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LETTERS

Synthesis, characterization and evaluation of prodrugs of ciprofloxacin clubbed with benzothiazoles through N-Mannich base approach

Mona Piplani, Avtar Chand Rana, Prabodh Chander Sharma

Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra-136119, India.

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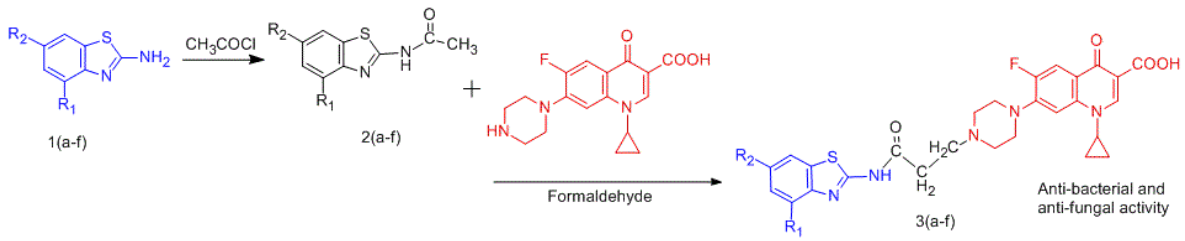
ABSTRACT



The present study aims towards the design and synthesis of N-Mannich base prodrugs of ciprofloxacin with benzothiazoles to improve the therapeutic potential of antibacterial agent. All of the prodrugs were evaluated for physicochemical characteristics and their structures were confirmed by IR, ^{1}H NMR as well as mass spectroscopy. *In vitro* dissolution studies of these synthesized prodrugs were carried out in physiological solutions (pH 1.2 and 7.4) resembling gastric and intestinal tract's environment to estimate their hydrolysis. The prodrugs were found to possess high partition coefficient compared to the parent drug. Prodrug 3c has been proved most potent antibacterial agent having MIC value of 12.5 and 25 µg/ml against *S. aureus* MTCC 96 and *S. pyogenus* MTCC 442 respectively when compared with ciprofloxacin (MIC value 50 µg/ml). Prodrugs 3e and 3f exhibited comparable antibacterial activity against selected bacterial strains to that of reference drugs. Some of the synthesized prodrugs showed good antifungal activity against selected strains compared to standard drugs.

Keywords: Antibacterial, Prodrug, Ciprofloxacin, Hydrophobicity, Hydrolysis

INTRODUCTION

Microbial infections have been the chief cause of death across the world since the time immemorial. Various pathogenic microorganisms are responsible for infectious disorders.[1] Fluoroquinolones, a family of synthetic, broad spectrum, chemotherapeutic agents has gained widespread use for treatment of a number of infections caused by Gram positive and Gram negative bacteria.[2] These agents have been found to exhibit their activities by impairing DNA gyrase (topoisomerase II) and topoisomerase IV, essential bacterial enzymes for DNA replication as well as transcription and causes cell death.[3,4] Ciprofloxacin, one of the important members of fluoroquinolone class is a potent and widely employed oral antimicrobial agent.[5,6] It exhibits less bioavailability owing to poor hydrophobic characteristics. The structure-activity-relationship of fluoroquinolones predicts that increase in hydrophobicity of the drug by some means results in modification of its antimicrobial property.[7] Various methods have been adopted by scientists to overcome the limitations associated with ciprofloxacin.[8-11] Prodrug design is one of the preferred strategies to solve this problem of less hydrophobicity and to improve the pharmacokinetic properties. Prodrugs, the chemically modified therapeutics agents that could be utilized to

Corresponding Author: Dr. Prabodh Chander Sharma
Tel: +91-9416025460
Email: sharma_prabodh@rediffmail.com

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improve the pharmacokinetic properties of parent drugs as well as their pharmacological potential.[12,13]

Benzothiazoles, a privileged class of bicyclic ring system have been proved as important and versatile scaffolds due to their assorted biological and pharmacological features. They have been extensively studied for their numerous biological activities such as antimicrobial, anticancer, antihelmintic, antidiabetic, *etc*. The compounds with benzothiazole nucleus have significant place in medicinal chemistry owing to possession of such biological potential.[14-17]

