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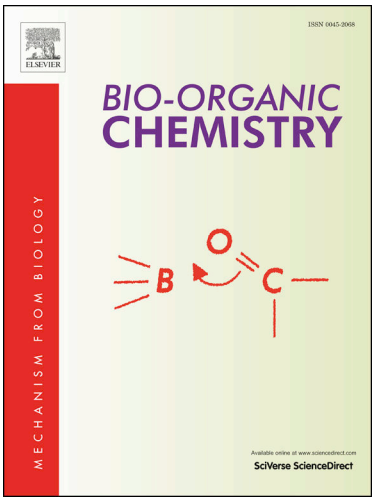

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Synthesis and biological assessment of ciprofloxacin-derived 1,3,4-thiadiazoles as anticancer agents

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Abstract

The quinolone-3-carboxylic acid scaffold is essential structure for antibacterial activity of fluoroquinolones such as ciprofloxacin. Modification of 3-carboxylic functionality in this structure can be used for switching its activity from antibacterial to anticancer. Accordingly, a series of C-3 modified ciprofloxacin derivatives containing *N*-(5-(benzylthio)-1,3,4-thiadiazol-2-yl)-carboxamide moiety was synthesized as novel anticancer agents. Most of compounds showed significant activity against MCF-7, A549 and SKOV-3 cancer cells in the MTT assay. In particular, compounds **13a-e** and **13g** were found to be as potent as standard drug doxorubicin against MCF-7 cell line (IC_{50}s = 3.26-3.90 μM). Furthermore, the 4-fluorobenzyl derivatives **13h** and **14b** with IC_{50} values of 3.58 and 2.79 μM exhibited the highest activity against SKOV-3 and A549 cells, being as potent as doxorubicin. Two promising compounds **13e** and **13g** were further tested for their apoptosis inducing activity and cell cycle arrest. Both compounds could significantly induce apoptosis in MCF-7 cells, while compound **13e** was more potent apoptosis inducer resulting in an 18-fold increase in the proportion of apoptotic cells at the IC_{50} concentration in MCF-7 cells. The cell cycle analyzing revealed that compounds **13e** and **13g** can increase cell portions in the sub-G1 phase, inducing oligonucleosomal DNA fragmentation and apoptosis confirmed by comet assay.

Keywords: Fluoroquinolones; Ciprofloxacin; 1,3,4-Thiadiazole; Anticancer activity; Apoptosis inducer; Cytotoxicity.

1. Introduction

Currently, anticancer agents have important position in the chemotherapy of malignancies. However, their potency and safety are not enough to overcome all kinds of cancers efficiently. Furthermore, there are some difficulties in the cancer chemotherapy, mainly due to the special characteristics of the disease such as resistance, diversity, different etiology and metastasis. This situation along with increasing number of cancer patients around the world instigate medicinal chemist to find new anticancer agents [1-3].

Fluoroquinolones are a large family of compounds with synthetic origin that are prominent in the field of antibacterial chemotherapy. These antibacterial agents prevent replication and transcription of DNA by targeting DNA gyrase and/or topoisomerase IV [4]. On the other hand, there are many reports on ability of fluoroquinolones to inhibit the division of cancer cells with diverse mechanisms including DNA intercalation, induction of apoptosis, and arresting of the cell cycle. Ciprofloxacin (CF, **1a**, Fig. 1), one of fluoroquinolone antibacterials that is used to treat diverse kinds of bacterial infections, including skin, bone, joint, respiratory and urinary tracts infections [5, 6]. Based on several reports, CF exhibits anticancer activity against some tumor cell lines including U87MG (Glioblastoma), MDA MB-231 (Breast), Colo829 (Melanoma), H460 (Lung cancer), Panc-1 (Pancreatic cancer), LOVO (Colon cancer), PC3, MLC9981 (Prostate cancer), HT-29 (Colorectal carcinoma), HTB9 (Bladder cancer), and K562 (Leukaemia cells) [4].

