European Journal of Medicinal Chemistry 68 (2013) 185–191

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Mannich bases of 7-piperazinylquinolones and kojic acid derivatives: Synthesis, *in vitro* antibacterial activity and *in silico* study

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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
15 July 2013
Accepted 18 July 2013
Available online 11 August 2013

Keywords:
Antibacterial activity
Quinolones
Kojic acid
Mannich bases
Docking study

ABSTRACT

Novel Mannich bases of 7-piperazinylquinolones with kojic acid and chlorokojic acid were designed as new quinolone antibacterials. All compounds showed significant *in vitro* antibacterial activity against both Gram-positive and Gram-negative bacteria. Particularly, chlorokojic derivative **2b** was the most potent compound against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC values $\leq$0.19 μg/mL). Its activity was 4–8 times more than that of standard drug norfloxacin. The molecular docking study of compound **2b** further supported the molecular basis of the designed compounds.

1. Introduction

Quinolones have emerged as a major class of chemotherapeutic agents widely used to treat Gram-negative and Gram-positive bacterial infections in both community and hospital settings [1]. Most of them currently in the market or under development are generally characterized by a broad spectrum of antibacterial activity, improved potency and excellent oral bioavailability [2]. However, their activity against clinically important Gram-positive cocci including Staphylococci, Streptococci and Enterococci is relatively moderate [3]. Moreover, the prevalence of newly emerging virulence traits and drug resistance of the pathogens toward the quinolones has become a serious problem [4]. Thus, the design and development of new agents are of critical importance in the field of antimicrobial chemotherapy.

Quinolone antibacterial agents act by inhibiting two type II bacterial topoisomerase enzymes, DNA gyrase and topoisomerase IV [5]. Type II bacterial topoisomerase enzymes control the topology and conformation of DNA during replication and transcription processes [6]. DNA gyrase is responsible for introducing negative supercoils into DNA in an ATP-dependent manner, while topoisomerase IV provides a potent decatenating activity and contributing to replication fork progression by relaxing positive supercoils [7,8]. The inhibition of these essential enzymes by quinolones results in the disruption of DNA synthesis and, subsequently cell death.

Structurally, the β-keto carboxylic acid part in the 1-alkyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acid skeleton of quinolones is essential for hydrogen bonding interaction with DNA bases in the single stranded region of double helix of DNA created by the action of the topoisomerase II enzymes [9]. It has been proposed that the basic group at the C-7 position of quinolones is might interact with the target enzyme [10–12]. In addition, structure–activity relationship studies of quinolones have shown that the cell permeability is dominantly controlled by C-7 side chains. Therefore, the

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http://dx.doi.org/10.1016/j.ejmech.2013.07.032

type of substituents at the C-7 position has determinative effects on their nature including antibacterial spectrum, potency, safety, and pharmacokinetics properties [3]. In general, 5- and 6-membered cyclic amines connected to the quinolone cycle by a C–N bond have been proven to be the optimal substituents at the C-7 position [13]. Numerous clinically important quinolones exemplified by norfloxacin, ciprofloxacin, enoxacin, lomefloxacin and gatifloxacin have piperazine substituent at C-7 position (Fig. 1). The previous studies revealed that the C-7 position of quinolones is capable to attach to the special bulky substituents [14–20].

Kojic acid is an antibiotic produced by some species of *Aspergillus* and *Penicillium* [21] and shows an inhibitory effect on the growth of *Escherichia coli* and *Staphylococcus aureus* [22,23]. Also, Wolf and Westveer have reported that chlorokojic acid (2-chloromethyl-5-hydroxy-4*H*-pyran-4-one) have antimicrobial activity [24]. Recently, Mannich bases of substituted piperazine and chlorokojic acid (Fig. 2) have been described as antimicrobial agents with significant activity against Gram-positive and Gram-negative bacteria [25,26].

In our effort to find new *N*-substituted piperazinylquinolones, we designed Mannich bases of 7-piperazinylquinolones with kojic acid and chlorokojic acid (Fig. 2). Thus we report here, synthesis, antibacterial activity evaluation and docking study of 7-[4-((3-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl)piperazin-1-yl]quinolone derivatives **2a**–**h**.

