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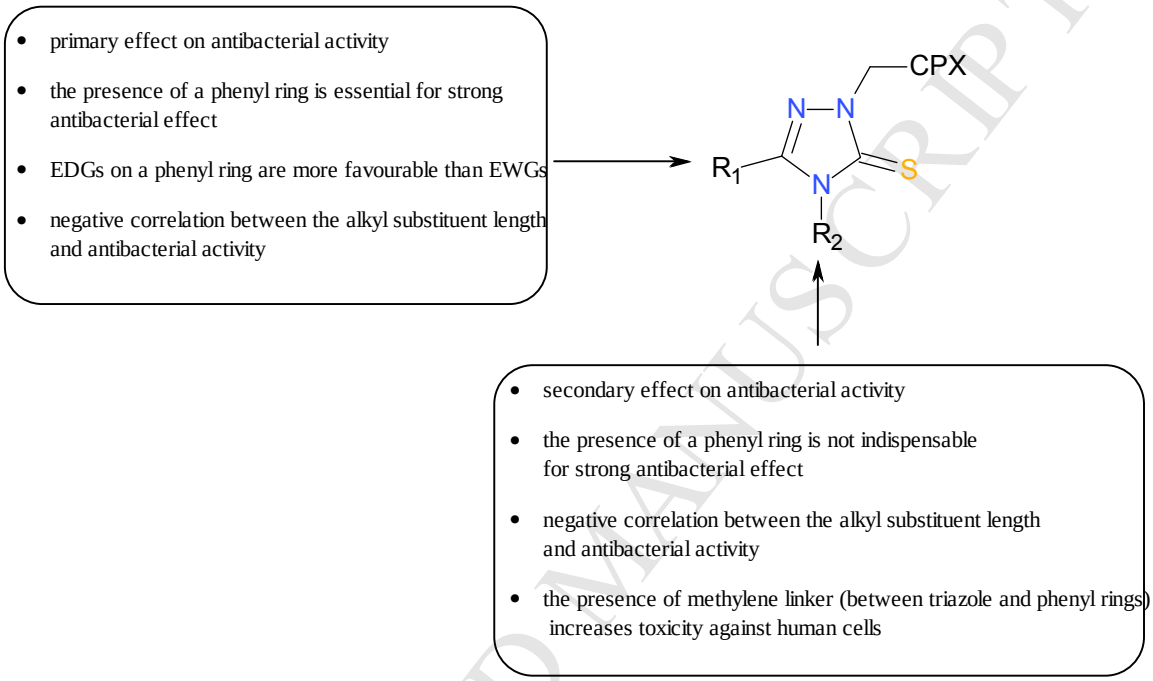

Search for factors affecting antibacterial activity and toxicity of 1,2,4-triazole-ciprofloxacin hybrids

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1,2,4-triazole-3-thiones; fluoroquinolones; gyrase DNA; topoisomerase IV; MTT assay

Abstract

A series of 1,2,4-triazole-based compounds was designed as potential antibacterial agents using molecular hybridization approach. The target compounds (**23-44**) were synthesized by Mannich reaction of 1,2,4-triazole-3-thione derivatives with ciprofloxacin (CPX) and formaldehyde. Their potent antibacterial effect on Gram-positive bacteria was accompanied by similarly strong activity against Gram-negative strains. The toxicity of the CPX-triazole hybrids for bacterial cells was even up to 18930 times higher than the toxicity for human cells. The results of enzymatic studies showed that the antibacterial activity of the CPX-triazole hybrids is not dependent solely on the degree of their affinity to DNA gyrase and topoisomerase IV.

1. Introduction

Bacterial type II topoisomerases (DNA gyrase and topoisomerase IV) are essential in the process of replication, transcription, repair and recombination of genetic material [1]. The action of topoisomerases consists in introducing changes to the spatial structure of DNA by catenation and decatenation of duplex DNA rings, relaxation of supercoiled DNA, and in the case of DNA gyrase – introduction of negative supercoils into DNA in an energy-dependent reaction [2]. Until today, fluoroquinolones (FQs) have been the most effective antibacterials, the molecular target of which is DNA gyrase (gyrDNA) and topoisomerase IV (topoIV) [3]. In the case of Gram-negative bacteria, a primary target of fluoroquinolones is gyrDNA whereas topoIV inhibition is merely an additional element that contributes to the total antibacterial action of FQs. DNA gyrase catalyses ATP-dependent reaction of introducing negative supercoils in the circular DNA of bacteria, which allows the double strand to be uncoiled during the replication process. In Gram-positive bacteria, however, the primary molecular target of FQs is topoIV. The main function of topoIV appears to be decatenation of the daughter circular chromosomes [4]. The aim of the research that we have conducted so far has been to design potential antibacterial drugs which inhibit bacterial topoisomerases [5, 6]. Our research revealed that by joining ciprofloxacin (CPX) with different 1,2,4-triazole derivatives, the antibacterial effect of CPX may be enhanced [6]. The rationale for combining these two molecules was clarified in our previous paper [6]. The utilization of the molecular hybridization strategy for obtaining novel CPX derivatives (hybrids) was also described by other authors [7, 8]. It was obvious that the differences in the activity of CPX-triazole hybrids

resulted from the structure of substituents connected to the 1,2,4-triazole ring. The most favourable antibacterial effect was obtained when the aforementioned five-membered heterocyclic ring was connected with a hydroxyphenyl fragment. By exploring this subject, we have attempted to trace the way in which further modification of the structure of CPX-triazole hybrids affects the antibacterial activity. Our aim was also to check whether there is an interrelation between the structure of the synthesized compounds and their toxicity for human cells. Finally, the enzymatic studies presented now are an attempt to gain a better understanding of the molecular mechanisms responsible for antibacterial activity of the CPX-triazole hybrids.

2. Results and discussion

2.1. Chemistry

4,5-Disubstituted-1,2,4-triazole-3-thiones (**1-22**) were obtained by dehydrocyclization of the respective thiosemicarbazides (described in Ref. [5]) in alkaline medium. Their chemical structures were confirmed on the basis of ^{1}H-NMR and ^{13}C-NMR spectra, and the results of elemental analysis. The CPX-triazole hybrids (**23-44**) were synthesized according to the method described previously [6], and presented in Scheme 1.

Scheme 1

2.2. Antibacterial activity

The microbiological tests conducted on the reference strains of Gram-positive and Gram-negative bacteria revealed that the newly obtained CPX-triazole hybrids were, in large majority, much more potent than CPX itself. In the group of the Gram-positive bacteria tested, the *Staphylococcus aureus* strain is of the greatest epidemiological relevance. Asymptomatic carrier state of these bacteria is quite common – even in 50% of the population, but the risk of developing symptoms mainly concerns persons with impaired immune system [9]. The greatest mortality rate is noted in individuals infected with methicillin-resistant *S. aureus* (MRSA). Treating MRSA infections is difficult due to the fact that these strains are resistant to β-lactam antibiotics, and additionally over 90% of MRSA strains exhibit cross-resistance with macrolides and fluoroquinolones (including CPX) [10]. In the group of the derivatives

tested, the compounds with 16-fold greater activity than CPX (i.e., **38**, **41**) with respect to *S. aureus* ATCC25923 were identified. Moreover, complete inhibition of the growth of the second methicillin-susceptible *S. aureus* strain (i.e. *S. aureus* ATCC6538) by compounds **30**, **34** and **38** occurred at about 8-fold lower concentrations when compared to CPX (Table 1). Surprisingly, the tested MRSA strain turned out to be more sensitive to the synthesized CPX-triazole hybrids than the MSSA strains. Merely 3 of 22 derivatives obtained exhibited activity that was comparable to or lower than vancomycin – a drug used for treatment of MRSA infections. The remaining compounds demonstrated more potent action than the aforementioned drug and, what must be emphasised, the minimal inhibitory concentrations (MICs) for compounds **26** and **38** were about 15 times lower than in the case of vancomycin (i.e., 0.046 μM vs. 0.68 μM). Furthermore, it must be noted that all CPX-triazole hybrids were characterised by bactericidal effects (MBC/MIC≤4) towards both MSSA and MRSA strains. Moreover, the fact that *Bacillus cereus* ATCC10876 was found to be highly sensitive to the synthesized compounds is also important from the microbiological point of view. It is crucial, as *Bacillus cereus* is phylogenetically related to *Bacillus anthracis* (i.e. an etiologic agent of anthrax) [11]. Due to this similarity, the screening tests on new candidates for anti-anthrax drugs are conducted using *B. cereus* strain since it is assumed that compounds which inhibit its growth will also be effective against *B. anthracis*. Thus, taking into account the MIC values, one can assume that the CPX-triazole hybrids could also be effective in the treatment of anthrax.

