Novel biologically active N-substituted benzimidazole derived Schiff bases: design, synthesis and biological evaluation

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Abstract

Herein we present the design and synthesis of novel *N*-substituted benzimidazole derived Schiff bases, and the evaluation of their antiviral, antibacterial and antiproliferative activity. One of the goals was to study the impact on the biological activity of substituents placed at the N atom of benzimidazole nuclei as well as the type of substituents placed at the phenyl ring. The synthesized Schiff bases were evaluated for their *in vitro* antiviral activity against different viruses, antibiotic activity against a panel of bacterial strains and antiproliferative activity on several human cancer cell lines, thus enabling the study of structure – activity relationships.

Some mild antiviral effects were noted, although at higher concentrations as compared to the included reference drugs. Additionally, some derivatives showed moderate antibacterial activity, with precursor **23** proving broadly active against most of the bacterial strains tested. Lastly, Schiff base **40**, a 4-*N,N*-diethylamino-2-hydroxy substituted derivative bearing a phenyl ring at the N atom on benzimidazole nuclei, displayed strong antiproliferative activity against several cancer cell lines (IC_{50} 1.1–4.4 μM). The strongest antitumoral effect was observed towards acute myeloid leukemia (HL-60).

1. Introduction

Schiff bases comprise a very important class of organic compounds [1], widely used in organic and medicinal chemistry as biologically important structural motifs being incorporated in the structure of many synthetic and semisynthetic organic compounds [2–5]. Also, some of naturally occurring Schiff bases play an important role in several physiological processes, for example rhodopsin, a photoreceptor present in the rod cells of the retina, which is essential in vision processing [6]. Schiff bases can be easily synthesized by a condensation reaction of different amino substituted compounds with versatile aldehydes or ketones [7]. Besides the fact that Schiff bases are important building blocks in medicinal chemistry due to their broad spectrum of biological activity [8–11], they also have potential applications in coordination chemistry [12] since they can coordinate to metal ions via azomethine nitrogen, in analytical chemistry [13–14], as dyes [15], optical chemosensors [16–17], polymers [18], in catalysis [19], metallurgy [20] and refining of metals, and as fungicidal and agrochemical compounds. Additionally, the interest of numerous scientists has been focused on evaluating Schiff bases as ligands for transition metals since such complexes possess diverse biological activities including anticancer [21–22], antimicrobial [23–24], antifungal [25] *etc*.

Nowadays, there is a growing interest in the synthesis of benzimidazole derived Schiff bases, primarily due to their significant biological activities but also due to the fact that they can easily form complexes with different types of metals giving rise to a variety of complexes with interesting electronic [26–27] and biological properties [28]. Nawrocka et al. have synthesized novel 2-benzimidazolyl substituted Schiff bases which were screened for their antiproliferative activity on several cancer cells. From the SAR study, they concluded that the replacement of the electron-withdrawing chlorine atom with electron-withdrawing bromine at the *ortho* position in the phenyl ring and with electron-withdrawing bromine at the *para* position in the phenyl substituent can increase the activity [29]. Another group designed and synthesized benzimidazole and 3-oxo-pyrimido[1,2-*a*]benzimidazole derived Schiff bases which showed antioxidant and cytotoxic activity being evaluated as inhibitors of lipoxygenase (LOX) and of lipid peroxidation (LPO) [30]. Another group described the synthesis of a series of Schiff bases bearing benzimidazole nuclei to evaluate their antimalarial and antitrypanosomal activity. One derivative showed minimal cytotoxic effects against HeLa cells and could be optimized to be a promising antiparasitic agent [31]. Furthermore, a group of authors explored the antimicrobial activity of a series of benzimidazole derived Schiff bases against *Staphylococcus aureus* and *Escherichia coli* [32]. Also, benzimidazole based Schiff base complexes with L-histidine were screened for their cytotoxic activity. The metal complexes could bind ct-DNA through intercalation and were found to exhibit cytotoxic effects against cancer cell lines with higher potency than that of the widely used drug cisplatin [33]. A group of authors from Colombia developed a series of lanthanum (III) and cerium (III) complexes, including Schiff base ligands derived from (1H-benzimidazol-2-yl)aniline, salicylaldehyde, and 2,4-dihydroxybenzaldehyde. These metal complexes were evaluated for their cytotoxic, antiparasitic and antibacterial activity as well as interaction with DNA [34]. Chinese authors studied the DNA interaction and antiproliferative activity of two Cu(II) complexes with Schiff base of benzimidazole [35].

Recently, we synthesized a series of novel benzimidazole substituted Schiff bases (Fig. 1a) which were tested for their *in vitro* antiproliferative activity. They exerted broad spectrum antiproliferative activity on different cancer cell lines, although only at high concentrations [36]. Compounds substituted with 4-*N,N*-diethylamino-2-hydroxyphenyl bearing either a methyl or a cyano group at the 5(6)-position on benzimidazole nuclei displayed the strongest antiproliferative effect on all tested cell lines, with a significant concentration-dependent effect on HeLa and MCF-7 cell lines.

As a continuation, we have now designed and synthesized novel *N*-substituted benzimidazole derived Schiff bases (Fig. 1b) to explore the influence of the side chain attached at the N atom on benzimidazole nuclei, both on antiproliferative and antiviral activity.

2. Results And Discussion

2.1. Chemistry

The synthesis of the novel *N*-substituted benzimidazole derived Schiff bases **28**–**45** is depicted in reaction Scheme 1. For the synthesis of the targeted Schiff bases, corresponding *N*-substituted 2-aminobenzimidazoles **17**–**24** were prepared as main precursors according to the modified reaction procedures previously described by our research group. Starting from 2-chloronitrobenzene **1** or 2-chloro-4-cyanonitrobenzene **2**, in the reaction of microwave assisted amination with an excess of amine, *N*-methyl **5**, *N*-phenyl **6**, *N*-isobutyl **3**–**4** and *N*-hexyl **7**–**8** substituted nitro precursors were

prepared without using any catalyst. After reduction with $SnCl_2 \times 2H_2O$ in acidic media, corresponding 1,2-diamino substituted benzenes **9**–**16** were obtained.

By cyclocondensation with cyanogen-bromide in methanol, substituted 2-amin benzimidazoles bearing either isobuthyl **17** and **21**, methyl **18** and **22**, phenyl **19** and **23** or *n*-hexyl **20** and **24** side chains at the N atom or cyano group placed at the 5(6)-position on benzimidazole nuclei were prepared in moderate reaction yields. Benzimidazole derived Schiff bases **28**–**45** were obtained by the reaction of *N*-substituted 2-aminobenzimidazoles **17**–**24** with chosen substituted benzaldehydes, namely 4-*N,N*-dimethylamino- **25**, 2-hydroxy-4-*N,N*-dimethylamino- **26** and 4-nitrobenzaldehyde **27**.

All Schiff bases were additionally purified either by recrystallization or through column chromatography on silica gel with dichloromethane/methanol as an eluent to yield the targeted compounds in low to moderate reaction yields (6–68%).

The structures of all newly prepared benzimidazole derived Schiff bases **28**–**45** were confirmed by the means of ^1H and ^{13}C NMR spectra and elemental analysis. The structural analysis was performed based on the chemical shifts in both ^1H and ^{13}C NMR spectra, and on the values of H–H coupling constants in the ^1H spectra. Amination of reactants **1**–**2** caused the appearance of the signals related to the amino group (8.12–9.90 ppm) as well as the signals for protons from isobuthyl, methyl, and *n*-hexyl sidechains (0.87–3.41 ppm) in the structure of compounds **3**–**8**. Reduction of the nitro group was confirmed within the singlet related to the protons from the amino group (4.44 to 5.25 ppm). The formation of the benzimidazole ring was confirmed by the disappearance of signals for amino groups in 1,2-diamino substituted benzenes **9**–**16** as well as the appearance of a singlet related to the proton of the amino group placed at position 2 on benzimidazole nuclei (**17**–**24**) which was downshifted in comparison to the singlet of the amino group in the diamino substituted benzenes **9**–**16**. The synthesis of targeted Schiff bases **28**–**45** was established by the observation of a singlet related to the proton of the imino group at 7.98–9.67 ppm in the ^1H NMR spectra as well as a signal for the C atom of the imino group in the ^{13}C NMR spectra.

2.2. Biological Evaluation

2.2.1. Cytotoxicity and antiviral activity

In Table 1 the results for the antiviral evaluation are depicted for all derivatives showing antiviral activity. All other compounds not included in the table were devoid of antiviral activity against this panel of viruses.