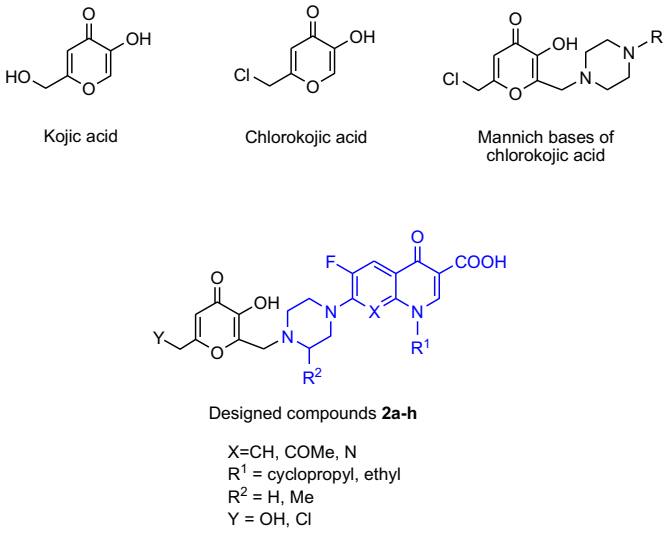


Fig. 2. Structures of kojic acid, chlorokojic acid and its Mannich bases with some antimicrobial activity, and designed Mannich bases of 7-piperazinylquinolones with kojic acid and chlorokojic acid as new antibacterial agents.

2. Chemistry

The title compounds **2a**–**h** were synthesized starting from 7-piperazinylquinolones **1a**–**e** and kojic acid derivatives as outlined in Scheme 1. Kojic acid was converted to chlorokojic acid by using thionyl chloride as reported in literature [27]. The Mannich bases **2a**–**h** (Table 1) were prepared by the reaction of appropriate 7-piperazinylquinolone with kojic acid or chlorokojic acid and formalin in methanol at room temperature. The structures of target compounds were confirmed by IR and NMR spectral data. For example, the ^{1}H NMR of compound **2a** showed a singlet at 8.65 ppm which assigned for H-2 proton of quinolone ring. The H-5 and H-8 protons of quinolone ring displayed two doublets at 7.88 and 7.55 ppm with coupling constants of 13.6 and 7.6 Hz, respectively. The spin–spin splitting of H-5 and H-8 protons were due to the presence of fluorine atom at C-6. The proton of pyran ring at C-5 was displayed at 6.34 ppm as a singlet peak. The broad singlet signal at 9.08 ppm was related to the hydroxyl group at C-3 of pyran ring. The alcoholic hydroxyl group was appeared at 5.70 ppm as a triplet due to the neighboring with methylene group. A doublet signal at 4.32 ppm was assigned for methylene connected to the C-6 of pyran ring and hydroxyl group. The resonance of C-1 proton of cyclopropyl was occurred at 3.79–3.82 ppm. Remaining protons related to the methylene groups of piperazine and cyclopropyl rings were appeared at the upfield of 3.34, 2.69 and 1.15–1.34 ppm, respectively.

3. Biology

The antibacterial activities of synthesized Mannich bases **2a**–**h** were evaluated by determination of their minimum inhibitory concentrations (MICs) by using agar dilution method [28,29]. A panel of Gram-positive (*S. aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633) and Gram-negative (*E. coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumonia* ATCC 10031) bacteria was used. The quinolone antibacterial agent norfloxacin was used as reference drug.

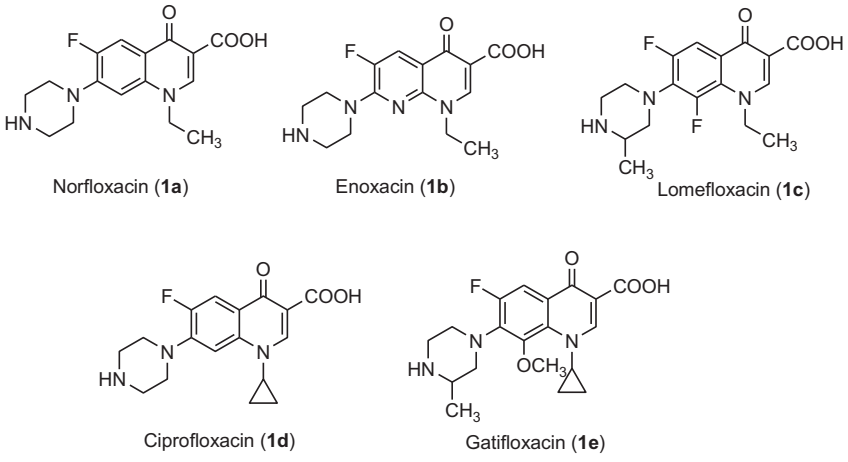
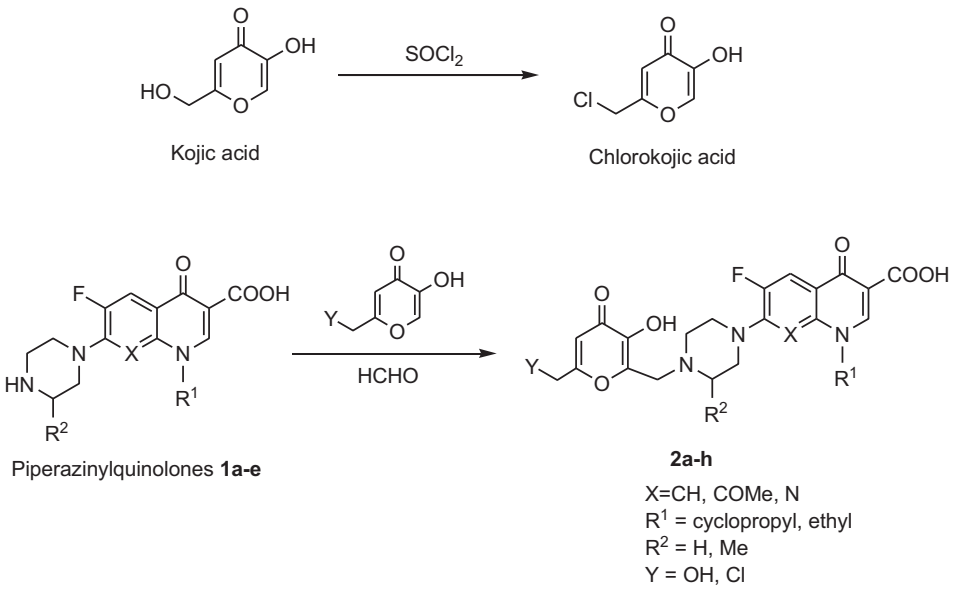


