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Synthesis, structure-activity relationships and preliminary mechanism of action of novel water-soluble 4-quinolone-3-carboxamides as antiproliferative agents

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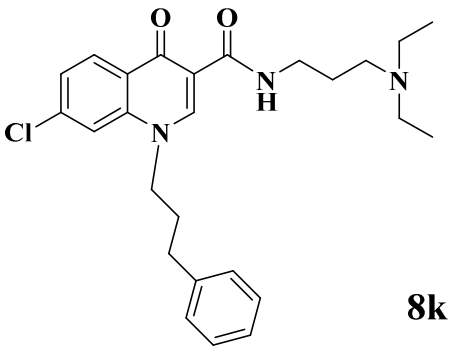
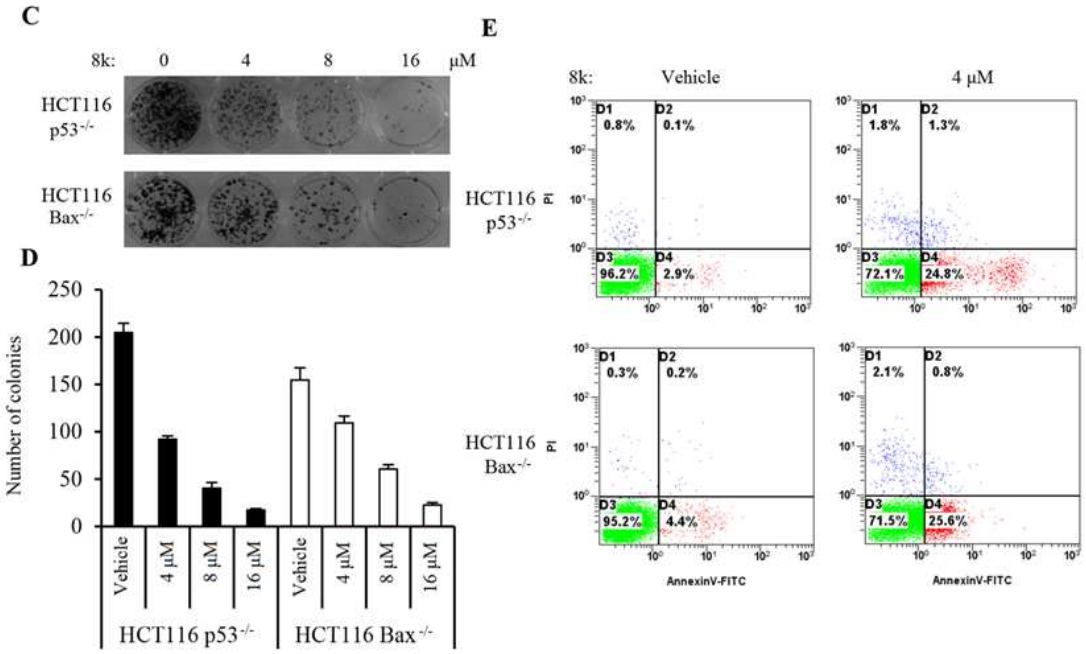
Graphic abstract



8k



A series of novel water-soluble 4-quinolone-3-carboxamides was prepared and evaluated as antitumor agents. The representative compound **8k** could trigger p53/Bax- independent colorectal cancer cell apoptosis *via* inducing ROS accumulation.

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Synthesis, structure-activity relationships and preliminary mechanism of action of novel water-soluble 4-quinolone-3-carboxamides as antiproliferative agents

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Abstract

A series of novel water-soluble 4-quinolone-3-carboxamides was prepared and evaluated as antiproliferative agents. Preliminary results indicated that most compounds tested in this study showed potent antiproliferative potencies against human tumor cell lines, and compound **8k** was found to be the most potent antiproliferative agents with IC_{50} value of lower than 10 μM against nine human tumor cell lines. These results suggested that (1) the alkylamino side chain substituent was the advisable pharmacophoric group for the enhanced antiproliferative activities; (2) the length of the alkylamino side chain moiety also affected their antiproliferative potencies, and three methylene units were more favorable; (3) introducing arylated alkyl substituent into N1-position of quinolone facilitated antiproliferative activities of this class of compounds. Further investigations on mechanism of action of this class of compound demonstrated that the representative compound **8k** could trigger p53/Bax- independent colorectal cancer cell apoptosis *via* inducing ROS accumulation.

Keywords: Synthesis; 4-quinolone; antiproliferative; structure-activity relationships; mechanism of action.

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1. Introduction

Cancer is a major public health problem worldwide. About 25% deaths in the United States is due to cancer[1]. Colorectal cancer (CRC) is the third most common cancer in the world and the fourth most frequent cause of cancer-related deaths[2]. Evasion of apoptosis is a key hallmark of cancer[3, 4]. Apoptosis is a genetically programmed cellular suicide process that can remove unwanted, redundant, or damaged cells, including cancer cells, from multicellular organisms. Induction of cell apoptosis has been a popular strategy for cancer therapy[5].

Quinolone nucleus is common to many natural and synthetic alkaloids with a broad spectrum of pharmacological properties including antibacterial, antitumor and anti-HIV activities[6-8]. Since the discovery of nalidixic acid by Lesher in 1962, many efforts have spent in searching for more potent antibacterial agents, and several generations of quinolones which exhibit excellent antibacterial activities have been discovered and used clinically in the past 40 years.

Quinolones are initially discovered to exert their antibacterial effects by inhibiting essential type II bacterial topoisomerases such as DNA gyrase[9]. Subsequently, topoisomerase IV[10] and penicillin-binding proteins (PBPs) were also found to be the pharmacological target of this class of compounds. Due to structural and functional similarities between bacterial DNA gyrase and mammalian topoisomerase II, some quinolones were found to inhibit both bacterial and mammalian topoisomerase II. Further investigation demonstrated that mammalian topoisomerase II inhibitory potency was correlated well with mammalian *in vitro*

cytotoxicity and *in vivo* antitumor activity. Consequently, numerous interests in quinolones have been stimulated by their potential antitumor activities, and a large number of quinolone derivatives acting as antitumor agents were reported [11-15].

7-Chloro-4(1H)-quinolone **1** (**Fig. 1**), a typical quinolone, was discovered as potent antitumor agent by inhibiting DNA synthesis and damaging DNA template of tumor cell. Its capsule was approved by the State Food and Drug Administration of China (SFDA) as antitumor drug for late mammary cancer and non-small cell lung cancer treatment in 2003[16]. Compound **1** is characteristic of low toxicity, ease of administration and cheap synthesis, however, the poor solubility of compound **1** restricted its utility for cancer therapy.

Our previous investigations[17-19] demonstrated that introducing a flexible amino side chain into many lead compounds contributed to the development of potential bioactive agents with good water solubility. As a part of our efforts to develop new quinolones as antitumor agents, we described the synthesis and antiproliferative evaluation of new quinolones bearing an alkyl substituent at N1-position and a flexible alkylamino side chain at position-3. Our primary objectives are (1) to improve the water solubility of quinolones; (2) to enhance the antitumor potency of this class of compounds; (3) to elucidate the structure-activity relationships of these compounds; (4) to disclose mechanism of action of this class of compounds. To the best of our knowledge, all quinolones bearing a flexible alkylamino side chain in position-3 are novel.

2. Chemistry

The synthetic routes of novel 4(1H)-quinolones **6a-d** are outlined **Scheme 1**. The reaction of anilines **2a-q** with ethoxymethylenemalonic ester **3** afforded Schiff bases **4a-d**. Cyclization of intermediate **4a-d** in refluxing diphenyl ether gave ethyl 4(1H)-quinolone-3-carboxylates **5a-d**[20]. Amination of carboxylates **5a-d** with an excess of diamines by subjecting to microwave irradiation (150℃, 30 min) provided the amidated derivatives **6a-d**, bearing a flexible alkylamine side chain in position-3[17-19].

The reaction of ethyl 7-chloro-4(1H)-quinolone-3-carboxylate **5b** with appropriate alkyl halogenide by the action of sodium hydride in anhydrous DMF furnished ethyl 7-chloro-1-alkyl-4-quinolone-3-carboxylate **7a-f**. Amination of carboxylates **7a-f** with an excess of diamines without solvent by subjecting to microwave irradiation (150℃, 30 min) gave the amidated derivatives **8a-k** in 53-68% yield (**Scheme 1**). The chemical structure of all the newly synthesized compounds was characterized by MS, HRMS, ^{1}H NMR and ^{13}C NMR spectra.

3. Results and Discussion

3.1 Antiproliferative activity and structure-activity relationships analysis

The antiproliferative potencies of quinolone derivatives **6a-d, 7a-f, 8a-k** against a panel of human tumor cell lines were investigated and compared with the reference drugs 7-chloro-4(1H)-quinolone **1** and cisplatin. In order to improve the solubility in aqueous solution, all compounds were prepared in the form of hydrochloride by the usual methods before use. As predicted, the hydrochloride salt of novel quinolones **6a-d** and **8a-k** bearing an alkylamino side chain showed excellent water solubility

(more than 10mg/ml). The tumor cell line panel was consisted of human laryngeal carcinoma (Hep-2), breast carcinoma (MCF-7), gastric carcinoma (BGC-823), liver carcinoma (HepG2), cervical carcinoma (Hela), prostate carcinoma (PC-3) and colorectal carcinoma (HCT-8, HCT-116 and RKO). The results are summarized in **Table 1**.

As shown in **Table 1**, the lead compound 7-chloro-4(1H)-quinolone **1** failed to display antiproliferative activities against nine human tumor cell lines at the concentration of 50.0 μM, and the poor water-soluble property might be contributed to its weak activity. While compounds **6a-d**, the condensation products of compounds **5a-d** with 3-(diethylamino) propylamine, showed potent antiproliferative potencies against most of human tumor cell lines with IC_{50} values lower than 50.0 μM. The influence of substituent in position-7 of quinolone on antiproliferative activities indicated that their antiproliferative potencies followed the tendency of 7-Cl > 7-Br > 7F >7-OCH_3.

Compounds **7a-d** bearing a short alkyl group at N1-position exhibited moderate or no antiproliferative activity at the concentration of 50.0 μM. Interestingly, introducing a benzyl or (3-phenyl) propyl substituent into N1-position of ethyl 7-chloro-4(1H)-quinolones-3-carboxylate **5b** led to compounds **7e** and **7f**, respectively, which showed remarkable antiproliferative activity against most of human tumor cell lines with IC_{50} values lower than 20.0 μM. These results suggested that the N1-arylated alkyl substituent of quinolones played a very important role in the modulation of the antiproliferative potencies.

