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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, antimicrobial, and cytotoxic activities of novel benzimidazole derivatives bearing cyanopyridine and 4-thiazolidinone motifs

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Abstract A series of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-yl)-4-(aryl)nicotinonitriles **5a–l** were synthesized and characterized by IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry techniques. These novel compounds **5a–l** were screened for their in vitro antimicrobial activity against different bacterial and fungal strains and in vitro cytotoxicity study (HeLa cell line) using MTT colorimetric assay. The results demonstrated that compounds **5c**, **5e,** and **5i–k** exhibited excellent antibacterial activity, while compounds **5d**, **5i**, and **5k** were found to be the most potent antifungal agents. From the standpoint of SAR studies, it was observed that the presence of electron donating groups remarkably enhanced the antimicrobial activity of newly synthesized compounds. Further, the results of preliminary MTT cytotoxicity studies on HeLa cells suggested that potent antimicrobial activity of **5c–e** and **5i–k** was accompanied by low cytotoxicity.

Keywords Antimicrobial activity · Benzimidazole · Cyanopyridine · Cytotoxicity · MTT assay · Thiazolidinone

Introduction

The increasing incidences of infection caused by rapid development of bacterial resistance to most of the known antibiotics are a serious health problem (Chu *et al*., 1996; Suree *et al*., 2007). Therefore, development of new types of antibacterial agents is a very important task, and much of the research effort is oriented to the design of new antibacterial agents with high efficiency (Cui *et al*., 2005). It is a well-known fact that more efficacious antibacterial agents can be designed by joining two or more biologically active heterocyclic systems together in a single molecular framework (Xie and Seto, 2007) i.e., benzimidazole, cyanopyridine, and thiazolidinone. These synergistic antimicrobial combinations have several major advantages, including the potential to slow down development of drug resistance, a broader antimicrobial spectrum, and potential reduction in the dose and undesired side effects of each drug (Bennett *et al*., 1979).

Benzimidazole scaffold is a useful structural motif for displaying chemical functionality in biologically active molecules, due to various activities like antihelmintic (Valdez *et al*., 2002), antihistaminic (Iemura *et al*., 1986), anticancer (Tong *et al*., 2009), antiviral (Li *et al*., 2006), anti-inflammatory (Achar *et al*., 2010), antiproliferative (Sann *et al*., 2006), antioxidant (Kálai *et al*., 2009), and anticoagulant (Mederski *et al*., 2004). This ring system is also present in numerous antiparasitic and antitumoral drugs (Boiani and Gonzalez, 2005; Iwao *et al*., 2004). Furthermore, pyridine derivatives occupy a pivotal position in modern heterocyclic chemistry, and consequently, pyridine substructure is one of the most important heterocycles found in natural products, pharmaceuticals, and functional materials (Teague, 2008; Movassaghi *et al*., 2007). Pyridine derivatives containing multi-functional groups such as streptonigrin, streptonigrone, and lavendamycin are reported as anticancer drugs, and cerivastatin is reported as HMGCoA enzyme inhibitor (Bringmann *et al*., 2004). Moreover, substituted pyridines are reported as leukotriene B-4 antagonists (Zhou *et al*., 2008). On the other hand,

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cyanopyridone and cyanopyridine derivatives have promising antimicrobial (Hammam *et al*., 2000) and anticancer activities (Abo-Ghalia *et al*., 2003).

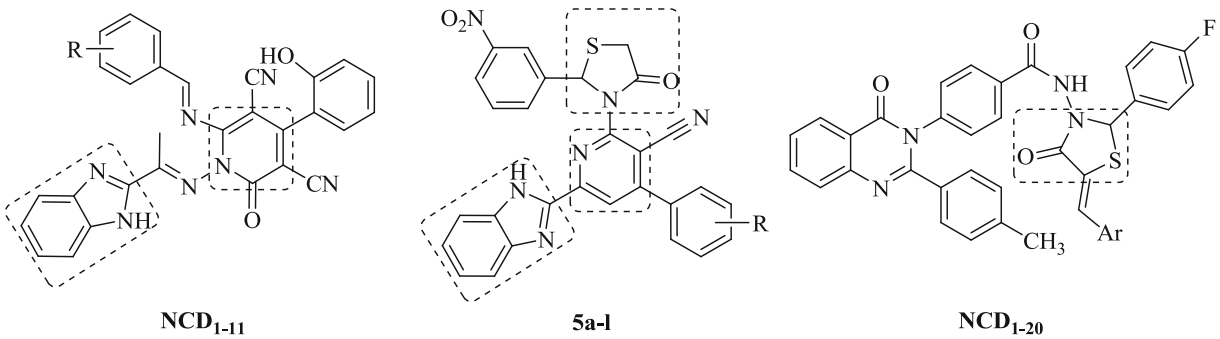
Numerous thiazolidinone derivatives have shown significant bioactivities such as antimicrobial (Gouveia and de Oliveira, 2009), antidiabetic (Noboyoshi and Heroaki, 2006), anticancer (Wu *et al*., 2006), anti HIV (Rawal *et al*., 2005), etc. Currently, 4-thiazolidinones are considered as a new class of antidiabetic (insulin-sensitizing) drug and potent aldose reductase inhibitors. In addition, 4-thiazolidinone nucleus appears frequently in compounds possessing cardiac and glycemic benefits such as troglitazone (Ghazzi *et al*., 1997). Mode of action of 4-thiazolidinones is a novel inhibition of the bacterial enzyme Mur B which is precursor acting during the biosynthesis of peptidoglycan (essential component of the cell wall of bacteria) (Andres *et al*., 2000).

In this context, our research group had reported the synthesis of 1-(1-(1*H*-benzo[*d*]imidazol-2-yl)ethylideneamino)-6-(arylideneamino)-4-(2-hydroxyphenyl)-2-oxo-1,2-di-hydropyridine-3,5-dicarbonitriles **NCD$_{1–11}$** (Desai *et al*., 2012) containing benzimidazole and 2-pyridone nucleus, and recently our group also reported the synthesis of *N*-(5-(aryl)-2-(4-fluorophenyl)-4-oxothiazolidin-3-yl)-4-(4-oxo-2-ptolylquinazolin-3(4*H*)-yl)benzamides **NCD$_{1–20}$** (Desai *et al*., 2013) and derived its antimicrobial activity. Considering this fact, in the present communication, we have reported the synthesis of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-4-(aryl)nicotinonitriles **5a–l** by introducing both the cyanopyridine and 4-thiazolidinone nucleus in the second position of benzimidazole nucleus. We envision our approach toward the design and synthesis of novel structurally diverse series of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-yl)-4-(aryl)nicotinonitriles **5a–l** derivatives bearing above afore mentioned moieties in single molecular framework in order to investigate their in vitro antimicrobial activity. Cytotoxicity studies were also conducted in HeLa cell lines to evaluate the ability of these compounds to inhibit cell growth. Structural relevance of title compounds **5a–l** with previously synthesized compounds is shown in Fig. 1.

