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Design, synthesis and biological evaluation of 5-fluorouracil-derived benzimidazoles as novel type of potential antimicrobial agents

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ABSTRACT

A series of 5-fluorouracil benzimidazoles as novel type of potential antimicrobial agents were designed and synthesized for the first time. Bioactive assay manifested that some of the prepared compounds exhibited good or even stronger antibacterial and antifungal activities against the tested strains in comparison with reference drugs norfloxacin, chloromycin and fluconazole. Noticeably, 3-fluorobenzyl benzimidazole derivative **5c** gave remarkable antimicrobial activities against *S. cerevisiae*, MRSA and *B. proteus* with MIC values of 1, 2 and 4 μg/mL, respectively. Experimental research revealed that compound **5c** could effectively intercalate into calf thymus DNA to form compound **5c**–DNA complex which might block DNA replication and thus exert antimicrobial activities. Molecular docking indicated that compound **5c** should bind with DNA topoisomerase IA through three hydrogen bonds by the use of fluorine atom and oxygen atoms in 5-fluorouracil with the residue Lys 423.

Pyrimidine ring is a six-membered aromatic heterocycle [1] bearing two nitrogen atoms, which extensively exists in biologically active natural products. The special heterocycle can readily interact with biological macromolecules like enzymes, receptors, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Thus, pyrimidine fragment has been prevalently employed in the design of new drugs and a lot of pyrimidines as drugs have been successfully developed and extensively used in clinic. Especially in antimicrobial aspects, many pyrimidine compounds such as sulfadiazine and voriconazole have been in widespread clinical use to treat diseases caused by bacteria and fungi. However, the increasing incidence of multidrug-resistant strains, intractable pathogenic microorganisms and newly emerging pathogens gradually limited their clinical application.[1] The development of new pyrimidine compounds have been an active topic in discovering highly potential antimicrobial agents. 5-Fluorouracil (5-FU), a known anticancer drug which target the nucleotide synthetic enzyme thymidylate synthase and disrupt DNA synthesis and thus inhibit the growth of cell,[2] is an antimetabolite of the pyrimidine derivative in which the fluoro and oxo groups play positive roles in exerting bioactivity. However, so far the 5-fluorouracil-based antimicrobial research has been seldom observed. Therefore, it is of great interest for us to employ pyrimidine-containing 5-FU as a constructing block to develop a series of hybrids of 5-FU and benzimidazole nucleus, and investigate their antimicrobial potency.

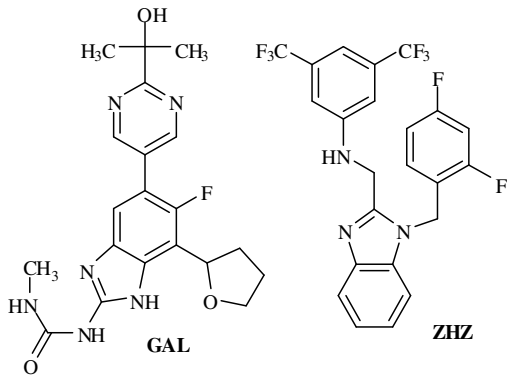

Figure 1. Structure of some antimicrobial benzimidazole derivatives.

Benzimidazoles exert various pharmacological activities such as antiparasitic, anticancer, antihistaminic, antihypertensive, antiulcer properties, and some of them have been successfully developed as clinical drugs.[3] This has drawn more and more concern to investigate the other medicinal application of benzimidazoles. Recently, extensive biochemical and pharmacological studies revealed that benzimidazoles possessed large potentiality to inhibit the growth of bacterial and fungal strains.[4] Benzimidazole is structurally similar to purine, and its derivatives could compete with purines, distinctly inhibiting the synthesis of nucleic acids and proteins, thereby killing bacterial strains or inhibiting their growth.[5] Several benzimidazoles have

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been found to easily interact with DNA and RNA.[6] Aminobenzimidazole urea **GAL** as dual inhibitors could target bacterial DNA gyrase and topoisomerase □ to exert broad-spectrum Gram-positive antibacterial activities.[7] Benzimidazole derivative **ZHZ** could effectively intercalate into calf thymus DNA and block DNA replication, thus exerting powerful antimicrobial activities (Fig. 1).[8] All these findings clearly pointed out that benzimidazole derivatives were promising candidates for developing new antimicrobial agents.