For evaluating the antiviral activity, following viruses were used; HCoV, influenza virus, RSV, HSV-1, yellow fever virus, Zika virus and Sindbis virus. The results are expressed as CC_{50} (50% cytotoxic concentration) and EC_{50} (50% effective concentration) values. Overall, the *N*-substituted 2-aminobenzimidazoles **17**–**24** displayed poor antiviral activity. Substituted 2-aminobenzimidazoles bearing methyl **18** and **22** were both able to inhibit Zika virus replication in Huh-7 cells with EC_{50} values of 43.1 μM and 46.4 μM, respectively. Among the *N*-substituted benzimidazole derived Schiff bases **28**–**45** some derivatives showed antiviral activity, although weak when compared to the included standard antiviral drugs (remdesivir, ribavirin, zanamivir, rimantadine and BVDU). The 4-*N,N*-diethylamino-2-hydroxy substituted derivative bearing a cyano and *N*-isobutyl side chain on benzimidazole nuclei **31** showed moderate activity against HCoV-NL63 in Huh-7 cells (EC_{50} 32 μM). Compound **42**, substituted with a *N,N*-dimethylamino and a *N*-hexyl side chain showed moderate activity against HCoV-229E in HEL299 cells (EC_{50} 34.7 μM). In summary, no significant antiviral effects were noted for the 4-*N,N*-diethylamino-2-hydroxyphenyl ring nor the 4-*N,N*-dimethylaminophenyl and 4-nitrophenyl rings.

2.2.2. Antibacterial activity in vitro

The *in vitro* antibacterial activity of the synthesized Schiff bases was evaluated against a panel of eight different bacterial strains. Gram positive bacterial strains comprised *S. aureus*, S. *pneumoniae* and *E. faecalis* and while the panel of Gram-negative bacteria included *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. As reference drugs, four antibiotics (*ampicillin*, *ceftazidime, ciprofloxacin*, and *meropenem*) were used.

As presented in Table 2, the majority of compounds lacked activity, while some Schiff bases showed moderate activity against certain bacterial strains.

Table 1
Cytotoxicity and antiviral activity of tested compounds **17–45**

Cpd	Cytotoxicity (CC_{50}/ µM)			Antiviral activity (EC_{50} /µM)										
	HEL 229	Huh-7	MDCK	HCoV 229E HEL 299	HCoV OC43 HEL 299	HCoV NL63 Huh-7	Influenza H1N1 MDCK	Influenza H3N2 MDCK	Influenza B MDCK	RSV A Long HEL 299	HSV-1 KOS HEL 299	YFV 17D Huh-7	Zika Mr776 Huh-7	Sindbis Huh-7
17	> 100	> 100	> 100	78.9	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
18	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	43.1	> 100
22	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	78.7	> 100	> 100	46.4	> 100
29	> 100	> 100	> 100	> 100	> 100	79.8	94.1	> 100	> 100	> 100	> 100	> 100	> 100	> 100
31	> 100	85.7	1.3	> 100	> 100	32	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
32	> 100	90.1	> 100	38.5	68.7	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
34	> 100	> 100	> 100	> 100	> 100	82.8	> 100	> 100	83.5	> 100	> 100	> 100	> 100	> 100
36	> 100	> 100	> 100	88.4	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
38	> 100	> 100	> 100	> 100	94.8	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
41	> 100	> 100	71.6	> 100	89.5	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
42	> 100	2.7	34.6	34.7	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
Remdesivir	> 10	> 10	-	0.06	0.06	0.03	-	-	-	0.03	-	6.2	0.7	> 10
Ribavirin	> 250	8.9	67.0	82.6	170.1	> 250	10.5	4.0	2.8	10.8	-	> 250	> 250	148.1
Zanamivir	-	-	> 100	-	-	-	0.1	16.8	0.05	-	-	-	-	-
Rimantadine	-	-	> 100	-	-	-	5.0	0.05	> 100	-	-	-	-	-
BVDU	> 100	-	-	-	-	-	-	-	-	-	0.05	-	-	-

Table 2
Antibacterial activity *in vitro* of tested compounds

Cpd	*S. aureus ATCC 29213*	*E. faecalis ATCC 29212*	*S. pneumoniae ATCC 49619*	*E. coli ATCC 25922*	*E. coli efflux del*	*P. aeruginosa ATCC 27853*	*K. pneumoniae ATCC 700603*	*A. baumannii ATCC 17978*
23	16	32	32	64	32	> 64	> 64	64
37	32	64	64	> 64	> 64	> 64	> 64	> 64
38	> 64	32	> 64	> 64	> 64	> 64	> 64	> 64
41	> 64	32	64	> 64	> 64	> 64	> 64	> 64
42	32	32	64	> 64	64	> 64	> 64	> 64
Ampicillin	0,5	2	2	< 0.125	0,5	> 64	> 64	> 64
Ceftazidime	8	< 0.125	0,25	0,5	> 64	1	64	32
Ciprofloxacin	0,125	< 0.125	< 0.125	0,5	1	0.25	0.25	4
Meropenem	< 0.125	< 0.125	< 0.125	< 0.125	8	1	0.25	< 0.125

Thus, *N,N*-dimethylamino **38** and 4-*N,N*-diethylamnino-2-hydroxy **41** derivatives, both substituted with a phenyl ring at the N atom on benzimidazole nuclei, showed activity against *E. faecalis* (MIC 32 μM). *p*-Nitro and cyano substituted Schiff base **37** with methyl group at N atom showed activity against *S. aureus* (32 μM). *N,N*-dimethylamino derivative **42** with a hexyl side chain placed at the N atom showed moderate activity against *S. aureus*, and *E. faecalis* (32 μM). In all, precursor *N*-hexyl-2-aminobenzimidazole **23** showed the most pronounced broad spectrum antibiotic activity against *E. faecalis*, *E. coli efflux dell*, and *K. pneumoniae* with MIC values of 32 μM, and against *S. aureus*, with MIC value of 16 μM.

2.2.3. Antiproliferative activity in vitro

All synthesized *N*-substituted 2-aminobenzimidazoles **9**–**16** and benzimidazole derived Schiff bases **28**–**45** were explored for their *in vitro* antiproliferative activity against several human cancer cell lines. The results are presented in Table 3 as IC_{50} values (50% inhibitory concentration). For the evaluation of antiproliferative activity, following human cancer cell lines were used: LN-229 – glioblastoma, Capan-1 – pancreatic adenocarcinoma, HCT-116 – colorectal carcinoma, NCI-H460 – lung carcinoma, DND-41 – acute lymphoblastic leukemia, HL-60 – acute myeloid leukemia, K-562 – chronic myeloid leukemia and Z-138 – non-Hodgkin lymphoma cancer cells. All obtained results are compared to *vincristine* and *docetaxel* which are used as standard chemotherapeutic agents.

The majority of the tested compounds showed low or no activity towards the selection of cancer cell lines (some inactive 2-aminobenzimidazoles were excluded from Table 3). Among all tested *N*-substituted 2-aminobenzimidazoles, the best activity was shown by *n*-hexyl substituted derivative **23** which showed moderate but broad antiproliferative activity against all tested cancer types.

N-phenyl substituted 2-aminobenzimidazole **21** showed mild but selective activity against lung carcinoma. Regarding the benzimidazole derived Schiff bases, the most potent one was the 4-*N,N*-diethylamino-2-hydroxy substituted Schiff base bearing a phenyl ring at the N atom on benzimidazole nuclei **40**. This derivative displayed pronounced antitumoral activity against Capan-1, DND-41, HL-60 and Z-138 cancer cells in the low micromolar range, with some selectivity towards HL-60 (acute myeloid leukemia) cancer cell line (IC_{50} 1.1 μM). In addition, derivative **40** showed moderate activity against all other cancer cells lines. Furthermore, 4-*N,N*-dimethylamino substituted Schiff base bearing an isobutyl side chain at the N atom **28** showed selective antiproliferative activity against colorectal carcinoma (HCT-116). Derivative **31** substituted with 2-hydroxy and 4-*N,N*-diethylamino groups at the phenyl ring as well as with an isobutyl sidechain on the N atom and a cyano group on benzimidazole nuclei displayed moderate activity against three cancer cell types (IC_{50} = 32.8–47.6 mM). The 4-*N,N*-diethylamino-2-hydroxy substituted Schiff base **41** bearing a phenyl ring at the N atom and a cyano group on the benzimidazole nuclei also displayed moderate activity against several of the tested cancer cell lines. Among *N*-hexyl substituted Schiff bases **42**–**45**, moderate activity was observed for compound **42** substituted with a 4-*N,N*-dimethylamino group at the phenyl ring as well as for compound **43** which also bears a cyano group at the benzimidazole nuclei.

