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Synthesis and evaluation of novel 1,3,4-thiadiazole–fluoroquinolone hybrids as antibacterial, antituberculosis, and anticancer agents

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Abstract: A series of 5-substituted-1,3,4-thiadiazole-based fluoroquinolone derivatives were designed as potential antibacterial and anticancer agents using a molecular hybridization approach. The target compounds **16–25** were synthesized by reacting the corresponding *N*-(5-substituted-1,3,4-thiadiazol-2-yl)-2-chloroacetamides with ciprofloxacin or norfloxacin. The purity and identity of the synthesized compounds were determined by the use of chromatographic and spectral techniques (NMR, IR, MS, etc.) besides elemental analysis. Antibacterial, antituberculosis, and anticancer activity of the target compounds were evaluated against selected strains and cancer cell lines. Compound **20** was appreciated as the most active agent representing antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with MIC values of 4 µg/mL and 2 µg/mL, respectively. Amongst the synthesized fluoroquinolone derivatives, compounds **19** and **20** were found to have modest antitubercular activity with 8 µg/mL MIC values for each. Most potent derivative, compound **20** was docked against *Staphylococcus aureus* and *Mycobacterium tuberculosis* DNA gyrase enzymes to visualize the possible conformation of the compound. Additionally, anticancer activities of target compounds were evaluated on seven different cancer cell lines.

Key words: Fluoroquinolones, 1,3,4-thiadiazoles, antibacterials, tuberculosis, DNA gyrase, molecular modeling, cytotoxicity

1. Introduction

Fluoroquinolones (FQs) are commonly used antibacterial agents that have been shown to possess a broad spectrum of antibacterial activity, great potency, and good oral bioavailability, as well as low side effects.¹ Moreover, the World Health Organization (WHO) approves FQs as second-line antituberculosis agents.² Despite the remarkable clinical success of FQs, new fluoroquinolone containing medicinal agents are needed immediately owing to increasing resistance against commonly prescribed antibacterials^{3,4} since resistance is a growing problem for treatment.³ Furthermore, the anticancer activity of FQs is a partly new and promising area for these agents.⁴

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Nalidixic acid is the first of the quinolone antibacterial agents, although technically it is a naphthyridine structure-containing compound, not quinolone.⁵ It was discovered as the synthetic by-product of the antimalarial agent chloroquine and indicated to have antibacterial activity towards gram-negative bacteria, 5 decades ago.⁵ Later, the quinolone ring has had many different modifications. The first one was the introduction of a fluorine atom to the sixth position of the quinolone ring; thereafter several fluoroquinolone-bearing antibacterial agents, namely norfloxacin, ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin, etc., have been discovered and gone into use in the clinic.⁶

FQs are most commonly prescribed broad spectrum antibacterial agents that are used against respiratory tract infections, urinary tract infections, gastrointestinal infections, and sexually transmitted diseases.⁷ Moreover, tuberculosis therapy is a relatively new indication of FQs approved by the WHO.² Ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin were demonstrated to show activity against *M. tuberculosis*;^{8,9} meanwhile, studies to generate new ones are going on globally.^{10–14}

FQ-containing agents possess antibacterial activity by inhibiting bacterial type II topoisomerase enzymes, also known as DNA gyrases. They preferably inhibit topoIV enzymes of gram-positive microorganisms and DNA gyrases of gram-negative microorganisms. Beyond, FQs inhibit the ParC domain of TopoII and the GyrA domain of DNA gyrase.¹⁵ Topoisomerases are well established essential enzymes for bacterial survival, DNA transcription, replication, and DNA repair. They are defined as crucial enzymes for every movement of DNA in cells¹⁶ so that inhibitors of topoisomerases are widely accepted rational candidates for antibacterial and anticancer agents.¹⁷

A breakthrough development concerning FQs and tuberculosis was the publication of the *M. tuberculosis* DNA gyrase crystal structure, in 2016.¹⁸ Crystal structures of DNA gyrase enzymes were established in 1997.¹⁹ Furthermore, co-crystals of DNA gyrases, derived from different species such as *S. pneumoniae* and *S. aureus*, and FQs have been published recently.^{20–22}

While FQs have been known as antibacterial agents for decades, there are several studies proposing FQs may be used for anticancer therapy. Although Hussy et al. showed prokaryotic topoisomerases are more responsive to FQs after they were first introduced into the clinic,²³ subsequent studies found that human topoisomerases could be inhibited by FQs and could be attractive targets for FQs during anticancer chemotherapy. In this manner, ciprofloxacin was found to affect cell proliferation of transitional cell carcinoma of the bladder.²⁴ Ciprofloxacin and ofloxacin were shown to enhance cytotoxicity of doxorubicin against bladder cancer.²⁵ Ciprofloxacin was screened to induce apoptosis in colon carcinoma cell lines time and dose dependently.²⁶ It was shown to be cytotoxic against ovarian cancer²⁷ and lung cancer²⁸ and was shown to inhibit proliferation of human lymphoidal cells by inducing apoptosis pathways.²⁹ Ciprofloxacin was also shown to have an antiproliferative and apoptosis-inducing effect on prostate cancer cells.³⁰ In addition, ciprofloxacin, norfloxacin, enoxacin, and levofloxacin were shown to inhibit growth of nonsmall lung cancer cells in a concentration- and time-dependent manner.³¹ It was important to see ciprofloxacin and moxifloxacin both inhibit topoI activity³² after knowing moxifloxacin inhibits human topoII, the considerable target for anticancer chemotherapy.³³ On the other hand, human breast cancer cells were screened to accumulate enoxacin at G₂/M phase when they were exposed to this agent,³⁴ whilst colon cancer cell accumulated ciprofloxacin at the S phase when they were exposed to ciprofloxacin.³⁵

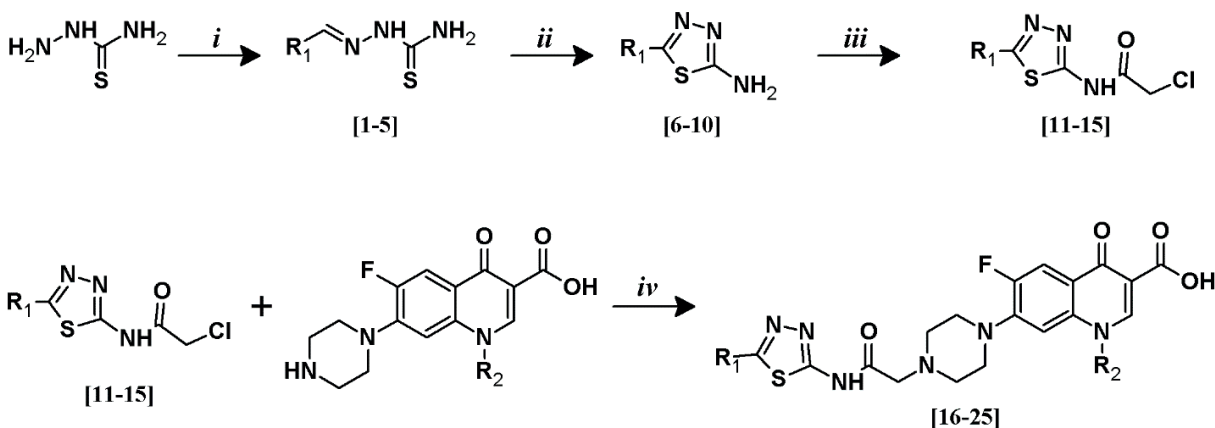
Modifying the already known FQ-containing agents is a widely used approach for drug discovery. To date, some studies have been performed to discover potent antibacterial agents,^{36–44} antimycobacterial

agents,^{10–14,45,46} and anticancer agents.^{47–53} Since it was already shown that the substituents at C7 determine the power and the preferential action target of the FQ, that is, the greater or lesser affinity for topoisomerase IV or gyrase,⁵⁴ we modified the piperazine ring at C7 of the FQ core by adding a 1,3,4-thiadiazole ring through $-\text{CH}_2-\text{CO}-$ linker to gain some insights into this existing relationship. Based on the above findings, several fluoroquinolone derivatives containing 1,3,4-thiadiazole moiety differing in the structure of substituents at C5 position have been designed, synthesized, and evaluated for their antibacterial, antimycobacterial, and anticancer activity.

2. Results and discussion

2.1. Chemistry

The synthetic route to achieve target molecules is shown in the Scheme. For this aim, selected aldehydes were converted to thiosemicarbazones **1–5** by reacting with thiosemicarbazide. 2-Amino-1,3,4-thiadiazole derivatives **6–10** were obtained by oxidative cyclization of thiosemicarbazones in the presence of ferric chloride. 2-Amino-1,3,4-thiadiazole derivatives **6–10** were converted to 2-chloro-*N*-(heteroaryl/alkyl)acetamide derivatives **11–15** by using α -chloroacetyl chloride in the presence of TEA. Finally, 2-chloro-*N*-(5-substituted-1,3,4-thiadiazole-2-yl)acetamide derivatives **11–15** and excess amount of ciprofloxacin/norfloxacin were reacted to yield the target compounds **16–25**. Following the isolation process, the crude products were crystallized from appropriate solvents. Purity of the synthesized compounds was checked by TLC and HPLC, and their structures were confirmed by IR, ^1H NMR, ^{13}C NMR, and mass spectral data besides elemental analysis.



