-H), 5.38–5.41 (t, *J* = 5.2 Hz, 1H, –NH), 4.73 (s, 2H, –NH₂), 3.83 (s, 3H, –OCH₃), 3.72 (s, 3H, –OCH₃), 3.26–3.33 (m, 2H, –CH₂), 2.85–2.89 (t, *J* = 7.6 Hz, 2H, –CH₂). ESI/MS (*m/z*): Calculated for [C₁₇H₂₀N₂O₃][M + H]⁺: 301.15. Found *m/z*: 301.2 [M + H]⁺. LCMS purity = 98.764%.

4.1.3.2. Synthesis of Methyl 3-Amino-4-((4-methoxybenzyl)amino)benzoate (4b). Following the general procedure B, compound 4b was obtained from compound 3b (4.60 g, 14.54 mmol, 1.0 equiv) using iron powder (0.92 g, 20% w/w) and saturated aqueous ammonium chloride solution (20 mL, 5 V) as an off-white solid with 84% yield (3.5 g).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 7.25–7.32 (m, 2H, Ar-H), 7.18–7.18 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.10–7.13 (m, 1H, Ar-H), 6.87–6.90 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.36–6.38 (d, *J* = 12 Hz, 1H, Ar-H), 5.80–5.84 (t, 1H, –NH), 4.81 (s, 2H, –NH₂), 4.29–4.31 (d, *J* = 5.4 Hz, 2H, –CH₂), 3.77–3.79 (d, 6H, –(OCH₃)₂). ESI/MS (*m/z*): Calculated for [C₁₆H₁₈N₂O₃][M + H]⁺: 287.13. Found *m/z*: 287.0 [M + H]⁺. LCMS purity = 91.361%.

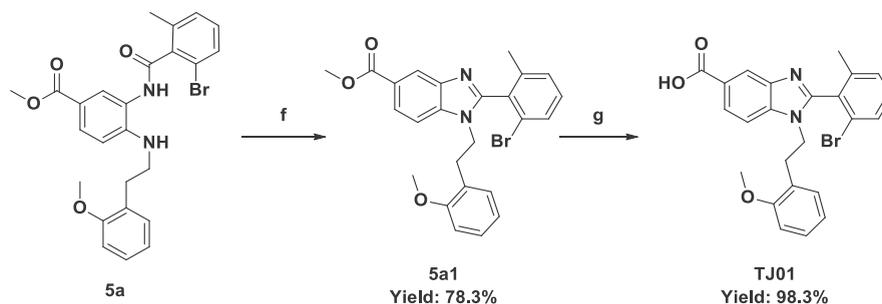
4.1.3.3. Synthesis of Methyl 3-Amino-4-(benzylamino)benzoate (4c). Following the general procedure B, compound 4c was obtained from compound 3c (4.0 g, 13.97 mmol, 1.0 equiv) using iron powder (0.8 g, 20% w/w) and saturated aqueous ammonium chloride solution (20 mL, 5 V) as an off-white solid with 86.5% yield (3.1 g). ¹H NMR (400 MHz, DMSO-*d*₆) δ: 7.30–7.36 (m, 4H, Ar-H), 7.21–7.25 (m, 2H, Ar-H), 7.19–7.20 (d, *J* = 2.0 Hz, 1H, Ar-H), 6.34–6.36 (d, *J* = 5.2 Hz, 1H, Ar-H), 5.91–5.93 (t, *J* = 5.8 Hz, 1H, Ar-H), 4.83 (s, 2H, –NH₂), 4.38–4.39 (d, *J* = 6.0 Hz, 2H, –CH₂), 3.69 (s, 3H, –OCH₃). ESI/MS (*m/z*): Calculated for [C₁₅H₁₆N₂O₂][M + H]⁺: 257.12. Found *m/z*: 257.2 [M + H]⁺. LCMS purity = 92.870%.

4.1.4. General Procedure C for the Synthesis of Compounds 5a–g. Substituted carboxylic acids 4a–c (1.1 equiv) were taken in separate round-bottomed flasks in toluene (10–20 V), thionyl chloride (1.5–1.8 equiv) was added at 0 °C under nitrogen atmosphere followed by a few drops of DMF, and then the reaction mixture was heated to 50 °C and stirred for 2 h. The reaction progress was monitored by TLC (esterification of aliquot reaction mixture with methanol). After completion of the reaction, the reaction mixture was concentrated *in vacuo* (toluene was added and redistilled to remove excess of thionyl chloride).

The obtained acid chlorides were further diluted with THF (10 V) and added to other round-bottom flasks containing the solutions of compounds 4a–c (1.0 equiv) in THF (10 V) at 0 °C under nitrogen atmosphere, *N,N*-diisopropylethylamine (DIPEA) base (2.0 equiv) was added to the reaction mixture at the same temperature, the reaction mixture (clear solution) was gradually warmed to room temperature and stirred for 3 h, and then the products were precipitated from the reaction mixtures.

Upon reaction completion, the reaction mixtures were concentrated partially (half of their volume) *in vacuo* and cooled at 0 °C, the products precipitated (5a and 5b) were filtered and washed with cold tetrahydrofuran (~0.5 mL) and dried *in vacuo*, and other intermediates 5c, 5d, 5e, 5f, and 5g were obtained. Upon reaction completion, the reaction mixture was poured into ice-cold water, the organic product was extracted with ethyl acetate (15 mL × 3), and the combined organic phase was washed with water and brine and dried over anhydrous sodium sulfate. The solvent was filtered and concentrated *in vacuo*, and the obtained crude intermediates 5a–g were directly used for the synthesis of final compounds TJ01–TJ15.

4.1.4.1. Synthesis of Methyl 3-(2-Bromo-6-methylbenzamide)-4-((2-methoxyphenylethyl)amino)benzoate (5a). Following the general procedure C, compound 5a was obtained

Scheme 2. Synthesis of TJ01 from 5a^a

^aReagents and reaction conditions (f): CH₃COOH (13 V), 100 °C, 3 h; (g) LiOH·H₂O (1.3 equiv), THF:CH₃OH:H₂O (2:2:1), 0 °C to rt, 2 h.

from **4a** (250 mg, 0.832 mmol, 1.0 equiv) using 2-bromo-6-methylbenzoic acid (200 mg, 0.930 mmol, 1.1 equiv) in toluene (4 mL), thionyl chloride (0.1 mL, 1.37 mmol, 1.6 equiv), and DIPEA (0.29 mL, 1.664 mmol, 2.0 equiv) as an off-white solid with 92.9% yield (430 mg).

4.1.4.2. Synthesis of Methyl 3-(4-Methoxy-3-nitrobenzamido)-4-((2-methoxyphenethyl)amino)benzoate (5b). Following the general procedure C, compound **5b** was obtained using **4a** (138 mg, 0.459 mmol, 1.0 equiv) using 4-methoxy-3-nitrobenzoic acid (100 mg, 0.507 mmol, 1.1 equiv) in toluene (2 mL), thionyl chloride (0.06 mL, 0.826 mmol, 1.8 equiv), and DIPEA (0.16 mL, 0.918 mmol, 2.0 equiv) as an off-white solid with 91.7% yield (223 mg).