Results and discussion

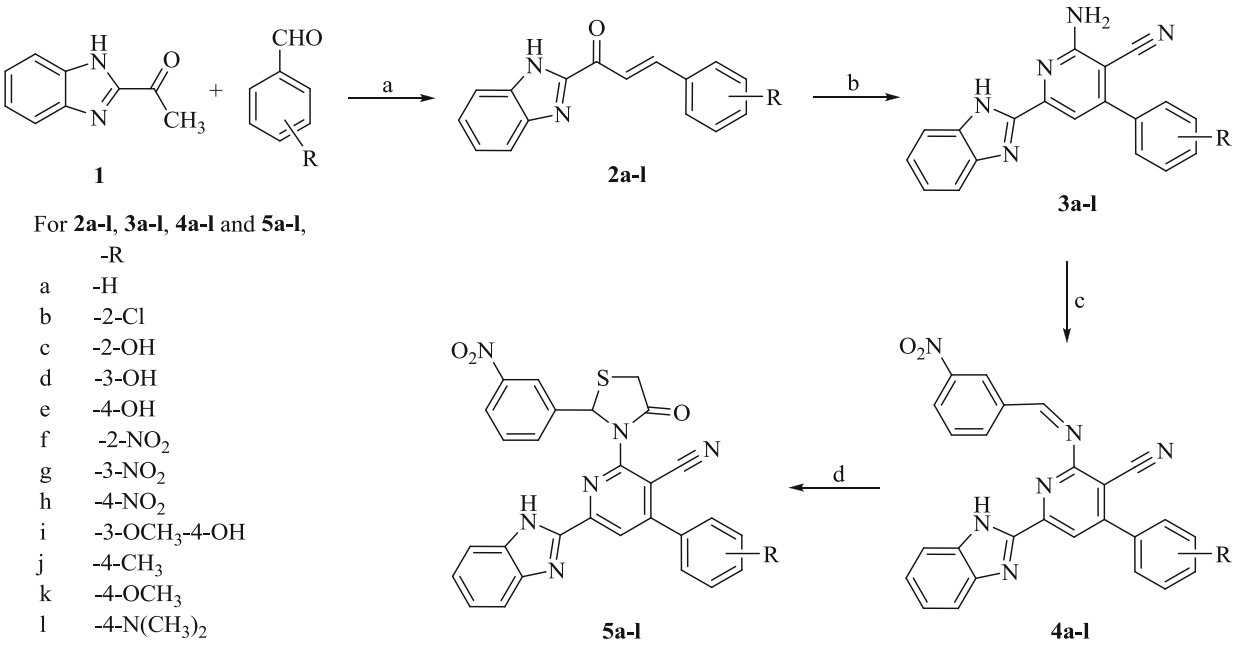
Chemistry

The synthetic strategies adopted to obtain the target compounds are depicted in Scheme 1. Compounds 1-(1*H*-benzo[*d*]imidazol-2-yl)-3-(aryl)prop-2-en-1-ones **2a–l** served as convenient intermediates in the synthesis, which were prepared according to the literature method (Shaharyar *et al*., 2010; Ouattara *et al*., 2011). Compounds **2a–l** were treated with ammonium acetate and malononitrile to achieve derivatives **3a–l**, produced via well-known Knoevenagel condensation reaction. In compounds **3a–l**, we observed primary amine and nitrile groups as well as the disappearance of α, β-unsaturated carbonyl group which was confirmed by IR and NMR spectra. Resultant compounds **3a–l** underwent simple condensation reaction with 3-nitrobenzaldehyde to provide good yield of Schiff bases **4a–l** which showed successful conversion of primary amine group into azomethine group. The disappearance of NH_2 signals in the FT-IR and ^{1}H NMR supported the condensation leading to the formation of compounds **4a–l**. It is interesting to note that the compounds having azomethine structure may exist as *E/Z* geometrical isomers about C=N double bond and as *cis/trans* imine conformers (Galic *et al*., 2001; Wyrzykiewicz and Prukah, 1998; Mentese *et al*., 2013). According to the literature (Wyrzykiewicz and Prukah, 1998), compounds containing >C=N– bond are present in higher percentage in dimethyl-d_6 sulfoxide solution in the form of geometrical *E* isomer about >C=N– double bond. The *Z* isomer can be stabilized in less polar solvents by an intramolecular hydrogen bond. In the present study, the spectral data were obtained in dimethyl-d_6 sulfoxide solution, and no signal belonging to *Z* isomer was observed. This azomethine proton appeared as a singlet at δ 8.31–8.42 ppm in the



NCD$_{1-11}$ **5a-l** **NCD$_{1-20}$**

Fig. 1 Structural relevance of title compounds **5a–l** with previously synthesized compounds **NCD$_{1–11}$** and **NCD$_{1–20}$**



Scheme 1 Synthetic route for the preparation of title compounds **5a–l**. Reagents and conditions: (*a*) EtOH, KOH, stirring, 10 h; (*b*) Malononitrile, ammonium acetate, EtOH, reflux, 10 h; (*c*) 3-Nitrobenzaldehyde, anhy. $ZnCl_2$, 1,4-dioxane, reflux, 6 h; (*d*) $SHCH_2COOH$, 1,4-dioxane, reflux, 12 h

^{1}H NMR spectra and the carbon of resulting compounds resonated at δ 158.5–158 ppm in the ^{13}C NMR spectra. The additional support for the formation of derivatives, **4a–l**, was obtained by the appearance of (M+1) ion peaks at corresponding *m/z* values confirming their molecular masses, and these compounds gave elemental analysis results consistent with the proposed structures. In the last step, azomethine derivatives **4a–l** generated final analogs **5a–l** using cyclization with thioglycolic acid.

Formation of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-yl)-4-(aryl)nicotinonitriles **5a–l** was confirmed on the basis of their IR, ^{1}H NMR, ^{13}C NMR spectroscopy, and mass spectrometry. IR spectrum of compound **5j** showed absorption band at 3,440 cm^{-1} which was due to N–H stretching. Strong absorption bands at 1,698 and 2,337 cm^{-1} were observed due to >C=O of thiazolidinone ring and cyanide group, respectively. Similarly, strong stretching vibrations at 3,030 and 1,535 cm^{-1} were responsible for C–H bond in reactive methylene group and N=O bond in $–NO_2$ group, respectively. In addition, the ^{1}H NMR spectrum of **5j** showed a singlet for three protons at δ 2.34 ppm due to CH_3 group. Two singlets appeared at δ 3.95 and 6.44 ppm due to protons of reactive methylene group and proton attached with asymmetric carbon, respectively. Aromatic protons displayed multiplets at δ 7.22–8.12 ppm. The singlet signal observed at δ 10.25 ppm integrating for one proton was assigned to –NH group. Moreover, in ^{13}C NMR spectra of compound **5j**, the carbon atoms of methyl, reactive methylene group, and methine carbon (asymmetric carbon) appeared at δ 21.4, 33.6, and 71.2 ppm, respectively. Carbonyl carbon of thiazolidinone ring showed a chemical shift at δ 171.3 ppm, whereas carbon of cyanide group showed chemical shift value at δ 113.8 ppm. The mass spectrum of **5j** showed molecular ion peak at *m/z* = 532.13 (M+1), which is in agreement with the molecular formula $C_{29}H_{20}N_6O_3S$. Similarly, spectral values for all the compounds and C, H, and N analysis are presented in the experimental part.

Antibacterial activity

All the newly synthesized compounds **5a–l** were initially screened for their in vitro antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* (MTCC-96), *Streptococcus pyogenes* (MTCC-442)) and Gram-negative bacteria (*Escherichia coli* (MTCC-443), *Pseudomonas aeruginosa* (MTCC-1688)) using conventional broth dilution method (Hannan, 2000). The MIC (minimum inhibitory concentration) values were determined by comparison to standard "chloramphenicol" as reference drug for evaluating antibacterial activity which showed MIC of 50 µg/mL against *E. coli, P.aeruginosa, S. aureus,* and *S. pyogenes*. The results of antibacterial activity are presented in Table 1. The obtained antimicrobial activity of tested compounds could be correlated to structural variations. Intermediates **3a–l** showed poor antimicrobial activity against all tested

Table 1 In vitro antimicrobial screening results of compounds **5a–l**

Entry	−R	Minimum inhibitory concentration (MIC [μg/mL])						
		Gram-positive bacteria		Gram-negative bacteria		Fungi		
		Sa	Sp	Ec	Pa	Ca	An	Ac
5a	−H	1000	1000	500	500	500	500	500
5b	−2-Cl	200	200	250	250	500	250	250
5c	−2-OH	100	62.5	62.5	50	250	100	100
5d	−3-OH	250	100	100	100	100	50	50
5e	−4-OH	100	50	50	50	250	100	100
5f	$-2\text{-}NO_2$	500	125	250	250	1000	250	250
5g	$-3\text{-}NO_2$	1000	250	250	500	500	250	250
5h	$-4\text{-}NO_2$	500	250	250	250	500	250	250
5i	$-3\text{-}OCH_3\text{-}4\text{-}OH$	50	25	12.5	25	50	25	12.5
5j	$-4\text{-}CH_3$	25	50	50	50	250	100	100
5k	$-4\text{-}OCH_3$	25	50	50	25	100	50	50
5l	$-4\text{-}N(CH_3)_2$	250	100	100	100	250	100	100
Chloramphenicol		50	50	50	50	–	–	–
Ketoconazole		–	–	–	–	50	50	50