Fig. 1. Structures of some 7-piperazinylquinolone antibacterial drugs.



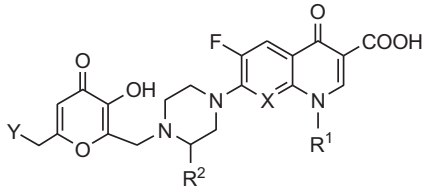
Scheme 1. Synthesis of designed compounds **2a**–**h**.

4. Docking study

Topoisomerase II DNA–gyrase atomic coordinates in complex with its bound inhibitor ciprofloxacin (PDB code: 2XCT) was retrieved from Brookhaven Protein Data Bank. To set the initial coordinates for the docking simulation, all water molecules, DNA fragments and three subunits of enzyme were removed and excluded from all calculations. The remaining subunit with its Mn^{2+} cofactor was considered for the following calculations. Ligand structure was sketched in HyperChem and optimized using semi-empirical AM1 method in Gaussian98. The resulting minimized conformation was then submitted to molecular docking simulation.

The lysine and arginine residues located in the binding site were protonated and the side chain of acidic amino acids, aspartate and glutamate, considered as carboxylate ion prior to any docking calculations. Gasteiger charges were assigned to the ligand and whole protein atoms. The binding site radius of the enzyme was set at any residues 12 Å distant from the X-ray crystallographic bound inhibitor (ciprofloxacin). LeadIT 2.0.2 (BiosolveIT GmbH, Sankt Augustin, Germany) was used for docking calculations. This program considers ligand flexibility by changing the conformation of the ligand in the active site while making the protein rigid. After docking calculation, the best pose with the highest rank was considered for visualization and further evaluation of the interactions. 2D map of the ligand-active site complex was made by LigPlot 1.4.4 [30].

Table 1
Chemical structures of synthesized compounds **2a**–**h**.



Compound	X	Y	R^1	R^2	MW	Yield (%)
2a	CH	OH	Cyclopropyl	H	485.46	83
2b	CH	Cl	Cyclopropyl	H	503.91	77
2c	COMe	OH	Cyclopropyl	CH_3	529.51	76
2d	COMe	Cl	Cyclopropyl	CH_3	547.96	82
2e	CH	OH	Ethyl	H	473.45	78
2f	CH	Cl	Ethyl	H	491.90	20
2g	CF	OH	Ethyl	CH_3	505.47	44
2h	N	OH	Ethyl	H	474.44	90

5. Results and discussion

5.1. Antibacterial activity

The MIC values of compounds **2a**–**h** in comparison with norfloxacin were presented in Table 2. At a glance, all compounds showed significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Compounds **2b**, **2c** and **2d** with MIC values ≤0.78 μg/mL exhibited potent inhibitory activity against *S. aureus*. Compound **2b** was the most potent compound against this strain (MIC = 0.097 μg/mL). Its activity was 4-fold more than that of standard drug norfloxacin. Moreover, compound **2b** showed the highest growth inhibitory activity against *S. epidermidis*, and *B. subtilis*, being as potent as norfloxacin. The MIC values of test compounds against *E. coli* were in the range of 0.39–1.56 μg/mL. The susceptibility of *E. coli* toward compounds **2a**–**h** was less than norfloxacin. In the case of *P. aeruginosa*, compound **2b** with MIC value of 0.19 μg/mL was the most potent compounds, being 8 times more potent than norfloxacin. In addition, the activity of compounds **2a**, **2c**, **2d** and **2f** against *P. aeruginosa* were comparable or superior to that of norfloxacin. The MIC values of test compounds against *K. pneumonia* revealed that compounds **2a**, **2c**–**g** showed relatively same inhibitory activity while compound **2h** was less active respect to the rest of compounds. The activity of the most potent compound **2b** (MIC = 0.19 μg/mL) was comparable to that of norfloxacin against the latter Gram-negative microorganism.