4.1.4.3. Synthesis of Methyl 3-(2-(4-Fluorophenyl)acetamido)-4-((2-methoxyphenethyl)amino)benzoate (5c). Following the general procedure C, compound **5c** was obtained from **4a** (176 mg, 0.585 mmol, 1.0 equiv) using 2-(4-fluorophenyl)acetic acid (100 mg, 0.648 mmol, 1.1 equiv) in toluene (2 mL), thionyl chloride (0.08 mL, 1.10 mmol, 1.8 equiv), and DIPEA (0.2 mL, 1.148 mmol, 2.0 equiv) as a pale-brown solid with 93.6% yield (265 mg).

4.1.4.4. Synthesis of Methyl 3-(4-Fluoro-3-nitrobenzamido)-4-((4-methoxybenzyl)amino)benzoate (5d). Following the general procedure C, compound **5d** was obtained from **4b** (209 mg, 0.729 mmol, 1.0 equiv) using 4-fluoro-3-nitrobenzoic acid (150 mg, 0.810 mmol, 1.1 equiv) in toluene (3 mL), thionyl chloride (0.1 mL, 1.37 mmol, 1.8 equiv), and DIPEA (0.26 mL, 1.492 mmol, 2.0 equiv) as a pale-brown solid with 88.4% yield (325 mg).

4.1.4.5. Synthesis of Methyl 3-(4-Methoxy-3-nitrobenzamido)-4-((4-methoxybenzyl)amino)benzoate (5e). Following the general procedure C, compound **5e** was obtained from **4b** (130 mg, 0.454 mmol) using 4-methoxy-3-nitrobenzoic acid (100 mg, 0.507 mmol) in toluene (2 mL), thionyl chloride (0.06 mL, 0.826 mmol, 1.8 equiv), and DIPEA (0.16 mL, 0.918 mmol, 2.0 equiv) as a pale-brown solid with 78.3% yield (185 mg).

4.1.4.6. Synthesis of Methyl 4-((4-Methoxybenzyl)amino)-3-(4-(trifluoromethoxy)benzamido)benzoate (5f). Following the general procedure C, compound **5f** was obtained from **4b** (188 mg, 0.656 mmol, 1.0 equiv) using 4-(Trifluoromethoxy)benzoic acid (150 mg, 0.727 mmol, 1.1 equiv) in toluene (3 mL), thionyl chloride (0.08 mL, 1.101 mmol, 1.7 equiv), and DIPEA (0.24 mL, 1.377 mmol, 2.0 equiv) as a pale-brown solid with 81.1% yield (280 mg).

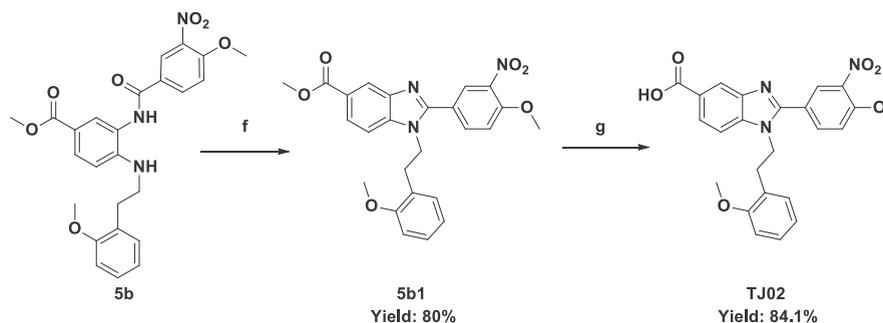
4.1.4.7. Synthesis of Methyl 3-(4-Fluoro-3-nitrobenzamido)-4-((4-methoxybenzyl)amino)benzoate (5g). Following the general procedure C, compound **5g** was obtained from **4c** (187 mg, 0.729 mmol, 1.0 equiv) using 4-Fluoro-3-nitro-

benzoic acid (150 mg, 0.810 mmol, 1.1 equiv) in toluene (3 mL), thionyl chloride (0.1 mL, 1.37 mmol, 1.8 equiv), and DIPEA (0.26 mL, 1.492 mmol, 2.0 equiv) as a pale-brown solid with 90.3% yield (310 mg).

4.1.5. General Procedure D for the Synthesis of 1,2-Disubstituted Benzimidazole-5-carboxylates TJ03, TJ07, TJ08, and TJ13 from 5c, 5d, 5g, and 5f. The intermediate compounds **5c**, **5d**, **5g**, and **5f** were dissolved in glacial acetic acid (10–15 V) in separate round-bottom flasks and stirred for 3–5 h at 100 °C under nitrogen atmosphere. The reaction progress was monitored by TLC. Upon reaction completion it was cooled to 0 °C and quenched with ice-cold water during which time the products were precipitated and then sonicated for 30–60 s, filtered, washed with cold water, and dried *in vacuo* to obtain compounds **TJ03**, **TJ07**, **TJ08**, and **TJ13**, respectively.

4.1.6. General Procedure E for the Synthesis of 1,2-Disubstituted Benzimidazole-5-carboxylic Acids TJ04, TJ09, TJ11, and TJ14 from TJ03, TJ07, TJ08, and TJ13. The 1,2-disubstituted benzimidazole-5-carboxylates **TJ03**, **TJ07**, **TJ08**, and **TJ13** were taken in separate round-bottomed flasks in a mixture of tetrahydrofuran:methanol (1:1 V/V, 10–20 V), a solution of lithium hydroxide monohydrate (1.1–3.2 equiv) in water was added at 0 °C, the reaction mixtures of **TJ03** and **TJ13** were gradually warmed to room temperature and stirred for 2 h, and the solutions of **TJ07** and **TJ08** (without methanol only with 1:1 tetrahydrofuran:water solvents) were stirred at 60 °C for 8 h. The reaction progress was monitored by TLC. Upon completion, the reaction mixture was concentrated partially (to remove tetrahydrofuran and methanol), and the residual aqueous layer was diluted with water and washed with 50% diethyl ether in *n*-pentane twice (10 mL × 2). The aqueous layer was acidified to pH ~ 6 (by adding saturated citric acid solution and in some cases 1.0 N aqueous HCl solution) during which product was precipitated which was sonicated for a minute then filtered and washed with water and dried *in vacuo* to obtain final compounds **TJ04**, **TJ09**, **TJ11**, and **TJ14**, respectively (During hydrolysis of esters **TJ07** and **TJ08**, along with hydrolysis of ester, the fluoro group was replaced by a hydroxyl group.)

4.1.7. General Procedure F for the Synthesis of 1,2-Disubstituted Benzimidazole-5-carboxamides TJ05, TJ06, TJ12, and TJ15 from Compounds TJ04, TJ02, TJ10, and TJ14. 1,2-Disubstituted benzimidazole-5-carboxylic acids **TJ04**, **TJ10**, and **TJ14** were taken in separate round-bottomed flasks, dissolved in DMF (10–15 V), and stirred at 0 °C under nitrogen atmosphere, carbonyldiimidazole (CDI, 1.5 equiv) was added, the reaction mixture was gradually warmed to room temperature and stirred for 1 h, and cooled at 0 °C, and

Scheme 3. Synthesis of TJ02 from 5b^a

^aReagents and reaction conditions (f): CH₃COOH (13 V), 100 °C, 5 h; (g) LiOH·H₂O (1.3 equiv), THF:CH₃OH (1:1), 0 °C to rt, 2 h.

aqueous ammonia (25% solution, 16–21 equiv) was added. After addition, the reaction mixture was warmed to room temperature and stirred for 6 h. The progress of the reaction was monitored by TLC. Upon completion of the reaction, ice-cold water was added to the reaction mixture, during which time product was precipitated out from the reaction mixture which was sonicated for 30–60 s, filtered, washed with water, and dried *in vacuo* to obtain final compounds TJ05, TJ06, TJ12, and TJ15, respectively.

