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Synthesis and biological evaluation of a new series of benzimidazole derivatives as antimicrobial, antiquorum-sensing and antitumor agents

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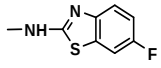
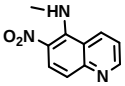
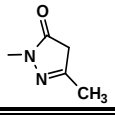
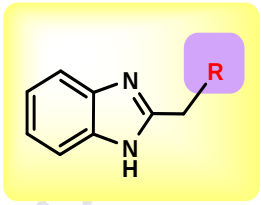
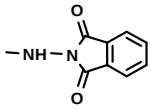
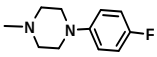
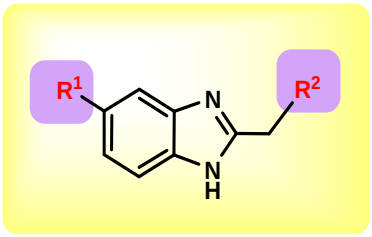
3c: R=		***C. albicans*:** MIC = 78.125 µg/mL (0.262 mM) ***S. aureus*:** MIC = 156.25 µg/mL (0.524 mM)
3i: R=		***B. cereus:*** MIC = 156.25 µg/mL (0.489 mM)
3n: R=		***S. aureus*:** MIC = 156.25 µg/mL (0.684 mM)



The most active antimicrobial analogs

Comp. No.	R^1	R^2	IC_{50} (mM)		
			HepG2	HCT-116	MCF-7
3f	H		0.032	0.031	0.037
3p	NO_2		0.022	0.014	0.015



The most potent antitumor analogs

Synthesis and biological evaluation of a new series of benzimidazole derivatives as antimicrobial, antiquorum-sensing and antitumor agents

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Abstract: New benzimidazole derivatives were synthesized and assessed for antimicrobial efficacy toward *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus fumigatus* 293. Results indicated that compounds **3c** and **3n** have promising activity toward *S. aureus*, whereas **3i** and **3j** exhibited remarkable efficacy toward *B. cereus*. Moreover, compound **3c** was proved to be the most active antifungal analog toward *C. albicans*. On the other hand, **3n** displayed the highest activity against *A. fumigatus* 293. Antiquorum-sensing activity of the same compounds was also tested against *Chromobacterium violacium* ATCC 12472, whereas compounds **3c-f, 3i-k** and **3m-o** showed acceptable activity. *In vitro* antitumor testing of these compounds toward liver cancer (HepG2), colon cancer (HCT-116) and breast cancer (MCF-7) cell lines revealed that compound **3p** has the highest potency against the three tested cell lines. Moreover, **3f**, **3m** and **3n** displayed promising activity toward all tested cell lines. Compounds **3f**, **3m, 3n** and **3p** were esteemed for their *in vivo* antitumor activity against EAC cells. The active antimicrobial and antitumor analogs, **3a, 3c, 3f, 3i-k, 3m, 3n** and **3p** were assessed for DNA-binding affinity, and results indicated that **3c, 3f, 3i, 3k** and **3n** have strong DNA-binding affinity. The computational studies affirmed that almost all of the inspected compounds meet the optimal requirements for good absorption and oral bioavailability.

Keywords: Synthesis, Benzimidazoles, Antimicrobial screening, Antiquorum-sensing screening, Antitumor screening, Cytotoxicity testing, DNA-binding assay, Computational studies

1. Introduction

High prevalence of antimicrobial resistance represents a serious concern worldwide [1]. The excessive use and misuse of antimicrobial drugs have contributed to a high extent in the development of microbial resistance, and hence the protective value of antimicrobial agents is decreased [2]. Therefore, new antimicrobial strategies are required to overcome pathogenesis and to prevent further development of drug resistance. A promising approach to combate bacterial resistance is through targeting virulence factors, e.g., quorum-sensing (QS). QS is a cell communication process, it controls different cellular activities like symbiosis, virulence, antibiotic production, motility and biofilm formation [3]. Therefore, it is an attractive target for treatment of bacterial pathogenicity [4]. QS inhibitors cause a significant reduction in the expression of QS-controlled genes without affecting cell growth and division, and hence the selective pressure for the evolution of resistance is minimized [5]. Based on these facts, many research groups are focused on the discovery of new antipathogenic drugs that inhibit QS.

In addition, cancer remains a major health threat, and it is considered to be the second leading cause of death globally after heart diseases [6]. It is characterized by the unregulated growth and metastasis of the abnormal cancer cells. Metastasis is the primary cause of death when cancer treatment fails [7]. Cancer therapeutics includes radiation therapy, cell based immunotherapy, gene therapy and chemotherapy. Ideal anticancer drugs would kill cancer cells and disrupt some aspects of cell division without harming normal tissues. Therefore, there is an imperious need for the development of new treatment approaches, especially the discovery of new potent chemotherapeutics with minimal adverse effects.

Compounds containing benzimidazole as a structural motif have been vastly utilized in medicinal chemistry and drug development. 2-Chloromethyl-1*H*-benzimidazoles are amongst the benzimidazole derivatives of considerable importance in biological chemistry. They are precious intermediates in the preparation of a wide variety of biologically active compounds such as antibacterial [8-12], antifungal [8-10,13-15], anthelmintic [10], antiviral [16], anti-inflammatory [17,18], analgesic [17] and anticancer agents [19,20]. In addition, literature survey revealed that substituted 2-(mercaptomethyl) benzimidazoles have been implemented as fungicidal [21] and anti-inflammatory agents [22]. Regarding these findings, and as a continuation to our previous work [23-27], a new series of benzimidazole derivatives **3a-p** was designed and synthesized. Our design strategy was based on introducing substituted aminomethyl, substituted pyrazol-1-yl methyl, and substituted piperazin-1-yl methyl groups at the 2-position of benzimidazole nucleus to study their effect on antimicrobial, antitumor and cytotoxic activities. DNA-binding affinity of compounds **3a, 3c, 3f, 3i-k, 3m, 3n** and **3p** was also examined to investigate their possible mode of action. A detailed study of the structure-activity relationship (SAR) of the new analogs will pave the road toward the design of more potent compounds.