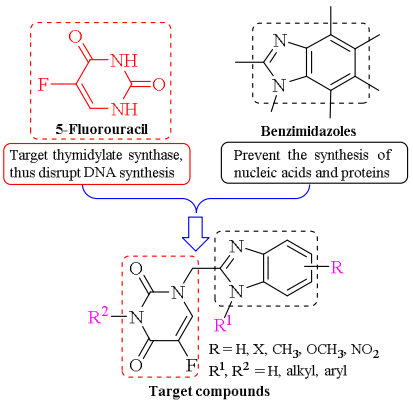


Figure 2. Design of novel 5-fluorouracil-derived benzimidazoles.

Inspired by special features of benzimidazole and 5-FU, and as a part of our ongoing research, it is worthwhile to introduce benzimidazole ring into the N^1-position of 5-FU core *via* methylene group to form a new hybrid skeleton, which was expected to show good antimicrobial activities through targeting DNA (Fig. 2). Here, we explored the role of fluorine atom at 5-position on benzimidazole ring due to its special structure and unique properties. Various alkyl or aryl groups were introduced into benzimidazole and 5-FU moiety to investigate the effect of different substituents on biological activities. In order to explore the importance of R^2 fraction, we synthesized several compounds without any substituents at 3-position of 5-FU. In view of the above observations, a novel series of 5-FU benzimidazole hybrids **3–5**, **8** and **9** were designed and prepared. Their antibacterial and antifungal activities were evaluated *in vitro* against four Gram-positive bacteria, four Gram-negative bacteria and four fungi strains. Moreover, the preliminary action mechanism of highly active compound **5c** was investigated by interacting with DNA.

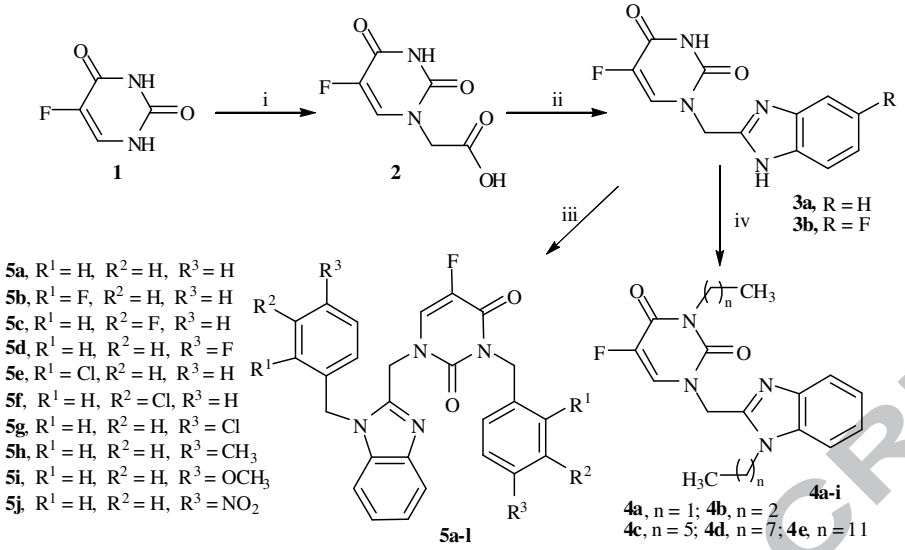
The target benzimidazole compounds and their intermediates were prepared according to the synthetic route outlined in Schemes 1 and 2. The reaction of commercially available 5-FU with chloroacetic acid in presence of aqueous potassium hydroxide afforded compound **2**.[9] Conjugates **3a** and **3b** were efficiently prepared by cyclization of compound **2** and *o*-phenylenediamine or its derivatives with yields of 80% and 78%, respectively. The *N*-alkylation of intermediate **3a** with a series of alkyl bromides in acetonitrile at 70 °C with potassium carbonate as base respectively afforded target alkyl benzimidazole compounds **4a–e** with yields ranging from 61% to 77% yields. It was found that the length of aliphatic chain in alkyl bromides played a slight role in forming target compounds. Halobenzyl halides and intermediate **3a** underwent *N*-alkylation to produce compounds **5a–j** with a large difference in yields of 47–78%.