4.1.8. Synthesis of Final Compounds TJ01–TJ15.

4.1.8.1. Synthesis of 2-(2-Bromo-6-methylphenyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxylic Acid (TJ01). Following the general procedure D, the intermediate **5a**₁ was obtained from compound **5a** (430 mg, 0.864 mmol, 1.0 equiv) using glacial acetic acid (4 mL) and refluxed at 100 °C for 3 h as a brown solid with 78% yield (325 mg). The obtained **5a**₁ converted to compound TJ01 by following the general procedure E using **5a**₁ (325 mg, 0.677 mmol, 1.0 equiv) in a mixture of tetrahydrofuran:methanol (4 mL, 12 V, 1:1 V/V) as the solvent and a solution of lithium hydroxide monohydrate (37 mg, 0.881 mmol, 1.3 equiv) in water (1 mL) at 0 °C to rt for 2 h. The pure compound TJ01 was obtained as a white solid with 98.3% yield (310 mg). (TLC *R*_f = 0.43 in dichloromethane:methanol 9:1 V/V.)

¹H NMR (400 MHz, DMSO-*d*₆) δ: 12.8 (s, 1H, -COOH), 8.24 (s, 1H, Ar-H), 7.95–7.97 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.78–7.80 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.63–7.65 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.31–7.33 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.16–7.20 (t, *J* = 7.4 Hz, 1H, Ar-H), 6.94 (s, 1H, Ar-H), 6.77–6.79 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.66–6.67 (t, *J* = 6.4 Hz, 1H, Ar-H), 6.55–6.56 (d, *J* = 7.2 Hz, 1H, Ar-H), 4.30 (s, 2H, -CH₂), 3.37 (s, 3H, -OCH₃), 2.9 (s, 2H, -CH₂), 1.95 (s, 3H, -CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.39, 157.63, 153.70, 142.68, 137.94, 137.19, 133.00, 132.58, 132.54, 132.08, 130.56, 128.83, 125.44, 125.31, 124.24, 121.48, 120.60, 118.80, 111.41, 110.98, 60.22, 55.11, 30.40, 19.08. FTIR ν_{max} (cm⁻¹): 3298.7, 3019.1, 2940.9, 2840.2, 1681.0, 1248.7. ESI/HRMS (*m/z*): Calculated for [C₂₄H₂₁BrN₂O₃][M + H]⁺ (⁷⁹Br): 465.07, [M + H]⁺ (⁸¹Br): 467.07; Found *m/z*: 465.0783 [M + H]⁺ (⁷⁹Br), 467.0755 [M + H]⁺ (⁸¹Br). ESI/MS (*m/z*): Calculated for [C₂₄H₂₁BrN₂O₃][M + H]⁺ (⁷⁹Br): 465.07, [M + H]⁺ (⁸¹Br): 467.07. Found *m/z*: 465.0 [M + H]⁺ (⁷⁹Br), 467.07 [M + H]⁺ (⁸¹Br). LCMS purity = 98.773%.

4.1.8.2. Synthesis of 2-(4-Methoxy-3-nitrophenyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxylic Acid (TJ02).

Following the general procedure D, the compound **5b**₁ was obtained from **5b** (223 mg, 0.465 mmol, 1.0 equiv) using

glacial acetic acid (3 mL) at 100 °C for 5 h as a brown solid with yield 80.0% (172 mg). The obtained compound **5b**₁ converted to TJ02 by following the general procedure E using **5b**₁ (172 mg, 0.372 mmol), a mixture of tetrahydrofuran:methanol (3 mL, 17 V, 1:1 V/V) as solvent, and lithium hydroxide monohydrate (21 mg 0.50 mmol) at 0 °C to rt for 2 h. The pure compound TJ02 obtained as a pale-yellow solid with 84.1% yield (140 mg). (TLC *R*_f = 0.39 in dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 12.67 (s, 1H, -COOH), 8.24 (s, 1H, Ar-H), 7.94–7.96 (d, *J* = 9.6 Hz, 1H, Ar-H), 7.77–7.83 (m, 3H, Ar-H), 7.45–7.48 (d, *J* = 9.2 Hz, 1H, Ar-H), 7.05–7.08 (t, *J* = 7.6 Hz, 1H, Ar-H), 6.65–6.68 (m, 2H, Ar-H), 6.55–6.63 (d, *J* = 7.2 Hz, 1H, Ar-H), 4.56–4.59 (t, *J* = 6.2 Hz, 2H, -CH₂), 4.03 (s, 3H, -OCH₃), 3.46 (s, 3H, -OCH₃), 2.91–2.94 (t, *J* = 6.2 Hz, 2H, -CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.32, 157.43, 153.36, 153.16, 142.49, 139.35, 138.95, 135.08, 130.53, 128.65, 125.97, 125.58, 125.29, 124.34, 122.43, 121.32, 120.48, 114.78, 111.26, 110.53, 57.52, 55.25, 44.44, 30.62. FTIR ν_{max} (cm⁻¹): 3067.6, 2937.1, 2836.5, 1684.8, 1244.9. ESI/HRMS (*m/z*): Calculated for [C₂₄H₂₁N₃O₆][M + H]⁺: 448.14; Found *m/z*: 448.1496 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₄H₂₁N₃O₆][M + H]⁺: 448.14. Found *m/z*: 448.20 [M + H]⁺. LCMS purity = 92.339%.

4.1.8.3. Synthesis of Methyl 2-(4-Fluorobenzyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxylate (TJ03). Following the general procedure D, the compound TJ03 was obtained from the intermediate compound **5c** (265 mg, 0.605 mmol, 1.0 equiv) using glacial acetic acid (3 mL), refluxed at 100 °C for 3 h. The obtained crude pale-brown solid was triturated with diethyl ether (3 mL) cooled to 0 °C, filtered, washed with cold diethyl ether, dried under *vacuo*. Pure TJ03 obtained as an off-white solid with 93.9% yield (230 mg). (TLC *R*_f = 0.2 in petroleum ether:ethyl acetate 6:4 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.15–8.16 (d, *J* = 1.2 Hz, 1H, Ar-H), 7.83–7.85 (t, *J* = 7.2 Hz, 1H, Ar-H), 7.51–7.53 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.14–7.28 (m, 5H, Ar-H), 6.92–6.94 (d, *J* = 8.0 Hz, 2H, Ar-H), 6.80–6.84 (t, *J* = 7.4 Hz, 1H, Ar-H), 4.33–4.37 (t, *J* = 7.0 Hz, 2H, CH₂), 4.08 (s, 2H, -CH₂), 3.86 (s, 3H, -OCH₃), 3.69 (s, 3H, -OCH₃), 2.83–2.87 (t, *J* = 7.0 Hz, 2H, -CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 167.24, 162.74 (d, *J*_{C-F} = 242 Hz), 157.76, 156.01, 142.25, 139.05, 133.05, 131.18, 131.10, 130.90, 128.82, 125.90, 123.43, 123.39, 120.81, 120.62, 115.84, 115.63, 111.06, 110.48, 55.63, 52.38, 43.85, 32.36, 30.60. FTIR ν_{max} (cm⁻¹): 3063.9, 2989.3, 2944.6, 2836.5, 1710.8, 1297.1. ESI/HRMS (*m/z*): Calculated for [C₂₅H₂₃FN₂O₃][M + H]⁺: 419.17; Found *m/z*: 419.1801

[M + H]⁺. **ESI/MS** (*m/z*): Calculated for [C₂₅H₂₃FN₂O₃][M + H]⁺: 419.17. Found *m/z*: 419.0 [M + H]⁺. **LCMS** purity = 99.539%.

