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Synthesis of Azole-containing Piperazine Derivatives and Evaluation of their Antibacterial, Antifungal and Cytotoxic Activities

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A series of azole-containing piperazine derivatives have been designed and synthesized. The obtained compounds were investigated *in vitro* for their antibacterial, antifungal and cytotoxic activities. The preliminary results showed that most compounds exhibited moderate to significant antibacterial and antifungal activities *in vitro*. 1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(1*H*-imidazol-1-yl)ethanone and 1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(2-phenyl-1*H*-imidazol-1-yl)ethanone gave remarkable and broad-spectrum antimicrobial efficacy against all tested strains with MIC values ranging from 3.1 to 25 μg/mL, and exhibited comparable activities to the standard drugs chloramphenicol and fluconazole in clinic. Moreover, 2-((4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)methyl)-1*H*-benzo[d]imidazole was found to be the most effective *in vitro* against the PC-3 cell line, reaching growth inhibition values (36.4, 60.1 and 76.5%) for each tested concentration: 25 μM, 50 μM and 100 μM in dose-dependent manner. The results also showed that the azole ring had noticeable effect on their antimicrobial and cytotoxic activities, and imidazole and benzimidazole moiety were much more favourable to biological activity than 1,2,4-triazole.

Key Words: Piperazine, Imidazole, Antibacterial, Antifungal, Cytotoxicity

Introduction

The number of patients suffering from cancer diseases or some life-threatening infections has continued to rise rapidly with years, though great progresses have been made in diagnosis, prevention, therapy and medicinal chemistry. Especially, the alarming rates of emerging drug resistant strains and cancer cell lines, leading to failure in therapy, continue to serve as impetus for the development of novel and more effective antimicrobial and anticancer agents.[1-3]

The piperazine-based research has attracted considerable attention in recent years. Piperazine and substituted piperazine nuclei had constituted an attractive pharmacological scaffold present in various potent marketed drugs. The incorporation of piperazine is an important synthetic strategy in drug discovery due to its easy modificability, proper alkality, water solubility, the capacity for the formation of hydrogen bonds and adjustment of molecular physicochemical properties.[4,5] A broad range of biological active compounds displaying antibacterial,[3,6-8] antifungal,[9,10] anticancer,[11-13] antiparasitic,[14,15] antihistamin,[16] psychotolytic,[17] and antidepressive activities[18] have been also found to contain this versatile core. In particular, structurally simple 1-(1-naphthylmethyl)-piperazine, as the efflux pump inhibitor, could exert positive effect on tetracyclines and ciprofloxacin against their resistant bacteria.[19,20] Moreover, benzotriazole-based piperazine derivatives and *N*,*N*'-bis(alkyloxymethyl)piperazines had moderate antibacterial and antifungal activities against pathogenic bacterial strains and fungal strains.[21,22] On the other hand, a novel microtubule depolymerizing piperazine derivative, 1-(5-chloro-2-methoxybenzoyl)-4-(3-chlorophenyl) piperazine, caused inhibition of proliferation of a wide range of cancer cell lines including a multidrug-resistant cell line, with an average IC_{50} of 85 nM.[23] These results once again highlighted that piperazine core was an important backbone and prompted us to design some active molecules with piperazine nucleus.

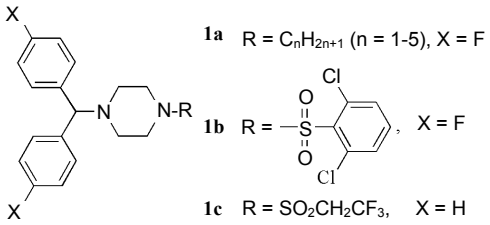


Figure 1. Structures of some biological active piperazine derivatives.

Several literatures provided evidence that the introduction of such bulky groups like diphenyl could increase antimicrobial activity by enhancing lipophilicity of the molecule, which may result in more penetration into cells.[24] Thus piperazine derivatives bearing diphenyl substitution at N1 position also have been introduced to design new antibacterial and antifungal agents, though this moiety has wide application in antihistamine like cetirizine, calcium antagonist (flunarizine) *etc*. Recently, Rangappa *et al.* [25] reported the synthesis and *in vitro* antimicrobial studies of medicinally important novel *N*-alkyl and *N*-sulfonyl derivatives of 1-[bis(4-fluorophenyl)-methyl]piperazine **1a** and **1b** (Figure 1), which demonstrated potent inhibition against representative strains of Gram-positive and Gram-negative bacteria when compared to the standard drug streptomycin. Furthermore, novel 1-benzhydryl piperazine derivative **1c** (Figure 1) gave interesting growth inhibitory effects against one normal cell line and four human cancer cell lines for the time period of 24 h.[26] Consequently, the optimization of diphenyl piperazine