The antiproliferative potency increased greatly after converting the quinolone-3-carboxylates **7a-f** to its 3-carboxamide derivatives **8a-k**. Of all 3-carboxamide derivatives, compounds **8i-k** bearing an arylated alkyl substituent at N1 of quinolone exhibited more potent antiproliferative effects than compounds **8a-i** having a short alkyl group at N1 of quinolone. Especially, compounds, bearing a (3-phenyl) propyl group in N1 of quinolone, were found to be the most potent antiproliferative agents with IC_{50} values lower than 10.0 μM against seven human tumor cell lines investigated. The results further confirmed that the N1-arylated alkyl substituent of quinolones facilitated the antiproliferative potencies. In addition, compounds **8b**, **8d**, **8f**, **8h**, **8j** and **8k** bearing a flexible amino side chain with three methylene spacer displayed more potent antiproliferative potencies than compounds **8a**, **8c**, **8e**, **8g** and **8i** having two methylene spacer. It is worth mentioning that compounds **8j** and **8k** exhibited more potent antiproliferative activity than the positive control drug cisplatin. These results indicated that (1) the alkylamino side chain substituent was the advisable pharmacophoric group for the enhanced antiproliferative activities; (2) the length of the alkylamino side chain moiety also affected their antiproliferative potencies, and three methylene units might be more favorable.

3.2 Growth inhibition of human cancer cells by compound 8k

To test the potential application of compound **8k** in the treatment of cancers, the inhibitory effect of compound **8k** on growth of human cancer cell lines, HCT116 and RKO, was investigated using the Cell Counting Kit-8(CCK-8) assay. Compared with the vehicle treated cells, HCT116 and RKO exhibited markedly inhibited growth in a

dose-dependent manner after treatment with compound **8k** for 24h (**Fig. 2A**). The IC_{50} values of compound **8k** (the concentrations for decreasing the growth rate of cells by 50%) were 5.79±0.5 μM for HCT116, 4.87±0.5 μM for RKO. To test whether compound **8k** affected tumorigenicity-correlated responses *in vitro*，plate colony formation assay was performed (**Fig. 2B**). The colony-forming ability of HCT116 and RKO was significantly ($p<0.05$) impaired when treated with compound **8k** for 24h dose-dependently (**Fig. 2C**).

3.3 Compound 8k induced apoptosis in HCT116 and RKO

In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in the intermediate stages of apoptosis, PS is translocated from the inner to the outer leaflet of the membrane. The translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane is an early event in apoptosis [21]. To determine whether the compound **8k** induced growth inhibition of cancer cells was associated with apoptosis, Annexin V-FITC/PI binding assay was performed to examine the integrity of cell membrane and the externalization of PS. As control, the binding of Annexin V to vehicle-treated HCT116 was 1.3%, which increased to 18.2% and 38.2% when treated with 2 μM and 4 μM compound **8k** for 24 h respectively (**Fig. 3A**).

At the late stage of apoptosis, some of the fragmentized DNA leaches out when cells are fixed in ethanol and subsequently rehydrated. So these cells contain less DNA and can be observed as a hypo-diploid or 'sub-G1' peak in a DNA histogram[22]. To further confirmed 8k killed cancer cells via inducing apoptosis, we performed PI

staining assay, and the results revealed that the proportion of 'sub-G1' cells increased when HCT116 was treated with increasing dosage of compound 8k (**Fig. 3B**). In addition, the cleaved PARP(c-PARP), a marker for apoptosis, was increased along with the increasing concentrations of compound **8k** treatment (**Fig. 3C**). Together, compound **8k** killed cancer cells, at least in part, by triggering apoptosis.

3.4 Compound 8k induce apoptosis via caspase-dependent pathway

As caspases are effector molecules of the extrinsic and intrinsic apoptotic pathways[23], we next examined whether compound **8k** induce cells apoptosis via a caspase-dependent pathway. As shown in **Fig. 4A**, the increasing of Annexin V positive cells in compound **8k** treated HCT116 WT or HCT116 $Bax^{-/-}$ could be reversed by pan-caspase inhibitor Z-VAD-FMK (CASPi) pretrement. In addition, the increasing c-PARP in compound 8k treated HCT116 WT or HCT116 $Bax^{-/-}$ also were blunted by CASPi pretrement (**Fig. 4B**). These results reveal that compound **8k** induce cancer cell apoptosis at least partially depend on caspase.

3.5 Compound 8k-induced cell apoptosis was through p53- and Bax-independent mechanisms

p53 and Bax are two of the most famous pro-apoptotic genes[24]. To verified whether they were required for compound **8k** induced apoptosis, HCT116 $p53^{-/-}$ and HCT116 $Bax^{-/-}$ cells were employed. First, the knock-out of these two genes was confirmed by western blotting as in our previously paper[25]. Then compound **8k** inhibited the viability of both these cell strains was revealed by the CCK-8 assay and colony formation (**Fig. 2B** and **Fig. 4C**). Furthermore, the proportion of Annexin V positive cells were increased when treated these two cells with dosage increasing

compound **8k** (**Fig. 4D**). At last, the c-PARP also increased in both cells treated with compound **8k** (**Fig. 4E**). Collectively, these results suggest that the compound **8k** inducing cell apoptosis is independent on both p53 and Bax.

3.6 Intracellular ROS are involved in compound 8k-induced apoptosis

Some chemotherapeutic agents can induce apoptosis through ROS generation in cancer cells[26-28]. Through H2DCFDA-based flow cytometry, ROS was detected remarkably accumulation in HCT116 treated with 8 μM compound **8k** for 24h, which could be reversed by the free radical scavengers L-NAC or GSH (**Fig. 5A**). To determine whether intracellular ROS accumulation were involved in compound **8k** induced cancer cells death, colony formation was performed. Compound **8k** potently inhibited colony formation of HCT116 in a dose-dependent manner, and ROS scavengers (both GSH and L-NAC) largely alleviated this inhibition (**Fig. 5B and C**). Furthermore, CCK-8 assay indicated that when treated with 4 μM, 16 μM and 32 μM for 24h, the viability of HCT116 were significantly higher in the GSH and L-NAC protecting groups in compared to the control group (**Fig. 5D**). As expected, the c-PARP increased when HCT116 was treated with increasing of compound **8k** dosage, and there were weaker increases of the c-PARP when cells were pretreated with ROS scavenger L-NAC (**Fig. 5E**). Together, these results suggest that compound **8k** induces intracellular ROS accumulation in HCT116 which is involved in compound **8k** induced apoptosis.

4. Conclusion

With the aim of improving the pharmacokinetic properties and enhancing the

antitumor potencies of quinolone derivatives, in the present investigation, a series of water-soluble quinolone derivatives bearing an alkyl substituent at N1 and a flexible alkyl amino side chain at position-3 was synthesized and their antiproliferative potential against seven human tumor cell line in culture were investigated. Preliminary results evidenced the importance of the alkylamino side chain group in position-3 and the nature of alkyl substituent at N1. The 3-phenylpropyl substituent in position N1-position was the suitable pharmacophore giving rise to the most potent antiproliferative agents and the optimal length of the alkylamino side chain moiety was three methylene units.

In addition, we presented evidence to show that compound **8k** was effective in killing colorectal colon (CRC) cancer cells. Cell death by compound **8k** was due to apoptosis as characterized by the increased Annexin V binding, 'sub-G1' fraction and cleavage of PARP-1. Moreover, our results suggested that compound **8k** induced ROS-triggering apoptosis depended on caspase, but not p53 and Bax. Many anti-cancer agents induce cancer cell apoptosis via the p53 modulated pathway[29, 30]. However, p53 mutations are frequent not only in colorectal cancer but also in majority of other human tumors[31]. Bax belongs to the bcl-2 family, which serves as an essential effector of the mitochondrial apoptotic pathway and p53-mediated apoptosis [24, 32]. Induction p53 and/or Bax-dependent apoptosis by drugs has been a strategy to kill cancer cell[33, 34]. Similarly, Bax is another most frequently inactivated genes in CRC, which resulted from MSI caused frameshift mutations[35, 36]. Hence, it's necessary to figure out whether the compound **8k** induced apoptosis is

p53- and/or Bax-dependent or not. In our study, we have demonstrated that compound **8k** could trigger apoptosis independent on p53 and Bax. So compound **8k** may serve as a potential therapeutic drug for treating cancer with p53 and/or Bax mutation.

The modulation of intracellular ROS levels has been a selective anticancer strategy for that cancer cells have a higher basal level of ROS[28, 37, 38]. Mechanistic insights showed that compound **8k** triggered CRC apoptosis via inducing ROS accumulation. And lowering the ROS level to mimic normal cells with low basal levels of ROS by free radical scavengers protected cells from compound **8k** inducing apoptosis. Hence, compound **8k** may be a potentially selective killer of CRC. Future studies are needed to test this possibility in primary cells and to clarify the more detailed molecular mechanisms of compound **8k** in inducing apoptosis in cancer cells.

5. Experimental protocols

5.1 Reagents and general methods

All reagents were purchased from commercial suppliers and were dried and purified when necessary. Melting points were determined with a Kofler micromelting point apparatus without correction. ESI-MS spectra were obtained from VG ZAB-HS spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury-Plus 300 spectrometer at 300 MHz and 75 MHz respectively, using TMS as internal standard and $CDCl_3$ or DMSO-d_6 as solvent and chemical shifts (δ) were expressed in ppm. HRMS were obtained from ESI-Q-TOF maxis 4G spectrometer. All reactions were monitored by TLC and spots were visualized with UV light or iodine. Silica gel F254 were used in analytical thin-layer chromatography (TLC) and silica gel were used in

column chromatography respectively.

5.2 General procedure for compounds 5a-d

A mixture of the respective aniline **2a-d** (100 mmol) and ethoxymethylenemalonic ester **3** (25.3 g, 110 mmol) was stirred at RT for 10 min, and then the mixture was stirred and heated at 110 ℃ for 1.5 h. The reaction mixture was evaporated in vacuum to remove the residual ethanol and provide the condensation products **4a-d**. Compounds **4a-d** prepared in these ways can be used directly in the next step without further purification.

The molten condensation products **4a-d** above (100 mmol) was poured slowly into boiling diphenyl ether (150 mL). The mixture was heated under reflux for 2 h. The solution was allowed to cool to room temperature and petroleum ether (75 mL) was added. The precipitate formed was collected by filtration, washed well with petroleum ether. It was found that the diphenyl ether could be satisfactorily removed from the product by resuspending the crude product in petroleum ether or ethanol (500 mL), stirring well, and refiltering. Compounds **5a-d** had very poor solubility even in DMSO-d_6, so it was too difficult to characterize their chemical structure by ^{1}H NMR and ^{13}C NMR spectra.

5.2.1 Ethyl 7-fluoro-4(1H)-quinolone-3-carboxylate (5a)

Starting from *m*-fluoroaniline **2a** (100 mmol), compound **5a** was obtained as white solid (16.9 g, 72%), Mp >250 ℃.

Ethyl 7-chloro-4(1H)-quinolone-3-carboxylate (5b)

Starting from *m*-chloroaniline **2b** (100 mmol), compound **5b** was obtained as

white solid (19.6 g, 78%), Mp >250 ℃.

5.2.2 Ethyl 7-bromo-4(1H)-quinolone-3-carboxylate (5c)

Starting from *m*-bromoaniline **2c** (100 mmol), compound **5c** was obtained as white solid (22.2 g, 75%), Mp >250 ℃.

5.2.3 Ethyl 7-methoxy-4(1H)-quinolone-3-carboxylate (5d)

Starting from *m*-methoxyaniline **2d** (100 mmol), compound **5d** was obtained as white solid (16.3 g, 66%), Mp >250 ℃.