The comparison of the MIC values of kojic acid and chlorokojic acid derivatives against different microorganisms revealed that in the most cases chlorokojic acid derivatives were more active than corresponding kojic acid analogues. The naphthyridine derivative **2h** was less potent than its corresponding quinolone congener **2e**.

Table 2
The minimum inhibitory concentrations (MICs, μg/mL) of compounds **2a**–**h** against different bacteria.

Compound	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Bacillus subtilis*	*E. coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*
2a	1.56	3.125	3.125	0.78	1.56	1.56
2b	0.097	0.39	0.39	0.78	0.195	0.195
2c	0.78	3.125	3.125	0.39	0.78	1.56
2d	0.39	1.56	1.56	1.56	0.78	1.56
2e	3.125	6.25	0.78	0.78	3.125	1.56
2f	1.56	1.56	1.56	0.39	1.56	0.78
2g	3.125	6.25	3.125	0.39	3.125	1.56
2h	6.25	12.5	12.5	1.56	12.5	12.5
Norfloxacin	0.39	0.39	0.195	0.195	1.56	0.39

Thus, replacement of CH at 8-position with N decreased the antibacterial activity against both Gram-positive and Gram-negative bacteria. By comparing the antibacterial activity of cyclopropyl derivative **2b** and related N1-ethyl compound **2f**, it is revealed that the cyclopropyl is more favorable group at N1-position. The introduction of the methoxy group at the C-8 position of quinolone and methyl at the piperazine ring could not improve the antibacterial activity (**2c** *vs.* **2a** or **2d** *vs.* **2b**). Similarly, the comparison of compounds **2e** and **2g** demonstrated that substitution of fluorine at C-8 and methyl at the 3-position of piperazine ring had no positive effect on activity.

5.2. Docking simulation

In order to evaluate the accuracy of our docking protocol, ciprofloxacin was docked into the topoisomerase II DNA–gyrase active site. The protein-inhibitor complex obtained from docking simulation showed that the docked ciprofloxacin was almost superimposed on the native co-crystallized one with RMSD being 0.862. The hydrogen bonds and interactions between the docked ciprofloxacin and the amino acids were the same as those between the native X-ray ciprofloxacin and the amino acids (Fig. 3A). The docked ciprofloxacin forms coordination bond with Mn^{2+} ion by the two oxygen atoms of carbonyl and carboxylate groups with 1.4 Å and 2 Å distance respectively while for the co-crystallized ciprofloxacin, 2.1 Å distance exists between the two oxygen atoms of carbonyl and carboxylate groups and the Mn^{2+} ion. Carboxylate group of the docked ciprofloxacin is hydrogen bonded to the hydroxyl group of Ser1084 and the nitrogen atom of piperazine ring forms hydrogen bonding with the side chain carboxylate group of Glu477.

Compound **2b** which is the most active compound exhibits very similar mode of binding compared to the both co-crystallized and docked ciprofloxacin. The carboxylate group of compound **2b** is hydrogen bonded to hydroxyl group of Ser1084 and NH_2 group of Ser1085. The 3-hydroxy group of pyrane-4-one ring makes hydrogen bond with side chain NH_2 group of Asn475. In addition, the carbonyl oxygen atom of pyran-4-one is hydrogen bonded to the amide nitrogen atom of Glu477. The carbonyl oxygen atom at the 4-position of quinolone is coordinated to the Mn^{2+} with 1.6 Å distance (Fig. 3B). The interactions and distances can be better visually inspected by the 2D map of compound **2b** in the binding site of topoisomerase II DNA gyrase in Fig. 4. Overall, compound **2b** can be docked in the binding site of enzyme in a similar manner to ciprofloxacin with additional hydrogen bonds related to the 3-hydroxy-pyran-4-one scaffold, supporting the molecular design of compound **2b** prototype.

6. Conclusion

We designed Mannich bases of 7-piperazinylquinolones with kojic acid and chlorokojic acid as new quinolone antibacterials. All conjugate compounds showed significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Particularly, compound **2b** with MIC value of 0.097 μg/mL was the most potent compound against *S. aureus*. Its activity was 4-fold more