As shown in Scheme 2, mono-substituted 5-FU derivatives **8** and **9** could be directly obtained from compound **2**. The reaction of *o*-phenylenediamine with alkyl halides or 1-(chloromethyl)-3-fluorobenzene efficiently provided compounds **6** and **7** in the presence of potassium carbonate and DMF, and the obtained products were subsequently reacted with acid **2** in polyphosphate (PPA) at 180 °C to produce target hybrids **8a–d** and **9** in 66–71%. All new compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, and HRMS spectra.

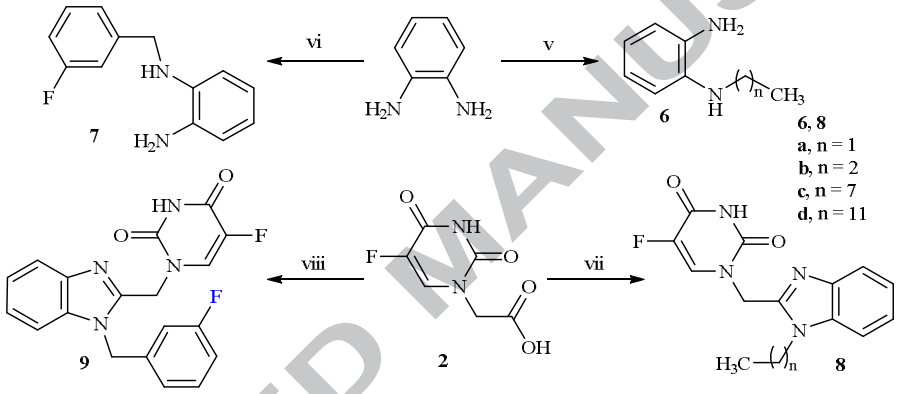
The *in vitro* antimicrobial activities for all synthesized novel benzimidazoles **3–5**, **8** and **9** were evaluated against four Gram-positive bacteria, four Gram-negative bacteria and four fungi using two fold serial dilution technique recommended by National Committee for Clinical Laboratory Standards (NCCLS).[10] Clinical drugs chloromycin, norfloxacin and fluconazole were taken as positive control. The results of antibacterial and antifungal screening are summarized in Table 1.

The antibacterial assay declared that most of the synthesized benzimidazole derivatives exhibited moderate to excellent activity against all the tested bacterial strains *in vitro*. Notably, compound **5c** displayed remarkable antibacterial activities compared with chloromycin and norfloxacin. Towards fungi *S. cerevisiae*, it displayed the highest antifungal activity (MIC = 1 μg/mL) among these prepared conjugates, which was 16-fold more potent than fluconazole. This revealed that compound **5c** could serve as a lead compound in the development of more effective board-spectrum antimicrobial agents.

As shown in Table 1, the length of alkyl chain had significant influence on biological activities. Generally, compounds bearing short alkyl chain could effectively inhibit the growth of tested microorganisms. Among alkyl derivatives, compound **4c** with pentyl groups showed strong anti-*B. proteus* activity with MIC value of 8 μg/mL, which was comparable to or even better than reference drugs norfloxacin (MIC = 8 μg/mL) and chloromycin (MIC = 32 μg/mL). The replacement of pentyl fragment in hybrid **4c** by ethyl or propyl group would lead to weaker activity against *B. proteus*. Towards MRSA and *S. aureus*, benzimidazole derivative **4c** exhibited equivalent potency to chloromycin (MIC = 16 μg/mL), which was more potentiality than compounds **4a** and **4b** (MIC = 32 μg/mL). Surprisingly, shortening the length of subsituents from pentyl to ethyl group led to the improvement of activities against *B. subtilis* and *E. coli*. With regard to mono-substituted 5-FU derivatives, compound **8a** showed 4-fold stronger activity than control drug chloromycin (MIC = 32 μg/mL) for *E. coli DH52*. When the length of alkyl chain was extended to heptyl or even dodecyl groups, benzimidazoles **4d**, **4e** and **8d** were not efficient to inhibit the growth of bacteria even at high concentration. This might be explained by the presence of long alkyl chain with good lipophilicity, which makes them unfavorable for being delivered to binding sites. These facts demonstrated that suitable side chain was necessary for better antibacterial activities in drug design.



