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Original article

Synthesis, antibacterial and antitubercular activities of benzimidazole bearing substituted 2-pyridone motifs

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ABSTRACT

A series of benzimidazole bearing 2-pyridones **5a**–**r** were synthesized and evaluated for their *in vitro* antibacterial and antitubercular activity. Further, all compounds were examined for their cytotoxic study on VERO cell line and characterized by well-known spectral techniques. It was observed that the compounds **5h**, **5i**, **5k**, **5q** and **5r** were found to possess significant broad spectrum antibacterial activity (12.5–100 μg/mL of MIC), while compounds **5g**-**5i**, **5k** and **5l** proved to be the most potent antitubercular activity in range of 2.76–20.4 μM of MIC at low level of cytotoxicity, indicating good selectivity. From SAR studies, lipophilic profile of compounds was remarkably vital for antibacterial activity, while MIC values of antitubercular activity could not be directly correlated with lipophilicity.

1. Introduction

The current first-line tuberculosis drug treatment is more than 40 years old and consists primarily of rifampicin and isoniazid. These antibiotics are drug-susceptible and require longer time and large number of doses, which are multi-drug resistant (MDR) and extensively drug resistant (XDR) to tuberculosis strains [1–3]. However, the rapid increase of multi-drug-resistant tuberculosis (MDR-TB) (resistant to at least isoniazid and rifampicin) and extensively drug-resistant tuberculosis (XDR-TB) (resistant to isoniazid, rifampicin in addition to fluoroquinolone, kanamycin, amikacin or capreomycin among second line anti-TB drugs) has led to an urgent need for the identification of new drug targets and the growth of novel anti-TB drugs. Furthermore, the most commonly encountered antibiotic-resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), has a major impact on infections in both hospitals and community settings [4,5]. Unfortunately, as antibiotic resistant organisms have become more commonplace, the pipeline for the discovery of new antimicrobial agents has decreased [6]. Thus, there is a pressing need for new antimicrobial agents that are capable of treating resistant bacterial strains.

Moreover, dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate that is essential for DNA synthesis. Inhibition of its activity leads to arrest of DNA synthesis and hence cell death [7]. *Mycobacterium tuberculosis* DHFR is an attractive novel drug target for developing anti-tuberculosis drugs. To overcome this problem, we have synthesized a series of benzimidazole bearing 2-pyridones by replacing the pyrazole motif in our previously synthesized compounds **NCD$_{1-20}$** [8] and screened them for their antibacterial property. In this attempt, we got excellent antibacterial results. Antibacterial studies impelled us to inspect **5a**–**r** for their *in vitro* antitubercular activity as well. Structural relevance of title compounds **5a**–**r** with previously synthesized compounds is shown in Fig. 1.

Benzimidazole nucleus is the key building block for numerous compounds that play beneficial roles in the functioning of biologically important molecules [9] and are remarkably effective both with respect to their inhibitory activity and favorable selectivity ratio [10–12]. Benzimidazoles are considered a promising class of bioactive heterocyclic compounds encompassing a diverse range of biological activities such as antiulcer [13], antihelminthic [14], antihypertensive [15], anticoagulant [16], anti-inflammatory [17], antimicrobial [18–20] and antiparasitic [21]. The azole group of heterocyclic compounds possesses significant pharmacokinetic profile and lipophilicity that influence the ability of drug to reach the target by transmembrane diffusion and along with promising

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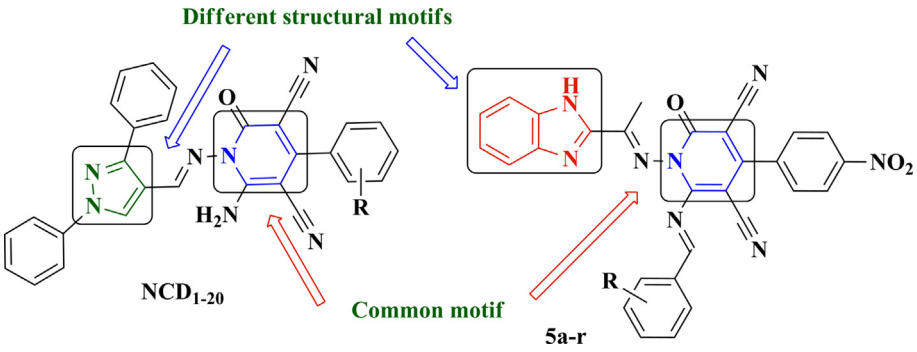


Fig. 1. Structural relevance of title compounds **5a**–**r** with previously synthesized compounds **NCD$_{1-20}$**.

activity against resistant TB by inhibiting the biosynthesis of lipids [22,23]. Further, 2-pyridones represent a unique class of pharmacophores, which are observed in various therapeutic agents [24]. In recent years, 2-pyridones have assimilated much importance as they exhibit several biological activities such as antitumoral [25], antimalarial [26], analgesic [27] and anti-HIV [28] properties. Moreover, 2-pyridones are a class of recently discovered potent antibacterial agents that are of particular interest due to their *in vitro* and *in vivo* antibacterial potencies against the bacterial type II DNA topoisomerases, which include two highly homologous enzymes-DNA gyrase and topoisomerase IV [29,30]. Moreover, among the pharmacokinetic properties, a low and highly variable bioavailability is indeed the main reason for stopping further development of the drug [31].

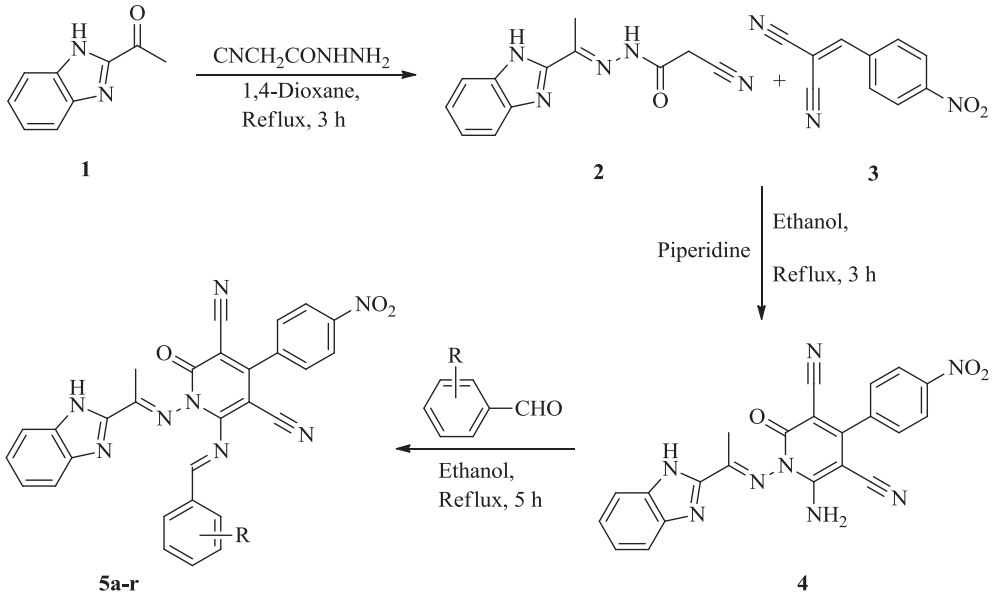
Motivated by the above findings and from our previous work [32,33], the main aim of the work is to obtain more active antibacterial and antitubercular agents with plausible novel mechanisms of action. It was thought worthwhile to synthesize some new benzimidazole bearing 2-pyridone derivatives comprising of the above aforementioned moieties in single molecular framework in order to investigate their *in vitro* antibacterial and antitubercular activity. In continuation to this, in our present communication, we have synthesized benzimidazole bearing 2-pyridones **5a**–**r** and evaluated them for their *in vitro* antibacterial and antitubercular activity. In addition, cytotoxicity studies were also conducted in VERO cell lines to evaluate the ability of these compounds to inhibit the cell growth. Most active compounds **5h**, **5q** and **5r** were also screened against MRSA strain.