5.3 General procedure for compounds 6a-d

The mixture of the corresponding ethyl 7-substituted-4(1H)-quinolone-3-carboxylates **5a-d** (5.0 mmol) and 3-(diethylamino) propylamine (5.0 ml) was stirred under microwave irradiation at 150 ℃ for 30 min. The resulting solution was partitioned between water and methylene chloride. The aqueous phase was extracted with methylene chloride and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, evaporated. The residue oil was purified by a column chromatography, using a mixture of chloroform and methanol 50:1 as an eluent, to successfully afford the desirable products.

5.3.1 N-[3-(diethylamino)propyl]-7-fluoro-4(1H)-quinolone-3-carboxamide (6a)

Starting from compound **5a** (5.0 mmol), compound **6a** was isolated as white solid (0.72 g, 45%). Mp 188.4-189.6 ℃. ESI-MS m/z 320 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.45 (s, 1H, CON***H***), 8.82 (s, 1H, Ar***H***), 8.35-8.40 (m, 1H, Ar***H***), 7.23-7.27 (m, 1H, Ar***H***), 7.08-7.14 (m, 1H, Ar***H***), 3.56 (q, J =5.1Hz, 2H, NHC***H***$_2$CH$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.53-2.60 (m, 6H, NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$),

1.78-1.88 (m, 2H, $NHCH_2C\boldsymbol{H}_2CH_2N(CH_2CH_3)_2$), 1.02 (t, *J* = 6.9 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$) ***δ*** 176.6, 166.4, 163.4, 144.6, 141.8, 129.1, 123.8, 114.2, 111.3, 104.8, 50.5, 47.0, 38.0, 27.4, 11.6. HRMS (ESI) calcd for $C_{17}H_{22}FN_3O_2$ $[M+H]^+$ 320.1769, found 320.1769.

5.3.2 7-Chloro-N-[3-(diethylamino)propyl]-4(1H)-quinolone-3-carboxamide (6b)

Starting from compound **5b** (5.0 mmol), compound **6b** was isolated as white solid (0.87 g, 52%). Mp 197.2-198.0 ℃. ESI-MS m/z 336 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$): *δ* 10.48 (s, 1H, CON***H***), 8.84 (s, 1H, Ar***H***), 8.34 (d, *J* = 8.7 Hz, 1H, Ar***H***), 7.63 (d, *J* = 1.5 Hz, 1H, Ar***H***), 7.36 (dd, *J* = 8.7, 1.5 Hz, 1H, Ar***H***), 3.55 (q, *J* =5.1 Hz, 2H, $NHC\boldsymbol{H}_2CH_2CH_2N(CH_2CH_3)_2$), 2.58-2.65 (m, 6H, $NHCH_2CH_2C\boldsymbol{H}_2N(C\boldsymbol{H}_2CH_3)_2$), 1.78-1.94 (m, 2H, $NHCH_2C\boldsymbol{H}_2CH_2N(CH_2CH_3)_2$), 1.07 (t, *J* = 7.2 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.7, 166.4, 144.6, 140.9, 138.8, 127.9, 125.8, 125.4, 118.9, 111.5, 50.5, 47.1, 38.1, 27.4, 11.6. HRMS (ESI) calcd for $C_{17}H_{22}ClN_3O_2$ $[M+H]^+$ 336.1473, found 336.1477.

5.3.3 7-Bromo-N-[3-(diethylamino)propyl]-4(1H)-quinolone-3-carboxamide (6c)

Starting from compound **5c** (5.0 mmol), compound **6c** was isolated as white solid (0.93 g, 49%). Mp 187.2-187.9 ℃. ESI-MS m/z 380 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$): *δ* 10.52 (s, 1H, CON***H***), 8.84 (s, 1H, Ar***H***), 8.26 (d, *J* = 8.7 Hz, 1H, Ar***H***), 7.81 (d, *J* = 1.5 Hz, 1H, Ar***H***), 7.51 (dd, *J* = 8.7, 1.5 Hz, 1H, Ar***H***), 3.56 (q, *J* =5.1 Hz, 2H, $NHC\boldsymbol{H}_2CH_2CH_2N(CH_2CH_3)_2$), 2.58-2.65 (m, 6H, $NHCH_2CH_2C\boldsymbol{H}_2N(C\boldsymbol{H}_2CH_3)_2$), 1.82-1.91 (m, 2H, $NHCH_2C\boldsymbol{H}_2CH_2N(CH_2CH_3)_2$), 1.06 (t, *J* = 7.2 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8, 166.5, 144.5, 141.1, 128.6, 127.9,

127.2, 125.8, 122.1, 111.5, 50.6, 47.1, 38.1, 27.4, 11.7. HRMS (ESI) calcd for $C_{17}H_{22}BrN_3O_2$ $[M+H]^+$ 380.0968, found 380.0973.

5.3.4 N-[3-(diethylamino)propyl]-7-methoxy-4-(1H)-quinolone-3-carboxamide (6d)

Starting from compound **5d** (5.0 mmol), compound **6d** was isolated as white solid (0.96 g, 58%). Mp 141.6-142.3 ℃. ESI-MS m/z 332 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.50 (s, 1H, CON***H***), 8.78 (s, 1H, Ar***H***), 8.30 (d, *J* = 9.0 Hz, 1H, Ar***H***), 7.00 (dd, *J* = 9.0, 1.8 Hz, 1H, Ar***H***), 6.91 (d, *J* = 1.8 Hz, 1H, Ar***H***), 3.89 (s, 3H, C***H***$_3$O), 3.54 (q, *J* =5.4Hz, 2H, NHC***H***$_2$CH$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.51-2.59 (m, 6H, NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 1.78-1.87 (m, 2H, NHCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 1.02 (t, *J* = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$) ***δ*** 176.6, 166.3, 162.9, 143.1, 141.1, 127.6, 120.7, 115.8, 110.8, 99.7, 55.6, 50.3, 46.7, 37.8, 27.2, 11.4. HRMS (ESI) calcd for $C_{18}H_{25}N_3O_3$ $[M+H]^+$ 332.1969, found 332.1977.

5.4 General procedure for compounds 7a-f

A mixture of ethyl 7-chloro-4(1H)-quinolone-3-carboxylate **5b** (10 mmol) and anhydrous DMF (60 mL) was stirred at RT for 10 min, and then 60% NaH (1.2 g, 30 mmol) and alkyl halogenide (15-30 mmol) were added. The mixture was continued to stir at RT. After completion of the reaction as indicated by TLC, the solution was poured into H_2O (150 mL), and extracted with ethyl acetate. The organic phase was made acidic with concentrated hydrochloric acid. Upon removal of solvent, the residue was crystallized from acetone to afford yellow solid. The solid was dissolved in water and made basic with sodium bicarbonate, and the aqueous mixture extracted with ethyl acetate. The organic phase was washed with water and brine, then dried

over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by a column chromatography, using a mixture of ethyl acetate and petroleum ether (3:1) as an eluent, to successfully afford the desirable products in good yield.

5.4.1 Ethyl 7-chloro-1-methyl-4-quinolone-3-carboxylate (7a)

Starting from compound **5b** (10 mmol) and iodomethane (15 mmol), compound **7a** was isolated as white solid (1.7 g, 65%). Mp 178.8-180.2 ℃. ESI-MS m/z 288 $[M+Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 8.42-8.45 (m, 2H, Ar***H***)), 7.38-7.41 (m, 2H, Ar***H***), 4.39 (q, J = 7.2 Hz, 2H, OC***H***$_2$CH$_3$), 3.86 (s, 3H, NC***H***$_3$), 1.42 (t, J = 7.2 Hz, 3H, OCH$_2$C***H***$_3$). ^{13}C NMR (75MHz, $CDCl_3$): δ 172.9, 164.9, 151.2, 141.3, 138.2, 128.8, 127.1, 125.8, 117.7, 110.8, 60.6, 41.8, 15.1. HRMS (ESI) calcd for $C_{13}H_{12}ClNO_3$ $[M+Na]^+$ 288.0398, found 288.0397.

5.4.2 Ethyl 7-chloro-1-ethyl-4-quinolone-3-carboxylate (7b)

Starting from compound **5b** (10 mmol) and iodoethane (15 mmol), compound **7c** was isolated as white solid (1.9 g, 69%). Mp 163.8-164.4 ℃. ESI-MS m/z 302 $[M+Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 8.44-8.47 (m, 2H, Ar***H***), 7.25-7.43 (m, 2H, Ar***H***), 4.39 (q, J =7.2Hz, NC***H***$_2$CH$_3$), 4.21(q, J =7.2Hz, OC***H***$_2$CH$_3$), 1.56 (t, J =7.2Hz, NCH$_2$C***H***$_3$), 1.41(t, J =7.2Hz, OCH$_2$C***H***$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 165.2, 148.9, 139.4, 139.1, 129.5, 127.5, 125.5, 115.8, 111.5, 61.1, 49.2, 14.7. HRMS (ESI) calcd for $C_{14}H_{14}ClNO_3$ $[M+Na]^+$ 302.0554, found 302.0564.

5.4.3 Ethyl 1-allyl-7-chloro-4-quinolone-3-carboxylate (7c)

Starting from compound **5b** (10 mmol) and allyl bromide (15 mmol), compound **7c** was isolated as white solid (2.2 g, 75%). Mp 188.0-189.6 ℃. ESI-MS m/z 314

$[M+Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 8.48 (s, 1H, Ar***H***), 8.42 (d, *J* =8.7Hz, 1H, Ar***H***), 7.34-7.39 (m, 2H, Ar***H***), 5.94-5.07(m, 1H, $CH_2C\boldsymbol{H}=CH_2$), 5.40 (d, *J* = 10.5 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 5.20 (d, *J* = 17.1 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 4.79 (m, 2H, $C\boldsymbol{H}_2CH=CH_2$), 4.38 (q, *J* = 6.9Hz, 2H, $OC\boldsymbol{H}_2CH_3$), 1.41 (t, *J* = 6.9 Hz, 3H, $OCH_2C\boldsymbol{H}_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.8, 165.2, 149.7, 140.0, 139.2, 130.5, 129.4, 127.3, 125.8, 119.8, 116.5, 111.6, 61.2, 56.2, 14.8. HRMS (ESI) calcd for $C_{15}H_{14}ClNO_3$ $[M+Na]^+$ 314.0565, found 314.0565.