Sa Staphylococcus aureus MTCC 96, *Sp Staphylococcus pyogenes* MTCC 442, *Ec Escherichia coli* MTCC 443, *Pa Pseudomonas aeruginosa* MTCC 1688, *Ca Candida albicans* MTCC 227, *An Aspergillus niger* MTCC 282, *Ac Aspergillus clavatus* MTCC 1323

bacterial and fungal strains, among which compounds **3c**, **3e**, and **3i** showed poor activity (MIC = 1 000 μg/mL against *P. aeruginosa* and *S. pyogenes*) and compounds **3d** and **3i** displayed poor antifungal activity (MIC = 1 000 μg/mL against *A. clavatus*). Precursors **3a–l** reacted with *m*-nitrobenzaldehyde to generate key scaffolds **4a–l.** Further, these intermediates were tested against different bacterial and fungal strains and were found to have mild to poor antimicrobial activity. Intermediate derivatives **4c**, **4e**, and **4i** displayed mild activity at MIC = 500 μg/mL against *P. aeruginosa* and *S. pyogenes*, while intermediates **4j** and **4k** exhibited mild activity against *E. coli* and *S. aureus* at MIC = 500 μg/mL. Similarly, compounds **4d**, **4i**, and **4k** exhibited mild activity against *A. niger* and *A. clavatus* at MIC = 500 μg/mL. Now, Schiff bases **4a–l** were treated with thioglycolic acid to afford final derivatives **5a–l** having 4-thiazolidinone motif, which were found to have broad spectrum antimicrobial activity. In addition, activity results revealed that some of the tested compounds showed excellent inhibition against various tested microbial strains compared to the standard drug. From antibacterial activity data, it was observed that compounds **5c** (2-OH), **5e** (4-OH), **5i** ($3\text{-}OCH_3$-4-OH), **5j** ($4\text{-}CH_3$), and **5k** ($4\text{-}OCH_3$) were most potent against all bacterial strains in the range of 12.5–100 μg/mL. On the basis of antibacterial evaluation, compound **5i** ($3\text{-}OCH_3$-4-OH) was the most active among the tested derivatives against *E. coli* at MIC = 12.5 μg/mL. Compounds **5d** (3-OH) and **5l** ($4\text{-}N(CH_3)_2$) showed good activity at MIC = 100 μg/mL against all the bacterial strains except *S. aureus*, whereas compounds **5c** (2-OH) and **5e** (4-OH) were found to exhibit good activity against *S. aureus* at MIC = 100 μg/mL. When electron releasing groups (OH, CH_3, OCH_3) at 4th position i.e., compounds **5e** (4-OH), **5j** ($4\text{-}CH_3$), and **5k** ($4\text{-}OCH_3$), activity increased and all compounds displayed very good activity at MIC = 50 μg/mL against both *E. coli* and *S. pyogenes*, while compounds **5e** (4-OH) and **5j** ($4\text{-}CH_3$) also flaunted very good inhibition at MIC = 50 μg/mL against *P. aeruginosa*. Compounds **5c** (2-OH) and **5i** ($3\text{-}OCH_3$-4-OH) displayed inhibition at MIC = 50 μg/mL against *P. aeruginosa* and *S. aureus,* respectively. Similarly, compound **5k** ($4\text{-}OCH_3$) showed excellent activity at MIC = 25 μg/mL against *P. aeruginosa* and *S. aureus*, while compound **5j** ($4\text{-}CH_3$) exhibited excellent inhibition against *S. aureus*. In addition, compound **5i** ($3\text{-}OCH_3$-4-OH) was found to possess excellent activity against *P. aeruginosa* and *S. pyogenes* at MIC = 25 μg/mL as compared to the standard chloramphenicol.

Antifungal activity

The in vitro antifungal activity of newly synthesized compounds **5a–l** was determined using conventional broth dilution method (Hannan, 2000). In this work, *C. albicans*, *A. niger,* and *A. clavatus* were used as standard strains to investigate the activity. The fungal activity of each compound was compared with ketoconazole as a standard drug, which showed 50 μg/mL MIC against *C. albicans*, *A. niger*, and *A. clavatus*. For fungal growth, in the present protocol, we have used Sabourauds dextrose broth at 28 °C in aerobic condition for 48 h. DMSO and sterilized distilled water were used as negative control, while ketoconazole (1 U strength) was used as a positive control. The results of antifungal activity are presented in Table 1. The results indicated that, among the tested compounds, **5c** (2-OH), **5e** (4-OH), **5j** ($4\text{-}CH_3$), and **5l** ($4\text{-}N(CH_3)_2$) showed good inhibition against all the fungal strains, whereas compounds **5d** (3-OH) and **5k** ($4\text{-}OCH_3$) exhibited very good activity against both *C. albicans* and *A. niger*, respectively. Compound **5i** ($3\text{-}OCH_3$-4-OH) showed the highest inhibition against *C. albicans* at MIC = 50 μg/mL, while compound **5i** ($3\text{-}OCH_3$-4-OH) showed enhanced activity against *A. niger* at MIC = 25 μg/mL. Compound **5i** ($3\text{-}OCH_3$-4-OH) exhibited MIC at 12.5 μg/mL against *A. clavatus* which showed the highest activity among the tested compounds of the series. The activity of the series of compounds was compared with the standard drug ketoconazole.

Table 2 Levels of cytotoxicity induced by compounds **5a–l** on HeLa cells

Entry	Cytotoxicity IC_{50} (μM) HeLa
5a	61.32
5b	74.20
5c	>100
5d	>100
5e	>100
5f	65.12
5g	78.34
5h	58.55
5i	>100
5j	>100
5k	>100
5l	91.70
Doxorubicin	3.24

IC_{50} is the concentration required to inhibit 50 % of cell growth
HeLa human cervical cancer cell line

In vitro cytotoxicity studies

In vitro cytotoxic activity of newly synthesized compounds **5a–l** was evaluated against human cervical cancer cell line (HeLa) by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983; Alley *et al*., 1988), which measures the reduction of tetrazolium bromide salt into a formazan dye by mitochondrial dehydrogenases in treated versus untreated cells. The IC50 values obtained for these compounds are shown in Table 2. Cytotoxicity results revealed that the derivatives **5c** (2-OH), **5d** (3-OH), **5e** (4-OH), **5i** (3-OCH_3-4-OH), **5j** (4-CH_3), and **5k** (4-OCH_3) accounted no toxicity at concentration of 100 μg/mL (IC_{50} > 100 μg/mL), while other derivatives showed moderate toxicity against HeLa cell lines. It was confirmed that none of the tested compounds exhibited any significant cytotoxic effects on HeLa cell line, suggesting that compounds were potential for their in vivo use as antimicrobial agents.

Experimental

All reactions except those in aqueous media were carried out by standard techniques for the exclusion of moisture. Melting points were determined on an electro thermal melting point apparatus and were reported uncorrected. TLC on silica gel plates (Merck, 60, F_{254}) was used for purity checking and reaction monitoring. Column chromatography on silica gel (Merck, 70–230 and 230–400 mesh ASTH for flash chromatography) was applied when necessary to isolate and purify the reaction products. Elemental analysis (% C, H, N) was carried out by a Perkin–Elmer 2400 CHN analyzer. IR spectra of all compounds were recorded on a Perkin–Elmer FT-IR spectrophotometer in KBr. ^{1}H NMR spectra were recorded on Varian Gemini 300 MHz and ^{13}C NMR spectra on Varian Mercury-400, 100 MHz in DMSO-d_6 as a solvent and tetramethylsilane (TMS) as an internal standard. Mass spectra were scanned on a Shimadzu LC–MS 2010 spectrometer. Anhydrous reactions were carried out in oven-dried glassware in nitrogen atmosphere.

General method for the preparation of 1-(1*H*-benzo[*d*]imidazol-2-yl)-3-(aryl)prop-2-en-1-ones (**2a–l**)

Compounds 1-(1*H*-benzo[*d*]imidazol-2-yl)-3-(aryl)prop-2-en-1-ones **2a–l** are prepared according to the literature method (Shaharyar *et al*., 2010; Ouattara *et al*., 2011).