16 (R_1 : cyclohexyl; R_2 : ethyl); **17** (R_1 : 4-fluorophenyl; R_2 : ethyl); **18** (R_1 : 2-chlorophenyl; R_2 : ethyl); **19** (R_1 : 4-chlorophenyl; R_2 : ethyl); **20** (R_1 : 2,4-dichlorophenyl; R_2 : ethyl); **21** (R_1 : cyclohexyl; R_2 : cyclopropyl); **22** (R_1 : 4-fluorophenyl; R_2 : cyclopropyl); **23** (R_1 : 2-chlorophenyl; R_2 : cyclopropyl); **24** (R_1 : 4-chlorophenyl; R_2 : cyclopropyl); **25** (R_1 : 2,4-dichlorophenyl; R_2 : cyclopropyl).

Scheme. Synthetic route for target compounds **16–25**. Reagents and conditions: *i*. $\text{R}_1\text{-CHO}$, EtOH, g.AcOH, reflux; *ii*. FeCl_3 , EtOH, reflux; *iii*. ClCH_2COCl , DCM, TEA; *iv*. DMF, NaHCO_3 .

In the FTIR spectra, N–H, C=N, and C=S stretching bands of thiosemicarbazone derivatives **1–5** were observed at 3444–3245, 1612–1587, and 1386–1244 cm^{-1} absorption values, respectively. After cyclization of thiosemicarbazones, C=N stretching bands of 1,3,4-thiadiazole rings **5–10** were detected at 1558–1587 cm^{-1} , while N–H stretches of amines were seen at 3288–3173 cm^{-1} . Stretching bands of C=O groups were monitored at 1701–1709 cm^{-1} values, which demonstrated the formation of chloracetamide derivatives **11–15**.⁵⁵

FTIR spectral data of the target compounds **16–25** showed 3607–3194, 3302–3182, 1732–1712, 1705–1681, and 1631–1624 cm^{-1} stretches, which were attributed to O–H, N–H, carboxylic acid, amide, and ketone groups, respectively.⁵⁶

In the analysis of the ^1H NMR spectra of the target compounds, protons belonging to piperazinyl moiety were observed at 2.76–3.54 ppm as multiplets.⁵⁷ Phenyl protons of compounds **17–20** and **21–25** were observed at 7.35–8.14 ppm. Cyclohexyl protons of compounds **16** and **21** were detected at 1.20–2.10 and 3.05 ppm. Cyclopropyl protons of compounds **21–25** were determined at 1.19–1.32 ppm. In the ^1H NMR spectra of the target compounds, there were no peaks attributable to NH protons of *N*-[5-substituted-1,3,4-thiadiazol-2-yl]acetamide residues since they were expected to be observed at around 9.28–11.10 ppm.⁴⁷ It was observed that the mentioned NH protons were exchanged with deuterium from DMSO- d_6 . Meanwhile, carboxylic acid protons were observed at 15.23–15.39 ppm. Methylene protons of *N*-[5-substituted-1,3,4-thiadiazol-2-yl]acetamide residues were detected at 2.49–2.52 ppm. When we analyzed the ^1H NMR spectra of the final compounds **16–25**, we identified H_2 , H_5 , and H_8 protons of the quinolone ring at 8.66–8.96, 7.60–7.91, and 7.19–7.92 ppm, respectively. Quinolone H_5 and H_8 protons coupled with fluorine atoms at the 6th position of the ring. Coupling constants were calculated for quinolone ring H_5 and H_8 protons as $J = 6.6\text{--}13.5$ Hz and $J = 3.0\text{--}7.5$ Hz, respectively. For the compounds **16–25**, ^1H NMR results were consistent with the literature.^{41,47,56–60}

Furthermore, in the ^{13}C NMR spectra of the selected compounds, carboxylic acid carbons displayed resonances at 166–167 ppm. Piperazine carbons were observed at 50–53 ppm, whilst conjugated ketone carbons were observed at 176–177 ppm. Other quinolone carbons were identified at 106–154 ppm. C_2 and C_5 carbon signals of 1,3,4-thiadiazole rings were detected at 158 and 160 ppm. Carbons that belong to acetylamino residue were determined at 169 ppm. Moreover, phenyl carbons were observed at 128–164 ppm. Similar to the ^1H NMR data, the ^{13}C NMR spectra also showed correlation with the literature.^{58–60}

Similar to the ^1H NMR findings, quinolone ring C_5 , C_6 , C_7 , and C_{4a} carbons interacted with fluorine atoms at the 6th position in the ^{13}C NMR spectra of the final compounds. In order of coupling constants for C_6 , C_5 , C_7 , and C_{4a} carbons were calculated as $J = 247.5$ Hz, $J = 22.5\text{--}27.0$ Hz, $J = 9.0\text{--}10.5$ Hz, and $J = 7.5$ Hz. These interactions are found to be consistent with the literature.⁶⁰

Low-resolution ESI mass spectra of compounds **16–25** were recorded in either positive or negative ionization mode and confirmed their molecular weights. The LC-MS/MS (ESI) analysis of the synthesized compounds gave correct molecular ion peaks corresponding to $[\text{M}+\text{H}]^+$ in positive ionization and $[\text{M}-\text{H}]^-$ in negative ionization mode in each case. All ESI negative LC-MS/MS analysis data revealed $[\text{M}-\text{H}]^-$ m/z values with 100% relative abundance, even as positive LC-MS/MS analysis data displayed $[\text{M}+\text{H}]^+$, $[\text{M}+\text{Na}]^+$, and $[\text{M}+\text{K}]^+$ m/z values of the target compounds with different relative abundances.

Of the synthesized fluoroquinolone–thiadiazole hybrids, compound **24** has been synthesized by a different method.⁶¹ As the melting point of this compound was different from the reported one, we presented full structural characterization data for this compound, including ^{13}C NMR and elemental analysis, which is not reported in the above mentioned study.⁶¹

2.2. Prediction of drug-likeness and ADME properties of compounds 16–25

Pharmacokinetic properties, which are specified as ADME (absorption, delivery, metabolism, elimination) and toxicity, are vital in the process of generating a new drug candidate. Desired physicochemical properties of

pharmacologically active drugs were summarized by Lipinski.⁶² Historical analyses of physicochemical properties of orally available marketed drugs that reached Phase II clinical trials demonstrated that 90% of them had fewer than five hydrogen bond donors and fewer than ten hydrogen bond acceptors. Their molecular masses were less than 500 Daltons, whereas log P values were scaled less than five. Listed properties then were stated as “Lipinski’s rule of five”.^{62,63}

Correlated to improvement in computational sciences, in silico programs can help to predict drug-gability of a small molecule. Physicochemical properties and ADME criteria can be estimated by these programs. Thus, the Molinspiration online calculation toolkit was used to predict drug-likeness aspects (<http://www.molinspiration.com/services/properties.html>). With the help of this technology, total polar surface area (TPSA), absorption% (ABS%), and Lipinski parameters were calculated as shown in Table 1.

Table 1. Drug-likeness properties* of compounds **16–25**.

Compound	MW	Vol	TPSA	ABS%	nROTB	nON	nOHNH	miLogP	nviol
16	541.65	471.61	120.66	67.37	7	10	2	1.41	1
17	554.58	457.95	120.66	67.37	7	10	2	1.88	1
18	571.03	466.55	120.66	67.37	7	10	2	2.34	1
19	571.03	466.55	120.66	67.37	7	10	2	2.39	1
20	605.48	480.09	120.66	67.37	7	10	2	3.00	1
21	554.65	477.83	120.66	67.37	7	10	2	1.40	1
22	566.59	464.18	120.66	67.37	7	10	2	1.86	1
23	583.04	472.78	120.66	67.37	7	10	2	2.33	1
24	583.04	472.78	120.66	67.37	7	10	2	2.38	1
25	617.49	486.32	120.66	67.37	7	10	2	2.98	1
Nor	319.33	279.26	74.57	83.27	3	6	2	-0.69	0
Cip	331.35	285.46	74.57	83.27	3	6	2	-0.70	0

*Nor: norloxacin, Cip: ciprofloxacin, MW: molecular weight, Vol: volume, TPSA: total polar surface area, ABS%: absorption%, nROTB: number of rotatable bonds, nOHNH: number of hydrogen bond donors, nON: number of hydrogen bond acceptors, miLogP: molinspiration partition coefficient *n*-octanol and water, nviol: number of violations.