5.4.4 Ethyl 1-butyl-7-chloro-4-quinolone-3-carboxylate (7d)

Starting from compound **5b** (10 mmol) and n-iodobutane (30 mmol), compound **7d** was isolated as white solid (1.9 g, 62%). Mp 105.8-106.7 ℃. ESI-MS m/z 330 $[M+Na]^+$. ^1H NMR (300MHz, DMSO-d_6): δ 8.62 (s, 1H, Ar***H***), 8.17 (d, *J* =8.7Hz, 1H, Ar***H***), 7.87 (d, *J* =1.8 Hz, 1H, Ar***H***), 7.47 (dd, *J* =8.7Hz, 1.8Hz, 1H, Ar***H***), 4.33 (t, *J*=7.2 Hz, 2H, $NC\boldsymbol{H}_2CH_2CH_2CH_3$), 4.20 (q, *J*=7.2Hz, 2H, $OC\boldsymbol{H}_2CH_3$), 1.64-1.73 (m, 2H, $NCH_2C\boldsymbol{H}_2CH_2CH_3$), 1.24-1.34 (m, 5H, $CH_2CH_2C\boldsymbol{H}_2CH_3$, $OCH_2C\boldsymbol{H}_3$), 0.88 (t, *J*=7.2 Hz, 3H, $NCH_2CH_2CH_2C\boldsymbol{H}_3$). ^{13}C NMR (75MHz, $CDCl_3$): δ 172.8, 165.0, 150.5, 140.3, 138.4, 129.2, 127.5, 125.8, 117.4, 111.1, 60.7, 53.3, 31.3, 19.9, 15.1, 14.3. HRMS (ESI) calcd for $C_{16}H_{16}ClNO_3$ $[M+Na]^+$ 330.0867, found 330.0870.

5.4.5 Ethyl 1-benzyl-7-chloro-4-quinolone-3-carboxylate (7e)

Starting from compound **5b** (5mmol) and benzyl bromide (15mmol), compound **7e** was isolated as white solid (2.9 g, 85%). Mp 147.9-148.8 ℃. ESI-MS m/z 364 $[M+Na]^+$. ^1H NMR (300MHz, DMSO-d_6): δ 8.54 (s, 1H, Ar***H***), 8.43 (d, *J*=8.1Hz, 1H, Ar***H***), 7.30-7.38 (m, 5H, Ar***H***), 7.14-7.18 (m, 2H, Ar***H***), 5.34 (s, 2H, $C\boldsymbol{H}_2Ph$), 4.38 (q,

J=7.2 Hz, 2H, OC***H***$_2$CH$_3$), 1.41 (t, *J*=7.2Hz, 3H, OCH$_2$C***H***$_3$). ^{13}C NMR (75MHz, $CDCl_3$) δ 173.6, 165.0, 150.0, 140.1, 139.1, 134.0, 129.5, 129.4, 128.8, 127.5, 126.4, 125.7, 116.7, 111.7, 61.1, 57.5, 14.8. HRMS (ESI) calcd for $C_{19}H_{16}ClNO_3$ $[M+Na]^+$ 364.0711, found 364.0714.

5.4.6 Ethyl 7-chloro-1-(3-phenylpropyl)-4-quinolone-3-carboxylate (7f)

Starting from compound **5b** (10 mmol) and 1-(3-bromopropyl)benzene (50 mmol), compound **7f** was isolated as white solid (2.4 g, 65%). Mp 136.6-137.8 ℃. ESI-MS m/z 392 $[M+Na]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.44 (d, *J* = 8.7 Hz, 1H, Ar***H***), 8.37 (s, 1H, Ar***H***), 7.43-7.15 (m, 7H, Ar***H***), 4.40 (q, *J* = 7.2 Hz, 2H, OC***H***$_2$CH$_3$), 4.10 (t, *J* = 7.2 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$Ph), 2.77 (t, *J* = 7.2 Hz, 2H, NCH$_2$CH$_2$C***H***$_2$Ph), 2.32–2.20 (m, 2H, NCH$_2$C***H***$_2$CH$_2$Ph), 1.42 (t, *J* = 7.2 Hz, 3H, OCH$_2$C***H***$_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2, 164.9, 149.1, 139.2, 139.1, 138.8, 129.4, 128.6, 128.1, 127.9, 127.3, 126.5, 125.3, 115.3, 111.2, 60.8, 52.9, 32.3, 29.6, 14.4. HRMS (ESI) calcd for $C_{21}H_{20}ClNO_3$ $[M+Na]^+$ 392.1024, found 392.1032.

5.5 General procedure for compounds 8a-k

The mixture of the corresponding 1-substituted ethyl 7-chloro-4-quinolone-3-carboxylate (5.0 mmol) and alkylamines (5.0 mL) was stirred under microwave irradiation at 150℃ for 15 min. The resulting solution was partitioned between water and methylene chloride. The aqueous phase was extracted with methylene chloride and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, evaporated. The crude oil was purified by a column chromatography, using a mixture of chloroform and methanol 50:1 as an eluent, to

successfully afford the desirable products.

5.5.1 7-Chloro-N-[2-(diethylamino)ethyl]-1-methyl-4-quinolone-3-carboxamide (8a)

Starting from compound **7a** (5.0 mmol) and N,N-diethylethylenediamine (5.0 mL), compound **8a** was isolated as white solid (1.1 g, 68%). Mp 163.6-163.7 ℃. ESI-MS m/z 336 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.92 (s, 1H, CON***H***), 8.73 (s, 1H, Ar***H***), 8.45 (d, J = 8.4 Hz, 1H, Ar***H***), 7.43-7.50 (m, 2H, Ar***H***), 3.91 (s, 3H, NC$\boldsymbol{H}_3$), 3.55 (q, J =6.3 Hz, 2H, NHC$\boldsymbol{H}_2$$CH_2N(CH_2CH_3)_2$), 2.61-2.67 (m, 6H, $NCH_2CH_2C\boldsymbol{H}_2N(C\boldsymbol{H}_2CH_3)_2$), 1.79-1.91(m, 2H, $NCH_2C\boldsymbol{H}_2CH_2N(CH_2CH_3)_2$), 1.08 (t, J = 7.2 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.0, 164.6, 148.8, 140.7, 139.5, 129.1, 126.3, 125.9, 116.0, 112.7, 52.1, 47.5, 41.8, 37.7, 12.2. HRMS (ESI) calcd for $C_{17}H_{22}ClN_3O_2$ $[M+H]^+$ 336.1473, found 336.1476.

5.5.2 7-Chloro-N-[3-(diethylamino)propyl]-1-methyl-4-quinolone-3-carboxamide (8b)

Starting from compound **7a** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8b** was isolated as white solid (0.9 g, 54%). Mp 142.7-143.1 ℃. ESI-MS m/z 350 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ .92 (s, 1H, CON***H***), 8.73 (s, 1H, Ar***H***), 8.45 (d, J = 8.7 Hz, 1H, Ar***H***), 7.43-7.50 (m, 2H, Ar***H***), 3.91 (s, 3H, NC$\boldsymbol{H}_3$), 3.55 (q, J =6.3 Hz, 2H, NHC$\boldsymbol{H}_2$$CH_2N(CH_2CH_3)_2$), 2.61-2.67 (m, 6H, $NCH_2CH_2C\boldsymbol{H}_2N(C\boldsymbol{H}_2CH_3)_2$), 1.79-1.91(m, 2H, $NCH_2C\boldsymbol{H}_2CH_2$ $N(CH_2CH_3)_2$), 1.08 (t, J = 7.2 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.1, 164.6, 148.9, 140.7, 139.6, 129.0, 125.9, 116.0, 112.7, 50.6, 47.1, 41.8, 38.0, 27.4, 11.9. HRMS (ESI) calcd for $C_{18}H_{24}ClN_3O_2$ $[M+H]^+$ 350.1630, found 350.1638.

5.5.3 7-Chloro-N-[2-(diethylamino)ethyl]-1-ethyl-4-quinolone-3-carboxamide (8c)

Starting from compound **7b** (5.0 mmol) and N,N-diethylethylenediamine (5.0 mL), compound **8c** was isolated as white solid (1.0 g, 58%). Mp 154.0-154.8 ℃. ESI-MS m/z 350 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.95 (s, 1H, CON***H***), 8.77 (s, 1H, Ar***H***), 8.44 (d, *J*=8.4Hz, 1H, Ar***H***), 7.50 (d, *J* =1.5Hz, Ar***H***), 7.43(d, *J* =8.4Hz, 1.5 Hz, Ar***H***), 4.29 (q, *J* =7.2Hz, 2H, NC***H***$_2$CH$_3$), 3.55 (q, *J* =6.6 Hz, 2H, NHC***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.82 (t, *J* =6.9Hz, 2H, NCH$_2$C***H***$_2$N(CH$_2$CH$_3$)$_2$), 2.64 (q, *J* =7.2Hz, 4H, N(C***H***$_2$CH$_3$)$_2$), 1.57 (t, *J* =7.2Hz, 3H, NCH$_2$C***H***$_3$), 1.10 (t, *J* =7.2Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.9, 164.7, 147.6, 139.6, 139.4, 129.3, 126.6, 125.6, 115.7, 112.9, 52.1, 49.3, 47.5, 37.7, 14.8, 12.3. HRMS (ESI) calcd for $C_{18}H_{24}ClN_3O_2$ $[M+H]^+$ 350.1630, found 350.1636.

5.5.4 7-Chloro-N-[3-(diethylamino)propyl]-1-ethyl-4-quinolone-3-carboxamide (8d)

Starting from compound **7b** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8d** was isolated as white solid (1.06 g, 57%). Mp 127.4-128.5 ℃. ESI-MS m/z 364 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.92 (s, 1H, CON***H***), 8.77 (s, 1H, Ar***H***), 8.45 (d, *J* = 8.7 Hz, 1H, Ar***H***), 7.51 (d, *J* = 1.8 Hz, 1H, Ar***H***), 7.43 (dd, *J* = 8.7, 1.8 Hz, 1H, Ar***H***), 4.28 (q, *J* = 7.2 Hz, 2H, NC***H***$_2$CH$_3$), 3.49 (q, *J* = 6.9 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.54 (m, 6H, NCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 1.86-1.74 (m, 2H, NCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 1.57 (t, *J* = 7.2 Hz, 3H, NCH$_2$C***H***$_3$), 1.04 (t, *J* = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.0, 164.6,

147.7, 139.5, 129.3, 126.6, 125.7, 115.7, 112.9, 50.7, 49.4, 47.2, 38.0, 27.6, 14.9, 12.1. HRMS (ESI) calcd for $C_{19}H_{26}ClN_3O_2$ $[M+H]^+$ 364.1786, found 364.1791.

5.5.5 1-Allyl-7-chloro-N-[2-(diethylamino)ethyl]-4-quinolone-3-carboxamide (8e)

Starting from compound **7c** (5.0 mmol) and N,N-diethylethylenediamine (5.0 mL), compound **8e** was isolated as white solid (0.96 g, 53%). Mp 149.8-150.8 ℃. ESI-MS m/z 362 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.00 (s, 1H, CON***H***), 8.75 (s, 1H, Ar***H***), 8.44 (d, J = 8.7 Hz, 1H, Ar***H***), 7.46 (d, J = 1.5 Hz, 1H, Ar***H***), 7.42 (dd, J = 8.7, 1.5 Hz, 1H, Ar***H***), 5.94-6.07 (m, 1H, $CH_2C\boldsymbol{H}=CH_2$), 5.40 (d, J = 10.2 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 5.20 (d, J = 17.1 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 4.81-4.84 (m, 2H, $C\boldsymbol{H}_2CH=CH_2$), 3.64 (q, J = 6.6 Hz, 2H, $NHC\boldsymbol{H}_2CH_2N(C\boldsymbol{H}_2CH_3)_2$), 2.82 (t, J = 6.6 Hz, 2H, $NHCH_2C\boldsymbol{H}_2N(CH_2CH_3)_2$), 2.74 (q, J = 6.9 Hz, 4H, $N(C\boldsymbol{H}_2CH_3)_2$), 1.17 (t, J = 7.2 Hz, 6H, $N(CH_2C\boldsymbol{H}_3)_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.0, 164.6, 148.3, 140.1, 139.4, 130.3, 129.1, 126.4, 125.8, 119.9, 116.4, 112.9, 56.2, 52.1, 47.5, 37.8. 12.2. HRMS (ESI) calcd for $C_{19}H_{24}ClN_3O_2$ $[M+H]^+$ 362.1630, found 362.1639.