Tables 1 & 2

As shown in Table 2, the potent antibacterial effect on Gram-positive bacteria exerted by the obtained CPX-triazole hybrids is also accompanied by similarly strong activity against Gram-negative bacteria. The Gram-negative strains tested included, among others, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC13883 and *Pseudomonas aeruginosa* ATCC9027. Epidemiological reports concerning hospital-acquired infections indicate that, next to the MRSA, these strains are the most common causes of such infections [12]. Due to the fact that the obtained CPX-triazole hybrids demonstrated more potent antibacterial effects on the three bacterial strains mentioned above as compared with CPX itself, these compounds might become efficacious treatment option in the management of hospital-acquired infections. In the

case of *P. aeruginosa*, which is responsible for approximately 10% of hospital-acquired infections, an alarming trend is observed consisting in the selection of strains resistant to antibiotics and chemotherapeutics, including fluoroquinolones [13]. It is assumed that this phenomenon results from a capacity of *P. aeruginosa* to produce membrane proteins, called efflux pumps, responsible for removing drugs from the inside of bacterial cells. In the group of the obtained derivatives (**23-44**), all compounds, with only one exception, were characterised by more potent activity than CPX. In the cases of 4-methoxybenzyl (**30**) and 4-methoxyphenyl (**43**) derivatives, an approximately 31-fold increase in antibacterial activity was observed when compared to CPX (MICs equalled 0.023 μM for **30** and **43** vs. 0.72 μM for CPX).

2.3. Toxicity evaluation

In the process of searching for new antibacterial drugs, it is important to differentiate between compounds toxic only to the bacterial cells from compounds that act non-selectively and are toxic both for bacterial and human cells. This is even more important in the cases of such compounds, the antibacterial effects of which result from inhibition of bacterial topoisomerases. There is a risk that bacterial topoisomerase inhibitors will also affect human topoisomerases leading to dysfunction of human cells. With the use of the MTT assay, it was investigated how the newly-obtained CPX-triazole hybrids affect the viability of human HEK-293 cells. For the purposes of the assay, we selected structurally diversified derivatives, including compounds **30**, **38** and **43** that were considered to be the most promising due to their potent antibacterial activity. The toxicity of the derivatives tested (i.e., **24**, **30**, **31**, **34**, **37**, **38**, **41**, **43**) was expressed in the form of concentrations that decrease the viability of cells by a half (EC_{50}). The EC_{50}/MIC ratio was used as a parameter representing the selectivity of the CPX-triazole hybrids towards bacterial cells [14]. The bigger the difference between EC_{50} and MIC values, the lesser the risk of toxic effects. EC_{50} values for the tested compounds ranged from 62.89 ± 0.43 μM to 139.55 ± 17.77 μM, and were considerably higher than doses needed to obtain the antibacterial effect (Table 3). The toxicity of the CPX-triazole hybrids for bacterial cells was even up to 18930 times higher than the toxicity for human HEK-293 cells. The most selective action of the tested compounds was observed for *E. coli* ATCC25922 strain.

Table 3

2.4. Enzymatic studies

Considerable increase in antibacterial activity of the obtained CPX-triazole hybrids may result from the fact that:

1) these derivatives inhibit bacterial topoisomerases in a more potent way than CPX itself;

2) CPX-triazole hybrids permeate inside the bacterial cells more easily than CPX;

3) CPX-triazole hybrids are not substrates for bacterial efflux pumps, thus, they are not removed from the bacterial cells;

4) there is an additional, so far unknown mechanism of antibacterial activity of CPX-triazole hybrids that is not associated with topoisomerases inhibition.

In order to verify the first hypothesis, the affinity of the selected compounds towards bacterial type II topoisomerases (i.e., DNA gyrase and topoisomerase IV) was analysed. For testing, the derivatives described in our earlier article [6] were selected (Table 4).

Table 4

The results obtained demonstrated that the antibacterial activity of the CPX-triazole hybrids is not dependent solely on the degree of their affinity to DNA gyrase and topoisomerase IV. Even though all three derivatives (A-C) inhibited DNA gyrase in a much weaker way than CPX itself, the compounds A and B were characterised by potent antibacterial activity (Figure 1). Moreover, the influence of other factors on the total antibacterial effect is confirmed by the fact that derivatives A and C differed considerably in their activity despite their nearly identical affinity to DNA gyrase (IC_{50} values for compounds A and C were 6.05 μM and 6.28 μM, respectively). The same example demonstrates to what degree the structure of the substituent in the C-5 position of 1,2,4-triazole-3-thione ring affects the activity of such compounds. The affinity of the derivatives tested was comparable to (in the cases of compounds A and B, containing hydroxyphenyl fragment) or approximately twofold lower (in the case of compound C, containing alkyl chain at the same position of the 1,2,4-triazole ring) than the affinity of CPX. However, there was no correlation between the affinity of the CPX-triazole hybrids towards topoisomerase IV and their antibacterial activity. Again, this also confirms that inhibition of bacterial type II topoisomerases in not the sole factor responsible for the antibacterial activity of the compounds discussed. Therefore, the first hypothesis has to

be refuted as untrue. Verification of the remaining hypotheses will require further microbiological studies during which the influence of the bacterial outer membranes permeability and efflux pumps on the antibacterial effect will be tested. Such studies, conducted with the use of so-called permeabilizers and efflux pumps inhibitors, have already been initiated and the results will be presented in due course.

Figure 1

2.5. Structure-activity observations

As can be seen from Scheme 1, compounds **23-44** differ in the position of the hydroxyl group in the phenyl ring and the structure of the substituent in the position 4 of the 1,2,4-triazole-3-thione core. The results of the MTT assay (Table 3) clearly show that the presence of the $-CH_2-$ linker between 1,2,4-triazole ring and aryl substituent negatively affects toxicity of the obtained CPX-triazole hybrids. EC_{50} values for the compounds in which aryl substituent was connected directly to the1,2,4-triazole ring ranged from 105.34 ± 2.75 μM (for compound **43**) to 139.55 ± 17.77 μM (for compound **31**). The toxicity of the respective benzyl derivatives was approximately twice as high. The data presented in Table 3 also shows that substitution pattern of the hydroxyphenyl moiety has no major effect on the toxicity profile of the compounds tested. Similarly, the change of hydroxyl group position affected antibacterial activity to a slight degree only. However, its presence alone seems to be crucial. It may be therefore concluded that it is not the position of the hydroxyl group that determines a potent antibacterial activity, but rather the particular properties of this group (e.g., the potential of forming hydrogen bonds, strong electrondonating character) and its influence on the electronic properties of the molecule that is the source of antibacterial properties. Nevertheless, having interpreted these results in connection with those presented previously [6], a number of structure – activity correlations were observed, as shown in Scheme 2.

Scheme 2

3. Conclusions

To sum up, thanks to combining 1,2,4-triazole-3-thione derivatives with the molecule of ciprofloxacin, new compounds endowed with potent antibacterial activity against Gram-

positive and Gram-negative strains were obtained. More importantly, it was observed that their antibacterial action was particularly potent in relation to strains that cause life-threatening infections. The concentrations that effectively inhibit bacterial growth were even several thousand times greater than respective toxic concentrations, which confirms that these derivatives may be treated as the lead compounds for novel chemotherapeutics. The enzymatic assay carried out for the selected compounds suggests that considerable increase in antibacterial activity of the obtained CPX-triazole hybrids does not result from an increase in their affinity to bacterial type II topoisomerases (DNA gyrase and topoisomerase IV).

4. Experimental

4.1. Chemistry

4.1.1. General comments

All reagents and solvents were purchased from Alfa Aesar (Ward Hill, USA) and Merck Co. (Darmstadt, Germany). Melting points were determined by using Fisher-Johns apparatus (Fisher Scientific, Schwerte, Germany) and are uncorrected. The ^{1}H-NMR and ^{13}C-NMR spectra (in DMSO-d_6) were recorded on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) using TMS as an internal standard. FT-IR spectra were recorded using ATR Platinum Diamond A 225 device. Elemental analyses were performed on an AMZ 851 CHX analyser (PG, Gdańsk, Poland) and the results were within ± 0.4% of the theoretical value.

4.1.2. General procedure for the synthesis of 1,2,4-triazole-3-thione derivatives (**1-22**)

Respective thiosemicarbazide derivatives, described in Ref. [5], were dissolved in 2% NaOH and refluxed for 2h. After cooling, the mixture was neutralized with 3M HCl. The precipitate formed was filtered and washed with distilled water. The compounds were crystallized from EtOH. In the cases of already known compounds, information about their properties may be retrieved in the Chemical Abstract Service database (CAS numbers are given below).

5-(2-Hydroxyphenyl)-4-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**1**)

Yield: 78%. CAS: 81518-26-5

4-Benzyl-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**2**)

Yield: 83%. CAS: 312319-66-7

4-(4-Fluorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**3**)

Yield: 81%, m.p. 246-248°C, ^{1}H-NMR (250 MHz): 6.76-6.92 (m, 2H, Ar-H), 7.22-7.42 (m, 6H, Ar-H), 10.06 (s, 1H, OH), 14.14 (s, 1H, NH). ^{13}C-NMR (75 MHz): 111.66, 113.92, 114.21, 114.28, 117.61, 128.72, 128.86, 129.24, 129.31, 130.17, 130.92, 148.42, 154.18, 158.32, 162.22, 166.18. Anal. calc. for $C_{14}H_{10}FN_3OS$ (287.31): C 58.53, H 3.51, N 14.63. Found: C 58.69, H 3.20, N 14.56.