2. Results and discussion

2.1. Chemistry

We have synthesized new analogues in which 2-pyridone motif is connected to the benzimidazole system. The synthetic strategies adopted for the synthesis of target benzimidazole bearing 2-pyridone derivatives **5a**–**r** are depicted in Schemes 1 and 2. In Scheme 1, condensation of 1-(1*H*-benzo[*d*]imidazol-2-yl)ethanone **1** with equimolar quantity of cyanoacetic acid hydrazide in refluxing 1,4-dioxane afforded a single product, that was identified as *N*′-(1-(1*H*-benzo[*d*]imidazol-2-yl)ethylidene)-2-cyanoacetohydrazide **2**. The elemental analysis and spectral data were in accordance with the proposed *N*′-(1-(1*H*-benzo[*d*]imidazol-2-yl)ethylidene)-2-cyanoacetohydrazide **2** structure. The IR spectrum of **2** showed strong absorption bands at 2248 and 1681 cm^{-1} due to cyanide and carbonyl group, respectively. Its ^{1}H NMR spectrum apart from the expected aromatic signals, showed two new singlets at δ 3.38 and 8.92 ppm due to the presence of reactive methylene protons and proton of secondary amine attached with carbonyl group respectively. The ^{13}C NMR spectrum displayed nine carbon signals, the most important signals appeared at δ 13.4, 27.3, 125.8, 171.3 ppm characteristic of methyl, methylene, cyanide and carbonyl carbons, respectively. The mass spectrum revealed a molecular ion peak at m/z = 241.11 (M^{+1}), in agreement with its proposed structure. The presence of the reactive methylene group in hydrazide **2** makes it a versatile precursor for the Michael type condensation with Knoevenagel product *p*-nitrobenzaldehyde and malononitrile compound **3** in presence of catalytic amount of piperidine. Utilizing Ethanol (95%) was used as a solvent to furnish 2-pyridone derivative identified as 1-((1-(1*H*-benzo[*d*]imidazol-2-yl)ethylidene)amino)-6-amino-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile **4**. Structure of the latter product was confirmed by IR spectra which showed characteristic absorption bands at 1688 and 3446 cm^{-1} for conjugated > C=O and primary amine group respectively. Their ^{1}H NMR spectra displayed a broad singlet at δ 8.78 ppm for primary amine protons, besides the disappearance of reactive methylene group and secondary amine singlets due to its involvement in cyclization. Condensation of 2-pyridone derivative **4** with appropriate aromatic aldehydes in boiling ethanol afforded the respective targeted benzimidazole bearing 2-pyridones acknowledged as 1-((1-(1*H*-benzo[*d*]imidazol-2-yl)ethylidene)amino)-6-((arylbenzyli-dene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitriles **5a**–**r**. The structures of the final analogues **5a**–**r** were established by IR spectra which showed disappearance of $-NH_2$ band in compound **4**. A strong absorption band between 1679 and 1688 cm^{-1} was assigned to conjugated > C=O group. Their ^{1}H NMR spectra revealed a singlet of methine proton at δ 9.40–9.57 ppm involved in azomethine formation, along with the vanishing of primary amine singlet. The ^{13}C NMR spectrum of compound **5l** displayed, besides the expected methyl and aromatic signals, three characteristic signals at δ 115.9, 160.1 and 163.8 ppm due to the carbons of CN, C=O and CH=N respectively. The mass spectrum of **5l** showed molecular ion peak at m/z = 540.17 (M^{+1}), in agreement with its proposed structure. Similarly, the spectral values for all the compounds and C, H, N analysis are presented in the experimental part.

A plausible mechanistic pathway for the formation of compounds **5a**–**r** is suggested in Scheme 2. Firstly, hydrazone (***A***) underwent Michael addition with Knoevenagel product (***B***) and furnished the intermediate (***C***), which further experienced intramolecular nucleophilic attack on cyanide carbon followed by annulation to yield intermediate (***D***). The intermediate (***D***) transformed to compound (***E***) by intramolecular electron transfer to nitrogen atom. In the last step, intermediate (***E***) was transformed to targeted compounds by intermolecular nucleophilic attack on carbonyl carbon of different aromatic aldehydes.



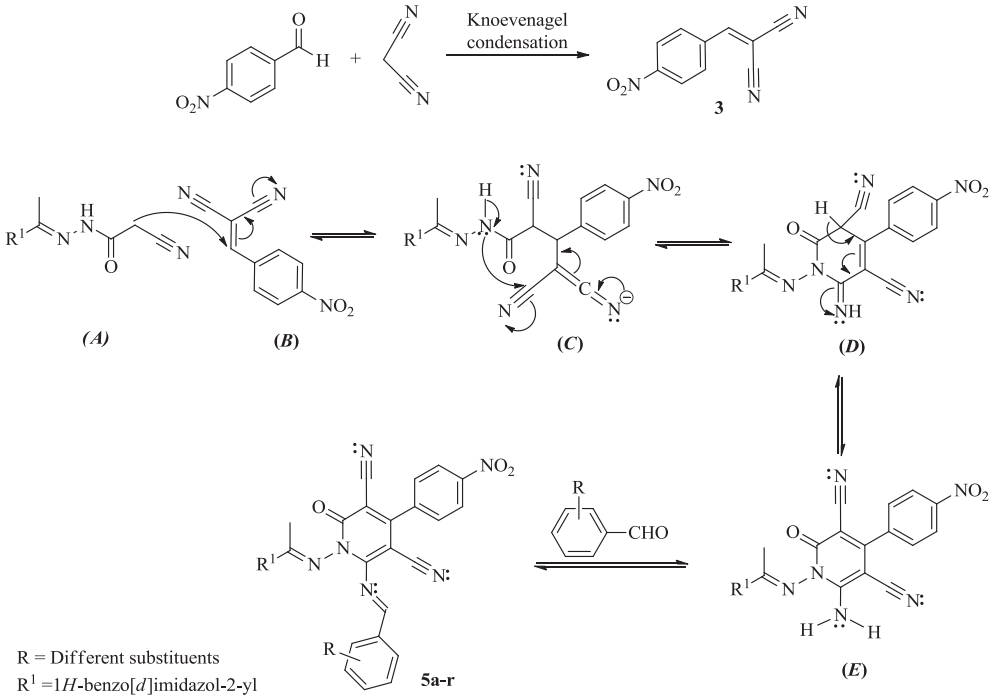
	-R		-R		-R
5a	-H	**5g**	-2-Cl	**5m**	-3-OCH_3
5b	-2-OH	**5h**	-3-Cl	**5n**	-4-OCH_3
5c	-3-OH	**5i**	-4-Cl	**5o**	-3-NO_2
5d	-4-OH	**5j**	-2-CH_3	**5p**	-4-NO_2
5e	-3-F	**5k**	-3-CH_3	**5q**	-3-Br
5f	-4-F	**5l**	-4-CH_3	**5r**	-4-Br

Scheme 1. Synthetic route for the preparation of title compounds **5a**–**r**.

2.2. Biological evaluation

2.2.1. Antibacterial activity

The intermediates **2**, **4** and target compounds **5a**–**r** were investigated for their *in vitro* antibacterial activity against two gram-positive and two gram-negative bacteria and yeast like pathogenic fungus *Candida albicans* using conventional broth-dilution method [34,35]. Minimum inhibitory concentration (MIC) is defined as concentration of the compound required to obtain complete inhibition of bacterial growth. MICs of the synthesized



Scheme 2. Plausible mechanistic pathway for the synthesis of compounds **5a**–**r**.

compounds were compared with ciprofloxacin and chloramphenicol and the results are depicted in Table 1. The antibacterial activity assessed (Table 1) for analogues **2**, **4** and **5a**–**r** against several strains flaunted good activity. From the bioassay, it was observed that the final analogues **5a**–**r** with substitutions of phenyl ring were most active against all the pathogenic strains studied than the intermediate hydrazone **2** and 2-pyridone derivative **4**. Intermediate **2** showed very poor antibacterial activity against all tested bacterial strains and it was observed that compound **2** with low lipophilicity (Clog P = 0.7220) displayed very low activity as compared to intermediate **4** and final analogues **5a**–**r**. Precursor **2** reacted with Knoevenagel compound **3** to generate intermediate **4** which was tested against different bacterial strains, and it showed more inhibitory efficacy than compound **2** due to high lipophilicity (Clog P = 1.4772) and less activity compared to all final analogues. Therefore, it was observed that formation of 2-pyridone nucleus was responsible for enhancing antibacterial potency. Now, intermediate **4** was treated with different aromatic aldehydes to afford targeted compounds **5a**–**r** which were found to have broad spectrum antimicrobial efficacy. This could be correlated to structural variations and different aromatic substitutions in phenyl ring.

Good bioavailability can be achieved by an appropriate balance between solubility and partitioning properties. Membrane permeability and bioavailability is always associated with some basic molecular descriptor such as Clog P (lipophilicity). Thus, prediction of bioavailability-related properties, such as lipophilicity is important before actual synthesis, in order to reduce enormous wastage of expensive chemicals and precious time. The computed Clog P values (P is the partition coefficient of the molecule in the water/octanol system) are shown in Tables 1 and 3. The ChemBioDraw Ultra, version 12.0, software by Cambridge Soft is a program used to predict lipophilicity of compounds.