5.5.6 1-Allyl-7-chloro-N-[3-(diethylamino)propyl]-4-quinolone-3-carboxamide (8f)

Starting from compound **7c** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8f** was isolated as white solid (1.24 g, 66%). Mp 121.9-122.3 ℃. ESI-MS m/z 376 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.91 (s, 1H, CON***H***), 8.76 (s, 1H, Ar***H***), 8.45 (d, J = 8.7 Hz, 1H, Ar***H***), 7.46 (d, J = 1.8 Hz, 1H, Ar***H***), 7.40-7.44 (m, 1H, Ar***H***), 5.94-6.07 (m, 1H, $CH_2C\boldsymbol{H}$=CH), 5.40 (d, J = 10.5 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 5.20 (d, J = 17.1 Hz, 1H, $CH_2CH=C\boldsymbol{H}_2$), 4.81-4.83 (m, 2H, $C\boldsymbol{H}_2CH=CH_2$), 3.50 (q, J = 6.6 Hz, 2H, $NHC\boldsymbol{H}_2CH_2CH_2N(CH_2CH_3)_2$), 2.56-2.63 (m, 6H,

NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 1.79-1.89 (m, 2H, NHCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 1.08 (t, *J* = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 171.4, 159.8, 143.4, 135.4, 134.7, 125.5, 124.4, 121.7, 121.1, 115.3, 111.7, 108.3, 51.5, 46.0, 42,5, 33.3, 22.8, 7.4. HRMS (ESI) calcd for $C_{20}H_{26}ClN_3O_2$ $[M+H]^+$ 376.1786, found 376.1793.

5.5.7 1-Butyl-7-chloro-N-[2-(diethylamino)ethyl]-4-quinolone-3-carboxamide (8g)

Starting from compound **7d** (5.0 mmol) and N,N-diethylethylenediamine (5.0 mL), compound **8g** was isolated as white solid (1.23g, 65%). Mp 168.3-168.5 ℃. ESI-MS m/z 378 $[M+H]^+$. ^{1}H NMR (300 MHz, CDCl$_3$): *δ* 9.96 (s, 1H, CON***H***), 8.72 (s, 1H, Ar***H***), 8.46 (d, *J* = 8.7 Hz, 1H, Ar***H***), 7.49 (d, *J* = 1.5 Hz, 1H, Ar***H***), 7.43 (dd, *J* = 8.7, 1.5 Hz, 1H, Ar***H***), 4.20 (t, *J* = 7.2 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$CH$_3$), 3.58 (q, *J* = 6.6 Hz, 2H, NHC***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.73 (t, *J* = 6.6 Hz, 2H, NHCH$_2$C***H***$_2$N(CH$_2$C*H*$_3$)$_2$), 2.66 (q, *J* =6.9Hz, 4H, N(C***H***$_2$CH$_3$)$_2$), 1.84-1.94 (m, 2H, NCH$_2$C***H***$_2$CH$_2$CH$_3$), 1.40-1.52 (m, 2H, NCH$_2$CH$_2$C***H***$_2$CH$_3$), 1.10 (t, *J* =7.2Hz, 6H, N(CH$_2$C***H***$_3$)$_2$), 1.01 (t, *J* = 7.2 Hz, 3H, NCH$_2$CH$_2$CH$_2$C***H***$_3$). ^{13}C NMR (75 MHz, CDCl$_3$): *δ* 175.9, 164.7, 148.2, 139.9, 139.4, 129.4, 126.7, 125.7, 115.8, 112.7, 54.3, 52.2, 47.6, 37.8, 31.2, 20.3, 13.9, 12.3. HRMS (ESI) calcd for $C_{20}H_{28}ClN_3O_2$ $[M+H]^+$ 378.1943, found 378.1951.

5.5.8 1-Butyl-7-chloro-N-[3-(diethylamino)propyl]-4-quinolone-3-carboxamide (8h)

Starting from compound **7d** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8h** was isolated as white solid (1.22 g, 63%). Mp 132.0-132.4 ℃. ESI-MS m/z 392 $[M+H]^+$. ^{1}H NMR (300 MHz, CDCl$_3$): *δ* 9.96 (s, 1H, CON***H***), 8.72 (s, 1H, Ar***H***), 8.45 (d, *J* = 8.7 Hz, 1H, Ar***H***), 7.49 (d, *J* = 1.5 Hz, 1H, Ar***H***), 7.43 (dd,

J = 8.7, 1.5 Hz, 1H, Ar***H***), 4.21 (t, *J* = 7.5 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$CH$_3$), 3.50 (q, *J*=6.6Hz, 2H, NHC***H***$_2$CH$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.65-2.71 (m, 6H, NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 1.84-1.94 (m, 4H, NCH$_2$C***H***$_2$CH$_2$CH$_3$, NHCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$,), 1.40-1.52 (m, 2H, NCH$_2$CH$_2$C***H***$_2$CH$_3$), 1.13 (t, *J* = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$), 1.02 (t, *J* = 7.2 Hz, 3H, NCH$_2$CH$_2$CH$_2$C***H***$_3$). ^{13}C NMR (75 MHz, CDCl$_3$): *δ* 176.0, 164.6, 148.2, 139.9. 139.4, 129.3, 126.6, 125.7, 115.9, 112.7, 54.3, 50.8, 47.2, 38.1, 31.2, 27.6, 20.3, 14.0, 12.1. HRMS (ESI) calcd for $C_{21}H_{30}ClN_3O_2$ $[M+H]^+$ 392.2099, found 392.2109.

5.5.9 1-Benzyl-7-chloro-N-[2-(diethylamino)ethyl]-4-quinolone-3-carboxamide (8i)

Starting from compound **7e** (5mmol) and N,N-diethylethylenediamine (5.0 mL), compound **8i** was isolated as white solid (1.4 g, 68%). Mp 204.2-205.2 ℃. ESI-MS m/z 412 $[M+H]^+$. ^{1}H NMR (300 MHz, CDCl$_3$): *δ* 9.94 (s, 1H, CON***H***), 8.88 (s, 1H, Ar***H***), 8.45 (d, *J* = 9.0 Hz, 1H, Ar***H***), 7.32-7.39 (m, 5H, Ar***H***), 7.15-7.17 (m, 2H, Ar***H***), 5.40 (s, 2H, C***H***$_2$Ph), 3.57 (q, *J* = 6.6 Hz, 2H, CONHC***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$)), 2.72 (t, *J* = 6.6 Hz, 2H, CONHCH$_2$C***H***$_2$ N(CH$_2$CH$_3$)$_2$), 2.63 (q, *J* = 7.2 Hz, 4H, N(C***H***$_2$CH$_3$)$_2$), 1.09 (t, *J* = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75MHz, CDCl$_3$): *δ* 176.1, 164.6, 148.9, 140.2, 139.4, 134.0, 129.6, 129.2, 128.9, 126.7, 126.4, 125.6, 116.7, 113.0, 57.8 52.1, 47.6, 37.8, 12.3. HRMS (ESI) calcd for $C_{23}H_{26}ClN_3O_2$ $[M+H]^+$ 412.1798, found 412.1798.

5.5.10 1-Benzyl-7-chloro-N-[2-(diethylamino)propyl]-4-quinolone-3-carboxamide (8j)

Starting from compound **7e** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8j** was isolated as white solid (1.2 g, 56%). Mp 182.3-183.2 ℃. ESI-MS m/z 426 $[M+H]^+$. ^{1}H NMR (300MHz, DMSO-d_6): δ 10.04 (s, 1H, CON***H***), 8.85(1H, s, Ar***H***), 8.41 (d, J=9.0Hz, 1H, Ar***H***), 7.34-7.40(m, 5H, Ar***H***), 7.14-7.16(m, 2H, Ar***H***), 5.42 (s, 2H, C***H***$_2$Ph), 3.55 (q, J =6.3Hz, 2H, NHC***H***$_2$CH$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 2.90-3.01 (m, 6H, NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 1.82-1.87(m, 2H, NHCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 1.29 (t, J =7.2Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75MHz, $CDCl_3$): δ 176.2, 164.5, 149.0, 140.2, 139.5, 133.9, 129.6, 129.2, 129.0, 126.6, 126.4, 125.9, 116.7, 113.0, 57.9, 50.7, 47.2, 38.1, 27.5, 12.0. HRMS (ESI) calcd for $C_{24}H_{28}ClN_3O_2$ $[M+H]^+$ 426.1943, found 426.1945.

5.5.11 7-Chloro-N-[3-(diethylamino)propyl]-1-(3-phenylpropyl)-4-quinolone-3-carboxamide (8k)

Starting from compound **7f** (5.0 mmol) and 3-(diethylamino)propylamine (5.0 mL), compound **8k** was isolated as white solid (1.4 g, 63%). Mp 136.2-136.7 ℃. ESI-MS m/z 454 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.91 (s, 1H, CON***H***), 8.71 (s, 1H, Ar***H***), 8.43 (d, J = 6.6 Hz, 1H, Ar***H***), 7.41-7.19 (m, 7H, Ar***H***), 4.16 (t, J =6.0 Hz, 2H, NC***H***$_2$CH$_2$CH$_2$Ph), 3.48 (q, J = 4.8 Hz, 2H, NHC***H***$_2$CH$_2$CH$_2$), 2.76 (t, J =5.4 Hz, 2H, NCH$_2$CH$_2$C***H***$_2$Ph), 2.51-2.56 (m, 6H, NHCH$_2$CH$_2$C***H***$_2$N(C***H***$_2$CH$_3$)$_2$), 2.19-2.27 (m, 2H, NCH$_2$C***H***$_2$CH$_2$Ph), 1.75-1.82 (m, 2H, NHCH$_2$C***H***$_2$CH$_2$N(CH$_2$CH$_3$)$_2$), 1.02 (t, J = 7.2 Hz, 6H, N(CH$_2$C***H***$_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.8, 164.5, 148.0, 139.6, 139.4, 129.1, 128.9, 128.3, 126.7, 126.4, 125.6, 115.6, 112.6, 53.4, 50.5, 47.0, 37.9, 32.7, 30.2, 27.3, 11.8. HRMS (ESI) calcd

for $C_{26}H_{32}ClN_3O_2$ $[M+H]^+$ 454.2256, found 454.2259.

5.6 General procedure for the hydrochloride salt of 6a-d, 7a-f and 8a-k.

The target compounds **6a-d**, **7a-f** and **8a-k 5b** (2 mmol) was dissolved in CH_2Cl_2 (20 mL) and stirred at RT for 5 min, respectively. Then 4M HCl/ethanol (10 mL) was added, and the mixture was stirred at RT for 30 min, then removed the solvent under reduced pressure to obtain the target hydrochloride salt as yellow solid.

