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Identification, biological activity, and mechanism of the anti-ischemic quinolone analog

Chan-Hee Park,[a] Jongwon Lee,[b] Hwi Young Jung,[b] Min Ji Kim,[b] Sun Ha Lim,[b] Hyung Tae Yeo,[c] Eung Chil Choi,[a] Eun Jeong Yoon,[a] Kyu Won Kim,[a] Jong Ho Cha,[a] Seok-Ho Kim,[a] Dong-Jo Chang,[a] Do-Yeon Kwon,[a] Funan Li[a] and Young-Ger Suh[a,*]

[a]*College of Pharmacy, Seoul National University, San 56-1 Shinrim-Dong, Kwanak-Gu, Seoul 151-742, Republic of Korea*
[b]*Department of Biochemistry, School of Medicine, Catholic University of Daegu, Daegu, Republic of Korea*
[c]*Department of Neurosurgery, School of Medicine, Catholic University of Daegu, Daegu, Republic of Korea*

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Abstract—The quinolone analog SQ-4004 has been identified as a potentially excellent anti-ischemic agent, which exhibited highly potent efficacy in reducing infarct volume size in vivo rat MCAO model (32.1% at 0.01 mg/kg) and potent cardioprotective effect at myocardial infarction in vivo model (26.6% at 0.01 mg/kg) while it exhibited highly reduced anti-bacterial activity. The mechanistic study revealed that the anti-ischemic activity might exert via an anti-apoptotic pathway, which implies its therapeutic uses against the ischemic cell injuries including ischemic stroke and ischemic heart disease.

1. Introduction

Ischemic cell injury under hypoxic conditions such as ischemic stroke and/or myocardial infarction, in many cases, leads to fatal situation or causes a long-lasting disability.[1] To the best of our knowledge, the currently approved treatment for the patients is only an administration of the tissue-type plasminogen activator (*t*-PA) as a thrombolytic therapy for the restoration of blood flow to the ischemic region. However, the thrombolytic therapy has some limitation to be treated within 3 h after a stroke and is applicable to a limited number of patients because of the risk of symptomatic intracranial hemorrhage. Moreover, during the reperfusion, cells are often endangered in a circumstance of oxidative stress, which results in further tissue injury. In this regard, antithrombolytic agents seem not effective enough to stop or retard the on-going tissue injury at a reperfusion phase. Therefore, development of the new anti-ischemic agents with different modes of action as well as with wider therapeutic window is in urgent need.

Current studies on the new anti-ischemic agents include glutamate antagonists, anti-inflammatory agents, anti-apoptotic agents, anti-oxidant agents, and ion-channel modulators.[2–4] In particular, the recent efforts focused on the evaluation of the currently available antibiotics on the basis of their potential role in reducing infarct size via an inhibition of inflammation and/or infection accompanied by ischemic injury. The tetracycline analogs,[5] the representative example, showed reasonable efficacy in in vivo animal model in reducing the infarct size during the ischemic insult. Several research groups also recently reported that the anti-ischemic effect by pretreatment of antibiotics is likely due to their anti-inflammatory or anti-apoptotic effect.[6–11]

In the course of our search for new anti-ischemic agents, we have also discovered that several antibiotics provide significant cell survivals under in vitro hypoxic condition.[12] In particular, among the fluoroquinolones tested, ciprofloxacin (CPFX) exhibited in vitro cell viability and furthermore considerably reduced infarct volume size in in vivo rat focal cerebral ischemic animal model (MCAO) at low concentration. During our extensive studies on anti-ischemic agent, Christian Meisel et al. also reported the moderate reduction of infarct volume size by moxifloxacin pretreatment in mouse MCAO

Keywords: Anti-ischemic agents; Quinolone analog; Structure–activity relationship; MCAO; MI; Anti-apoptotic activity.
* Corresponding author. Tel.: +82 2 880 7875; fax: +82 2 888 0649; e-mail: ygsuh@snu.ac.kr

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model (about 14% reduction in infarct size after six times intravenous administration of 100 mg/kg) and its anti-infection mechanism for the anti-ischemic activity.[13]

On the basis of the excellent anti-ischemic activity of CPFX at both in vitro and in vivo experiments, our initial efforts for new anti-ischemic agents included the systematic structural modification of CPFX in an anticipation of minimization of the anti-bacterial activity for the separation of anti-ischemic activity from the anti-bacterial activity of CPFX as well as enhancement of its anti-ischemic activity. As our preliminary work to understand structural requirements for the anti-ischemic effect of CPFX, we manipulated the functional groups of CPFX associated with the anti-bacterial effect. Consequently, we were able to identify SQ-4004, which exhibited potent cell viability with highly reduced anti-bacterial efficacy among the CPFX analogs. Moreover, SQ-4004 showed high anti-ischemic efficacy in further in vivo animal model studies. Thus, we herein communicate our initial success to identify the quinolone-based anti-ischemic agent, SQ-4004, which envisions separation of the potent neuroprotective and cardioprotective activity from the anti-bacterial effect. In addition, we describe the preliminary structure–activity relationship of CPFX and the mechanism of the anti-ischemic activity associated with the anti-apoptotic activity.

2. Results and discussion

2.1. Structural modification of CPFX

According to the structure–activity relationship for the anti-bacterial activity of fluoroquinolones, N1, C2–H, C3-Carboxylic acid, C4-carbonyl, C6-F, and C7-piperazine are essential or beneficial for the anti-bacterial activity.[14,15] Thus, modification or elimination of these groups would give us the valuable structural information for separation from the anti-bacterial activity as well as further improvement of anti-ischemic effect of CPFX. The structural modification of CPFX and the rationale for the modification are summarized in Figure 1. The CPFX analogs prepared for the preliminary structure–activity relationship of CPFX appeared in a few literatures except SQ-4007 and SQ-4008 as synthetic intermediates without biological property (SQ-4001,[16] SQ-4004,[17] and SQ-4005[18]) or with anti-bacterial activity (SQ-4002,[19,20] SQ-4003,[21] and SQ-4006[22]). All the analogs were synthesized by modification (method A or method B) of the established procedure as shown in Scheme 1. In method A, the *cis*-isomer of **2** was directly subjected to acylation right after purification by column chromatography on NH–silica gel to afford the desired product **3** with a minimum isomerization to the corresponding *trans*-isomer. Cyclization of **3** under basic condition and further hydrolysis gave the quinolone intermediate **4**. The analogs, SQ-4003 and SQ-4004,