From the bioassay, final analogues with electron withdrawing halogen substitution were found to be more active than the remaining final analogues against all the bacterial strains. Among all the final active analogues, compound **5q** (3-Br, Clog P = 3.9272) endowed with bromine exerted highest inhibition against all the bacterial strains and showed highest efficacy against *S. aureus* at 12.5 μg/mL of MIC as compared to standards ciprofloxacin (50 μg/mL, Clog P = −0.7252) and chloramphenicol (50 μg/mL, Clog P = 1.293). In addition, compound **5q** (Clog P = 3.9272) with higher lipophilicity flaunted potential inhibitory action against *Escherichia coli* and *Streptococcus pyogenes* at MIC of 25 μg/mL. Moreover, the above mentioned derivative **5q** displayed excellent inhibition against *Pseudomonas aeruginosa* at 25 μg/mL of MIC. Furthermore, among the second line active compounds, **5h** (3-Cl, Clog P = 3.7772) and **5r** (4-Br, Clog P = 3.9272) indicated MIC of 50 μg/mL, in which more lipophilic compound **5r** showed better results against both *E. coli* and *S. pyogenes*. In addition, the above mentioned derivatives **5h** and **5r** were also found to demonstrate excellent efficacy against *S. aureus* at 25 μg/mL of MIC and analogue **5r** also demonstrated MIC of 50 μg/mL against *P. aeruginosa.* Derivatives **5e** (3-F, Clog P = 3.2072) with fluoro, **5g** (2-Cl, Clog P = 3.7772) with chloro and **5k** (3-CH_3, Clog P = 3.5632) with methyl group to the aromatic nucleus bequeathed appreciable potency towards *E. coli* at 100 μg/mL of MIC, in which compound **5g** showing highest lipophilicity gave better results. Compound **5h** (3-Cl, Clog P = 3.7772) with electron withdrawing chlorine substituent indicated diminished activity at 62.5 μg/mL of MIC against *P. aeruginosa*. Another analogue **5i** (4-Cl, Clog P = 3.7772) with chlorine and **5k** (3-CH_3, Clog P = 3.5632) endowed with electron donating methyl group exerted MIC of 100 μg/mL against *P. aeruginosa* as compared to standard ciprofloxacin (25 μg/mL, Clog P = −0.7252) and chloramphenicol (50 μg/mL, Clog P = 1.293) antibiotics. Derivative **5g** (2-Cl, Clog P = 3.7772) displayed more potency against *S. aureus* than *E. coli* at MIC of 50 μg/mL and diminished activity at MIC of 62.5 μg/mL against *S. pyogenes*. Final derivatives **5c** (3-OH, Clog P = 3.2982), **5e** (3-F, Clog P = 3.2072), **5k** (3-CH_3, Clog P = 3.5632) and **5m** (3-OCH_3, Clog P = 3.4082) displayed remarkable inhibitory effects at MIC of 100 μg/mL against *S. aureus*. Among them compound **5k** contemplated same action against *S. pyogenes*. In addition, compound **5i** (4-Cl, Clog P = 3.7772) exhibited activity at MIC of 50 and 62.5 μg/mL against *S. pyogenes* and *S. aureus* respectively. Moreover, compound **5e** possessing fluorine atom at 3rd position of phenyl ring increased the potency and showed inhibition at 62.5 μg/mL of MIC against *S. pyogenes.* Furthermore, the inhibitory activity of all the synthesized compounds against *C. albicans* was rather lower than their antibacterial activity, only compound **5q** (3-Br, MIC = 250 μg/mL) exhibited moderate activity as compared to standard ketoconazole.

Furthermore, the most active compounds **5h**, **5q** and **5r** against *S. aureus* and *S. pyogenes* (MIC = 12.5–50 μg/mL) were also tested against methicillin-resistant *S. aureus* (MRSA isolate ATCC 43300) and results are given in Table 2. Compound **5r** exhibited more potent activity than the standard drugs against methicillin-resistant *S. aureus*. Compound **5r**, with MIC value of 6.25 μg/mL against MRSA showed four-fold more potency than ciprofloxacin (MIC = 25 μg/mL) and eight-fold more activity than chloramphenicol (MIC = 50 μg/mL). In addition, compound **5q** endowed

Table 1
Results of *in vitro* antibacterial screening of tested compounds.

Entry	-R	Clog P[a]	Minimum inhibitory concentration (MIC) μg/mL			
			Gram-negative[b]		Gram-positive[c]	
			Ec	Pa	Sa	Sp
2	–	0.7220	>1000	>1000	1000	1000
4	–	1.4772	500	500	500	500
5a	-H	3.0642	500	500	500	500
5b	-2-OH	3.2982	500	500	500	250
5c	-3-OH	3.2982	500	250	100	500
5d	-4-OH	3.2982	250	500	500	250
5e	-3-F	3.2072	100	250	100	125
5f	-4-F	3.2072	250	250	250	100
5g	-2-Cl	3.7772	100	125	**50**	62.5
5h	-3-Cl	3.7772	**50**	62.5	**25**	**50**
5i	-4-Cl	3.7772	100	100	62.5	**50**
5j	-2-CH_3	3.5632	250	250	125	100
5k	-3-CH_3	3.5632	100	100	100	100
5l	-4-CH_3	3.5632	250	250	250	125
5m	-3-OCH_3	3.4082	250	500	100	250
5n	-4-OCH_3	3.4082	500	500	250	250
5o	-3-NO_2	2.8072	250	500	250	500
5p	-4-NO_2	2.8072	1000	1000	500	500
5q	-3-Br	3.9272	**25**	**25**	**12.5**	**25**
5r	-4-Br	3.9272	**50**	**50**	**25**	**50**
Ciprofloxacin		−0.7252	25	25	50	50
Chloramphenicol		1.293	50	50	50	50

[a] Clog P calculated using the ChemBioDraw Ultra, version 12.0, software by Cambridge Soft.
[b] Ec: *Escherichia coli* (MTCC-443); Pa: *Pseudomonas aeruginosa* (MTCC-1688).
[c] Sa: *Staphylococcus aureus* (MTCC-96), Sp: *Streptococcus pyogenes* (MTCC-442).

Table 2
Inhibitory activity (MIC, μg/mL) of compounds **5h**, **5q** and **5r** against methicillin-resistant *S. aureus*.

Entry	MRSA[a]
5h	>50
5q	12.5
5r	6.25
Ciprofloxacin	25
Chloramphenicol	50

[a] Methicillin-resistant *S. aureus* (ATCC 43300).

Table 3
Results of antitubercular and cytotoxic activities of the tested compounds.

Entry	-R	Clog *P*[a]	MIC (μg/mL)[b]	IC_{50} (μg/mL)[c]
2	–	0.7220	>39.42	n.d.
4	–	1.4772	>39.42	n.d.
5a	-H	3.0642	35.23	17.61
5b	-2-OH	3.2982	31.62	19.83
5c	-3-OH	3.2982	30.18	17.00
5d	-4-OH	3.2982	30.85	13.73
5e	-3-F	3.2072	25.37	23.65
5f	-4-F	3.2072	27.10	24.76
5g	-2-Cl	3.7772	**2.65**	>39.42
5h	-3-Cl	3.7772	**0.85**	>39.42
5i	-4-Cl	3.7772	**1.79**	>39.42
5j	-2-CH_3	3.5632	9.17	34.55
5k	-3-CH_3	3.5632	**2.07**	>39.42
5l	-4-CH_3	3.5632	**6.28**	>39.42
5m	-3-OCH_3	3.4082	17.98	24.22
5n	-4-OCH_3	3.4082	23.46	25.39
5o	-3-NO_2	2.8072	28.33	27.63
5p	-4-NO_2	2.8072	32.21	16.05
5q	-3-Br	3.9272	10.28	>39.42
5r	-4-Br	3.9272	13.79	>39.42
Isoniazid		−0.668	0.24	–

n.d. not determined.
[a] *C*log *P* calculated using the ChemBioDraw Ultra, version 12.0, software by Cambridge Soft.
[b] Minimum inhibitory concentration against $H_{37}Rv$ strain of *M. Tuberculosis* (μg/mL).
[c] Measurement of cytotoxic activity in VERO cell lines: 50% inhibitory concentrations (μg/mL).

with bromo group showed two-fold more inhibition at MIC value of 12.5 μg/mL than ciprofloxacin and four-fold higher potency than chloramphenicol against MRSA.