Scheme 1. Reagents and conditions: i) chloroacetic acid, 10% potassium hydroxide, reflux, 76%; ii) *o*-phenylenediamine, polyphosphoric acid, 180 °C, 78–80%; iii) substituted halobenzyl halide, potassium carbonate, acetonitrile, 70 °C, 47–78%; iv) alkyl bromide, potassium carbonate, acetonitrile, 70 °C, 61–77%.



Scheme 2. Reagents and conditions: v) alkyl bromide, potassium carbonate, DMF, r.t., 30–46%; vi) substituted halobenzyl halide, potassium carbonate, DMF, r.t., 38%; vii) corresponding *o*-phenylenediamines, polyphosphoric acid, 180 °C, 66–77%; viii) corresponding *o*-phenylenediamine, polyphosphoric acid, 180 °C, 69%.

In comparison with alkyl benzimidazole compounds **4a–e** and **8a–d**, derivatives **5a–j** and **9** bearing a variety of halobenzyl groups exerted relatively better activities in inhibiting the growth of tested strains. The activities of compound **5c** with 3-fluorobenzyl groups against *S. aureus*, *B. subtilis*, *E. coli DH52* and *E. coli JM109* were better than chloromycin. Moreover, its anti-MRSA activity (MIC = 2 μg/mL) was 4-fold higher than norfloxacin (MIC = 8 μg/mL) and 8-fold higher than chloromycin (MIC = 16 μg/mL). For Gram-negative bacteria *B. typhi*, it was the most sensitive to compound **5c** among all synthesized novel benzimidazole compounds. Removal of 3-fluorobenzyl group at 3-position of 5-FU core in compound **5c** would produce benzimidazole **9**, which afforded the best anti-*B. proteus* activity with MIC value of 2 μg/mL, 4-fold higher than norfloxacin. Considering the MIC values of **5b–d**, it could be found that biological activities largely depended on the position of fluorine atom. Incorporation of methoxyl residue into benzene ring of compound **3a** generated benzimidazole **5i**, which gave improved potency towards the tested bacteria. The activity of compound **5j** with nitro group at 4-position of benzene ring against *B. subtilis* (MIC = 32 μg/mL) and *B. typhi* (MIC = 32 μg/mL) was comparable to chloromycin. All the facts demonstrated that not only the type and position of substitutions at benzene ring but also the number of halobenzyl group exerted important effect.

Antifungal screening revealed that some compounds also showed good inhibition against various tested microbial strains compared with standard drug fluconazole. Among the synthesized compounds, derivatives **4c**, **8a** and **8b** showed potent activities against *S. cerevisiae*, which were superior to fluconazole. When the length of alkyl side chain in compound **4c** was altered, the resulting derivatives **4a** and **4b** displyed weak activities against *S. cerevisiae*. Halophenyl fragments also played key role in antifungal potency. Fluorobenzyl derivative **5c** exhibited the highest antifungal activity (MIC = 1 μg/mL) among these prepared conjugates of 5-FU and benzimidazole towards *S. cerevisiae*, which was 16-fold higher than fluconazole. Regrettably, all other benzimidazole compounds showed weak antifungal activities against *C. albicans* and *C. mycoderma.*

It was found that intermediate **3b** displayed fairly good activities against most of the tested bacterial and fungal strains in comparison with compound **3a** (Table 1). Particularly, compound **3b** exerted 16-fold more active than intermediate **3a** against *S. aureus*, which was superior to reference drug chloromycin. Towards *S. cerevisiae*, benzimidazole **3b** exerted more inhibitory activity than fluconazole. Consequently, the introduction of fluorine on benzimidazole ring seemed to be favorable for their antimicrobial efficacy.