The 4-*N,N*-diethylamino-2-hydroxy substituted derivatives **44**–**45** were less active in comparison to 4-*N,N*-dimethylamino substituted analogues. Regarding the *N*-methyl substituted Schiff bases, only the 4-*N,N*-diethylamino-2-hydroxy substituted derivative bearing a cyano group showed moderate but selective activity against DND-41 cells. When comparing all *N*-isobutyl substituted Schiff bases **28**–**31**, we can conclude that the derivative bearing a 4-*N,N*-diethylamino-2-hydroxyphenyl as well as a cyano group **31** was the most active one.

Table 1
Antiproliferative activity *in vitro* of tested compounds **28**–**45** against a broad panel of cancer cell types

Cpd	IC_{50} /μM							
	Cell line							
	LN-229	Capan-1	HCT-116	NCI-H460	DND-41	HL-60	K-562	Z-138
19	> 100	> 100	> 100	> 100	98.3	> 100	> 100	> 100
21	> 100	> 100	> 100	69.2	> 100	> 100	> 100	> 100
23	45.2	46.5	57.4	60.2	47.7	44.9	46.7	44.7
28	> 100	> 100	15.2	> 100	> 100	> 100	> 100	> 100
29	> 100	> 100	> 100	> 100	> 100	42.8	> 100	> 100
30	> 100	> 100	> 100	> 100	> 100	70.4	> 100	> 100
31	> 100	32.8	> 100	> 100	34.4	47.6	> 100	> 100
32	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
33	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
34	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
35	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
36	> 100	> 100	> 100	> 100	41.4	> 100	> 100	> 100
37	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
38	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
39	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
40	21.5	2.4	9.6	43.7	2.2	1.1	12.9	4.4
41	> 100	69.5	> 100	> 100	76.3	49.6	> 100	35.2
42	> 100	> 100	> 100	64.4	> 100	73.2	90.8	> 100
43	> 100	> 100	> 100	91.6	> 100	33.5	> 100	> 100
44	> 100	> 100	> 100	> 100	> 100	83.9	> 100	> 100
45	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
Docetaxel	0.0041	0.0038	0.0025	0.0024	0.0025	0.0022	0.0085	0.0023
Vincristine	0.0027	0.0088	0.0079	-	0.010	0.0033	0.017	0.031

In conclusion, we observed that the most significant impact on the antiproliferative activity was seen for the 4-*N,N*-diethylamino-2-hydroxyphenyl ring in comparison to 4-*N,N*-dimethylaminophenyl and 4-nitrophenyl rings. A cyano group placed at the 5(6) position on benzimidazole nuclei increased the antiproliferative activity, with the exception of *N*-phenyl substituted derivatives. The 4-*N,N*-diethylamino-2-hydroxy substituted Schiff base bearing a phenyl ring at the N atom on benzimidazole nuclei **40**, which showed the most promising activity among all tested derivatives, was chosen as a lead compound for further optimization in order to get more selective and potent antiproliferative agents (Fig. 2).

3. Conclusion

Herein we present the design and synthesis of novel, *N*-substituted benzimidazole derived Schiff bases, bearing isobutyl, methyl, and *n*-hexyl sidechains or a phenyl group at the N atom on the benzimidazole nuclei as well as a 4-*N,N*-dimethylamino- or 4-*N,N*-diethylamino-2-hydroxyphenyl ring attached directly to the imino bond. Within this research, our main focus was to study the impact on the biological activity of the substituents placed on the phenyl ring and also on the N atom of benzimidazole nuclei. All Schiff bases were tested for their *in vitro* antiviral activity against a broad selection of viruses, antibiotic activity against Gram positive and Gram negative bacterial strains and antiproliferative activity on a diverse panel of human cancer cell lines.

The series of newly synthesized derivatives lacked pronounced antiviral activity against the panel of selected viruses. Schiff base **31** substituted with a 4-*N,N*-diethylamino-2-hydroxyphenyl and bearing a cyano and *N*-isobutyl side chain on the benzimidazole nuclei showed moderate activity against HCoV-NL63 virus on Huh-7 cells, although with low selectivity (EC_{50} 32 μM and CC_{50} 85.7 μM).

The majority of tested compounds were inactive against the used Gram positive and Gram negative bacterial strains. A selection of Schiff bases showed moderate activity against one and/or two bacterial strains, while the most active compound was *N*-hexyl-2-aminobenzimidazole **23** with moderate but

broad activity against *E. faecalis*, *E. coli efflux dell*, and *K. pneumoniae* (MIC 32 μM), and against *S. aureus* (MIC 16 μM).

Additionally, all prepared 2-aminobenzimidazoles **17–24** and Schiff bases **28–45** were tested for their antiproliferative activity against several cancer cell lines. The obtained results revealed that Schiff base **31** substituted with a 4-*N,N*-diethylamino-2-hydroxyphenyl and with an isobutyl sidechain on the N atom and a cyano group on the benzimidazole nuclei displayed moderate activity against three cancer types (IC_{50} 32.8–47.6 μM). Also, among the tested *N*-substituted-2-aminobenzimidazoles, *N*-hexyl substituted derivative **23** showed broad but modest activity against all the tested cancer cell lines. Furthermore, the 4-*N,N*-diethylamino-2-hydroxyphenyl ring as well as the phenyl ring attached to the N atom on benzimidazole nuclei had the most significant impact on the activity. Schiff base **40** bearing the above mentioned substituents showed the most promising selective antiproliferative activity against Capan-1, DND-41, HL-60 and Z-138 cancer cell lines (IC_{50} = 1.1–4.4 μM), and moderate activity against all other tested cell lines.

In conclusion, we have shown that out of the prepared *N*-substituted benzimidazole derived Schiff bases, derivative **40** showed the most interesting biological potential with promising antiproliferative activity, making it a promising candidate for further design and optimization.

4. Experimental Part

4.1. Chemistry

4.1.1. General methods

All chemicals were purchased from commercial suppliers. Melting points were recorded on Büchi 535 melting apparatus. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Bruker Advance III HD 400 MHz/54 mm Ascend instrument. All NMR spectra were measured in DMSO-d6 solutions using TMS as an internal standard. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates and the spots were detected under UV light. Microwave-assisted synthesis was performed in a Milestone start S microwave oven using quartz cuvettes under a pressure of 40 bar. Elemental analysis for carbon, hydrogen and nitrogen were performed on a PerkineElmer 2400 elemental analyzer. Where analyses are indicated only as symbols of elements, analytical results obtained are within 0.4% of the theoretical value.

4.1.2. General method for preparation of compounds 7–8

Compounds **7–8** were prepared using microwave irradiation, at optimized reaction time at 170°C with power 800 W and 40 bar pressure, from **1** or **2** in acetonitrile (10 mL) with excess of added corresponding amine. After cooling, resulting product was purified by column chromatography on SiO_2 using dichlormethane/methanol as eluent.

N-hexyl-2-nitroaniline 7

7 was prepared from **1** (0.50 g, 3.2 mmol) and hexylamine (2.90 mL, 22.2 mmol) after 2 h of irradiation to yield 0.60 g (94%) of orange oil. ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 8.12 (t, 1H, J = 5.07 Hz, NH), 8.06 (dd, 1H, J_1 = 1.58 Hz, J_2 = 8.57 Hz, H_{arom}), 7.56–7.51 (m, 1H, H_{arom}), 7.05 (d, 1H, J = 7.99 Hz, H_{arom}), 6.70–7.65 (m, 1H, H_{arom}), 3.37–3.33 (m, 2H, CH_2), 1.67–1.58 (m, 2H, CH_2), 1.41–1.27 (m, 6H, CH_2), 0.87 (t, 3H, J = 7.06 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 145.7, 137.1, 131.3, 126.7, 115.5, 115.0, 42.7, 31.4, 28.7, 26.5, 22.5, 14.4; Anal. Calcd. for $C_{12}H_{18}N_2O_2$: C, 64.84; H, 8.16; N, 12.60; O, 14.39. Found: C, 64.89; H, 8.23; N, 12.51; O, 14.29%.

3-N-(hexylamino)-4-nitrobenzonitrile 8

8 was prepared from **2** (0.50 g, 2.7 mmol) and hexylamine (1.80 mL, 13.7 mmol) after 2 h of irradiation to yield 0.50 g (97%) of yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 8.58 (t, 1H, J = 5.38 Hz, NH), 8.50 (d, 1H, J = 2.00 Hz, H_{arom}), 7.81 (dd, 1H, J_1 = 1.60 Hz, J_2 = 9.08 Hz, H_{arom}), 7.18 (d, 1H, J = 9.10 Hz, H_{arom}), 3.41 (q, 2H, J = 6.72 Hz, CH_2), 1.65–1.55 (m, 2H, CH_2), 1.38–1.23 (m, 6H, CH_2), 0.87 (t, 3H J = 6.70 Hz, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ/ppm = 146.8, 130.5, 118.2, 96.0, 42.3, 30.8, 27.9, 25.8, 21.9; Anal. Calcd. for $C_{13}H_{17}N_3O_2$: C, 63.14; H, 6.93; N, 16.99; O, 12.94. Found: C, 63.20; H, 6.88; N, 16.91; O, 12.89%.