2.2.2. Antitubercular activity

The encouraging results from the antibacterial studies impelled us to go for the screening of title compounds for their *in vitro* antitubercular activity. Intermediates (**2** and **4**) and all the final compounds along with the standard drug for comparison were firstly evaluated for their activity against the *M. tuberculosis* $H_{37}Rv$ strain in Middlebrook 7H12 medium using Microplate Alamar Blue Assay (MABA) MIC method [36]. The drug in clinical use, isoniazid was used as a reference drug. The results of actual MICs of tested compounds were reported in Table 3. It was observed that final analogues **5a**–**r** displayed superior anti-tubercular activity compared to both the intermediates (**2** and **4**). Among the eighteen synthesized compounds, compounds **5g**–**5i**, **5k** and **5l** endowed with inductively electron withdrawing chlorine and electron donating methyl groups afforded maximum MICs ranging from 0.85 to 6.28 μg/mL against *M. tuberculosis*. For final derivatives, introduction of chlorine substituent in **5g**–**5i** was tolerated, 3-chloro yielding the best activity (3-Cl > 4-Cl > 2-Cl). Furthermore, among the second line active compounds, **5k** and **5l** brandished MICs in range of 2.07–6.28 μg/mL, in which compound **5k** having methyl in *meta* position showed better result at MIC of 2.07 μg/mL whereas compound **5l** displayed inhibition at MIC of 6.28 μg/mL. Compound **5h** (3-Cl) possessed highest inhibition at MIC of 0.85 μg/mL amongst all the tested derivatives as compared to standard isoniazid, while their *ortho* and *para* derivatives **5i** (4-Cl) and **5g** (2-Cl) showed MICs of 1.79 and 2.65 μg/mL respectively. Here, we have discussed and compared antitubercular activity based on first line drug Isoniazid (0.24 μg/mL, *C*log $P = -0.668$). From the antitubercular activity results, it may be concluded that final derivatives **5a**–**o** were mixedly active and no specific relationship was observed in case of *C*log *P* or lipophilicity verses antitubercular activity profiles.

2.2.3. Cytotoxicity study

After having identified numerous active antibacterial and antimycobacterial benzimidazole bearing 2-pyridones, the next step was to examine the toxicity of the drug contenders. *In vitro* cytotoxicity of compounds **5a**–**r** was determined against mammalian VERO cell lines [37]. After 72 h of exposure, viability was considered on the basis of cellular conversion of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and phenylmethasulfazone] (100:20) into formazan product using Promega Cell Titer 96 non-radioactive cell proliferation assay. The cytotoxicity results presented in Table 3 are expressed as the concentration inhibiting 50% of the cell growth IC_{50}. The compounds exhibited moderate to low level of cytotoxicity with the IC_{50} values in the range of 13.73 to >39.42 μg/mL. It was observed that none of the tested compounds exhibited any significant cytotoxic effects on VERO cells, suggesting a great potential for their *in vivo* use as antibacterial and anti-tubercular agents. As for activity against VERO cell lines, the highest cytotoxic activity was flaunted by compound **5d** which showed percentage viability IC_{50} at 13.73 μg/mL, whereas, the second line cytotoxic activity was displayed by compounds **5a**, **5c** and **5p** which showed the percentage viability IC_{50} at 17.61, 17.00 and 16.05 μg/mL respectively. Moreover, final analogues **5g**–**5i**, **5k**, **5l**, **5q** and **5r** showed no toxicity for the percentage viability IC_{50} > 39.42 μg/mL.

2.3. Structure–activity relationship

The results of antibacterial and antitubercular screening demonstrated the following assumptions about the structural activity relationship (SAR): in the present study, we investigated the effects of the substitution pattern of the hybrid benzimidazole and 2-pyridone derivatives were carefully selected to impart different electronic environment on the molecules. By comparing the antibacterial activity of the synthesized compounds, it was found that the tested compounds were more effective against Gram-positive bacteria. In addition, the antibacterial activity was considerably affected by substitution pattern on the phenyl ring and lipophilic profile of the compounds. It is believed that strong lipophilic character of the molecule plays an essential role in producing antibacterial effect. These properties are seen as an important parameter related to membrane permeation in biological system. Many processes of drug disposition depend on the capability to cross membranes and hence there is a high correlation with measures of lipophilicity. Lipophilicity plays a major role in determining where drugs are distributed within the body after adsorption and as a consequence how rapidly they are metabolized and excreted. In this context, the presence of lipophilic moiety would be important for such activity. In case of antibacterial activity, some analogues of this series were found to have even more potency than the standard drug 'chloramphenicol' while some of them exhibited comparable potency. Compounds **5h**, **5i**, **5k**, **5q** and **5r** bearing Cl, Br, CH_3 groups were found to be most potent antibacterial agents due to its high lipophilicity. Among them, compound **5q** endowed with inductively electron withdrawing bromine at *meta* position emerged as the most effective antibacterial agent at MIC of 12.5 μg/mL. The MIC values of these novel compounds confirmed that the presence of bromine, chlorine and methyl substituent at *meta* position gave rise to better pharmacological potency rather than the same substituent present at *ortho* or *para* positions. Whereas, the opposite trend was observed for antitubercular activity results and there was no specific relationship observed between MIC profiles versus lipophilicity. Among the eighteen synthesized compounds, **5g**–**5i**, **5k** and **5l** endowed with inductively electron withdrawing chlorine and electron donating methyl group illustrated maximum MICs in the range of 0.85–6.28 μg/mL against *M.*

tuberculosis. Out of them, 3-chloro yielded the best activity at MIC of 0.85 μg/mL as compared to first line drug isoniazid (0.24 μg/mL).

3. Conclusion

The focal point of the current work was the development of new bioactive scaffolds based on benzimidazole clubbed 2-pyridone compact system with the hope of generating new bioactive chemical entities that could be useful as potent antibacterial and antitubercular agents. Many of the synthesized motifs (**5h**, **5i**, **5k**, **5q** and **5r**), possessing atom/group such as bromo, chloro and methyl at *meta* or *para* positions were identified as the most compelling antibacterial agents. Albeit, it was observed that the strong lipophilic character of the molecule plays an imperative role in producing antibacterial effect. Compounds **5g**–**5i**, **5k** and **5l** came out as the most promising antitubercular agents. In addition, the relationship between activity profiles and lipophilicity of the newer analogues was also discussed in which higher lipophilic compounds showed higher bioacitivity, while, in case of antitubercular activity, there was no specific relationship observed between MIC profiles versus lipophilicity. Moreover, the potent antibacterial and antitubercular activity of most active compounds **5g**–**5i**, **5k**, **5l**, **5q** and **5r** were accompanied with relatively low level of cytotoxicity. Consequently, the degree of activity and the encouraging physicochemical parameters displayed by a novel innovative structural combination system of benzimidazole and 2-oxopyridine rings make these compounds a privileged structure to achieve more active derivatives in ongoing studies. Synthesized compounds were screened against *C. albicans*, but it was our observation that none of the compounds exhibited significant activity against this fungi.

4. Experimental

4.1. General methods

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 mesh 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 and spectra were recorded on Varian Gemini 400 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.

4.2. General procedure for the synthesis of N′-(1-(1H-benzo[d] imidazol-2-yl)ethylidene)-2-cyanoacetohydrazide (**2**)

A mixture of equimolar amount of acetyl benzimidazole compound **1** (0.01 mol) and cyanoacetic acid hydrazide (0.01 mol) in 1,4-dioxane (50 mL) was refluxed for 3 h. The reaction mixture was concentrated by evaporating to dryness under reduced pressure and then cooled down to room temperature. The separated crystals were filtered, air dried and recrystallized from absolute alcohol. Yield 70%; mp 165 °C. IR (ν_{max}, cm^{-1}, KBr): 3371 (–NH, benzimidazole), 3278 (–NH, –CONH–), 3028 (C–H, aromatic), 2917 (C–H, CH_3), 2248 (CN), 1681 (CO), 1641 (C=C), 1532 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 3.38 (s, 2H, $-CH_2CN$), 7.20 (d, 2H, J = 8.0 Hz, Ar-H), 7.61 (d, 2H, J = 8.2 Hz, Ar-H), 8.92 (s, 1H, –NHCO– D_2O exch.), 10.32 (s, 1H, –NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 171.3, 155.7, 151.6, 134.9, 125.8, 123.2, 115.1, 27.3, 13.4. LCMS (ESI): M/Z = 241.11 [M$^+$]. Anal. Calcd. for $C_{12}H_{11}N_5O$: C, 59.74; H, 4.60; N, 29.03. Found: C, 59.69; H, 4.55; N, 29.09.