Table 1 ClogP values and *in vitro* antibacterial and antifungal activities as MIC (μg/mL) for compounds **3–5, 8, 9** [a,b,c,d]

Compds	clogP	Gram-positive bacteria				Gram-negative bacteria				Fungi			
		MRSA	*S. aureus*	*B. subtilis*	*M. luteus*	*E. coli* (DH52)	*E. coli* (JM109)	*B. proteus*	*B. typhi*	*C. albicans*	*C. mycoderma*	*S. cerevisiae*	*A. flavus*
3a	0.47	128 ± 68.66	128 ± 68.66	64 ± 6.34	64 ± 6.34	64 ± 6.34	32 ± 3.47	16 ± 1.75	32 ± 3.47	32 ± 3.47	64 ± 6.34	64 ± 6.34	128 ± 68.66
3b	0.69	16 ± 1.75	8 ± 1.16	128 ± 68.66	16 ± 1.75	32 ± 3.47	128 ± 68.66	16 ± 1.75	32 ± 3.47	2 ± 0.21	128 ± 68.66	8 ± 1.16	128 ± 68.66
4a	2.2	64 ± 6.34	32 ± 3.47	32 ± 3.47	32 ± 3.47	32 ± 3.47	16 ± 1.75	32 ± 3.47	64 ± 6.34	64 ± 6.34	32 ± 3.47	32 ± 3.47	64 ± 6.34
4b	3.26	64 ± 6.34	32 ± 3.47	64 ± 6.34	32 ± 3.47	32 ± 3.47	16 ± 1.75	128 ± 68.66	64 ± 6.34	64 ± 6.34	32 ± 3.47	128 ± 68.66	64 ± 6.34
4c	6.43	16 ± 1.75	16 ± 1.75	512 ± 49.63	32 ± 3.47	512 ± 49.63	16 ± 1.75	8 ± 1.16	512 ± 49.63	>512	8 ± 1.16	8 ± 1.16	> 512
4d	8.55	> 512	> 512	512 ± 49.63	512 ± 49.63	512 ± 49.63	512 ± 49.63	>512	512 ± 49.63	64 ± 6.34	512 ± 49.63	> 512	512 ± 49.63
4e	-	> 512	> 512	512 ± 49.63	512 ± 49.63	512 ± 49.63	256 ± 137.24	512 ± 49.63	512 ± 49.63	256 ± 137.24	32 ± 3.47	> 512	512 ± 49.63
5a	4.58	> 512	> 512	64 ± 6.34	128 ± 68.66	> 512	> 512	> 512	128 ± 68.66	128 ± 68.66	128 ± 68.66	> 512	64 ± 6.34
5b	4.86	512 ± 49.63	16 ± 1.75	128 ± 68.66	512 ± 49.63	128 ± 68.66	128 ± 68.66	512 ± 49.63	512 ± 49.63	> 512	512 ± 49.63	128 ± 68.66	> 512
5c	4.86	2 ± 0.21	4 ± 0.59	16 ± 1.75	64 ± 6.34	2 ± 0.21	8 ± 1.16	4 ± 0.59	8 ± 1.16	32 ± 3.47	32 ± 3.47	1 ± 0.13	8 ± 1.16
5d	4.86	512 ± 49.63	> 512	> 512	> 512	> 512	> 512	> 512	512 ± 49.63	>512	> 512	> 512	512 ± 49.63
5e	6.00	> 512	> 512	> 512	> 512	> 512	> 512	> 512	512 ± 49.63	512 ± 49.63	512 ± 49.63	> 512	512 ± 49.63
5f	6.00	128 ± 68.66	512 ± 49.63	64 ± 6.34	128 ± 68.66	16 ± 1.75	128 ± 68.66	64 ± 6.34	64 ± 6.34	64 ± 6.34	64 ± 6.34	64 ± 6.34	64 ± 6.34
5g	6.00	> 512	> 512	> 512	> 512	> 512	> 512	> 512	512	4 ± 0.59	> 512	> 512	> 512
5h	5.58	> 512	> 512	> 512	> 512	128 ± 68.66	> 512	> 512	512 ± 49.63	> 512	> 512	> 512	> 512
5i	4.42	32 ± 3.47	128 ± 68.66	64 ± 6.34	64 ± 6.34	128 ± 68.66	16 ± 1.75	32 ± 3.47	64 ± 6.34	64 ± 6.34	512 ± 49.63	64 ± 6.34	32 ± 3.47
5j	4.06	> 512	> 512	32 ± 3.47	> 512	> 512	>512	> 512	32 ± 3.47	>512	>512	>512	> 512
8a	1.02	16 ± 1.75	16 ± 1.75	64 ± 6.34	16 ± 1.75	8 ± 1.16	64 ± 6.34	32 ± 3.47	32 ± 3.47	2 ± 0.21	16 ± 1.75	4 ± 0.59	32 ± 3.47
8b	1.54	16 ± 1.75	16 ± 1.75	16 ± 1.75	16 ± 1.75	64 ± 6.34	64 ± 6.34	64 ± 6.34	64 ± 6.34	2 ± 0.21	16 ± 1.75	8 ± 1.16	32 ± 3.47
8c	4.19	512 ± 49.63	512 ± 49.63	512 ± 49.63	512 ± 49.63	64 ± 6.34	256 ± 137.24	512 ± 49.63	512 ± 49.63	32 ± 3.47	64 ± 6.34	32 ± 3.47	32 ± 3.47
8d	6.31	> 512	> 512	> 512	> 512	> 512	>512	> 512	> 512	>512	512 ± 49.63	64 ± 6.34	> 512
9	2.40	64 ± 6.34	4 ± 0.59	8 ± 1.16	16 ± 1.75	2 ± 0.21	64 ± 6.34	2 ± 0.21	128 ± 68.66	16 ± 1.75	16 ± 1.75	4 ± 0.59	32 ± 3.47
A	-	16 ± 1.75	16 ± 1.75	32 ± 3.47	8 ± 1.16	32 ± 3.47	32 ± 3.47	32 ± 3.47	32 ± 3.47	-	-	-	-
B	-	8 ± 1.16	2 ± 0.21	4 ± 0.59	2 ± 0.21	1 ± 0.13	1 ± 0.13	8 ± 1.16	4 ± 0.59	-	-	-	-
C	-	-	-	-	-	-	-	-	-	1 ± 0.13	4 ± 0.59	16 ± 1.75	256 ± 137.24