4-(4-Fluorobenzyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**4**)

Yield: 85%, m.p. 210-211°C, ^{1}H-NMR (250 MHz): 5.20 (s, 2H, -CH_2-), 6.80-7.16 (m, 7H, Ar-H), 7.38-7.47 (m, 1H, Ar-H), 10.55 (s, 1H, OH), 14.05 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.50, 111.81, 113.52, 113.86, 114.60, 117.94, 127.86, 127.99, 129.91, 130.56, 131.23, 148.59, 154.19, 158.07, 161.94, 165.54. Anal. calc. for $C_{15}H_{12}FN_3OS$ (301.34): C 59.79, H 4.01, N 13.94. Found: C 59.65, H 3.86, N 14.08.

4-(4-Chlorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**5**)

Yield: 87%. CAS: 26131-65-7

4-(4-Chlorobenzyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**6**)

Yield: 77%, m.p. 218-219°C, ^{1}H-NMR (250 MHz): 5.20 (s, 2H, -CH_2-), 6.84-6.93 (m, 1H, Ar-H), 6.98 (dd, 2H, Ar-H, J = 1.9 Hz, 6.5 Hz), 7.03-7.17 (m, 2H, Ar-H), 7.30 (dd, 2H, Ar-H, J = 1.9 Hz, 6.5 Hz), 7.40-7.47 (m, 1H, Ar-H), 10.56 (s, 1H, OH), 14.18 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.56, 111.67, 114.60, 117.94, 126.90, 127.63, 129.95, 130.68, 131.21, 133.33, 148.64, 154.17, 165.57. Anal. calc. for $C_{15}H_{12}ClN_3OS$ (317.79): C 56.69, H 3.81, N 13.22. Found: C 56.49, H 3.95, N 13.12.

5-(2-Hydroxyphenyl)-4-(4-methoxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**7**)

Yield: 80%, m.p. 288-290°C, ^{1}H-NMR (250 MHz): 3.76 (s, 3H, OCH_3), 6.77-6.90 (m, 2H, Ar-H), 6.95 (dd, 2H, Ar-H, J = 2.2 Hz, 6.8 Hz), 7.22 (dd, 2H, Ar-H, J = 2.2 Hz, 6.8 Hz), 7.26-7.38 (m, 2H, Ar-H), 10.04 (s, 1H, OH), 14.06 (s, 1H, NH). ^{13}C-NMR (75 MHz): 53.90, 111.94, 112.34, 114.20, 114.30, 117.51, 125.58, 127.79, 130.14, 130.74, 148.56, 154.34,

154.50, 157.69, 166.32, 166.48. Anal. calc. for $C_{15}H_{13}N_3O_2S$ (299.35): C 60.18, H 4.38, N 14.04. Found: C 60.10, H 4.21, N 13.86.

5-(2-Hydroxyphenyl)-4-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**8**)

Yield: 75%, m.p. 186-187°C, ^{1}H-NMR (250 MHz): 3.70 (s, 3H, OCH_3), 5.16 (s, 2H, -CH_2-), 6.76 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.7 Hz), 6.86-6.95 (m, 3H, Ar-H), 7.05-7.15 (m, 2H, Ar-H), 7.41-7.50 (m, 1H, Ar-H), 10.55 (s, 1H, OH), 14.01 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.64, 53.64, 112.01, 112.22, 114.61, 117.98, 126.40, 127.38, 129.98, 131.14, 148.64, 154.10, 157.20, 165.45. Anal. calc. for $C_{16}H_{15}N_3O_2S$ (313.37): C 61.32, H 4.82, N 13.41. Found: C 61.20, H 5.05, N 13.26.

5-(3-Hydroxyphenyl)-4-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**9**)

Yield: 85%. CAS: 26028-80-8

4-Benzyl-5-(3-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**10**)

Yield: 81%, m.p. 161-163°C, ^{1}H-NMR (250 MHz): 5.39 (s, 2H, -CH_2-), 6.91-7.42 (m, 9H, Ar-H), 9.98 (s, 1H, OH), 14.18 (s, 1H, NH). ^{13}C-NMR (75 MHz): 45.40, 113.72, 116.38, 117.46, 125.14, 125.61, 126.13, 127.18, 128.73, 134.36, 150.12, 156.04, 156.18, 166.44, 166.60. Anal. calc. for $C_{15}H_{13}N_3OS$ (283.35): C 63.58, H 4.62, N 14.83. Found: C 63.51, H 4.50, N 14.67.

4-(4-Fluorophenyl)-5-(3-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**11**)

Yield: 74%. CAS: 727982-83-4

4-(4-Fluorobenzyl)-5-(3-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**12**)

Yield: 81%, m.p. 161-163°C, ^{1}H-NMR (250 MHz): 5.28 (s, 2H, -CH_2-), 6.84-6.93 (m, 3H, Ar-H), 7.02-7.14 (m, 4H, Ar-H), 7.21-7.28 (m, 1H, Ar-H), 9.89 (s, 1H, OH), 14.10 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.75, 113.70, 113.84, 114.19, 116.40, 117.54, 125.55, 127.33, 127.46, 128.78, 130.54, 150.01, 156.03, 156.18, 158.11, 162.00, 166.41. Anal. calc. for $C_{15}H_{12}FN_3OS$ (301.34): C 59.79, H 4.01, N 13.94. Found: C 59.60, H 3.80, N 14.16.

4-(4-Chlorophenyl)-5-(3-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**13**)

Yield: 84%, m.p. 220-221°C, ^{1}H-NMR (250 MHz): 6.71-6.90 (m, 3H, Ar-H), 7.20 (t, 1H, Ar-H, *J* = 8.0 Hz), 7.44 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.6 Hz), 7.62 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.6

Hz), 9.84 (s, 1H, OH), 14.18 (s, 1H, NH). ^{13}C-NMR (75 MHz): 113.70, 116.07, 117.62, 125.27, 128.02, 128.42, 129.24, 132.10, 132.65, 149.07, 155.74, 166.97. Anal. calc. for $C_{14}H_{10}ClN_3OS$ (303.77): C 55.35, H 3.32, N 13.83. Found: C 55.23, H 3.12, N 13.98.

4-(4-Chlorobenzyl)-5-(3-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**14**)

Yield: 80%, m.p. 160-162°C, ^{1}H-NMR (250 MHz): 5.34 (s, 2H, -CH_2-), 6.91-7.00 (m, 3H, Ar-H), 7.06-7.14 (m, 2H, Ar-H), 7.27-7.35 (m, 1H, Ar-H), 7.40 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.6 Hz), 9.94 (s, 1H, OH), 14.17 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.86, 113.67, 116.48, 117.53, 125.48, 127.09, 127.19, 128.81, 130.73, 133.36, 150.00, 156.04, 166.38. Anal. calc. for $C_{15}H_{12}ClN_3OS$ (317.79): C 56.69, H 3.81, N 13.22. Found: C 56.78, H 3.62, N 13.04.

5-(3-Hydroxyphenyl)-4-(4-methoxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**15**)

Yield: 73%, m.p. 238-240°C, ^{1}H-NMR (250 MHz): 3.85 (s, 3H, OCH_3), 6.71-6.90 (m, 3H, Ar-H), 7.10 (dd, 2H, Ar-H, *J* = 2.1 Hz, 6.9 Hz), 7.18 (t, 1H, Ar-H, *J* = 7.7 Hz), 7.30 (dd, 2H, Ar-H, *J* = 2.1 Hz, 6.9 Hz), 9.84 (s, 1H, OH), 14.11 (s, 1H, NH). ^{13}C-NMR (75 MHz): 54.04, 113.09, 113.61, 115.89, 117.51, 125.56, 125.74, 128.31, 128.45, 149.29, 155.67, 158.15, 167.29. Anal. calc. for $C_{15}H_{13}N_3O_2S$ (299.35): C 60.18, H 4.38, N 14.04. Found: C 60.23, H 4.50, N 14.20.

5-(3-Hydroxyphenyl)-4-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**16**)

Yield: 78%, m.p. 188-190°C, ^{1}H-NMR (250 MHz): 3.74 (s, 3H, OCH_3), 5.31 (s, 2H, -CH_2-), 6.82-7.04 (m, 7H, Ar-H), 7.28-7.36 (m, 1H, Ar-H), 9.94 (s, 1H, OH), 14.08 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.84, 53.66, 112.53, 113.76, 116.37, 117.60, 125.70, 126.30, 126.72, 128.75, 150.01, 156.04, 157.18, 166.32, 166.48. Anal. calc. for $C_{16}H_{15}N_3O_2S$ (313.37): C 61.32, H 4.82, N 13.41. Found: C 61.10, H 4.73, N 13.23.