CF has good penetration into different tissues and glands. However, in vitro studies have demonstrated that high concentration of CF is required for anticancer activity [7]. Thus CF could be a candidate scaffold for structural modification in order to obtain potential anticancer agents. From a structure-activity relationship point of view, the carboxylic acid group at 3-position of fluoroquinolones is imperative for antibacterial activity and is a good choice for structural modification to switch into the effective anticancer activity [8, 9]. Accordingly, several strategies such as derivertization, bioisosteric replacement and cyclization of 3-carboxylic acid of quinolone antibacterials were applied to design of quinolone-based anticancer agents. There have already been several instances of conversion of 3-COOH in CF to hydrazide functionality. For instance,

Hu et al., have showed that some hydrazide-hydrazone of CF (**2**, Fig. 1) can inhibit the growth of cancer cell lines such as CHO (Chinese hamster ovary), HL60 and L1210 (murine leukemia) [10]. In a similar work by Shi et al., ciprofloxacin-piperonal hydrazone (**3**) has been described as cytotoxic agent against SMMC-7721, MCF-7 and HCT-8 cancer cells [11].

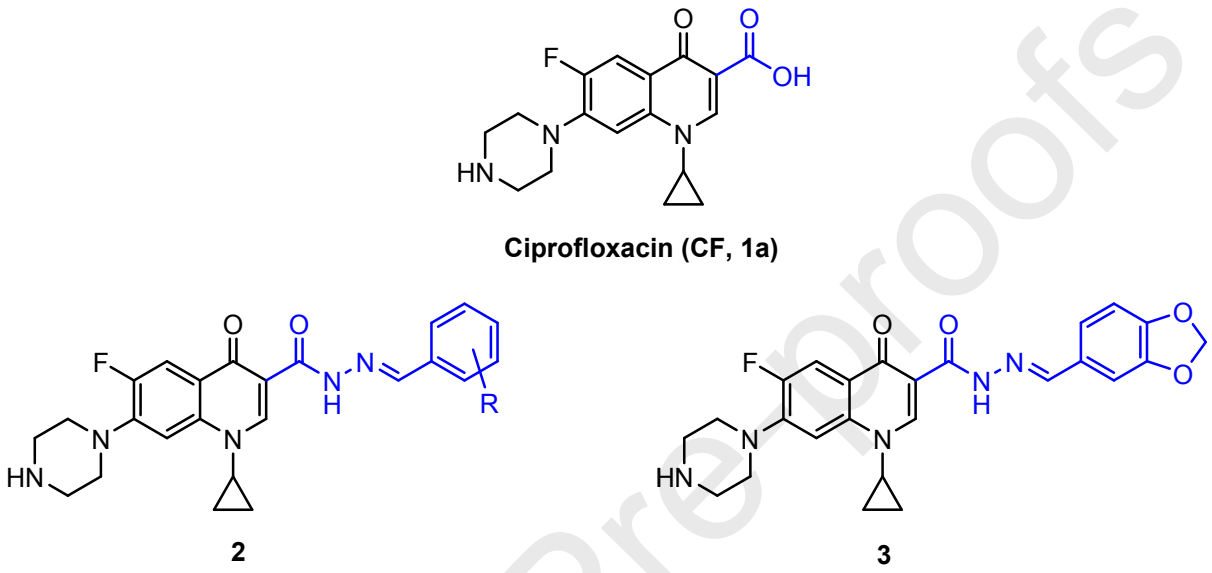


Figure 1. Structure of ciprofloxacin and related derivatives as reported anticancer agents.