5.7 Cell lines and cell culture

The Human colon cancer cells (Hep-2, MCF-7, BGC-823, HepG2, Hela, PC-3, HCT-8, HCT116 and RKO) were purchase from American type culture collection (ATCC, Rockville, MD). HCT116 *p53*$^{-/-}$ and HCT116 *Bax*$^{-/-}$ were kindly provided by Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University Medical Institutions, Baltimore). All cells were cultured in McCoy's 5A medium (AppliChem) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 mg·mL^{-1} streptomycin and 100 units·mL^{-1} penicillin at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air.

5.8 Cell viability assay

Cells were seeded into 96-well flat-bottom plates at a density of 3×10^4 per well and cultured for 24 h, then treated with increasing dosage of compound **8k** for the 24 h. The cells viability was assayed using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) cells were incubated with 10 μl of CCK-8 solution for 1 h at 37, according to the manufacturer's instruction. Cell viability was then determined by a spectrophotometer set (ELx800, BioTek, USA) at a wavelength of

450 nm.

5.9 Colony formation assay

After being seeded into 12-well plates at a density of 300 per well for 48 h, cells were treated with indicated does of compound **8k**. After 24 h, the medium with drug were replaced with fresh medium. About 10 days later, cell colonies were stained with crystal violet for 15 min and photographed. Finally, the numbers of colonies were scored.

5.10 Annexin V-FITC/PI binding assay

Cells were plated in 6-well plates at a density of 5×10^5, and the cells were treated with compound **8k** after 80% confluence. After 24 h, cells were collected with trypsin and washed with ice-cold PBS buffer (137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for twice and resuspended in 100 μL binding buffer. Then, 5 μL Annexin V-FITC and 5 μL PI (FITC Annexin V Apoptosis Detection Kit I (BD, CAT#556547) were added to the cells. After incubated for 15 min at room temperature in the dark, the cells were added an additional 400 μL binding buffer and subjected to FACS cytometry. The results were analyzed with Summit version 4.3 software.

5.11 PI staining

5×10^5 Cells were seeded in 6-well plates to reach 80% confluence and treat with compound **8k**. After exposure to 8k for 24 h, cells were collected with trypsin and washed with PBS buffer, then fixed with 75% ethanol at -20 ^oC overnight. The fixed cells were re-suspended in 500 μL PBS buffer containing 0.2 mg/mL RNase A

for 30min at 37 ^{o}C to digest RNA, and then add PI to 50 μg/mL to stain the cells for 30min in the dark. Finally, the cells were analyzed with flow cytometer. The results were analyzed with Summit version 4.3 software.

5.12 Intracellular ROS generation examination

Treated cells were collected and washed twice with PBS. Then the cells were re-suspended in serum-free McCoy's 5A medium, followed by the addition of 10 nM H2DCFDA (carboxy-20,70-dichlorodihydrofluorescin diacetate; Invitrogen) and incubation at 37 ^{o}C in the dark for 20 min. The cells were then washed twice with PBS and the relative ROS levels of the cells were measured through flow cytometry analysis. The experimental data were analyzed using FCS Express version 3 software (DeNovo, Los Angeles, CA, USA).

5.13 Western Blot Analysis

Cells seeded in 60 mm plates were applied with indicated experimental treatments. Treated cells were collected and washed with PBS. Then the cell lysates were extracted using SDS sample buffer(2% SDS; 50 mM Tris-HCl, pH 6.8; 10% glycerol; 12.5 mM EDTA) and the protein concentration was detected using bicinchoninic acid protein assay kit (Pierce). Identical quantities of proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (PVDF, Millipore, cat # IPVH00010, Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk and the probed with indicated primary antibodies overnight at 4 ^{o}C. After incubated with specific horseradish peroxidase conjugated secondary antibodies, the bands were revealed by ECL detection system (Millipore).

Antibodies used in this study are as follows: the primary antibodies, c-PARP (1:1000) and GAPDH (1:5000) were all purchased from Cell Signaling Technology. HRP conjugated secondary antibodies, Pierce(R) Goat Anti-Rabbit IgG, (H&L) (#31460) and Pierce(R) Goat Anti-Mouse IgG, (H&L) (#31430) were purchased from Thermo.

5.14 Statistical analysis

The data given in the text were expressed as means ± SD. The significance of the difference between compared groups was determined with the Student's t test. The differences were considered significant at * $p < 0.05$ and ** $p<0.01$.

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Table and Figure captions

Table 1 Cytotoxic activities of 4-quinolones derivatives *in Vitro*[c] (IC_{50}, μM [a])

[a]Cytotoxicity as IC_{50} for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay. Values with standard deviations (SD) are averages of at least three independent determinations.

[b]Cell lines include human laryngeal carcinoma (Hep-2), breast carcinoma (MCF-7), gastric carcinoma (BGC-823), liver carcinoma (HepG2), cervical carcinoma (Hela), prostate carcinoma (PC-3) and colorectal carcinoma (HCT-8, HCT-116 and RKO).

[c] ClogP represent the calculated n-octanol/water partition coefficient (log Pow), and the values produced by Chemdraw software.

Fig. 1 The chemical structure of the lead compound 7-chloro-4(1H)-quinolone 1.

Fig. 2 Compound 8k inhibited viability of human colorectal cancer cells. (A) Human CRC cells (HCT116, RKO, HCT116 $p53^{-/-}$ and HCT116 $Bax^{-/-}$) were treated with **8k** at indicated concentrations. After 24 h, the cell viability was measured by CCK-8 assay (n=3). (B and C) The number of colonies was counted after the treatment of HCT116 cells with the indicated concentration of compound **8k** for 24 h. The experiment was performed in triplicate, and representative wells were shown.

Fig. 3 Compound 8k induced apoptosis in HCT116 and RKO. (A, B) For flow cytometry analysis, HCT116 cells were treated with indicated concentration of compound **8k** for 48 h. Cell apoptosis and cell cycle was assayed respectively by Annexin V-FITC/PI binding assay (A) and PI staining (B). (C) The protein levels of c-PARP were detected by western blot, GAPDH was used as an internal control.

Fig. 4 Compound 8k induced apoptosis in CRC is depend on caspase, but not p53 and Bax. (A, B) Compound **8k** induced apoptosis of HCT116 and HCT116 $Bax^{-/-}$ via caspase-dependent pathway. HCT116 and HCT116 $Bax^{-/-}$ cells were pre-treated with DMSO or 20 μM CASPi for 5 h,

followed by exposure with indicated concentration of compound **8k** for 48 h. Annexin V/PI binding assay was perform to detect the apoptosis (A) and western blot was carried out to detect c-PARP (B). (C, D) Compound **8k** inhibited the colony formation in a p53- and Bax-independent manner. The colony formation inhibition by compound **8k** treatment was detected by colony formation assay. The experiment was performed in triplicate, and representative wells were shown. (E) Compound **8k** induced apoptosis of HCT116 $p53^{-/-}$ and HCT116 $Bax^{-/-}$. After treated with vehicle or 4 μM, the apoptosis was detected by Annexin V/PI assay. (F) c-PARP protein levels were detected by western blot, GAPDH was used as an internal control.

Fig. 5 Compound 8k triggers apoptosis of HCT116 by inducing ROS accumulation. (A) Compound **8k** induced ROS accumulation in HCT116. Cells were pre-treated with H_2O, 7.5 mM LNAC or GSH for 5 h, followed by treatment with indicated concentration of compound **8k** 24 h. ROS was assayed by H_2DCFDA-based flow cytometry. (B, C) Colony formation inhibition by compound **8k** was alleviated by L-NAC or GSH. About 300 cells per well were seeded in 12-well plates and grown for 2 h, then cells were pre-treated with H_2O, 7.5 mM LNAC or GSH for 2h, followed by exposure to indicated concentration of compound **8k** for 24 h and the colonies were counted after 10 days growth in fresh medium. The experiment was performed in triplicate, and representative wells were shown (B). The bar chart (C) showed the statistics data (** $p<0.01$). (D) GSH and L-NAC pre-treatment blunted the compound **8k** inducing HCT116 cell death. CCK-8 assay was performed to detect the cell viability of H_2O, 7.5 mM L-NAC or 7.5 mM GSH pre-treatment HCT116 followed by exposure to 4 μM, 16 μM and 32 μM for 24 h (n=3, ** $p<0.01$). (E) ROS scavengers blunted compound 8k triggering apoptosis detected by c-PARP. HCT116 cells were pre-treated with vehicle (H_2O) or 7.5 mM L-NAC for 6h followed by exposure to increasing dosage compound **8k** (0 μM, 4 μM and 8 μM) for 24 h, then cell lysates were extracted to detect c-PARP by compound **8k,** GAPDH was used as an internal control.

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Table 1 Cytotoxic activities of 4-quinolones derivatives *in Vitro*

Compds	IC_{50} (μM)±SD [a]									ClogP [c]
	Hep-2 [b]	MCF-7 [b]	BGC-823 [b]	HepG2 [b]	HeLa [b]	PC-3 [b]	HCT-8 [b]	HCT116 [b]	RKO [b]	
6a	45.1±5.0	36.8±5.2	46.3±6.8	>50.0	39.4±4.6	>50.0	48.5±6.2	38.6±4.2	29.8±2.5	1.725
6b	25.1±4.0	22.4±2.4	23.4±4.0	28.2±3.0	22.4±3.2	39.8±4.5	28.8±4.0	23.3±2.5	25.2±3.0	2.295
6c	44.7±3.8	21.1±2.0	44.7±5.4	45.7±6.0	>50.0	>50.0	>50.0	44.3±3.8	38.7±2.5	2.445
6d	40.8±4.8	34.7±2.8	40.8±5.6	47.9±6.0	39.8±4.0	>50.0	44.6±6.0	38.9±3.6	42.5±5.2	1.516
7a	25.0±3.0	>50.0	30.2±4.0	38.0±4.6	>50.0	>50.0	>50.0	>50.0	>50.0	1.591
7b	>50.0	>50.0	40.7±3.6	47.9±4.2	>50.0	>50.0	>50.0	>50.0	>50.0	2.120
7c	>50.0	49.8±4.0	>50.0	43.8±5.2	>50.0	>50.0	45.2±3.9	>50.0	48.0±6.8	2.365
7d	35.5±3.2	>50.0	43.6±5.0	28.3±4.2	>50.0	41.7±5.6	>50.0	39.8±5.0	45.2±5.4	3.178
7e	19.1±2.0	15.8±2.6	13.8±2.5	22.4±3.0	16.2±1.4	27.9±2.6	27.4±3.2	21.3±1.8	16.3±1.2	4.149
7f	7.63±0.64	14.1±2.0	18.6±3.0	15.1±2.0	14.8±1.6	16.1±3.0	17.0±2.2	10.8±2.0	9.86±0.82	4.067
8a	17.8±1.6	22.4±2.0	27.6±4.0	10.2±0.81	28.9±3.0	31.6±2.8	32.6±3.2	15.6±2.4	24.6±3.4	2.246
8b	4.51±0.56	10.0±0.81	17.8±0.83	8.72±0.62	9.50±1.2	15.8±2.0	22.4±3.2	9.85±0.64	11.2±1.1	2.525
8c	42.3±6.0	25.1±2.0	12.3±0.92	9.52±1.0	13.8±1.8	15.9±2.4	25.1±3.0	19.8±1.2	23.4±2.0	2.776
8d	9.82±1.2	14.1±2.0	11.7±0.81	10.5±0.64	15.1±1.2	12.6±1.5	16.9±2.0	8.78±0.44	10.2±0.68	3.054