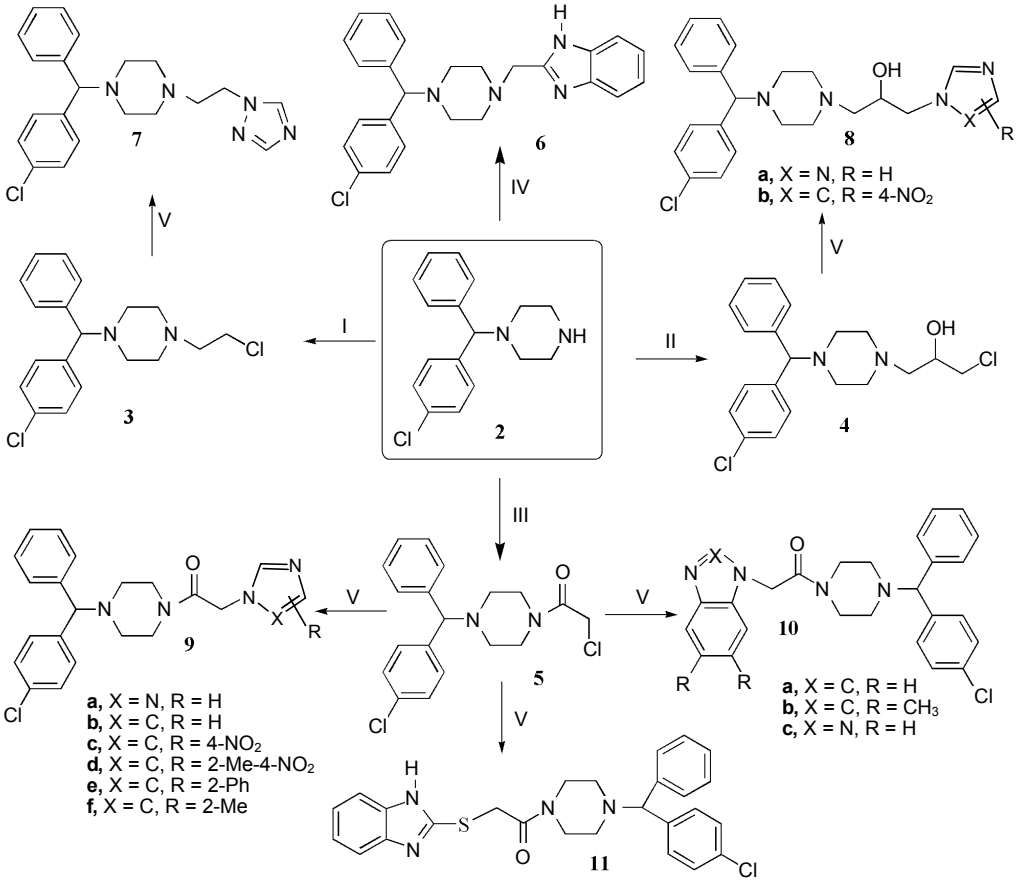
derivatives may lead to a new route to potential pharmaceutical molecules as antimicrobial and anticancer agents.

It is well-known that azole moieties, such as triazole, imidazole, benzimidazole ring, *etc*. as important pharmacophores are present extensively in diverse types of drugs in clinical use.[27-29] More importantly, azole derivatives as antimicrobial agents, especially as antifungal drugs represent a novel emerging major chemical entity. For instance, ketoconazole, itraconazole, fluconazole and its ester prodrug fosfluconazole, posaconazole and ravuconazole are widely used in the treatment of systemic fungal infections, and particularly, miconazole could exhibit remarkable antibacterial activity against MRSA.[30] On the other hand, the azole compounds also have attracted much attention due to their prominent utilization as antitumor agents.[31-33] Therefore, these progressive findings about the biological activities of azole derivatives led numerous efforts to develop some azole derivatives as new antimicrobial and anticancer agents.

In view of this and in the continuation of previous researches on synthesis and antimicrobial studies of bioactive heterocycles,[34,35] it is worthwhile to synthesize new 1-((4-chlorophenyl)(phenyl)methyl) piperazine derivatives bearing various heterocyclic nuclei such as triazole, imidazole, benzimidazole *etc*. and to evaluate for their antimicrobial activities against *S. aureus*, *Methicillin-resistant Staphylococcus aureus* (MRSA), *B. subtilis*, *M. luteus*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, *S. typhi*, *S. dysenteriae*, *C. albicans*, *S. cerevisiae* and *A. fumigatus* as well as antitumor activity against human prostatic carcinoma cell (PC-3).

Results and Discussion

Chemistry. The general route for the synthesis of diphenyl-attached piperazine derivatives is outlined in Scheme 1. The intermediate 1-((4-chlorophenyl)(phenyl)methyl) piperazine **2** was prepared by a known procedure in three steps, including $NaBH_4$ reduction, chlorination and N-aralkylation from the commercially available (4-chlorophenyl)(phenyl)methanone as starting materials.[26] The chloride **3** was obtained by the reaction of **2** with a large excess of 1,2-dichloroethane using triethylamine as base.[37] The reaction of **2** with 2-(chloromethyl)oxirane at room temperature without base using anhydrous ethanol as solvent produced another important chloride **4** with the yield of 40%.[38] The key intermediate **5** was obtained in 45% yield by the *N*-4 acylation of **2** with chloroacetyl chloride in the presence of triethylamine in ice-bath.[39] The subsequent treatment of **3**, **4** and **5** with a series of azole compounds such as triazole, imidazole, substituted imidazole and benzimidazole *etc* in the presence of a mild base, anhydrous K_2CO_3 gave the azole 1-((4-chlorophenyl)(phenyl)methyl) piperazine derivatives **7**, **8a-b** **9a-f**, **10a-c** and **11** in good yields after chromatographic purifica-



Scheme 1. Synthetic route of azole-containing piperazine derivatives **6-11**. Reagents and conditions: (I) $ClCH_2CH_2Cl$, Et_3N, reflux, 20 h. (II) 2-(Chloromethyl)oxirane, CH_3CH_2OH, rt, 24 h. (III) $ClCOCH_2Cl$, CH_2Cl_2, Et_3N, 0 - 5 °C, 5 h. (IV) 2-(Chloromethyl)-1*H*-benzo[*d*]imidazole, CH_3COCH_3, rt, 12 h. (V) Azoles, CH_3CN, 45 - 70 °C.

Table 1. Antibacterial activity of compounds **3-11** expressed as MIC (μg/mL)

Compd.	*S. aureus*	MRSA	*B. subtilis*	*M. luteus*	*E. coli*	*P. vulgaris*	*S. typhi*	*S. dysenteriae*
3	200	200	100	100	400	200	200	200
4	50	25	50	50	100	100	100	100
5	25	25	25	25	25	25	50	50
6	25	25	6.2	25	50	25	25	12.5
7	100	100	100	100	200	100	200	100
8a	100	100	100	100	200	100	200	100
8b	50	50	50	50	25	25	50	50
9a	200	200	50	100	400	100	100	50
9b	3.1	6.2	6.2	6.2	25	12.5	12.5	6.2
9c	50	50	25	50	100	50	25	6.2
9d	50	50	25	50	200	50	25	6.2
9e	6.2	12.5	12.5	6.2	25	25	12.5	6.2
9f	50	50	25	12.5	50	25	50	25
10a	25	25	25	25	200	25	25	25
10b	50	50	25	25	200	50	25	25
10c	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 400
11	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 400
Chl[a]	3.1	6.2	3.1	1.6	6.2	3.1	3.1	1.6

[a]chl, chloramphenicol.