5-(4-Hydroxyphenyl)-4-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**17**)

Yield: 82%. CAS: 26028-88-6

4-Benzyl-5-(4-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**18**)

Yield: 84%. CAS: 174573-91-2

4-(4-Fluorophenyl)-5-(4-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**19**)

Yield: 75%. CAS: 451502-00-4

4-(4-Chlorophenyl)-5-(4-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**20**)

Yield: 77%. CAS: 54918-96-6

5-(4-Hydroxyphenyl)-4-(4-methoxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**21**)

Yield: 83%. CAS: 69626-14-8

5-(4-Hydroxyphenyl)-4-(4-methoxybenzyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**22**)

Yield: 86%, m.p. 178-180°C, ^{1}H-NMR (250 MHz): 3.66 (s, 3H, OCH_3), 5.24 (s, 2H, -CH_2-), 6.75-6.84 (m, 4H, Ar-H), 6.94 (m, 2H, Ar-H), 7.33 (dd, 2H, Ar-H, *J* = 1.9 Hz, 6.7 Hz), 10.11 (s, 1H, OH), 13.95 (s, 1H, NH). ^{13}C-NMR (75 MHz): 44.72, 53.66, 112.53, 114.20, 115.18, 126.42, 126.72, 128.67, 150.23, 157.17, 157.92, 166.07. Anal. calc. for $C_{16}H_{15}N_3O_2S$ (313.37): C 61.32, H 4.82, N 13.41. Found: C 61.19, H 4.69, N 13.30.

4.1.3. General procedure for the synthesis of 1,2,4-triazole-ciprofloxacin hybrids (**23-44**)

1 mmol of the respective 1,2,4-triazole derivative (**1-22**) was dissolved (with heating) in 40 ml of anhydrous ethanol and then equimolar amount of ciprofloxacin and formaldehyde solution were added. The obtained suspension was stirred at room temperature for 12 hours. The precipitate formed was filtered off, dried, and crystallized from ethanol to give compounds **23-44**.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(2-hydroxyphenyl)-4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**23**)

Yield: 69%, m.p. 241-243°C, ^{1}H-NMR (250 MHz): 1.13-1.17 (m, 2H, cyclopropyl), 1.28-1.32 (m, 2H, cyclopropyl), 2.88 (bs, 4H, piperazine), 3.30 (bs, 4H, piperazine), 3.74-3.80 (m, 1H, cyclopropyl), 4.67 (s, 2H, CH_2), 7.04-8.10 (m, 11H, Ar-H), 8.65 (s, 1H, Ar-H), 10.35 (s, 1H, OH), 14.94 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 7.68, 36.21, 46.11, 50.47, 61.07, 106.32, 106.80, 110.72, 111.33, 114.20, 116.89, 117.84, 126.14, 126.87, 127.14, 129.45, 130.11, 132.02, 139.20, 144.17, 147.20, 148.31, 152.42, 154.67, 164.92, 166.20, 176.19. Elemental analysis for $C_{32}H_{29}FN_6O_4S$ (612.67). Calculated: C 62.73, H 4.77, N 13.72. Found: C 62.85, H 4.62, N 13.87.

1-Cyclopropyl-6-fluoro-7-[4-{[4-benzyl-3-(2-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**24**)

Yield: 74%, m.p. 204-205°C, ^{1}H-NMR (250 MHz): 1.20-1.23 (m, 2H, cyclopropyl), 1.27-1.30 (m, 2H, cyclopropyl), 2.70 (s, 4H, piperazine), 3.27 (bs, 4H, piperazine), 3.94-3.97 (m, 1H, cyclopropyl), 4.65 (s, 2H, CH_2), 5.14 (s, 2H, CH_2), 7.06-8.01 (m, 11H, Ar-H), 8.65 (s, 1H, Ar-H), 10.50 (s, 1H, OH), 14.88 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 8.14, 37.81, 45.23, 46.18, 51.73, 62.40, 106.38, 107.17, 111.06, 112.18, 113.52, 116.25, 117.62, 125.14, 125.69, 126.90, 127.09, 128.52, 134.17, 139.66, 145.60, 147.82, 148.71, 152.92, 156.04, 164.71, 166.43, 178.50. Elemental analysis for $C_{33}H_{31}FN_6O_4S$ (626.70). Calculated: C 63.24, H 4.99, N 13.41. Found: C 63.11, H 4.82, N 13.47.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-fluorophenyl)-3-(2-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**25**)

Yield: 70%, m.p. 233-235°C, ^{1}H-NMR (250 MHz): 1.09-1.11 (m, 2H, cyclopropyl), 1.15-1.18 (m, 2H, cyclopropyl), 2.78 (bs, 4H, piperazine), 3.26 (bs, 4H, piperazine), 3.77-3.88 (m, 1H, cyclopropyl), 4.58 (s, 2H, CH_2), 7.11-7.98 (m, 10H, Ar-H), 8.67 (s, 1H, Ar-H), 10.10 (s, 1H, OH), 15.06 (s, 1H, COOH). IR (ATR): 3405 (O-H, stretch), 3121, 2946, 2867 (C-H, stretch), 1724 (C=O, stretch), 1573 (C=N, stretch), 1426 (C-O, stretch), 1303 (C=S, stretch), 1289 (O-H, bend), 1048 (C-F, stretch). Elemental analysis for $C_{32}H_{28}F_2N_6O_4S$ (630.66). Calculated: C 60.94, H 4.48, N 13.33. Found: C 60.83, H 4.42, N 13.37.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-fluorobenzyl)-3-(2-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**26**)

Yield: 65%, m.p. 160-161°C, ^{1}H-NMR (250 MHz): 1.20-1.23 (m, 2H, cyclopropyl), 1.28-1.31 (m, 2H, cyclopropyl), 2.70 (bs, 4H, piperazine), 3.42 (bs, 4H, piperazine), 3.72-3.76 (m, 1H, cyclopropyl), 4.70 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 7.18-7.85 (m, 10H, Ar-H), 8.69 (s, 1H, Ar-H), 10.53 (s, 1H, OH), 14.98 (s, 1H, COOH). IR (ATR): 3446 (O-H, stretch), 3101, 2946, 2782 (C-H, stretch), 1714 (C=O, stretch), 1580 (C=N, stretch), 1406 (C-O, stretch), 1294 (C=S, stretch), 1256 (O-H, bend), 1021 (C-F, stretch). Elemental analysis for $C_{33}H_{30}F_2N_6O_4S$ (644.69). Calculated: C 61.48, H 4.69, N 13.04. Found: C 61.57, H 4.62, N 13.17.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-chlorophenyl)-3-(2-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**27**)

Yield: 75%, m.p. 235-237°C, ^{1}H-NMR (250 MHz): 1.12-1.14 (m, 2H, cyclopropyl), 1.20-1.23 (m, 2H, cyclopropyl), 2.86 (bs, 4H, piperazine), 3.32 (bs, 4H, piperazine), 3.80-3.85 (m, 1H, cyclopropyl), 4.48 (s, 2H, CH_2), 6.94 (dd, 2H, Ar-H, *J* = 1.8 Hz, 6.7 Hz), 7.11-7.75 (m, 8H, Ar-H), 8.68 (s, 1H, Ar-H), 10.41 (s, 1H, OH), 15.09 (s, 1H, COOH). IR (ATR): 3482 (O-H, stretch), 3062, 2861 (C-H, stretch), 1738 (C=O, stretch), 1618 (C=N, stretch), 1426 (C-O, stretch), 1321 (C=S, stretch), 1273 (O-H, bend), 1012 (C-F, stretch). Elemental analysis for $C_{32}H_{28}ClFN_6O_4S$ (647.12). Calculated: C 59.39, H 4.36, N 12.99. Found: C 59.46, H 4.50, N 13.10.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-chlorobenzyl)-3-(2-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**28**)

Yield: 69%, m.p. 208-210°C, ^{1}H-NMR (250 MHz): 1.15-1.21 (m, 2H, cyclopropyl), 1.30-1.34 (m, 2H, cyclopropyl), 2.86 (bs, 4H, piperazine), 3.35 (bs, 4H, piperazine), 3.86-3.95 (m, 1H, cyclopropyl), 4.62 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 6.94-7.75 (m, 10H, Ar-H), 8.67 (s, 1H, Ar-H), 10.50 (s, 1H, OH), 14.98 (s, 1H, COOH). IR (ATR): 3452 (O-H, stretch), 3185, 2971, 2732 (C-H, stretch), 1726 (C=O, stretch), 1582 (C=N, stretch), 1439 (C-O, stretch), 1323 (C=S, stretch), 1281 (O-H, bend), 1054 (C-F, stretch). Elemental analysis for $C_{33}H_{30}ClFN_6O_4S$ (661.14). Calculated: C 59.95, H 4.57, N 12.71. Found: C 60.13, H 4.68, N 12.90.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(2-hydroxyphenyl)-4-(4-methoxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**29**)

Yield: 74%, m.p. 246-247°C, ^{1}H-NMR (250 MHz): 1.12-1.15 (m, 2H, cyclopropyl), 1.21-1.24 (m, 2H, cyclopropyl), 2.94 (s, 4H, piperazine), 3.37 (s, 4H, piperazine), 3.61 (s, 3H, OCH_3), 3.82-3.86 (m, 1H, cyclopropyl), 4.60 (s, 2H, CH_2), 6.99 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.7 Hz), 7.10-7.85 (m, 8H, Ar-H), 8.66 (s, 1H, Ar-H), 10.16 (s, 1H, OH), 14.86 (s, 1H, COOH). IR

(ATR): 3427 (O-H, stretch), 3162, 2861, 2782 (C-H, stretch), 1753 (C=O, stretch), 1604 (C=N, stretch), 1430 (C-O, stretch), 1274 (C=S, stretch), 1250 (O-H, bend), 1052 (C-F, stretch). Elemental analysis for $C_{33}H_{31}FN_6O_5S$ (642.70). Calculated: C 61.67, H 4.86, N 13.08. Found: C 61.55, H 4.72, N 13.19.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(2-hydroxyphenyl)-4-(4-methoxybenzyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**30**)