4.1.8.4. Synthesis of 2-(4-Fluorobenzyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxylic acid (TJ04). Following the [general procedure E](#), the compound **TJ04** was obtained from compound **TJ03** (190 mg, 0.45 mmol, 1.0 equiv) using a mixture of tetrahydrofuran:methanol (2 mL, 10 v, 1:1 V/V) as solvent and lithium hydroxide monohydrate (25 mg, 0.595 mmol, 1.3 equiv) in water (1 mL) at 0 °C to rt for 2 h. The pure compound **TJ04** was obtained as a white solid with 93.9% yield (171 mg). (TLC *R_f* = 0.41, dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 12.59 (s, 1H, -COOH), 8.12 (s, 1H, Ar-H), 7.81–7.83 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.48–7.50 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.20–7.27 (m, 3H, Ar-H), 7.13–7.17 (t, *J* = 8.8 Hz, 2H, Ar-H), 6.91–6.95 (t, *J* = 6.8 Hz, 2H, Ar-H), 6.79–6.83 (t, *J* = 7.2 Hz, 1H, Ar-H), 4.31–4.35 (t, *J* = 7.0 Hz, 2H, -CH₂), 4.06 (s, 2H, -CH₂), 3.70 (s, 3H, -OCH₃), 2.82–2.85 (t, *J* = 7.0 Hz, 2H, -CH₂). **¹³C NMR (100 MHz, DMSO-*d*₆)** δ: 168.40, 162.76 (d, *J_{C-F}* = 241 Hz), 157.79, 155.75, 142.25, 138.81, 133.14, 131.20, 131.12, 130.94, 128.86, 125.93, 124.57, 123.73, 120.85, 120.79, 115.88, 115.67, 111.09, 110.27, 55.68, 43.82, 32.39, 30.66. **FTIR** ν_{max} (cm⁻¹): 3447.8, 3011.7, 2937.1, 1677.3, 1308.3. **ESI/HRMS** (*m/z*): Calculated for [C₂₄H₂₁FN₂O₃][M + H]⁺: 405.15; Found *m/z*: 405.1613 [M + H]⁺. **ESI/MS** (*m/z*): Calculated for [C₂₄H₂₁FN₂O₃][M + H]⁺: 405.15; Found *m/z*: 405.0 [M + H]⁺. **LCMS** purity = 98.938%.

4.1.8.5. Synthesis of 2-(4-Fluorobenzyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxamide (TJ05). Following the [general procedure F](#), the compound **TJ05** was obtained from compound **TJ04** (110 mg, 0.271 mmol, 1.0 equiv) in DMF (1.5 mL) as solvent, CDI (67 mg, 0.413 mmol, 1.5 equiv), and aqueous ammonia (~25% solution, 0.4 mL, 16 equiv) at 0 °C to rt for 7 h. The compound **TJ05** was obtained as a white solid with 86.7% yield (95 mg) after purification (TLC *R_f* = 0.47, dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.14 (s, 1H, Ar-H), 7.92 (s, 1H, -NH₂), 7.76–7.78 (t, *J* = 2.2 Hz, 1H, Ar-H), 7.46–7.48 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.13–7.27 (m, 5H, Ar-H), 6.91–6.97 (m, 2H, Ar-H), 6.80–6.84 (t, *J* = 7.2 Hz, 1H, Ar-H), 4.31–4.34 (t, *J* = 7.2 Hz, 2H, -CH₂), 4.05 (s, 2H, -CH₂), 3.73 (s, 3H, -OCH₃), 2.83–2.86 (t, *J* = 7.2 Hz, 2H, -CH₂). **¹³C NMR (100 MHz, DMSO-*d*₆)** δ: 168.85, 162.69 (d, *J_{C-F}* = 241 Hz), 157.74, 155.16, 142.17, 137.59, 131.12, 131.05, 130.90, 128.80, 128.20, 125.96, 122.23, 120.81, 118.68, 115.80, 115.59, 111.08, 109.98, 55.68, 43.68, 32.35, 30.68. **FTIR** ν_{max} (cm⁻¹): 3309.9, 3134.7, 2978.1, 2836.5, 1677.3, 1244.9. **ESI/HRMS** (*m/z*): Calculated for [C₂₄H₂₂FN₃O₂][M + H]⁺: 404.17. Found *m/z*: 404.1691 [M + H]⁺. **ESI/MS** (*m/z*): Calculated for [C₂₄H₂₂FN₃O₂][M + H]⁺: 404.17. Found *m/z*: 404.0 [M + H]⁺. **LCMS** purity = 99.876%.

4.1.8.6. Synthesis of 2-(4-Methoxy-3-nitrophenyl)-1-(2-methoxyphenethyl)-1H-benzo[d]imidazole-5-carboxamide (TJ06). Following the [general procedure F](#), the compound **TJ06** was obtained from compound **TJ02** (90 mg, 0.201 mmol, 1.0 equiv) in DMF (1.2 mL) as a solvent, CDI (49 mg, 0.302 mmol, 1.5 equiv), and aqueous ammonia (~25% solution, 0.3 mL, 21 equiv) at 0 °C to rt for 7 h. **TJ06** was obtained as a white solid with 90.2% yield (81 mg). (TLC *R_f* = 0.47, dichloromethane: methanol 9:1 V/V).

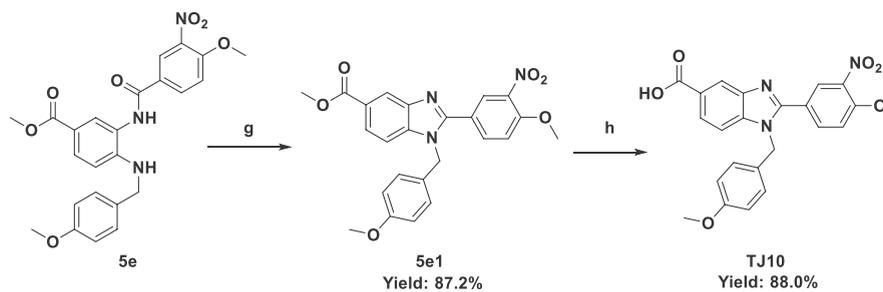
¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.23 (s, 1H, Ar-H), 8.01 (s, 1H, -NH₂); 7.88–7.90 (d, *J* = 8.4 Hz, 1H, -Ar-H), 7.72–7.80 (m, 2H, Ar-H), 7.43–7.46 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.29 (s, 1H, Ar-H), 7.04–7.07 (t, *J* = 7.4 Hz, 1H, Ar-H), 6.61–6.67 (m, 2H, Ar-H), 6.52–6.54 (d, *J* = 7.2 Hz, 1H, -Ar-H), 4.53–4.56 (t, *J* = 6.8 Hz, 2H, -CH₂), 4.01 (s, 3H, -OCH₃), 3.46 (s, 3H, -OCH₃), 2.89–2.92 (t, *J* = 6.2 Hz, 2H, -CH₂). **¹³C NMR (100 MHz, DMSO-*d*₆)** δ: 168.89, 157.28, 153.40, 153.21, 142.54, 139.40, 138.98, 135.12, 130.58, 128.69, 126.01, 125.70, 125.34, 124.39, 122.48, 121.37, 120.53, 114.81, 111.28, 110.57, 57.55, 55.28, 44.47, 30.65. **FTIR** ν_{max} (cm⁻¹): 3451.5, 3332.2, 3283.8, 3179.4, 2944.6, 1669.8, 1244.9. **ESI/HRMS** (*m/z*): Calculated for [C₂₄H₂₂N₄O₅][M + H]⁺: 447.16. Found *m/z*: 447.1580 [M + H]⁺. **ESI/MS** (*m/z*): Calculated for [C₂₄H₂₂N₄O₅][M + H]⁺: 447.16. Found *m/z*: 447.0 [M + H]⁺. **LCMS** Purity = 98.866%.