Table 2. Antifungal activity of compounds **3-11** expressed as MIC (μg/mL)

Compd.	*C. albicans*	*S. cerevisiae*	*A. fumigatus*	Compd.	*C. albicans*	*S.cerevisiae*	*A. fumigatus*
3	200	200	200	**9c**	25	25	50
4	100	100	100	**9d**	100	25	100
5	25	25	50	**9e**	12.5	3.1	25
6	25	25	50	**9f**	25	25	50
7	100	100	200	**10a**	25	25	100
8a	100	100	200	**10b**	25	25	100
8b	25	25	50	**10c**	> 400	> 400	> 400
9a	100	100	100	**11**	> 400	> 400	> 400
9b	6.2	12.5	25	**flu**[a]	0.8	1.6	256

[a]flu, fluconazole.

tion on silica gel. Besides, the compound **6** was synthesized by direct reaction of intermediate **2** and 2-(chloromethyl)-1*H*-benzo[*d*]imidazole in acetone at room temperature.

In the preparation of intermediate **5**, the reaction was related with the dropwise rate of chloroacetyl chloride. The slow dropwise addition of the chloroacetyl chloride instead of addition at once would increase the yield and decrease the possibility of decomposition. The nucleophilic substitution reactions of the intermediate **4** or **5** with different azoles were found to depend on the acidity of the azole rings and the reactivity of the intermediates to some extent. The azole rings with stronger acidity (triazole, 4-nitro-imidazole, 2-methyl-5-nitro-imidazole) need shorter reaction time and lower temperature than those with weaker acidity (imidazole, benzimidazole and the electron-donor group substituted imidazole), and compound **5** reacted far more readily with azoles than **4** under the same conditions. Furthermore, the presence of weak base potassium carbonate rather than strong base such as NaH in the reaction of compound **5** with imidazole, 2-methyl, 2-phenyl imidazole and benzimidazole gave good yields of title compounds, which made the procedure more convenient and safe.

All the synthesized compounds were confirmed by MS, IR and ^{1}H NMR spectra as well as elemental analyses. The spectral analyses were in accordance with the assigned structures, and all the characterization data were given in the experimental section. The mass spectra showed that compounds **6-11** gave a $[M]^+$ or $[M+Na]^+$ peak, in agreement with their molecular formula. Since $NaBH_4$ used for the (4-chlorophenyl)(phenyl)methanone reduction would in no way result in a stereochemical preference on the formation of benzhydrol,[40] the synthesized piperazine derivatives were racemates, which were confirmed by polarimeter.

Pharmacology. The desired compounds **6-11** and the intermediates **3-5** were provided for their antibacterial and antifungal

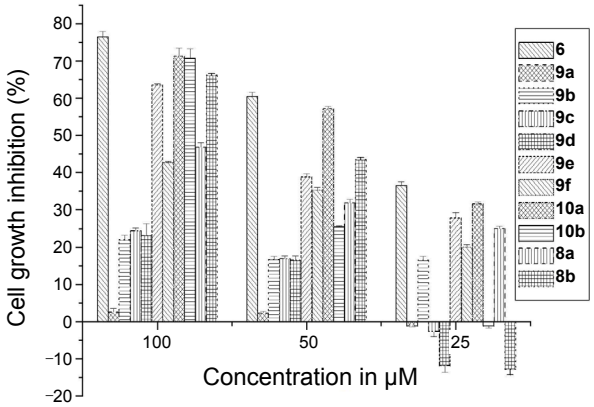


Figure 2. The cytotoxic activities of the tested compounds.

assaying as racemates against Gram positive, Gram negative bacteria and fungi, in comparison with chloramphenicol and fluconazole as standard drugs, and their antimicrobial results were reported as minimal inhibitory concentrations (MICs, μg/mL), and were shown in Table 1 and Table 2.

The newly synthesized compounds were also evaluated for their cytotoxic activity towards human tumour cell line PC-3 (human, prostatic carcinoma cell) by MTT assay. Results of each tested agents were reported as the growth inhibition percentage of treated cells when compared to the untreated control ones. The cell growth inhibition rates were summarized in Figure 2.

Antibacterial activity: The antibacterial tests indicated that all the tested compounds were able to inhibit the growth of the selected microorganisms *in vitro*, whereas the compounds **10c** and **11** were inactive even at the highest tested concentration against both Gram-negative and Gram-positive bacteria. In addition, it turned out that Gram-negative strain *E. coli* was less sensitive to most azole-containing compounds compared to other tested bacterial strains.

As was seen in Table 1, the three intermediates **3-5** showed weak to moderate antibacterial activities, and the chloride **5** with amide linkage, which had been reported as hypocholesteremic agents at the first time in 1968,[39] exhibited best antibacterial activities among the three intermediates **3-5**. The replacement of chlorine atom with the different azole rings resulted in variation of the antibacterial activities. It was observed that the heterocyclic moiety had noticeable effect on the antibacterial activities. The biological activities of azole-containing piperazine derivatives decreased as the following orders: imidazole and substituted imidazoles **9b-f** ≥ benzimidazoles **6**, **10a** and 5,6-dimethyl-benzimidazole **10b** > triazoles **7**, **8a** and **9a** > benzotriazole **10c**, 2-thiol benzimidazole **11**. These facts suggested that imidazole and benzimidazole residues should have the synergistic effect on biological activities, but the benzotriazole and 2-thiol benzimidazole nucleus may exert negative efficacy on their activities.