In the view of this background, it would be beneficial to join the antibacterial ciprofloxacin and benzothiazoles in single structure by synthesizing prodrug through N-Mannich base linkage. As per studies by several researchers, N-Mannich bases have been found to hydrolyze rapidly to parent drug in aqueous solutions (pH dependent) in association with improving the hydrophobicity of drug[18,19]. They are also known to exhibit diverse biological potential[20]. In continuation of our efforts for synthesizing fluoroquinolone derivatives and their evaluation in laboratory[21-23], the present study describes the synthesis of prodrugs of antibacterial drug ciprofloxacin and benzothiazoles by N-Mannich base linkage in order to improve the pharmacokinetic characteristics and consequently therapeutic efficacy. Various benzothiazoles with electron withdrawing groups (Cl and NO_2) and weak to moderate electron donating groups (OCH_3, OC_2H_5, CH_3) substituted at 4th and 6th positions were selected for the study to understand and ascertain the effect of such functional groups on biological properties.

EXPERIMENTAL

Materials and methods

Ciprofloxacin was received as a gift sample from Combitic Global Caplet Private Limited, Sonepat, Haryana, India. All chemicals and solvents used for synthesis and crystallization were purchased from SD Fines (Mumbai, India), Sigma Aldrich (Banglore, India), Rankem Lab (New Delhi, India) and were of high purity grade. Melting point was determined by open capillary method on MR-VIS Labindia, melting visual range apparatus and uncorrected. IR spectra were recorded on Perkin Elmer IR spectrophotometer (KBr disc pellets). ^{1}H NMR was recorded on Bruker Avance II 400 NMR spectrometer. The λ_{max} and absorbance of sample solution was recorded on Systronic double beam UV, spectrophotometer. Buffers were prepared according to the procedure given in Indian Pharmacopoeia, 1996.

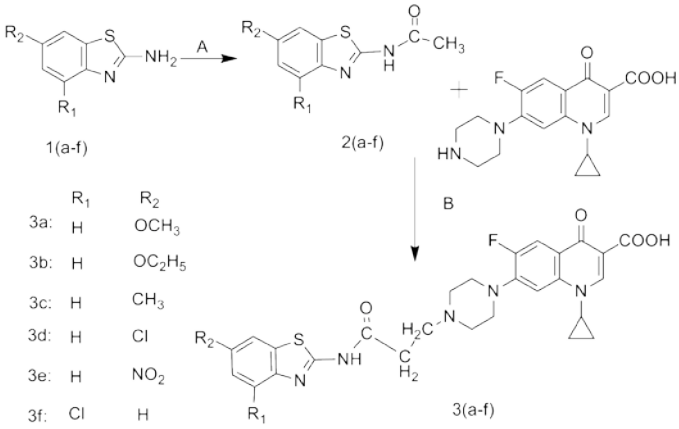
Chemistry

General procedure for synthesis of acetylated benzothiazoles (2a-f)

A mixture of equimolar quantity of benzothiazole (0.01 M), (1a-f) in chloroform, and acetyl chloride/acetic anhydride (0.01 M) were taken in round bottom flask and allowed to reflux for 10-12 hrs in presence of K_2CO_3. Then, reaction mixture was reduced to about half of its original volume and filtered. Sodium bicarbonate solution (4-5% w/v) followed by water was used for washing the residue. After washing, the resultant product was dried and collected. The crude product was purified from methanol. Purity of the synthesized compounds was checked by TLC[24].

General procedure for synthesis of N-Mannich base of fluoroquinolone (3a-3f)

Solution of ciprofloxacin (0.005 M) in methanol was taken in round bottom flask containing 37% formalin (0.25 ml). The mixture was allowed to stir for 30 min. After addition of acetylated benzothiazole (2a-f, 0.005 M), the reaction mixture was refluxed and cooled at room temperature. Crystals were appeared after refrigeration for 24-48 h. The crystallized product were separated by filtration, washed with cold water and dried. Employing the above described procedure six (3a-3f) N-Mannich bases have been synthesized. Physical and analytical data of synthesized prodrugs has been given. The synthetic route for these compounds is outlined in scheme **1**.



Scheme 1 Synthetic route of prodrug synthesis via N-Mannich base, Reagents and reaction conditions: (A) (i) CH_3COCl, (ii) $CHCl_3$, reflux for 10-12 hrs; (B) (i) methanol, (ii) formalin, reflux for 1-2 hrs.