4.1.3. General method for preparation of compounds 10–11

Derivative **7** or benzonitrile derivative **8** and a solution of $SnCl_2 \times 2H_2O$ in MeOH and concentrated HCl were refluxed for 0.5 hours. The resulting solution was treated with 20% NaOH to pH = 14. The resulting precipitate was filtered off, washed with hot ethanol and filtered. The filtrate was evaporated at a reduced pressure and extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$ and concentrated at reduced pressure.

N^1-hexylbenzene-1,2-diamine 10

10 was prepared from **7** (3.52 g, 15.8 mmol), $SnCl_2 \times 2H_2O$ (29.70 g, 131.5 mmol), $HCl_{conc.}$ (49 mL) and MeOH (49 mL) to yield 2.13 g (70%) of red oil. ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 6.52 (dd, 1H, J_1 = 1.46 Hz, J_2 = 7.78 Hz, H_{arom}), 6.48 (td, 1H, J_1 = 1.48 Hz, J_2 = 7.56 Hz, H_{arom}), 6.40–6.37 (m 2H, H_{arom}), 4.44 (bs, 2H, NH_2), 4.28 (t, 1H, J = 5.18 Hz, NH), 2.99 (q, 2H, J = 6.88 Hz, CH_2), 1.61–1.55 (m, 2H CH_2), 1.41–1.35 (m, 2H, CH_2), 1.32–1.28 (m, 4H, CH_2), 0.88 (t, 3H, J = 6.98 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 136.1, 135.0, 117.5, 116.5, 114.0, 109.6, 43.4, 31.2, 28.8, 26.5, 22.1, 13.9; Anal. Calcd. for $C_{12}H_{20}N_2$: C, 74.95; H, 10.48; N, 14.57. Found: C, 74.88; H, 10.54; N, 14.66%.

3-amino-4-N-(hexylamino)benzonitrile 11

11 was prepared from **8** (2.71 g, 10.9 mmol), $SnCl_2 \times 2H_2O$ (14.85 g, 68.8 mmol), $HCl_{conc.}$ (29 mL) and MeOH (29 mL) to yield 1.65 g (69%) of pink powder. m.p. 157–161°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 6.91 (dd, 1H, J_1 = 1.90 Hz, J_2 = 8.18 Hz, H_{arom}), 6.76 (d, 1H, J = 2.00 Hz, H_{arom}), 6.44 (d, 1H, J = 8.16 Hz, H_{arom}), 5.32 (t, 1H, J = 5.00 Hz, NH), 4.96 (s, 2H, NH_2), 3.08 (q, 2H, J = 6.42 Hz, CH_2), 1.64–1.51 (m, 2H, CH_2), 1.41–1.25 (m, 6H, CH_2), 0.88 (t, 3H, J = 6.58 Hz, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ/ppm = 140.5, 135.5, 123.4, 121.6, 115.2, 108.8, 96.7, 43.2, 31.6, 28.8, 26.8, 22.6, 14.4; Anal. Calcd. for $C_{13}H_{19}N_3$: C, 71.85; H, 8.81; N, 19.34. Found: C, 71.94; H, 8.87; N, 19.41%.

4.1.4. General method for preparation of compounds 21 and 25

BrCN was added dropwise to a solution of *o*-phenylenediamine or in 20 mL H_2O and 5 mL acetonitrile. The reaction mixture was refluxed for 2 hours and NH_4OH was added to adjust to pH = 9. After cooling, the resulting precipitate was filtered off.

2-amino-1-hexylbenzimidazole 21

21 was prepared from **10** (1.59 g, 8.3 mmol) and BrCN (0.88 g, 8.3 mmol) to to yield 1.45 g (81%) of brown powder. m.p. 245–250°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 7.96 (bs, 2H, NH2), 7.43–7.41 (m, 1H, Harom), 7.32–7.30 (m, 1H, Harom), 7.18–7.14 (m, 2H, Harom), 4.08 (t, 2H, J = 7.37 Hz, CH2), 1.68–1.63 (m, 2H, CH2), 1.33–1.24 (m, 6H, CH2), 0.84 (t, 3H, J = 7.07 Hz, CH3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 151.6, 131.9, 122.9, 121.9, 112.9, 109.9, 42.4, 31.3, 28.2, 26.0, 22.5, 14.3; Anal. Calcd. for $C_{13}H_{19}N_3$: C, 71.85; H, 8.81; N, 19.34. Found: C, 71.87; H, 8.79; N, 19.29%.

2-amino-6-cyano-1-hexylbenzimidazole 25

13 was prepared from **11** (0.03 g, 1.4 mmol) and BrCN (0.14 g, 1.4 mmol) to to yield 0.28 g (83%) of grey powder. m.p. 215–220°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 7.49 (s, 1H, H_{arom}), 7.33–7,28 (m, 2H, H_{arom}), 6.84 (s, 1H, H_{arom}), 4.01 (t, 2H, J = 7.18 Hz, CH_2), 1.63–1.58 (m, 2H, CH_2), 1.27–1.22 (m, 6H, CH_2), 0.83 (t, 3H, J = 6.68 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 157.1, 143.2, 138.3, 121.2, 102.5, 42.1, 31.4, 28.7, 26.1, 22.5; Anal. Calcd. for $C_{14}H_{18}N_4$: C, 69.39; H, 7.49; N, 23.12. Found: C, 69.45; H, 7.40; N, 23.07%.

4.1.5. General Method For Preparation Of Schiff Bases 28–45

Solutions of equimolar amounts of corresponding N-substituted-2-aminobenzimidazole and aromatic aldehyde in absolute ethanol, were refluxed for 24–48 h. After cooling, the

obtained products were filtered off and recrystallized from ethanol. If necessary, products were purified by column chromatography on SiO_2 using a gradient elution of dichloromethane/methanol/TEA. Basic TEA was used to prevent the decomposition of the Schiff base conjugates in the silica gel column.

(E)-4-(((1-isobutyl-1H-benzo[d]imidazol-2-yl)imino)methyl)-N,N-dimethylaniline 28

Compound **28** was prepared from 2-amino-1-isobutylbenzimidazole **17** (0.10 g, 0.5 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.08 g, 0.5 mmol) in absolute ethanol (3 mL) after refluxing for 48 h to obtain 0.02 g (14%) of yellow powder. m.p. 277–279°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.26 (s, 1H, H_{arom}), 7.91 (d, 2H, J = 8.79 Hz, H_{arom}), 7.55–7.50 (m, 2H, H_{arom}), 7.19–7.14 (m, 2H, H_{arom}), 6.85 (d, 2H, J = 8.88 Hz, H_{arom}), 4.17 (d, 2H, J = 7.27 Hz, CH_2), 3.07 (s, 6H, CH_3), 2.24–2.16 (m, 1H, CH), 0.89 (t, 6H, J = 6.66 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 150.4, 131.2 (2C), 129.1 (2C), 124.0, 123.4, 112.0 (2C), 111.2 (3C), 49.1, 27.7, 19.7 (2C); Anal. Calcd. for $C_{20}H_{24}N_4$: C, 74.97; H, 7.55; N, 17.48. Found: C, 74.95; H, 7.59; N, 17.41%.

(E)-2-((4-(dimethylamino)benzylidene)amino)-1-isobutyl-1H-benzo[d]imidazole-6-carbonitrile 29

Compound **29** was prepared from 2-amino-6-cyano-1-isobutylbenzimidazole **21** (0.10 g, 0.5 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.07 g, 0.5 mmol) in absolute ethanol (4 mL) after refluxing for 24 h to obtain 0.03 g (22%) of yellow powder in the form of a mixture of *E*- and *Z*-isomers at the ratio of **29a**/**29b** = 5:3. m.p. 283–285°C; **29a**: ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 9.28 (s, 1H, H_{arom}), 8.03 (d, 1H, J = 1.01 Hz, H_{arom}), 7.94 (d, 2H, J = 8.78 Hz, H_{arom}), 7.74 (d, 1H, J = 8.37 Hz, H_{arom}), 7.69 (d, 1H, J = 9.02 Hz, H_{arom}), 7.57 (dd, 1H, J_1 = 8.31, J_2 = 1.38 Hz, H_{arom}), 6.85 (d, 2H, J = 9.03 Hz, H_{arom}), 4.21 (d, 2H, J = 7.27 Hz, CH_2), 3.08 (s, 6H, CH_3), 2.23–2.16 (m, 1H, CH), 0.89 (d, 6H, J = 6.66 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 166.2, 159.0, 154.3, 141.6, 138.7, 132.6, 125.2, 122.7, 120.6, 112.1, 104.1, 49.8, 29.3, 20.3; **29b**: ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 9.67 (s, 1H, H_{arom}), 7.69 (d, 1H, J = 9.01 Hz, H_{arom}), 7.49 (d, 1H, J = 1.09 Hz, H_{arom}), 7.33 (d, 1H, J = 8.07 Hz, H_{arom}), 7.28 (dd, 1H, J_1 = 8.11, J_2 = 1.37 Hz, H_{arom}), 6.84–6.78 (m, 3H, H_{arom}), 3.85 (d, 2H, J = 7.56 Hz, CH_2), 3.05 (s, 6H, CH_3), 2.13–2.04 (m, 1H, CH), 0.86 (d, 6H, J = 6.67 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 190.3, 157.4, 143.2, 132.0, 121.2, 111.9, 111.5, 109.3, 102.4, 48.9, 28.4, 20.0; Anal. Calcd. for $C_{21}H_{23}N_5$: C, 73.02; H, 6.71; N, 20.27. Found: C, 73.10; H, 6.68; N, 20.32%.