For the tested imidazole and its substituted derivatives **9b-f**, all of them showed broad antibacterial spectrum. Especially, compounds **9b** and **9e** with imidazole and 2-phenyl imidazole ring, respectively, had the strongest antibacterial activities against pathogenic bacterial strains with the MIC values in the range of 3.1 - 25 μg/mL. Compound **9b** exhibited significant activity against *S. aureus* with the MIC value of 3.1 μg/mL, which was equivalent to that of chloramphenicol as the reference drug. This compound also revealed good activity against MRSA, *B. subtilis*, *M.luteus* and *S. dysenteriae* (MIC = 6.2 μg/mL), as well as moderate activity against *E. coli, P. vulgaris* and *S. typhi*, with the MIC values of 25, 12.5 and 12.5 μg/mL, respectively. The results suggested that it was the most active compound among the azole-containing piperazine derivatives. Besides, compound **9e** also showed excellent activity against *S. aureus*, *M. luteus* and *S. dysenteriae*, and it could effectively inhibit the growth of the three strains at the same concentration of 6.2 μg/mL. Meanwhile, this compound also displayed moderate activity against the rest tested strains. On the other hand, other compounds with substitution on imidazole ring, such as **9c** with 4-NO_2, **9d** with 2-CH_3-4-NO_2, **9f** with 2-CH_3 group also gave moderate to excellent antibacterial activities with the MIC values ranging from 6.2 to 50 μg/mL, but poor activity against *E. coli*. Furthermore, it's noteworthy that the three compounds almost have the same antibacterial potential against Gram positive bacterial strains, whereas they had slight difference on their antibacterial activities against Gram negative bacterial strains. Compound **9f** with electron-donor methyl group exhibited best antibacterial activities against the four Gram negative bacterial strains among the three compounds, the next one was the compound **9d** with 2-CH_3-5-NO_2, and the lowest antibacterial activity was the compound **9c** with 4-NO_2. These results suggested that their antibacterial activities had been ascribed to the nature of the substituents to some extent.

Benzimidazoles bearing derivatives **6** and **10a-b** gave relatively good activities against most tested bacterial strains with the MIC values ranging from 25 to 100 μg/mL. Additionally, we can see from the data that the substitution with electron releasing methyl group at the 5- and 6-position of benzimidazole nucleus only result in a little decrease in potency.

Compared to imidazole derivatives, triazole ones **7**, **8a** and **9a** gave weaker antibacterial activities against *S. aureus*, MRSA, *B. subtilis*, *M. luteus*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, *S. typhi* and *S. dysenteriae* with the MIC values ranging from 50 to 400 μg/mL.

Antifungal activities: The antifungal evaluation exhibited that the intermediates **3-5** and most of azole-containing compounds showed antifungal efficacy against *C. albicans* and *S. cerevisiae* to some extent. Unexpectedly, these compounds showed less antifungal activities against *A. fumigatu*s, especially triazole ones, which may be due to the intrinsic mechanism resistant to triazole antifungal agents.[41] Despite of this, title compounds **9b** and **9e** showed desirable antifungal activities against *C. albicans* and *S. cerevisiae* with the MIC values between 3.1 and 12.5 μg/mL, which were nearly close to fluconazole being in clinical use. Moreover, the two compounds also displayed excellent activity against *A. fumigatu*s (MIC = 25 μg/mL), which were superior to the first-line antifungal drug fluconazole. This suggested that it is necessary to further investigate compounds **9b** and **9e** as antifungal agents.

***In vitro* assay for cytotoxic activity:** The results of preliminary cytotoxic activity showed that several compounds exhibited low growth inhibition against PC-3 cell line (Figure 2). Compounds **6** and **10a** with benzimidazole were found to be most effective

in vitro against the PC-3 cell line. The former compound **6** reached growth inhibition values (36.4, 60.1 and 76.5%) for each tested concentration: 25 μM(10.4 μg/mL), 50 μM(20.8 μg/mL) and 100 μM (41.6 μg/mL), and the latter with growth inhibition values (31.5, 54 and 71.2%) for each tested concentration: 25 μM (11.1 μg/mL), 50 μM (22.2 μg/mL) and 100 μM (44.4 μg/mL). Compounds **8b**, **9e**, and **10b** also showed comparative inhibitory activity against PC-3 cell line at the concentration of 100 μM, with the cell growth inhibition rate of 66.2%, 63.5% and 70.6% respectively, and compound **10b** also could effectively inhibit the cell growth at the mediate dose (50 μM). In addition, compounds **8a** and **9f** exhibited moderate inhibitory activity, and the antiproliferative effect achieved at 46.7% and 42.7% at the highest dose used. Compared to the good inhibitory activity of above compounds, compounds **9b**, **9c** and **9d** showed less antiproliferative activity, and compound **9a** with triazole nearly lost the antiproliferative properties. Therefore, it is rational to deduce that the nature of the azole ring exerts an effect on the antiproliferative activity. The presence of benzimidazole group afforded a clear beneficial effect with regard to antiproliferative properties. Meanwhile, the antiproliferative results indicated that all the compounds showed dose-dependent antiproliferative activity in the tested cell line. Further studies are in progress to define the important mechanisms of action of the above mentioned compounds.

Conclusion

In conclusion, a series of azole-containing piperazine derivatives were designed and synthesized by a convenient and efficient method. All new compounds were characterized by MS, IR, ^{1}H NMR and elemental analyses. The assessment of *in vitro* antibacterial and antifungal activities which were determined against eight bacterial strains and three fungal strains showed that some intermediates and target compounds exhibited significant antibacterial and antifungal activities, especially imidazole and 2-phenyl imidazole bearing compounds **9b** and **9e** presented better antibacterial and antifungal activities than other compounds against all tested bacterial strains. Moreover, the azole ring had noticeable effect on their antimicrobial efficacy, and imidazoles and benzimidazoles were much more favourable to biological activity than 1,2,4-triazole. These preliminary results suggested that the remarkable and broad-spectrum antibacterial and antifungal efficacy made compounds **9b** and **9e** as promising antimicrobial agents. Further evaluations are necessary on the way to determine the antimicrobial activities of these title compounds *in vivo* which will help us to optimize these new leading compounds.

On the other hand, compounds **6** and **10a** with benzimidazole were found to be most effective *in vitro* against the PC-3 cell line. Further studies are essential to define the important mechanisms of action of the above mentioned compounds and to deduce the structure-activity relationships.

Experimental Section

General. Melting points were determined on a X-6 melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Bio-Rad FTS-185 (Bio-Rad, Cambridge, MA, USA) by using KBr disks. NMR spectra were recorded by using $CDCl_3$ as solvent and TMS as an internal reference standard on Varian 400 spectrometer or Bruker AV 300 spectrometer. Chemical shifts are reported in parts per million (ppm). Coupling constants (*J*) are reported in hertz (Hz). The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; m, multiplet, bs, broad singlet. The piperazine was abbreviated as pip. The mass spectra were recorded on FINIGAN TRACE GC/MS. Elemental analyses were carried out on a Carlo Erba model EA 1106 elemental analyzer. TLC analyses were done using precoated silica gel plates. Column chromatographies were performed on silica gel (300 - 400 mesh) column. All chemicals and solvents were of AR grade and, when necessary, were purified and dried by standard methods.