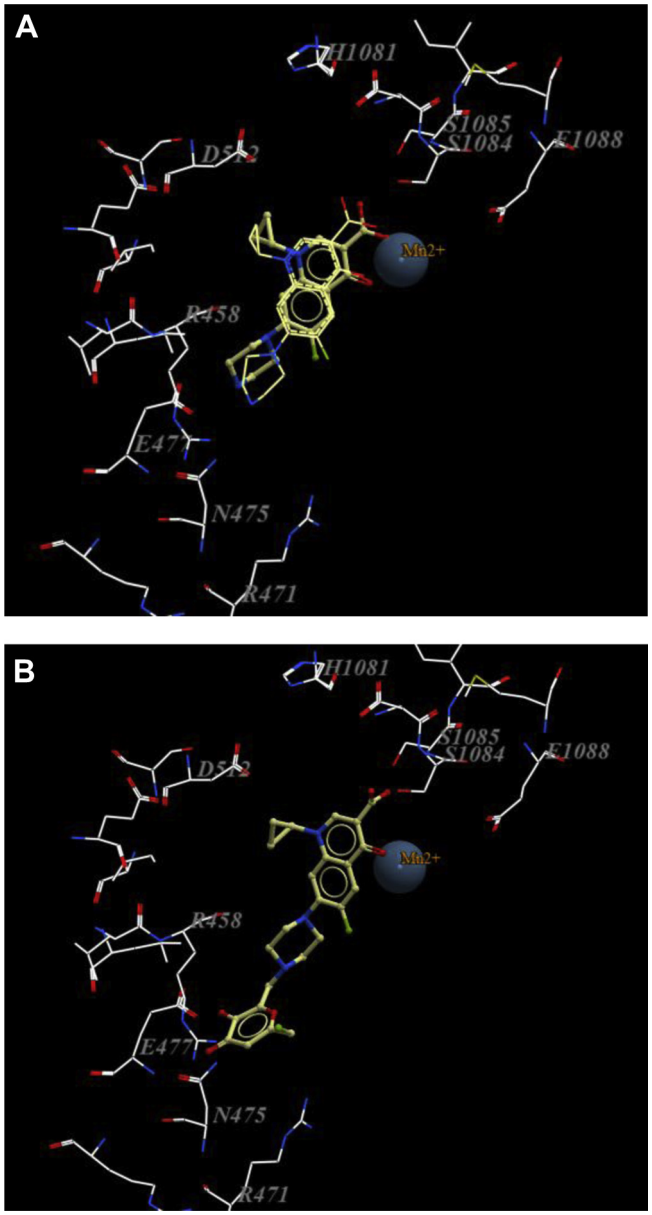


Fig. 3. (A) Docked conformation of ciprofloxacin (ball and stick) and the co-crystallized one (wire) in the active site of topoisomerase II DNA–gyrase; (B) Binding mode of compound **2b** in the binding site of topoisomerase II DNA–gyrase. For clarity only amino acids within 8 Å distant from the docked ligand are shown.

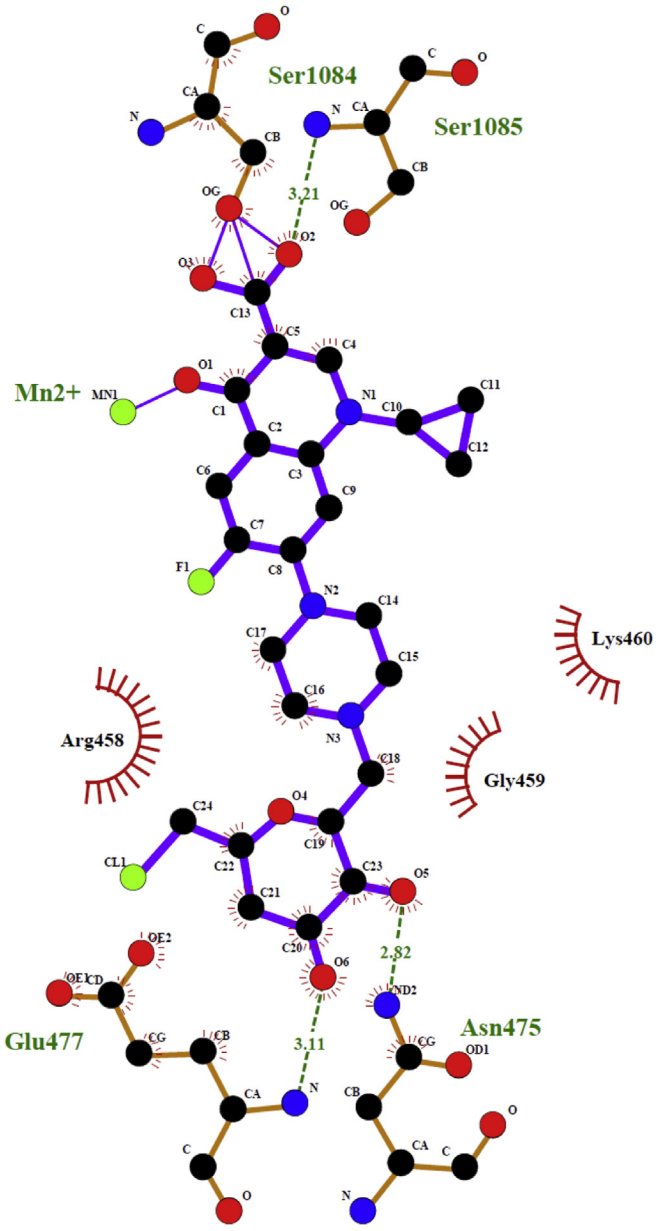


Fig. 4. 2D map of compound **2b** in the binding site of topoisomerase II DNA–gyrase. Distances are in angstrom.