(E)-5-(diethylamino)-2-(((1-isobutyl-1H-benzo[d]imidazol-2-yl)imino)methyl)phenol 30

Compound **30** was prepared from 2-amino-1-isobutylbenzimidazole **17** (0.15 g, 0.8 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.12 g, 0.8 mmol) in absolute ethanol (6 mL) after refluxing for 48 h to obtain 0.10 g (36%) of yellow powder. m.p. 254–258°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 12.79 (s, 1H, OH), 9.35 (s, 1H, H_{arom}), 7.55–7.50 (m, 2H, H_{arom}), 7.42 (d, 1H, J = 8.97 Hz, H_{arom}), 7.20–7.15 (m, 2H, H_{arom}), 6.42 (dd, 1H, J_1 = 2.27 Hz, J_2 = 8.87 Hz, H_{arom}), 6.17 (d, 1H, J = 2.09 Hz, H_{arom}), 4.06 (d, 2H, J = 7.18 Hz, CH_2), 3.46 (q, 4H, J = 7.08, CH_2), 2.22–2.12 (m, 1H, CH), 1.12 (t, 6H, J = 7.09, CH_3), 0.91 (d, 6H, J = 6.55 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 163.5, 153.8, 152.9, 135.0, 121.8, 121.3, 117.9, 111.2, 110.0, 108.5, 105.0, 104.4, 96.6, 95.9, 49.4, 44.1 (2C), 28.9, 19.9 (2C), 12.5 (2C); Anal. Calcd. for $C_{22}H_{28}N_4O$: C, 72.50; H, 7.74; N, 15.37; O, 4.39. Found: C, 72.48; H, 7.70; N, 15.32; O, 4.44%.

(E)-2-((4-(diethylamino)-2-hydroxybenzylidene)amino)-1-isobutyl-1H-benzo[d]imidazole-6-carbonitrile 31

Compound **31** was prepared from 2-amino-6-cyano-1-isobutylbenzimidazole **21** (0.15 g, 0.7 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.13 g, 0.7 mmol) in absolute ethanol (7 mL) after refluxing for 48 h to obtain 0.11 g (40%) of orange powder. m.p. 205–208°C; ^{1}H NMR (600 MHz, DMSO-d_6): δ/ppm = 12.62 (s, 1H, OH), 9.37 (s, 1H, H_{arom}), 8.01 (d, 1H, J = 1.09 Hz, H_{arom}), 7.74 (d, 1H, J = 8.26 Hz, H_{arom}), 7.59 (d, 1H, J = 8.97 Hz, H_{arom}), 7.56 (dd, 1H, J_1 = 1.48 Hz, J_2 = 8.98 Hz, H_{arom}), 6.44 (dd, 1H, J_1 = 2.29 Hz, J_2 = 8.99 Hz, H_{arom}), 6.18 (d, 1H, J = 2.27 Hz, H_{arom}), 4.10 (d, 2H, J = 7.36 Hz, CH_2), 3.45 (q, 4H, J = 6.97 Hz, CH_2), 2.20–2.12 (m, 2H, CH), 1.15 (t, 6H, J = 7.08 Hz, CH_3), 0.90 (d, 6H, J = 6.69 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 164.3, 153.9 (2C), 141.6, 138.7, 125.3, 122.6, 120.6, 111.8 (2C), 109.1, 105.9 (2C), 104.1, 97.0 (2C), 50.0, 44.7, 29.4, 20.3, 13.0; Anal. Calcd. for $C_{23}H_{27}N_5O$: C, 70.92; H, 6.99; N, 17.98; O, 4.11. Found: C, 70.97; H, 6.91; N, 17.93; O, 4.08%.

(E)-1-isobutyl-2-((4-nitrobenzylidene)amino)-1H-benzo[d]imidazole-6-carbonitrile 32

Compound **32** was prepared from 2-amino-6-cyano-1-isobutylbenzimidazole **21** (0.10 g, 0.5 mmol) and 4-nitrobenzaldehyde **27** (0.08 g, 0.5 mmol) in absolute ethanol (3 mL) after refluxing for 24 h to obtain 0.02 g (14%) of yellow powder. m.p. 277–279°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.66 (s, 1H, H_{arom}), 8.41–8.38 (m, 5H, H_{arom}), 8.19 (d, 1H, J = 0.98 Hz, H_{arom}), 7.88 (d, 1H, J = 8.46 Hz, H_{arom}), 7.67 (dd, 1H, J_1 = 1.47 Hz, J_2 = 8.38 Hz, H_{arom}), 4.31 (d, 2H, J = 7.28 Hz, CH_2), 2.25–2.13 (m, 1H, CH), 0.90 (d, 6H, J = 6.69 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 165.2, 156.2, 149.8, 140.6, 140.2, 138.2, 130.9 (2C), 125.7, 124.2 (2C), 123.9, 119.8, 112.4, 104.6, 49.6, 29.0, 19.8 (2C); Anal. Calcd. for $C_{19}H_{17}N_5O_2$: C, 65.69; H, 4.93; N, 20.16; O, 9.21. Found: C, 65.72; H, 4.87; N, 20.09; O, 9.34%.

(E)-N,N-dimethyl-4-(((1-methyl-1H-benzo[d]imidazol-2-yl)imino)methyl)aniline 33

Compound **33** was prepared from 2-amino-1-methylbenzimidazole **18** (0.10 g, 0.7 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.10 g, 0.7 mmol) in absolute ethanol (4 mL) after refluxing for 48 h to obtain 0.01 g (6%) of yellow powder. m.p. 186–188°C; ^{1}H NMR (600 MHz, DMSO-d_6): δ/ppm = 7.98–7.94 (m, 2H, H_{arom}), 7.41–7.36 (m, 2H, H_{arom}), 7.32–7.29 (m, 2H, H_{arom}), 7.18–7.15 (m, 3H, H_{arom}), 3.59 (s, 6H, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 190.4, 154.7, 132.0, 125.0, 123.8, 123.1, 112.2, 112.1, 111.6, 111.0, 110.4, 19.8, 9.1 (2C); Anal. Calcd. for $C_{17}H_{18}N_4$: C, 71.27; H, 5.65; N, 23.09. Found: C, 71.30; H, 5.61; N, 23.16%.

(E)-2-((4-(dimethylamino)benzylidene)amino)-1-methyl-1H-benzo[d]imidazole-6-carbonitrile 34

Compound **34** was prepared from 2-amino-6-cyano-1-methylbenzimidazole **22** (0.10 g, 0.6 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.09 g, 0.6 mmol) in absolute ethanol (3 mL) after refluxing for 48 h to obtain 0.05 g (27%) of yellow powder. m.p. 222–226°C; ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 9.27 (s, 1H, H_{arom}), 8.01 (d, 1H, J = 1.26 Hz, H_{arom}), 7.95 (d, 2H, J = 8.81 Hz, H_{arom}), 7.68 (d, 1H, J = 8.32 Hz, H_{arom}), 7.58 (dd, 1H, J_1 = 8.28, J_2 = 1.37 Hz, H_{arom}), 6.84 (d, 2H, J = 8.91 Hz, H_{arom}), 3.88 (s, 3H, CH_3), 3.08 (s, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 166.4, 158.9, 154.3, 141.7, 139.0, 132.8, 125.2, 122.9, 122.8, 122.6, 120.7, 118.2, 112.0, 111.6, 108.8, 104.1, 29.5 (2C), 29.1; Anal. Calcd. for $C_{18}H_{17}N_5$: C, 71.27; H, 5.65; N, 23.09. Found: C, 71.30; H, 5.61; N, 23.16%.