1-(2-Chloroethyl)-4-[(4-chlorophenyl)(phenyl)methyl]piperazine (3): A mixture of 1-((4-chlorophenyl)(phenyl)methyl) piperazine (7.0 g, 24.4 mmol), triethylamine (2.828 g, 28 mmol) and a large excess of 1,2-dichloroethane was stirred at reflux for 20 h. The progress of the reaction was monitored by TLC. Upon completion, the mixture was filtered to remove the salts. The filtrate was concentrated and then directly purified by chromatographic column (petroleum ether/ethyl acetate, 5/1, v/v) to afford intermediate **3** (2.6 g, 30% yield) as yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.38-7.18 (m, 9H, Ar-H), 4.25 (s, 1H, Ar_2-CH), 3.69 (t, J = 6.8 Hz, 2H, $ClCH_2CH_2$), 2.91 (t, J = 6.8 Hz, 2H, $ClCH_2CH_2$), 2.77 (bs, 4H, pip), 2.55 (bs, 4H, pip).

1-Chloro-3-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)propan-2-ol (4): A mixture of 1-[(4-chlorophenyl)(phenyl) methyl]piperazine (5.0 g, 17.4 mmol) and 2-(chloromethyl)oxirane (4.8 g, 52.2 mmol) in anhydrous ethanol was stirred at room temperature for 24 h. After the reaction completed (monitored by TLC, eluent, petroleum ether/ethyl acetate, 5/1, v/v), the solvent was removed by a rotary evaporator, and then the mixture was cooled. Distilled water (30 mL) was added. The resulting solution was extracted with CH_2Cl_2 (30 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. The residue was purified *via* silica gel column chromatography (petroleum ether/ethyl acetate, 5/1, v/v) to give compound **4** (2.64 g) as white solid. Yield 40%; mp 70 - 72 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.5-7.35 (m, 9H, Ar-H), 4.23 (s, 1H, Ar_2CH), 3.65 (m, 2H), 2.91 (m, 2H), 2.74-2.62 (m, 8H, pip).

2-Chloro-1-[4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl]ethanone (5): To a solution of 1-((4-chlorophenyl)(phenyl) methyl)piperazine (7.0 g, 24.4 mmol) and triethylamine (2.828 g, 28 mmol) in dry dichloromethane at 0 °C was added 2-chloroacetyl chloride (6.00 g, 53.0 mmol) dropwise. The reaction mixture was stirred at 0 °C about 2 h and the stirring was continued at room temperature for about 5 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction was quenched with distilled water and extracted with chloroform (100 mL × 3). The organic layer was washed with 10% ammonium chloride solution and then water and dried over anhydrous Na_2SO_4. Chromatographic purification (petroleum ether/ethyl acetate, 4/1, v/v) afforded intermediate **5** (4.0 g, 45% yield) as yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.43-7.22 (m, 9H, Ar-H), 4.33 (s, 1H, Ar_2CH), 4.04 (s, 2H, $COCH_2$),

3.68 (bs, 2H, pip), 3.58 (bs, 2H, pip), 2.52-2.48 (bs, 4H, pip).

2-((4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-methyl)-1*H*-benzo[*d*]imidazole (6): To a stirred suspension of potassium carbonate (96 mg, 0.70 mmol) in 5 mL of acetone was added 1-((4-chlorophenyl)(phenyl)methyl)piperazine (200 mg, 0.70 mmol). The mixture was stirred at room temperature for 30 minutes, and then 2-(chloromethyl)-1*H*-benzo[*d*]imidazole (116 mg, 0.70 mmol) was added and the resulting mixture was stirred after 12 h. After the reaction came to the end (monitored by TLC, eluent, ethyl acetate/petroleum ether, 2/1, v/v), the solvent was evaporated. The residue was diluted with water (30 mL), extracted with CH_2Cl_2 (30 mL × 3), and the combined organic phase was dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. The product was purified by silica gel column chromatography (ethyl acetate/petroleum ether, 2/1, v/v) to give the final compound **6** (180 mg, 61% yield) as yellow solid; mp 109 - 110 °C; IR (KBr) 3419 (NH), 3085, 3059, 3026, 2956, 2927, 1487, 1453, 1427, 1405, 1134, 745 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.57 (s, 2H, benimidazole-H), 7.37-7.19 (m, 11H, Ar-H, benimidazole-H), 4.28 (s, 1H, Ar_2CH), 3.90 (s, 2H, benimidazole-CH_2), 2.67 (bs, 4H, pip), 2.49 (bs, 4H, pip); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 151.9, 141.7, 140.9, 132.7, 129.2, 128.7, 128.6, 127.8, 127.3, 122.5, 75.2, 56.4, 53.7, 51.6; MS (*m*/*z*) 417 $[M]^+$; Anal. Calcd. for $C_{25}H_{25}ClN_4$: C, 72.02; H, 6.04; N, 13.44; Found: C, 71.90; H, 5.96; N, 13.31.