4.3. General procedure for the synthesis of 1-((1-(1H-benzo[d] imidazol-2-yl)ethylidene)-amino)-6-amino-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**4**)

A mixture containing compound **2** (0.01 mol) and Knoevenagel compound 2-(4-nitrobenzylidene)malononitrile **3** (0.01 mol) in ethanol (50 mL) was refluxed for 3 h using 2 drops of piperidine as catalyst. The excess of solvent was distilled out and the mixture was then cooled down to room temperature. The crystals formed were filtered, air dried and recrystallized from aqueous DMF. Yield 71%; mp 208–210 °C. IR (ν_{max}, cm^{-1}, KBr): 3446 (NH_2), 3376 (–NH, benzimidazole), 3030 (C–H, aromatic), 2921 (C–H, CH_3), 2232 (CN), 1688 (CO), 1642 (C=C), 1536 (C=N), 1516, 1332 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.91 (s, 3H, CH_3), 7.22 (d, 2H, J = 8.0 Hz, Ar-H), 7.44 (d, 2H, J = 7.6 Hz, Ar-H), 7.62 (d, 2H, J = 8.4 Hz, Ar-H), 8.21 (d, 2H, J = 7.7 Hz, Ar-H), 8.78 (s, 2H, NH_2 D_2O exch.), 10.36 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 160.2, 159.4, 155.8, 151.7, 147.2, 138.7, 134.9, 130.2, 123.1, 123.8, 115.1, 115.5, 115.9, 76.6, 13.8. LCMS (ESI): M/Z = 438.11 [M$^+$]. Anal. Calcd. for $C_{22}H_{14}N_8O_3$: C, 60.27; H, 3.22; N, 25.56. Found: C, 60.34; H, 3.25; N, 25.51.

4.4. General procedure for the synthesis of 1-((1-(1H-benzo[d] imidazol-2-yl)ethylidene)amino)-6-((arylbenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydro-pyridine-3,5-dicarbonitriles (**5a**–**r**)

Compound **4** (0.01 mol), different substituted aromatic aldehydes (0.01 mol) and ethanol (50 mL) were taken in a round bottom flask and refluxed for 5 h. After 5 h, the reaction mass was poured onto crushed ice and separated solid was filtered, dried and recrystallized from DMSO.

4.4.1. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-(benzylideneamino)-4-(4-nitro-phenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5a**)

Yield 66%; mp 221 °C. IR (ν_{max}, cm^{-1}, KBr): 3380 (–NH, benzimidazole), 3034 (C–H, aromatic), 2992 (C–H, CH=N), 2924 (C–H, CH_3), 2228 (CN), 1682 (CO), 1641 (C=C), 1532 (C=N), 1520, 1331 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.90 (s, 3H, CH_3), 7.20 (d, 2H, J = 8.1 Hz, Ar-H), 7.41 (d, 2H, J = 7.5 Hz, Ar-H), 7.47 (d, 2H, J = 7.6 Hz, Ar-H), 7.54 (t, 1H, J = 7.4 Hz, Ar-H), 7.63 (d, 2H, J = 8.3 Hz, Ar-H), 7.84 (d, 2H, J = 7.7 Hz, Ar-H), 8.24 (d, 2H, J = 7.6 Hz, Ar-H), 9.51 (s, 1H, CH=N), 10.41 (s, 1H, –NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.8, 160.1, 155.7, 153.3, 151.6, 147.1, 138.6, 134.8, 133.8, 131.2, 130.1, 129.4, 128.9, 123.2, 123.8, 115.0, 115.5, 115.9, 114.7, 13.5. LCMS (ESI): M/Z = 526.14 [M$^+$]. Anal. Calcd. for $C_{29}H_{18}N_8O_3$: C, 66.16; H, 3.45; N, 21.28. Found: C, 66.22; H, 3.49; N, 21.21.

4.4.2. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((2-hydroxybenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5b**)

Yield 69%; mp 242 °C. IR (ν_{max}, cm^{-1}, KBr): 3441 (OH), 3382 (–NH, benzimidazole), 3031 (C–H, aromatic), 2990 (C–H, CH=N), 2924 (C–H, CH_3), 2225 (CN), 1681 (CO), 1637 (C=C), 1531 (C=N),

1522, 1330 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.92 (s, 3H, CH_3), 7.01 (d, 1H, J = 7.5 Hz, Ar-H), 7.11 (t, 1H, J = 7.6 Hz, Ar-H), 7.21 (d, 2H, J = 8.2 Hz, Ar-H), 7.44 (d, 2H, J = 7.6 Hz, Ar-H), 7.53 (t, 1H, J = 7.4 Hz, Ar-H), 7.62 (d, 2H, J = 8.4 Hz, Ar-H), 7.71 (d, 1H, J = 7.6 Hz, Ar-H), 8.21 (d, 2H, J = 7.6 Hz, Ar-H), 8.74 (s, 1H, OH D_2O exch.), 9.54 (s, 1H, CH=N), 10.42 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.6, 163.7, 160.2, 155.6, 153.1, 151.5, 147.0, 138.5, 134.8, 134.1, 133.5, 130.1, 127.9, 127.4, 123.8, 123.1, 115.9, 115.5, 115.0, 115.9, 114.8, 59.4, 13.7, 13.2. LCMS (ESI): M/Z = 542.16 [M^+]. Anal. Calcd. for $C_{29}H_{18}N_8O_4$: C, 64.20; H, 3.34; N, 20.65. Found: C, 64.28; H, 3.39; N, 20.71.

4.4.3. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-hydroxybenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5c**)

Yield 68%; mp 256 °C. IR (ν_{max}, cm^{-1}, KBr): 3440 (OH), 3384 (−NH, benzimidazole), 3034 (C−H, aromatic), 2992 (C−H, CH=N), 2927 (C−H, CH_3), 2223 (CN), 1684 (CO), 1639 (C=C), 1534 (C=N), 1524, 1334 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.90 (s, 3H, CH_3), 7.02 (d, 1H, J = 7.6 Hz, Ar-H), 7.18 (d, 2H, J = 8.0 Hz, Ar-H), 7.27 (t, 1H, J = 7.6 Hz, Ar-H), 7.39 (d, 1H, J = 7.4 Hz, Ar-H), 7.46 (d, 2H, J = 7.6 Hz, Ar-H), 7.53 (s, 1H, Ar-H), 7.63 (d, 2H, J = 8.4 Hz, Ar-H), 8.22 (d, 2H, J = 7.6 Hz, Ar-H), 8.71 (s, 1H, OH D_2O exch.), 9.55 (s, 1H, CH=N), 10.44 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.4, 163.8, 160.1, 158.6, 155.5, 153.2, 151.5, 147.1, 138.7, 135.2, 134.9, 130.5, 130.1, 123.9, 123.1, 121.8, 118.2, 115.9, 115.6, 115.0, 114.8, 114.3, 13.6. LCMS (ESI): M/Z = 542.14 [M^+]. Anal. Calcd. for $C_{29}H_{18}N_8O_4$: C, 64.20; H, 3.34; N, 20.65. Found: C, 64.29; H, 3.38; N, 20.72.

4.4.4. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-hydroxybenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5d**)

Yield 63%; mp 236 °C. IR (ν_{max}, cm^{-1}, KBr): 3444 (OH), 3386 (−NH, benzimidazole), 3036 (C−H, aromatic), 2992 (C−H, CH=N), 2928 (C−H, CH_3), 2224 (CN), 1685 (CO), 1637 (C=C), 1531 (C=N), 1522, 1332 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.89 (s, 3H, CH_3), 6.88 (d, 2H, J = 7.8 Hz, Ar-H), 7.20 (d, 2H, J = 7.9 Hz, Ar-H), 7.44 (d, 2H, J = 7.5 Hz, Ar-H), 7.62 (d, 2H, J = 8.3 Hz, Ar-H), 7.82 (d, 2H, J = 7.4 Hz, Ar-H), 8.24 (d, 2H, J = 7.5 Hz, Ar-H), 8.70 (s, 1H, OH D_2O exch.), 9.57 (s, 1H, CH=N), 10.44 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.9, 160.8, 160.0, 155.6, 153.1, 151.6, 147.2, 138.5, 134.8, 130.7, 130.1, 126.2, 123.8, 123.0, 116.1, 115.9, 115.4, 115.0, 114.8, 13.7. LCMS (ESI): M/Z = 542.15 [M^+]. Anal. Calcd. for $C_{29}H_{18}N_8O_4$: C, 64.20; H, 3.34; N, 20.65. Found: C, 64.30; H, 3.38; N, 20.73.