On the other hand, thiadiazole is a key five-membered heterocyclic core, found in many anticancer compounds [12-15]. Sulfur containing heterocycles such as thiadiazoles have distinct chemical features due to the presence of electron deficient, bivalent sulfur atom with two areas of positive electrostatic potential, in which the low lying C−S σ* orbitals can interact with electron donors (such as O and N atoms). The ability of thiadiazoles for non-covalent interactions involving sulfur atom in the compound conformation and ligand−protein interactions make them as an interesting scaffold in drug design and development [16,17]. Among the thiadiazole derivatives, the most attractive ones are those containing alkyl(aryl)amido- and/or benzylthio moieties at positions 2 and 5, respectively [18]. In the search for new lead compounds targeting cancer cells including chronic myeloid leukemia (CML), Radi group described thiadiazole derivatives **4** and **5** (Fig. 2) as Bcr-Abl inhibitors [19]. Moreover, some analogs of **4** and **5** namely *N*-(5-(benzylthio)-1,3,4-thiadiazol-2-yl)-2-phenylacetamides (**6**) were synthesized by Aliabadi and coworkers. The in vitro

results indicated that the breast cancer cell line MCF-7 had the highest susceptibility towards compounds **6** [20].

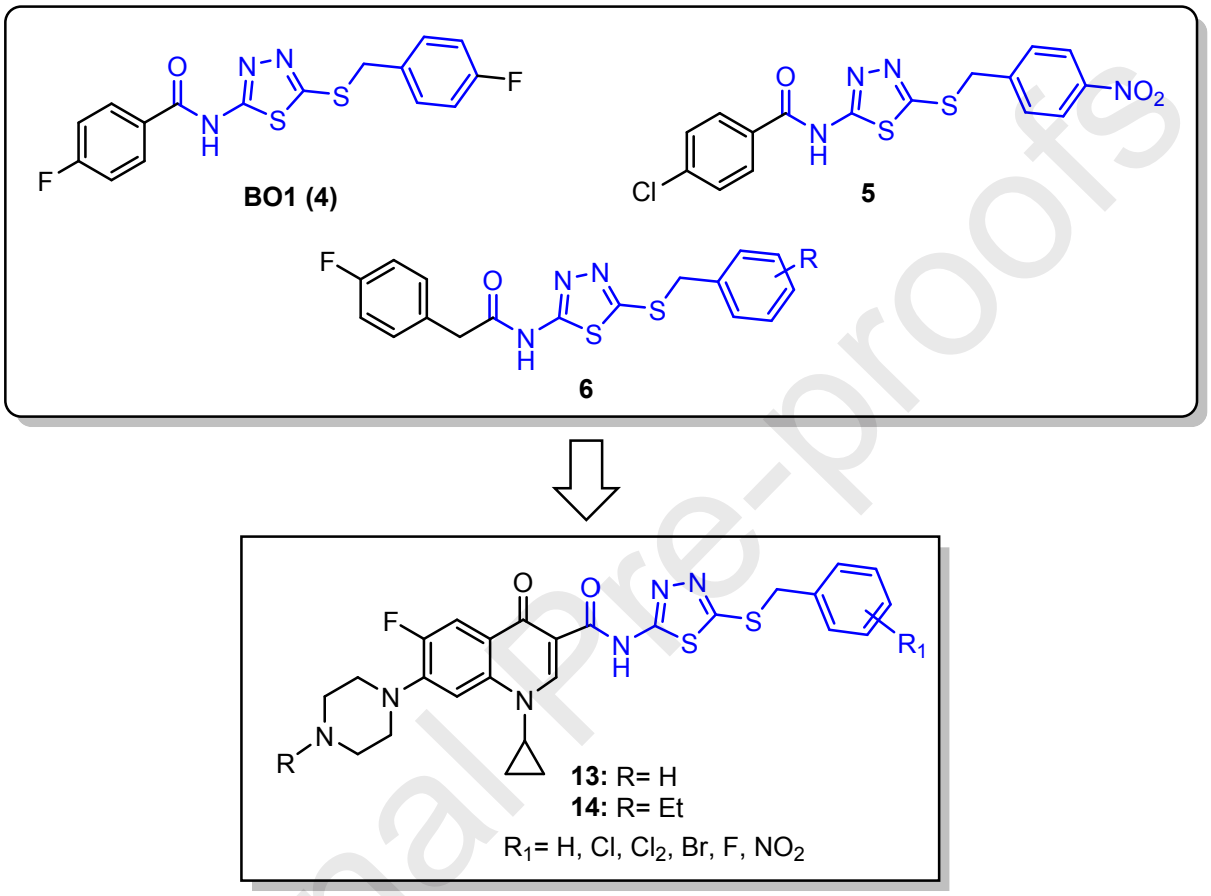


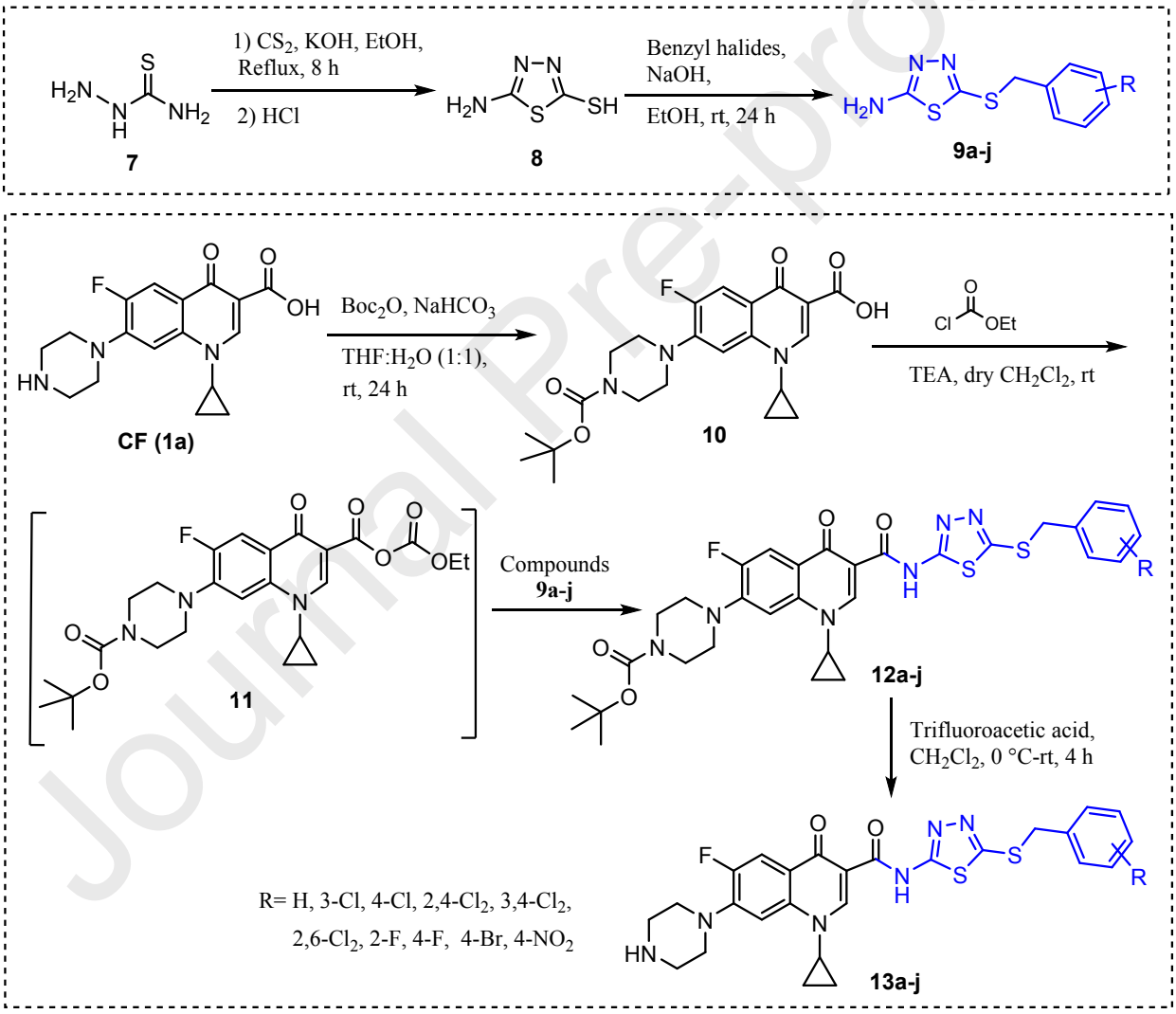
Figure 2. Structures of 1,3,4-thiadiazole-based compounds **4-6** with anticancer activity and designed compounds **13-14** as ciprofloxacin-thiadiazole hybrids.