1-(2-(1*H*-1,2,4-Triazol-1-yl)ethyl)-4-((4-chlorophenyl)(phenyl)methyl)piperazine (7): To a stirred suspension of potassium carbonate (353 mg, 2.56 mmol) in 10 mL of acetonitrile was added triazole (177 mg, 2.56 mmol). The mixture was stirred at 45 °C for 1 h. After cooled to room temperature, the compound **3** (891 mg, 2.04 mmol) was added and the mixture was stirred at 45 - 70 °C after 24 h. After the reaction came to the end (monitored by TLC, eluent, chloroform/methanol, 100/1, v/v), the solvent was evaporated and then water (30 mL) was added. The resulting mixture was extracted with CH_2Cl_2 (30 mL × 3), the combined organic phase was dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure. The product was purified by silica gel column chromatography (methanol/chloroform, 1/150 - 1/100, v/v) to give the final compound **7** (466 mg, 60% yield) as yellow oil; IR (KBr) 3146, 3027, 2941, 2881, 1600, 1519, 1489, 1451, 1239, 855, 757, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.19 (s, 1H, triazole 3-H), 7.91 (s, 1H, triazole 5-H), 7.36-7.17 (m, 9H, Ar-H), 4.32 (t, *J* = 6.4 Hz, 2H, triazole-C*H*$_2$CH$_2$), 4.20 (s, 1H, Ar_2CH), 2.87 (t, *J* = 6.4 Hz, 2H, triazole-CH$_2$C*H*$_2$), 2.56 (s, 4H, pip), 2.42 (s, 4H, pip); MS (*m*/*z*) 405 $[M+Na]^+$, 382 $[M]^+$; Anal. Calcd. for $C_{21}H_{24}ClN_5$: C, 66.04; H, 6.33; N, 18.34; Found: C, 66.14; H, 6.30; N, 18.31.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-3-(1*H*-1,2,4-triazol-1-yl)propan-2-ol (8a): Compound **8a** was prepared according to the procedure reported for **7**, starting from triazole (110 mg, 1.6 mmol), potassium carbonate (221 mg, 1.6 mmol) and compound **4** (529 mg, 1.4 mmol). The pure compound **8a** (345 mg, 60% yield) was obtained as yellow oil after 24 h. IR (KBr) 3417, 3119, 3026, 2941, 2860, 1600, 1540, 1519, 1489, 1451, 1404, 1238, 854, 760, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.17 (s, 1H, triazole 3-H), 7.88 (s, 1H, triazole 5-H), 7.34-7.14 (m, 9H, Ar-H), 4.29-4.05 (m, 5H), 2.59 (s, 2H), 2.38-2.13 (m, 8H, pip); MS (*m*/*z*) 412 $[M]^+$; Anal. Calcd. for $C_{22}H_{26}ClN_5O$: C, 64.15; H, 6.36; N, 17.00; Found: C, 64.40; H, 6.30; N, 16.89.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-3-(4-nitro-1*H*-imidazol-1-yl)propan-2-ol (8b): Compound **8b** was prepared according to the procedure reported for **7**, starting from 4-nitro-imidazole (147 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **4** (529 mg, 1.4 mmol). The pure compound **8b** (296 mg, 50% yield) was obtained as white solid after 24 h. mp 92 - 94 °C; IR (KBr) 3417, 3146, 3027, 2941, 2881, 1620, 1543, 1519, 1489, 1451, 1404, 1238, 855, 757, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, *J* = 1.6 Hz, 1H, imidazole 5-H), 7.45 (d, *J* = 1.6 Hz, 1H, imidazole 2-H), 7.35-7.16 (m, 9H, Ar-H), 4.19 (s, 1H), 4.15-4.11 (m, 1H), 3.97-3.88 (m, 2H), 2.61 (m, 2H), 2.40-2.15 (m, 8H, pip); MS (*m*/*z*) 478 $[M+Na]^+$, 456 $[M]^+$; Anal. Calcd. for $C_{23}H_{26}ClN_5O_3$: C, 60.59; H, 5.75; N, 15.36; Found: C, 60.40; H, 5.68; N, 15.29.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(1*H*-1,2,4-triazol-1-yl)ethanone (9a): Compound **9a** was prepared according to the procedure reported for **7**, starting from triazole (110 mg, 1.6 mmol), potassium carbonate (221 mg, 1.6 mmol) and compound **5** (471 mg, 1.3 mmol). The pure compound **9a** (308 mg, 60% yield) was obtained as white solid after 16 h. mp 112 - 113 °C; IR (KBr) 3119, 3060, 3026, 2960, 2920, 2857, 1661 (C=O), 1511, 1488, 1469, 1451, 1239, 851, 760, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.22 (s, 1H, triazole 3-H), 7.94 (s, 1H, triazole 5-H), 7.36-7.26 (m, 9H, Ar-H), 5.01 (s, 2H, $COCH_2$), 4.23 (s, 1H, Ar_2CH), 3.64 (bs, 2H, pip), 3.53 (bs, 2H, pip), 2.40 (bs, 4H, pip); MS (*m*/*z*) 418 $[M+Na]^+$, 396 $[M]^+$; Anal. Calcd. for $C_{21}H_{22}ClN_5O$: C, 63.71; H, 5.60; N, 17.69; Found: C, 63.54; H, 5.55; N, 17.55.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(1*H*-imidazol-1-yl)ethanone (9b): Compound **9b** was prepared according to the procedure reported for **7**, starting from imidazole (109 mg, 1.6 mmol), potassium carbonate (221 mg, 1.6 mmol) and compound **5** (471 mg, 1.3 mmol). The pure compound **9b** (380 mg, 74% yield) was obtained as yellow solid after 48 h. mp: 80 - 81 °C; IR (KBr) 3050, 3027, 2920, 2859, 1660 (C=O), 1540, 1490, 1452, 1426, 1408, 1238, 852, 756, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.50 (s, 1H, imidazole 2-H), 7.35-7.20 (m, 9H, Ar-H), 7.08 (s, 1H, imidazole 4-H), 6.92 (s, 1H, imidazole 5-H), 4.74 (s, 2H, $COCH_2$), 4.24 (s, 1H, Ar_2CH), 3.63 (bs, 2H, pip), 3.44 (bs, 2H, pip), 2.39 (bs, 4H, pip); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.4, 141.2, 140.4, 137.8, 132.9, 131.4, 129.1, 128.9, 128.8, 127.7, 127.5, 120.2, 75.1, 51.5, 51.2, 48.1, 45.2, 42.4; MS (*m*/*z*) 417 $[M+Na]^+$, 395 $[M]^+$; Anal. Calcd. for $C_{22}H_{23}ClN_4O$: C, 66.91; H, 5.87; N, 14.19; Found: C, 66.71; H, 5.82; N, 14.11.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(4-nitro-1*H*-imidazol-1-yl)ethanone (9c): Compound **9c** was prepared according to the procedure reported for **7**, starting from 4-nitro-1*H*-imidazole (147 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **9c** (404 mg, 71% yield) was obtained as white solid after 16 h. mp 150 - 152 °C; IR (KBr) 3061, 3027, 2961, 2920, 2859, 1661 (C=O), 1545, 1521, 1490, 1452, 1426, 1408, 1238, 851, 760, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.80 (s, 1H, imidazole 5-H), 7.42 (s, 1H, imidazole 2-H), 7.35-