4.1.8.7. Synthesis of Methyl 2-(4-Fluoro-3-nitrophenyl)-1-(4-methoxybenzyl)-1H-benzo[d]imidazole-5-carboxylate (TJ07). Following the [general procedure D](#), the compound **TJ07** was obtained from intermediate compound **5d** (325 mg, 0.716 mmol, 1.0 equiv) using glacial acetic acid (4 mL) and stirred at 100 °C for 3 h. The compound **TJ07** was obtained as a pale yellow solid with 73.7% yield (230 mg). (TLC *R_f* = 0.25, petroleum ether:ethyl acetate 6:4 V/V).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 8.47–8.49 (t, *J* = 3.6 Hz, 1H, Ar-H), 8.47 (s, 1H, Ar-H), 8.18–8.19 (d, *J* = 4.5 Hz, 1H, Ar-H), 7.91–7.94 (d, *J* = 8.7 Hz, 1H, Ar-H), 7.69–7.75 (t, *J* = 9.45 Hz, 2H, Ar-H), 6.94–6.97 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.82–6.85 (t, *J* = 8.4 Hz, 2H, Ar-H), 5.60 (s, 2H, -CH₂), 3.88 (s, 3H, -OCH₃), 3.68 (s, 3H, -OCH₃). **¹³C NMR (100 MHz, DMSO-*d*₆)** δ: 167.01, 159.18, 157.23 (d, *J_{C-F}* = 264 Hz), 152.71, 142.46, 139.77, 137.52, 137.44, 137.37, 137.27, 128.53 (d, *J* = 35 Hz), 127.47 (d, *J* = 32 Hz), 127.46, 127.19, 124.65, 121.62, 119.96, 119.75, 114.72, 112.08, 55.52, 52.59, 47.80. **FTIR** ν_{max} (cm⁻¹): 3082.5, 3004.2, 2832.8, 1714.6, 1297.1, 1215.1. **ESI/HRMS** (*m/z*): Calculated for [C₂₃H₁₈FN₃O₅][M + H]⁺: 436.12. Found *m/z*: 436.1216 [M + H]⁺. **ESI/MS** (*m/z*): Calculated for [C₂₃H₁₈FN₃O₅][M + H]⁺: 436.12. Found *m/z*: 436.0 [M + H]⁺. **LCMS** purity = 93.457%.

4.1.8.8. Synthesis of Methyl 1-Benzyl-2-(4-fluoro-3-nitrophenyl)-1H-benzo[d]imidazole-5-carboxylate (TJ08). Following the [general procedure D](#), the final compound **TJ08** was obtained from intermediate compound **5g** (310 mg, 0.732 mmol, 1.0 equiv) using glacial acetic acid (4 mL), stirred at 100 °C for 3 h. **TJ08** was obtained as a pale-yellow solid with 75.8% yield (225 mg) after purification of crude product by flash column chromatography using 25–33% ethyl acetate in petroleum ether as a mobile phase. (TLC *R_f* = 0.31, petroleum ether: ethyl acetate 6:4 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.45–8.48 (m, 1H, Ar-H), 8.363–8.365 (d, *J* = 0.8 Hz, 1H, Ar-H), 8.16–8.20 (m, 1H, Ar-H), 7.92–7.95 (m, 1H, Ar-H), 7.75–7.78 (m, 1H, Ar-H), 7.69–7.71 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.25–7.32 (m, 3H, Ar-H), 7.01–7.03 (d, *J* = 6.8 Hz, 2H, Ar-H), 5.70 (s, 2H, -CH₂), 3.89 (s, 3H, -OCH₃). **¹³C NMR (100 MHz, DMSO-*d*₆)** δ: 167.00, 157.23 (d, *J_{C-F}* = 264 Hz), 152.75, 142.45, 139.86, 137.48 (d, *J_{C-F}* = 25 Hz), 137.40, 137.33, 136.72, 129.37, 128.20, 127.39, 127.37, 127.11, 127.07, 126.69, 124.74, 124.71, 121.66, 119.95 (d, *J_{C-F}* = 21 Hz), 111.99, 52.57, 48.28. **FTIR** ν_{max} (cm⁻¹): 3029.5, 2952.8, 2844.0, 1716.68, 1545.95, 1285.01. **ESI/HRMS** (*m/z*): Calculated for [C₂₂H₁₆FN₃O₄][M + H]⁺: 406.11. Found *m/z*: 406.1218 [M + H]⁺. **ESI/MS**

Scheme 4. Synthesis of TJ10 from 5e^a

^aReagents and reaction conditions: (g) CH₃COOH (16 V), 100 °C, 3 h; (h) LiOH·H₂O (1.1 equiv), THF:CH₃OH:H₂O (1:1:1), 0 °C to rt, 2 h.

(*m/z*): Calculated for [C₂₂H₁₆FN₃O₄] [M + H]⁺: 406.11. Found *m/z*: 406.0 [M + H]⁺. LCMS purity = 98.344%.

4.1.8.9. Synthesis of 2-(4-Hydroxy-3-nitrophenyl)-1-(4-methoxybenzyl)-1H-benzodimidazole-5-carboxylic Acid (TJ09). Following the general procedure E, the compound TJ09 was obtained from compound TJ07 (100 mg, 0.229 mmol, 1.0 equiv) using THF (1 mL) as a solvent and a solution of lithium hydroxide monohydrate (31 mg, 0.738 mmol, 3.2 equiv) in water (1 mL) at 60 °C and stirred for 8 h. The pure TJ09 was obtained as a pale-yellow solid with 57.2% yield (55 mg) after purification of crude TJ09 by flash column chromatography using 1–2% methanol in dichloromethane as a mobile phase. (TLC *R_f* = 0.21, dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 12.81 (s, 1H, –COOH), 11.65 (s, 1H, –OH); 8.25–8.28 (d, *J* = 15.2 Hz, 2H, Ar-H), 7.87–7.95 (m, 2H, Ar-H), 7.61–7.64 (d, *J* = 8.8 Hz, 1H, Ar-H), 7.26–7.28 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.95–6.97 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.84–6.86 (d, *J* = 8.0 Hz, 2H, Ar-H), 5.58 (s, 2H, –CH₂), 3.68 (s, 3H, –OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.14, 159.07, 153.77, 153.53, 142.49, 139.58, 137.35, 135.77, 128.70, 128.00, 126.47, 125.54, 124.48, 121.37, 120.87, 120.10, 114.67, 111.50, 55.48, 47.70. FTIR ν_{\max} (cm⁻¹): 3092.3, 3068.4, 2964.51, 1629.42, 1257.23. ESI/HRMS (*m/z*): Calculated for [C₂₂H₁₇N₃O₆] [M + H]⁺: 420.11; Found *m/z*: 420.1067 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₂H₁₇N₃O₆] [M + H]⁺: 420.11; Found *m/z*: 420.2 [M + H]⁺. LCMS purity = 87.992%.