Oral bioavailability is a desirable feature for drug candidates.⁶⁴ Due to the poor pharmacokinetic profiles, about 30% of oral drugs are eliminated in the area of drug development.⁶⁵ Log P calculation gives us an idea about oral bioavailability relevant to absorption, solubility, and permeability. A drug candidate should be neither too hydrophilic to cross the gastrointestinal wall nor too lipophilic to be absorbed. According to Log P calculation results, synthesized compounds **16–25** do not exceed the lipophilicity limitation.

The equation $109 - (0.345 \times \text{TPSA}) = \text{ABS}\%$ gives predicted percentage absorption.⁶⁶ Calculated absorption percentages of compounds **16–25** offered average results close to 70%. TPSA was calculated with the help of the Molinspiration online property calculation toolkit using the parameters originally proposed by Ertl et al.⁶⁷ Similar to lipophilicity, polar surface area is substantial for drug candidates to cross biological membranes. Too high TPSA results in poor absorption and bioavailability.⁶⁴

Numbers of hydrogen bond donors and hydrogen bond acceptors for compounds **16–25** amounted in the range of Lipinski’s rule of five. Due to Molinspiration analyses, only one violation was molecular weight, which

is not an important problem, since there are pharmacologically effective marketed and FDA approved molecules that have molecular weight over 500 Da, for example bedaquiline offering tuberculosis treatment.⁶⁸

2.3. Osiris calculations/prediction of toxicity, solubility, drug-likeness, and drug score for compounds 16-25

Potential toxicity, solubility, drug-like properties, and drug scores of the synthesized compounds **16–25** were estimated by Osiris Property Explorer (<http://www.organic-chemistry.org/prog/peo/>). Table 2 represents possibilities of mutagenicity, tumorigenicity, irritation, and reproductive toxicity of target compounds depending upon this predictor tool. Compounds **16–25** do not possess these undesirable features, according to the Osiris calculation. Calculated drug score for a lead molecule is expected to be over 0.5. According to this claim we may propose our candidates are close to being good candidates.

Table 2. Osiris calculations* for compounds **16–25**.

Compound	Toxicity risks				cLogP	Sol	MW	TPSA	DL	DS
	Mut	Tum	Irrit	Rep						
16	■	■	■	■	1.51	-4.74	542.0	147.2	2.30	0.51
17	■	■	■	■	1.30	-4.59	554.0	147.2	7.73	0.53
18	■	■	■	■	1.81	-5.01	570.0	147.2	7.69	0.48
19	■	■	■	■	1.81	-5.01	570.0	147.2	7.99	0.48
20	■	■	■	■	2.41	-5.74	604.0	147.2	8.14	0.39
21	■	■	■	■	1.64	-5.21	554.0	147.2	2.06	0.45
22	■	■	■	■	1.42	-5.05	566.0	147.2	7.45	0.48
23	■	■	■	■	1.93	-5.47	582.0	147.2	7.41	0.43
24	■	■	■	■	1.93	5.47	582.0	147.2	7.71	0.43
25	■	■	■	■	2.54	-6.21	616.0	147.2	7.66	0.35
Nor	■	■	■	■	-1.65	-2.96	319.0	72.88	2.24	0.86
Cip	■	■	■	■	-1.53	-3.32	331.0	72.88	2.07	0.82

*Nor: norloxacin, Cip: ciprofloxacin, ■: nontoxic, ■: slightly toxic, ■: highly toxic, Mut: mutagenicity, Tum: tumorigenicity, Irrit: irritation, Rep: reproductive, cLogP: partition coefficient *n*-octanol and water, Sol: solubility, MW: molecular weight, TPSA: total polar surface area, Sol: solubility, DL: drug likeness, DS: drug score.

2.4. Biological studies

2.4.1. Antimicrobial activity

Antibacterial activity of compounds **16–25** was tested against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Antimicrobial activities were determined as minimal inhibitory concentrations (MICs) and minimum bactericidal or (fungicidal) concentrations (MBCs or MFCs) by microwell dilution method and designated in Table 3. Since lower MIC values were observed with reference drugs, it might be predicted that introduction of 2-(heteroaryl-amino)-2-oxoethyl moiety at the *N*-4 position of the piperazine ring causes diminution in antibacterial potency.

Table 3. Antimicrobial activity results of compounds **16–25**.

Compound	MIC ($\mu\text{g/mL}$)			MBC/MFC ($\mu\text{g/mL}$)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
16	256	512	512	512	512	512
17	256	512	512	512	512	512
18	128	128	64	512	512	256
19	512	256	64	512	512	128
20	4	2	256	8	4	512
21	128	512	512	256	512	512
22	256	512	512	512	512	512
23	256	512	64	512	512	128
24	128	256	256	512	512	256
25	128	256	64	512	512	128
Norfloxacin	0.5	0.06	-	-	-	-
Ciprofloxacin	0.125	0.008	-	-	-	-
Fluconazole	-	-	1	-	-	-

Compound **20** bearing a 2,4-dichlorophenyl moiety at the R₁ position and an ethyl group at the R₂ position was appreciated as the most potent compound, representing MIC values of 4 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$ against *E. coli* and *S. aureus*, respectively. Thus, compound **20** drastically differs from other designed fluoroquinolones, depending on antibacterial activity results. The lack of antifungal activity observed with compound **20** clearly shows the selectivity of the antibacterial activity of this compound. Another noteworthy feature of this compound is that it has the highest Log P value amongst compounds **16–25**, which might indicate the influence of lipophilicity.

2.4.2. Antituberculosis activity

Target compounds **16–25** were initially screened for their in vitro antituberculosis activity against *M. tuberculosis* H₃₇Rv strain. The minimal inhibitory concentration vs. *M. tuberculosis* H₃₇Rv was determined by a broth microdilution method in the range of 8–64 $\mu\text{g/mL}$ (Table 4). Antituberculosis activity results of the compounds were compared to those of ciprofloxacin and norfloxacin as reference drugs.^{69,70}

Norfloxacin-derived compounds **19** and **20** carrying 4-chlorophenyl and 2,4-dichlorophenyl substituents on the 1,3,4-thiadiazole ring scored the best results with 8 $\mu\text{g/mL}$ MIC value against *M. tuberculosis*. Compounds **21** and **22** with ciprofloxacin core and cyclohexyl and 4-fluorophenyl substituents on the 1,3,4-thiadiazole ring were found to show MIC values of 16 and 32 $\mu\text{g/mL}$ respectively. Other target compounds are considered as weakly active against *M. tuberculosis* H₃₇Rv strain with the same MIC value of 64 $\mu\text{g/mL}$.

It has also been reported that MICs inhibiting 50% and 90% of the *M. tuberculosis* isolates for norfloxacin were 4 and 8 $\mu\text{g/mL}$, while MIC₅₀ and MIC₉₀ values for ciprofloxacin were 0.5 and 1 $\mu\text{g/mL}$.⁷¹

According to the results that we were able to experimentally observe it might be concluded that the presence of a bulky group at the N-4 position of the piperazine ring of either norfloxacin or ciprofloxacin decreases the antituberculosis activity.

Table 4. Antituberculosis activity and cytotoxicity results of compounds **16–25**.

Compound	MIC ($\mu\text{g/mL}$)	Cytotoxicity-IC ₅₀ ($\mu\text{g/mL}$)		Selectivity index*
		VERO	L929	
	<i>M. tuberculosis H37Rv</i>			
16	64	192	191	3.0
17	64	160	259	2.5
18	64	265	561	4.1
19	8	274	433	34.3
20	8	236	469	29.5
21	16	125	85	7.8
22	32	280	318	8.8
23	64	130	146	2.0
24	64	221	96	3.5
25	64	311	540	4.9
Ciprofloxacin	0.5 ⁶⁹	311	250	622.0
Norfloxacin	2.0 ⁷⁰	375	128	187.5

*Selectivity index was calculated as $SI = IC_{50} (VERO)/MIC (Mtb)$

2.4.3. Anticancer activity

Cytotoxic properties of the synthesized compounds **16–25** were tested against A579 (lung cancer), PC3 (prostate cancer), and SK MEL1 (melanoma) cell lines. Cell viability was measured by the MTS assay. However, no significant activity was observed against the mentioned cancer cell lines. Percentage viability results of the cell lines exposed to reference drugs and synthesized compounds **16–25** are presented in Table 5.

Table 5. Percentage viability of the cell lines exposed to compounds **16–25** at 10 μM .