(E)-5-(diethylamino)-2-(((1-methyl-1H-benzo[d]imidazol-2-yl)imino)methyl)phenol 35

Compound **35** was prepared from 2-amino-1-methylbenzimidazole **18** (0.15 g, 1.0 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.19 g, 1.0 mmol) in absolute ethanol (7 mL) after refluxing for 24 h to obtain 0.04 g (13%) of orange powder. m.p. 169–173°C; ^{1}H NMR (600 MHz, DMSO-d_6): δ/ppm = 12.61 (s, 1H, OH), 9.36 (s, 1H, H_{arom}), 7.58 (d, 1H, J = 8.87 Hz, H_{arom}), 7.54–7.51 (m, 1H, H_{arom}), 7.50–7.47 (m, 1H, H_{arom}), 7.21–7.15 (m, 2H, H_{arom}), 6.42 (dd, 1H, J_1 = 2.38 Hz, J_2 = 8.99 Hz, H_{arom}), 6.16 (d, 1H, J = 2.26 Hz, H_{arom}), 3.78 (s, 3H, CH_3), 3.45 (q, 4H, J = 7.08 Hz, CH_2), 1.15 (t, 6H, J = 7.07 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 164.5, 163.9, 153.4, 141.9 (2C), 135.8, 122.3, 121.7, 118.3, 110.2, 109.1, 105.5, 97.0, 44.6 (2C), 29.1, 13.0 (2C); Anal. Calcd. for $C_{19}H_{22}N_4O$: C, 70.78; H, 6.88; N, 17.38; O, 4.96. Found: C, 70.71; H, 6.79; N, 17.44; O, 5.03%.

(E)-2-((4-(diethylamino)-2-hydroxybenzylidene)amino)-1-methyl-1H-benzo[d]imidazole-6-carbonitrile 36

Compound **36** was prepared from 2-amino-6-cyano-1-methylbenzimidazole **22** (0.20 g, 1.2 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.22 g, 1.2 mmol) in absolute ethanol (10 mL) after refluxing for 48 h to obtain 0.27 g (67%) of yellow powder. m.p. 227–231°C; ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 12.40 (s, 1H, OH), 9.37 (s, 1H, H_{arom}), 7.99 (d, 1H, J = 1.08 Hz, H_{arom}), 7.68 (d, 1H, J = 8.29 Hz, H_{arom}), 7.61 (d, 1H, J = 8.97 Hz, H_{arom}), 7.57 (dd, 1H, J_1 = 1.45 Hz, J_2 = 8.27 Hz, H_{arom}), 6.43 (dd, 1H, J_1 = 2.36 Hz, J_2 = 9.00 Hz, H_{arom}), 6.15 (d, 1H, J = 2.26 Hz, H_{arom}), 3.80 (s, 3H, CH_3), 3.45 (q,

4H, J = 6.98 Hz, CH_2), 1.15 (t, 6H, J = 6.95 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 165.3, 164.2, 157.6, 153.9, 141.7, 138.9, 125.2, 122.4, 120.7, 111.5, 109.2, 105.9, 104.1, 96.9, 44.7 (2C), 29.5, 13.0 (2C); Anal. Calcd. for $C_{20}H_{21}N_5O$: C, 69.14; H, 6.09; N, 20.16; O, 4.61. Found: C, 69.19; H, 5.98; N, 20.13; O, 4.71%.

(E)-1-methyl-2-((4-nitrobenzylidene)amino)-1H-benzo[d]imidazole-6-carbonitrile 37

Compound **37** was prepared from 2-amino-6-cyano-1-methylbenzimidazole **22** (0.10 g, 0.5 mmol) and 4-nitrobenzaldehyde **27** (0.08 g, 0.5 mmol) in absolute ethanol (3 mL) after refluxing for 24 h to obtain 0.02 g (14%) of yellow powder. m.p. 277–279°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.67 (s, 1H, H_{arom}), 8.44–8.41 (m, 4H, H_{arom}), 8.20 (d, 1H, J = 0.97 Hz, H_{arom}), 7.83 (d, 1H, J = 8.38 Hz, H_{arom}), 7.69 (dd, 1H, J_1 = 1.49 Hz, J_2 = 8.37 Hz, H_{arom}), 3.99 (s, 3H, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 165.9, 156.7, 150.4, 141.2, 140.7, 139.1, 131.5 (2C), 126.2, 124.7 (2C), 124.3, 120.3, 112.6, 105.1, 29.9; Anal. Calcd. for $C_{16}H_{11}N_5O_2$: C, 62.95; H, 3.63; N, 22.94; O, 10.48. Found: C, 62.91; H, 3.69; N, 22.88; O, 10.41%.

(E)-N,N-dimethyl-4-(((1-phenyl-1H-benzo[d]imidazol-2-yl)imino)methyl)aniline 38

Compound **38** was prepared from 2-amino-1-phenylbenzimidazole **19** (0.10 g, 0.5 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.07 g, 0.5 mmol) in absolute ethanol (3 mL) after refluxing for 48 h to obtain 0.11 g (68%) of yellow powder. m.p. 204–207°C; ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 9.29 (s, 1H, H_{arom}), 7.76 (d, 2H, J = 8.86 Hz, H_{arom}), 1.66–1.57 (m, 5H, H_{arom}), 1.54–1.49 (m, 1H, H_{arom}), 7.30 (d, 1H, J = 7.89 Hz, H_{arom}), 7.28–7.24 (m, 1H, H_{arom}), 7.22–7.17 (m, 1H, H_{arom}), 6.79 (d, 2H, J = 8.87 Hz, H_{arom}), 3.04 (s, 6H, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 165.5, 156.2, 153.9, 142.1, 135.9, 135.6, 132.3, 129.7 (2C), 128.2, 127.4 (2C), 123.1, 122.9, 122.6, 118.9, 112.0, 110.4; Anal. Calcd. for $C_{22}H_{20}N_4$: C, 77.62; H, 5.92; N, 16.46. Found: C, 77.68; H, 5.98; N, 16.39%.

(E)-2-((4-(dimethylamino)benzylidene)amino)-1-phenyl-1H-benzo[d]imidazole-6-carbonitrile 39

Compound **39** was prepared from 2-amino-6-cyano-1-phenylbenzimidazole **23** (0.13 g, 0.6 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.09 g, 0.6 mmol) in absolute ethanol (6 mL) after refluxing for 24 h to obtain 0.04 g (17%) of yellow powder. m.p. 247–251°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.29 (s, 1H, H_{arom}), 8.14 (d, 1H, J = 1.09 Hz, H_{arom}), 7.75 (d, 2H, J = 8.97 Hz, H_{arom}), 7.66–7.62 (m, 2H, H_{arom}), 7.62–7.59 (m, 2H, H_{arom}), 7.57 (dd, 1H, J_1 = 1.47 Hz, J_2 = 8.28 Hz, H_{arom}), 7.56–7.54 (m, 1H, H_{arom}), 7.41 (d, 1H, J = 8.27 Hz, H_{arom}), 6.78 (d, 2H, J = 8.98 Hz, H_{arom}), 3.05 (s, 6H, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 167.1, 158.6, 154.4, 141.9, 138.6, 135.0, 129.8 (2C), 128.8, 127.5 (2C), 126.1, 123.2, 122.6, 120.4, 112.0, 111.7, 105.0; Anal. Calcd. for $C_{23}H_{19}N_5$: C, 75.59; H, 5.24; N, 19.16. Found: C, 75.65; H, 5.30; N, 19.09%.

(E)-5-(diethylamino)-2-(((1-phenyl-1H-benzo[d]imidazol-2-yl)imino)methyl)phenol 40

Compound **40** was prepared from 2-amino-1-phenylbenzimidazole **19** (0.10 g, 0.5 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.09 g, 0.5 mmol) in absolute ethanol (7 mL) after refluxing for 48 h to obtain 0.10 g (52%) of yellow powder. m.p. 197–201°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ/ppm = 12.40 (s, 1H, OH), 9.37 (s, 1H, H_{arom}), 7.67–7.62 (m, 3H, H_{arom}), 7.60–7.53 (m, 3H, H_{arom}), 7.50 (d, 1H, J = 8.97 Hz, H_{arom}), 7.29–7.22 (m, 2H, H_{arom}), 7.21–7.17 (m, 1H, H_{arom}), 6.39 (dd, 1H, J_1 = 2.37 Hz, J_2 = 8.99 Hz, H_{arom}), 6.04 (d, 1H, J = 2.27 Hz, H_{arom}), 3.41 (q, 4H, J = 7.54 Hz, CH_2), 1.11 (t, 6H, J = 6.88 Hz, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 164.4, 163.5, 153.0, 141.6, 135.6, 135.1, 135.0, 129.7 (2C), 128.2, 126.9 (2C), 122.6, 122.1, 118.3, 109.7, 108.5, 105.0, 96.5, 44.1, 12.5; Anal. Calcd. for $C_{24}H_{24}N_4O$: C, 74.97; H, 6.29; N, 14.57; O, 4.16. Found: C, 74.89; H, 6.19; N, 14.51; O, 4.20%.