[a] Minimum inhibitory concentrations were determined by micro broth dilution method for microdilution plates.
[b] **A** = Chloromycin, **B** = Norfloxacin, **C** = Fluconazole.
[c] MRSA, Methicillin-Resistant *Staphylococcus aureus* N315; *S. aureus, Staphylococcus aureus* ATCC25923; *B. subtilis, Bacillus subtilis* ATCC6633; *M. luteus, Micrococcus luteus* ATCC4698; *E. coli* DH52, *Escherichia coli*; *E. coli* JM109, *Escherichia coli*; *B. proteus, Bacillus proteus* ATCC13315; *B. typhi*, *Bacillus typhi*; *C. albicans*, *Candida albicans* ATCC10231; *C. mycoderma*, *Candida mycoderma* ATCC9888; *S. cerevisia*, *Saccharomyces cerevisia*; *A. flavus*, *Aspergillus flavus* ATCC204304.
[d] ClogP values were calculated by ChemDraw Ultra 10.0.

Lipophilicity/hydrophilicity properties had significant effect on exerting biological activity.[11] Accordingly, the calculated liposome/water partition coefficients (ClogP) by ChemDraw Ultra 10.0 software could be applied to analyze antimicrobial activities. The calculated values of ClogP were shown in Table 1. The data demonstrated that most of compounds with lower values of ClogP showed better antimicrobial activities. As seen from Table 1, the ClogP values of compounds **4a–d** and **8a–d** generally increased with the increase of alkyl length, and their potencies were gradually reduced, which might be explained by the possibility that higher lipophilic compounds were unfavourable for being delivered to the binding sites in organism. However, these were not available for compounds **5h–j**. All those facts confirmed the significant role of suitable lipophilicity in drug design.