Compound	A549	MRC5	PC3	PNT1	SK MEL 1	HACAT	HEK 293
16	91.6	91.8	108.3	102.1	102.2	76.8	71.8
17	91.3	98.9	110.6	104.5	100.4	95.4	73.6
18	91.4	88.5	106.9	103.2	99.9	86.8	67.8
19	93.3	94.8	107.4	109.6	100.7	94.8	68.3
20	93.6	84.5	107.2	100.7	102.7	90.8	67.4
21	81.0	63.2	105.8	103.2	98.7	78.4	66.8
22	91.8	87.0	104.6	101.1	105.4	86.0	60.1
23	91.3	95.4	106.6	106.1	104.9	79.0	59.8
24	88.1	83.9	105.3	103.7	99.9	67.1	72.4
25	94.7	75.2	103.6	99.5	104.8	86.5	69.7

2.5. Molecular modeling studies

Molecular docking studies concerning the synthesized compounds were performed to simulate potential inhibition profiles of related bacterial and mycobacterial targets. *M. tuberculosis* DNA gyrase enzyme and *S. aureus* DNA

gyrase enzyme were used for docking studies (Figure 1). Synthesized compounds as ligands revealed promising results according to docking calculations. Binding affinity (kcal/mol) of each compound was calculated and all docked poses were evaluated.

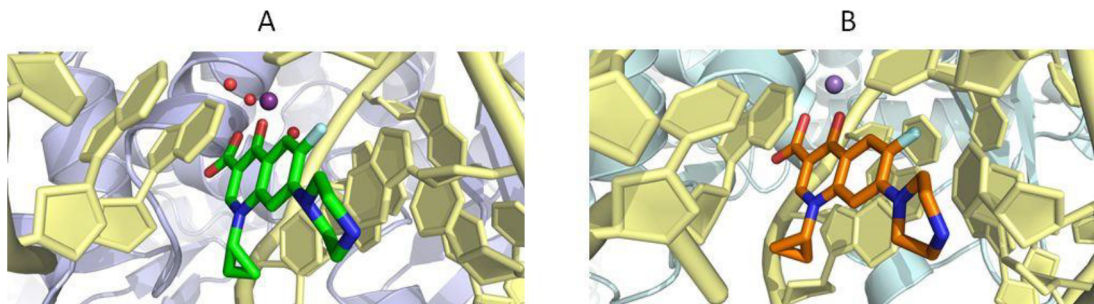


Figure 1. DNA Gyrase ciprofloxacin binding site. A. *M. tuberculosis* DNA gyrase (PDB code: 5BTC) B. *S. aureus* DNA gyrase (PDB code: 2XCT).

In order to evaluate the accuracy of our docking studies, the co-crystallized structure of ciprofloxacin was re-docked first into both DNA gyrase active sites with RMSD, being 0.586 and 0.840 values (Figure 2). Newly synthesized compounds were docked afterwards. Molecular docking studies showed that all synthesized fluoroquinolone derivatives adopt a similar binding mode in both DNA gyrase enzymes as already known fluoroquinolone derivative compounds.

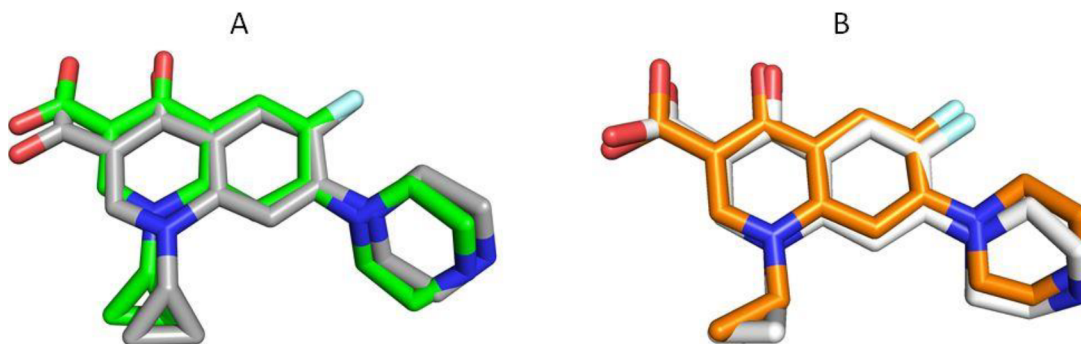


Figure 2. A. Superimposition of re-docked ciprofloxacin (gray) into *M. tuberculosis* DNA gyrase on the co-crystallized one (green). B. Superimposition of re-docked ciprofloxacin (white) into *S. aureus* DNA gyrase on the co-crystallized one (orange).

Mycobacterium tuberculosis DNA gyrase Ser90, Arg128, Arg482, Gly483, Thr500, and Glu501 amino acid residues were detected within 4 Å area of the docked pose of compound **20**. The carboxylate group of compound **20** forms a hydrogen bond with Arg128 residue. Interactions with Ser90, Arg482, Gly483, Thr500, and Glu501 amino acid residues were also observed. Possible conformation of compound **20** in *M. tuberculosis* DNA gyrase is presented in Figure 3A. The Mg^{2+} and oxygen atom of compound **20** quinolone ring interact, and water molecules of the crystal structure and carboxylate group of compound **20** interact as well.

Docking studies with *Staphylococcus aureus* DNA gyrase indicate that compound **20** makes interactions with Arg458, Asp 1083, Ser1084, and Arg1122 amino acid residues. Interactions with Mg^{2+} and oxygen atom of compound **20** quinolone ring were also observed. Interactions between water molecules of crystal structure and carboxylate group of compound **20** were detected as well. *Staphylococcus aureus* DNA gyrase amino acid

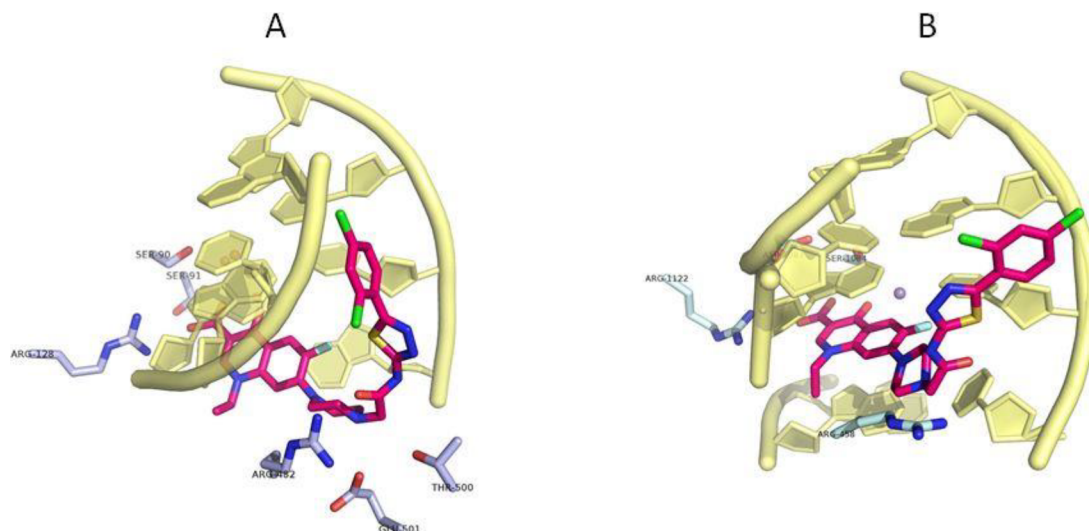


Figure 3. Possible conformation of compound **20** in complex with DNA gyrase residues around the ligand within 4 Å distance. A. *M. tuberculosis* DNA gyrase. B. *S. aureus* DNA gyrase.

residues and DNA coil within the 4 Å area of the docked pose of compound **20** are shown in Figure 3B.

Residues of *M. tuberculosis* DNA gyrase and residues of *S. aureus* DNA gyrase around 4 Å of compound **20** docked poses are presented in Table 6.

Table 6. Residues around compound **20** within 4 Å distance at DNA gyrase binding site.

Protein	Residues
<i>M. tuberculosis</i> DNA gyrase	DNA, H ₂ O molecules, Mg ²⁺
	Ser90, Arg128, Arg482, Gly483, Thr500, Glu501
<i>S. aureus</i> DNA gyrase	DNA, H ₂ O molecules, Mg ²⁺
	Arg458, Asp 1083, Ser1084, Arg1122

In conclusion, amongst newly synthesized thiadiazole–fluoroquinolone hybrids, only one representative (compound **20**) exhibited significant antibacterial activity towards *S. aureus* and *E. coli*. This unpredictable activity could be attributed to poor solubility of the compounds. This study also revealed two active fluoroquinolone derivatives (compounds **19** and **20**) against *M. tuberculosis* H37 Rv. Docking studies showed that compounds **16–25** are capable of binding DNA-gyrase B enzyme of *S. aureus* and *M. tuberculosis*. Further studies on newer fluoroquinolones with better solubility are in progress.