(E)-2-((4-(diethylamino)-2-hydroxybenzylidene)amino)-1-phenyl-1H-benzo[d]imidazole-6-carbonitrile 41

Compound **41** was prepared from 2-amino-6-cyano-1-phenylbenzimidazole **23** (0.20 g, 0.9 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.16 g, 0.9 mmol) in absolute ethanol (10 mL) after refluxing for 48 h to obtain 0.23 g (66%) of yellow powder. m.p. 111–114°C; ^{1}H NMR (400 MHz, DMSO-d_6) (δ/ppm): 12.28 (bs, 1H, OH), 9.37 (s, 1H, H_{arom}), 8.13–8.11 (m, 1H, H_{arom}), 7.68–7.64 (m, 2H, H_{arom}), 7.61–7.59 (m, 3H, H_{arom}), 7.56 (dd, 1H, J_1 = 8.28 Hz, J_2 = 1.56 Hz, H_{arom}), 7.51 (d, 1H, J = 9.09 Hz, H_{arom}), 7.35 (d, 1H, J = 8.37 Hz, H_{arom}), 6.40 (dd, 1H, J_1 = 9.08 Hz, J_2 = 2.40 Hz, H_{arom}), 6.04 (d, 1H, J = 2.28 Hz, H_{arom}), 3.44–3.39 (m, 1H, CH_2), 1.11 (t, 6H, J = 7.01 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 165.8, 164.4, 156.8, 154.0, 142.0, 138.6, 136.4, 134.7, 130.3, 129.3, 127.8, 126.2, 122.9, 120.4, 111.5, 109.1, 105.9, 105.0, 96.9, 44.7 (2C), 13.0 (2C); Anal. Calcd. for $C_{25}H_{23}N_5O$: C, 73.33; H, 5.66; N, 17.10; O, 3.91. Found: C, 73.36; H, 5.69; N, 17.15; O, 4.02%.

(E)-4-(((1-hexyl-1H-benzo[d]imidazol-2-yl)imino)methyl)-N,N-dimethylaniline 42

Compound **42** was prepared from 2-amino-1-hexylbenzimidazole **20** (0.20 g, 0.9 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.13 g, 0.9 mmol) in absolute ethanol (5 mL) after refluxing for 24 h to obtain 0.09 g (31%) of yellow powder in the form of a mixture of *E*- and *Z*-isomers at the ratio of **42a**/**42b** = 3:1. m.p. 231–234°C; **42a**: ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.26 (s, 1H, H_{arom}), 7.91 (d, 2H, J = 8.88 Hz, H_{arom}), 7.55–7.51 (m, 1H, H_{arom}), 7.51–7.47 (m, 1H, H_{arom}), 7.19–7.14 (m, 2H, H_{arom}), 6.84 (d, 2H, J = 8.99 Hz, H_{arom}), 4.35 (t, 2H, J = 6.88 Hz, CH_2), 3.07 (s, 6H, CH_3), 1.80–1.75 (m, 2H, CH_2), 1.29–1.17 (m, 6H, CH_2), 0.79 (t, 3H, J = 7.27 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 165.8, 153.8, 137.8, 124.7, 122.4 (2C), 122.4, 122.2, 120.1, 117.7, 111.5 (2C), 111.1 (2C), 108.4, 103.6, 41.9, 30.5, 28.9, 25.5, 21.9, 13.8; **42b**: ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.67 (s, 1H, H_{arom}), 7.69 (d, 2H, J = 8.97 Hz, H_{arom}), 7.35 (d, 1H, J = 8.36 Hz, H_{arom}), 7.26 (dd, 1H, J_1 = 1.98 Hz, J_2 = 6.67 Hz, H_{arom}), 7.13–7.08 (m, 2H, H_{arom}), 6.79 (d, 2H, J =

8.86 Hz, H_{arom}), 4.03 (t, 2H, *J* = 7.29 Hz, CH_2), 3.05 (s, 6H, CH_3), 1.67–1.62 (m, 2H, CH_2), 1.29–1.17 (m, 6H, CH_2), 0.84 (t, 3H, *J* = 6.97 Hz, CH_3); ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 156.6, 142.8, 141.2 (2C), 132.1, 123.0, 128.1, 122.4 (2C), 120.7, 117.7 (2C), 111.1, 111.0, 102.0, 41.6, 30.9, 28.2, 25.6, 22.0, 13.8; Anal. Calcd. for $C_{22}H_{28}N_4$: C, 75.82; H, 8.10; N, 16.08. Found: C, 75.78; H, 8.16; N, 16.14%.

(E)-2-((4-(dimethylamino)benzylidene)amino)-1-hexyl-1H-benzo[d]imidazole-6-carbonitrile 43

Compound **43** was prepared from 2-amino-6-cyano-1-hexylbenzimidazole **24** (0.15 g, 0.6 mmol) and 4-*N,N*-dimethylamino-benzaldehyde **25** (0.09 g, 0.6 mmol) in absolute ethanol (5 mL) after refluxing for 48 h to obtain 0.09 g (43%) of yellow powder. m.p. 165–170°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.27 (s, 1H, H_{arom}), 8.02 (d, 1H, *J* = 0.88 Hz, H_{arom}), 7.95 (s, 1H, H_{arom}), 7.92 (s, 1H, H_{arom}), 7.72 (d, 1H, *J* = 8.37 Hz, H_{arom}), 7.56 (dd, 1H, J_1 = 1.39 Hz, J_2 = 8.28 Hz, H_{arom}), 7.30 (d, 1H, *J* = 1.38 Hz, H_{arom}), 6.86–6.82 (m, 2H, H_{arom}), 4.39 (t, 2H, *J* = 6.79 Hz, CH_2), 3.08 (s, 6H, CH_3), 1.83–1.72 (m, 2H, CH_2), 1.27–1.20 (m, 6H, CH_2), 0.77 (t, 3H, *J* = 6.97 Hz, CH_3); Anal. Calcd. for $C_{23}H_{27}N_5$: C, 73.96; H, 7.29; N, 18.75. Found: C, 74.01; H, 7.24; N, 18.70%.

(E)-5-(diethylamino)-2-(((1-hexyl-1H-benzo[d]imidazol-2-yl)imino)methyl)phenol 44

Compound **44** was prepared from 2-amino-1-hexylbenzimidazole **20** (0.15 g, 0.7 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.13 g, 0.7 mmol) in absolute ethanol (5 mL) after refluxing for 48 h to obtain 0.03 g (11%) of yellow powder. m.p. 201–204°C; ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 12.68 (s, 1H, OH), 9.36 (s, 1H, H_{arom}), 7.56 (d, 1H, *J* = 8.96 Hz, H_{arom}), 7.54–7.48 (m, 2H, H_{arom}), 7.21–7.13 (m, 2H, H_{arom}), 6.42 (dd, 1H, J_1 = 2.37 Hz, J_2 = 8.97 Hz, H_{arom}), 6.16 (d, 1H, *J* = 2.27 Hz, H_{arom}), 4.24 (t, 2H, *J* = 7.07 Hz, CH_3), 3.44 (q, 4H, *J* = 7.09 Hz, CH_2), 1.78–1.73 (m, 2H, CH_2), 1.29–1.20 (m, 6H, CH_2), 1.15 (t, 6H, *J* = 7.08 Hz, CH_3), 0.80 (t, 3H, *J* = 7.07 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) (δ/ppm): 164.0, 163.9, 154.6, 154.3, 153.4, 141.9, 135.1, 122.2, 121.8, 118.4, 111.7, 110.2, 109.0, 105.5, 104.9, 97.0, 96.4, 44.6, 44.6, 42.6, 31.2, 29.6, 26.4, 22.5, 14.2, 13.0, 12.9; Anal. Calcd. for $C_{24}H_{32}N_4O$: C, 73.43; H, 8.22; N, 14.27; O, 4.08. Found: C, 73.37; H, 8.09; N, 14.15; O, 4.02%.