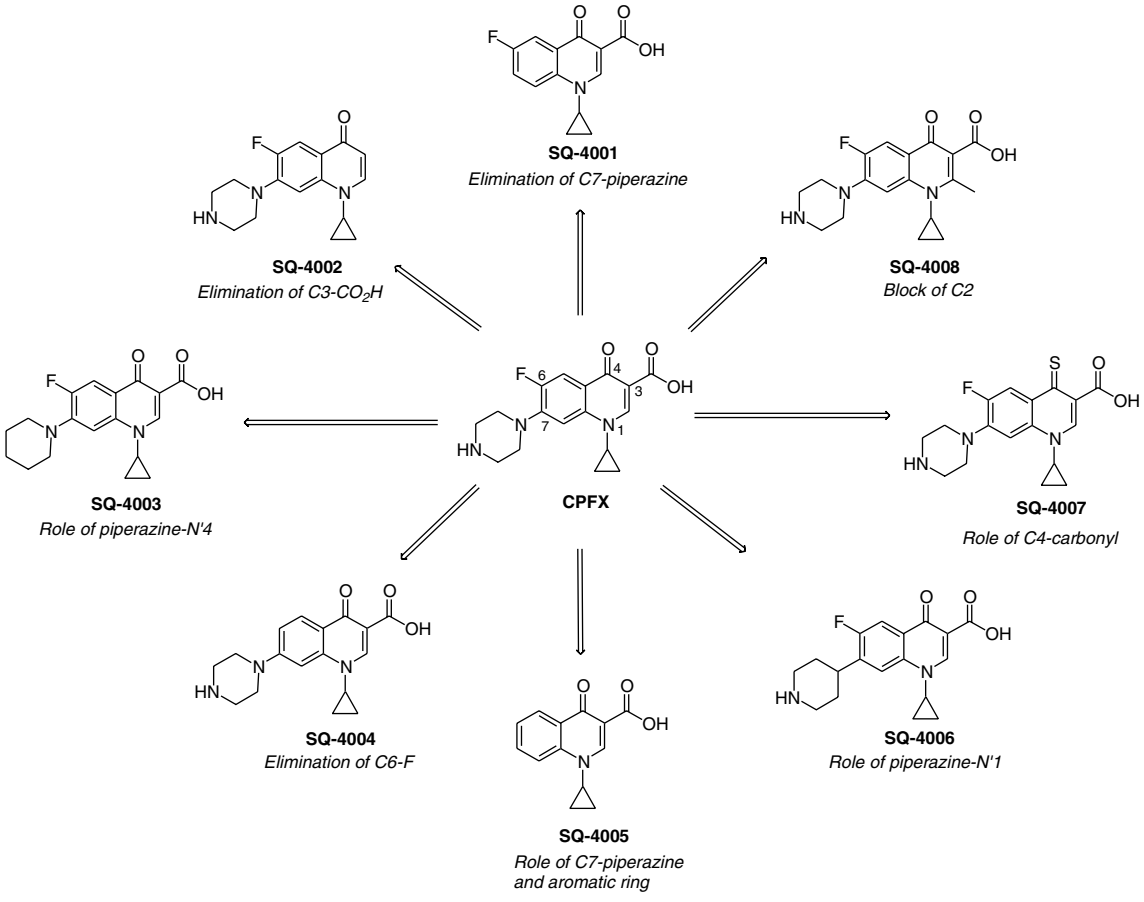
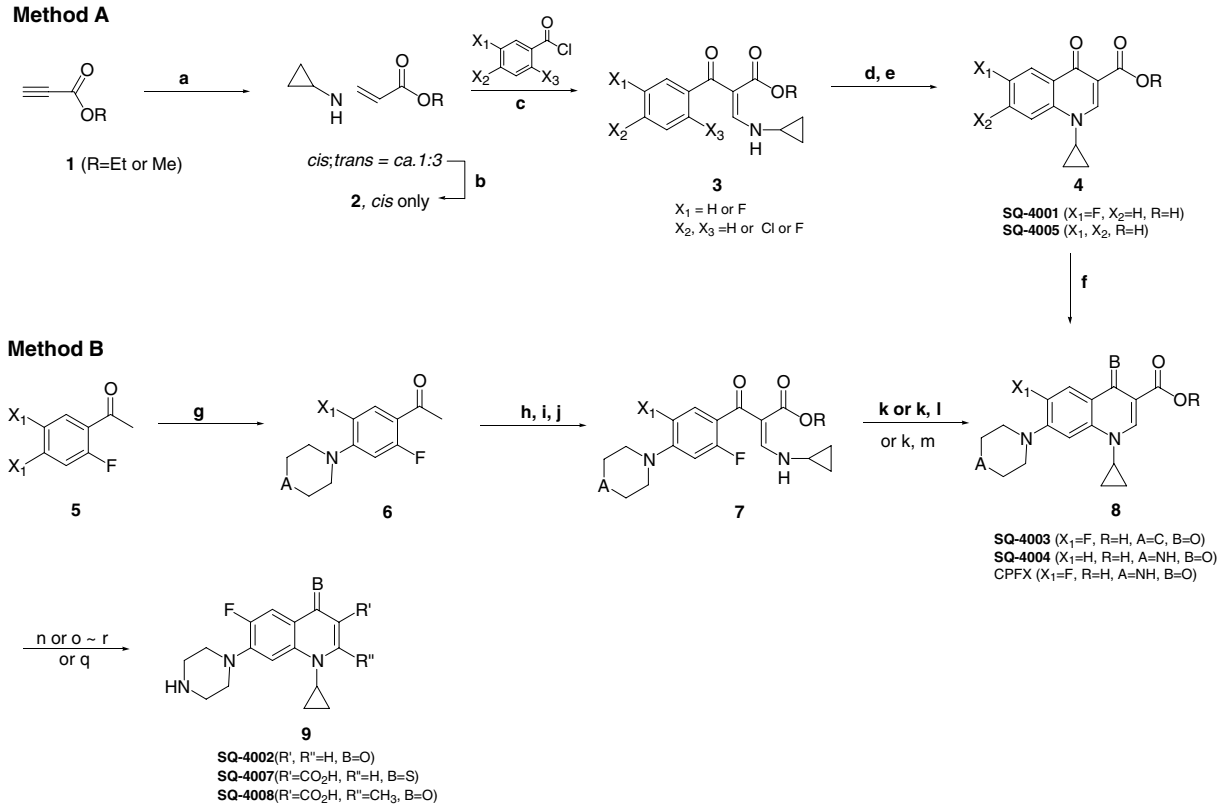


Figure 1. Structure of CPFX and its structural modification.