3. Experimental

3.1. Chemistry

All solvents and reagents were obtained from commercial sources and used without further purification. The purity of the compounds was confirmed by thin-layer chromatography (TLC) performed on Merck silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany), using developing systems: S₁: petroleum ether/ethyl acetate (50:50 v/v) and S₂: chloroform/methanol/acetic acid (93:5:2 v/v/v). Spots were detected under UV light at λ = 254 and 366 nm. All melting points were determined using a Kleinfeld SMP-II basic model point

apparatus and are uncorrected. Elemental analyses were obtained using a Leco CHNS-932 and are consistent with the assigned structures. ESI positive and ESI negative ionization (low resolution) mass spectra of the synthesized compounds were obtained using an AB SCIEX API 2000 LC-MS/MS instrument. FT-infrared spectra were recorded on a Shimadzu FT-IR Affinity-1 and data are expressed in wavenumbers (cm^{-1}) . ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE DPX at 150, 300, and 600 MHz. The chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane (TMS) using DMSO- d_6 as solvent.

The high-performance liquid chromatographic system consisted of an Agilent 1100 series instrument equipped with a quaternary solvent delivery system and a model Agilent series G1315 B photodiode array detector. A Rheodyne syringe loading sample injector with 50 μL sample loop was used for the injection of the analytes. Chromatographic data were collected and processed using Agilent ChemStation plus software. The separation was performed at ambient temperature by using a reversed phase ACE C₁₈ (100 \times 4 mm; 5 μm particle size) column. All experiments were performed in gradient mode. The mobile phase was prepared by mixing pH 4.50 phosphate buffer containing 0.1% TEA and acetonitrile (75:25 v/v during 0–2 min, 50:50 v/v during 2–4 min, 0:100 v/v during 4–12 min, 75:25 v/v during 12–15 min) and filtered through a 0.45- μm pore filter and subsequently degassed by ultrasonication, prior to use. Solvent delivery was employed at a flow rate of 1.0 mL/min. Detection of the analytes was carried out at 210, 230, 254, and 280 nm. SMILES codes were generated from the structures using the ACD/ChemSketch freeware version 12.0 molecular editor (<http://www.acdlabs.com>) and then pharmacokinetic properties were calculated using Molinspiration and Osiris web tools (<http://www.molinspiration.com/services/properties.html>, <http://www.organic-chemistry.org/prog/peo/>). The calculated log P values for the compounds are given in Tables 4 and 5.

General procedure for the synthesis of hydrazinecarbothioamides 1–5

Ethanol solution of thiosemicarbazide (30 mmol) was heated under reflux with various aromatic aldehydes (30 mmol) in the presence of a few drops of acetic acid. The crude products **1–5** precipitated on cooling were filtered and crystallized from ethanol.

2-(Cyclohexylmethylidene)hydrazinecarbothioamide (1)

Yield 90%. mp 90 °C (EtOH) (lit. 84–86 °C).⁷²

2-(4-Fluorobenzylidene)hydrazinecarbothioamide (2)

Yield 69%. mp 195–196 °C (EtOH) (lit. 197–198 °C).⁷³

2-(2-Chlorobenzylidene)hydrazinecarbothioamide (3)

Yield 75%. mp 221 °C (EtOH) (lit. 220 °C).⁵⁵

2-(4-Chlorobenzylidene)hydrazinecarbothioamide (4)

Yield 72%. mp 216 °C (EtOH) (lit. 217–220 °C).⁷⁴

2-(2,4-Dichlorobenzylidene)hydrazinecarbothioamide (5)

Yield 62%. mp 240 °C (EtOH) (lit. 240 °C).⁵⁵

General procedure for the synthesis of 1,3,4-thiadiazol-2-amines 6–10

Compounds **1–5** (1 mmol) were dissolved in ethanol and ethanolic ferric chloride solution (4 mmol) was added. The reaction mixtures were heated under reflux for 16–20 h. The mixtures were neutralized using ammonia solution, filtered and washed with water, dried, and crystallized from ethanol to obtain compounds **6–10**.

5-Cyclohexyl-1,3,4-thiadiazol-2-amine (6)

Yield 77%. mp 237 °C (EtOH) (lit. 238–240 °C).⁷⁵

5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-amine (7)

Yield 65%. mp 235 °C (EtOH) (lit. 240 °C).⁷⁶

5-(2-Chlorophenyl)-1,3,4-thiadiazol-2-amine (8)

Yield 58%. mp 192 °C (EtOH) (lit. 190–192 °C).⁵⁵

5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-amine (9)

Yield 68%. mp 225 °C (EtOH) (lit. 230 °C).⁷⁶

5-(2,4-Dichlorophenyl)-1,3,4-thiadiazol-2-amine (10)

Yield 45%. mp 232 °C (EtOH) (lit. 229 °C).⁵⁵

General procedure for the synthesis of 2-chloro-N-(heteroaryl/alkyl)acetamides 11–15

Compounds **6–10** (5 mmol) were dissolved in DCM and TEA (6 mmol) was added to the reaction mixtures. α -Chloroacetyl chloride (10 mmol) was slowly added to the reaction mixtures. The reaction mixtures were heated for 2 h under reflux. The reaction was checked with TLC. The crude products were filtered, dried, and crystallized from 1,4-dioxane to obtain products **11–15**.

2-Chloro-N-[5-(cyclohexyl)-1,3,4-thiadiazol-2-yl]acetamide (11)

Yield 64%. mp 218 °C.⁷⁷ TLC Rf: 0.58 (S₁). HPLC t_R (min): 6.1. IR (cm⁻¹): 3182 (N–H str), 1701 (amide C=O), 1566 (C=N str).

2-Chloro-N-[5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (12)

Yield 72%. mp 252 °C (lit. 252 °C).⁷⁶ TLC Rf: 0.68 (S₁). HPLC t_R (min): 5.8. IR (cm⁻¹): 3182 (N–H str), 1705 (amide C=O), 1567 (C=N str).

2-Chloro-N-[5-(2-chlorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (13)

Yield 61%. mp 215–217 °C (lit. 215–217 °C).⁵⁵ TLC Rf: 0.66 (S₁). HPLC t_R (min): 6.1. IR (cm⁻¹): 3192 (N–H str), 1709 (amide C=O), 1575 (C=N str).

2-Chloro-N-[5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (14)

Yield 72%. mp 251 °C (lit. 251 °C).⁷⁶ TLC Rf: 0.70 (S₁). HPLC t_R (min): 6.3. IR (cm⁻¹): 3180 (N–H str), 1705 (amide C=O), 1554 (C=N str).

2-Chloro-N-[5-(2,4-dichlorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (15)

Yield 28%. mp 248–250 °C (lit. 248–250 °C).⁵⁵ TLC Rf: 0.75 (S₁). HPLC t_R (min): 10.4. IR (cm⁻¹): 3174 (N–H str), 1708 (amide C=O), 1581 (C=N str).

General procedure for the synthesis of fluoroquinolone derivatives 16–25

Compounds **11–15** (1 mmol) and norfloxacin/ciprofloxacin (1.5 mmol) were dissolved in DMF. The reaction mixtures were stirred at room temperature in the presence of NaHCO₃ (1.5 mmol) for 24 h. The crude products were filtered, dried, and crystallized from appropriate solvent to obtain final products **16–25**.

1-Ethyl-6-fluoro-7-[4-(2-{[5-(cyclohexyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (16)

Yield 57%; mp 256 °C; TLC Rf: 0.54 (S₁); HPLC t_R (min): 6.3; IR (cm⁻¹): 3198 (O–H and N–H str), 1732 (c. acid C=O str), 1681 (amide C=O str), 1627 (ketone C=O str), 1510, 1477 (C=N str, N–H b); LC/MS ESI⁻ m/z (%): 541.30 ([M–H]⁻, 100); LC/MS ESI⁺ m/z (%): 581.07 ([M+K]⁺, 85), 565.14 ([M+Na]⁺, 100), 543.33 ([M+H]⁺, 37); ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.20–2.10 (m, 13H, 5 × CH₂ for cyclohexyl

and methyl), 2.51 (s, 2H, $-\text{COCH}_2-$), 2.76 (m, 4H, piperazine \mathbf{H}_3 , \mathbf{H}_5), 3.01–3.08 (m, 1H, cyclohexyl $-\text{CH}-$), 3.45 (m, 4H, piperazine \mathbf{H}_2 , \mathbf{H}_6), 4.59 (q, 2H, $-\text{CH}_2\text{CH}_3$), 7.19 (s, 1H, quinolone \mathbf{H}_8), 7.90 (d, 1H, $J = 13.2$ Hz, quinolone \mathbf{H}_5), 8.95 (s, 1H, quinolone \mathbf{H}_2), 15.36 (bs, 1H, $-\text{COOH}$); Elemental analysis, Calcd. for $\text{C}_{27}\text{H}_{32}\text{FN}_5\text{O}_4\text{S}\cdot\text{H}_2\text{O}$: C 55.70; H 5.93; N 14.99; S 5.72. Found: C 56.29; H 5.78; N 15.00; S 5.49.