2. Results and Discussion

2.1. Chemistry

The reaction of 2-chloromethylbenzimidazoles with aromatic or heteroaromatic amines was reported in *N*,*N*-dimethylformamide (DMF) in presence of K_2CO_3 [8], in DMF in presence of K_2CO_3 and KI [13], in ethanol [16], in ethanol in presence of KOH [28], or in ethanol in presence of KOH and KI [14,15,17,20] to yield the 2-(aryl/heteroarylamino)methylbenzimidazoles. In the current study, a straightforward, efficient and reproducible method with simple work-up procedure was followed for the preparation of the new benzimidazoles **3a-p** using 2-chloromethyl-1*H*-benzimidazoles **2a,b** [13] as starting materials (Scheme 1). This method involves the reaction of the appropriate 2-chloromethyl-1*H*-benzimidazole **2a,b** with the appropriate arylamine, ethyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate [29], 3-methyl-1*H*-pyrazol-5(4*H*)-one [30] or 1-(4-fluorophenyl)piperazine in DMF in presence of triethylamine to produce 2-(arylamino)methyl-5-(un)substituted benzimidazoles **3a-l** in moderate to good yields (55-85%), ethyl cyclohepta[*b*]thiophene-3-carboxylate analog **3m** in reasonable yield (60%), pyrazol-5(4*H*)-one derivatives **3n,o** in 50% and 65% yields, respectively, and benzimidazole derivative **3p** in good yield **(**75%). The structures of the new benzimidazoles **3a-p** were ascertained by elemental analyses, ^{1}H & ^{13}C NMR and mass spectroscopy. ^{1}H NMR spectra showed a characteristic singlet at δ 3.66-4.78 ppm integrated for two protons of CH_2 group at 2-position of benzimidazole. In addition, ^{13}C NMR spectra displayed a significant signal at δ 40.4-56.0 ppm for CH_2 carbon at 2-position of benzimidazole.

2.2. Biological evaluation

2.2.1. Antimicrobial and antiquorum-sensing evaluation

Compounds **3a-p** were estimated for *in vitro* antimicrobial efficacy toward Gram -ve bacterium (*Escherichia coli*), Gram +ve bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and pathogenic fungi (*Candida albicans* and *Aspergillus fumigatus* 293) using amoxicillin as a standard antibacterial antibiotic and fluconazole as a standard antifungal agent.

The screening was done employing the two-fold serial dilution method [25,26,31-33] and the minimal concentrations of the compounds that inhibit microbial growth (MICs, µg/mL and mM) was detected visually (no turbidity).

Results (Table 1) illustrated that *C. albicans* and *A. fumigatus* 293 are sensitive to majority of the tested compounds. Particularly, compound **3c** showed remarkable antifungal efficacy toward *C. albicans*, while **3n** displayed the highest efficacy toward *A. fumigatus*. It is noteworthy that compounds **3c** and **3n** have promising efficacy toward *S. aureus*, whereas **3i** and **3j** exhibited prominent efficacy toward *B. cereus*.

The same analogs were assessed for antiquorum-sensing efficacy toward *Chromobacterium violaceum* ATCC 12472 using catechin as a positive control [24,25,34]. QS system of *C. violaceum* releases violacein (a violet pigment) in response to signaling molecules known as acyl homoserine lactones [35,36]. Therefore, drugs that disrupt the activity of QS in *C. violaceum* will inhibit violacein production. QS inhibition was calculated by subtracting the radius of bacterial growth inhibition (r_1) from the total radius of both growth and pigment inhibition (r_2); therefore, QS inhibition = (r_2-r_1) in mm. Results illustrated that compounds **3c-f, 3i-k** and **3m-o** have anti-QS efficacy (Table 2).

2.2.1.1. Structure-activity relationship

Correlation of the obtained results of antimicrobial activity of compounds **3a-p** and structure variations was studied and revealed that introduction of thiadiazol-2-yl moiety into the unsubstituted benzimidazole nucleus enhanced the efficacy toward *E. coli, S. aureus* and *C. albicans* compared to its counterpart 5-nitrobenzimidazole (**3a** versus **3b**). Moreover, incorporation of benzothiazol-2-yl moiety into the unsubstituted benzimidazole nucleus led to an obvious increase in activity toward *S. aureus, C. albicans* and *A. fumigatus* compared to its counterpart 5-nitrobenzimidazole (**3c** versus **3d**). In addition, replacement of thiadiazol-2-yl moiety in **3a** with benzothiazol-2-yl counterpart led to improved activity toward *S. aureus, C. albicans* and *A. fumigatus* (compound **3c**). On the other hand, replacing thiadiazol-2-yl moiety in **3b** with benzothiazol-2-yl counterpart led to increased activity against *A. fumigatus* (compound **3d**). Also, incorporation of 6-nitroquinolin-5-yl moiety into the unsubstituted benzimidazole nucleus **3i** increased the activity toward all tested microorganisms compared to its 5-nitrobenzimidazole counterpart **3j**. Similarly, incorporation of 3-methyl-5-oxopyrazol-1-yl or 2,3-dimethyl-5-oxo-1-phenylpyrazol-4-yl moieties into the unsubstituted benzimidazole nucleus **3k** and **3n**, respectively resulted in remarkable activity against *S. aureus, C. albicans* and *A. fumigatus* compared to the corresponding 5-nitrobenzimidazole derivatives **3l** and **3o**, respectively. These results emphasized the significant contribution of the unsubstituted benzimidazole nucleus to the antimicrobial activity compared to the 5-nitrobenzimidazole counterpart. On contrary, incorporation of cyclohepta[*b*]thiophen-2-yl moiety into the unsubstituted benzimidazole nucleus did not contribute to the antimicrobial activity (compound **3m**).

2.2.2. In vitro antitumor evaluation

Compounds **3a-p** were subjected to *in vitro* antitumor testing against three different cell lines; liver cancer (HepG2), colon cancer (HCT-116) and breast cancer (MCF-7) cell lines employing MTT assay [37-39]. The concentrations of the compounds and 5-fluorouracil (reference drug) required to inhibit 50% of cell viability (IC_{50}, mM) were calculated. Results (Table 3) indicated that compound **3p** has the highest potency against the three selected cell lines. Also, **3f, 3m** and **3n** displayed promising activity toward all tested cell lines, whereas **3f** and **3m** were proved to be relatively more potent than 5-

fluorouracil against the three tested cell lines. In addition, **3g**, **3h**, **3k** and **3l** exhibited eminent efficacy toward HCT-116 cell line, while **3g** and **3k** showed reasonable activity toward MCF-7.