4.1.8.10. Synthesis of 2-(4-Methoxy-3-nitrophenyl)-1-(4-methoxybenzyl)-1H-benzodimidazole-5-carboxylic Acid (TJ10). Following the general procedure D, the intermediate compound 5e₁ was obtained from compound 5e (185 mg, 0.397 mmol, 1.0 equiv) using glacial acetic acid (3 mL) at 100 °C for 3 h as a pale-brown solid with 87.2% yield (155 mg). The obtained 5e₁ was converted to TJ10 by following the general procedure E using the precursor ester compound 5e₁ (155 mg, 0.346 mmol, 1.0 equiv) in a mixture of tetrahydrofuran:methanol (3 mL, 20 V, 1:1 V/V) as a solvent and a solution of lithium hydroxide monohydrate (16 mg, 0.381 mmol, 1.1 equiv) in water (1 mL) at 0 °C to rt for 2 h. The crude pale-brown solid was further purified by flash column chromatography using 2–3% methanol in dichloromethane mobile phase, and pure compound TJ10 was obtained as a yellow solid with 88.0% yield (132 mg). (TLC *R_f* = 0.30, dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 12.80 (s, 1H, –COOH), 8.26–8.28 (d, *J* = 9.6 Hz, 2H, Ar-H), 8.02–8.04 (d, *J* = 9.2 Hz, 1H, Ar-H), 7.81–7.84 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.52–7.59 (m, 2H, Ar-H), 6.93–6.95 (d, *J* = 8.0 Hz, 2H,

Ar-H), 6.83–6.85 (d, *J* = 8.4 Hz, 2H, Ar-H), 5.59 (s, 2H, –CH₂), 4.01 (s, 3H, –OCH₃), 3.68 (s, 3H, –OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.12, 159.08, 153.54, 153.22, 142.48, 139.56, 137.35, 135.72, 135.24, 128.66, 127.98, 126.17, 125.62, 124.58, 122.23, 121.44, 115.35, 114.68, 111.48, 57.49, 55.48, 47.70. 27.25. FTIR ν_{\max} (cm⁻¹): 3075.2, 2938.46, 2838.5, 1686.27, 1247.17. ESI/HRMS (*m/z*): Calculated for [C₂₃H₁₉N₃O₆] [M + H]⁺: 434.13. Found *m/z*: 434.1151 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₃H₁₉N₃O₆] [M + H]⁺: 434.13. Found *m/z*: 434.2 [M + H]⁺. LCMS purity = 99.309%.

4.1.8.11. Synthesis of 1-Benzyl-2-(4-hydroxy-3-nitrophenyl)-1H-benzodimidazole-5-carboxylic Acid (TJ11). Following the general procedure E, the compound TJ11 was obtained from compound TJ08 (100 mg, 0.246 mmol, 1.0 equiv) using THF (1 mL) as a solvent and a solution of lithium hydroxide monohydrate (30 mg, 0.714 mmol, 2.9 equiv) in water (1 mL) at 60 °C and stirred for 8 h. The obtained crude TJ11 was further purified by flash column chromatography using 50–70% ethyl acetate in dichloromethane, and pure TJ11 was obtained as a pale-yellow solid with 65.7% (63 mg). (TLC *R_f* = 0.32, dichloromethane:methanol 9:1 V/V).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 12.80 (s, 1H, –COOH), 11.62 (s, 1H, OH), 8.30 (s, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 7.86–7.94 (m, 2H, Ar-H), 7.60–7.63 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.25–7.30 (t, *J* = 8.4 Hz, 4H, Ar-H), 7.00–7.03 (d, *J* = 6.6 Hz, 2H, –CH₂), 5.66 (s, 2H, –CH₂). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.17, 153.80, 153.60, 142.52, 139.70, 137.36, 136.94, 135.80, 129.35, 128.89, 128.11, 127.12, 126.60, 126.45, 125.66, 124.59, 121.44, 120.86, 120.14, 111.48, 48.22. FTIR ν_{\max} (cm⁻¹): 3091.7, 3064.9, 3029.8, 2934.32, 1661.64, 1214.55. ESI/HRMS (*m/z*): Calculated for [C₂₁H₁₅N₃O₅] [M + H]⁺: 390.10. Found *m/z*: 390.1065 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₁H₁₅N₃O₅] [M + H]⁺: 390.10. Found *m/z*: 390.1 [M + H]⁺. LCMS purity = 88.982%.

4.1.8.12. Synthesis of 2-(4-Methoxy-3-nitrophenyl)-1-(4-methoxybenzyl)-1H-benzodimidazole-5-carboxamide (TJ12). Following the general procedure F, the final compound TJ12 was obtained from TJ10 (70 mg, 0.161 mmol, 1.0 equiv) using DMF (1.0 mL) as a solvent, CDI (40 mg, 0.246 mmol, 1.5 equiv), and aqueous ammonia (~25% solution, 0.2 mL, 18 equiv) at 0 °C to rt and stirred for 7 h. The compound TJ12 was obtained as a pale-yellow solid with 77.5% yield (54 mg). (TLC *R_f* = 0.52, dichloromethane:methanol 9:1 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.27–8.28 (d, *J* = 3.2 Hz, 2H, –NH₂), 7.99–8.03 (t, *J* = 8.2 Hz, 2H, –Ar-H), 7.81–7.84 (d, *J* = 15.6 Hz, 1H, Ar-H), 7.57–7.59 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.51–7.53 (d, *J* = 8.8 Hz, 1H, Ar-H), 7.29 (s, 1H, Ar-H), 6.93–6.95 (d, *J* = 8.0 Hz, 2H, Ar-H), 6.82–6.84 (d, *J* = 8.0 Hz, 2H, Ar-H), 5.57 (s, 2H, –CH₂), 4.00 (s, 3H, –OCH₃),

3.67 (s, 3H, -OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.66, 159.11, 153.49, 152.79, 142.50, 139.60, 138.50, 135.24, 129.33, 128.85, 128.02, 126.13, 123.22, 122.49, 119.37, 115.38, 114.70, 111.19, 57.52, 55.51, 47.66. FTIR ν_{max} (cm⁻¹): 3452.96, 3185.60, 2949.51, 1669.33, 1247.30. ESI/HRMS (*m/z*): Calculated for [C₂₃H₂₀N₄O₅] [M + H]⁺: 433.14. Found *m/z*: 433.1477 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₃H₂₀N₄O₅] [M + H]⁺: 433.14. Found *m/z*: 433.2 [M + H]⁺. LCMS purity = 92.826%.