7-(4-(2-(6-Methoxybenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3a**). Yield 68%; m.p.: 277-279 °C; IR (KBr, cm^{-1}) 3370 (NH str), 3086-2854 (C-H str), 1712 (C=O str), 1668(CONH str), 1631 (C=O str), 1251 (C-N str); ^{1}H NMR (DMSO-*d6*) δ ppm: 1.28 (m, 4H, -CH_2CH_2- cyclopropyl), 3.32-3.88 (m, 9H, piperazine-H and cyclopropyl-H), 3.93 (3H, s, OH_3 benzothiazole), 4.42 (t, 2H, -CH_2 methylene bridge), 4.83 (s, 1H, -NH), 6.86-7.91 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{7'}$, $H_{8'}$- benzothiazole)}, 8.61 (s, 1H, H_2-quinolone), 13.07 (s br, 1H, -COOH); MS: Calcd. for $C_{28}H_{28}FN_5O_5S$ found m/z = 565.6 (M^+).

7-(4-(2-(6-Ethoxybenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3b**). Yield 67%; m.p.: 281-283 °C; IR (KBr, cm^{-1}) 3350 (NH str), 3073-2885 (C-H str), 1717 (C=O str), 1673 (CONH str), 1624 (C=O str), 1253 (C-N str); ^{1}H NMR (DMSO-*d6*) δ ppm: 1.32 (m, 4H, -CH_2CH_2- cyclopropyl), 1.41 (3H, t, CH_3 ethoxy), 3.27-3.82 (m, 9H, piperazine-H and cyclopropyl-H), 3.88 (2H, q, CH_2 ethoxy), 4.37 (t, 2H, -CH_2 methylene bridge), 4.78 (s, 1H, -NH), 6.81-

7.84 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{7'}$, $H_{8'}$-benzothiazole)}, 8.36 (s, 1H, H_2-quinolone), 13.34 (s br, 1H, -COOH). MS: Calcd. for $C_{29}H_{30}FN_5O_5S$ found m/z = 579.6 (M^+).

7-(4-(2-(6-Methylbenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3c**). Yield 71%; m.p.: 293-295 °C; IR (KBr, cm^{-1}) 3380 (NH str), 3069-2879 (C-H str), 1723 (C=O str), 1658 (CONH str), 1621 (C=O str), 1272 (C-N str); ^{1}H NMR (DMSO-*d6*) δ ppm: 1.35 (m, 4H, $-CH_2CH_2-$ cyclopropyl), 1.87 (3H, s, CH_3 benzothiazole), 3.18-3.74 (m, 9H, piperazine-H and cyclopropyl-H), 4.18 (t, 2H, $-CH_2$ methylene bridge), 4.71 (s, 1H, -NH), 6.72-7.91 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{7'}$, $H_{8'}$- benzothiazole)}, 8.63 (s, 1H, H_2-quinolone), 14.2 (s br, 1H, -COOH). MS: Calcd. for $C_{28}H_{28}FN_5O_4S$ found m/z = 549.6 (M^+).

7-(4-(2-(6-Chlorobenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3d**). Yield 69%; m.p.: 272-275 °C; IR (KBr, cm^{-1}) 3370 (NH str), 3084-2869 (C-H str), 1736 (C=O str), 1665 (CONH str), 1637 (C=O str), 1268 (C-N str); ^{1}H NMR (DMSO-*d6*) δ ppm: 1.38 (m, 4H, $-CH_2CH_2-$ cyclopropyl), 3.26-3.91 (m, 9H, piperazine-H and cyclopropyl-H), 3.98 (t, 2H, $-CH_2$ methylene bridge), 4.82 (s, 1H, -NH), 7.05-7.89 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{7'}$, $H_{8'}$-benzothiazole)}, 8.35 (s, 1H, H_2-quinolone), 13.80 (s br, 1H, -COOH). MS: Calcd. for $C_{27}H_{25}ClFN_5O_4S$ found m/z = 570.03 (M^+).

7-(4-(2-(6-Nitrobenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3e**). Yield 63%; m.p.: 237-239 °C; IR (KBr, cm^{-1}) 3370 (NH str), 3096-2867 (C-H str), 1726 (C=O str), 1692 (CONH str), 1628 (C=O str), 1265 (C-N str); ^{1}H NMR (DMSO-d6) δ ppm: 1.26 (m, 4H, $-CH_2CH_2-$ cyclopropyl), 3.31-3.92 (m, 9H, piperazine-H and cyclopropyl-H), 4.31 (t, 2H, $-CH_2$ methylene bridge), 4.91 (s, 1H, -NH), 7.18-7.90 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{7'}$, $H_{8'}$-benzothiazole)}, 8.56 (s, 1H, H_2-quinolone), 14.46 (s br, 1H, -COOH). MS: Calcd. for $C_{27}H_{25}FN_6O_6S$ found m/z = 580.6 (M^+).