than that of standard drug norfloxacin. Moreover, compound **2b** showed potent activity against *P. aeruginosa* as important Gram-negative bacteria (MIC = 0.19 μg/mL). It was 8 times more potent than norfloxacin. The molecular docking study of compound **2b** further supported the molecular basis of our design by introducing 3-hydroxy-pyran-4-one scaffold into the7-pi perazinylquinolones.

7. Experimental protocols

7.1. Chemistry

7.1.1. General methods

All starting materials, reagents and solvents were purchased from Merck Company. Chlorokojic acid was prepared from kojic acid according to the literature method [27]. The progress of reactions was checked by thin-layer chromatography (TLC) using precoated silica gel 60 F254 plastic sheets. The UV lamp (254 nm) was used for TLC visualization and detection of spots. Melting points were determined in open glass capillaries using Bibby Stuart Scientific SMP3 apparatus (Stuart Scientific, Stone, UK) and are uncorrected. The IR spectra were recorded on a PerkinElmer FT-IR spectrophotometer using KBr disks. The NMR spectra were recorded using Bruker 400 or 500 spectrometers and chemical shifts are expressed as δ (ppm) with tetramethylsilane (TMS) as internal standard.

7.1.2. General procedure for the synthesis of Mannich bases **2a–h**

To a mixture of 7-piperazinylquinolone **1a–e** (0.5 mmol) in methanol (4 mL), 37% formalin (0.05 mL) and kojic acid or chlorokojic acid (0.5 mmol) were added, respectively. The mixture was stirred at room temperature for 48–72 h. The resulting precipitated solid was collected by filtration and washed with cold methanol. The product was recrystallized from methanol to give pure compounds **2a–h**.

*7.1.2.1. 1-cyclopropyl-6-fluoro-1,4-dihydro-7-[4-((3-hydroxy-6-hydroxymethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2a**).* Yield 83%; mp 206–207 °C; IR (KBr, cm^{-1}) ν_{max}: 3372 (O–H), 1724 (C=O), 1630 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15–1.34 (m, 4H, cyclopropyl), 2.69 (br s, 4H, piperazine), 3.34 (br s, 4H, piperazine), 3.62 (s, 2H, CH_2N), 3.79–3.82 (m, 1H, cyclopropyl), 4.32 (d, 2H, J = 5.6 Hz, $\underline{CH_2}$-OH), 5.70 (t, 1H, J = 6.0 Hz, CH_2-$\underline{OH}$), 6.34 (s, 1H, H_5–pyran), 7.55 (d, 1H, $J_{H,F}$ = 7.6 Hz, H_8–quinolone), 7.88 (d, 1H, $J_{H,F}$ = 13.6 Hz, H_5–quinolone), 8.65 (s, 1H, H_2–quinolone), 9.08 (br s, 1H, 3-OH pyran). Anal. Calcd for $C_{24}H_{24}FN_3O_7$: C, 59.38; H, 4.98; N, 8.66. Found: C, 59.22; H, 5.05; N, 8.81.

*7.1.2.2. 1-cyclopropyl-6-fluoro-1,4-dihydro-7-[4-((3-hydroxy-6-chloromethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2b**).* Yield 77%; mp 209–210 °C; IR (KBr, cm^{-1}) ν_{max}: 3421 (O–H), 1730 (C=O), 1650 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15–1.34 (m, 4H, cyclopropyl), 2.71 (br s, 4H, piperazine), 3.34 (br s, 4H, piperazine), 3.68 (s, 2H, CH_2N), 3.80–3.83 (m, 1H, cyclopropyl), 4.68 (s, 2H, CH_2Cl), 6.58 (s, 1H, H_5–pyran), 7.56 (d, 1H, $J_{H,F}$ = 7.6 Hz, H_8–quinolone), 7.89 (d, 1H, $J_{H,F}$ = 13.2 Hz, H_5–quinolone), 8.66 (s, 1H, H_2–quinolone), 9.34 (br s, 1H, 3-OH pyran). Anal. Calcd for $C_{24}H_{23}ClFN_3O_6$: C, 57.20; H, 4.60; N, 8.34. Found: C, 57.14; H, 4.42; N, 8.55.