Yield: 66%, m.p. 166-167°C, ^{1}H-NMR (250 MHz): 1.20-1.24 (m, 2H, cyclopropyl), 1.32-1.35 (m, 2H, cyclopropyl), 2.92 (bs, 4H, piperazine), 3.30 (bs, 4H, piperazine), 3.61 (s, 3H, OCH_3), 3.86-3.93 (m, 1H, cyclopropyl), 4.54 (s, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.90 (dd, 2H, Ar-H, J = 2.1 Hz, 6.7 Hz), 6.94-7.75 (m, 8H, Ar-H), 8.67 (s, 1H, Ar-H), 10.50 (s, 1H, OH), 14.94 (s, 1H, COOH). IR (ATR): 3405 (O-H, stretch), 3167, 3052, 2861 (C-H, stretch), 1719 (C=O, stretch), 1582 (C=N, stretch), 1440 (C-O, stretch), 1307 (C=S, stretch), 1289 (O-H, bend), 1042 (C-F, stretch). Elemental analysis for $C_{34}H_{33}FN_6O_5S$ (656.73). Calculated: C 62.18, H 5.06, N 12.80. Found: C 62.10, H 5.17, N 12.94.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(3-hydroxyphenyl)-4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**31**)

Yield: 78%, m.p. 232-233°C, ^{1}H-NMR (250 MHz): 1.13-1.16 (m, 2H, cyclopropyl), 1.27-1.30 (m, 2H, cyclopropyl), 2.94 (bs, 4H, piperazine), 3.35 (bs, 4H, piperazine), 3.76-3.81 (m, 1H, cyclopropyl), 4.65 (s, 2H, CH_2), 7.10-7.86 (m, 11H, Ar-H), 8.69 (s, 1H, Ar-H), 10.09 (s, 1H, OH), 14.99 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 7.50, 35.00, 45.19, 51.21, 59.27, 105.78, 106.26, 111.02, 111.64, 113.86, 116.74, 117.64, 125.54, 126.04, 127.32, 128.64, 129.13, 131.64, 138.22, 144.32, 147.20, 148.80, 151.62, 154.64, 164.74, 165.87, 178.37. Elemental analysis for $C_{32}H_{29}FN_6O_4S$ (612.67). Calculated: C 62.73, H 4.77, N 13.72. Found: C 62.80, H 4.65, N 13.77.

1-Cyclopropyl-6-fluoro-7-[4-{[4-benzyl-3-(3-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**32**)

Yield: 62%, m.p. 244-246°C, ^{1}H-NMR (250 MHz): 1.15-1.22 (m, 2H, cyclopropyl), 1.27-1.31

(m, 2H, cyclopropyl), 2.84-2.93 (m, 4H, piperazine), 3.45-3.52 (m, 4H, piperazine), 3.88-3.93 (m, 1H, cyclopropyl), 4.52 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 7.04-7.64 (m, 11H, Ar-H), 8.69 (s, 1H, Ar-H), 10.02 (s, 1H, OH), 14.93 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 7.59, 35.87, 44.65, 46.16, 52.34, 60.64, 105.97, 106.27, 110.34, 111.68, 113.22, 115.26, 117.62, 124.34, 126.60, 126.90, 128.06, 128.68, 135.17, 138.16, 144.61, 148.71, 147.34, 151.14, 155.64, 165.64, 166.46, 179.61. Elemental analysis for $C_{33}H_{31}FN_6O_4S$ (626.70). Calculated: C 63.24, H 4.99, N 13.41. Found: C 63.18, H 4.81, N 13.33.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-fluorophenyl)-3-(3-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**33**)

Yield: 59%, m.p. 247-249°C, ^{1}H-NMR (250 MHz): 1.13-1.18 (m, 2H, cyclopropyl), 1.21-1.25 (m, 2H, cyclopropyl), 2.85 (bs, 4H, piperazine), 3.35 (bs, 4H, piperazine), 3.82-3.88 (m, 1H, cyclopropyl), 4.42 (s, 2H, CH_2), 6.92-7.68 (m, 10H, Ar-H), 8.66 (s, 1H, Ar-H), 10.34 (s, 1H, OH), 14.87 (s, 1H, COOH). IR (ATR): 3479 (O-H, stretch), 3042, 2804 (C-H, stretch), 1723 (C=O, stretch), 1621 (C=N, stretch), 1445 (C-O, stretch), 1327 (C=S, stretch), 1286 (O-H, bend), 1027 (C-F, stretch). Elemental analysis for $C_{32}H_{28}F_2N_6O_4S$ (630.66). Calculated: C 60.94, H 4.48, N 13.33. Found: C 60.80, H 4.35, N 13.27.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-fluorobenzyl)-3-(3-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**34**)

Yield: 66%, m.p. 238-240°C, ^{1}H-NMR (250 MHz): 1.20-1.23 (m, 2H, cyclopropyl), 1.27-1.31 (m, 2H, cyclopropyl), 2.86 (bs, 4H, piperazine), 3.31 (bs, 4H, piperazine), 3.74-3.80 (m, 1H, cyclopropyl), 4.67 (s, 2H, CH_2), 5.25 (s, 2H, CH_2), 6.90-7.54 (m, 10H, Ar-H), 8.64 (s, 1H, Ar-H), 9.92 (s, 1H, OH), 14.98 (s, 1H, COOH). IR (ATR): 3505 (O-H, stretch), 2963, 2861 (C-H, stretch), 1703 (C=O, stretch), 1591 (C=N, stretch), 1417 (C-O, stretch), 1329 (C=S, stretch), 1270 (O-H, bend), 1011 (C-F, stretch). Elemental analysis for $C_{33}H_{30}F_2N_6O_4S$ (644.69). Calculated: C 61.48, H 4.69, N 13.04. Found: C 61.50, H 4.56, N 13.07.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-chlorophenyl)-3-(3-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**35**)

Yield: 75%, m.p. 250-252°C, ^{1}H-NMR (250 MHz): 1.20-1.23 (m, 2H, cyclopropyl), 1.27-1.30 (m, 2H, cyclopropyl), 2.90 (bs, 4H, piperazine), 3.35 (bs, 4H, piperazine), 3.80-3.85 (m, 1H, cyclopropyl), 4.65 (s, 2H, CH_2), 6.82-7.53 (m, 8H, Ar-H), 7.60 (dd, 2H, Ar-H, J = 2.0 Hz, 6.5 Hz), 8.67 (s, 1H, Ar-H), 9.81 (s, 1H, OH), 14.89 (s, 1H, COOH). IR (ATR): 3440 (O-H, stretch), 3103, 2943, 2767 (C-H, stretch), 1736 (C=O, stretch), 1595 (C=N, stretch), 1428 (C-O, stretch), 1319 (C=S, stretch), 1260 (O-H, bend), 1032 (C-F, stretch). Elemental analysis for $C_{32}H_{28}ClFN_6O_4S$ (647.12). Calculated: C 59.39, H 4.36, N 12.99. Found: C 59.52, H 4.43, N 13.11.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-chlorobenzyl)-3-(3-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**36**)

Yield: 68%, m.p. 250-251°C, ^{1}H-NMR (250 MHz): 1.15-1.19 (m, 2H, cyclopropyl), 1.27-1.32 (m, 2H, cyclopropyl), 2.92-3.30 (m, 8H, piperazine), 3.85-3.92 (m, 1H, cyclopropyl), 4.62 (s, 2H, CH_2), 5.30 (s, 2H, CH_2), 6.94-7.61 (m, 10H, Ar-H), 8.67 (s, 1H, Ar-H), 10.05 (s, 1H, OH), 14.82 (s, 1H, COOH). IR (ATR): 3457 (O-H, stretch), 3067, 2974, 2861 (C-H, stretch), 1735 (C=O, stretch), 1605 (C=N, stretch), 1418 (C-O, stretch), 1315 (C=S, stretch), 1267 (O-H, bend), 1017 (C-F, stretch). Elemental analysis for $C_{33}H_{30}ClFN_6O_4S$ (661.14). Calculated: C 59.95, H 4.57, N 12.71. Found: C 59.90, H 4.58, N 12.82.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(3-hydroxyphenyl)-4-(4-methoxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**37**)

Yield: 76%, m.p. 224-226°C, ^{1}H-NMR (250 MHz): 1.23-1.27 (m, 2H, cyclopropyl), 1.30-1.34 (m, 2H, cyclopropyl), 2.94 (bs, 4H, piperazine), 3.37 (bs, 4H, piperazine), 3.80 (s, 3H, OCH_3), 3.82-3.86 (m, 1H, cyclopropyl), 4.60 (s, 2H, CH_2), 6.92-7.65 (m, 10H, Ar-H), 8.65 (s, 1H, Ar-H), 9.85 (s, 1H, OH), 14.92 (s, 1H, COOH). IR (ATR): 3483 (O-H, stretch), 3052, 2939, 2842 (C-H, stretch), 1737 (C=O, stretch), 1597 (C=N, stretch), 1429 (C-O, stretch), 1328 (C=S,

stretch), 1257 (O-H, bend), 1022 (C-F, stretch). Elemental analysis for $C_{33}H_{31}FN_6O_5S$ (642.70). Calculated: C 61.67, H 4.86, N 13.08. Found: C 61.60, H 4.93, N 13.05.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(3-hydroxyphenyl)-4-(4-methoxybenzyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**38**)