7-(4-(2-(4-Chlorobenzo[d]thiazol-2-ylcarbamoyl) ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid, (**3f**). Yield 61%; m.p.: 285-287 °C; IR (KBr, cm^{-1}) 3330 (NH str), 3125-2849 (C-H str), 1712 (C=O str), 1675 (CONH str), 1632 (C=O str), 1255 (C-N str); ^{1}H NMR (DMSO-d6) δ ppm: 1.33 (m, 4H, $-CH_2CH_2-$ cyclopropyl), 3.22-3.87 (m, 9H, piperazine-H and cyclopropyl-H), 4.39 (t, 2H, $-CH_2$ methylene bridge), 4.88 (s, 1H, -NH), 7.12-7.86 {m, 5H, aromatic (H_5, H_8-quinolone and $H_{5'}$, $H_{6'}$, $H_{7'}$-benzothiazole)}, 8.77 (s, 1H, H_2-quinolone), 13.74 (s br, 1H, -COOH). MS: Calcd. for $C_{27}H_{25}ClFN_5O_4S$ found m/z = 570.03 (M^+).

In vitro dissolution studies

The synthesized prodrugs were subjected to *in vitro* dissolution studies in both HCl buffer (pH 1.2) and phosphate buffer (pH 7.4) at 37 ± 0.1 ºC. The prodrug (10 mg) in suspension form was poured in dialysis membrane bag and dipped in buffer (100 ml) contained in beaker. The whole assembly was placed on magnetic stirrer and allowed to rotate at 100 rpm. Cumulative percent drug release was determined by taking out an aliquot of 2 ml at fixed time intervals and replacing it with same volume of buffer to maintain the sink conditions. The sample was filtered by whatmann filter paper, diluted with buffer and assayed at 275 nm against buffer taken as blank using UV/VIS spectrophotometer.[25,26] The dissolution studies were carried out in triplicate.

Partition coefficient

Shake flask method was employed to determine partition coefficient of prodrugs. 10 mg of sample prodrug was added into separating funnel having 10 ml of octanol and 10 ml of water. After shaking for 30 minutes the separating funnel was kept undisturbed for few minutes. The aqueous phase was separated and absorbance of this phase was taken using UV/VIS spectrophotometer at the respective $\boldsymbol{\lambda}_{max}$ of each compound. The standard plots of each prodrugs were plotted in water as well as octanol individually and used to calculate the unknown concentration of prodrugs in each phase[27,28]. The partition coefficient was calculated according to equation given below:

$PC = C_o/C_w$

C_o= the concentration of prodrug in octanol

C_w= the concentration of prodrug in water.

Antimicrobial screening

The antibacterial activity of prodrugs was carried out against selected bacterial strains, Gram positive bacteria: *Streptococcus aureus* MTCC 96, *Streptococcus pyogenus* MTCC 442, and Gram negative bacteria: *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 1688. The antifungal activities of prodrugs were tested against three fungal strains: *Candida albicans* MTCC 227, *Aspergillus niger* MTCC 282 and *Aspergillus clavatus* MTCC 1323. Tube dilution method was employed for antimicrobial screening of synthesized prodrugs. The bacterial and fungal cultures were incubated at 37 °C for 24 h and at 25 °C for 7 days respectively. The antibacterial and antifungal activities were noted in terms of MIC and compared with standard drugs.[29]

RESULTS AND DISCUSSION

Development and characterization of ciprofloxacin prodrugs (3a-3f). The synthesis of prodrugs of ciprofloxacin and benzothiazoles was carried out as outlined in scheme **1**. Benzothiazoles were acetylated according to standard protocol. They were refluxed with acetyl chloride in chloroform as solvent and in presence of potassium carbonate to prepare acetylated benzothiazoles. The prodrugs **3a-3f** were prepared by reacting acetylated benzothiazoles, ciprofloxacin along with formalin in presence of few drops of acetic acid and methanol as solvent. All the synthesized prodrugs were characterized by suitable spectral methods *i.e.* IR, ^{1}H NMR and mass spectroscopy. The ^{1}H-NMR spectrum of synthesized prodrugs **3(a-f)** in DMSO-*d6* displayed $-CH_2$ methylene bridge at 3.98-4.42 ppm as triplet. The signals of NH proton and COOH proton were obtained as singlet at 4.71-4.91 ppm and broad

singlet at 13.07-14.46 ppm respectively. The IR spectrum of prodrugs showed characteristics absorption peaks approximately at 1600, 1700, 1250, 3000 and 3300. Mass spectra of all prodrugs were found to be in accordance with its proposed molecular formula.