4.4.5. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-fluorobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5e**)

Yield 62%; mp 192 °C. IR (ν_{max}, cm^{-1}, KBr): 3388 (−NH, benzimidazole), 3038 (C−H, aromatic), 2994 (C−H, CH=N), 2930 (C−H, CH_3), 2226 (CN), 1688 (CO), 1638 (C=C), 1532 (C=N), 1524, 1334 (N=O, Ar-NO_2), 1122 (C−F). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.87 (s, 3H, CH_3), 7.21 (d, 2H, J = 8.0 Hz, Ar-H), 7.37 (d, 1H, J = 7.4 Hz, Ar-H), 7.45 (d, 2H, J = 7.4 Hz, Ar-H), 7.58 (d, 2H, J = 8.3 Hz, Ar-H), 7.65 (d, 1H, J = 7.5 Hz, Ar-H), 7.71 (t, 1H, J = 7.6 Hz, Ar-H), 7.84 (d, 1H, J = 7.4 Hz, Ar-H), 8.22 (d, 2H, J = 7.5 Hz, Ar-H), 9.54 (s, 1H, CH=N), 10.41 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.3, 163.8, 163.1, 160.1, 155.7, 153.1, 151.5, 147.1, 138.5, 135.2, 134.9, 130.5, 130.1, 124.8, 123.9, 123.2, 117.7, 115.9, 115.5, 115.0, 114.8, 114.1, 13.7. LCMS (ESI): M/Z = 544.12 [M^+]. Anal. Calcd. for $C_{29}H_{17}FN_8O_3$: C, 63.97; H, 3.15; N, 20.58. Found: C, 63.90; H, 3.19; N, 20.52.

4.4.6. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-fluorobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5f**)

Yield 64%; mp 208 °C. IR (ν_{max}, cm^{-1}, KBr): 3386 (−NH, benzimidazole), 3037 (C−H, aromatic), 2993 (C−H, CH=N), 2931 (C−H, CH_3), 2227 (CN), 1686 (CO), 1638 (C=C), 1531 (C=N), 1522, 1332 (N=O, Ar-NO_2), 1125 (C−F). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 7.22 (d, 2H, J = 8.1 Hz, Ar-H), 7.37 (d, 2H, J = 7.6 Hz, Ar-H), 7.44 (d, 2H, J = 7.5 Hz, Ar-H), 7.62 (d, 2H, J = 8.4 Hz, Ar-H), 7.83 (d, 2H, J = 7.7 Hz, Ar-H), 8.23 (d, 2H, J = 7.5 Hz, Ar-H), 9.51 (s, 1H, CH=N), 10.45 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 165.3, 163.7, 160.1, 155.7, 153.3, 151.7, 147.2, 138.7, 134.8, 130.9, 130.2, 129.2, 123.8, 123.1, 115.9, 115.6, 115.2, 114.9, 114.2, 13.5. LCMS (ESI): M/Z = 544.12 [M^+]. Anal. Calcd. for $C_{29}H_{17}FN_8O_3$: C, 63.97; H, 3.15; N, 20.58. Found: C, 63.91; H, 3.20; N, 20.51.

4.4.7. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((2-chlorobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5g**)

Yield 67%; mp 223 °C. IR (ν_{max}, cm^{-1}, KBr): 3384 (−NH, benzimidazole), 3034 (C−H, aromatic), 2991 (C−H, CH=N), 2929 (C−H, CH_3), 2226 (CN), 1684 (CO), 1634 (C=C), 1530 (C=N), 1521, 1331 (N=O, Ar-NO_2), 756 (C−Cl). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.92 (s, 3H, CH_3), 7.19 (d, 2H, J = 8.2 Hz, Ar-H), 7.38 (t, 1H, J = 7.4 Hz, Ar-H), 7.44 (d, 2H, J = 7.6 Hz, Ar-H), 7.50 (t, 1H, J = 7.5 Hz, Ar-H), 7.57 (d, 1H, J = 7.5 Hz, Ar-H), 7.68 (d, 2H, J = 8.4 Hz, Ar-H), 7.81 (d, 1H, J = 7.6 Hz, Ar-H), 8.22 (d, 2H, J = 7.5 Hz, Ar-H), 9.48 (s, 1H, CH=N), 10.42 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.4, 163.6, 160.1, 155.7, 153.3, 151.6, 147.2, 138.7, 134.8, 133.9, 133.3, 132.5, 130.6, 130.1, 127.3, 126.8, 123.8, 123.2, 115.9, 115.5, 115.0, 114.9, 13.6. LCMS (ESI): M/Z = 561.12 [M^+]. Anal. Calcd. for $C_{29}H_{17}ClN_8O_3$: C, 62.09; H, 3.05; N, 19.98. Found: C, 62.19; H, 3.09; N, 19.92.

4.4.8. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-chlorobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5h**)

Yield 64%; mp 199 °C. IR (ν_{max}, cm^{-1}, KBr): 3384 (−NH, benzimidazole), 3035 (C−H, aromatic), 2992 (C−H, CH=N), 2930 (C−H, CH_3), 2228 (CN), 1682 (CO), 1632 (C=C), 1528 (C=N), 1520, 1330 (N=O, Ar-NO_2), 758 (C−Cl). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.90 (s, 3H, CH_3), 7.21 (d, 2H, J = 8.1 Hz, Ar-H), 7.41 (d, 2H, J = 7.4 Hz, Ar-H), 7.47 (t, 1H, J = 7.4 Hz, Ar-H), 7.58 (d, 1H, J = 7.5 Hz, Ar-H), 7.69 (d, 2H, J = 8.3 Hz, Ar-H), 7.74 (d, 1H, J = 7.5 Hz, Ar-H), 7.92 (d, 1H, J = 7.6 Hz, Ar-H), 8.24 (d, 2H, J = 7.7 Hz, Ar-H), 9.44 (s, 1H, CH=N), 10.43 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.8, 160.2, 155.6, 153.4, 151.4, 147.1, 138.6, 135.1, 134.9, 134.3, 131.0, 130.6, 130.0, 127.7, 127.2, 123.9, 123.1, 115.8, 115.5, 115.0, 114.7, 13.5. LCMS (ESI): M/Z = 561.11 [M^+]. Anal. Calcd. for $C_{29}H_{17}ClN_8O_3$: C, 62.09; H, 3.05; N, 19.98. Found: C, 62.18; H, 3.10; N, 19.93.

4.4.9. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-chlorobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5i**)

Yield 67%; mp 229 °C. IR (ν_{max}, cm^{-1}, KBr): 3386 (−NH, benzimidazole), 3036 (C−H, aromatic), 2994 (C−H, CH=N), 2932 (C−H, CH_3), 2229 (CN), 1682 (CO), 1634 (C=C), 1528 (C=N), 1522, 1331 (N=O, Ar-NO_2), 760 (C−Cl). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 7.22 (d, 2H, J = 8.2 Hz, Ar-H), 7.44 (d, 2H, J = 7.5 Hz, Ar-H), 7.57 (d, 2H, J = 7.6 Hz, Ar-H), 7.68 (d, 2H, J = 8.2 Hz, Ar-H), 7.79 (d, 2H, J = 7.6 Hz, Ar-H), 8.22 (d, 2H, J = 7.7 Hz, Ar-H), 9.49 (s, 1H, CH=N), 10.44 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.6, 163.7, 160.1, 155.5, 153.2, 151.5, 147.0, 138.5, 136.7, 134.8, 131.9, 130.7, 130.0, 128.9, 123.8,

123.2, 115.9, 115.6, 115.2, 114.8, 13.7. LCMS (ESI): $M/Z = 561.11$ [M^+]. Anal. Calcd. for $C_{29}H_{17}ClN_8O_3$: C, 62.09; H, 3.05; N, 19.98. Found: C, 62.19; H, 3.09; N, 19.92.

4.4.10. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((2-methylbenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5j**)

Yield 63%; mp 244 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3381 (-NH, benzimidazole), 3032 (C–H, aromatic), 2990 (C–H, CH=N), 2928 (C–H, CH_3), 2224 (CN), 1679 (CO), 1630 (C=C), 1523 (C=N), 1520, 1328 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.91 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 7.17 (d, 2H, J = 8.1 Hz, Ar-H), 7.24 (t, 1H, J = 7.4 Hz, Ar-H), 7.30 (d, 1H, J = 7.5 Hz, Ar-H), 7.38 (t, 1H, J = 7.6 Hz, Ar-H), 7.46 (d, 2H, J = 7.9 Hz, Ar-H), 7.67 (d, 2H, J = 8.3 Hz, Ar-H), 7.74 (d, 1H, J = 7.4 Hz, Ar-H), 8.23 (d, 2H, J = 7.6 Hz, Ar-H), 9.43 (s, 1H, CH=N), 10.45 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.6, 160.1, 155.7, 153.3, 151.6, 147.1, 138.6, 135.3, 134.9, 131.2, 130.8, 130.1, 129.1, 126.6, 125.9, 123.9, 123.1, 115.9, 115.5, 115.0, 114.7, 18.8, 13.7. LCMS (ESI): $M/Z = 540.18$ [M^+]. Anal. Calcd. for $C_{30}H_{20}N_8O_3$: C, 66.66; H, 3.73; N, 20.73. Found: C, 66.60; H, 3.77; N, 20.78.