Scheme 1. General synthetic routes for SQ-analogs. Reagents and conditions, Method A: (a) cyclopropylamine, CH_3CN, 5–10 °C, 5 h, 100%; (b) column chromatography on NH–silica gel, 20%; (c) Et_3N, dioxane, reflux, 12 h, 60–96%; (d) K_2CO_3, DMF, 110 °C, 12 h, 70–88%; (e) 6 N HCl, EtOH, reflux, 6 h, 78–96%; (f) piperazine or piperidine, DMSO, 130–140 °C, 5 h, 40–67%. Method B: (g) piperidine or *N*-Boc-piperazine, pyridine, Et_3N, rt, 48 h, 95–100%; (h) diethylcarbonate, NaH, rt → 80 °C, 3 h, 60–79%; (i) triethylorthoformate, Ac_2O, 110 °C, 2 h; (j) cyclopropylamine, CH_2Cl_2, rt, 3 h, 79–87% for two steps; (k) NaH, THF, reflux, 2 h, 90–98%; (l) 6 N HCl, EtOH, reflux, 6 h, 96%; (m) P_2S_{10}, pyridine, reflux, 2 h, 33%; (n) NaCN, DMSO, 170–180°C, 2 h, 30%; (o) CH_3MgBr, CuI, THF, −78 °C, 3 h, 62%; (p) NaH, PhSeCl, THF, 0 °C, 10 min then H_2O_2, CH_2Cl_2, rt, 30 min, 70% for two steps; (q) concd HCl, EtOH/CH_2Cl_2, rt, 2 h, 77%; (r)1 N NaOH, THF/EtOH, rt, 1 day, 54%.

were synthesized by aromatic nucleophilic substitution of the corresponding C7-fluorobenzenes with piperazine or piperidine. The analogs SQ-4003 and SQ-4004 could be also synthesized by method B under the much milder reaction condition. The nucleophilic substitution took place even at room temperature with an excellent isolation yield and then facile alkoxycarbonylation of the resulting substitution product, followed by aminomethylenation, gave the intermediate **7** with good yields. Finally, sequential cyclization, Grignard reaction or conversion of carbonyl to thiocarbonyl, and then hydrolysis provided SQ-4007 and SQ-4008 with moderate yields. The analogs, SQ-4002 and SQ-4006, were synthesized by the reported procedure.[20,22]

2.2. Evaluation of the pharmacophoric parts of CPFX (in vitro cell viability and anti-bacterial effect)

Anti-ischemic effects of CPFX-analogs were assessed preliminarly at 0.1 μM concentration in vitro in SH-SY5Y, neuroblastoma cell line. Ischemic insult was induced by 200 μM of H_2O_2 and the level of cell viability was determined using MTS assay and was expressed as a percentage compared to that of the control. As shown in Table 1, among the tested CPFX-analogs, SQ-4002 and SQ-4004 exhibited 33% and 42% enhancement of cell viability, respectively, compared to the control. However, SQ-4007 seems to show cell cytotoxicity even at low concentration (Table 1). The anti-bacterial activity for both SQ-4002 and SQ-4004, which exhibited excellent cell viability, was also tested against four representative gram-positive and three gram-negative strains. As anticipated, SQ-4002 and SQ-4004 apparently exhibited the highly reduced anti-bacterial activities and especially, SQ-4004 exhibited 8- to 32-fold

Table 1. Cell viability for SQ-analogs by MTS assay in SH-SY5Y cell line

Analogs	Cell viability (%) (mean ± SD)
Positive control	43.3 ± 5.4
CPFX	46.5 ± 5.3
SQ-4001	45.1 ± 7.4
SQ-4002	57.4 ± 4.3*
SQ-4003	44.1 ± 7.9
SQ-4004	61.7 ± 4.6**
SQ-4005	39.0 ± 1.2
SQ-4006	42.4 ± 0.9
SQ-4007	27.1 ± 2.2
SQ-4008	49.9 ± 3.9

* $P < 0.1$.
** $P < 0.05$ as compared to the control.

Table 2. Anti-bacterial activity of SQ-4002, SQ-4004, and CPFX against Gram-positive and Gram-negative strains

Analogs	MIC (μg/mL)						
	S. aureus	*S. epidermidis*	*E. faecalis*	*B. subtilis*	*E. coli*	*E. clocaes*	*S. marcesens*
CPFX	2	1	2	1	0.031	0.063	0.125
SQ-4002	16	8	8	8	<0.12	0.5	1
SQ-4004	32	16	64	8	0.5	1	4

reduced anti-bacterial activities compared to CPFX against all the tested strains (Table 2). Obviously, elimination of carboxyl and fluoro groups of CPFX, which are essential or beneficial for the anti-bacterial activity, respectively, seems to enhance the anti-ischemic activity. On the other hand, modification of C7-piperazine group (SQ-4001, 4003, 4005, and 4006), which is beneficial for the anti-bacterial activity of CPFX, slightly reduced the anti-ischemic activity. Thiocarbonyl substitution of C4-carbonyl group of the enone moiety, which is essential for the anti-bacterial effect significantly dropped the anti-ischemic activity. However, methyl introduction at C2 slightly increased the anti-ischemic activity.

2.3. In vivo MCAO model

To assess the anti-ischemic activity of SQ-4004 in vivo, SQ-4004 was evaluated in rat MCAO model under both pre- and post-ischemic conditions (ischemia/reperfusion = 2 h/22 h) and CPFX was used as a control drug. Both compounds were tested by intraperitoneal administration three times (pre-ischemic condition; 3 h, 1 h prior, and 1 h after occlusion of MCA) at the doses of 0.001, 0.01 and 0.1 mg/kg, respectively. As a result, the significant reduction of the infarct size of brain was observed as shown in Table 3 and Figure 2. The infarct volumes for the group of rats treated with SQ-4004 were 54.6 ± 5.0 mm^3 (0.001 mg/kg, $N = 4$; $P < 0.05$),

Table 3. Anti-ischemic effect of CPFX and SQ-4004 in the rat MCAO model

Condition	Drugs	Dose (mg/kg, ip)	Infarct volume (mm^3 ± SD)	Infarct size reduction (%)
Pre-ischemia	Vehicle	($n = 28$)	60.4 ± 7.6	
	CPFX	0.001 ($n = 4$)	52.0 ± 8.0	13.9
		0.01 ($n = 8$)	49.0 ± 8.3*	18.8
		0.1 ($n = 5$)	58.8 ± 8.5	2.7
	SQ-4004	0.001 ($n = 4$)	54.6 ± 5.0*	9.6
		0.01 ($n = 9$)	41.0 ± 7.3**	32.1
		0.1 ($n = 7$)	57.2 ± 8.7	5.3
Post-ischemia	SQ-4004	0.01 ($n = 5$)	55.1 ± 5.6	8.9

* $P < 0.05$.
** $P = 0$ as compared to vehicle treated.