The above mentioned findings prompted us to design a novel series of quinolone-based thiadiazoles derived from ciprofloxacin. Thus we report here, the synthesis and biological evaluation of compounds **13-14** as anticancer agents (Fig. 2).

2. Results and discussion

2.1. Chemistry

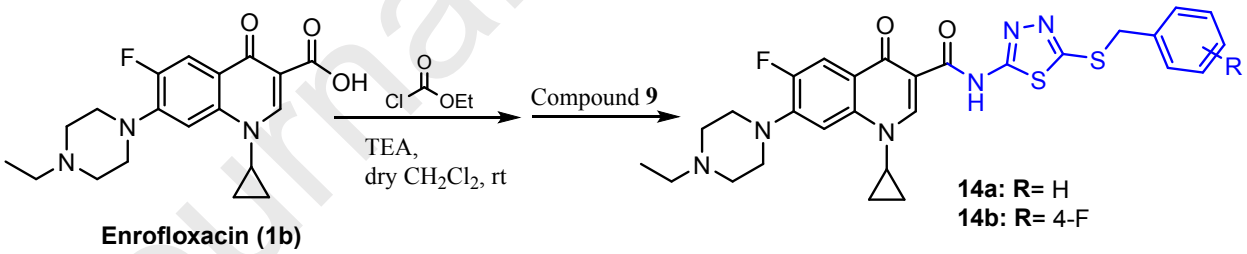
The sequence of the reactions used for the synthesis of novel quinolone-based thiadiazoles is illustrated in Scheme 1. Initially, 5-amino-1,3,4-thiadiazole-2-thiol (**8**) was synthesized by heterocyclization of thiosemicarbazide (**7**) in the presence of carbon disulfide and KOH in absolute ethanol. The compound **8** was subjected to the reaction with benzyl chloride or bromide derivatives to afford *S*-benzyl derivatives **9a-j** in good yields [21].



Scheme 1. Synthesis of compounds **13a-j**.

On the other hand, in order to modify the 3-carboxylic acid functionality of CF, the NH of piperazinyl group was protected with *tert*-butyloxycarbonyl (Boc) group. Thus, treatment of CF with Boc_2O in the presence of sodium bicarbonate in THF-H_2O gave the *N*-Boc-CF (**10**). For activation of 3-carboxilic acid, compound **10** was reacted with ethyl chloroformate and triethylamine (TEA) as a base in dry dichloromethane (DCM) to obtain intermediate **11**. The reaction of compound **11** with aminothiadiazoles **9a-j** afforded the corresponding amides **12a-j**. Finally, deprotection of the *N*-Boc-amides (**12a-j**) with trifluoroacetic acid (TFA) in DCM at room temperature produced compounds **13a-j**. Furthermore, for obtaining compounds **14**, enrofloxacin (**1b**) the commercially available derivative of CF, was subjected to coupling with appropriate aminothiadiazole **9**. Similarly, activation of COOH functional group was conducted by using ethyl chloroformate, as described for the preparation of compounds **12** (Scheme 2).