1-Ethyl-6-fluoro-7-[4-(2-{[5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (17)

Yield 51%. mp 245 °C (dec.). TLC Rf: 0.75 (S_1). HPLC t_R (min): 6.2. IR (cm^{-1}): 3288 (O–H and N–H str), 1697 (c. acid C=O str, amide C=O str), 1625 (ketone C=O str), 1558, 1447 (C=N str, N–H b). LC/MS ESI[−] m/z (%): 553.24 ($[\text{M}-\text{H}]^-$, 100). LC/MS ESI⁺ m/z (%): 593.04 ($[\text{M}+\text{K}]^+$, 100 LC), 577.03 ($[\text{M}+\text{Na}]^+$, 28), 555.14 ($[\text{M}+\text{H}]^+$, 11). ¹H NMR δ ppm (300 MHz, DMSO- d_6): 1.43 (t, $J = 7.0$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 2.51 (s, 2H, $-\text{COCH}_2-$), 2.79 (m, 4H, piperazine \mathbf{H}_3 , \mathbf{H}_5), 3.51 (m, 4H, piperazine \mathbf{H}_2 , \mathbf{H}_6), 4.60 (q, 2H, $-\text{CH}_2\text{CH}_3$), 7.20 (d, $J = 7.2$ Hz, 1H, quinolone \mathbf{H}_8), 7.36–7.41 (m, 2H, Ar \mathbf{H}_3' , \mathbf{H}_5'), 7.89 (d, 1H, $J = 13.2$ Hz, quinolone \mathbf{H}_5), 7.99–8.04 (m, 2H, Ar \mathbf{H}_2' , \mathbf{H}_6'), 8.96 (s, 1H, quinolone \mathbf{H}_2), 15.39 (bs, 1H, $-\text{COOH}$). ¹³C NMR δ ppm (150 MHz, DMSO- d_6): 14.83 ($-\text{N}-\text{CH}_2\text{CH}_3$), 49.73 ($-\text{N}-\text{CH}_2\text{CH}_3$), 52.65 and 52.36 (piperazine \mathbf{C}_3 , \mathbf{C}_5), 52.50 and 52.56 (piperazine \mathbf{C}_2 , \mathbf{C}_6), 60.20 (1,3,4-thiadiazole–NH–CO– CH_2-), 106.44 (quinolone \mathbf{C}_8), 107.54 (quinolone \mathbf{C}_3), 111.66 (quinolone \mathbf{C}_5 , $J = 27.0$ Hz), 119.75 (quinolone \mathbf{C}_{4a} , $J = 7.5$ Hz), 116.94, 117.09, 127.26, 129.69, 129.85, and 164.64 (phenyl C), 137.67 (quinolone \mathbf{C}_{8a}), 145.94 (quinolone \mathbf{C}_7 , $J = 9.0$ Hz), 149.01 (quinolone \mathbf{C}_2), 153.38 (quinolone \mathbf{C}_6 , $J = 247.5$ Hz), 158.29 (1,3,4-thiadiazole \mathbf{C}_2), 162.15 (1,3,4-thiadiazole \mathbf{C}_5), 166.59 ($-\text{COOH}$), 169.24 (amide C=O), 176.63 (quinolone $\mathbf{C}_4 = \text{O}$). Elemental analysis, Calcd. for $\text{C}_{26}\text{H}_{24}\text{F}_2\text{N}_6\text{O}_4\text{S}\cdot 3/2\text{H}_2\text{O}$: C 55.12; H 4.51; N 14.83; S 5.66. Found: C 55.87; H 4.65; N 15.01; S 5.75.

1-Ethyl-6-fluoro-7-[4-(2-{[5-(2-chlorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (18)

Yield 62%. mp 272 °C (dec.). TLC Rf: 0.54 (S_1). HPLC t_R (min): 6.3. IR (cm^{-1}): 3452 (O–H str), 3182 (N–H str), 1728 (c. acid C=O str), 1701 (amide C=O str), 1624 (ketone C=O str), 1554, 1447 (C=N str, N–H b). LC/MS ESI[−] m/z (%): 569.11 ($[\text{M}-\text{H}]^-$, 100). LC/MS ESI⁺ m/z (%): 609.08 ($[\text{M}+\text{K}]^+$, 100), 593.07 ($[\text{M}+\text{Na}]^+$, 42), 571.02 ($[\text{M}+\text{H}]^+$, 9). ¹H NMR δ ppm (300 MHz, DMSO- d_6): 1.43 (t, $J = 7.2$ Hz, 3H, $-\text{CH}_2\text{CH}_3$), 2.52 (s, 2H, $-\text{COCH}_2-$), 2.80 (m, 4H, piperazine \mathbf{H}_3 , \mathbf{H}_5), 3.52 (m, 4H, piperazine \mathbf{H}_2 , \mathbf{H}_6), 4.60 (q, 2H, $-\text{CH}_2\text{CH}_3$), 7.20 (d, $J = 7.2$ Hz, 1H, quinolone \mathbf{H}_8), 7.51–7.61 (m, 2H, Ar \mathbf{H}_4' , \mathbf{H}_5'), 7.68–7.71 (m, 1H, Ar \mathbf{H}_6'), 7.93 (d, 1H, $J = 13.2$ Hz, quinolone \mathbf{H}_5), 8.10–8.14 (m, 1H, Ar \mathbf{H}_3'), 8.96 (s, 1H, quinolone \mathbf{H}_2), 15.38 (s, 1H, COOH). ¹³C NMR δ ppm (150 MHz, DMSO- d_6): 14.82 ($-\text{N}-\text{CH}_2\text{CH}_3$), 49.75 ($-\text{N}-\text{CH}_2\text{CH}_3$), 49.94 and 49.96 (piperazine \mathbf{C}_3 , \mathbf{C}_5), 52.48 (piperazine \mathbf{C}_2 , \mathbf{C}_6), 60.16 (1,3,4-thiadiazole–NH–CO– CH_2-), 106.39 (quinolone \mathbf{C}_8), 107.54 (quinolone \mathbf{C}_3), 111.65 (quinolone \mathbf{C}_5 , $J = 22.5$ Hz), 119.75 (quinolone \mathbf{C}_{4a} , $J = 7.5$ Hz), 128.38, 129.42, 131.08, 131.35, 131.53, and 132.33 (phenyl C), 137.68 (quinolone \mathbf{C}_{8a}), 145.95 (quinolone \mathbf{C}_7 , $J = 9.0$ Hz), 149.01 (quinolone \mathbf{C}_2), 153.37 (quinolone \mathbf{C}_6 , $J = 247.5$ Hz), 158.29 (1,3,4-thiadiazole \mathbf{C}_2), 160.21 (1,3,4-thiadiazole \mathbf{C}_5), 166.60 ($-\text{COOH}$), 169.41 (amide C=O), 176.64 (quinolone $\mathbf{C}_4 = \text{O}$). Elemental analysis, Calcd. for $\text{C}_{26}\text{H}_{24}\text{ClFN}_6\text{O}_4\text{S}\cdot\text{H}_2\text{O}$: C 53.01; H 4.45; N 14.27; S 5.44. Found: C 53.34; H 4.66; N 14.19; S 5.37.

1-Ethyl-6-fluoro-7-[4-(2-{[5-(4-chlorophenyl)-1,3,4-thiadiazole-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (19)

Yield 23%. mp 273 °C (dec.). TLC Rf: 0.53 (S₁). HPLC t_R (min): 6.5. IR (cm⁻¹): 3282 (O–H and N–H str), 1728 (c. acid C=O str), 1697 (amide C=O str), 1627 (ketone C=O str), 1498, 1475 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 569.22 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 609.10 ([M+K]⁺, 67), 593.09 ([M+Na]⁺, 100), 571.21 ([M+H]⁺, 11). ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.42 (t, J = 7.0 Hz, 3H, –CH₂CH₃), 2.51 (s, 2H, –COCH₂–), 2.79 (m, 4H, piperazine H₃, H₅), 3.51 (m, 4H, piperazine H₂, H₆), 4.60 (q, 2H, –CH₂CH₃), 7.20 (d, J = 7.2 Hz, 1H, quinolone H₈), 7.60 (d, 2H, J = 8.7 Hz, Ar H₃', H₅'), 7.91 (d, 1H, J = 13.5 Hz, quinolone H₅), 7.96 (d, 2H, J = 8.7 Hz, Ar H₂', H₆'), 8.95 (s, 1H, quinolone H₂), 15.35 (bs, 1H, –COOH). Elemental analysis, Calcd. for C₂₆H₂₄ClFN₆O₄S: C 54.69; H 4.24; N 14.72; S 5.62. Found: C 54.59; H 4.47; N 14.56; S 5.46.