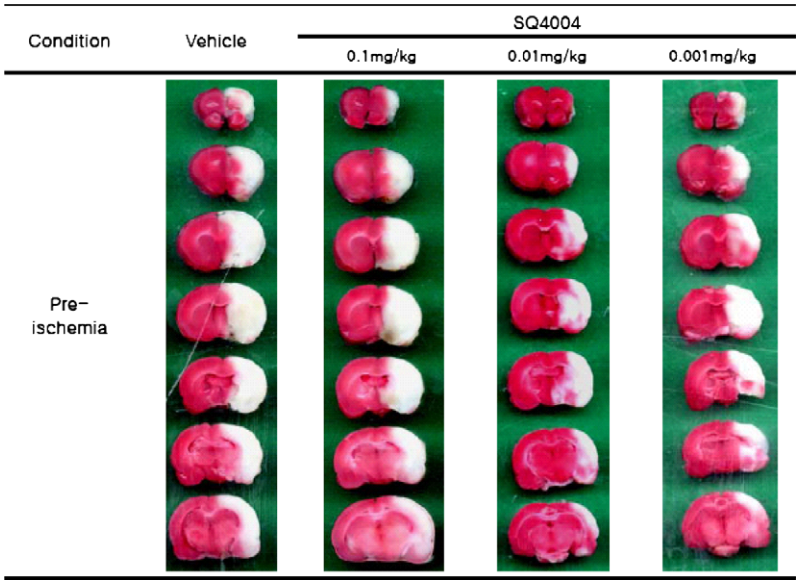


Figure 2. Effect of SQ-4004 under the condition of ischemia/reperfusion (2/22 h) in the rat MCAO model. Brains were dissected into seven slices, stained with triphenyltetrazolium chloride, and assessed for infarct size.

$41.0 \pm 7.3\ mm^3$ (0.01 mg/kg, $N = 9$; $P = 0$) and $57.2 \pm 8.7\ mm^3$ (0.1 mg/kg, $N = 7$), respectively, compared to $60.4 \pm 7.6\ mm^3$ ($N = 28$) for the vehicle group.

The analog SQ-4004 exhibited a highly potent anti-ischemic activity of 32.1% infarct reduction compared to the vehicle at 0.01 mg/kg and 1.7-fold potency compared to CPFX. Based on the high efficacy at pre-ischemic condition, SQ-4004 were also tested by intraperitoneal administration three times under the post-ischemic condition (right after, 2 h after occlusion of MCA and 2 h after reperfusion) at the dose of 0.01 mg/kg. However, only 8.9% reduction of the infarct size was observed under this condition. No reduction of the infarct area at 0.1 mg/kg of SQ-4004 impinged on dose-dependent manner seems to imply the possible rebounding aspect of SQ-4004.

Considering the highly potent anti-ischemic effect of SQ-4004, its in vivo evaluation was extended to myocardial infarction animal model. The reduction in myocardial infarction was examined after intraperitoneal administrations of SQ-4004 1 h prior to the local ischemia at the doses of 0.001, 0.01 and 0.1 mg/kg, respectively. After transient ischemia/reperfusion (30 min/3 h) insult, the infarct area of the hearts was assessed and the highly potent cardioprotective effect at 0.01 mg/kg was also observed as shown in Table 4. Again, no reduction of the infarct area at 0.1 mg/kg was observed in MI model.

Table 4. Cardioprotective efficacy of SQ-4004 in the myocardial infarction (MI) model in rats

Condition	Drugs	Dose (mg/kg, ip)	Infarct area (% of area at risk)	Infarct size reduction (%)
Myocardial infarction	Vehicle	($n = 37$)	72.4 ± 9.8	
	SQ-4004	0.001 ($n = 5$)	62.1 ± 6.0*	14.2
		0.01 ($n = 10$)	53.1 ± 14.3*	26.6
		0.1 ($n = 6$)	78.3 ± 13.1	—

* $P < 0.05$ as compared to the vehicle treated.

2.4. Anti-apoptotic activity of SQ-4004

On the basis of potent anti-ischemic activities of SQ-4004 at both MCAO and MI models, we investigated its modes of action associated with the inhibitory activity of programmed cell death during hypoxic injury. Histopathological examination of the heart tissue was carried out after a transient ischemic/reperfusion insult followed by assessing the degree of apoptotic cell death via TUNEL (terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end-labeling) staining. As a result, the significant apoptosis reduction compared to that of the vehicle was observed as shown in Figure 3. These results strongly implicate the possible anti-apoptotic activity for the anti-ischemic activity of SQ-4004.

3. Conclusion

In conclusion, starting from an observation of the highly potent anti-ischemic activity of CPFX at both in vitro and in vivo assays, we have established the preliminary structure–activity relationship for anti-ischemic effect of CPFX. In addition, we have identified two potent anti-ischemic quinolones. In particular, SQ-4004 exhibited highly enhanced in vitro anti-ischemic activity with

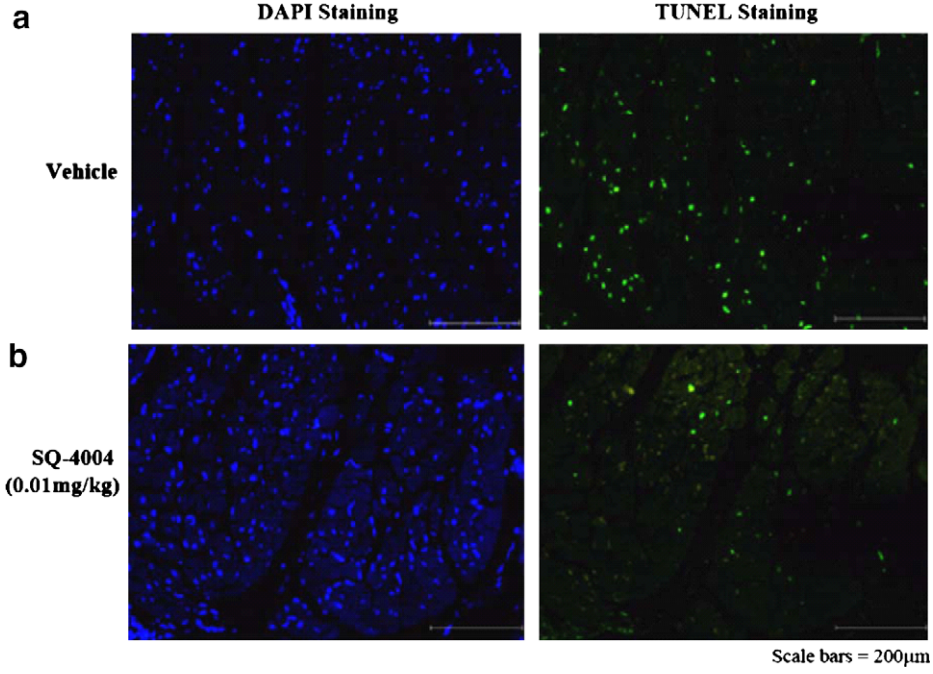


Figure 3. Histological examination on the apoptotic pathway of SQ-4004, TUNEL staining and DAPI staining: (a) vehicle, (b) SQ-4004 (0.01 mg/kg).

significantly reduced anti-bacterial activity compared to that of CPFX. Moreover, SQ-4004 showed a highly potent neuroprotective effect as well as an excellent cardioprotective effect. The anti-apoptotic mechanism for the anti-ischemic activity was also elucidated, which provides the valuable information for further studies on the novel anti-ischemic quinolones. Currently, the intensive studies on SQ-4004 including the detailed structure–activity relationship and the anti-ischemic mechanism are in good progress. The successful results will be reported in due course.