2.2.2.1. Structure-activity relationship

The distance between the benzimidazole nucleus and the aryl moiety is critical for antitumor activity. Extending the spacer length between the 5-nitrobenzimidazole nucleus and the aryl moiety enhances the antitumor activity, compound **3p** that possesses five atoms spacer has optimal broad spectrum activity (higher than that of 5-fluorouracil) compared to the other members of the new series. Moreover, incorporation of thiadiazol-2-yl, benzothiazol-2-yl, 2,3-dihydronaphthalen-4-ylidene, 6-nitroquinolin-5-yl, 2,3-dimethyl-5-oxo-1-phenylpyrazol-4-yl and 3-methyl-5-oxopyrazol-1-yl moieties into the unsubstituted benzimidazole nucleus increased the effectiveness toward all tested cell lines compared to the corresponding 5-nitrobenzimidazole analogs (compounds **3a, 3c, 3g, 3i, 3k** and **3n** versus **3b, 3d, 3h, 3j, 3l** and **3o**, respectively). This confirms the high contribution of the unsubstituted benzimidazole moiety to the antitumor activity compared to the 5-nitrobenzimidazole counterpart. Replacement of thiadiazol-2-yl moiety in compounds **3a** and **3b** with benzothiazol-2-yl counterpart led to decreased efficacy toward all tested cell lines (**3c** and **3d**, respectively). In addition, incorporation of 1,3-dioxoisoindolin-2-yl, cyclohepta[*b*]thiophen-2-yl and 3-methyl-5-oxopyrazol-1-yl moieties into the unsubstituted benzimidazole nucleus boosted the activity toward all tested cell lines compared to the other moieties (compounds **3f**, **3m** and **3n** versus **3a**, **3c**, **3g**, **3i** and **3k**).

2.2.3. In vivo antitumor evaluation

Compounds **3f, 3m, 3n** and **3p** (exhibiting the highest *in vitro* antitumor activity) were evaluated for *in vivo* antitumor activity against EAC in mice and results are listed in Tables 4-6. Three important measures have been determined for assessment of antitumor efficacy of the active compounds and 5-fluorouracil (reference drug) [40-42]. % Increase in lifespan (%ILS) was determined by the equation, %ILS = [(MST of treated group/MST of positive control group)-1] x 100, where MST = days of each mouse in a group/total no. of mice. Compounds **3f** and **3p** exhibited an obvious ILS of mice inoculated with EAC cells (Table 4). The viable cell count of EAC was determined. Compounds **3f** and **3p** displayed prominent drop in viable tumor cell count (Table 5). Effects on blood profile, hemoglobin (Hb) content, total red blood cell (RBC) count and white blood cell (WBC) count were determined. Compounds **3f** and **3p** displayed higher Hb and RBC levels and lower WBC count than 5-fluorouracil (Table 6).

2.2.4. In vitro cytotoxicity testing

Compounds **3f, 3m, 3n** and **3p** were assessed for *in vitro* cytotoxicity toward human normal lung fibroblast (W138) cell line employing MTT assay [37-39] and utilizing 5-fluorouracil as a standard cytotoxic drug. IC_{50} values (mM) were calculated and listed in Table 7. The results disclosed that the four screened compounds are less cytotoxic than 5-fluorouracil.

2.2.5. DNA-binding assay

A wide variety of known antimicrobial and antitumor agents exert their effect through binding with DNA. Therefore, DNA-binding assay [43,44] was adopted for evaluation of DNA-binding affinity of the active analogs in this study.

2.2.5.1. DNA-binding assay on TLC-plates

It is well established that when DNA was applied to RP-18 TLC plates, it migrates using methanol/water (8:2) as an eluent. However, upon mixing DNA with compounds with which it binds (e.g., ethidium bromide), it forms a complex that remains at the baseline utilizing the same eluent. On contrary, compounds with no affinity to DNA did not cause the DNA to be remained at the baseline [44]. Results from DNA-binding assay of benzimidazoles **3a, 3c, 3f, 3i-k, 3m, 3n** and **3p** are illustrated in Table 8 and revealed that **3c, 3f, 3i, 3k** and **3n** have strong DNA-binding affinity. On the other hand, compounds **3a, 3j** and **3p** displayed moderate affinity, whereas the complexes were migrated for short distances. Guided by these results, most of the tested active analogs have either strong or moderate DNA-binding affinity, and therefore they are predicted to exert their biological activity through interaction with DNA.

3. Computational tools

In silico techniques are utilized for studying the substantial parameters that assist medicinal chemists in estimating the physicochemical properties of a compound. The principal goal of *in silico* studies is to overcome the dispensable costs associated with biological screening of the compounds [45].

Lipophilicity and solubility are amongst the properties of drugs that influence their absorption. Thus, the new analogs **3a-p** were studied for the anticipation of Lipinski's rule [46] and other properties [47]. Results of computational studies are provided in the supplementary data.

4. Conclusion

In conclusion, an interesting series of benzimidazole derivatives **3a-p** bearing substituted aminomethyl, substituted pyrazol-1-yl methyl, and substituted piperazin-1-yl methyl groups at the 2-position of benzimidazole nucleus was prepared. Antimicrobial evaluation indicated different pharmacological profiles of these new compounds, the benzimidazoles **3c** and **3n** displayed promising efficacy toward *S. aureus*. Furthermore, compound **3c** showed good antifungal efficacy toward *C. albicans*, whereas **3i** demonstrated wonderful activity against *B. cereus*. Switching to the antitumor evaluation, SAR studies demonstrated the significance of the spacer length between the benzimidazole nucleus and the aryl moiety for the antitumor activity, whereas compound **3p** that possesses five atoms spacer exhibited the highest potency against all tested cell lines. Moreover, benzimidazoles **3f**, **3m** and **3n** displayed promising activity toward all tested cell lines. *In vivo* antitumor evaluation showed that **3f** and **3p** have the highest activity. Cytotoxicity testing against W138 human normal cell line proved that the active antitumor analogs, **3f, 3m, 3n** and **3p** are less cytotoxic than 5-fluorouracil. Taken together, **3f** and **3p** have the highest *in vitro* and *in vivo* antitumor activities as well as the least cytotoxic activity. Results of DNA-binding assay confirmed that the active antimicrobial and/or antitumor compounds, **3c, 3f, 3i, 3j, 3k, 3n** and **3p** are thought to exert their biological activities through interaction with DNA. Referring to the computational studies, almost all the benzimidazoles prepared in the current work are foreseen to have good absorption and oral bioavailability. Inspired by these auspicious results, the active compounds, particularly **3c, 3f, 3i, 3n** and **3p** will be further examined against a variety of microbial strains and cancer cell lines to explore their broad spectrum antimicrobial and/or antitumor activities. In addition, these active compounds may serve as promising candidates for future design, modification, and investigation to obtain new potent antimicrobial and/or antitumor analogs.