The structures of the synthesized compounds **12-14** were confirmed on the basis of their spectral data (IR, ^{1}H-NMR, ^{13}C-NMR, MS and elemental analyses). Particularly, in the ^{1}H-NMR spectra of compounds **12**, the presence of singlet in the range of 13.99-14.01 ppm is related to the NH of amide, indicating the involvement of NH in the hydrogen bonding with 4-oxo of quinolone (Fig. 3). The full interpretation of spectroscopic data could be found in Experimental section.



Scheme 2. Synthesis of compounds **14a,b**.

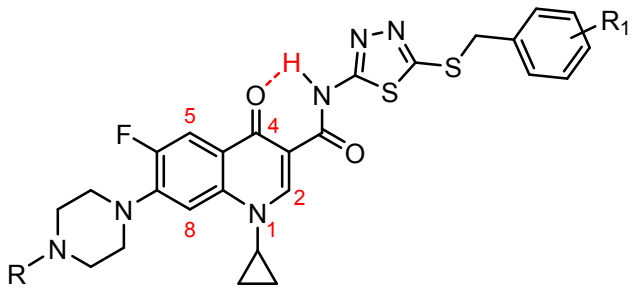


Figure 3. Presentation of the possible intramolecular hydrogen bond in compounds **12-14** and the atom numbering of the core structure.

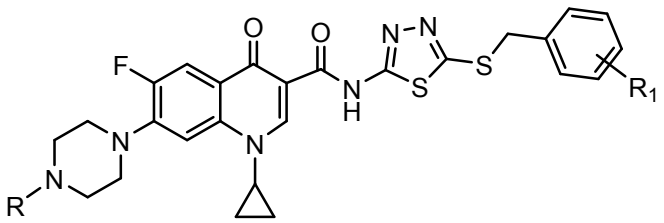
*2.2. Effect of compounds **13-14** on the viability of cancer cells*

All the synthesized ciprofloxacin-based compounds **13a-j**, **14a,b** and two *N*-Boc protected compounds **12**, were evaluated for their in vitro activity against three human cancerous cell lines; breast MCF-7, lung A549 and ovarian SKOV-3 cancer cells. The cell viability was determined by using MTT assay after 48 h incubation. The results were expressed by median growth inhibitory concentration (IC_{50} in μM) as shown in Table 1. Ciprofloxacin (CF) and doxorubicin were included in the bioassay as parent and reference drugs, respectively.

As seen in Table 1, while the parent quinolone (CF) showed no activity against tested cancer cells (IC_{50} > 100 μM), all the quinolone-based compounds had significant activities at least against one cell line, displaying IC_{50} values in the range of 2.79-26.28 μM. The obtained IC_{50} values for standard drug doxorubicin were 2.25-3.48 μM.

Notably, the *N*-Boc derivatives **12b** and **12h** and *N*-ethyl analog **14a** were found to be inactive against MCF-7 and SKOV-3 cells (IC_{50} >50 μM). In the unsubstituted piperazine series **13a-g**, MCF-7 cells were more sensitive than A549 and SKOV-3 cells. In particular, compounds **13a-e** and **13g** with IC_{50} values ≤3.90 μM were as potent as doxorubicin against MCF-7. Interestingly, all tested compounds were active against A549 cells. While compounds **14a** and **14b** bearing *N*-ethylpiperazine moiety were the most active compounds against A549, the *N*-Boc-piperazine derivative **12b** was the less potent one. The activity of compound **14b** (IC_{50} A549= 2.79 μM) was comparable to that of standard drug doxorubicin. In the case of SKOV-3 cancer cells, the unsubstituted piperazine derivatives **13a-h** showed potent activity (IC_{50} <10 μM). The most potent compound was **13h** (IC_{50} SKOV-3= 3.58 μM), being as potent as doxorubicin.

Table 1. The IC_{50} values (μM) of final compounds **13** and **14**, and intermediate compounds **12b** and **12h** against three different cell lines.