4. Experimental

4.1. Materials

All reagents including starting materials and solvents were purchased from Aldrich Chemical Co. or TCI and used without further purification. Reaction was routinely checked with thin-layer chromatography (Kiesegel $60F_{254}$, Merck) and column chromatography was performed with Kiesegel 60, 230–400 mesh (Merck) or NH–silica gel, Chromatorex, 100–200 mesh (Fuji Silysia), respectively. NMR spectra were recorded on a JEOL LNM-LA 300 (300 MHz), Bruker ARX 300 and TMS (tetramethylsilane) was used as an internal standard. Chemical shifts (δ) were recorded in ppm and coupling constants (J) in hertz (Hz). IR (infrared) spectra were recorded on a Jasco FT/IR-300E and Perkin-Elmer 1710 FT spectrometer. High resolution mass spectra were obtained on a JEOL JMS-AX 505wA and JEOL JMS-HX/HX 110A spectrometer.

4.2. Chemistry

The representative synthetic procedure was selected for the preparation of SQ-4004, SQ-4007 and SQ-4008.

4.2.1. Method A

4.2.1.1. Synthesis of *cis*-3-cyclopropylaminoacrylic acid methyl ester (2). To a stirred solution of *c*-propylamine, **1** (1.14 mL, 16.5 mmol) in 10.0 mL CH_3CN was added dropwise ethylpropiolate (1.06 g, 12.6 mmol) at 5–10 °C and stirred for 3 h. The reaction mixture was concentrated in vacuo, and then the residue was purified by NH–silica column chromatography using EtOAc/*n*-hexane (1:5) as an eluent to give the title compound, **2** (600 mg, 22%): colorless oil; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.91 (br s, 1H), 7.44 (dd, 1H, J = 13.0, 8.2 Hz), 4.50 (d, 1H, J = 8.4 Hz), 3.63 (s, 3H), 2.69 (m, 1H), 0.50–0.75 (complex m, 4H). Bp 65–68 °C/0.09 mm Hg.

4.2.1.2. For *trans*-3-cyclopropylaminoacrylic acid methyl ester (2). Colorless crystal; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.44 (dd, 1H, J = 13.2, 7.6 Hz), 5.04 (d, 1H, J = 13.3 Hz), 4.74 (br s, 1H), 3.67 (s, 3H), 2.43 (m, 1H), 0.50–0.75 (complex m, 4H).

4.2.1.3. 3-Cyclopropylamino-2-(2,4-dichlorobenzoyl) acrylic acid methyl ester (3). A mixture of **2** (464 mg, 3.29 mmol), Et_3N (0.24 mL, 3.39 mmol), and 2-chloro-5-fluorobenzoyl chloride (666 mg, 1.69 mmol) in 5.00 mL dioxane was heated at reflux for 1 h and then cooled down to room temperature. The reaction mixture was diluted with CH_2Cl_2 (10.0 mL) and washed with water and brine. After drying over anhydrous $MgSO_4$, removal of the solvent in vacuo gave a residue, which was chromatographed over SiO_2 using EtOAc/*n*-hexane (1:8) to give the title compound, **3** (434 mg, 44%). Pale yellowish solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.10 (br s, 1H), 8.20 (d, 1H, J = 13.8 Hz), 7.36 (s, 1H), 7.25 (d, 1H, J = 7.7 Hz), 7.11 (d, 1H, J = 7.9 Hz), 3.55 (s, 3H), 3.00 (m, 1H), 0.80–1.00 (m, 4H). ESI-MS: m/z 314 $[M+H]^+$.

4.2.1.4. 7-Chloro-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (4). A mixture of **3** (355 mg, 1.13 mmol), K_2CO_3 (171 mg, 1.24 mmol) in 5.00 mL dimethylformamide was heated at 110–120 °C for 1 h and then cooled down to room temperature. The reaction mixture was diluted with cold water (10.0 mL) and stirred for 1 h at room temperature. The resulting solid was filtered, washed with cold water, and dried under vacuum to give 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid methyl ester (245 mg, 78%). Pale yellowish solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.58 (s, 1H), 8.40 (d, 1H, J = 8.3 Hz), 7.90 (d, 1H, J = 1.9 Hz), 7.40 (dd, 1H, J = 8.4, 1.9 Hz), 3.92 (s, 3H), 3.47 (m, 1H), 1.33 (m, 2H), 1.17 (m, 2H). ESI-MS: m/z 278 $[M+H]^+$.

A mixture of 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid methyl ester (37 mg, 0.13 mmol) in 6 N HCl / EtOH (8.00 mL, 1 : 1 v/v) was heated at reflux for 4 h and then cooled down to room temperature. The reaction mixture was diluted with cold water (5.00 mL) and stirred for 1 h at room temperature. The resulting solid was filtered, washed with cold water, and dried under vacuum to give the title compound, **4** (31 mg, 91%). Yellowish solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 15.00 (br s, 1H), 9.00 (s, 1H), 8.57(d, 1H, J = 8.9 Hz), 8.51 (d, 1H, J = 1.6 Hz), 7.95 (dd, 1H, J = 8.4, 1.9 Hz), 4.10 (m, 1H), 1.38–1.56 (complex m, 4H).

4.2.1.5. 1-Cyclopropyl-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid (8a, SQ-4004). A mixture of **4** (233 mg, 0.88 mmol) and piperazine (227 mg, 3.00 mmol) in anhydrous dimethylsulfoxide (2.00 mL) was heated at 130–140 °C for 5 h. The reaction mixture was diluted with water and treated conc. HCl to adjust pH 4–5 and stirred for 1 h under steam bath. The resulting solid was filtered, washed with hot water several times and dried under vacuum to give the title compound, **8a** (SQ-4004, 100 mg, 40%) (Table 5).