General method for the preparation of 2-amino-6-(1*H*-benzo[*d*]imidazol-2-yl)-4-(aryl)nicotinonitriles (**3a–l**)

A mixture of an equimolar amount of compound **2a–l** and malononitrile (0.01 mol) was refluxed in ethanol containing ammonium acetate (0.01 mol) for 10 h. The reaction mixture was cooled and poured onto crushed ice. The product obtained was isolated and crystallized from benzene.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-phenylnicotinonitrile (**3a**)*

White crystals (MeOH); m.p.: 235 °C; IR (KBr) υ_{max} 3462, 3380, 2922, 2350, 1528, 1565, 1610 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.25 (s, 1H, –NH), 8.25 (s, 1H, C_5–H pyridine ring), 7.70 (s, 2H, Ar–NH_2), 7.15–7.80 (m, 9H, Ar–H); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 158.8 (C, C=N), 154.7 (C, C-2 benzimidazole), 113.5 (C, –C≡N), 85.8 (C, C–C≡N); LCMS: *m/z* = 311.12 (M^+); Anal. Calcd. for $C_{19}H_{13}N_5$: C, 73.30; H, 4.21; N, 22.49. Found: C, 73.42; H, 4.34; N, 22.65.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(2-chlorophenyl)nicotinonitrile (**3b**)*

Yellowish crystals (MeOH); m.p.: 219 °C, IR (KBr) υ_{max} 3460, 3380, 2919, 2347, 1528, 1565, 1615, 724 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.24 (1H, s, –NH), 8.26 (1H, s, C_5–H pyridine ring), 7.72 (2H, s, Ar–NH_2), 7.25–7.60 (8H, m, Ar–H); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 158.7 (C, C=N), 154.7 (C, C-2 benzimidazole), 132.0 (C, C–Cl), 113.6 (C, –C≡N), 85.6 (C, C–C≡N); LCMS: *m/z* = 345.08 (M^+);

Anal. Calcd. for $C_{19}H_{12}N_5Cl$: C, 66.00; H, 3.50; N, 20.25. Found: C, 66.40; H, 4.24; N, 22.59.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(2-hydroxyphenyl)nicotinonitrile (**3c**)*

Yellowish crystals (MeOH); m.p.: 219 °C, IR (KBr) υ_{max} 3635, 3435, 3373, 2923, 2340, 1528, 1565, 1598 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.24 (1H, s, C_5–H pyridine ring), 7.21–8.40 (8H, m, Ar–H), 7.73 (2H, s, Ar–NH_2), 5.34 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 155.1 (C, C–OH), 154.7 (C, C-2 benzimidazole), 113.7 (C, –C≡N), 85.6 (C, C–C≡N); LCMS: *m/z* = 327.34 (M^+); Anal. Calcd. for $C_{19}H_{13}N_5O$: C, 69.71; H, 4.00; N, 21.39. Found: C, 69.68; H, 4.14; N, 21.37.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(3-hydroxyphenyl)nicotinonitrile (**3d**)*

White crystals (MeOH); m.p.: 245 °C, IR (KBr) υ_{max} 3621, 3440, 3350, 2943, 2350, 1524, 1569, 1587 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.28 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 7.22–8.65 (8H, m, Ar–H), 7.73 (2H, s, Ar–NH_2), 5.35 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 157.5 (C, C–OH), 154.7 (C, C-2 benzimidazole), 113.7 (C, –C≡N), 85.6 (C, C–C≡N); LCMS: *m/z* = 327.11 (M^+); Anal. Calcd. for $C_{19}H_{13}N_5O$: C, 69.71; H, 4.00; N, 21.39. Found: C, 69.70; H, 4.14; N, 21.41.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxyphenyl)nicotinonitrile (**3e**)*

Light yellow crystals (MeOH); m.p.: 249 °C, IR (KBr) υ_{max} 3623, 3430, 3370, 2920, 2342, 1527, 1565, 1591 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.26 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 7.73 (2H, s, Ar–NH_2), 6.86–7.62 (8H, m, Ar–H), 5.36 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 159.0 (C, C–OH), 154.7 (C, C-2 benzimidazole), 113.7 (C, –C≡N), 85.6 (C, C–C≡N); LCMS: *m/z* = 327.11 (M^+); Anal. Calcd. for $C_{19}H_{13}N_5O$: C, 69.71; H-4.00; N, 21.39. Found: C, 69.68; H, 4.14; N, 21.37.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(2-nitrophenyl)nicotinonitrile (**3f**)*

Yellow amorphous; m.p.: 255 °C, IR (KBr) υ_{max} 3439, 3373, 2932, 2345, 1529, 1568, 1595, 1515 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.27 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 7.22–8.05 (8H, m, Ar–H), 7.74 (2H, s, Ar–NH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 154.7 (C, C–2 benzimidazole), 145.4 (C, C–NO_2), 113.8 (C, –C≡N), 85.5 (C, C–C≡N); LCMS: *m/z* = 356.10 (M^+); Anal. Calcd. for $C_{19}H_{12}N_6O_2$: C, 64.04; H, 3.39; N, 23.58. Found: C, 64.47; H, 3.34; N, 23.61.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(3-nitrophenyl)nicotinonitrile (**3g**)*

Yellow crystals (MeOH); m.p.: 225 °C, IR (KBr) υ_{max} 3436, 3368, 2923, 2342, 1527, 1565, 1591, 1512 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 7.22–8.27 (8H, m, Ar–H), 7.74 (2H, s, Ar–NH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 154.7 (C, C-2 benzimidazole), 148.4 (C, C–NO_2), 113.9 (C, –C≡N), 85.5 (C, C–C≡N); LCMS: *m/z* = 356.10 (M^+); Anal. Calcd. for $C_{19}H_{12}N_6O_2$: C, 64.04; H, 3.39; N, 23.58. Found: C, 64.47; H, 3.34; N, 23.61.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(4-nitrophenyl)nicotinonitrile (**3h**)*

Light yellow crystals (MeOH); m.p.: 225 °C, IR (KBr) υ_{max} 3437, 3370, 2928, 2340, 1525, 1564, 1593, 1516 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.24 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 7.22–8.32 (8H, m, Ar–H), 7.74 (2H, s, Ar–NH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 154.7 (C, C-2 benzimidazole), 148.4 (C, C–NO_2), 113.8 (C, –C≡N), 85.6 (C, C–C≡N); LCMS: *m/z* = 356.10 (M^+); Anal. Calcd. for $C_{19}H_{12}N_6O_2$: C, 64.04; H, 3.39; N, 23.58. Found: C, 64.47; H, 3.34; N, 23.61.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxy-3-methoxyphenyl)nicotinonitrile (**3i**)*

White crystals ($CHCl_3$); m.p.: 231 °C, IR (KBr) υ_{max} 3621, 3436, 3368, 2829, 2923, 2342, 1527, 1565, 1591 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 6.89–8.16 (7H, m, Ar–H), 7.74 (2H, s, Ar–NH_2), 5.36 (1H, s, Ar–OH), 3.83 (3H, s, Ar–O–CH_3–); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 154.7 (C, C-2 benzimidazole), 149.2 (C, C–OH), 148.0 (C, C–OCH_3), 113.7 (C, –C≡N), 85.6 (C, C–C≡N), 56.1 (C, –OCH_3); LCMS: *m/z* = 357.12 (M^+); Anal. Calcd. for $C_{20}H_{15}N_5O_2$: C, 67.22; H, 4.23; N, 19.60. Found: C, 67.27; H, 4.27.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-p-tolylnicotinonitrile (**3j**)*

Brown amorphous; m.p.: 240–243 °C, IR (KBr) υ_{max} 3463, 3370, 2951, 2922, 2348, 1528, 1565, 1610 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.19 (1H,

s, C_5–H pyridine ring), 7.20–7.80 (8H, m, Ar–H), 7.72 (2H, s, Ar–NH_2), 2.34 (3H, s, Ar–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 154.7 (C, C-2 benzimidazole), 132.2 (C, C–CH_3), 113.8 (C, –C≡N), 85.8 (C, C–C≡N), 21.3 (C, –CH_3); LCMS: *m/z* = 325.13 (M^+); Anal. Calcd. for $C_{20}H_{15}N_5$: C, 73.83; H, 4.65; N, 21.52. Found: C, 73.42; H, 4.60; N, 21.65.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(4-methoxyphenyl)nicotinonitrile (**3k**)*

Pale yellow crystals (MeOH); m.p.: 260–262 °C, IR (KBr) υ_{max} 3466, 3365, 2922, 2828, 2342, 1530, 1566, 1613 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.23 (1H, s, –NH), 8.15 (1H, s, C_5–H pyridine ring), 7.05–7.70 (8H, m, Ar–H), 7.74 (2H, s, Ar–NH_2), 3.83 (3H, s, Ar–O–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 161.2 (C, C–OCH_3), 154.8 (C, C-2 benzimidazole), 113.8 (C, –C≡N), 85.5 (C, C–C≡N), 55.7 (C, –OCH_3); LCMS: *m/z* = 341.13 (M^+); Anal. Calcd. for $C_{20}H_{15}N_5O$: C, 70.37; H, 4.43; N, 20.52. Found: C, 70.42; H, 4.45; N, 20.56.