1-Ethyl-6-fluoro-7-[4-(2-{[5-(2,4-dichlorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (20)

Yield 40%. mp 258 °C (dec.). TLC Rf: 0.55 (S₁). HPLC t_R (min): 6.8. IR (cm⁻¹): 3282 (O–H and N–H str), 1728 (c. acid C=O str), 1710 (amide C=O str), 1622 (ketone C=O str), 1552, 1469 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 603.07 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 626.97 ([M+Na]⁺, 100), 605.09 ([M+H]⁺, 40). ¹H NMR δ ppm (600 MHz, DMSO-d₆) δ ppm: 1.42 (t, J = 7.2 Hz, 3H, –CH₂CH₃), 2.50 (s, 2H, –COCH₂–, DMSO), 2.79 (m, 4H, piperazine H₃, H₅), 3.52 (m, 4H, piperazine H₂, H₆), 4.59 (q, 2H, –CH₂CH₃), 7.20 (d, J = 3.0 Hz, 1H, quinolone H₈), 7.63 (d, J = 4.2 Hz, 1H, Ar H₅'), 7.90 (s, 1H, Ar H₃'), 7.94 (d, J = 6.6 Hz, 1H, quinolone H₅), 8.14 (d, 1H, J = 4.8 Hz, Ar H₆'), 8.96 (s, 1H, quinolone H₂), 15.38 (bs, 1H, –COOH). Elemental analysis, Calcd. for C₂₆H₂₃Cl₂FN₆O₄S·3/2H₂O: C 49.37; H 4.14; N 13.29; S 5.07. Found: C 49.10; H 4.31; N 12.92; S 4.65.

1-Cyclopropyl-6-fluoro-7-[4-(2-{[5-(cyclohexyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (21)

Yield 59%. mp 255–258 °C (dec.). TLC Rf: 0.82 (S₁). HPLC t_R (min): 6.3. IR (cm⁻¹): 3452 (O–H and N–H str), 1722 (c. acid C=O str), 1701 (amide C=O str), 1627 (ketone C=O str), 1558, 1506 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 553.34 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 593.12 ([M+K]⁺, 100), 577.21 ([M+Na]⁺, 67), 555.18 ([M+H]⁺, 57). ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.13–2.10 (m, 14H, 5 × CH₂ for cyclohexyl, 2 × CH₂ for cyclopropyl), 2.51 (s, 2H, –COCH₂–), 2.90 (m, 4H, piperazine H₃, H₅), 3.05 (m, 1H, cyclohexyl –CH–), 3.45 (m, 4H, piperazine H₂, H₆), 3.83 (m, 1H, cyclopropyl –CH–), 7.58 (d, J = 7.2 Hz, 1H, quinolone H₈), 7.89 (d, J = 13.2 Hz, 1H, quinolone H₅), 8.66 (s, 1H, quinolone H₂). Elemental analysis, Calcd. for C₂₇H₃₁FN₆O₄S: C 58.47; H 5.63; N 15.15; S 5.78. Found: C 58.31; H 5.75; N 15.02; S 5.58.

1-Cyclopropyl-6-fluoro-7-[4-(2-{[5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (22)

Yield 41%. mp 245 °C (dec.). TLC 0.75 (S₁). HPLC t_R (min): 6.2. IR (cm⁻¹): 3288 (O–H and N–H str), 1722 (c. acid C=O str), 1701 (amide C=O str), 1627 (ketone C=O str), 1558, 1506 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 565.23 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 605.07 ([M+K]⁺, 100), 589.15 ([M+Na]⁺, 57), 567.22 ([M+H]⁺, 32). ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.19 (s, 2H, cyclopropyl –CH₂–), 1.32 (d, J = 6.2 Hz, 2H, cyclopropyl –CH₂–), 2.51 (s, 2H, –COCH₂–), 2.81 (m, 4H, piperazine H₃, H₅), 3.51 (m, 4H, piperazine H₂, H₆), 3.83 (m, 1H, cyclopropyl –CH–), 7.35–7.41 (m, 2H, Ar H₃', H₅'), 7.58 (d, J = 7.5 Hz, 1H, quinolone H₈), 7.90 (d, J = 13.2 Hz, 1H, quinolone H₅), 7.99–8.04 (m, 2H, Ar H₂'

H₆'), 8.86 (s, 1H, quinolone **H₂**). ¹³C NMR δ ppm (150 MHz, DMSO-d₆): 8.04 (–N–CH(CH₂)₂), 36.34 (–N–CH(CH₂)₂), 49.84 and 49.87 (piperazine **C₃**, **C₅**), 52.49 (piperazine **C₂**, **C₆**), 60.20 (1,3,4-thiadiazole–NH–CO–CH₂–), 106.91 (quinolone **C₈**), 107.20 (quinolone **C₃**), 111.42 (quinolone **C₅**, *J* = 24.0 Hz), 119.05 (quinolone **C_{4a}**, *J* = 7.5 Hz), 116.85, 117.00, 127.26, 129.71, 129.77, and 164.64 (phenyl **C**), 139.64 (quinolone **C_{8a}**), 145.65 (quinolone **C₇**, *J* = 10.5 Hz), 148.47 (quinolone **C₂**), 153.50 (quinolone **C₆**, *J* = 247.5 Hz), 158.58 (1,3,4-thiadiazole **C₂**), 162.99 (1,3,4-thiadiazole **C₅**), 166.41 (–COOH), 169.38 (amide C=O), 176.82 (quinolone **C₄** =O). Elemental analysis, Calcd. for C₂₇H₂₄F₂N₆O₄S.1/2H₂O: C 56.34; H 4.38; N 14.60; S 5.57. Found: C 56.37; H 4.29; N 14.44; S 5.17.

1-Cyclopropyl-6-fluoro-7-[4-(2-{[5-(2-chlorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (23)

Yield 32%. mp 265–275 °C (dec.). TLC Rf: 0.75 (S₁). HPLC t_R (min): 6.3. IR (cm⁻¹): 3190 (O–H and N–H str), 1712 (c. acid C=O str), 1693 (amide C=O str), 1624 (ketone C=O str), 1543, 1446 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 581.10 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 621.03 ([M+K]⁺, 57), 605.09 ([M+Na]⁺, 100), 583.13 ([M+H]⁺, 54). ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.19 (s, 2H, cyclopropyl –CH₂–), 1.32 (d, *J* = 6.0 Hz, 2H, cyclopropyl –CH₂–), 2.51 (s, 2H, –COCH₂–), 2.81 (m, 4H, piperazine **H₃**, **H₅**), 3.53 (m, 4H, piperazine **H₂**, **H₆**), 3.83 (m, 1H, cyclopropyl –CH–), 7.51–7.60 (m, 3H, Ar **H₄'**, **H₅'**, quinolone **H₈**), 7.68–7.71 (m, 1H, Ar **H₆'**), 7.90 (d, *J* = 13.2 Hz, 1H, quinolone **H₅**), 8.10–8.14 (m, 1H, Ar **H₃'**), 8.66 (s, 1H, quinolone **H₂**), 15.23 (bs, 1H, –COOH). ¹³C NMR δ ppm (150 MHz, DMSO-d₆): 8.03 (–N–CH(CH₂)₂), 36.33 (–N–CH(CH₂)₂), 49.86 and 49.89 (piperazine **C₃**, **C₅**), 52.46 (piperazine **C₂**, **C₆**), 60.14 (1,3,4-thiadiazole–NH–CO–CH₂–), 106.87 (quinolone **C₈**), 107.19 (quinolone **C₃**), 111.40 (quinolone **C₅**, *J* = 24.0 Hz), 119.05 (quinolone **C_{4a}**, *J* = 7.5 Hz), 129.37, 129.40, 131.08, 131.33, 131.53, and 132.33 (phenyl **C**), 139.64 (quinolone **C_{8a}**), 145.64 (quinolone **C₇**, *J* = 10.5 Hz), 148.44 (quinolone **C₂**), 153.49 (quinolone **C₆**, *J* = 247.5 Hz), 158.30 (1,3,4-thiadiazole **C₂**), 160.15 (1,3,4-thiadiazole **C₅**), 166.41 (–COOH), 169.38 (amide C=O), 176.82 (quinolone **C₄** =O). Elemental analysis, Calcd. for C₂₇H₂₄ClF₂N₆O₄S.3/2H₂O: C 53.95; H 4.36; N 13.98; S 5.33. Found: C 54.42; H 4.52; N 14.19; S 5.43.