5. Experimental

Fisher-Johns melting point apparatus was used for determining melting points °C. The IR spectra (KBr disc) were obtained on a Unicam SP 1000 IR spectrometer (ν in cm^{-1}). Varian Gemini 300 MHz spectrometer was utilized for recording ^{1}H & ^{13}C NMR spectra using DMSO-d_6 as solvent and TMS as internal standard. Mass spectra were recorded on JEOL JMS-600H spectrometer (70 eV). The new analogs were analyzed for C, H & N and agreed with the suggested structures, Microanalytical Center, Cairo University, Egypt. TLC plates precoated with silica gel 60 F_{254} (E. Merck) were employed for controlling the progress of reactions, and UV (366 nm) was utilized for visualization of the spots. Chloroform/methanol (9:1) was utilized as an eluent. Benzimidazoles **2a,b** [13], ethyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate [29] and 2-methyl-1*H*-pyrazol-5(4*H*)-one [30] were synthesized following the literature procedures. *S. aureus*, *B. cereus*, *E. coli* and *C. albicans* were acquired from the Department of Microbiology, Faculty of Pharmacy, Mansoura University, Egypt. *A.*

fumigatus 293 was supplied by Prof. Nancy Keller, Department of Medical Microbiology and Immunology, Wisconsin-Madison University, USA. *C. violaceum* ATCC 12472 was provided by Prof. Bob Mclean, Department of Biology, Texas State University, USA. The cell lines and EAC cells were procured from NCI, Cairo, Egypt.

5.1. Chemistry

5.1.1. Preparation of 2-(arylamino)methyl-5-(un)substituted-1H-benzimidazoles 3a-m, 1-((un)substituted-1H-benzimidazol-2-yl)methyl-3-methyl-1H-pyrazol-5(4H)-ones 3n,o and 2-((2-(4-fluorophenyl)piperazin-1-yl)methyl)-5-nitro-1H-benzimidazole (3p)

A mixture of 2-chloromethyl-1*H*-benzimidazole derivative **2a,b** (0.002 mol), the appropriate arylamine, ethyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate, 2-methyl-1*H*-pyrazol-5(4*H*)-one or 1-(4-fluorophenyl)piperazine (0.002 mol) and triethylamine (0.3 mL) in DMF (10 mL) was refluxed for 6-12 hours. The mixture was poured onto ice and the precipitate formed was filtered, dried and crystallized from ethanol/water (2:1).

5.1.1.1. 2-[((5-Sulfamoyl-[1,3,4]thiadiazol-2-yl)amino)methyl]-1H-benzimidazole ***(3a)***
65%, m.p. 173-175 °C. IR (KBr, *v*, cm^{-1}): 3446, 3422 (NH_2, 2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 4.38 (s, 2H, CH_2), 7.14 (s, 3H, NH_2, NH), 7.22-7.60 (m, 2H, Ar-H), 7.67-7.91 (m, 2H, Ar-H), 15.05 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 46.7, 120.2, 124.0, 139.2, 140.2, 155.6, 168.0. MS *m/z* (%): 312 (4.81, M^++2), 311 (3.46, M^++1), 310 (5.26, M^+), 63 (100.00). Anal. Calcd for $C_{10}H_{10}N_6O_2S_2$ (310.36): C, 38.70; H, 3.25; N, 27.08%. Found: C, 38.42; H, 3.16; N, 27.32%.
5.1.1.2. 5-Nitro-2-[((5-sulfamoyl-[1,3,4]thiadiazol-2-yl)amino)methyl]-1H-benzimidazole ***(3b)***
70%, m.p. 147-148 °C. IR (KBr, *v*, cm^{-1}): 3383, 3102 (NH_2, 2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 4.32 (s, 2H, CH_2), 7.20 (d, 1H, Ar-H), 7.50 (d, 1H, Ar-H), 7.75 (s, 1H, Ar-H), 8.15 (s, 2H, NH_2), 11.25 (s, 1H, NH), 12.70 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 43.4, 111.8, 115.3, 118.7, 139.3, 139.5, 143.4, 152.7, 156.0, 172.9. MS *m/z* (%): 357 (3.09, M^++2), 356 (1.99, M^++1), 355 (10.60, M^+), 193 (100.00). Anal. Calcd for $C_{10}H_9N_7O_4S_2$ (355.35): C, 33.80; H, 2.55; N, 27.59%. Found: C, 33.71; H, 2.79; N, 27.26%.
5.1.1.3. 2-[((6-Fluorobenzothiazol-2-yl)amino)methyl]-1H-benzimidazole ***(3c)***
60%, m.p. 197-198 °C. IR (KBr, *v*, cm^{-1}): 3420 (2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 4.32 (s, 2H, CH_2), 7.02-7.13 (m, 2H, Ar-H), 7.29-7.39 (m, 5H, Ar-H), 8.51 (s, 1H, NH), 9.59 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 46.0, 111.6, 111.9, 116.8, 118.7, 120.6, 128.2, 136.4, 138.2, 148.4, 148.8, 160.7, 169.6. MS *m/z* (%): 298 (0.87, M^+), 297 (0.36, M^+-1), 91 (100.00). Anal. Calcd for $C_{15}H_{11}FN_4S$ (298.34): C, 60.39; H, 3.72; N, 18.78%. Found: C, 60.11; H, 3.95; N, 18.43%.
5.1.1.4. 2-[((6-Fluorobenzothiazol-2-yl)amino)methyl]-5-nitro-1H-benzimidazole ***(3d)***
68%, m.p. 121-122 °C. IR (KBr, *v*, cm^{-1}): 3448 (2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 4.78 (s, 2H, CH_2), 7.01-8.52 (m, 7H, Ar-H, NH), 12.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 45.5, 113.4, 116.6, 118.3, 121.4, 123.4, 128.7, 129.0, 129.3, 139.3, 139.8,

151.4, 164.4, 165.5, 165.6. MS *m/z* (%): 345 (0.6, M^++2), 344 (1.05, M^++1), 343 (3.07, M^+), 216 (100.00). Anal. Calcd for $C_{15}H_{10}FN_5O_2S$ (343.34): C, 52.47; H, 2.94; N, 20.40%. Found: C, 52.74; H, 2.62; N, 20.73%.