7.23 (m, 9H, Ar-H), 4.81 (s, 2H, $COCH_2$), 4.28 (s, 1H, Ar_2CH), 3.66 (bs, 2H, pip), 3.48 (bs, 2H, pip), 2.45 (d, J = 14 Hz, 4H, pip); MS (*m*/*z*) 462 $[M+Na]^+$; Anal. Calcd. for $C_{22}H_{22}ClN_5O_3$: C, 60.07; H, 5.04; N, 15.92; Found: C, 60.21; H, 5.00; N, 15.88.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(2-methyl-4-nitro-1*H*-imidazol-1-yl)et-hanone (9d): Compound **9d** was prepared according to the procedure reported for **7**, starting from 2-methyl-5-nitro-1*H*-imidazole (165 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **9d** (312 mg, 53% yield) was obtained as white solid after 16 h. mp 190 - 192 °C; IR (KBr) 3145, 3061, 3026, 2963, 2925, 2862, 1662 (C=O), 1541, 1492, 1471, 1451, 1425, 1239, 835, 756, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.68 (s, 1H, imidazole 5-H), 7.35-7.23 (m, 9H, Ar-H), 4.72 (s, 2H, $COCH_2$), 4.28 (s, 1H, Ar_2CH), 3.64 (bs, 2H, pip), 3.50 (bs, 2H, pip), 2.47 (bs, 2H, pip), 2.42 (bs, 2H, pip), 2.34 (s, 3H, CH_3); MS (*m*/*z*) 476 $[M+Na]^+$, 454 $[M]^+$; Anal. Calcd. for $C_{23}H_{24}ClN_5O_3$: C, 60.86; H, 5.33; N, 15.43; Found: C, 60.61; H, 5.29; N, 15.50.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(2-phenyl-1*H*-imidazol-1-yl)ethanone (9e): Compound **9e** was prepared according to the procedure reported for **7**, starting from 2-phenyl-1*H*-imidazole (187 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **9e** (415 mg, 68% yield) was obtained as yellow solid after 48 h. mp 132 - 134 °C; IR (KBr) 3060, 3027, 2965, 2920, 2859, 1659 (C=O), 1477, 1450, 1413, 1237, 850, 758, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.48-7.21 (m, 14H, Ar-H), 7.15 (s, 1H, imidazole 4-H), 7.00 (s, 1H, imidazole 5-H), 4.71 (s, 2H, $COCH_2$), 4.23 (s, 1H, Ar_2CH), 3.64 (bs, 2H, pip), 3.28 (bs, 2H, pip), 2.37 (bs, 2H, pip), 2.27 (bs, 2H, pip); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.9, 148.0, 141.1, 140.3, 133.0, 129.1, 128.9, 128.8, 128.7, 128.5, 127.7, 127.5, 122.0, 98.4, 75.0, 51.6, 51.3, 48.0, 45.1, 42.5; MS (*m*/*z*) 471 $[M]^+$; Anal. Calcd. for $C_{28}H_{27}ClN_4O$: C, 71.40; H, 5.78; N, 11.90; Found: C, 71.23; H, 5.26; N, 15.45.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(2-methyl-1*H*-imidazol-1-yl)ethanone (9f): Compound **9f** was prepared according to the procedure reported for **7**, starting from 2-methyl-1*H*-imidazole (131 mg, 1.6 mmol), potassium carbonate (221 mg, 1.6 mmol) and compound **5** (471 mg, 1.3 mmol). The pure compound **9f** (263 mg, 50% yield) was obtained as yellow solid after 48 h. mp 115 - 116 °C; IR (KBr) 3027, 2964, 2922, 2859, 1657 (C=O), 1531, 1487, 1471, 1422, 1237, 837, 759, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.35-7.21 (m, 9H, Ar-H), 6.90 (s, 1H, imidazole 4-H), 6.76 (s, 1H, imidazole 5-H), 4.60 (s, 2H, $COCH_2$), 4.23 (s, 1H, Ar_2CH), 3.62 (bs, 2H, pip), 3.44 (bs, 2H, pip), 2.38 (bs, 4H, pip), 2.29 (s, 3H, CH_3); MS (*m*/*z*) 431 $[M+Na]^+$, 409 $[M]^+$; Anal. Calcd. for $C_{23}H_{25}ClN_4O$: C, 67.55; H, 6.16; N, 13.70; Found: C, 67.32; H, 5.22; N, 15.39.

2-(1*H*-Benzo[*d*]imidazol-1-yl)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)ethanone (10a): Compound **10a** was prepared according to the procedure reported for **7**, starting from benzimidazole (153 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **10a** (226 mg, 39% yield) was obtained as yellow solid after 48 h. mp 156 - 157 °C; IR (KBr) 3059, 3027, 2967, 2921, 2860, 1658 (C=O), 1495, 1457, 1423, 1238, 850, 744, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.90 (s, 1H, benzimidazole 2-H), 7.80 (s, 1H, benzimidazole 4-H), 7.34-7.22 (m, 12H, Ar-H), 4.92 (s, 2H, $COCH_2$), 4.24 (s, 1H, Ar_2CH), 3.65 (bs, 2H, pip), 3.52 (bs, 2H, pip), 2.40 (bs, 4H, pip); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.3, 143.7, 141.3, 140.5, 133.1, 129.2, 129.0, 128.9, 127.8, 127.6, 123.3, 122.4, 120.5, 109.4, 75.1, 51.7, 51.3, 45.9, 45.4, 42.6; MS (*m*/*z*) 445 $[M]^+$; Anal. Calcd. for $C_{26}H_{25}ClN_4O$: C, 70.18; H, 5.66; N, 12.59; Found: C, 70.35; H, 5.72; N, 12.55.