8e	24.0±3.0	47.9±4.0	12.0±2.0	19.1±3.0	38.9±5.2	44.5±3.6	30.2±2.2	35.6±4.0	30.1±2.2	3.021
8f	14.5±2.0	35.5±4.2	15.1±1.0	12.6±0.81	19.1±1.2	14.1±0.84	30.0±4.0	18.7±1.0	16.3±0.86	3.299
8g	22.6±3.2	35.5±4.0	7.11±0.55	6.92±0.46	21.6±1.6	44.6±5.4	46.8±6.0	21.0±1.2	16.8±0.94	3.834
8h	11.2±0.82	12.3±1.0	9.12±0.52	5.63±0.34	15.4±2.0	8.31±0.45	28.8±4.2	7.84±0.84	9.65±1.1	4.112
8i	7.02±0.53	15.8±2.0	10.2±0.81	4.32±0.24	5.83±0.36	9.50±0.54	23.1±2.0	11.2±0.8	14.3±1.2	4.805
8j	4.51±0.38	4.54±0.56	5.21±0.41	4.43±0.51	4.90±0.36	10.2±0.82	19.5±2.0	8.65±1.2	7.89±0.82	5.083
8k	3.53±0.54	5.02±0.32	4.92±0.43	3.94±0.51	3.52±0.28	6.41±0.56	6.82±0.41	5.79±0.52	4.87±0.65	5.001
1	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	1.054
Cisplatin	11.6±0.86	12.6±2.0	6.83±0.53	7.60±0.52	10.4±1.2	11.9±2.0	9.62±0.61	10.8±1.6	8.92±1.0	-

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Fig. 1 The chemical structure of the lead compound 7-chloro-4(1H)-quinolone

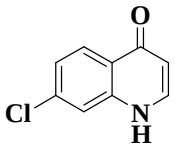

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Fig. 2 Compound 8k inhibited viability of human colorectal cancer cells.

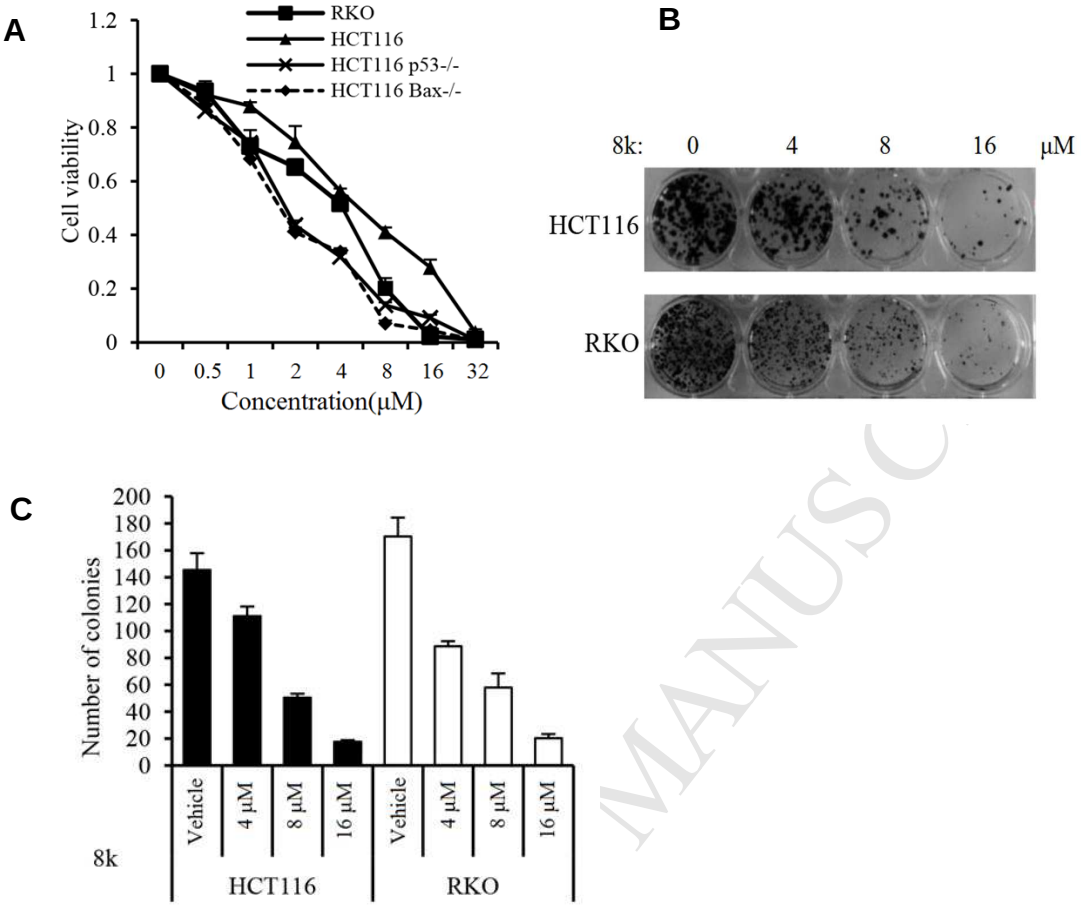

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Fig. 3 Compound 8k induced apoptosis in HCT116 and RKO.

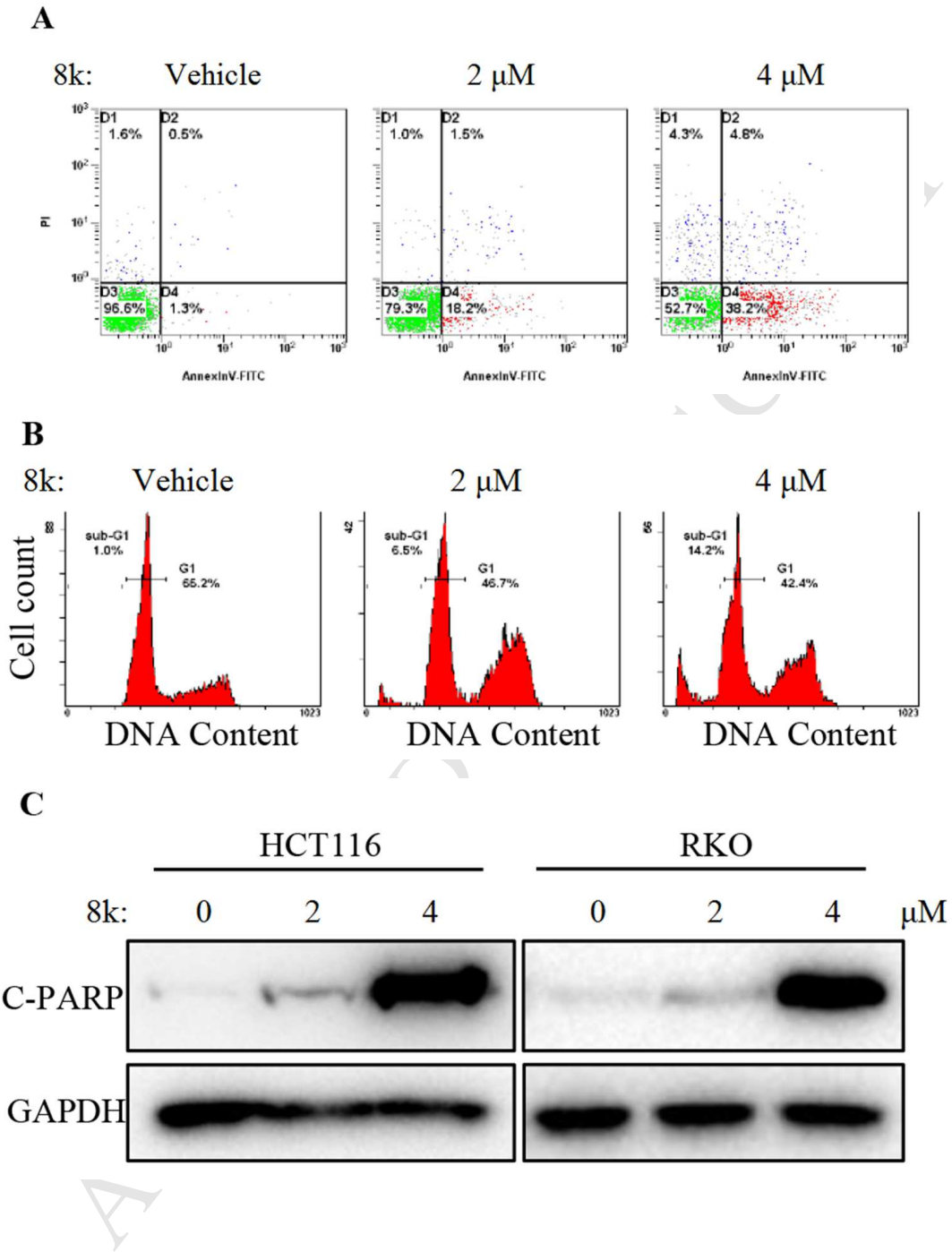


Fig. 4 Compound 8k induced apoptosis in CRC is depend on caspase, but not p53 and Bax.