5.1.1.5. 5-Nitro-2-[((4-phenylthiazol-2-yl)amino)methyl]-1H-benzimidazole ***(3e)***

65%, 106-107 °C. IR (KBr, *v*, cm^{-1}): 3420 (2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 4.35 (s, 2H, CH_2), 6.61 (s, 1H, C_4-H of thiazole), 7.10-7.38 (m, 5H, Ar-H), 7.45-7.68 (m, 3H, Ar-H), 8.85 (s, 1H, NH), 9.75 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 46.7, 110.4, 115.6, 118.6, 120.1, 128.9, 129.0, 129.2, 130.7, 137.9, 139.3, 140.1, 148.5, 151.6, 153.4. MS *m/z* (%): 352 (10.61, M^++1), 351 (18.54, M^+), 55 (100.00). Anal. Calcd for $C_{17}H_{13}N_5O_2S$ (351.38): C, 58.11; H, 3.73; N, 19.93%. Found: C, 58.36; H, 3.98; N, 20.21%.

5.1.1.6. 2-((1H-Benzimidazol-2-yl)methylamino)isoindoline-1,3-dione ***(3f)***

70%, m.p. 185-186 °C. IR (KBr, *v*, cm^{-1}): 3398, 3301 (2NH), 1652 (2C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 3.98 (s, 2H, CH_2), 7.19-7.67 (m, 8H, Ar-H), 8.86 (s, 1H, NH), 13.00 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 47.9, 114.4, 119.4, 122.7, 130.9, 133.3, 138.8, 142.1, 166.5. MS *m/z* (%): 294 (37.79, M^++2), 293 (6.50, M^++1), 263 (100.00). Anal. Calcd for $C_{16}H_{12}N_4O_2$ (292.29): C, 65.75; H, 4.14; N, 19.17%. Found: C, 65.47; H, 4.46; N, 19.38%.

5.1.1.7. 1-((1H-Benzimidazol-2-yl)methyl)-2-(2,3-dihydro-7-methoxynaphthalen-4(1H)-ylidene)hydrazine ***(3g)***

55%, m.p. 182-183 °C. IR (KBr, *v*, cm^{-1}): 3421 (2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 1.28-1.35 (m, 2H, CH_2), 1.57 (t, 2H, CH_2), 1.81 (t, 2H, CH_2), 3.96 (s, 3H, OCH_3), 4.52 (s, 2H, CH_2), 6.82-7.08 (m, 3H, Ar-H), 7.14-7.28 (m, 2H, Ar-H), 7.38-7.52 (m, 2H, Ar-H), 7.68 (s, 1H, NH), 11.45 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 26.0, 26.1, 31.7, 51.5, 52.2, 114.5, 116.8, 120.6, 123.7, 124.2, 126.0, 131.7, 139.8, 143.2, 147.1, 164.6. MS *m/z* (%): 321 (6.67, M^++1), 320 (65.00, M^+), 64 (100.00). Anal. Calcd for $C_{19}H_{20}N_4O$ (320.39): C, 71.23; H, 6.29; N, 17.49%. Found: C, 71.56; H, 6.52; N, 17.13%.

5.1.1.8. 2-(2,3-Dihydro-7-methoxynaphthalen-4(1H)-ylidene)-1-((5-nitro-1H-benzimidazol-2-yl)methyl)hydrazine ***(3h)***

62%, m.p. 142-143 °C. IR (KBr, *v*, cm^{-1}): 3419 (2NH). ^{1}H NMR (DMSO-d_6, δ ppm): 1.65-2.16 (m, 6H, $3CH_2$), 3.79 (s, 3H, OCH_3), 4.35 (s, 2H, CH_2), 6.74-6.90 (m, 2H, Ar-H), 7.08-7.35 (m, 2H, Ar-H), 7.52-7.70 (m, 2H, Ar-H), 8.61 (s, 1H, NH), 9.78 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 22.4, 22.8, 32.2, 46.0, 56.0, 106.7, 111.6, 111.9, 116.8, 117.4, 120.7, 130.4, 136.4, 138.2, 146.0, 150.1, 153.2, 154.6, 168.9. MS *m/z* (%): 366 (1.83, M^++1), 365 (2.75, M^+), 64 (100.00). Anal. Calcd for $C_{19}H_{19}N_5O_3$ (365.39): C, 62.46; H, 5.24; N, 19.17%. Found: C, 62.71; H, 5.43; N, 18.89%.

5.1.1.9. 5-Amino-N-((1H-benzimidazol-2-yl)methyl)-6-nitroquinoline ***(3i)***

85%, m.p. 214-215 °C. ^{1}H NMR (DMSO-d_6, δ ppm): 3.66 (s, 2H, CH_2), 6.92-7.05 (m, 2H, Ar-H), 7.26-7.51 (m, 3H, Ar-H), 7.60-7.72 (m, 2H, Ar-H), 8.05-8.20 (m, 2H, Ar-H), 8.82 (s, 1H, NH), 11.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 43.9, 117.3, 119.6, 121.5, 125.4, 125.7, 133.6, 135.7, 138.6, 138.8, 141.1, 146.4, 151.6, 153.9. MS *m/z* (%): 321 (60.19, M^++2), 320 (63.11, M^++1), 319 (79.61, M^+), 55 (100.00). Anal. Calcd for $C_{17}H_{13}N_5O_2$ (319.32): C, 63.94; H, 4.10; N, 21.93%. Found: C, 63.67; H, 3.79; N, 21.67%.