*7.1.2.3. 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[3-methyl-4-((3-hydroxy-6-hydroxymethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2c**).* Yield 76%; mp 190–191 °C; IR (KBr, cm^{-1}) ν_{max}: 3421 (O–H), 1732 (C=O), 1623 (C=O); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.9–1.12 (m, 4H, cyclopropyl), 1.12–1.3 (m, 3H, CH_3–piperazine), 2.5–2.64 (m, 2H, piperazine), 2.85–2.90 (m, 1H, piperazine), 2.92–3.03 (m, 1H, piperazine), 3.66–3.69 (m, 1H, piperazine), 3.72 (s, 3H, OCH_3), 3.80–3.86 (m, 1H, piperazine), 4.15 (br s, 1H, cyclopropyl), 4.31 (br s, 2H, $\underline{CH_2}$OH), 5.70 (br s, 1H, $CH_2\underline{OH}$), 6.33 (s, 1H, H_5–pyran), 7.2 (d, $J_{H,F}$ = 11.75 Hz, H_5–quinolone), 8.68 (s, 1H, H_2–quinolone), 9.09 (br s, 1H, 3-OH pyran), 14.93 (br s, 1H, COOH). Anal. Calcd for $C_{26}H_{28}FN_3O_8$: C, 58.97; H, 5.33; N, 7.94. Found: C, 59.11; H, 5.28; N, 8.26.

*7.1.2.4. 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[3-methyl-4-((3-hydroxy-6-chloromethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2d**).* Yield 82%; mp

186–187 °C; IR (KBr, cm^{-1}) ν_{max}: 3424 (O–H), 1723 (C=O), 1622 (C=O); ^1H NMR (500 MHz, DMSO-d_6), δ 0.96–1.14 (m, 4H, cyclopropyl), 1.17 (d, 3H, J = 6.1 Hz, CH_3–piperazine), 2.60–2.70 (m, 2H, piperazine), 2.89 (d, 1H, J = 11.4 Hz, piperazine), 2.98 (t, 1H, piperazine), 3.16 (br s, 1H, piperazine), 3.77 (d, J = 14.7 Hz, 1H, piperazine), 3.87 (d, J = 14.6 Hz, 1H, piperazine), 3.72 (s, 3H, OCH_3), 4.15 (m, 1H, cyclopropyl), 4.68 (s, 2H, CH_2Cl), 6.57 (s, 1H, H_5–pyran), 7.72 (d, 1H, $J_{H,F}$ = 11.95 Hz, H_5–quinolone), 8.68 (s, 1H, H_2–quinolone), 9.3 (br s, 1H, 3-OH pyran), 14.96 (s, 1H, COOH). Anal. Calcd for $C_{26}H_{27}ClFN_3O_7$: C, 56.99; H, 4.97; N, 7.67. Found: C, 56.70; H, 5.17; N, 7.66.

*7.1.2.5. 1-ethyl-6-fluoro-1,4-dihydro-7-[4-((3-hydroxy-6-hydroxymethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2e**).* Yield 78%; mp 232–234 °C; IR (KBr, cm^{-1}) ν_{max}: 3430 (O–H), 1722 (C=O), 1630 (C=O); ^1H NMR (500 MHz, DMSO-d_6), δ 1.40 (m, 3H, CH_3), 2.67 (m, 4H, piperazine), 3.62 (s, 2H, CH_2N), 4.31 (m, 2H, $\underline{CH_2}OH$), 4.55–4.65 (m, 2H, $\underline{CH_2}CH_3$), 5.69 (br s, 1H, $CH_2\underline{OH}$), 6.34 (s, 1H, H_5–pyran), 7.16 (d, 1H, J = 5.85 Hz, H_8–quinolone), 7.90 (d, $J_{H,F}$ = 13.0 Hz, H_5–quinolone), 8.95 (s, 1H, H_2–quinolone), 9.07 (br s, 1H, 3-OH pyran), 15.35 (s, 1H, COOH). ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.20, 49.02, 49.39, 52.05, 53.37, 59.56, 105.89, 107.01, 108.93, 111.11 (d, $J_{C,F}$ = 22.7 Hz), 137.12, 143.75, 146.08, 146.26, 148.48, 152.9 (d, $J_{C,F}$ = 241 Hz), 166.08, 167.62, 173.57, 176.11. Anal. Calcd for $C_{23}H_{24}FN_3O_7$: C, 58.35; H, 5.11; N, 8.88. Found: C, 58.37; H, 5.02; N, 8.49.