Hydrolytic behavior of prodrugs at 1.2 pH (HCl buffer). *In vitro* drug release studies of prodrugs (**3a-3f**) were carried out to determine the ability of prodrugs to release ciprofloxacin in HCl (1.2) as well as phosphate buffer (7.4). The percentage cumulative drug release from prodrugs **3a-3f** was determined at λ_{max} 275 nm in predetermined time intervals (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 min) and illustrated in Fig. **1**. From the graph it has been indicated that the prodrugs **3c** (methyl group at 6th position) and **3e** (chloro group at 4th position) started releasing ciprofloxacin after 30 minutes. Both of these prodrugs liberated only about 2.5 % ciprofloxacin in first 2.5 hrs. All of other prodrugs were also found to hydrolyze slowly and liberated approximately 5% of ciprofloxacin in 2.5 hrs.

Hydrolytic behavior of prodrugs at 7.4 pH (phosphate buffer). The extent of ciprofloxacin release in different time intervals for 6 hrs at pH 7.4 (phosphate buffer) has been displayed in Fig. **2**. The results indicates that in phosphate buffer (pH 7.4) prodrugs **3(a-f)** were found to release about 6-13% of parent drug in 2.5 hrs in comparison to its release of approximately 2-5% in HCl buffer (pH 1.2) showing good stability of prodrugs in later. The maximum amount of drug release over 6 hrs was 15-40% in phosphate buffer.

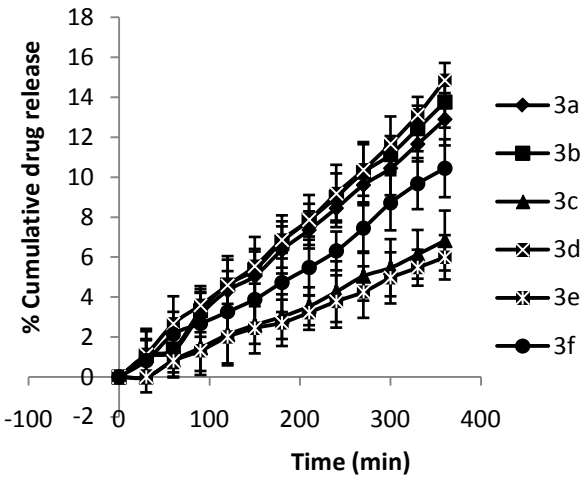


Figure 1 Comparative release of ciprofloxacin from prodrugs (3a-3f) in HCl buffer (1.2 pH). Each value is mean ±S.D, n = 3.

Determination of partition coefficient. Partition coefficient of synthesized prodrugs was determined by shake flask method in octanol and water. The quantity of prodrug in each part *i.e.* octanol and water was calculated by UV/VIS spectrophotometer and calibration curve of these prodrugs in both phases at their respective λ_{max}. The results of partition coefficient and experimentally calculated log P have been given in Table **1** and revealed the improved partition coefficients of all synthesized prodrugs (0.33-0.46) than those of parent drug ciprofloxacin (0.28). The prodrug **3c** substituted with the CH_3 group on 6th position of benzothiazoles showed maximum partition coefficient (0.46). This improved partition coefficients and stability of prodrugs in HCl buffer can lead to augment the movement of whole prodrug across the bacterial cellular membrane by passive diffusion process.

Table 1 Partition coefficients, Log P of synthesized prodrugs (**3a-3f**)

Prodrugs	Partition Coefficient	Log P
3a	0.37	-0.4318
3b	0.33	-0.4815
3c	0.46	-0.3372
3d	0.35	-0.4559
3e	0.40	-0.3979
3f	0.41	-0.3872
Ciprofloxacin	0.28	-0.5528

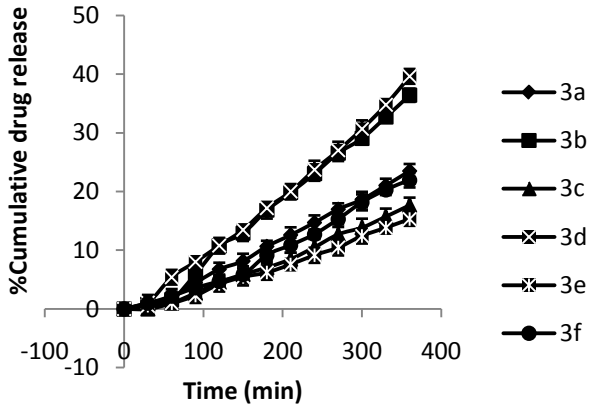


Figure 2 Comparative release of ciprofloxacin from prodrugs (**3a-3f**) in 7.4 pH buffer. Each value is mean ±S.D, n = 3.