5.1.1.10. 5-Amino-N-((5-nitro-1H-benzimidazol-2-yl)methyl)-6-nitroquinoline ***(3j)***
80%, m.p. 229-230 °C. ^{1}H NMR (DMSO-d_6, δ ppm): 4.11 (s, 2H, CH_2), 7.15 (d, 1H, Ar-H), 7.55 (d, 1H, Ar-H), 8.22 (d, 1H, Ar-H), 8.46 (s, 1H, Ar-H), 8.68-8.80 (m, 2H, Ar-H), 8.95-9.05 (m, 2H, Ar-H), 10.74 (s, 1H, NH), 13.05 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 49.1, 108.5, 114.3, 118.8, 120.5, 123.0, 127.9, 128.9, 134.1, 137.6, 145.8, 146.0, 154.2, 156.8, 156.9, 160.2, 163.0. MS *m/z* (%): 365 (9.68, M^++1), 364 (14.71, M^+), 55 (100.00). Anal. Calcd for $C_{17}H_{12}N_6O_4$ (364.32): C, 56.05; H, 3.32; N, 23.07%. Found: C, 56.39; H, 3.56; N, 22.77%.

5.1.1.11. 4-((1H-Benzimidazol-2-yl)methylamino)-1,2-dihydro-2,3-dimethyl-1-phenylpyrazol-5-one ***(3k)***
55%, m.p. 210-212 °C. IR (KBr, *v*, cm^{-1}): 3422 (2NH), 1649 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.76 (s, 3H, CH_3), 2.48 (s, 3H, NCH_3), 4.08 (s, 2H, CH_2), 7.00-7.18 (m, 2H, Ar-H), 7.25-7.58 (m, 3H, Ar-H), 7.62-7.96 (m, 4H, Ar-H), 8.10 (s, 1H, NH), 8.70 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 12.6, 37.0, 48.9, 111.1, 112.5, 113.3, 120.5, 121.7, 128.5, 132.0, 133.6, 144.1, 145.6, 166.1. MS *m/z* (%): 335 (4.95, M^++2), 334 (4.91, M^++1), 333 (6.78, M^+), 291 (100.00). Anal. Calcd for $C_{19}H_{19}N_5O$ (333.39): C, 68.45; H, 5.74; N, 21.01%. Found: C, 68.61; H, 5.51; N, 21.33%.

5.1.1.12. 1,2-Dihydro-2,3-dimethyl-4-((5-nitro-1H-benzimidazol-2-yl)methylamino)-1-phenylpyrazol-5-one ***(3l)***
60%, m.p. 220-222 °C. IR (KBr, *v*, cm^{-1}): 3446, 3420 (2NH), 1680 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.65 (s, 3H, CH_3), 2.74 (s, 3H, NCH_3), 4.32 (s, 2H, CH_2), 7.13-7.65 (m, 8H, Ar-H), 8.51 (s, 1H, NH), 9.56 (s, 1H, NH). MS *m/z* (%): 380 (19.39, M^++2), 379 (23.81, M^++1), 69 (100.00). Anal. Calcd for $C_{19}H_{18}N_6O_3$ (378.38): C, 60.31; H, 4.79; N, 22.21%. Found: C, 60.11; H, 4.63; N, 22.57%.

5.1.1.13. Ethyl 2-[((1H-benzimidazol-2-yl)methyl)amino]-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxylate ***(3m)***
60%, m.p. 75-76 °C. IR (KBr, *v*, cm^{-1}): 3420 (2NH), 1742 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.30 (t, 3H, $CH_2C\underline{H_3}$), 1.55-1.75 (m, 6H, $3CH_2$), 2.55-2.90 (m, 4H, $2CH_2$), 3.80 (s, 2H, CH_2), 4.25-4.29 (q, 2H, $C\underline{H_2}CH_3$), 7.05-7.21 (m, 2H, Ar-H), 7.36-7.58 (m, 2H, Ar-H), 7.69 (s, 1H, NH), 10.65 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 14.6, 25.8, 26.9, 27.9, 30.5, 32.1, 40.4, 61.1, 112.4, 113.6, 127.3, 128.2, 137.8, 139.5, 140.8, 152.1, 163.3. MS *m/z* (%): 370 (48.57, M^++1), 369 (38.57, M^+), 80 (100.00). Anal. Calcd for $C_{20}H_{23}N_3O_2S$ (369.48): C, 65.02; H, 6.27; N, 11.37%. Found: C, 65.36; H, 5.91; N, 11.53%.

5.1.1.14. 1-((1H-Benzimidazol-2-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one ***(3n)***
50%, m.p. 191-192 °C. IR (KBr, *v*, cm^{-1}): 3308 (NH), 1657 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.56 (s, 3H, CH_3), 2.31 (s, 2H, C_4-H of pyrazolone), 4.25 (s, 2H, CH_2), 7.29-7.39 (m, 4H, Ar-H), 9.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 20.9, 37.2, 50.8, 114.5, 120.7, 139.8, 147.1, 164.6, 195.6. MS *m/z* (%): 229 (70.76, M^++1), 91 (100.00). Anal. Calcd for $C_{12}H_{12}N_4O$ (228.25): C, 63.15; H, 5.30; N, 24.55%. Found: C, 63.41; H, 5.63; N, 24.76%.

5.1.1.15. 3-Methyl-1-((5-nitro-1H-benzimidazol-2-yl)methyl)-1H-pyrazol-5(4H)-one ***(3o)***
65%, m.p. 189-190 °C. IR (KBr, *v*, cm^{-1}): 3412 (NH), 1622 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.35 (s, 3H, CH_3), 2.15 (s, 2H, C_4-H of pyrazolone), 4.45 (s, 2H, CH_2), 7.39 (d, 1H, Ar-H), 7.70 (d, 1H, Ar-H), 8.10 (s, 1H, Ar-H), 11.15 (s, 1H, NH).^{13}C NMR (DMSO-

d_6, δ ppm): 22.5, 43.3, 56.0, 119.0, 121.0, 124.9, 140.3, 149.9, 152.1, 159.0, 167.6, 176.4. Anal. Calcd for $C_{12}H_{11}N_5O_3$ (273.25): C, 52.75; H, 4.06; N, 25.63%. Found: C, 52.42; H, 4.38; N, 25.27%.