*7.1.2.6. 1-ethyl-6-fluoro-1,4-dihydro-7-[4-((3-hydroxy-6-chloromethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2f**).* Yield 20%; mp 208–209 °C; IR (KBr, cm^{-1}) ν_{max}: 3430 (O–H), 1738 (C=O), 1652 (C=O), 1626 (C=O); ^1H NMR (500 MHz, DMSO-d_6), δ 1.41 (m, 3H, CH_3), 2.7 (m, 4H, piperazine), 3.67 (s, 2H, CH_2N), 4.58 (q, 2H, J = 6.3 Hz, $\underline{CH_2}CH_3$), 4.68 (s, 2H, CH_2Cl), 6.58 (s, 1H, H_5–pyran), 7.17 (d, 1H, J = 6.0 Hz, H_8–quinolone), 7.91 (d, 1H, $J_{H,F}$ = 13.1 Hz, H_5–quinolone), 8.95 (s, 1H, H_2–quinolone), 9.33 (br s, 1H, 3-OH pyran), 15.35 (s, 1H, COOH). Anal. Calcd for $C_{23}H_{23}ClFN_3O_6$: C, 56.16; H, 4.71; N, 8.54. Found: C, 56.42; H, 4.85; N, 8.43.

*7.1.2.7. (RS)-1-ethyl-6,8-difluoro-1,4-dihydro-7-[3-methyl-4-((3-hydroxy-6-hydroxymethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxoquinoline-3-carboxylic acid (**2g**).* Yield 44%; mp 191–192 °C; IR (KBr, cm^{-1}) ν_{max}: 3309 (O–H), 1726 (C=O), 1624 (C=O); ^1H NMR (500 MHz, DMSO-d_6), δ 1.14 (d, 3H, CH_3-piperazine), 1.42 (m, 3H, $\underline{CH_3}CH_2$), 2.59 (m, 3H, piperazine), 2.89 (m, 1H, piperazine), 3.00 (m, 1H, piperazine), 3.68 (d, J = 14.2 Hz, 1H, piperazine), 3.80 (d, J = 14.25, 1H, piperazine), 4.31 (br s, 2H, $\underline{CH_2}OH$), 4.5 (br s, 2H, $\underline{CH_2}CH_3$), 5.7 (br s, 1H, $CH_2\underline{OH}$), 6.33 (s, 1H, H_5–pyran), 7.82 (d, 1H, J = 11.3 Hz, H_5–quinolone), 8.91 (s, 1H, H_2–quinolone), 14.7 (br s, 1H, COOH). Anal. Calcd for $C_{24}H_{25}F_2N_3O_7$: C, 57.03; H, 4.99; N, 8.31. Found: C, 57.01; H, 5.16; N, 8.23.

*7.1.2.8. 1-ethyl-6-fluoro-1,4-dihydro-7-[4-((3-hydroxy-6-hydroxymethyl-4-oxo-4H-pyran-2-yl)methyl)piperazin-1-yl]-4-oxo-1,8-naphthyridine-3-carboxylic acid (**2h**).* Yield 90%; mp 232–234 °C; IR (KBr, cm^{-1}) ν_{max}: 3261 (O–H), 1711 (C=O), 1634 (C=O); ^1H NMR (400 MHz, DMSO-d_6), δ 1.37 (t, 3H, J = 7.0 Hz, $\underline{CH_3}CH_2$), 2.63 (t, 4H, J = 4.4 Hz, piperazine), 3.59 (s, 2H, CH_2N), 3.81 (t, 4H, J = 4.4 Hz, piperazine), 4.31 (d, 2H, J = 6 Hz, $\underline{CH_2}OH$), 4.47 (q, 2H, J = 6.8 Hz, $\underline{CH_2}CH_3$), 5.69 (t, 1H, J = 6.0 Hz, $CH_2\underline{OH}$), 6.33 (s, 1H, H_5–pyran), 8.06 (d, 1H, $J_{H,F}$ = 13.6 Hz, H_5–quinolone), 8.96 (s, 1H, H_2–quinolone), 9.07 (br s, 1H, 3-OH pyran). Anal. Calcd for $C_{22}H_{23}FN_4O_7$: C, 55.69; H, 4.89; N, 11.81. Found: C, 55.44; H, 5.02; N, 11.69.

7.2. Agar dilution method for MICs determination

The antibacterial activity of compounds **2a**–**h** were determined against Gram-positive (*S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *B. subtilis* ATCC 6633) and Gram-negative (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031) bacteria. The minimum inhibitory concentrations (MICs) of compounds were determined by the conventional agar dilution method [31]. For preparation of stock solution, the test compounds (10.0 mg) were dissolved in DMSO (1 mL) and then diluted with distilled water (9 mL). Further progressive two-fold serial dilution with molten sterile Mueller–Hinton agar was performed to obtain the required concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19, 0.098, 0.049, and 0.025 μg/mL. The medium containing the test compounds was dispensed into a sterile Petri-dish and allowed to solidify. Petri-dishes were inoculated with 1–5 × 10^4 CFU and incubated at 37 °C for 18 h. The MIC was defined as the lowest concentration of the test compound, which resulted in no visible growth.

Acknowledgments

This work was supported by a grant from the Research Council of Mazandaran University of Medical Sciences, Sari, Iran. A part of this work was related to the Pharm.D thesis of Ebrahim Ghafouri (Faculty of Pharmacy, Mazandaran University of Medical Sciences).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.07.032.

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