1-Cyclopropyl-6-fluoro-7-[4-(2-{[5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (24)

Yield 43%. mp 272 °C (dec.) (lit. 330–333 °C). ⁶¹ TLC Rf: 0.74 (S₁). HPLC t_R (min): 6.6. IR (cm⁻¹): 3607 (O–H str), 3302 (N–H str), 1728 (c. acid C=O str), 1701 (amide C=O str), 1627 (ketone C=O str), 1554, 1447 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 581.10 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 621.07 ([M+K]⁺, 100), 605.13 ([M+Na]⁺, 47), 583.15 ([M+H]⁺, 43). ¹H NMR δ ppm (300 MHz, DMSO-d₆): 1.19 (s, 2H, cyclopropyl –CH₂–), 1.32 (d, *J* = 6.2 Hz, 2H, cyclopropyl –CH₂–), 2.51 (s, 2H, –COCH₂–), 2.81 (m, 4H, piperazine **H₃**, **H₅**), 3.52 (m, 4H, piperazine **H₂**, **H₆**), 3.83 (m, 1H, cyclopropyl –CH–), 7.57–7.59 (m, 3H, quinolone **H₈**, Ar **H₂'**, **H₆'**) 7.91 (d, *J* = 13.2 Hz, 1H, quinolone **H₅**), 7.96 (d, 2H, *J* = 8.7 Hz, Ar **H₃'**, **H₅'**), 8.67 (s, 1H, quinolone **H₂**), 15.24 (bs, 1H, –COOH). ¹³C NMR δ ppm (150 MHz, DMSO-d₆): 8.03 (–N–CH(CH₂)₂), 36.26 and 36.34 (–N–CH(CH₂)₂), 49.82 and 49.85 (piperazine **C₃**, **C₅**), 52.48 (piperazine **C₂**, **C₆**), 60.20 (1,3,4-thiadiazole–NH–CO–CH₂–), 106.90 (quinolone **C₈**), 107.18, (quinolone **C₃**), 111.43 (quinolone **C₅**, *J* = 22.5 Hz), 119.07 (quinolone **C_{4a}**, *J* = 7.5 Hz), 129.09, 129.46, 129.91, and 135.68 (phenyl **C**), 139.67 (quinolone **C_{8a}**), 145.66 (quinolone **C₇**, *J* = 10.5 Hz), 148.48 (quinolone **C₂**), 153.50 (quinolone **C₆**, *J* = 247.5 Hz), 158.79 (1,3,4-thiadiazole **C₃**), 162.82 (1,3,4-thiadiazole **C₅**), 166.47 (–COOH), 169.31

(amide C=O), 176.84 (quinolone C₄ =O). Elemental analysis, Calcd. for C₂₇H₂₄ClFN₆O₄S.1/2H₂O: C 54.77; H 4.26; N 14.19; S 5.42. Found: C 54.38; H 4.43; N 14.14; S 5.30.

1-Cyclopropyl-6-fluoro-7-[4-(2-{[5-(2,4-dichlorophenyl)-1,3,4-thiadiazol-2-yl]amino}-2-oxo-ethyl)piperazine-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (25)

Yield 46%. mp 252 °C (dec.). TLC Rf: 0.78 (S₁). HPLC t_R (min): 6.9. IR (cm⁻¹): 3288 (O–H and N–H str), 1730 (c. acid C=O str), 1701 (amide C=O str), 1627 (ketone C=O str), 1487 (C=N str, N–H b). LC/MS ESI⁻ m/z (%): 615.17 ([M–H]⁻, 100). LC/MS ESI⁺ m/z (%): 639.18 ([M+Na]⁺, 100), 617.09 ([M+H]⁺, 48). ¹H NMR δ ppm (600 MHz, DMSO-d₆): 1.19 (m, 2H, cyclopropyl –CH₂–), 1.32 (m, 2H, cyclopropyl –CH₂–), 2.50 (m, 2H, –COCH₂–, DMSO), 2.81 (m, 4H, piperazine H₃, H₅), 3.54 (m, 4H, piperazine H₂, H₆), 3.83 (m, 1H, cyclopropyl –CH–), 7.60-7.92 (m, 5H, quinolone H₅, quinolone H₈, Ar H₃', Ar H₅', Ar H₆'), 8.67 (s, 1H, quinolone H₂), 15.24 (s, 1H, –COOH). Elemental analysis, Calcd. for C₂₇H₂₃Cl₂FN₆O₄S.3/2H₂O: C 50.32; H 4.07; N 13.04; S 4.98. Found: C 50.43; H 4.09; N 13.00; S 5.20.

3.2. Biological studies

3.3. Antimicrobial activity

The antimicrobial activity of the compounds was tested against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *C. albicans* (ATCC 10231). The minimal inhibitory concentration (MIC) and minimum bactericidal or (fungicidal) concentration (MBC or MFC) for *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *C. albicans* (ATCC 10231) were determined by a microbroth dilution method depicted below.⁷⁸

For MIC determination, the compounds were dissolved in dimethyl sulfoxide and serial twofold dilutions were done in Luria-Bertani (LB) broth. Microorganisms were suspended in LB broth to match the turbidity of 0.5 McFarland (1.5 × 10⁸ cfu/mL) and 1/10 dilution was prepared from this suspension and used as inoculum. The tested final concentrations ranged between 512 and 0.5 μg/mL. To make sure that dimethyl sulfoxide did not show any inhibitory activity, controls prepared with serial dilutions of dimethyl sulfoxide were also tested. The tubes were incubated at 37 °C for 24 h and then examined for turbidity. MIC was determined if turbidity was observed in the positive control tube containing no compound and no turbidity in the negative control tube containing no microorganism.

After MIC determination, aliquots of 10 μL from all tubes in which no visible bacterial growth was observed were inoculated in agar plates for determination of MBC. The plates were then incubated overnight at 37 °C. MBC was identified as the lowest concentration of the compound that completely eliminated the growth of the microorganism. Ciprofloxacin, norfloxacin, and fluconazole were used as the positive sensitivity reference standard for bacteria and yeast.

The antimicrobial activity study was carried out at the Department of Medical Microbiology, School of Medicine, Acıbadem University, İstanbul, Turkey.

3.3.1. Antituberculosis activity

Antimycobacterial activity of the synthesized compounds was tested against *M. tuberculosis* H₃₇RV strain. For the MIC determination, the compounds were dissolved in dimethyl sulfoxide and serial twofold dilutions were done in Middlebrook 7H9 Broth containing glycerol. Microorganisms were suspended in Middlebrook 7H9 Broth to match the turbidity of 0.5 McFarland (1.5 × 10⁸ cfu/mL) and 1/10 dilution was prepared from this suspension and used as inoculum. The tested final concentrations ranged between 512 and 0.5 μg/mL. To

make sure that dimethyl sulfoxide did not show any inhibitory activity, controls prepared with serial dilutions of dimethyl sulfoxide were also tested. The tubes were incubated at 37 °C for 24 h and then examined for turbidity. MIC was determined if turbidity was observed in the positive control tube containing no compound and no turbidity in the negative control tube containing no microorganism.^{79–81} Isoniazid and rifampicine were used as the positive sensitivity reference standard for mycobacteria.

The antimycobacterial activity study was performed at the Department of Medical Microbiology, School of Medicine, Acıbadem University, İstanbul, Turkey.

3.3.2. Anticancer activity

A549, MRC5, PC3, PNT1, SK MEL 1, HACAT, and HEK 293 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSA (Invitrogen, Gibco, UK). After sufficient confluence was achieved (about ~70–80), cells were trypsinized using 0.25% (v/v) trypsin/EDTA (Invitrogen, Gibco, UK) and seeded on a T-75 flask (Zelkultur Flaschen, Switzerland). The cells were maintained at 37 °C and 5% CO₂ in a humidified incubator.

Next 50 μM, 100 μM, 150 μM, 200 μM, and 250 μM concentrations of compounds and references were prepared in DMEM. Cell lines were seeded at a concentration of 5000 cells/well onto 96-well plates (BIOFIL; TCP, Switzerland). The following day, cells were treated with different concentrations of synthesized compounds and references besides 20% (v/v) DMSO as lethal dose (positive control). Cell viability was measured by the MTS assay (CellTiter96 Aqueous One Solution, Promega, UK) according to the manufacturer's instructions. After incubating the cells in the presence of pluronics for 24, 48, and 72 h, 10 μL of MTS reagent was added to the growth medium followed by further incubation for 2 h. Thereafter, the absorbance at 490 nm was measured by an ELISA plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

The anticancer activity studies were carried out at the Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Yeditepe University, İstanbul, Turkey.

3.4. Docking studies

Since DNA gyrase enzyme was selected as target, *M. tuberculosis* DNA gyrase in complex with ciprofloxacin (PDB ID: 5BTC) and *S. aureus* DNA gyrase in complex with ciprofloxacin (PDB ID: 2XCT) were obtained from the Protein Data Bank.

Biovia Discovery Studio Visualizer and MGLTools software were used to prepare data before docking. DNA, Mg²⁺, and conserved water molecules within the receptor were kept during calculations. Gasteiger charges were assigned to the ligands and the receptors. AutoDock Vina docking software was used to calculate binding affinities (kcal/mol) of each compound.

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