5.1.1.16. 2-((4-(4-Fluorophenyl)piperazin-1-yl)methyl)-5-nitro-1H-benzimidazole ***(3p)***

75%, m.p. 217-219 °C. IR (KBr, v, cm^{-1}): 3421 (NH). ^{1}H NMR (DMSO-d_6, δ ppm): 2.66 (s, 4H, $2CH_2$), 3.18 (s, 4H, $2CH_2$), 3.75 (s, 2H, CH_2), 6.92 (d, 2H, Ar-H), 7.38 (d, 2H, Ar-H), 7.62-7.72 (m, 3H, Ar-H), 10.04 (s, 1H, NH). MS *m/z* (%): 357 (0.48, M^++2), 356 (0.68, M^++1), 355 (1.69, M^+), 93 (100.00). Anal. Calcd for $C_{18}H_{18}FN_5O_2$ (355.37): C, 60.84; H, 5.11; N, 19.71%. Found: C, 60.53; H, 5.36; N, 19.46%.

5.2. Biology

5.2.1. Antimicrobial and antiquorum-sensing screening

5.2.1.1. Antibacterial screening

The new derivatives were assessed for *in vitro* antibacterial efficacy as reported [25,26,31].

5.2.1.2. Antifungal screening

The new analogs were examined for *in vitro* antifungal activity following the literature procedure [25,26,32,33].

5.2.1.3. Antiquorum-sensing screening

Antiquorum-sensing efficacy of the new compounds was esteemed employing the literature method [25,26,34].

5.2.2. In vitro antitumor screening

In vitro antitumor screening of the new derivatives was done adopting the reported method [37-39].

5.2.3. In vivo antitumor assay

The detailed procedure of *in vivo* antitumor assay is provided in the supplementary data.

5.2.4. In vitro cytotoxicity testing

In vitro cytotoxicity testing of **3f, 3m, 3n** and **3p** was performed adopting the MTT assay [37-39].

5.2.5. DNA-binding assay on TLC-plates

DNA-binding assay was performed adopting the literature procedure [43].

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Captions of Tables and Scheme

Table 1. Antibacterial and antifungal activities of compounds **3a-p**

Table 2. Antiquorum-sensing activity of compounds **3a-p**

Table 3. *In vitro* antitumor activity of compounds **3a-p** toward HepG2, HCT-116 and MCF-7 cancer cell lines

Table 4. Effect of compounds **3f, 3m, 3n** and **3p** on mean survival time and % increase in lifespan of mice inoculated with EAC cells

Table 5. Effect of compounds **3f, 3m, 3n** and **3p** on tumor volume and viable tumor cell count of mice inoculated with EAC cells

Table 6. Effect of compounds **3f, 3m, 3n** and **3p** on blood profile of mice inoculated with EAC cells

Table 7. *In vitro* cytotoxic activity of compounds **3f, 3m, 3n** and **3p** toward W138 human normal cell line

Table 8. DNA-binding affinity of compounds **3a, 3c, 3f, 3i-k, 3m, 3n** and **3p**

Scheme 1. Synthesis of compounds **3a-p**

Table 1. Antibacterial and antifungal activities of compounds **3a-p**

Comp. No.	MIC, µg/mL (mM)[a]				
	E. coli	***B. cereus***	***S. aureus***	***C. albicans***	***A. fumigatus***
3a	625 (2.01)	-	625 (2.01)	**312.5 (1.007)**	-
3b	-	-	-	625 (1.76)	-
3c	-	-	**156.25 (0.524)**	**78.125 (0.262)**	1250 (4.19)
3d	-	-	-	625 (1.82)	1250 (3.64)
3e	-	-	-	625 (1.78)	1250 (3.56)
3f	-	-	-	625 (2.14)	1250 (4.28)
3g	-	-	1250 (3.90)	1250 (3.90)	-
3h	-	-	-	1250 (3.42)	2500 (6.84)
3i	625 (1.96)	**156.25 (0.489)**	1250 (3.91)	1250 (3.91)	1250 (3.91)
3j	1250 (3.43)	**312.5 (0.858)**	2500 (6.86)	2500 (6.86)	2500 (6.86)
3k	2500 (7.50)	1250 (3.75)	**312.5 (0.937)**	625 (1.87)	625 (1.87)
3l	2500 (6.60)	1250 (3.30)	625 (1.65)	2500 (6.60)	2500 (6.60)
3m	-	-	-	-	-
3n	-	1250 (5.48)	**156.25 (0.684)**	625 (2.74)	**312.5 (1.37)**
3o	-	1250 (4.57)	2500 (9.15)	1250 (4.57)	625 (2.29)
3p	2500 (7.03)	2500 (7.03)	1250 (3.52)	2500 (7.03)	2500 (7.03)
Ampicillin	19.53 (0.056)	1250 (3.58)	312.5 (0.894)	nt	nt
Fluconazole	nt	nt	nt	2500 (8.16)	-

Bold values refer to the best results.
MICs (mM) are shown between parentheses.
[a] -, MIC > 2500 µg/mL.
nt, not tested.

Table 2. Antiquorum-sensing activity of compounds **3a-p**[a,b,c]

Comp. No.	Diameter of pigment inhibition (mm)	Comp. No.	Diameter of pigment inhibition (mm)
	Ch. violaceum		*Ch. violaceum*
3a	-	**3j**	4
3b	-	**3k**	6
3c	11	**3l**	-
3d	6	**3m**	3
3e	6	**3n**	4
3f	4	**3o**	3
3g	-	**3p**	-
3h	-	**Catechin**	2
3i	8	**------**	**------**

[a] Sample concentration: 5000 µg/mL, Sample volume: 0.1 mL/well.
[b] Results were calculated after subtraction of DMSO activity.
[c] No activity (-, inhibition zone < 2 mm); weak activity (2-9 mm); moderate activity (10-15 mm); strong activity (>15 mm).