4.2.2. Method B

4.2.2.1. Synthesis of 4-(4-acetyl-2, 5-difluorophenyl)piperazine-1-carboxylic acid *tert*-butyl ester (6). To a stirred solution of commercially available 2,4,6-difluoro acetophenone (1.00 g, 5.74 mmol) and Et_3N (1.10 mL, 8.04 mmol) in pyridine (6.00 mL) was added *N*-Boc-piperazine (1.18 g, 6.34 mmol) at room temperature, and the reaction mixture was stirred for 2 days.

Table 5. Physicochemical and spectral properties of the CPFX analogs

SQ-analogs	Empirical formula	^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm), J (Hz)	IR (KBr, cm^{-1})	HR-MS (FAB+)	Appearance
SQ-4001	$C_{13}H_{10}FNO_3$	14.90 (br s, 1H), 8.77 (s, 1H), 8.38 (dd, 1H, J = 9.6, 4.3 Hz), 8.04 (dd, 1H, J = 8.8, 3.1 Hz), 7.94 (td, 1H, J = 7.9, 3.0 Hz), 3.95 (m, 1H), 1.34 (m, 2H), 1.20 (m, 2H)	2918, 1731, 1617, 1548, 1459, 1079, 936, 829**	Calcd for $C_{13}H_{11}FNO_3$ 248.0723, found 248.0719	White solid
SQ-4002	$C_{16}H_{18}FN_3O$	8.00 (d, 1H, J = 13.3 Hz), 7.60 (d, 1H, J = 8.1 Hz), 7.26 (d, 1H, J = 7.2 Hz), 6.15 (d, 1H, J = 7.8 Hz), 3.35 (m, 1H), 3.25 (m, 4H), 3.13 (m, 4H), 1.30 (m, 2H), 1.05 (m, 2H)*	3436, 1623, 1484, 1382, 1295, 1261, 1129, 823, 737, 620	Calcd for $C_{16}H_{19}N_3OF$ 288.1512, found 288.1515	White solid
SQ-4003	$C_{18}H_{19}FN_2O_3$	15.10 (s, 1H), 8.76 (s, 1H), 8.00 (d, 1H, J = 13.4 Hz), 7.35 (d, 1H, J = 7.3 Hz), 3.60 (m, 1H), 3.35 (m, 4H), 1.60–1.95 (complex m, 6H), 1.40 (m, 2H), 1.20 (m, 2H)	3441, 2935, 1725, 1626, 1505, 1469, 1383, 1338, 1252, 1101, 957, 887, 858, 827	Calcd for $C_{18}H_{20}FN_2O_3$ 331.1458, found 331.1458	White solid
SQ-4004	$C_{17}H_{19}N_3O_3$	8.61 (S, 1H), 8.14 (d, 1H, J = 8.9 Hz), 7.35 (m, 2H), 3.60 (m, 1H), 3.50 (m, 4H), 3.10 (m, 4H), 1.35 (m, 2H), 1.14 (m, 2H)	2819, 1681, 1619, 1515, 1463, 1271, 1156, 1044, 944, 839, 800. 625	Calcd for $C_{17}H_{20}N_3O_3$ 314.1505, found 314.1496	Yellowish solid
SQ-4005	$C_{13}H_{11}NO_3$	14.90 (s, 1H), 8.91 (s, 1H), 8.53 (dd, 1H, J = 8.3, 1.5 Hz), 8.10 (d, 1H, J = 8.8 Hz), 7.90 (m, 1H), 7.60 (m, 1H), 3.60 (m, 1H), 1.45 (m, 2H), 1.20 (m, 2H)*	2918, 1722, 1616, 1542, 1451, 1336, 1235, 1171, 1050, 908, 810, 757**	Calcd for $C_{13}H_{12}NO_3$ 230.0817, found 230.0818	White solid
SQ-4006	$C_{18}H_{19}FN_2O_3$	8.93 (s, 1H), 8.38 (d, 1H, J = 6.1 Hz), 8.20 (d, 1H, J = 10.2 Hz), 4.10 (m, 1H), 3.50–3.65 (m, 3H), 3.21–3.49 (m, 2H), 2.15–2.42 (m, 4H), 1.57 (m, 2H), 1.39 (m, 2H)	3439, 2935, 2712, 2499, 1730, 1615, 1543, 1509, 1468, 1394, 1338, 1261, 808, 555	Calcd for $C_{18}H_{20}FN_2O_3$ 331.1458, found 331.1461	White solid
SQ-4007	$C_{17}H_{18}FN_3O_2S$	9.29 (br s, 1H), 9.00 (s, 1H), 8.58 (d, 1H, J = 14.7 Hz), 7.73 (d, 1H, J = 7.8 Hz), 4.11 (m, 1H), 3.69 (m, 4H), 3.48 (m, 4H), 1.50 (m, 2H), 1.30 (m, 2H)	3426, 2496, 1704, 1623, 1494, 1455, 1319, 1268, 1138, 1027, 938, 775, 536	Calcd for $C_{17}H_{19}FN_3O_2S$ 348.1182, found 348.1178	Yellowish solid
SQ-4008	$C_{18}H_{20}FN_3O_3$	7.85 (d, 1H, J = 13.2 Hz), 7.53 (d, 1H, J = 7.5 Hz), 3.65 (m, 1H), 3.33 (m, 4H), 3.09 (s, 3H), 3.01 (m, 4H), 1.48 (m, 2H), 1.04 (m, 2H)	3443, 2677, 1602, 1478, 1404, 1293, 1262, 1142, 848, 728, 609	Calcd for $C_{18}H_{21}FN_3O_3$ 346.1567, found 346.1562	White solid

* ^{1}H NMR (300 MHz, $CDCl_3$).

** IR ($CHCl_3$, neat).

The resulting solution was concentrated and the crude residue was partitioned between CH_2Cl_2 (20.0 mL) and water (2× 20.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by recrystallization (EtOAc/*n*-hexane, 1:5) to give the title compound, **6** (1.96 g, 100%). White solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.58 (dd, 1H, J = 13.6, 6.8 Hz), 6.58 (dd, 1H, J = 12.7, 6.9 Hz), 3.60 (m, 4H), 3.17 (m, 4H), 2.59 and 2.57 (s, 3H), 1.49 (s, 9H). ESI-MS: *m*/*z* 341 $[M+H]^+$, 363 $[M+Na]^+$.