Yield: 73%, m.p. 233-235°C, ^{1}H-NMR (250 MHz): 1.18-1.23 (m, 2H, cyclopropyl), 1.29-1.34 (m, 2H, cyclopropyl), 2.90 (bs, 4H, piperazine), 3.35 (bs, 4H, piperazine), 3.61 (s, 3H, OCH_3), 3.86-3.93 (m, 1H, cyclopropyl), 4.54 (s, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.90 (dd, 2H, Ar-H, J = 2.1 Hz, 6.7 Hz), 6.94-7.75 (m, 8H, Ar-H), 8.67 (s, 1H, Ar-H), 10.50 (s, 1H, OH), 14.94 (s, 1H, COOH). IR (ATR): 3403 (O-H, stretch), 3067, 2885 (C-H, stretch), 1707 (C=O, stretch), 1582 (C=N, stretch), 1440 (C-O, stretch), 1327 (C=S, stretch), 1295 (O-H, bend), 1010 (C-F, stretch). Elemental analysis for $C_{34}H_{33}FN_6O_5S$ (656.73). Calculated: C 62.18, H 5.06, N 12.80. Found: C 62.13, H 5.00, N 12.92.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(4-hydroxyphenyl)-4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**39**)

Yield: 65%, m.p. 254-255°C, ^{1}H-NMR (250 MHz): 1.13-1.16 (m, 2H, cyclopropyl), 1.26-1.30 (m, 2H, cyclopropyl), 2.90 (bs, 4H, piperazine), 3.33 (bs, 4H, piperazine), 3.80-3.85 (m, 1H, cyclopropyl), 4.63 (s, 2H, CH_2), 7.06-7.66 (m, 11H, Ar-H), 8.67 (s, 1H, Ar-H), 10.13 (s, 1H, OH), 14.84 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 8.21, 34.32, 45.19, 52.25, 58.47, 106.78, 107.72, 111.02, 112.63, 114.81, 115.82, 117.95, 124.84, 125.94, 127.52, 128.21, 129.93, 130.27, 137.44, 145.17, 147.20, 149.06, 152.62, 154.64, 165.83, 166.22, 176.54. Elemental analysis for $C_{32}H_{29}FN_6O_4S$ (612.67). Calculated: C 62.73, H 4.77, N 13.72. Found: C 62.85, H 4.67, N 13.67.

1-Cyclopropyl-6-fluoro-7-[4-{[4-benzyl-3-(4-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**40**)

Yield: 71%, m.p. 258-259°C, ^{1}H-NMR (250 MHz): 1.22-1.27 (m, 2H, cyclopropyl), 1.30-1.34 (m, 2H, cyclopropyl), 2.74 (bs, 4H, piperazine), 3.30 (bs, 4H, piperazine), 3.76-3.82 (m, 1H, cyclopropyl), 4.54 (s, 2H, CH_2), 5.20 (s, 2H, CH_2), 6.91-7.69 (m, 11H, Ar-H), 8.63 (s, 1H, Ar-

H), 10.22 (s, 1H, OH), 15.11 (s, 1H, COOH). ^{13}C-NMR (75 MHz): 7.66, 36.89, 45.34, 46.59, 50.14, 61.10, 106.16, 107.22, 110.34, 111.28, 114.72, 115.66, 117.12, 125.58, 126.07, 126.91, 127.82, 129.18, 135.27, 137.02, 145.28, 147.93, 148.34, 152.54, 156.14, 164.21, 165.71, 177.82. Elemental analysis for $C_{33}H_{31}FN_6O_4S$ (626.70). Calculated: C 63.24, H 4.99, N 13.41. Found: C 63.20, H 4.85, N 13.49.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-fluorophenyl)-3-(4-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**41**)

Yield: 71%, m.p. 256-257°C, ^{1}H-NMR (250 MHz): 1.24-1.28 (m, 2H, cyclopropyl), 1.33-1.39 (m, 2H, cyclopropyl), 2.72 (bs, 4H, piperazine), 3.31 (bs, 4H, piperazine), 3.82-3.86 (m, 1H, cyclopropyl), 4.52 (s, 2H, CH_2), 7.07-7.70 (m, 10H, Ar-H), 8.69 (s, 1H, Ar-H), 9.76 (s, 1H, OH), 14.94 (s, 1H, COOH). IR (ATR): 3502 (O-H, stretch), 3021, 2917 (C-H, stretch), 1727 (C=O, stretch), 1593 (C=N, stretch), 1448 (C-O, stretch), 1336 (C=S, stretch), 1275 (O-H, bend), 1048 (C-F, stretch). Elemental analysis for $C_{32}H_{28}F_2N_6O_4S$ (630.66). Calculated: C 60.94, H 4.48, N 13.33. Found: C 60.83, H 4.28, N 13.49.

1-Cyclopropyl-6-fluoro-7-[4-{[4-(4-chlorophenyl)-3-(4-hydroxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**42**)

Yield: 67%, m.p. 222-224°C, ^{1}H-NMR (250 MHz): 1.21-1.24 (m, 2H, cyclopropyl), 1.28-1.32 (m, 2H, cyclopropyl), 2.91 (bs, 4H, piperazine), 3.32 (bs, 4H, piperazine), 3.82-3.86 (m, 1H, cyclopropyl), 4.55 (s, 2H, CH_2), 6.92 (dd, 2H, Ar-H, J = 1.9 Hz, 6.4 Hz), 7.10-7.48 (m, 6H, Ar-H), 7.62 (dd, 2H, Ar-H, J = 2.0 Hz, 6.5 Hz), 8.68 (s, 1H, Ar-H), 9.92 (s, 1H, OH), 14.73 (s, 1H, COOH). IR (ATR): 3432 (O-H, stretch), 3121, 2921 (C-H, stretch), 1717 (C=O, stretch), 1565 (C=N, stretch), 1436 (C-O, stretch), 1317 (C=S, stretch), 1274 (O-H, bend), 1020 (C-F, stretch). Elemental analysis for $C_{32}H_{28}ClFN_6O_4S$ (647.12). Calculated: C 59.39, H 4.36, N 12.99. Found: C 59.45, H 4.46, N 13.06.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(4-hydroxyphenyl)-4-(4-methoxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**43**)

Yield: 74%, m.p. 224-226°C, ^{1}H-NMR (250 MHz): 1.24-1.27 (m, 2H, cyclopropyl), 1.31-1.34 (m, 2H, cyclopropyl), 2.94 (bs, 4H, piperazine), 3.34 (bs, 4H, piperazine), 3.56 (s, 3H, OCH_3), 3.84-3.87 (m, 1H, cyclopropyl), 4.61 (s, 2H, CH_2), 6.97 (dd, 2H, Ar-H, *J* = 2.1 Hz, 6.4 Hz), 7.13-7.56 (m, 6H, Ar-H), 7.66 (dd, 2H, Ar-H, *J* = 2.0 Hz, 6.5 Hz) 8.69 (s, 1H, Ar-H), 10.13 (s, 1H, OH), 14.96 (s, 1H, COOH). IR (ATR): 3409 (O-H, stretch), 3117, 2917, 2783 (C-H, stretch), 1725 (C=O, stretch), 1604 (C=N, stretch), 1439 (C-O, stretch), 1321 (C=S, stretch), 1268 (O-H, bend), 1011 (C-F, stretch). Elemental analysis for $C_{33}H_{31}FN_6O_5S$ (642.70). Calculated: C 61.67, H 4.86, N 13.08. Found: C 61.56, H 4.98, N 13.15.

1-Cyclopropyl-6-fluoro-7-[4-{[3-(4-hydroxyphenyl)-4-(4-methoxybenzyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-1-yl]methyl}piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**44**)

Yield: 76%, m.p. 249-251°C, ^{1}H-NMR (250 MHz): 1.19-1.23 (m, 2H, cyclopropyl), 1.26-1.30 (m, 2H, cyclopropyl), 2.93 (s, 4H, piperazine), 3.34 (s, 4H, piperazine), 3.67 (s, 3H, OCH_3), 3.89-3.92 (m, 1H, cyclopropyl), 4.58 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 6.97 (dd, 2H, Ar-H, *J* = 2.1 Hz, 6.7 Hz), 7.10-7.69 (m, 8H, Ar-H), 8.67 (s, 1H, Ar-H), 10.21 (s, 1H, OH), 14.87 (s, 1H, COOH). IR (ATR): 3452 (O-H, stretch), 3060, 2849 (C-H, stretch), 1707 (C=O, stretch), 1582 (C=N, stretch), 1435 (C-O, stretch), 1330 (C=S, stretch), 1267 (O-H, bend), 1028 (C-F, stretch). Elemental analysis for $C_{34}H_{33}FN_6O_5S$ (656.73). Calculated: C 62.18, H 5.06, N 12.80. Found: C 62.29, H 5.21, N 12.68.