Compound	**R**	**R_1**	**MCF-7**	**A549**	**SKOV-3**
13a	H	H	3.84	10.21	9.66
13b	H	3-Cl	3.58	9.97	7.17
13c	H	4-Cl	3.90	6.49	8.50
13d	H	2,4-Cl_2	3.31	8.52	7.6
13e	H	3,4-Cl_2	3.26	10.53	5.08
13f	H	2,6-Cl_2	5.71	14.8	4.14
13g	H	2-F	3.34	9.69	5.43
13h	H	4-F	9.48	6.95	3.58
13i	H	4-Br	7.71	5.50	10.57
13j	H	4-NO_2	15.79	23.51	16.58
14a	Et	H	>50	4.02	>50
14b	Et	4-F	4.99	2.79	10.29
12b	Boc	3-Cl	>50	26.28	>50
12h	Boc	4-F	>50	11.09	>50
Ciprofloxacin	-	-	>100	>100	>100
Doxorubicin	-	-	3.48	2.25	3.47

Each experiment was performed in triplicate and the results were showed as mean values

By comparing IC_{50} values of the series, some structure-activity relationships could be concluded. The simplest compound **13a** (R= R_1= H) showed good activity against all cell lines especially MCF-7 cells. While the introduction of halogens (Cl or F) on benzyl group could not improved the activity against MCF-7, but significantly increased the inhibitory potency against SKOV-3. Particularly, the better result on SKOV-3 was obtained with 4-fluoro- substitution. Conversely, this substituent (R_1= 4-F) decreased the activity against MCF-7 (compare **13h** with **13a**). The comparison of 2-fluorobenzyl derivative **13g** with the unsubstituted benzyl analog **13a** revealed that the 2-fluoro substitution can improve the activity against SKOV-3 while serving the level of activity on MCF-7 cells. Among the halobenzyl compounds (**13b-i**), the 4-bromo- analog showed the greater activity against A549. These findings indicate that the effect of substituent on the benzyl group depends on the type of cell line. The introduction of strong electron-withdrawing NO_2 group on the *para*-position of benzyl group resulted in compound **13j** with diminished activity against all cell lines.

The comparison of *N*-ethylpiperazine derivatives (**14a** and **14b**) with their parent counterparts (**13a** and **13h**, respectively) indicates that the ethyl group has positive effect on cytotoxic activity against A549. Indeed, the *N*-ethylpiperazine derivatives **14b** was the most potent compound against this cell line. However, the introduction of ethyl on piperazine ring remarkably diminished the activity on SKOV-3 as observed in compounds **14a** and **14b**.

*2.3. Apoptosis inducing activity of compounds **13e** and **13g***

Actually, many of the anticancer drugs commonly treat malignancies by induction of apoptosis in cancer cells. Nowadays, apoptotic pathways have currently been important targets for development of new anticancer agents [22]. In order to check the potential of our compounds for induction of apoptosis in cancer cells, we use Annexin V/PI double staining technique for analyzing of MCF-7 treated with selected compounds **13e** and **13g**. It should be noted that these two compounds were the most active ones against MCF-7 cells thus we selected them for further studies on MCF-7 cells. As shown in Fig. 4, the results demonstrated that the percentages of the cell in regions Q3 and Q2 for treated cells significantly increased in comparison with those of control cells. After 48 h of treatment with **13e** and **13g** the number of early apoptotic cells increased from 1.82 to 53.2 and

18.6%, respectively. Also the percentage of cell in the late apoptotic region reached from 2.14 to 21.3 and 13%, respectively. Overall, both compounds could significantly induce apoptosis in MCF-7 cells, while compound **13e** was more potent apoptosis inducer. An 18-fold increment in the proportion of apoptotic cells occurred after treating with the IC_{50} concentration (3.26 µM) of **13e** in MCF-7 cells.