*2-amino-6-(1H-benzo[d]imidazol-2-yl)-4-(4-(dimethylamino)phenyl)nicotinonitrile (**3l**)*

Light brown amorphous; m.p.: 240 °C, IR (KBr) υ_{max} 3466, 3365, 2927, 2338, 1537, 1560, 1615 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.27 (1H, s, –NH), 8.16 (1H, s, C_5–H pyridine ring), 6.82–7.61 (8H, m, Ar–H), 7.74 (2H, s, Ar–NH_2), 3.06 (6H, s, Ar–$N(CH_3)_2$); ^{13}C NMR (DMSO-d_6, 100 MHz): = 161.9 (C, C–NH_2), 155.8 (C, C–$N(CH_3)_2$), 154.8 (C, C-2 benzimidazole), 113.5 (C, –C≡N), 85.5 (C, C–C≡N), 41.3 (C, >NCH_3); LCMS: *m/z* = 354.16 (M^+); Anal. Calcd. for $C_{21}H_{18}N_6$: C, 71.17; H, 5.12; N, 23.71. Found: C, 71.23; H, 5.14; N, 23.70.

General method for the preparation of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-((3-nitrobenzylidene)amino)-4-(aryl)nicotinonitriles (**4a–l**)

In a round bottom flask, compound **(3a–l)** (0.01 mol) was taken in 1,4-dioxane (20 mL), and m-nitrobenzaldehyde (0.01 mol) was added. To this mixture, a pinch of anhydrous zinc chloride was added and refluxed for 6 h and poured onto crushed ice. The product was filtered and washed with cold water. The product was dried and recrystallized from methanol.

*6-(1H-benzo[d]imidazol-2-yl)-2-((3-nitrobenzylidene)amino)-4-phenylnicotinonitrile (**4a**)*

Yellowish crystals (MeOH); m.p.: 248 °C, IR (KBr) υ_{max} 3454, 2950, 2346, 1520, 1561, 1621, 1519 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.27 (1H, s, –NH), 8.90 (1H, s, C_5–H pyridine ring), 8.35 (1H, s, CH=N), 7.22–8.52 (13H, m, Ar–H); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.7 (C, –N–C–N), 158.8 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.0 (C, C–NO_2), 117.3 (C, –C≡N), 98.2 (C, C–C≡N); LCMS: *m/z* = 444.13 (M^+); Anal. Calcd. for $C_{26}H_{16}N_6O_2$: C, 70.26; H, 3.63; N, 18.91. Found: C, 70.30; H, 3.68; N, 19.0.

*6-(1H-benzo[d]imidazol-2-yl)-4-(2-chlorophenyl)-2-((3-nitrobenzylidene)amino)nicotino-nitrile (**4b**)*

Yellow crystals ($CHCl_3$); m.p.: 219 °C, IR (KBr) υ_{max} 3462, 2919, 2347, 1528, 1565, 1615, 1518, 734 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.98 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.36 (1H, s, CH=N); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.6 (C, –N–C–N), 158.7 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.3 (C, C–NO_2), 132.1 (C, C–Cl), 117.4 (C, –C≡N), 98.3 (C, C–C≡N); LCMS: *m/z* = 478.09 (M^+); Anal. Calcd. for $C_{26}H_{15}N_6O_2Cl$: C, 65.21; H, 3.16; N, 17.55. Found: C, 65.50; H, 3.68; N, 17.46.

*6-(1H-benzo[d]imidazol-2-yl)-4-(2-hydroxyphenyl)-2-((3-nitrobenzylidene)amino)nico-tinonitrile (**4c**)*

Light yellow crystals (MeOH); m.p.: 252 °C, IR (KBr) υ_{max} 3627, 3459, 2929, 2351, 1523, 1569, 1627, 1525 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.20 (1H, s, –NH), 8.92 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.32 (1H, s, CH=N), 5.33 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.8 (C, –N–C–N), 158.5 (C, –C=N), 155.2 (C, C–OH), 154.5 (C, C-2 benzimidazole), 147.9 (C, C–NO_2), 117.1 (C, –C≡N), 98.4 (C, C–C≡N); LCMS: *m/z* = 460.13 (M^+); Anal. Calcd. for $C_{26}H_{16}N_6O_3$: C, 67.82; H, 3.50; N, 18.25. Found: C, 67.78; H, 3.58; N, 18.46.

*6-(1H-benzo[d]imidazol-2-yl)-4-(3-hydroxyphenyl)-2-((3-nitrobenzylidene)amino)nicotino-nitrile (**4d**)*

Pale yellow crystals (MeOH); pale yellow crystals; m.p.: 247–249 °C, IR (KBr) υ_{max} 3627, 3449, 2927, 2356, 1527, 1562, 1625, 1524 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz): = 10.28 (1H, s, –NH), 8.94 (1H, s, C_5–H pyridine ring), 6.91–8.52 (12H, m, Ar–H), 8.36 (1H, s, CH=N), 5.32 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.9 (C, –N–C–N), 158.5 (C, –C=N), 157.7 (C, C–OH), 154.7 (C, C-2 benzimidazole), 148.1 (C, C–NO_2), 117.2 (C, –C≡N), 98.3 (C, C–C≡N); LCMS: *m/z* = 460.13 (M^+); Anal. Calcd. for $C_{26}H_{16}N_6O_3$: C, 67.82; H, 3.50; N, 18.25. Found: C, 67.78; H, 3.58; N, 18.46.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxyphenyl)-2-((3-nitrobenzylidene)amino)nicotino-nitrile (**4e**)*

Light brown crystals ($CHCl_3$); m.p.: 241–243 °C, IR (KBr) υ_{max} 3638, 3450, 2925, 2349, 1525, 1562, 1629, 1518 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.29 (1H, s, –NH), 8.98 (1H, s, C_5–H pyridine ring), 6.86–8.53 (12H, m, Ar–H), 8.31 (1H, s, CH=N), 8.36 (1H, s, Ar–OH); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.9 (C, –N–C–N), 159.5 (C, C–OH), 158.9 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.4 (C, C–NO_2), 117.3 (C, –C≡N), 98.6 (C, C–C≡N); LCMS: *m/z* = 460.13 (M^+); Anal. Calcd. for $C_{26}H_{16}N_6O_3$: C, 67.82; H, 3.50; N, 18.25. Found: C, 67.85; H, 3.60; N, 18.45.

*6-(1H-benzo[d]imidazol-2-yl)-2-((3-nitrobenzyllidene)amino)-4-(2-nitrophenyl)nicotino-nitrile (**4f**)*

Light yellow crystals (MeOH); m.p.: 240 °C, IR (KBr) υ_{max} 3452, 2936, 2357, 1524, 1559, 1635, 1531 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.27 (1H, s, –NH), 8.94 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.36 (1H, s, CH=N); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.8 (C, –N–C–N), 158.6 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.0 (C, C–NO_2), 117.1 (C, –C≡N), 98.2 (C, C–C≡N); LCMS: *m/z* = 489.12 (M^+); Anal. Calcd. for $C_{26}H_{15}N_7O_4$: C, 63.80; H, 3.09; N, 20.03. Found: C, 63.78; H, 3.11; N, 20.10.

*6-(1H-benzo[d]imidazol-2-yl)-2-((3-nitrobenzyllidene)amino)-4-(3-nitrophenyl)nicotino-nitrile (**4g**)*

Yellowish crystals (MeOH); m.p.: 251 °C, IR (KBr) υ_{max} 3450, 2934, 2356, 1522, 1558, 1631, 1529 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.92 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.35 (1H, s, CH=N); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.9 (C, –N–C–N), 158.5 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.7 (C, C–NO_2), 117.1 (C, –C≡N), 98.6 (C, C–C≡N); LCMS: *m/z* = 489.12 (M^+); Anal. Calcd. for $C_{26}H_{15}N_7O_4$: C, 63.80; H, 3.09; N, 20.03. Found: C, 63.78; H, 3.11; N, 20.10.