Antimicrobial study of prodrugs. The antimicrobial activity of synthesized prodrugs was determined in terms of MIC by tube dilution method. The antibacterial activity of prodrugs was evaluated against Gram positive strains: *Streptococcus aureus* MTCC 96, *Streptococcus pyogenus* MTCC 442, Gram negative bacteria: *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 1688 and results of standard drugs and synthesized prodrugs are given in Table **2**. The MIC values of few prodrugs were found to be less in comparison to standard drugs. The prodrugs **3c** with CH_3 group substituted at 6th position of benzothiazole (MIC value 12.5, 25 µg/ml) and **3f** with Cl group at 4th position of benzothiazole (MIC value 50, 25 µg/ml) showed significantly more or almost comparable potency against *S. aureus* MTCC 96 and *S. pyogenus* MTCC 442 when compared with ciprofloxacin (MIC value 50 µg/ml). Prodrug **3e** (substituted with NO_2 group at 6th position of benzothiazole)

Table 2 Antibacterial activity of synthesized prodrugs (3a-3f)

Prodrugs	Antibacterial Activity (Minimum inhibitory concentration in µg/ml)			
	Gram Positive Bacteria		Gram Negative Bacteria	
	S. aureus (MTCC 96)	*S. pyogenus* (MTCC 442)	*P. aeruginosa* (MTCC 1688)	*E. coli* (MTC C 443)
3a	250	250	500	500
3b	250	250	200	200
3c	**12.5**	**25**	100	100
3d	125	250	100	100
3e	250	500	**25**	250
3f	**50**	**25**	250	100
Norfloxacin	10	10	10	10
Ciprofloxacin	50	50	25	25

Table 3 *In vitro* antifungal activity of synthesized prodrugs

Prodrugs	Antifungal Activity (Minimum inhibitory concentration in µg/ml)		
	C. albicans (MTCC 227)	*A. niger* (MTCC 282)	*A. clavatus* (MTCC 1323)
3a	**250**	500	500
3b	**200**	500	500
3c	500	**50**	500
3d	**500**	1000	1000
3e	**500**	**100**	500
3f	**500**	1000	1000
Greseofulvin	500	100	100
Nystatin	100	100	100

exhibited comparable antibacterial profile (MIC value 25 µg/ml) against *P. aeruginosa* MTCC 1688 to that of ciprofloxacin (MIC value 25 µg/ml).

The same synthesized prodrugs were evaluated for antifungal activities against selected strains and compared with standard drugs, nystatin and greseofulvin. The antifungal activity of each prodrug in terms of MIC is described in Table **3**. The prodrug **3a, 3b** (OCH_3, OC_2H_5 groups substituted at 6^{th} position of benzothiazole respectively) (MIC value 250, 200 µg/ml) and **3c** (CH_3 group substituted at 6^{th} position of benzothiazole) (MIC value 50 µg/ml) was found to be more potent against *C. albicans* MTCC 227 and *A. niger* MTCC 282 respectively than those of standard drugs. Particularly, prodrugs **3d, 3e, 3f** exhibited similar potency (MIC value 500 µg/ml) compared to the reference drug greseofulvin ((MIC value 500 µg/ml) against *C. albicans* MTCC 227. Prodrug **3e** also possessed comparable potency against *A. niger* MTCC 282 to standard drugs.

CONCLUSION

In summary, a novel series of ciprofloxacin prodrugs with high hydrophobicity were designed, synthesized and evaluated for antimicrobial activity. All of the synthesized prodrugs demonstrated better partition coefficient than that of parent drug. Based on the results obtained from *in vitro* dissolution studies, all of the synthesized prodrugs showed less hydrolysis in acidic environment (gastric pH) as compared to alkaline (intestinal pH). The data of antimicrobial activity revealed that some of synthesized prodrugs exhibit promising biological potential against selected microbial strains. Therefore, taking into account the data presented herein, it can be inferred that the improvement of absorption characteristics owing to increased hydrophobicity can be useful in furtherance of endeavors towards reduction in doses of drug.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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