DNA is a significant information molecule encoding genetic instructions used in the development and function of almost all known living organisms. It has been regarded as an important target for studies of bioactive molecules like antimicrobial drugs.[12] Thus, the study of the interaction of small molecules with DNA is propitious to understand action mechanism of drugs at molecular level, and to rationally design and construct new and efficient drugs targeting DNA. In order to explore possible antimicrobial action mechanism, binding behavior of highly active compound **5c** with calf thymus DNA (a DNA model with medical importance, low cost and ready availability properties) was studied *in vitro* by UV−vis spectroscopic and fluorescent methods.[13-16] The results indicated that compound **5c** could effectively intercalate into calf thymus DNA by forming compound **5c**–DNA complex which might block DNA replication and thus exert antimicrobial activities (Supplementary data).

To rationalize the observed antibacterial activity and understand the possible mechanism of the hybrids, a flexible ligand receptor docking investigation was undertaken. The crystal structure data (DNA topoisomerase IA) was obtained from the Protein Data Bank, which was a representative target to investigate the antibacterial mechanism. Target compound **5c** was found to dock into the DNA topoisomerase IA (Topo IA) through three hydrogen bonds by the use of fluorine atom and oxygen atoms in 5-fluorouracil with the residue Lys 423 (Supplementary data).

In summary, a new series of 5-FU benzimidazoles were successfully developed for the first time *via* an easy, convenient and efficient synthetic procedure starting from commercial 5-fluorouracil. All the new compounds were confirmed by ^{1}H NMR, ^{13}C NMR, IR and HRMS. The *in vitro* antimicrobial activities manifested that some prepared compounds exhibited good or even superior antibacterial and antifungal activities against the tested strains to the reference drugs chloromycin, norfloxacin and fluconazole. Hybrids **4a**, **8a** and **8b** showed significant inhibition against all tested strains with low inhibitory concentrations in range of 2–64 μg/mL. Especially, benzimidazole derivative **5c** was efficacious in inhibiting the growth of MRSA (MIC = 2 μg/mL) and *B. proteus* (MIC = 4 μg/mL). Towards fungi *S. cerevisiae*, it gave the highest antifungal activity (MIC = 1 μg/mL) among these prepared conjugates, which was 16-fold higher than fluconazole. Experimental research revealed that compound **5c** could effectively intercalate into calf thymus DNA to form compound **5c**–DNA complex which might block DNA replication and thus exert antimicrobial activities. Molecular docking indicated that compound **5c** should bind with DNA topoisomerase IA through three hydrogen bonds by the use of fluorine atom and oxygen atoms in 5-fluorouracil with the residue Lys 423. Further researches, including the *in vivo* bioactive evaluation, bacterial membrane permeabilization, time–kill kinetic assay, toxicity, and some effect factors on antimicrobial activities such as other heterocyclic azole rings (benzotriazole, imidazole, triazole and their derivatives), various functional groups (amino, halogen and nitro groups, etc.) linked to benzimidazole fragment as well as other substituents on 5-fluorouracil backbone are now in progress in our group.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/

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Graphical Abstract

Design, synthesis and biological evaluation of 5-fluorouracil-derived benzimidazoles as novel type of potential antimicrobial agents

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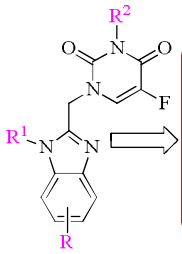

5c, R = H, $R^1 = R^2$ = 3-fluorobenzyl
MIC: 1 μg/mL (*S. cerevisiae*)
2 μg/mL (MRSA)
4 μg/mL (*B. proteus*)
Experiment: intercalate into DNA for blocking DNA replication
Molecular docking: bind with DNA topoisomerase IA by three hydrogen bonds