4.2. Antimicrobial activity evaluation

The antimicrobial activity of the compounds was tested on the Gram-positive strains (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* Microbank 14001, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240), and on the Gram-negative strains (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027). Ciprofloxacin and

vancomycin were used as control antibacterial agents. Microbial suspensions with an optical density of 0.5 McFarland standard 150 x 10^6 CFU/mL (CFU – colony forming units) were prepared in sterile 0.85 % NaCl. All stock solutions of the tested compounds were dissolved in dimethyl sulfoxide (DMSO). The medium with DMSO in the final concentration and without the tested compounds served as a control - no microbial growth inhibition was observed. Preliminary antibacterial *in vitro* potency of the tested compounds was screened using an agar dilution method on the basis of the growth inhibition on a Mueller-Hinton agar to which the tested compounds in concentration of 1000 µg/mL were added. In the next assay *in vitro* antibacterial activity of the compounds with inhibitory effect was determined by a broth microdilution method. The 96-well microplates were used; 198 µL of Mueller–Hinton broth with a series of two-fold dilutions of the tested compound in the range of the final concentrations from 0.24 to 1000 µg/mL was inoculated with 2 µL of microbial suspension. After incubation (at 37°C for 18 h), spectrophotometric measurements of optical density (OD_{600}) of the bacterial cultures with the tested compounds were performed in order to determine MIC. OD_{600} of bacterial cultures in the medium without the tested compounds was used as a control. The activity was expressed as the minimal concentration of the compound that inhibits the visible growth of the bacteria (MIC, minimal inhibitory concentration). The MBC (minimal bactericidal concentration), defined as the lowest concentration of each compound that resulted in >99.9% reduction in CFU of the initial inoculum, was also assessed.

4.3. Cytotoxicity assay

HEK-293 (human embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC CRL-1573) and were grown in Eagle's Minimal Essential Medium (MEM; Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma). 100 U/mL of penicillin and 100 μg/mL of streptomycin were added to the media. The cell cultures were incubated at 37˚C in a humidified atmosphere with 5% CO2. The investigated compounds were dissolved in dimethyl sulfoxide (50 mg/mL) and then diluted in cell culture media supplemented with 2% FBS. HEK-293 cells were placed into 96-well plastic plates (Nunc, Roskilde, Denmark) at a cell density of 3 x 10^5 cells per well. After 24 h of incubation at 37˚C, the media were removed and cells treated with the derivatives, diluted in media at final concentrations of 2-500 µg/mL. Cell cultures were incubated at 37˚C for 48 h. The cytotoxicity was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that is cleaved into a coloured formazan product by metabolically active cells, according to the assay

described by Takenouchi and Munekata [15]. The quantity of the formazan product was measured in an automatic plate reader. From the obtained results the EC50 (concentration of the substance which inhibits cells growth in 50% in proportion to the growth of control cells) values were calculated. The results were given as mean ± SD of three independent experiments.

4.4. Enzymatic assays

The inhibitory activity of DNA gyrase and topoisomerase IV from *E. coli* was evaluated using gyrase supercoling assay kit and topoisomerase IV decatenation kit (both kits obtained from Inspiralis). Briefly, supercoiled pBR322 plasmid DNA (0.5μg) was incubated with 1 U gyrase, in the dedicated supercoiling assay buffer supplied by the manufacturer, in the presence of varying concentrations of the test compounds. Reactions were carried out at 37 °C for 1 h and then terminated by the addition of equal volume of 2x STOP Buffer (40% sucrose,100 mM Tris Cl pH 7.5, 1 mM EDTA, 0.5 mg/ml bromophenol blue) and chloroform/isoamyl alcohol. Samples were vortexed, centrifuged and run through a 15 cm 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) for 3 h at 50 V. Gels were stained with ethidium bromide and visualized under UV light. The decatenation assay was performed using *E. coli* topoisomerase IV decatenation kit (Inspiralis). Interlinked kDNA substrate (0.5 μg) was incubated with 1 U topoisomerase IV (Inspiralis), in the dedicated decatenation assay buffer supplied by the manufacturer, in the presence of varying concentrations of the test compounds. Reactions were carried out at 37°C for 1 h and then terminated by the addition of equal volume of 2x STOP Buffer (40% sucrose, 100 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.5 mg/ml bromophenol blue) and chloroform/isoamyl alcohol. Samples were vortexed, centrifuged and run through a 15 cm 1% agarose gel in TAE buffer for 1.5 h at 80 V. Gels were stained with ethidium bromide and visualized under UV light. Concentrations of inhibitor that prevented 50% of the kinetoplast DNA from being converted into decatenated minicircles (IC_{50} values) were determined by plotting the results obtained from the densytometric analyses of the gel images using Quantity One software (BioRad).

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Table 1. Antibacterial activity of compounds **23-44** against Gram-positive strains

			Minimal inhibitory concentrations (μM)						
	R_1	**R_2**	***MSSA-1****	***MSSA-2*****	***MRSA*****	***S. epidermidis ATCC 12228***	***B. subtilis ATCC 6638***	***B. cereus ATCC 10876***	***M. luteus ATCC 10240***
23	2-OH	C_6H_5-	0.80	0.39	0.19	0.098	0.39	0.098	1.59
24	2-OH	C_6H_5-CH_2-	0.38	0.19	0.38	0.38	0.048	0.19	1.56
25	2-OH	4-F-C_6H_4-	1.55	0.38	0.19	0.38	0.19	0.19	3.09
26	2-OH	4-F-C_6H_4-CH_2-	0.76	0.19	0.046	0.046	0.011	0.093	0.76
27	2-OH	4-Cl-C_6H_4-	0.76	0.18	0.093	0.18	0.093	0.18	1.51
28	2-OH	4-Cl-C_6H_4-CH_2-	0.74	0.18	0.36	0.091	0.045	0.091	1.48
29	2-OH	4-OCH_3-C_6H_4-	0.76	0.37	0.18	0.093	0.093	0.37	1.52
30	2-OH	4-OCH_3-C_6H_4-CH_2-	0.36	0.091	0.091	0.046	0.011	0.091	1.49
31	3-OH	C_6H_5-	0.80	0.39	0.39	0.19	0.098	0.19	1.60
32	3-OH	C_6H_5-CH_2-	0.38	0.19	0.19	0.096	0.048	0.096	1.56
33	3-OH	4-F-C_6H_4-	0.76	0.38	0.38	0.19	0.095	0.19	1.55
34	3-OH	4-F-C_6H_4-CH_2-	0.37	0.093	0.37	0.19	0.023	0.093	0.76
35	3-OH	4-Cl-C_6H_4-	0.76	0.37	0.37	0.093	0.093	0.18	1.51
36	3-OH	4-Cl-C_6H_4-CH_2-	1.48	1.48	1.48	0.18	0.18	0.18	2.95
37	3-OH	4-OCH_3-C_6H_4-	0.74	0.37	0.093	0.19	0.37	0.19	1.52
38	3-OH	4-OCH_3-C_6H_4-CH_2-	0.18	0.091	0.046	0.091	0.091	0.18	1.49
39	4-OH	C_6H_5-	1.60	0.39	0.39	0.098	0.024	0.098	1.60
40	4-OH	C_6H_5-CH_2-	3.11	1.56	1.56	0.19	0.78	0.19	6.24
41	4-OH	4-F-C_6H_4-	0.19	0.19	0.19	0.095	0.048	0.095	1.55

42	4-OH	4-Cl-C_6H_4-	0.76	0.37	0.093	0.18	0.046	0.18	1.51
43	4-OH	4-OCH_3-C_6H_4-	0.76	0.19	0.093	0.37	0.023	0.19	1.52
44	4-OH	4-OCH_3-C_6H_4-CH_2-	2.97	0.75	0.75	1.49	0.18	0.091	5.95
CPX			2.96	0.72	-	1.48	0.09	0.36	5.88
VCN			-	-	0.68	-	-	-	-

„*" – *S. aureus* ATCC 25923, „**" – *S. aureus* ATCC 6538, „***" – *S. aureus* MICROBANK 14001; CPX – ciprofloxacin; VCN – vancomycin .