(E)-2-((4-(diethylamino)-2-hydroxybenzylidene)amino)-1-hexyl-1H-benzo[d]imidazole-6-carbonitrile 45

Compound **45** was prepared from 2-amino-6-cyano-1-hexylbenzimidazole **24** (0.15 g, 0.6 mmol) and 4-*N,N*-diethylamino-2-hydroxybenzaldehyde **26** (0.11 g, 0.6 mmol) in absolute ethanol (6 mL) after refluxing for 48 h to obtain 0.02 g (8%) of yellow powder. m.p. 233–237°C; ^{1}H NMR (600 MHz, DMSO-d_6): δ/ppm = 12.47 (s, 1H, OH), 9.38 (s, 1H, H_{arom}), 8.00 (d, 1H, *J* = 1.19 Hz, H_{arom}), 7.72 (d, 1H, *J* = 8.27 Hz, H_{arom}), 7.60 (d, 1H, *J* = 8.99 Hz, H_{arom}), 7.56 (dd, 1H, J_1 = 1.45 Hz, J_2 = 8.27 Hz, H_{arom}), 6.44 (dd, 1H, J_1 = 2.37 Hz, J_2 = 8.96 Hz, H_{arom}), 6.16 (d, 1H, *J* = 2.28 Hz, H_{arom}), 4.28 (t, 2H, *J* = 7.06 Hz, CH_2), 3.45 (q, 4H, *J* = 6.98 Hz, CH_2), 1.76–1.72 (m, 2H, CH_2), 1.28–1.25 (m, 4H, CH_2), 1.23–1.20 (m, 2H, CH_2), 1.15 (t, 6H, *J* = 7.09 Hz, CH_3), 0.80 (t, 3H, *J* = 7.08 Hz, CH_3), ^{13}C NMR (151 MHz, DMSO-d_6): δ/ppm = 164.3, 153.9, 141.7, 138.3, 125.2, 122.6, 120.6, 111.5, 109.1, 105.9 (2C), 104.1, 96.9 (2C), 44.7 (2C), 42.9, 31.1, 29.5, 26.3, 22.4, 14.2, 13.0; Anal. Calcd. for $C_{25}H_{31}N_5O$: C, 71.91; H, 7.48; N, 16.77; O, 3.83. Found: C, 71.94; H, 7.42; N, 16.71; O, 3.88%.

4.2. Biology

4.2.1. Antiviral activity

HEL 299 (ATCC CCL-137; human lung fibroblast), Huh-7 (CLS – 300156; human hepatoblastoma), and MDCK (Madin-Darby canine kidney cells; a kind gift from M. Matrosovich, Marburg, Germany) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) supplemented with 8% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences), 0.075% sodium bicarbonate (Gibco Life Technologies) and 1mM sodium pyruvate (Gibco Life Technologies), and maintained at 37°C under 5% CO_2. Antiviral assays towards herpes simplex virus-1 (HSV-1 KOS), human coronavirus (HCoV-229E and -OC43) and respiratory syncytial virus A in HEL 299 cell cultures, sindbis virus, yellow fever virus, Zika virus and human coronavirus (HCoV-NL63) in Huh-7 cell cultures and influenza A/H1N1 (A/Ned/378/05), influenza A/H3N2 (A/HK/7/87), influenza B (B/Ned/537/05) in MDCK cell cultures were performed. On the day of the infection, growth medium was aspirated and replaced by serial dilutions of the test compounds. The virus was then added to each well, diluted to obtain a viral input of 100 $CCID_{50}$ ($CCID_{50}$ being the virus dose that is able to infect 50% of the cell cultures). Mock-treated cultures receiving solely the test compounds were included, to determine the cytotoxicity.

After 3 to 7 days of incubation, the virus-induced cytopathogenic effect was measured colorimetrically by the formazan-based MTS cell viability assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega, Madison, WI), and the antiviral activity was expressed as the 50% effective concentration (EC_{50}). In parallel, the 50% cytotoxic concentration (CC_{50}) was derived from the mock-infected cells. The activities were compared with the activities of reference antiviral drugs: remdesivir, ribavirin, zanamivir, rimantadine and brivudine (BVDU).

4.2.2. Antibacterial Activity

4.2.2.1. Materials

In addition to the synthesized compounds, standard antibiotics ampicillin, ceftazidime, ciprofloxacin and meropenem from USP were tested. Selected bacterial strains were gram negative *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* and gram positive *S. aureus*, S. *pneumoniae* and *E. faecalis*. Synthesized compounds were prepared as 10 mM DMSO solutions and tested in a final concentration range of 100 − 0.2 μM [37].

Standard antibiotics were prepared as 5 mg/mL DMSO solutions and tested in a final concentration range of 64–0.125 µg/mL.

4.2.2.2. Methods

Broth microdilution testing was performed according to CLSI (Clinical Laboratory Standards Institute) guidelines. MIC (minimal inhibitory concentration) value was defined as the last tested concentration of compound at which there is no visible growth of bacteria. Inoculums for each microorganism were prepared using the direct colony suspension method where broth solutions that achieved turbidity equivalent to 0.5 McFarland standard were additionally diluted 100x with Ca adjusted MH media (Becton Dickinson). All test plates were incubated for 16–24h at 37°C.

MIC values for reference antibiotics against quality control strains were used for confirming the validity of the screen according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically M07, 11th edition, 2018. and Clinical and Laboratory Standards Institute (CLSI) guidelines. Performance standards for antimicrobial susceptibility testing M100, 28th edition, 2018.

4.2.3 Cell Culture And Reference Compounds

Human cancer cells used in this manuscript, namely Capan-1, HCT-116, NCI-H460, LN-229, HL-60, K-562 and Z-138 were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the DND-41 cell line was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ Leibniz-Institut, Germany). Culture media were purchased from Gibco Life Technologies, USA, and supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences, USA). Vincristine and *docetaxel*, which were used as reference inhibitors, were purchased from Selleckchem (Munich, Germany). Stock solutions were prepared in DMSO.

4.2.4 Proliferation Assays

Adherent cell lines LN-229, HCT-116, NCI-H460 and Capan-1 cells were seeded at a density between 500 and 1500 cells per well, in 384-well tissue culture plates (Greiner). After overnight incubation, cells were treated with seven different concentrations of the test compounds, ranging from 100 to 0.006µM. Suspension cell lines HL-60, K-562, Z-138 and DND-41 were seeded at densities ranging from 2500 to 5500 cells per well in 384-well culture plates containing the test compounds at the same concentration points. Cells were incubated for 72 hours with compounds and were then analysed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) reagent (Promega) according to the manufacturer's instructions.

Absorbance of the samples was measured at 490 nm using a SpectraMax Plus 384 (Molecular Devices), and OD values were used to calculate the 50% inhibitory concentration (IC_{50}). Compounds were tested in two independent experiments.

Declarations

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Conflict of interests

The authors declare no conflict of interest.

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Scheme

Scheme 1 is available in supplementary section.

Figures

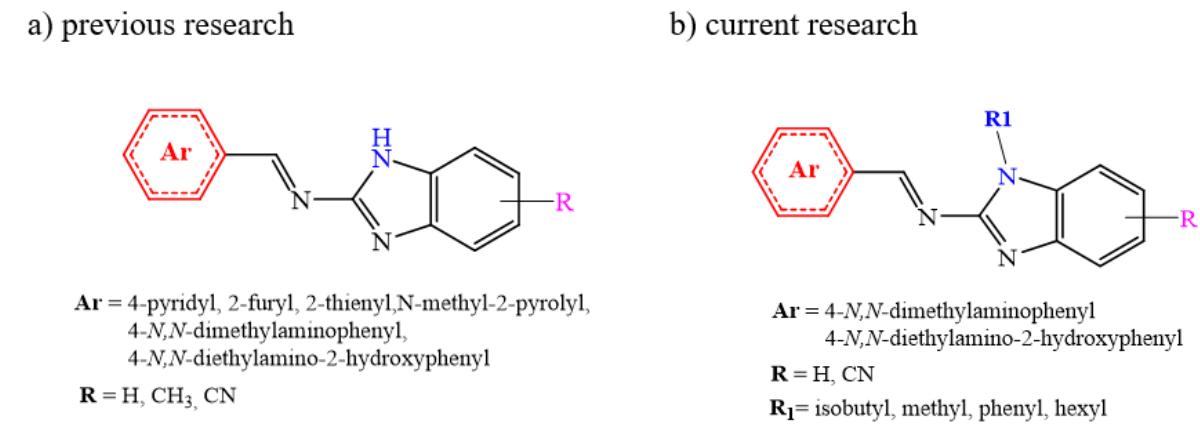

Figure 1

Synthesized benzimidazole derived Schiff bases

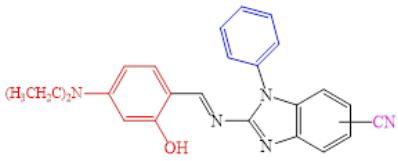

Figure 2

Selected Schiff base **40** as a lead compound for further optimization

Supplementary Files

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- Scheme1.png

- SupportingMaterial.docx