1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-2-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-1-yl)ethanone (10b): Compound **10b** was prepared according to the procedure reported for **7**, starting from benzimidazole (190 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **10b** (166 mg, 27% yield) was obtained as yellow solid after 48 h. mp 200 - 201 °C; IR (KBr) 3059, 3027, 2967, 2921, 2860, 2680, 1659 (C=O), 1495, 1457, 1423, 1238, 852, 750, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.77 (s, 1H, benzimidazole-H), 7.55 (s, 1H, benzimidazole-H), 7.34-7.22 (m, 9H, Ar-H), 7.04 (s, 1H, benzimidazole-H), 4.86 (s, 2H, $COCH_2$), 4.23 (s, 1H, Ar_2CH), 3.65 (bs, 2H, pip), 3.49 (bs, 2H, pip), 2.39 (bs, 4H, pip), 2.35 (s, 6H, CH_3); MS (*m*/*z*) 473 $[M]^+$; Anal. Calcd. for $C_{28}H_{29}ClN_4O$: C, 71.10; H, 6.18; N, 11.84; Found: C, 70.90; H, 6.12; N, 12.00.

2-(1*H*-Benzo[*d*][1,2,3]triazol-1-yl)-1-(4-((4-chlorophenyl)-(phenyl)methyl)piperazin-1-yl)ethanone (10c): Compound **10c** was prepared according to the procedure reported for **7**, starting from benzotriazole (155 mg, 1.3 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (507 mg, 1.4 mmol). The pure compound **10c** (465 mg, 80% yield) was obtained as white solid after 48 h. mp 189 - 190 °C; IR (KBr) 3166, 3061, 3026, 2962, 2921, 2858, 1632 (C=O), 1448, 1439, 1403, 1224, 849, 758, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.51-7.49 (m, 2H, benzotriazole-H), 7.35-7.17 (m, 11H, Ar-H, benzotriazole-H), 4.24 (s, 1H, Ar_2CH), 3.96 (s, 2H, $COCH_2$), 3.71 (bs, 2H, pip), 3.60 (bs, 2H, pip), 2.45 (d, J = 23.2 Hz, 4H, pip); MS (*m*/*z*) 468 $[M+Na]^+$, 446 $[M]^+$; Anal. Calcd. for $C_{25}H_{24}ClN_5O$: C, 67.33; H, 5.42; N, 15.70; Found: C, 67.60; H, 5.38; N, 15.60.

2-(1*H*-Benzo[*d*]imidazol-2-ylthio)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)ethanone (11): Compound **11** was prepared according to the procedure reported for **7**, starting from 1*H*-benzo[*d*]imidazole-2-thiol (127 mg, 0.847 mmol), potassium carbonate (179 mg, 1.3 mmol) and compound **5** (217 mg, 0.598 mmol). The pure compound **11** (268 mg, 85% yield) was obtained as white solid after 48 h. mp 185 - 187 °C; IR (KBr) 3425(NH), 3063, 3027, 2959, 2923, 2861, 1662 (C=O), 1454, 1405, 1234, 850, 747, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.04 (d, J = 8.0 Hz, 1H, benzimidazole-H), 7.56 (d, J = 8.0 Hz, 1H, benzimidazole-H), 7.48 (t, J = 8.0 Hz, 1H, benzimidazole-H), 7.38-7.20 (m, 10H, benzimidazole-H, Ar-*H*), 5.46 (s, 2H, $COCH_2$), 4.19 (s, 1H, Ar_2CH), 3.61 (bs, 4H, pip), 2.33 (m, 4H, pip); MS (*m*/*z*) 477 $[M]^+$. Anal. Calcd. for $C_{26}H_{25}ClN_4OS$: C, 65.46; H, 5.28; N, 11.75; Found: C, 65.24; H, 5.20; N, 11.62.

Antibacterial and antifungal assays. The *in vitro* minimal inhibitory concentrations (MICs) of the compounds **3-11** were determined by the twofold broth dilution method against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Methicillin-resistant Staphylococcus aureus* N315 (MRSA), *Bacillus*

subtilis ATCC 6633 and *Micrococcus luteus* ATCC 4698), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Salmonella typhi ATCC 9484* and *Shigella dysenteriae*) and fungi (*Candida albicans* ATCC 76615; *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus fumigatus* ATCC) 96918. The minimal inhibitory concentration (MIC, μg/mL) was defined as the lowest concentration of compounds that completely inhibited the growth of each strain.[36] Antibacterial drug chloramphenicol and antifungal drug fluconazole were used as standard drugs. Test compounds were dissolved in H_2O containing 1% DMSO and diluted into different concentrations from 0.8 to 400 μg/mL with liquid medium. The bacterial inocula was at the concentration of 1×10^5 CFU, and the fungi spore $1 - 5 \times 10^3$ spore/mL. These dilutions were inoculated and incubated at 37 °C for 24 h for bacteria in Mueller-Hinton broth and at 30 °C for 48 h for the fungi in improved Sabourasud medium. Microbial growth was monitored visually and spectrophotometrically. The H_2O/1% DMSO was used as a blank, inoculation bacterial not medicine as positive control. All experiments were performed in triplicate.

MTT assay for cytotoxic activity. The human prostatic carcinoma cell line (PC-3) was purchased from the Shanghai Institutes for Biological Sciences, China. Ham's F12 Nutrient Mixture, enriched with 10% heat inactivated foetal bovine serum (FBS) and 1% of penicillin-streptomycin was used for cell cultivation and to perform the tests. The cytotoxic activity was investigated using the MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide).[26] Stock solutions (100 mM) of tested compounds **6**, **8a-b**, **9a-f** and **10a-b** were prepared in dimethylsulfoxide (DMSO) and stored at −20 °C prior to dilution into the biological assay. Cell suspensions were diluted to 10^5 cells/mL, suitably prepared and distributed in plates of culture with 96 wells (225 μL in each well), then incubated at 37 °C in a humid atmosphere with 5% of CO_2. After 24 h, 25 μL of the test compounds were added to each well. The plates were incubated again at 37 °C for 24 h. Then, 25 μL of MTT solution (5 mg/mL) was added to each well, and the mixture was incubated at 37 °C for 2 h. At the end of this period, the culture medium with the MTT excess was aspirated and after that, 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) of the wells was measured at 490 nm. Results were evaluated by comparing the absorbance of the wells containing compound treated cells with the absorbance of wells containing 0.1% DMSO alone (solvent control). Conventionally, cell viability was estimated to be 100% in the solvent control. All assays were performed in triplicate and mean ± SD values were used to estimate cell growth inhibition rate.

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