4.2.2.2. 4-[4-(3-Cyclopropylamino-2-ethoxycarbonyl-acryloyl)-2,5-difluorophenyl]piperazine-1-carboxylic acid *tert*-butyl ester (7). To a stirred solution of **6** (653 mg, 1.92 mmol) in diethylcarbonate (10.0 mL) was added NaH (60% in dispersion oil, 307 mg, 7.68 mmol) at room temperature, and the reaction mixture was stirred at 80 °C for 2 h. The resulting solution was concentrated by evaporation of diethylcarbonate and the crude mixture was partitioned between Et_2O (20.0 mL) and water (2× 20.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane, 1:7) to give 4-[4-(2-ethoxycarbonylacetyl)-2,5-difluorophenyl]piperazine-1-carboxylic acid *tert*-butyl ester (394 mg, 50%). Beige solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 12.72 (s, 1/3H), 7.60 and 7.58 (dd, 1H, J = 13.6, 6.8 Hz), 6.59 and 6.58 (dd, 1H, J = 12.7, 6.9 Hz), 5.81 (s, 1/3H), 4.24 (q, 2H, J = 7.1 Hz), 3.90 (d, 1 and 2/3H, J = 3.8 Hz), 3.58 (m, 4H), 3.12 and 3.20 (m, 4H), 1.43 (s, 9H), 1.23 (m, 3H). ESI-MS: *m*/*z* 413 $[M+H]^+$, 435 $[M+Na]^+$.

To a stirred solution of 4-[4-(2-Ethoxycarbonylacetyl)-2,5-difluorophenyl]piperazine-1-carboxylic acid *tert*-butyl ester (394 mg, 0.96 mmol) in acetic anhydride (1.00 mL) was added triethylorthoformate (0.32 mL, 1.92 mmol) at room temperature and the reaction mixture was stirred at 110 °C for 2 days. The solution was concentrated by evaporation of acetic anhydride and triethylorthoformate under vacuum and the resulting crude mixture was dissolved in anhydrous CH_2Cl_2 (1.00 mL) and then cyclopropylamine (80.0 μL) was added. The mixture was concentrated under reduced pressure and the resulting residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane, 1:4) to give the title compound, **7** (398 mg, 87%). Yellowish amorphous; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 10.76 (br d, 2/3H, J = 13.1 Hz), 9.21 (br d, 1/3H, J = 13.2 Hz), 8.14 and 8.05 (d, 1H, J = 13.7 Hz), 7.10 (m, 1H), 6.50 (m, 1H), 4.02 (m, 2H), 3.60 (m, 4H), 3.05 (m, 4H), 2.98 (m, 1H), 1.50 (s, 9H), 0.80–1.20 (complex m, 7H). ESI-MS: *m*/*z* 480 $[M+H]^+$, 502 $[M+Na]^+$.

4.2.2.3. 7-(4-*tert*-Butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (8b). To a stirred solution of compound **7** (275 mg, 0.57 mmol) in THF (2.00 mL) was added NaH (60% in dispersion oil, 27.4 mg, 0.68 mmol) at room temperature and the mixture was heated at reflux for 2 h. The resulting crude mixture was partitioned between CH_2Cl_2 (10.0 mL) and water (2× 10.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 40:1) to give title compound, **8b** (220 mg, 84%). Yellowish solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.53 (s, 1H), 8.04(d, 1H, J = 13.2 Hz), 7.26 (br s, 1H), 4.40(q, 2H, J = 7.2 Hz), 3.65 (m, 4H), 3.45 (m, 4H), 3.20 (m, 1H), 1.49 (s, 9H), 1.40 (t, 3H, J = 7.1 Hz), 1.35 (m, 2H), 1.25 (m, 2H). ESI-MS: *m*/*z* 460 $[M+H]^+$, 482 $[M+Na]^+$.

4.2.2.4. 7-(4-*tert*-Butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-4-thioxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (8c). A mixture of **8b** (163 mg, 0.35 mmol) and P_4S_{10} (101 mg, 0.23 mmol) in pyridine (1.20 mL) was heated at reflux for 2 h. The resulting solution was concentrated by evaporation of pyridine and the crude mixture was partitioned between $CHCl_3$ (20.0 mL) and water (2× 20.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane, 3:1) to give title compound, **8c** (54 mg, 33%). Orange yellowish solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.63 (d, 1H, J = 11.1 Hz), 7.97 (s, 1H), 4.38 (q, 2H, J = 5.4 Hz), 3.64 (m, 4H), 3.50 (m, 1H), 3.23 (m, 4H), 1.39 (s, 9H), 1.36 (m+t, 5H), 1.11 (m, 2H).

4.2.2.5. 1-Cyclopropyl-6-fluoro-2-methyl-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid (9a, SQ-4008). To a stirred solution of **8b** (615 mg, 1.34 mmol) and CuI (76.6 mg, 0.40 mmol) in anhydrous THF (7.00 mL) was added CH_3MgBr (3 M in ether, 0.67 mL, 2.00 mmol) at −78 °C dropwise and then stirred for 3 h. The reaction mixture was quenched with satd $NaHCO_3$ (aq) and concentrated under reduced pressure. The crude mixture was partitioned between $CHCl_3$ (10.0 mL) and water (2× 20.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane, 1:5) to give 7-(4-*tert*-butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-2-methyl-4- oxo 1,2,3,4-tetrahydroquinoline-3-carboxylic acid ethyl ester (274 mg, 70%). White solid; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.54 (d, 1H, J = 13.4 Hz), 6.54 (d, 1H, J = 7.1 Hz), 4.26 (m, 1H), 4.10 (qd, 2H, J = 7.1, 0.9 Hz), 3.59 (m, 4H), 3.19 (m, 5H), 2.40 (m, 1H), 1.60 (s, 9H), 0.92–1.35 (m, 6H), 0.79–1.12 (m, 4H). ESI-MS: *m*/*z* 476 $[M+H]^+$.

To a stirred solution of 7-(4-*tert*-butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-2-methyl-4-oxo 1,2,3,4-tetrahydroquinoline-3-carboxylic acid ethyl ester (393 mg, 0.83 mmol) in anhydrous THF (4.00 mL) was added NaH (60% dispersion in oil, 40.0 mg, 0.99 mmol) portionwise and then added PhSeCl (175 mg, 0.91 mmol) in anhydrous THF (0.50 mL) rapidly at room temperature. The crude mixture was partitioned between pentane/ether (6.00 mL, 1/1 v/v) and satd $NaHCO_3$ (aq) (3.00 mL), then the organic layer was separated. The organic layer was treated with H_2O_2 (30%

w/w, 0.20 mL) in CH_2Cl_2 (11.0 mL). After stirring for 1 h at room temperature, the reaction mixture was diluted with CH_2Cl_2 (10.0 mL) and washed with 10% w/w $NaHCO_3$ (aq), water, and brine. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane, 2:1) to give 7-(4-*tert*-butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-2-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (274 mg, 70%). White solid; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.90 (d, 1H, $J = 13.0$ Hz), 7.22 (d, 1H, $J = 7.0$ Hz), 4.39 (q, 2H, $J = 7.1$ Hz), 3.66 (m, 4H), 3.20 (m, 4H), 2.61 (s, 3H), 1.49 (s, 9H), 1.35 (m+t, 5H), 0.88 (m, 2H). ESI-MS: *m/z* 474 $[M+H]^+$, 496 $[M+Na]^+$.