*6-(1H-benzo[d]imidazol-2-yl)-2-((3-nitrobenzyllidene)amino)-4-(4-nitrophenyl)nicotino-nitrile (**4h**)*

Pale yellow crystals (MeOH); m.p.: 231 °C, IR (KBr) υ_{max} 3454, 2932, 2357, 1525, 1559, 1634, 1527 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.26 (1H, s, –NH), 8.95 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.33 (1H, s, CH=N); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.8 (C, –N–C–N), 158.7 (C, –C=N), 154.7 (C, C-2 benzimidazole), 148.6 (C, C–NO_2), 117.2 (C, –C≡N), 98.2 (C, C–C≡N); LCMS: *m/z* = 489.12 (M^+); Anal. Calcd. for $C_{26}H_{15}N_7O_4$: C, 63.80; H, 3.09; N, 20.03. Found: C, 63.78; H, 3.11; N, 20.10.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxy-3-methoxyphenyl)-2-((3-nitrobenzylidene)-amino)nicotinonitrile (**4i**)*

White crystals (MeOH); m.p.: 259 °C, IR (KBr) υ_{max} 3622, 2839, 3423, 2929, 2351, 1520, 1548, 1628, 1523 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.22 (1H, s, –NH), 8.88 (1H, s, C_5–H pyridine ring), 6.90–8.52 (11H, m, Ar–H), 8.41 (1H, s, CH=N) 5.34 (1H, s, Ar–OH), 3.84 (3H, s, Ar–O–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.9 (C, –N–C–N), 158.5 (C, –C=N), 154.4 (C, C-2 benzimidazole), 149.7 (C, C–OH), 148.2 (C, C–NO_2), 148.0 (C, C–OCH_3), 117.3 (C, –C≡N), 98.4 (C, C–C≡N), 56.1 (C, –OCH_3); LCMS: *m/z* = 490.14 (M^+); Anal. Calcd. for $C_{27}H_{18}N_6O_4$: C, 66.12; H, 3.70; N, 17.13. Found: C, 66.15; H, 3.78; N, 13.10.

*6-(1H-benzo[d]imidazol-2-yl)-2-((3-nitrobenzylidene)amino)-4-p-tolylnicotinonitrile (**4j**)*

White crystals (MeOH); m.p.: 244 °C, IR (KBr) υ_{max} 3450, 2925, 2349, 2951, 1527, 1553, 1634, 1520 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.29 (1H, s, –NH), 8.94 (1H, s, C_5–H pyridine ring), 7.22–8.52 (12H, m, Ar–H), 8.40 (1H, s, CH=N) 2.35 (3H, s, Ar–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 180.0 (C, –N–C–N), 158.6 (C, –C=N), 154.3 (C, C-2 benzimidazole), 148.5 (C, C–NO_2), 132.7 (C, C–CH_3), 117.3 (C, –C≡N), 98.3 (C, C–C≡N), 21.4 (C, –CH_3); LCMS: *m/z* = 460.13 (M^+); Anal. Calcd. for $C_{27}H_{18}N_6O_2$: C, 70.73; H, 3.96; N, 18.33. Found: C, 70.85; H, 3.60; N, 18.40.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-methoxyphenyl)-2-((3-nitrobenzylidene)amino)nico-tinonitrile (**4k**)*

Pale white crystals (MeOH); m.p.: 251–253 °C, IR (KBr) υ_{max} 3450, 2841, 2931, 2330, 1522, 1557, 1631, 1520, 1325 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH–), 8.90 (1H, s, C_5–H pyridine ring), 7.05–8.52 (12H, m, Ar–H), 8.41 (1H, s, CH=N) 3.84 (3H, s, Ar–O–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.6 (C, –N–C–N), 161.4 (C, C–OCH_3), 158.7 (C, –C=N), 154.9 (C, C-2 benzimidazole), 148.2 (C, C–NO_2), 117.3 (C, –C≡N), 98.0 (C, C–C≡N), 55.9 (C, –OCH_3); LCMS: *m/z* = 474.14 (M^+); Anal. Calcd. for $C_{27}H_{18}N_6O_3$: C, 68.35; H, 3.82; N, 17.71. Found: C, 68.40; H, 3.88; N, 17.76.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-dimethylaminophenyl)-2-((3-nitrobenzylidene)amino)-nicotinonitrile (**4l**)*

Yellowish crystals (MeOH); m.p.: 248 °C, IR (KBr) υ_{max} 3468, 2927, 2338, 1537, 1560, 1615, 1523 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.93 (1H, s, C_5–H pyridine ring), 6.82–8.54 (12H, m, Ar–H), 8.42 (1H, s, CH=N), 3.06 (6H, s, Ar–N<); ^{13}C NMR (DMSO-d_6, 100 MHz): = 178.1 (C, –N–C–N), 158.6 (C, –C=N), 155.9 (C, C–N(CH_3)$_2$), 154.7 (C, C-2 benzimidazole), 148.2 (C, C–NO_2), 116.9 (C, –C≡N), 98.4 (C, C–C≡N), 41.3 (C, >NCH_3); LCMS: *m/z* = 487.18 (M^+); Anal. Calcd. for $C_{28}H_{18}N_7O_2$: C, 68.98; H, 4.34; N, 20.11. Found: C, 69.0; H, 4.38; N, 20.16.

General method for the preparation of 6-(1*H*-benzo[*d*]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-4-(aryl)nicotinonitriles (**5a–l**)

Compound (**4a–l**) (0.01 mol) in an anhydrous 1,4-dioxane (25 mL) was added to 2-mercaptoacetic acid (0.012 mol). The mixture was refluxed for 12 h, cooled, and poured into aqueous saturated solution of sodium bicarbonate to remove unreacted 2-mercaptoacetic acid. The residue was filtered, dried, and recrystallized from ethanol (99.5 %).

*6-(1H-benzo[d]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-4-phenylnico- tinonitrile (**5a**)*

Brownish crystals (EtOH); m.p.: 318 °C; IR (KBr) υ_{max} 3421, 3024, 2950, 2346, 1698, 1528, 1619, 1597, 1519 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.29 (1H, s, –NH), 8.73 (1H, s, C_5–H pyridine ring), 7.22–8.12 (13H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 3.95 (2H, s, –CH_2 group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 154.6 (C, C-2 benzimidazole), 148.0 (C, C–NO_2), 113.7 (C, –C≡N), 91.4 (C, C–C≡N), 71.2 (C, –S–CH–N), 33.6 (C, –CH_2); LCMS: *m/z* = 518.12 (M^+); Anal. Calcd. for $C_{28}H_{18}N_6O_3S$: C, 64.85; H, 3.50; N, 16.21. Found: C, 64.77; H, 3.41; N, 16.12.

*6-(1H-benzo[d]imidazol-2-yl)-4-(2-chlorophenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)nicotinonitrile (**5b**)*

White crystals (EtOH); m.p.: 327 °C; IR (KBr) υ_{max} 3423, 3034, 2938, 2343, 1703, 1520, 1625, 1600, 1527, 724 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.74 (1H, s, C_5–H pyridine ring), 7.22–8.12 (12H, m, Ar–H), 6.45 (1H, s, S–CH–N group), 3.96 (2H, s, –CH_2 group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.2 (C, –C=O), 154.7 (C, C-2 benzimidazole), 147.8 (C, C–NO_2), 132.2 (C, C–Cl), 113.8 (C, –C≡N), 91.4 (C, C–C≡N), 71.2 (C, –S–CH–N), 33.5 (C, –CH_2); LCMS: *m/z* = 522.08 (M^+); Anal. Calcd. for $C_{28}H_{17}N_6O_3ClS$: C, 60.81; H, 3.10; N, 15.20. Found: C, 60.90; H, 3.16; N, 15.29.

*6-(1H-benzo[d]imidazol-2-yl)-4-(2-hydroxyphenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)nicotinonitrile (**5c**)*

Light brown amorphous; m.p.: 352 °C; IR (KBr) υ_{max} 3629, 3434, 3022, 2939, 2340, 1699, 1520, 1618, 1629, 1526 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.26 (1H, s, –NH), 8.75 (1H, s, C_5–H pyridine ring), 7.01–8.12 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 5.35 (1H, s, Ar–OH), 3.95 (2H, s, –CH_2 group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.2 (C, –C=O), 155.1 (C, C–OH), 154.7 (C, C-2 benzimidazole), 147.9 (C, C–NO_2), 113.9 (C, –C≡N), 91.5 (C, C–C≡N), 71.3 (C, –S–CH–N), 33.2 (C, –CH_2); LCMS: *m/z* = 534.11 (M^+); Anal. Calcd. for $C_{28}H_{18}N_6O_4S$: C, 62.91; H, 3.39; N, 15.75. Found: C, 62.98; H, 3.43; N, 15.85.