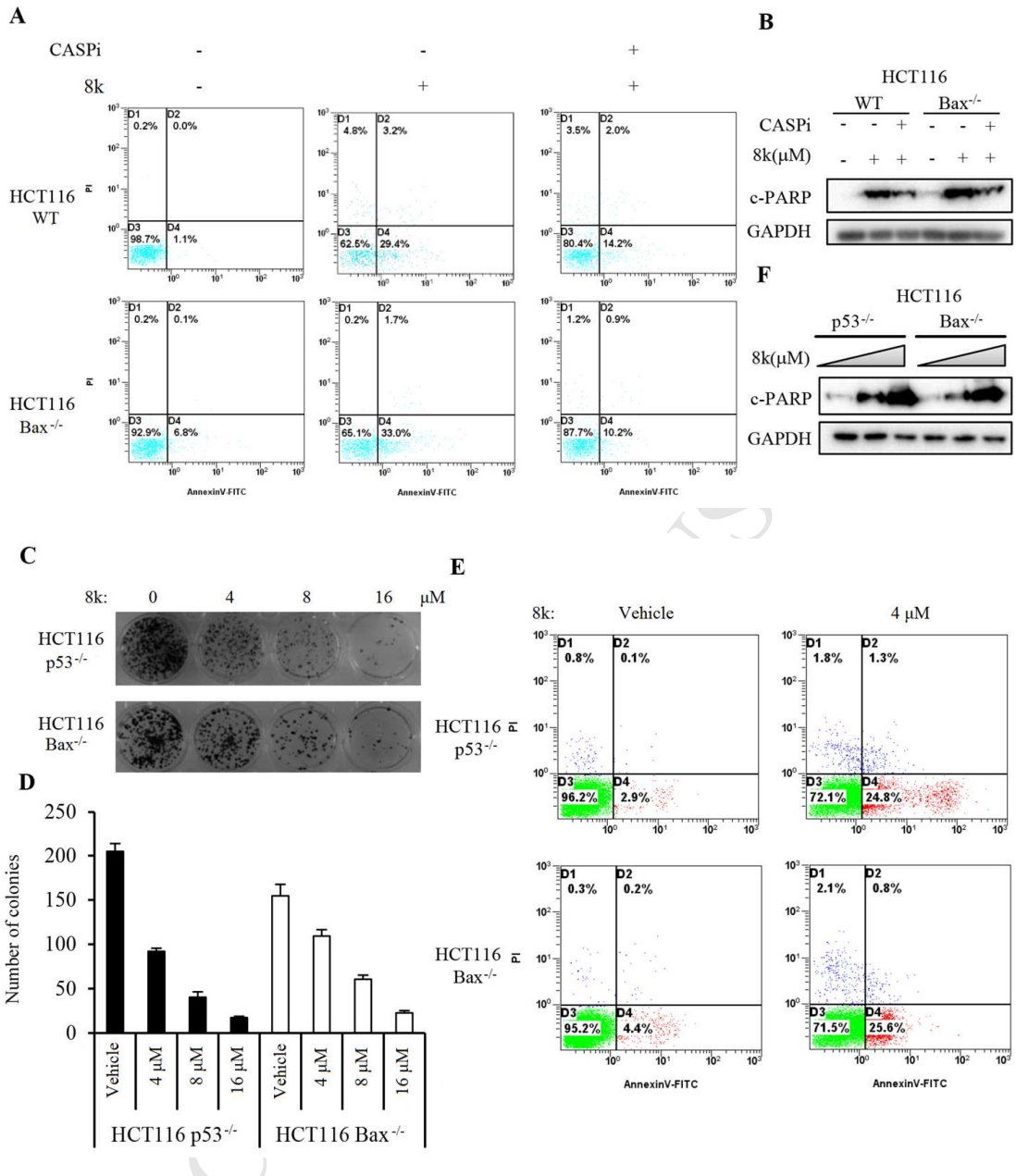
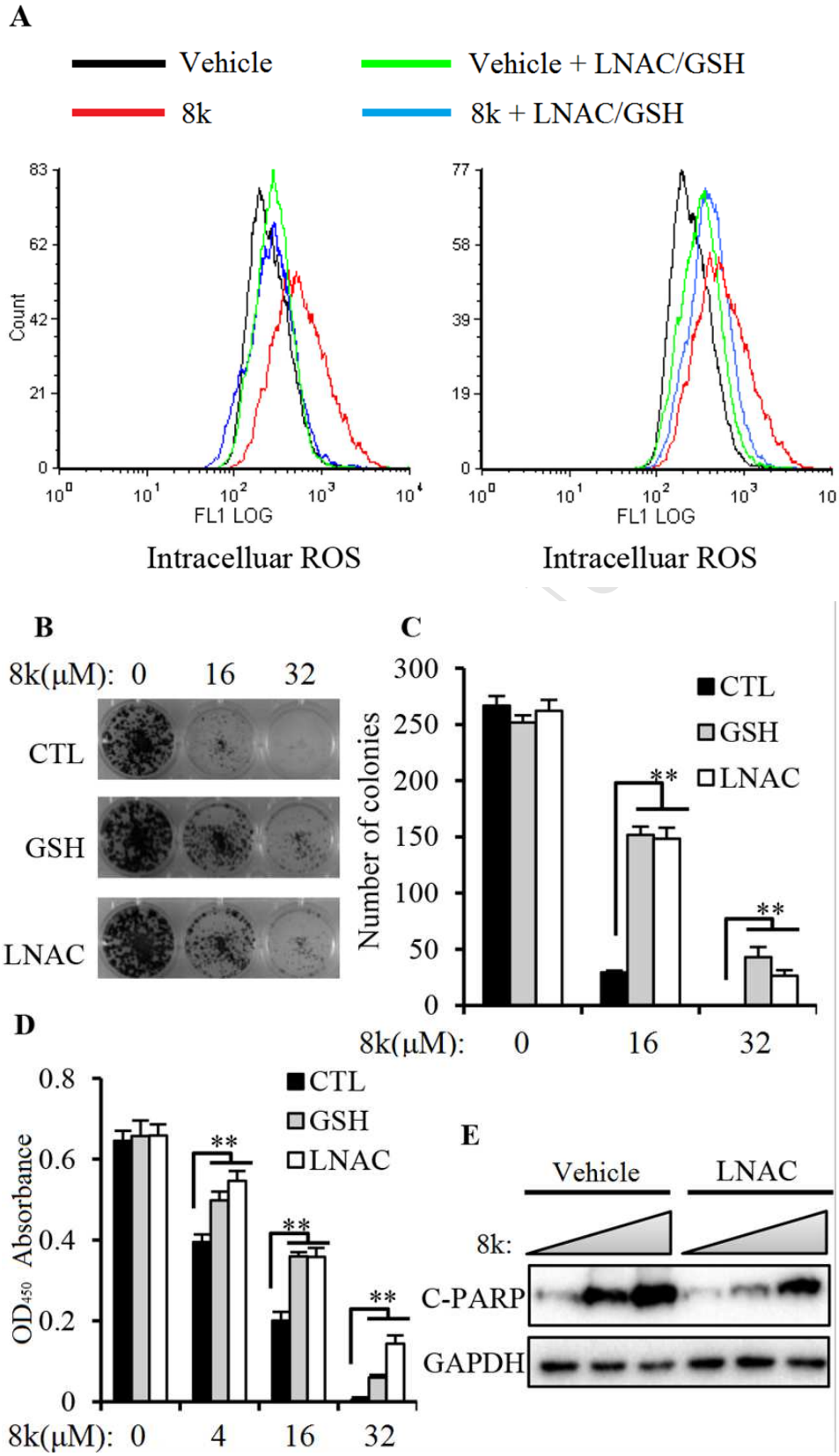
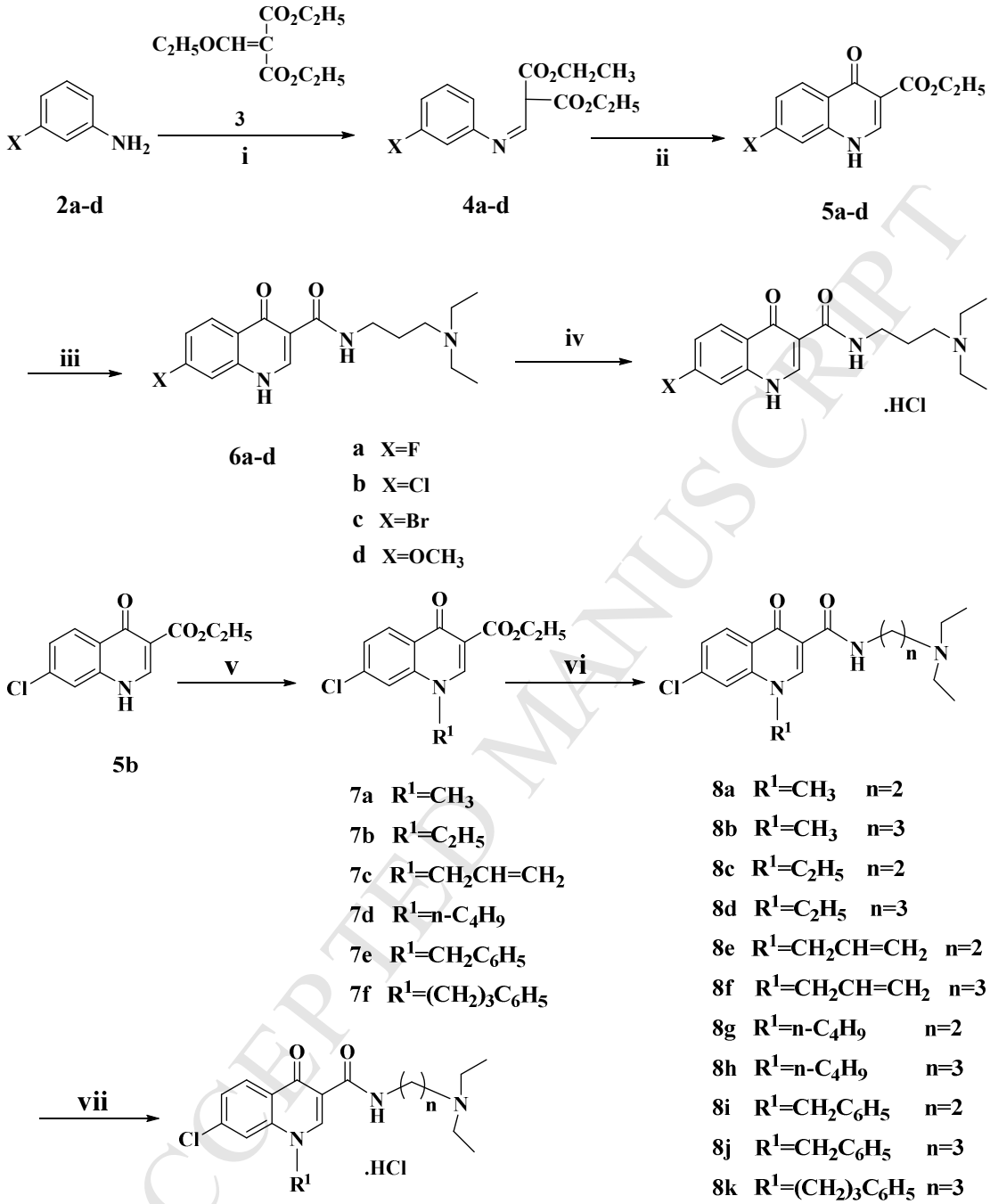


Fig. 5 Compound 8k triggers apoptosis of HCT116 by inducing ROS accumulation.



Scheme 1 Synthesis of compounds **6a-d**, **7a-f** and **8a-k**.



Reagents and conditions: (**i**) stirred at 100 ℃; (**ii**) diphenyl ether, reflux; (**iii**) $NH_2CH_2CH_2CH_2N(CH_2CH_3)_2$, microwave irradiation, 150 ℃, 30 min; (**iv**) 4M HCl/ethanol solution, stirred at RT; (**v**) DMF, NaH, alkyl halogenide, stirred at RT. stirred at 100 ℃; (**vi**) $NH_2CH_2CH_2N(CH_2CH_3)_2$ or $NH_2CH_2CH_2CH_2N(CH_2CH_3)_2$, microwave irradiation, 150℃, 30 min. (**vii**) 4M HCl/ethanol solution, stirred at RT.

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Research highlights

- ✓ A series of novel 4-quinolone-3-carboxamides was prepared and evaluated as antitumor agents.
- ✓ Compound **8k** was found to be the most potent antitumor agent.
- ✓ Compound **8k** could trigger p53/Bax- independent colorectal cancer cell apoptosis *via* inducing ROS accumulation.

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