4.1.8.13. Synthesis of Methyl 1-(4-methoxybenzyl)-2-(4-(trifluoromethoxy) phenyl)-1H-benzo[d]imidazole-5-carboxylate (TJ13). Following the general procedure D, the compound TJ13 was obtained from intermediate compound 5f (280 mg, 0.590 mmol, 1.0 equiv) using glacial acetic acid (3 mL) and stirred at 100 °C for 3 h. TJ13 was obtained as a pale-brown solid with 81.6% yield (220 mg). (TLC R_f = 0.25, petroleum ether: ethyl acetate 5:5 V/V).

¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.33 (s, 1H, Ar-H), 7.90–7.92 (d, *J* = 6.4 Hz, 1H, Ar-H), 7.72–7.77 (m, 2H, Ar-H), 7.66–7.68 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.57–7.63 (m, 2H, Ar-H), 6.86–6.88 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.77–6.79 (d, *J* = 7.6 Hz, 2H, Ar-H), 5.33 (s, 2H, -CH₂), 3.88 (s, 3H, -OCH₃), 3.67 (s, 3H, -OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 166.46, 164.56, 158.72, 147.12, 145.41, 132.30, 131.35, 131.11, 130.66, 129.25, 128.71, 128.17, 128.01, 122.34, 122.14, 116.46, 114.45, 114.23, 110.75, 55.47, 51.87, 45.84. FTIR ν_{max} (cm⁻¹): 2960.23, 1649.21, 1245.78, 1176.00. ESI/HRMS (*m/z*): Calculated for [C₂₄H₁₉F₃N₂O₄] [M + H]⁺: 457.13. Found *m/z*: 457.1238 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₄H₁₉F₃N₂O₄] [M + H]⁺: 457.13. Found *m/z*: 457.1 [M + H]⁺. LCMS purity = 97.681%.

4.1.8.14. Synthesis of 1-(4-Methoxybenzyl)-2-(4-(trifluoromethoxy) phenyl)-1H-benzo[d]imidazole-5-carboxylic Acid (TJ14). Following the general procedure E, the compound TJ14 was obtained from TJ13 (170 mg, 0.372 mmol, 1.0 equiv) using a mixture of tetrahydrofuran:methanol (2 mL, 12 V, 1:1 V/V) as solvent and a solution of lithium hydroxide monohydrate (21 mg, 0.500 mmol, 1.3 equiv) in water (1 mL) at 0 °C to rt for 2 h. The pure TJ14 obtained as a white solid with 89.7% yield (148 mg). (TLC R_f = 0.39, dichloromethane:methanol 9:1 V/V).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 12.73 (s, 1H, -COOH), 8.30 (s, 1H, Ar-H), 7.88–7.90 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.70–7.79 (m, 2H, Ar-H), 7.56–7.64 (m, 3H, Ar-H), 6.85–6.88 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.76–6.79 (d, *J* = 8.4 Hz, 2H, Ar-H), 5.32 (s, 2H, -CH₂), 3.66 (s, 3H, -OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.14, 159.16, 151.07, 146.84, 146.83, 142.81, 138.41, 133.09, 133.06, 128.47, 128.21, 125.53, 124.57, 124.16 (q, *J*_{C-F} = 257 Hz, 257 Hz, 256 Hz), 123.84, 121.74, 121.59, 121.46, 119.02, 116.46, 114.45, 111.79, 55.49, 47.44. FTIR ν_{max}(cm⁻¹): 3093.7, 2959.5, 2937.1, 2840.2, 1692.2, 1244.9. ESI/HRMS (*m/z*): Calculated for [C₂₃H₁₇F₃N₂O₄] [M + H]⁺: 443.11. Found *m/z*: 443.1077 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₃H₁₇F₃N₂O₄] [M + H]⁺: 443.11. Found *m/z*: 443.2 [M + H]⁺. LCMS purity = 97.377%.

4.1.8.15. Synthesis of 1-(4-Methoxybenzyl)-2-(4-(trifluoromethoxy) phenyl)-1H-benzo[d]imidazole-5-carboxamide (TJ15). Following the general procedure F, the compound TJ15 was obtained from TJ14 (80 mg, 0.180 mmol, 1.0 equiv) using DMF (1.0 mL) as a solvent, CDI (44 mg, 0.271 mmol, 1.5 equiv), and aqueous ammonia (~25% solution, 0.2 mL, 16 equiv) at 0 °C to rt and stirred for 7 h. Pure TJ15 compound

was obtained as an off-white solid with 78.7% yield (63 mg) after purification. (TLC R_f = 0.47, dichloromethane:methanol 9:1 V/V).

¹H NMR (300 MHz, DMSO-*d*₆) δ: 8.29 (s, 1H, Ar-H), 7.99 (s, 1H, -NH), 7.69–7.83 (m, 3H, Ar-H), 7.59–7.62 (d, *J* = 9 Hz, 3H, Ar-H), 7.29 (s, 1H, -NH), 6.85–6.88 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.76–6.79 (d, *J* = 8.7 Hz, 2H, Ar-H), 5.30 (s, 2H, -CH₂), 3.66 (s, 3H, -OCH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 168.67, 159.13, 150.56, 146.85, 142.75, 137.29, 133.09, 132.95, 129.16, 128.45, 128.42, 128.22, 124.17 (q, *J*_{C-F} = 257 Hz, 256 Hz, 257 Hz), 124.07, 123.19, 121.60, 121.53, 119.57, 119.04, 116.47, 114.43, 111.39, 55.49, 47.36. FTIR ν_{max} (cm⁻¹): 3567.1, 3306.1, 3134.7, 2952.1, 2832.8, 1613.9, 1162.9. ESI/HRMS (*m/z*): Calculated for [C₂₃H₁₈F₃N₃O₃] [M + H]⁺: 442.13. Found *m/z*: 442.1346 [M + H]⁺. ESI/MS (*m/z*): Calculated for [C₂₃H₁₈F₃N₃O₃] [M + H]⁺: 442.13. Found *m/z*: 442.0 [M + H]⁺. LCMS purity = 96.380%.

4.2. Biological Activities. 4.2.1. Cell Lines and Culture Conditions. Human chronic myeloid leukemia cells, K-562; human leukemic T cell lymphoblast cell lines, Jurkat; human acute lymphoblastic leukemia cells, MOLT-4; human colorectal carcinoma cells, HCT116; and human pancreatic adenocarcinoma cells, MIA PaCa-2 were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Human cervical cancer cell lines, HeLa, and human normal kidney cell lines, HEK-293 were generously gifted by Prof. Sathees C. Raghavan, Dept of Biochemistry, Indian Institute of Sciences (IISc), Bengaluru. Cells were cultured in RPMI 1640, McCoy's 5A, DMEM low glucose media, and DMEM high glucose media, respectively, with 2 mM L-glutamine (Thermo Fisher Scientific, Inc.; Waltham, MA USA) containing 10% FBS (Gibco; Grand Island, NY, USA) and some are supplemented with 2 mM Sodium pyruvate (Thermo Fisher Scientific, Inc.; Waltham, MA USA). Cells were cultured in humidified incubator with 5% CO₂ at 37 °C.