*6-(1H-benzo[d]imidazol-2-yl)-4-(3-hydroxyphenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)nicotinonitrile (**5d**)*

Pale yellow crystals (MeOH); m.p.: 325 °C; IR (KBr) υ_{max} 3630, 3434, 3025, 2937, 2337, 1706, 1521, 1613, 1630, 1528, 1327 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.74 (1H, s, C_5–H pyridine ring), 6.91–8.12 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 5.36 (1H, s, Ar–OH), 3.94 (2H, s, –CH_2 group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.2 (C, –C=O), 157.7 (C, C–OH), 154.3 (C, C-2 benzimidazole), 147.9 (C, C–NO_2), 113.7 (C, –C≡N), 91.6 (C, C–C≡N), 71.3 (C, –S–CH–N), 33.5 (C, –CH_2); LCMS: *m/z* = 534.11 (M^+); Anal. Calcd. for $C_{28}H_{18}N_6O_4S$: C, 62.91; H, 3.39; N, 15.75. Found: C, 62.98; H, 3.43; N, 15.85.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxyphenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)nicotinonitrile (**5e**)*

White crystals (EtOH); m.p.: 334 °C; IR (KBr) υ_{max} 3624, 3440, 3029, 2937, 2339, 1710, 1529, 1615, 1632, 1531 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.28 (1H, s, –NH), 8.73 (1H, s, C_5–H pyridine ring), 6.86–8.12 (12H, m, Ar–H), 6.45 (1H, s, S–CH–N group), 5.35 (1H, s, Ar–OH), 3.95 (2H, d, –CH_2 group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 157.9 (C, C–OH), 154.4 (C, C-2 benzimidazole), 147.9 (C, C–NO_2), 115.5 (C, –C≡N), 91.5 (C, C–C≡N), 71.3 (C, –S–CH–N), 33.4 (C, –CH_2); LCMS: *m/z* = 534.11 (M^+); Anal. Calcd. for $C_{28}H_{18}N_6O_4S$: C, 62.91; H, 3.39; N, 15.72. Found: C, 62.98; H, 3.43; N, 15.85.

*6-(1H-benzo[d]imidazol-2-yl)-4-(2-nitrophenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-nicotinonitrile (**5f**)*

Light brown amorphous (EtOH); m.p.: 310 °C; IR (KBr) υ_{max} 3438, 3031, 2932, 2333, 1711, 1527, 1619, 1635, 1536 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.75 (1H, s, C_5–H pyridine ring), 7.22–8.12 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 3.95 (2H, s, $–CH_2$ group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 154.7 (C, C-2 benzimidazole), 147.8 (C, C–NO_2), 113.8 (C, –C≡N), 91.5 (C, C–C≡N), 71.1 (C, –S–CH–N), 33.5 (C, $–CH_2$); LCMS: *m/z* = 563.10 (M^+); Anal. Calcd. for $C_{28}H_{17}N_7O_5S$: C, 59.68; H, 3.04; N, 17.40. Found: C, 59.72; H, 3.14; N, 17.53.

*6-(1H-benzo[d]imidazol-2-yl)-4-(3-nitrophenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-nicotinonitrile (**5g**)*

Yellowish crystals (EtOH); m.p.: 325 °C; IR (KBr) υ_{max} 3438, 3029, 2927, 2330, 1709, 1526, 1616, 1633, 1532 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.73 (1H, s, C_5–H pyridine ring), 7.22–8.67 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 3.95 (2H, s, $–CH_2$ group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.6 (C, –C=O), 154.5 (C, C-2 benzimidazole), 147.9 (C, C–NO_2), 113.9 (C, –C≡N), 91.7 (C, C–C≡N), 71.1 (C, –S–CH–N), 33.7 (C, $–CH_2$); LCMS: *m/z* = 563.10 (M^+); Anal. Calcd. for $C_{28}H_{17}N_7O_5S$: C, 59.68; H, 3.04; N, 17.40. Found: C, 59.72; H, 3.14; N, 17.53.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-nitrophenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-nicotinonitrile (**5h**)*

Yellowish crystals (MeOH); m.p.: 298 °C; IR (KBr) υ_{max} 3434, 3027, 2932, 2331, 1711, 1527, 1615, 1631, 1533 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.47 (1H, s, C_5–H pyridine ring), 7.06–8.32 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 3.95 (2H, s, $–CH_2$ group); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 155.1 (C, C-2 benzimidazole), 149.0 (C, C–NO_2), 113.6 (C, –C≡N), 94.7 (C, C–C≡N), 71.1 (C, –S–CH–N), 34.5 (C, $–CH_2$); LCMS: *m/z* = 563.10 (M^+); Anal. Calcd. for $C_{28}H_{17}N_7O_5S$: C, 59.68; H, 3.04; N, 17.40. Found: C, 59.72; H, 3.14; N, 17.53.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-hydroxy-3-methoxyphenyl)-2-(2-(3-nitrophenyl)-4-oxo-thiazolidin-3-yl)nicotinonitrile (**5i**)*

White crystals (EtOH); m.p.: 345 °C; IR (KBr) υ_{max} 3640, 3434, 3021, 2929, 2835, 2329, 1697, 1522, 1619, 1633, 1534 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.27 (1H, s, –NH), 8.76 (1H, s, C_5–H pyridine ring), 6.89–8.12 (11H, m, Ar–H), 6.45 (1H, s, S–CH–N group), 5.35 (1H, s, Ar–OH), 3.96 (2H, s, $–CH_2$ group), 3.84 (3H, s, Ar–O–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 154.8 (C, C-2 benzimidazole), 148.0 (C, C–OCH_3), 147.3 (C, C–NO_2), 113.9 (C, –C≡N), 91.6 (C, C–C≡N), 71.1 (C, –S–CH–N), 56.2 (C, $–OCH_3$), 33.5 (C, $–CH_2$); LCMS: *m/z* = 564.12 (M^+); Anal. Calcd. for $C_{29}H_{20}N_6O_5S$: C, 61.69; H, 3.57; N, 14.89. Found: C, 61.72; H, 3.64; N, 14.33.

*6-(1H-benzo[d]imidazol-2-yl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)-4-(p-tolyl)-nico-tinonitrile (**5j**)*

White crystals (EtOH); m.p.: 318 °C; IR (KBr) υ_{max} 3440, 3030, 2952, 2930, 2337, 1698, 1529, 1616, 1639, 1535 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.73 (1H, s, C_5–H pyridine ring), 7.22–8.12 (12H, m, Ar–H), 6.44 (1H, s, S–CH–N group), 3.95 (2H, d, $–CH_2$ group), 2.34 (3H, s, Ar–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 154.7 (C, C-2 benzimidazole), 147.8 (C, C–NO_2), 132.3 (C, C–CH_3), 113.8 (C, –C≡N), 91.5 (C, C–C≡N), 71.2 (C, –S–CH–N), 33.6 (C, $–CH_2$), 21.4 (C, $–CH_3$); LCMS: *m/z* = 532.13 (M^+); Anal. Calcd. for $C_{29}H_{20}N_6O_3S$: C, 65.40; H, 3.79; N, 15.78. Found: C, 65.48; H, 3.73; N, 15.85.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-methoxyphenyl)-2-(2-(3-nitrophenyl)-4-oxothiazolidin-3-yl)nicotinonitrile (**5k**)*

Yellowish crystals (EtOH); crystals (EtOH); m.p.: 348 °C; IR (KBr) υ_{max} 3441, 3040, 2927, 2843, 2331, 1705, 1533, 1627, 1634, 1531 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): = 10.25 (1H, s, –NH), 8.75 (1H, s, C_5–H pyridine ring), 7.05–8.12 (12H, m, Ar–H), 6.45 (1H, s, S–CH–N group), 3.95 (2H, s, $–CH_2$ group), 3.84 (3H, s, Ar–O–CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 161.1 (C, C–OCH_3), 153.8 (C, C-2 benzimidazole), 147.8 (C, C–NO_2), 113.7 (C, –C≡N), 91.6 (C, C–C≡N), 71.2 (C, –S–CH–N), 55.8 (C, $–OCH_3$), 33.4 (C, $–CH_2$); LCMS: *m/z* = 548.13 (M^+); Anal. Calcd. for $C_{29}H_{20}N_6O_4S$: C, 63.49; H, 3.67; N, 15.32. Found: C, 63.56; H, 3.73; N, 15.45.