4.4.11. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-methylbenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5k**)

Yield 64%; mp 218 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3380 (−NH, benzimidazole), 3031 (C–H, aromatic), 2990 (C–H, CH=N), 2927 (C–H, CH_3), 2222 (CN), 1678 (CO), 1630 (C=C), 1523 (C=N), 1521, 1328 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 7.18 (d, 2H, J = 8.2 Hz, Ar-H), 7.29 (d, 1H, J = 7.5 Hz, Ar-H), 7.39 (t, 1H, J = 7.6 Hz, Ar-H), 7.45 (d, 2H, J = 7.5 Hz, Ar-H), 7.61 (d, 1H, J = 7.4 Hz, Ar-H), 7.67 (d, 2H, J = 8.4 Hz, Ar-H), 7.76 (s, 1H, Ar-H), 8.21 (d, 2H, J = 7.4 Hz, Ar-H), 9.41 (s, 1H, CH=N), 10.44 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.4, 163.8, 160.0, 155.6, 153.2, 151.7, 147.2, 138.8, 138.2, 134.8, 133.7, 131.4, 130.2, 129.5, 128.8, 126.3, 123.9, 123.2, 115.9, 115.5, 115.0, 114.7, 21.4, 13.6. LCMS (ESI): $M/Z = 540.17$ [M^+]. Anal. Calcd. for $C_{30}H_{20}N_8O_3$: C, 66.66; H, 3.73; N, 20.73. Found: C, 66.58; H, 3.78; N, 20.77.

4.4.12. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-methylbenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5l**)

Yield 62%; mp 247 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3381 (−NH, benzimidazole), 3030 (C–H, aromatic), 2990 (C–H, CH=N), 2928 (C–H, CH_3), 2224 (CN), 1680 (CO), 1631 (C=C), 1524 (C=N), 1520, 1326 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.87 (s, 3H, CH_3), 2.37 (s, 3H, Ar-CH_3), 7.19 (d, 2H, J = 8.1 Hz, Ar-H), 7.29 (d, 2H, J = 7.7 Hz, Ar-H), 7.44 (d, 2H, J = 7.5 Hz, Ar-H), 7.67 (d, 2H, J = 8.3 Hz, Ar-H), 7.74 (d, 2H, J = 7.4 Hz, Ar-H), 8.24 (d, 2H, J = 7.6 Hz, Ar-H), 9.44 (s, 1H, CH=N), 10.46 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.8, 160.1, 155.7, 153.1, 151.6, 147.0, 140.7, 138.5, 134.8, 130.7, 130.1, 129.7, 129.2, 123.8, 123.1, 115.9, 115.5, 115.1, 114.7, 21.5, 13.7. LCMS (ESI): $M/Z = 540.17$ [M^+]. Anal. Calcd. for $C_{30}H_{20}N_8O_3$: C, 66.66; H, 3.73; N, 20.73. Found: C, 66.58; H, 3.78; N, 20.78.

4.4.13. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-methoxybenzylidene)-amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5m**)

Yield 66%; mp 201 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3380 (−NH, benzimidazole), 3027 (C–H, aromatic), 2988 (C–H, CH=N), 2928 (C–H, CH_3), 2851 (C–H, OCH_3), 2221 (CN), 1678 (CO), 1630 (C=C), 1522 (C=N), 1518, 1322 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.87 (s, 3H, CH_3), 3.86 (s, 3H, OCH_3), 7.07 (d, 1H, J = 7.5 Hz, Ar-H), 7.18 (d, 2H, J = 8.1 Hz, Ar-H), 7.32 (t, 1H, J = 7.6 Hz, Ar-H), 7.39 (d, 1H, J = 7.4 Hz, Ar-H), 7.45 (d, 2H, J = 7.6 Hz, Ar-H), 7.54 (s, 1H, Ar-H), 7.66 (d, 2H, J = 8.4 Hz, Ar-H), 8.21 (d, 2H, J = 7.5 Hz, Ar-H), 9.41 (s, 1H, CH=N), 10.42 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.7, 160.8, 160.1, 155.7, 153.1, 151.5, 147.1, 138.7, 134.9, 134.3, 130.1, 129.7, 123.9, 123.2, 121.6, 116.7, 115.8, 115.4, 115.0, 114.7, 111.3, 55.6, 13.6. LCMS (ESI): $M/Z = 556.15$ [M^+]. Anal. Calcd. for $C_{30}H_{20}N_8O_4$: C, 64.74; H, 3.62; N, 20.13. Found: C, 64.68; H, 3.67; N, 20.18.

4.4.14. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-methoxybenzylidene)-amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5n**)

Yield 67%; mp 211 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3381 (−NH, benzimidazole), 3028 (C–H, aromatic), 2990 (C–H, CH=N), 2929 (C–H, CH_3), 2854 (C–H, OCH_3), 2223 (CN), 1679 (CO), 1632 (C=C), 1524 (C=N), 1520, 1323 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.86 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 7.07 (d, 2H, J = 7.5 Hz, Ar-H), 7.19 (d, 2H, J = 8.0 Hz, Ar-H), 7.41 (d, 2H, J = 7.4 Hz, Ar-H), 7.67 (d, 2H, J = 8.4 Hz, Ar-H), 7.84 (d, 2H, J = 7.3 Hz, Ar-H), 8.20 (d, 2H, J = 7.5 Hz, Ar-H), 9.40 (s, 1H, CH=N), 10.42 (s, 1H, −NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.4, 163.9, 162.8, 160.0, 155.7, 153.2, 151.7, 147.2, 138.6, 134.9, 130.8, 130.3, 126.1, 123.8, 123.1, 115.9, 115.5, 115.0, 114.8, 114.3, 55.9, 13.7. LCMS (ESI): $M/Z = 556.14$ [M^+]. Anal. Calcd. for $C_{30}H_{20}N_8O_4$: C, 64.74; H, 3.62; N, 20.13. Found: C, 64.68; H, 3.68; N, 20.19.

4.4.15. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-nitrobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5o**)

Yield 69%; mp 251 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3385 (−NH, benzimidazole), 3031 (C–H, aromatic), 2994 (C–H, CH=N), 2932 (C–H, CH_3), 2226 (CN), 1684 (CO), 1634 (C=C), 1527 (C=N), 1524, 1326 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.90 (s, 3H, CH_3), 7.21 (d, 2H, J = 8.0 Hz, Ar-H), 7.42 (d, 2H, J = 7.5 Hz, Ar-H), 7.69 (d, 2H, J = 8.3 Hz, Ar-H), 7.80 (t, 1H, J = 7.5 Hz, Ar-H), 8.14 (d, 1H, J = 7.5 Hz, Ar-H), 8.20 (d, 2H, J = 7.6 Hz, Ar-H), 8.27 (d, 1H, J = 7.7 Hz, Ar-H), 8.51 (s, 1H, Ar-H), 9.44 (s, 1H, CH=N), 10.48 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.4, 163.6, 160.1, 155.7, 153.2, 151.6, 148.1, 147.0, 138.5, 135.4, 134.8, 134.4, 130.2, 129.6, 126.3, 123.8, 123.2, 121.7, 115.8, 115.5, 115.0, 114.8, 13.7. LCMS (ESI): $M/Z = 571.12$ [M^+]. Anal. Calcd. for $C_{29}H_{17}N_9O_5$: C, 60.95; H, 3.00; N, 22.06. Found: C, 60.89; H, 3.04; N, 22.11.