A mixture of 7-(4-*tert*-butoxycarbonylpiperazin-1-yl)-1-cyclopropyl-6-fluoro-2-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (274 mg, 0.58 mmol) in EtOH/CH_2Cl_2/concd HCl (2.00/2.00/0.20 mL) was stirred for 3 h at room temperature. The reaction mixture was evaporated under reduced pressure. The residue was diluted with CH_2Cl_2 (10.0 mL) and washed with water and brine. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 15:1) to give 1-cyclopropyl-6-fluoro-2-methyl-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (167 mg, 77%). White solid; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.95(d, 1H, $J = 12.9$ Hz), 7.23 (d, 1H, $J = 7.2$ Hz), 4.41 (q, 2H, $J = 6.9$ Hz), 3.23 (m, 4H), 3.18 (m, 4H), 2.62 (s, 3H), 1.43 (m+t, 5H), 1.00 (m, 2H). ESI-MS: *m/z* 374 $[M+H]^+$.

A mixture of 1-cyclopropyl-6-fluoro-2-methyl-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (100 mg, 0.27 mmol) in EtOH/THF/1 N NaOH (aq) (0.50/0.50/0.20 mL) was stirred for 12 h at room temperature. The reaction mixture was acidified with concd HCl adjust to pH 5–6. The resulting solid was filtered, washed with cold water, and dried under vacuum to give the title compound **9a**, SQ-4008 (50 mg, 54%) (Table 5).

4.2.2.6. 1-Cyclopropyl-6-fluoro-7-piperazin-1-yl-4-thioxo-1,4-dihydroquinoline-3-carboxylic acid (9b, SQ-4007). A mixture of **8c** (55.0 mg, 0.11 mmol) in 6 N HCl/EtOH (1.00 mL/1.00 mL) was heated at reflux for 12 h. The reaction mixture was cooled down to room temperature and the resulting solid was filtered, washed with cold EtOH, and dried under vacuum to give the title compound **9b**, SQ-4007 (19 mg, 50%) (Table 5).

4.3. Biology

4.3.1. In vitro MTS assay. Human neuroblastoma cell line, SH-SY5Y (ATCC, CRL-2262), was cultured under 1:1 mixture of EMEM (Eagle's minimum essential medium) and F12K (Ham's F12 medium) supplemented by 10% heat-inactivated FBS (fetal bovine serum) at 37.0 °C in 5% CO_2. Cells were plated at 5×10^4 cells per well in a 96-well plate in a 1:1 mixture of FBS-free EMEM and F12K medium at a humidified CO_2 incubator. After one day, cells were pre-treated with 0.1 μM of the test compounds for 2 h and then treated with 200 μM of hydrogen peroxide (Sigma Aldrich Co.) followed by incubation for 24 h. To assess the cell viability, cells were incubated with 20 μL of MTS solution (CelTiter 96 Aqueous one solution cell proliferation assay, Promega) for 3 h at 37.0°C, and OD was determined at 490 nm using a spectrophotometer. All values are averages of at least two independent experiments, each done in triplicate.

4.3.2. In vitro anti-bacterial activity. Minimal Inhibitory Concentrations (MICs) were determined by a standardized agar dilution method according to CLSI guidelines. *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Enterobacter clocae* ATCC 13047, and *Serratia marcescens* ATCC 27117 were tested on Mueller Hinton agar and *Enterococcus faecalis* ATCC 29212 was on Brain–Heart Infusion agar. The stock solutions of test compounds were diluted in dimethylsulfoxide (DMSO) or 0.1 M NaOH solution to give a serial and twofold series yielding final drug concentrations in a range of 0.06–128 μg/mL. A microinoculator (Sakuma Co. Ltd, Tokyo, Japan) was used to inoculate the bacterial suspensions (104 cfu/spot). The inoculated plates were incubated in ambient air at 36 °C for 16–18 h. The MIC of each compound was defined as the lowest concentration that inhibited visible growth of the organism.[23]

4.3.3. In vivo MCAO animal model. Transient focal ischemic stroke was modeled in rats using the intraluminal thread procedure according to Longa's protocol with a minor modification.[24] The male Sprague–Dawley rats (240–270 g, Charles River, USA) were anesthetized with 5% enflurane. The rectal temperature was maintained in the range of 37 ± 0.5 °C with a heating pad. A 4–0 nylon suture was inserted through the internal carotid artery into the origin of the middle cerebral artery (MCA). The suture was carefully removed after 2 h of MCA occlusion. Drugs were administered total three times divided into two groups under pre-ischemic condition and post-ischemic condition as mentioned in the main text. Then, brains were isolated at 24 h after ischemia, sliced, and processed for TTC (2,3,5-tripenyltetrazolium chloride) histochemistry. The infarcts were measured using the computer-assisted planimetry, Gel Documentation System, Bio-Rad. The effect of drug treatment is expressed as the percent reduction in infarct volume compared to the vehicle controls. Statistical significance was determined using the Student's *t*-test with a confidence interval accepted when $P < 0.05$.

4.3.4. In vivo MI animal model. In vivo rat MI model was performed according to Ju's protocol with a slight modification.[25] The male Sprague–Dawley rats (260–290 g, Charles River, USA) were anesthetized with ketamine (10 mg/kg) and xylazine (5 mg/kg) and then SQ-4004 was intraperitoneally administered 1 h before regional ischemia. The heart was exposed by a median sternot-

omy and the left anterior descending artery was snare-occluded for 30 min and then reperfused for 3 h. After injection of 1% Evans blue, rats were sacrificed and the hearts were separated. The left ventricle was cut into two slices and then processed for TTC histochemistry. The infarct size was calculated as a percentage of the area at risk. Statistical significance was determined using the Student's *t*-test with a confidence interval accepted when $P < 0.05$.

4.3.5. TUNEL staining. Cardiomyocyte apoptosis was analyzed by the terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining using Apop-Tag (POD Roche Mannheim, Germany). After 3 h reperfusion, the heart was separated and subjected to the general process (dehydration, clearing, impregnation, and embedding) for the tissue preparation. The tissue was washed with 0.02 M phosphate buffered saline, at pH 7.4 and treated with proteinase K. The prepared tissue slides were treated with a mixture of 1:9 of enzyme solution of Apop-Tag kit and label solution and then left for 1 h at dark room, followed by DAPI (4,6-diamino-2-phenylindole) staining. Cells, which lay in the defined rectangular field area (20× objective) and exhibited positive staining for apoptosis, were examined and photographed using a fluorescent microscope.

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