*6-(1H-benzo[d]imidazol-2-yl)-4-(4-dimethylaminophenyl)-2-(2-(3-nitrophenyl)-4-oxo-thiazolidin-3-yl) nicotinonitrile (**5l**)*

White crystals (MeOH);m.p.: 271 °C; IR (KBr) υ_{max} 3441, 3033, 2937, 2333, 1700, 1523, 1629, 1630, 1534 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): 10.26 (1H, s, –NH), 8.73 (1H, s, C_5–H pyridine ring), 6.82–8.12 (12H, m, Ar–H), 6.43 (1H, s, S–CH–N group), 3.94 (2H, s, $–CH_2$ group),

3.06 (6H, s, Ar–N<); ^{13}C NMR (DMSO-d_6, 100 MHz): = 171.3 (C, –C=O), 155.9 (C, C–N(CH_3)$_2$), 154.8 (C, C-2 benzimidazole), 147.8 (C, C–NO_2), 113.7 (C, –C≡N), 91.6 (C, C–C≡N), 71.2 (C, –S–CH–N), 41.2 (C, >NCH_3), 33.4 (C, –CH_2); LCMS: *m/z* = 561.16 (M^+); Anal. Calcd. for $C_{30}H_{23}N_7O_3S$: C, 64.16; H, 4.13; N, 17.46. Found: C, 64.22; H, 4.43; N, 17.49.

Biological assay

Antibacterial assay

Antibacterial studies of newly synthesized compounds **5a–l** were carried out against the representative panel of Gram-positive (*S. aureus* (MTCC-96), *S. pyogenes* (MTCC-442)) and Gram-negative (*E. coli* (MTCC-443), *P. aeruginosa* (MTCC-1688)) bacteria. All MTCC cultures were collected from Institute of Microbial Technology, Chandigarh. Primary screening was done first for antibacterial activity in six sets at different concentrations of 1,000, 500, and 250 μg/mL. The compounds found to be active in primary screening were similarly diluted to obtain 200, 125, 100, 62.5, 50, 25, and 12.5 μg/mL concentrations for secondary screening to test in a second set of dilution against all microorganisms. Inoculum size for test strain was adjusted to 10^6 CFU/mL (Colony Forming Unit per milliliter) by comparing the turbidity (turbidimetric method). Mueller–Hinton Broth was used as a nutrient medium to grow and dilute the compound suspension for test organisms. 2 % DMSO was used as a diluent/vehicle to obtain the desired concentration of synthesized compounds and standard drugs to test upon standard microbial strains. Synthesized compounds were diluted to 1000 μg/mL concentration, as stock solution. The control tube containing no antibiotic was immediately subcultured [before inoculation] by spreading a loopful evenly over quarter of a plate of medium suitable for the growth of test organisms. The culture tubes were then incubated for 24 h at 37 °C, and the growth was monitored visually and spectrophotometrically. 10 μg/mL suspensions were further inoculated on an appropriate media, and growth was noted after 24 and 48 h. The lowest concentration (the highest dilution) required to arrest the growth of bacteria was regarded as minimum inhibitory concentration (MIC) i.e., the amount of growth from the control tube before incubation (which represents the original inoculum) was compared. DMSO and sterilized distilled water were used as negative control, while chloramphenicol antibiotic (1 U strength) was used as positive control. Standard drug used in the present study was "chloramphenicol" for evaluating antibacterial activity which showed 50 μg/mL MIC against *E. coli, P. aeruginosa, S. aureus*, and *S. pyogenes*.

Antifungal assay

The newly prepared compounds **5a–l** were screened for their antifungal activity as primary screening in six sets against *C. albicans*, *A. niger*, and *A. clavatus* at various concentrations of 1,000, 500, and 250 μg/mL. The primary active compounds were similarly diluted to obtain 200, 125, 100, 62.5, 50, 25, and 12.5 μg/mL concentrations for secondary screening to test in a second set of dilution against all fungi. The fungal activity of each compound was compared with ketoconazole as a standard drug, which showed 50 μg/mL MIC against *C. albicans*, *A. niger*, and *A. clavatus*. For fungal growth, in the present protocol, we have used Sabourauds dextrose broth at 28 °C in aerobic condition for 48 h. DMSO and sterilized distilled water were used as negative control, while ketoconazole (1 U strength) was used as positive control.

Preliminary cytotoxic activities (MTT assay)

In vitro cytotoxicity activity was measured by means of the IC_{50} using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay method. The IC_{50} determination was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations. All compounds were dissolved in 0.1 % DMSO with the stock concentration of 10 g/L and diluted with medium freshly before drug administration. Cell lines were seeded into 96-well plates at density of 8,000 cells/well. After 24 h seeding, each compound dilution was added in duplicate, and incubation continued at 37 °C in a humidified atmosphere containing 10 % FBS, 1 % glutamine, and 50 μM/mL gentamicin sulfate in a 5 % CO_2 and 95 % air. After 24 h, 20 μL MTT reagent at 5 mg/mL in PBS (filter sterilized, light protected, and stored at 4 °C) per well was added , and after 4 h of incubation at 37 °C, MTT is converted to a blue formazan product by mitochondrial succinate dehydrogenase. This product was eluted from cells by addition of 150 mL of DMSO. The absorbance at 570 nm was determined by an ELISA using a ELX800 micro plate spectrophotometer. The IC_{50} value was defined as the concentration at which 50 % of the cells could survive.

Structure activity relationship

The results of the antimicrobial screening were demonstrated following assumptions about the structural activity relationship (SAR): substitution pattern of the three biolabile benzimidazole, pyridine, and 4-thiazolidinone derivatives was carefully selected to bestow different electronic environments on the molecules. Antimicrobial activity was considerably affected by substitution pattern on phenyl ring

and the most active compounds contained electron donating substituents. The highest antibacterial activity was observed when electron donating substituents were present at *ortho* or *para* positions (**5c**, **5e**, **5k**) of phenyl ring. In addition, the highest antifungal activity was observed when electron donating substituents were placed at *meta* position of phenyl ring. In general, *ortho*- and *para*-substituted derivatives showed higher antibacterial activity than *meta* substituted, while *meta*-substituted derivatives exhibited higher antifungal potency than *ortho*- and *para*-substituted compounds. Compounds **5c** and **5e** substituted with hydroxyl group at *ortho* and *para* positions, respectively, showed higher antibacterial activity than the presence of the same functional group at *meta* position. In case of antifungal activity, compound **5d** proved to be much more potent than compounds **5c** and **5e**. Moreover, on the basis of activity results, it was observed that introduction of 4-thiazolidinone was also responsible for enhancing antimicrobial potency, which was confirmed by comparison of antimicrobial activity of intermediates **3a–l** and **4a–l** with final compounds **5a–l**. Compounds **5c**, **5e**, **5–k** were found to be the most potent antibacterial agents, whereas compounds **5d**, **5i**, and **5k** emerged as most effective antifungal agents indicating that structural requirements are different for binding of drug to bacterial or fungal targets, respectively (Sortino *et al*., 2007).

Conclusion

Our main objective of the present study was to synthesize and screening of antimicrobial activity of some new 4-thiazolidinone derivatives of pyridyl benzimidazoles with the hope of generating new bioactive molecules that could be useful as potent antimicrobial agents. All the synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, and mass spectral analysis. The newly synthesized compounds were investigated for their in vitro antimicrobial and antifungal activity by broth dilution method. The final compounds **5a–l** showed antimicrobial efficacy considerably greater than the intermediate compounds **3a–l** and **4a–l.** Among the newly synthesized compounds **5a–l**, analogs **5c**, **5e**, **5–k** showed the highest inhibition against nearly all of the tested bacteria, while compounds **5d**, **5i**, and **5k** displayed the highest antifungal potency, superior to the reference drugs at low level of cytotoxic concentrations. In addition, compound **5i** showed the highest inhibition at MIC = 12.5 μg/mL against both *E. coli* and *A. clavatus*. As discussed from the SAR and toxicity studies, we were encouraged to make modifications in electronic diversity on the basic structure of the final compounds **5a–l** for generation of non-toxic antimicrobial agents. Moreover, the presence of different substituents causes a certain change of biological activity. It may be concluded that the presence of three different pharmacophore scaffolds enhanced the biological activity. We have achieved our goal of the present study by exploring the antimicrobial activity of some new structural motifs of benzimidazole, cyanopyridine, and 4-thiazolidinone with anticipation of generating new leads with potent activity against various gram-positive, gram-negative bacterial, and fungal strains.

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