4.4.16. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-nitrobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5p**)

Yield 66%; mp 261 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3387 (−NH, benzimidazole), 3034 (C–H, aromatic), 2996 (C–H, CH=N), 2934 (C–H, CH_3), 2229 (CN), 1688 (CO), 1637 (C=C), 1529 (C=N), 1526, 1327 (N=O, Ar-NO_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.90 (s, 3H, CH_3), 7.21 (d, 2H, J = 8.0 Hz, Ar-H), 7.42 (d, 2H, J = 7.5 Hz, Ar-H), 7.69 (d, 2H, J = 8.3 Hz, Ar-H), 7.80 (t, 1H, J = 7.5 Hz, Ar-H), 8.14 (d, 1H, J = 7.5 Hz, Ar-H), 8.20 (d, 2H, J = 7.6 Hz, Ar-H), 8.27 (d, 1H, J = 7.7 Hz, Ar-H), 8.51 (s, 1H, Ar-H), 9.44 (s, 1H, CH=N), 10.48 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.6, 163.8, 160.1, 155.7, 153.1, 151.7, 150.4, 147.2, 139.9, 138.6, 134.9, 130.1, 127.8, 124.1, 123.9, 123.3, 115.9, 115.5, 115.0, 114.7, 13.5. LCMS (ESI): $M/Z = 571.14$ [M^+]. Anal. Calcd. for $C_{29}H_{17}N_9O_5$: C, 60.95; H, 3.00; N, 22.06. Found: C, 60.87; H, 3.06; N, 22.12.

4.4.17. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((3-bromobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5q**)

Yield 70%; mp 193 °C. IR ($\nu_{max,}$ cm^{-1}, KBr): 3386 (−NH, benzimidazole), 3033 (C–H, aromatic), 2992 (C–H, CH=N), 2931 (C–H,

CH_3), 2224 (CN), 1684 (CO), 1635 (C=C), 1525 (C=N), 1526, 1324 (N=O, Ar-NO_2), 543 (C–Br). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.89 (s, 3H, CH_3), 7.21 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.39 (t, 1H, $J = 7.5$ Hz, Ar-H), 7.45 (d, 2H, $J = 7.4$ Hz, Ar-H), 7.57 (d, 1H, $J = 7.5$ Hz, Ar-H), 7.67 (d, 2H, $J = 7.6$ Hz, Ar-H), 7.79 (d, 1H, $J = 7.4$ Hz, Ar-H), 7.85 (s, 1H, Ar-H), 8.21 (d, 2H, $J = 7.6$ Hz, Ar-H), 9.49 (s, 1H, CH=N), 10.43 (s, 1H, -NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.7, 160.2, 155.7, 153.3, 151.6, 147.1, 138.9, 135.8, 134.7, 133.7, 132.8, 130.2, 129.8, 128.3, 123.9, 123.5, 123.0, 115.8, 115.5, 115.1, 114.8, 13.7. LCMS (ESI): M/$Z = 604.08$ [M^+]. Anal. Calcd. for $C_{29}H_{17}BrN_8O_3$: C, 57.53; H, 2.83; N, 18.51. Found: C, 57.60; H, 2.87; N, 18.57.

4.4.18. 1-((1-(1H-benzo[d]imidazol-2-yl)ethylidene)amino)-6-((4-bromobenzylidene)amino)-4-(4-nitrophenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (**5r**)

Yield 71%; mp 223 °C. IR (ν_{max}, cm^{-1}, KBr): 3387 (–NH, benzimidazole), 3034 (C–H, aromatic), 2994 (C–H, CH=N), 2932 (C–H, CH_3), 2225 (CN), 1686 (CO), 1636 (C=C), 1526 (C=N), 1527, 1325 (N=O, Ar-NO_2), 546 (C–Br). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 7.20 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.42 (d, 2H, $J = 7.5$ Hz, Ar-H), 7.58 (d, 2H, $J = 7.7$ Hz, Ar-H), 7.68 (d, 2H, $J = 7.6$ Hz, Ar-H), 7.75 (d, 2H, $J = 7.6$ Hz, Ar-H), 8.20 (d, 2H, $J = 7.6$ Hz, Ar-H), 9.46 (s, 1H, CH=N), 10.45 (s, 1H, –NH benzimidazole D_2O exch.). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.5, 163.7, 160.2, 155.5, 153.2, 151.6, 147.1, 138.7, 134.8, 132.6, 131.8, 130.2, 128.6, 125.5, 123.8, 123.0, 115.9, 115.4, 115.0, 114.8, 13.7. LCMS (ESI): M/$Z = 604.07$ [M^+]. Anal. Calcd. for $C_{29}H_{17}BrN_8O_3$: C, 57.53; H, 2.83; N, 18.51. Found: C, 57.60; H, 2.87; N, 18.58.

4.5. Biological assay

4.5.1. Antibacterial assay

Antibacterial studies of newly synthesized compounds **5a-r** were carried out against the representative panel of bacteria such as *S. aureus* MTCC-96, *S. pyogenes* MTCC-442, *E. coli* MTCC-443, *P. aeruginosa* MTCC-1688 and methicillin-resistant *S. aureus* (MRSA isolate ATCC 43300). Antifungal activity was carried out against the yeast-like pathogenic fungus *C. albicans* MTCC 227. All MTCC and ATCC cultures were collected from Institute of Microbial Technology, Chandigarh. The activity of compounds was determined as per National Committee for Clinical Laboratory Standards (NCCLS) protocol using Mueller Hinton Broth (Becton Dickinson, USA). Primary screening was done first for antibacterial activity in six sets against *E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes* at different concentrations of 1000, 500, 250 μg/mL. The compounds found to be active in primary screening were similarly diluted to obtain 200, 125, 100, 62.5, 50, 25, 12.5 and 6.25 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 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 a quarter of plate in a suitable medium 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 h and 48 h. The lowest concentration (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. Solvent had no influence on strain growth. The result of this was greatly affected by the size of the inoculums. The test mixture should contain 10^6 CFU/mL organisms. DMSO and sterilized distilled water were used as negative control while chloramphenicol (1 U strength) was used as positive control. Standard drugs used in the present study were 'ciprofloxacin' and 'chloramphenicol' for evaluating antibacterial activity.

4.5.2. Antitubercular assay

Antitubercular activity was determined using the modified radiometric 7H12 broth (BACTEC 12B system) in which stock solutions as test compounds were prepared in dimethylsulfoxide (DMSO) at a concentration of 12.8 mM and the final test concentrations ranged from 39.42 to 0.15 μg/mL. Controls received 50 μL DMSO. INH was included as a positive drug control. INH was solubilized and diluted in DMSO and added to BACTEC-12 broth to achieve a range of concentration for determination of minimum inhibitory concentration (MIC, lowest concentration inhibiting ≥ 90% of the inoculums, MIC value of INH is 0.24 μg/mL at 95% inhibition of H_{37}Rv strain). M. tuberculosis H_{37}Rv strain (ATCC 27294) was cultured at 37 °C in 100 mL of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (vol/vol) glycerol, 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase) and 0.05% (vol/vol) Tween 80. The complete medium was referred to as 7H9GC-Tween. Cultures were incubated in 500-mL nephelometer flasks on a rotary shaker at 150 rpm and 37 °C until they reached an optical density of 0.4–0.5 at 550 nm. Bacteria were washed and suspended in 20 mL of phosphate-buffered saline and passed through an 8-mm pore size filter to eliminate clumps. The filtrates were aliquoted, stored at 280 °C and used within 30 days. Cultures were prepared and an appropriate dilution performed such that a BACTEC-12B vial inoculated with 0.1 mL would reach a growth index (GI) of 999 in five days. Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water and subsequent two fold dilutions were performed in 0.1 mL of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC, and 0.1 mL was added to wells. Subsequent determination of bacterial titers yielded 1×10^6 CFU/mL in plate wells for H_{37}Rv. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Addition of 1/10 mL to wells resulted in final bacterial titers of 2.0×10^5 for H_{37}Rv. Wells containing drug only were used to detect auto fluorescence of compounds. Cultures were incubated at 37 °C and the Growth of Inhibition (GI) determined daily until control cultures achieved a GI of 999. Assays were usually completed in 5–8 days. Percent inhibition was defined as 1 – (GI of test sample/GI of control) 100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC.

4.5.3. Cytotoxicity assay

VERO cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 2 mM Na_2CO_3 supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were incubated at 37 °C under 5% CO_2 and 95% air in a humidified atmosphere until confluent and then diluted with phosphate-buffered saline to 10^6 cells/mL. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The concentration of DMSO in the final culture medium was 1%, which had no effect

on the cell viability. In a transparent 96-well plate (Falcon Micro test 96), three fold serial dilutions of the macrolide stock solutions resulted in final concentrations of 31.53 to 0.12 μg/mL in a final volume of 200 μL. After incubation at 37 °C for 72 h, medium was removed and monolayer was washed twice with 100 μL of warm Hanks' balanced salt solution (HBSS). One hundred microliters of warm medium and 20 μL of freshly made MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and phenylmethasulfazone] (100:20) (Promega) were added to each well, plates were incubated for 3 h, and absorbance was determined at 490 nm using a plate reader. Each concentration was repeated in three wells and control cell viability was considered as 100%. The same experimental conditions were provided for all compounds and analysis was repeated three times for each cell line.

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