Table 3. *In vitro* antitumor activity of compounds **3a-p** toward HepG2, HCT-116 and MCF-7 cancer cell lines

Comp. No.	IC_{50} (mM)		
	HepG2	**HCT-116**	**MCF-7**
3a	0.162	0.156	0.228
3b	0.184	0.176	0.230
3c	0.233	0.238	0.242
3d	0.263	0.253	0.291
3e	0.226	0.198	0.254
3f	**0.032**	**0.031**	**0.037**
3g	0.08	**0.041**	**0.063**
3h	0.096	**0.045**	0.078
3i	0.259	0.204	0.237
3j	0.265	0.256	0.274
3k	0.063	**0.038**	**0.053**
3l	0.093	**0.044**	0.078
3m	**0.043**	**0.031**	**0.04**
3n	**0.054**	**0.042**	**0.062**
3o	0.265	0.20	0.281
3p	**0.022**	**0.014**	**0.015**
5-Fluorouracil	0.061	0.041	0.0415

Bold values refer to the best results.

Table 4. Effect of compounds **3f, 3m, 3n** and **3p** on mean survival time and % increase in lifespan of mice inoculated with EAC cells

Group	Mean survival time (day)	% Increase in lifespan
Normal	nd[a]	nd[a]
EAC cells only	14.5	nd[a]
5-Fluorouracil	49	237.93
3f	**46.7**	**222.07**
3m	39	168.9
3n	31.5	110.34
3p	**44.5**	**206.98**

[a]nd: not determined.
Bold values refer to the best results.

Table 5. Effect of compounds **3f, 3m, 3n** and **3p** on tumor volume and viable tumor cell count of mice inoculated with EAC cells

Group	Tumor volume (mL)	Viable tumor cell count/100 µL
Normal	nd[a]	nd[a]
EAC cells only	9.85	83.20 x10^6
5-Fluorouracil	1.60	20.17 x10^6
3f	**2.15**	**21.95 x10^6**
3m	3.23	29.65 x10^6
3n	4.16	45.38 x10^6
3p	**2.32**	**23.54 x10^6**

[a] nd: not determined.
Bold values refer to the best results.

Table 6. Effect of compounds **3f, 3m, 3n** and **3p** on blood profile of mice inoculated with EAC cells

Group	Hb (g/dl)	RBC Count 10^6/mm^3	WBC Count 10^3/mm^3
Normal	13.73	5.84	5.99
EAC cells only	8.15	3.69	23.96
5-Fluorouracil	12.96	5.21	8.86
3f	**13.21**	**5.52**	**7.11**
3m	12.13	4.89	9.11
3n	11.47	4.55	11.26
3p	**13.15**	**5.36**	**7.34**

Bold values refer to the best results.

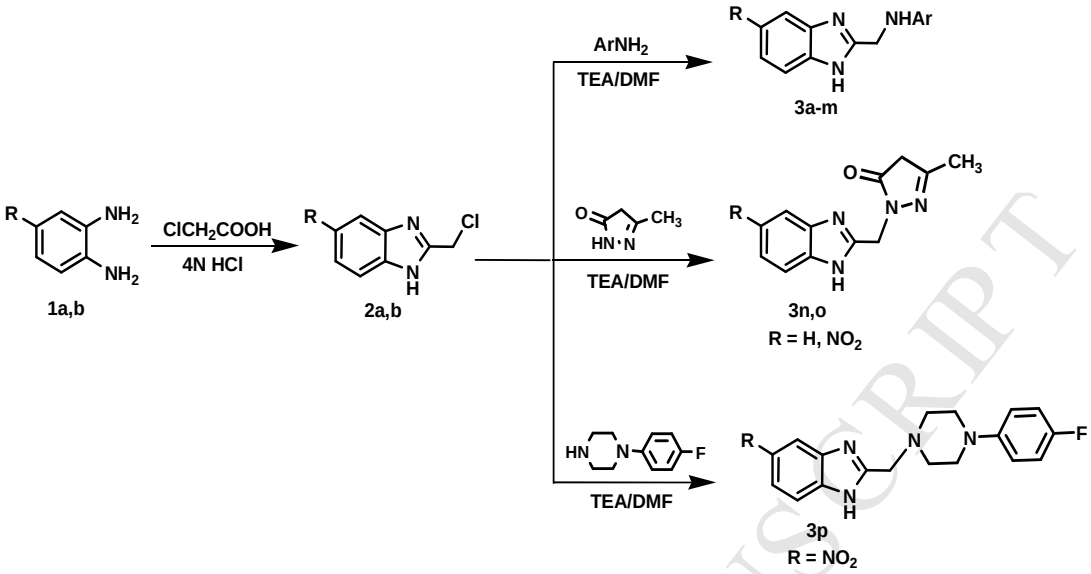
Table 7. *In vitro* cytotoxic activity of compounds **3f, 3m, 3n** and **3p** toward W138 human normal cell line

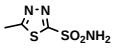
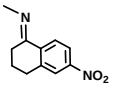
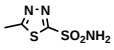
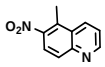
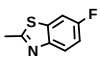
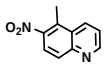
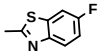
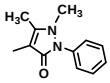
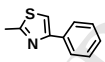
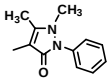
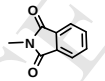
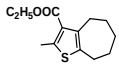
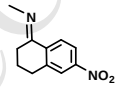
Comp. No.	IC_{50} (mM)
3f	0.322
3m	0.058
3n	0.246
3p	0.298
5-Fluorouracil	0.051

Table 8. DNA-binding affinity of compounds **3a**, **3c, 3f, 3i-k, 3m, 3n** and **3p**

Comp. No.	DNA-binding affinity
3a	++
3c	+++
3f	+++
5i	+++
3j	++
3k	+++
3m	+
3n	+++
3p	++
Ethidium bromide	+++

+++ Strong affinity.
++ Moderate affinity.
+ Weak affinity.



Comp. No.	Ar	R	Comp. No.	Ar	R
3a		**H**	**3h**		**NO_2**
3b		**NO_2**	**3i**		**H**
3c		**H**	**3j**		**NO_2**
3d		**NO_2**	**3k**		**H**
3e		**NO_2**	**3l**		**NO_2**
3f		**H**	**3m**		**H**
3g		**H**	-----	-----	-----

Scheme 1. Synthesis of compounds **3a-p**

- New benzimidazole analogs were synthesized.
- The newly synthesized compounds were screened for antimicrobial and antitumor activities.
- Compounds **3c**, **3i** and **3n** are the most active antimicrobial agents.
- Compounds **3p** and **3f** are the most potent analogs against all tested cancer cell lines.