4.2.2. MTT Cell Proliferation Assay. MTT assay was performed as described previously.⁴⁰ Cells treated with an increased concentration of TJ08 were subjected to MTT assay after 48 and 72 h of incubation with addition of thiazolyl blue tetrazolium bromide (MTT; Sigma; St Louis, MO, USA) and incubated additionally for 4 h. Insoluble formazan crystals were solubilized using 100 μL of DMSO, and absorbance was recorded at 570 nm using a Tecan Microplate Reader (Tecan Instruments, Switzerland). Error bars were calculated based on a minimum of three independent experiments and data represented as histogram.

4.2.3. Trypan Blue Dye Exclusion Assay. TBDE assay was performed as described. Cells treated with an increased concentration of TJ08 were incubated for 48 and 72 h, and cells were harvested and subjected to trypan blue dye exclusion assay. Error bars were calculated based on a minimum of three independent experiments and data represented as histogram.

4.2.4. Colony Formation Assay. CFA was performed as described. HeLa, HCT116, and MiaPaca2 cells were used to assess the colony formation capability after TJ08 treatment. Cells were incubated for 2 weeks and allowed to form colonies and cells were washed with PBS, fixed in methanol:acetic acid (7:1) and stained with 0.5% crystal violet stain and allowed to dry and imaged.

4.2.5. Cell Migration and Cell Invasion Assay. 4.2.5.1. Cell Migration. HeLa cells were seeded at 5 × 10⁴ per 96 well plate, allowed to attach overnight, treated with increased concentration of TJ08, incubated for 12 h, and subjected for

migration assay according to the manufacturer's protocol (ECM510, Merck, Milipore).

4.2.5.2. Cell Invasion. HeLa cells were serum starved for 18 h, cells were seeded into a 96-well plate and incubated with increased concentration of **TJ08** for 12 h, and the lower chamber was supplemented with and without FBS (Chemo attractant) and incubated for 8 h and then subjected for cell invasion assay according to the manufacturer's protocol (ECM555, Merck, Milipore).

4.2.6. Mitochondrial Membrane Potential Assay. HeLa, Jurkat, and HCT116 cells were used for the assay, and the assay was performed as described previously.⁴⁰ The treated cells were allowed to incubate for 24 h and subjected for MMP assay as per the manufacturer's protocol (MAK159, Sigma-Aldrich). Spectrophotometric absorbance of the plate was recorded at ($\lambda_{\text{ex}} = 490/\lambda_{\text{em}} = 525$ nm) and ($\lambda_{\text{ex}} = 540/\lambda_{\text{em}} = 590$ nm) for ratio analysis using Tecan Microplate Reader (Tecan Instruments, Switzerland). Cells were centrifuged at 800 rpm and imaged using an Olympus CKX53 inverted fluorescence microscope.

4.2.7. Annexin V-FITC/PI Staining. Jurkat cells were seeded at 1×10^5 cells/mL in 6-well dishes and allowed to grow overnight. Cells were treated with different concentrations of **TJ08** 10 and 20 μM and allowed to incubate for 48 h. Cells were harvested, washed with cold PBS, and subjected for Annexin V FITC/PI double staining assay. Samples were resuspended in 1 \times binding buffer and incubated with Annexin V FITC and PI according to the manufacturer's protocol (Roche) and imaged using Olympus CKX53 inverted fluorescence microscope.

4.2.8. Cell Cycle Analysis. Cell cycle analysis by flow cytometer was performed as described previously. Synchronized cells by serum starvation were treated with different concentrations of **TJ08**, incubated for 24 h, harvested, fixed with 70% ethanol, subjected to propidium iodide staining, and analyzed using flow cytometer Beckman cell counter at an excitation with 488 nm laser and emission at 560/570 nm.

4.2.9. Western Blotting. Western blot was performed as described previously. Proteins were separated in different percentage gels and subjected to Western blotting to identify expressed proteins. Blots were blocked and treated with primary and secondary antibodies to detect pro-apoptotic, anti-apoptotic and loading control proteins. HRP-conjugated secondary antibodies were developed by using chemiluminescent solution and scanned using chemi doc (UViTech, UK).

4.2.10. Statistical Analysis. Data was analyzed by GraphPad prism software (GraphPad Software Inc., San Diego, CA, USA) for elucidating IC_{50} of synthesized small molecules. Each experiment was repeated a minimum of 3 times. Error bars indicate the SEM and *P* values and were calculated by comparing the mean of control group with the mean of **TJ08**-treated group *p* < 0.05.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c06057>.

¹H NMR spectra of intermediates and final compounds **2**, **3a**, **3b**, **3c**, **4a**, **4b**, **4c**, and **TJ01–TJ15**; ¹³C NMR spectra of the final compounds **TJ01–TJ15**; HRMS spectra of the final compounds **TJ01–TJ15**; LC–MS spectra of intermediates and final compounds **2**, **3a**, **3b**,

3c, **4a**, **4b**, **4c**, and **TJ01–TJ15**; FT-IR spectra of the final compounds **TJ01–TJ15** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Thimmegowda Naraganahalli Ramegowda – Government. S. K. S. J. Technological Institute (Affiliated to Visvesvaraya Technological University), Bangalore 560001 Karnataka, India; Email: nrthimmegowda@gmail.com

Shobith Rangappa – Adichunchanagiri Institute for Molecular Medicine, Adichunchanagiri Institute of Medical Sciences, Adichunchanagiri University, BG Nagara 571448 Karnataka, India; School of Natural Sciences, Adichunchanagiri University, BG Nagara 571448 Karnataka, India; Email: shobithrangappa@gmail.com

Authors

Jagadeesha Gullahalli Swathantraiah – Government. S. K. S. J. Technological Institute (Affiliated to Visvesvaraya Technological University), Bangalore 560001 Karnataka, India

Sudhanva Muddenahalli Srinivasa – Adichunchanagiri Institute for Molecular Medicine, Adichunchanagiri Institute of Medical Sciences, Adichunchanagiri University, BG Nagara 571448 Karnataka, India; School of Natural Sciences, Adichunchanagiri University, BG Nagara 571448 Karnataka, India

Anil Kumar Belagal Motatis – Adichunchanagiri Institute for Molecular Medicine, Adichunchanagiri Institute of Medical Sciences, Adichunchanagiri University, BG Nagara 571448 Karnataka, India

Akshay Uttarkar – Department of Biotechnology, R V College of Engineering, Bengaluru 560059 Karnataka, India

Shwetha Bettaswamygowda – Department of applied science, CPGS, Visvesvaraya Technological University, Muddenahalli 562101 Karnataka, India

Sridhar Bilgumba Thimmaiah – Department of Chemistry, Maharani's Science College for Women, Maharani cluster University, Bangalore 560001 Karnataka, India

Vidya Niranjana – Department of Biotechnology, R V College of Engineering, Bengaluru 560059 Karnataka, India; orcid.org/0000-0001-9187-7753

Rangappa Kanchugarakoppal Subbegowda – Institution of Excellence, University of Mysore, Mysore 570006 Karnataka, India

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsomega.2c06057>

Author Contributions

#J.G.S. and S.M.S. contributed equally.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CDI, carbonyldiimidazole; THF, tetrahydrofuran; DIPEA, *N,N*-diisopropylethylamine; DMSO, dimethyl sulfoxide-*d*₆; *R*_f, retention factor; rt, room temperature; V, volume; V/V, volume/volume; W/W, weight/weight; equiv, equivalent; h, hours; ESI, electron spray ionization; HRMS, high resolution mass spectrometry; LCMS, Liquid chromatography-mass spectrometry; MHz, megahertz; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared

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