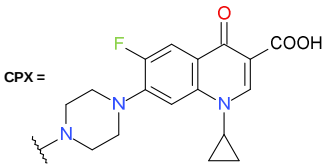
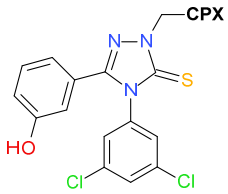
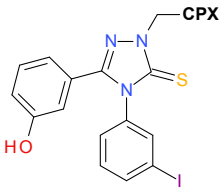
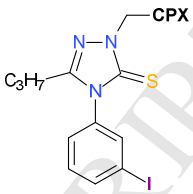
Table 2. Antibacterial activity of compounds **23-44** against Gram-negative strains

	Minimal inhibitory concentrations (μM)			
Compounds	***E. coli* *ATCC 25922***	***K. pneumoniae* *ATCC 13883***	***P. mirabilis* *ATCC 12453***	***P. aeruginosa* *ATCC 9027***
23	0.012	0.049	0.024	0.39
24	0.006	0.096	0.024	0.096
25	0.011	0.048	0.024	0.095
26	0.011	0.093	0.023	0.046
27	0.011	0.023	0.011	0.37
28	0.023	0.023	0.046	0.36
29	0.011	0.047	0.047	0.76
30	0.011	0.023	0.023	0.023
31	0.012	0.049	0.024	0.19
32	0.006	0.048	0.012	0.19
33	0.012	0.048	0.024	0.38
34	0.006	0.046	0.006	0.093
35	0.012	0.046	0.024	0.37
36	0.023	0.18	0.023	0.36
37	0.006	0.093	0.047	0.19
38	0.011	0.091	0.023	0.18
39	0.024	0.049	0.049	0.20
40	0.048	0.19	0.024	0.38
41	0.024	0.19	0.048	0.38
42	0.011	0.093	0.012	0.18
43	0.012	0.093	0.023	0.023
44	0.046	0.091	0.091	0.36
Ciprofloxacin	0.024	0.36	0.045	0.72

Table 3. Toxic effects of the selected triazole-CPX hybrids towards human HEK-293 cells

Compounds	EC_{50} ± SD (μM)
24	69.09 ± 10.15
30	78.72 ± 9.18
31	139.55 ± 17.77
34	69.49 ± 5.04
37	113.58 ± 2.43
38	62.89 ± 0.43
41	106.24 ± 16.60
43	105.34 ± 2.75

Table 4. Affinity of the selected triazole-CPX hybrids towards bacterial type II topoisomerases

		CPX = 			
		A 	**B** 	**C** 	**CPX**
Affinity IC_{50} (µM)	*gyrDNA*	6.05	9.78	6.28	0.15
	topoIV	78.70	78.09	154.84	80.00

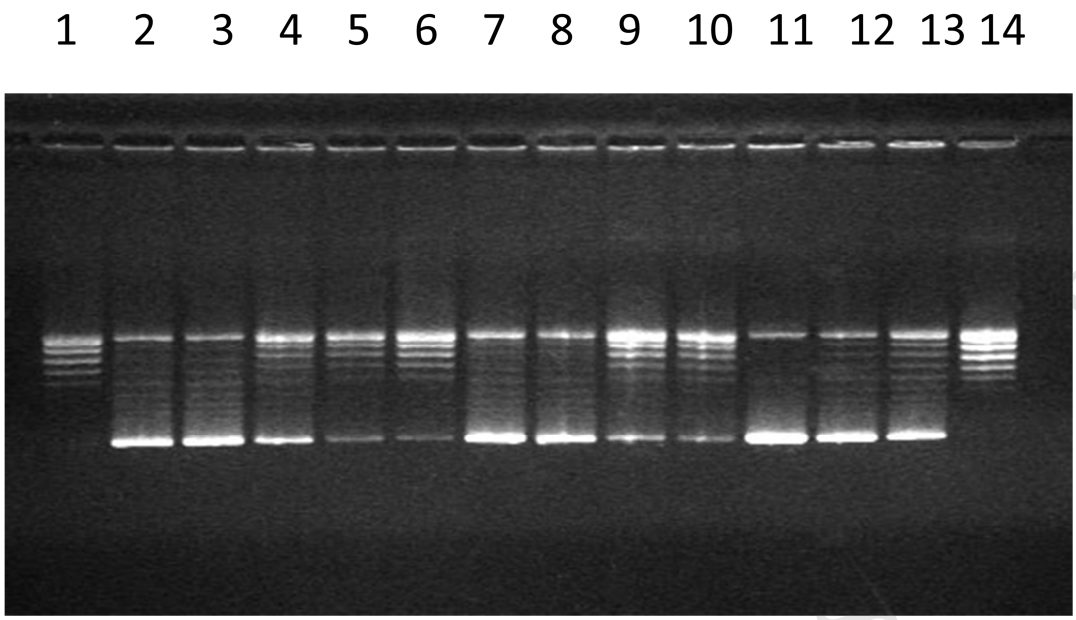
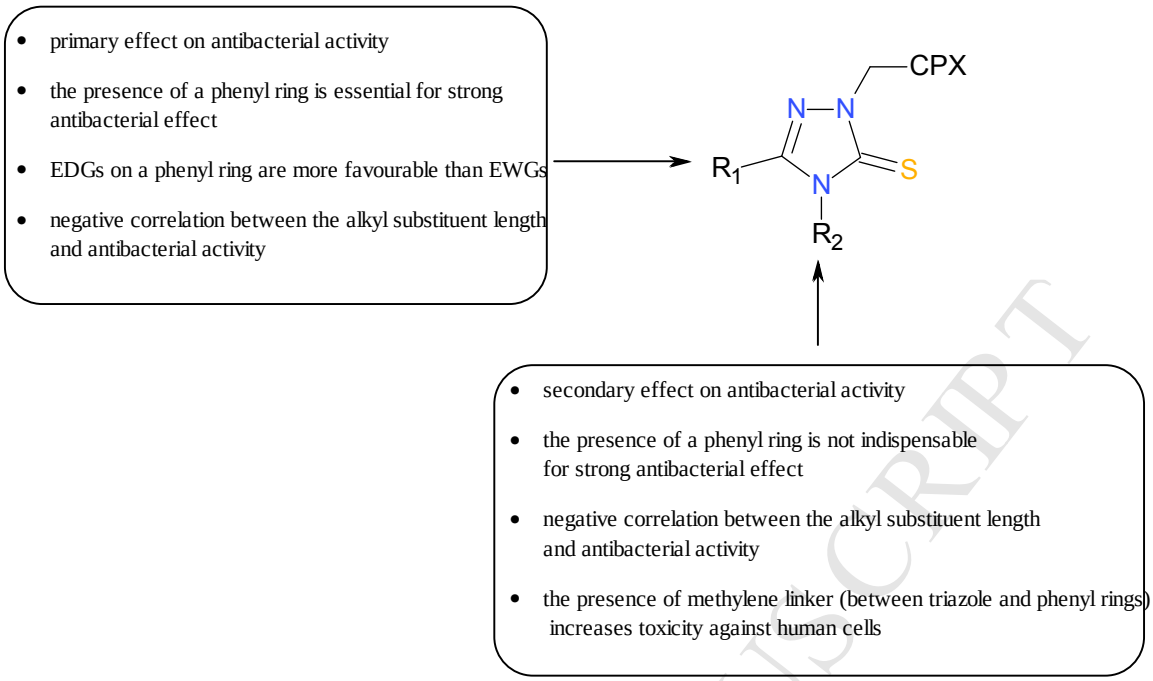
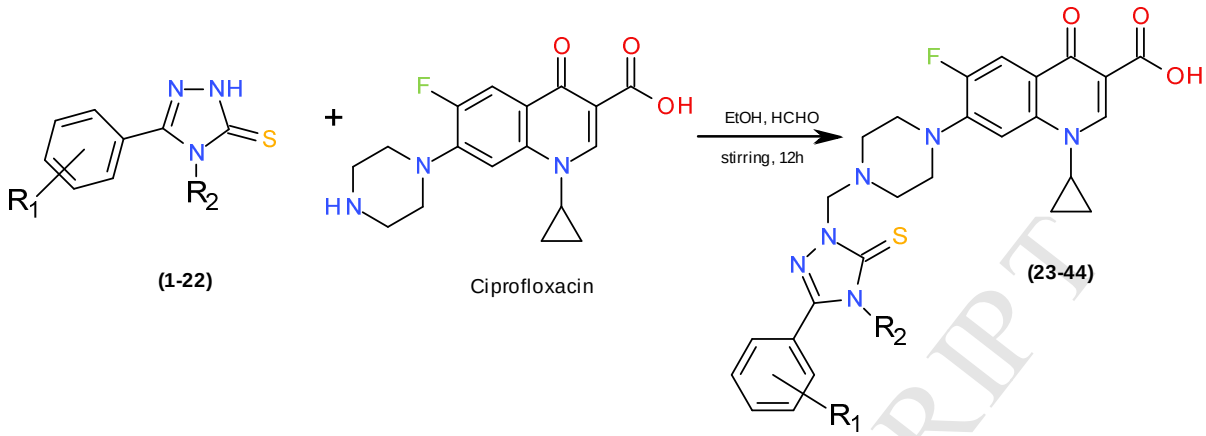


Figure 1. Assay results of gyrase DNA from *E. coli* with compound **A-C**. [1 – relaxed pBR; 2 – gyrase DNA control; 3 – compound C (0.5 μM); 4 – compound C (1.0 μM); 5 – compound C (5.0 μM); 6 – compound C (10.0 μM); 7 – compound A (0.5 μM); 8 – compound A (1.0 μM); 9 – compound A (5.0 μM); 10 – compound A (10.0 μM); 11 – compound B (0.5 μM); 12 – compound B (1.0 μM); 13 – compound B (5.0 μM); 14 – compound B (10.0 μM)]



Scheme 2. SAR observations for the triazole-CPX hybrids



Scheme 1. Synthetic route to compounds **23-44**. R_1 and R_2 substituents are presented in Table 1.

Research highlights

- Molecular hybridization strategy was employed to design novel antibacterials
- Ciprofloxacin-triazole hybrids were synthesized using Mannich reaction
- The obtained hybrids were active at completely non-toxic doses for human cells