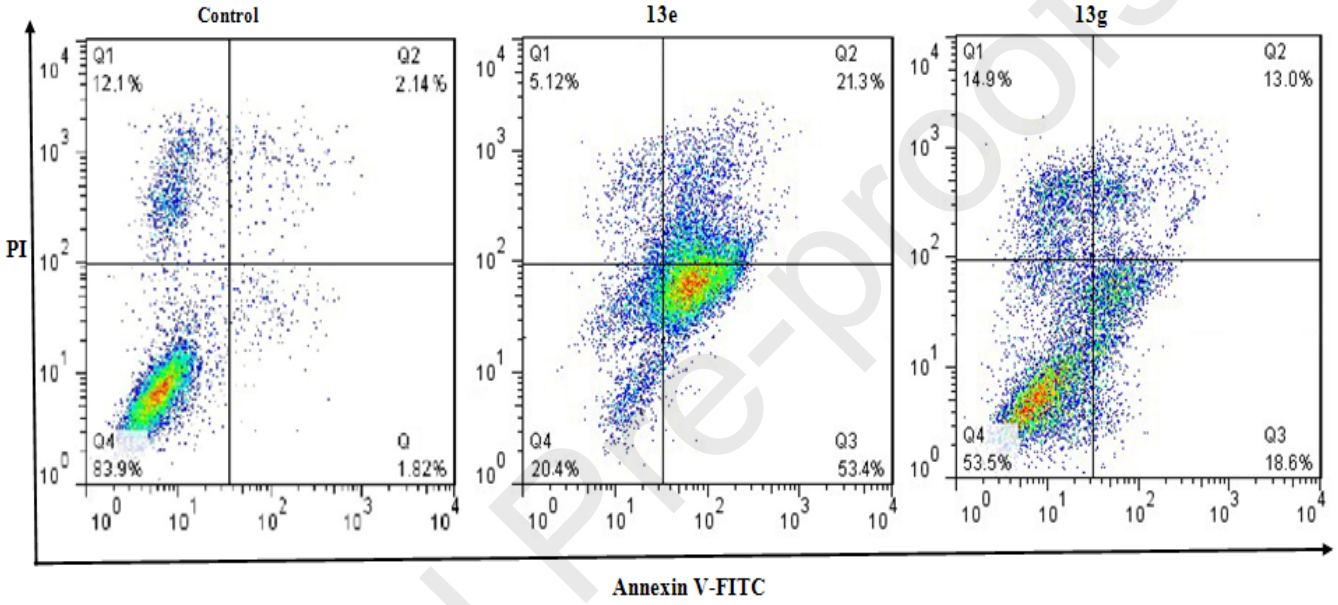


Figure 4. Induction of apoptosis in MCF-7 cells after 48 h treatment with **13e** and **13g** (at IC_{50} concentrations). Quadrant panels have been separated into four gates that labeled Q1 (necrotic cells, AnnexinV^-/PI^+), Q2 (late apoptotic cells, Annexin V^+/PI^+), Q3 (early apoptotic cells, Annexin V^+/PI^-) and Q4 (viable cells, Annexin V^-/PI^-). The control group was untreated cells.

*2.4. Cell cycle arrest by compounds **13e** and **13g***

The cell cycle regulation is an important way for the control of cell growth and anticancer therapy [23]. Therefore, study of the effect of new cytotoxic agents on the proportion of cells in the cell cycle phases can help to highlight the anticancer properties and involved mechanisms.

The effect of representative compounds **13e** and **13g** on the cell cycle of MCF-7 cells was analyzed by using flow cytometric method and the distribution of cells in different phases was determined. As presented in Fig. 5, after treating with **13e** and **13g**, the percentages of cells in the sub-G1 phase

remarkably elevated from 0.94 to 34.68 and 35.72%, respectively. On the other hand, the obtained results showed that the percentage of cells in G1, S and G2/M phases decreased when comparing with control cells. These results suggested that compounds **13e** and **13g** (at the concentration of IC_{50} values) can increase the proportion of sub-G1 phase, inducing oligonucleosomal DNA fragmentation and apoptosis.

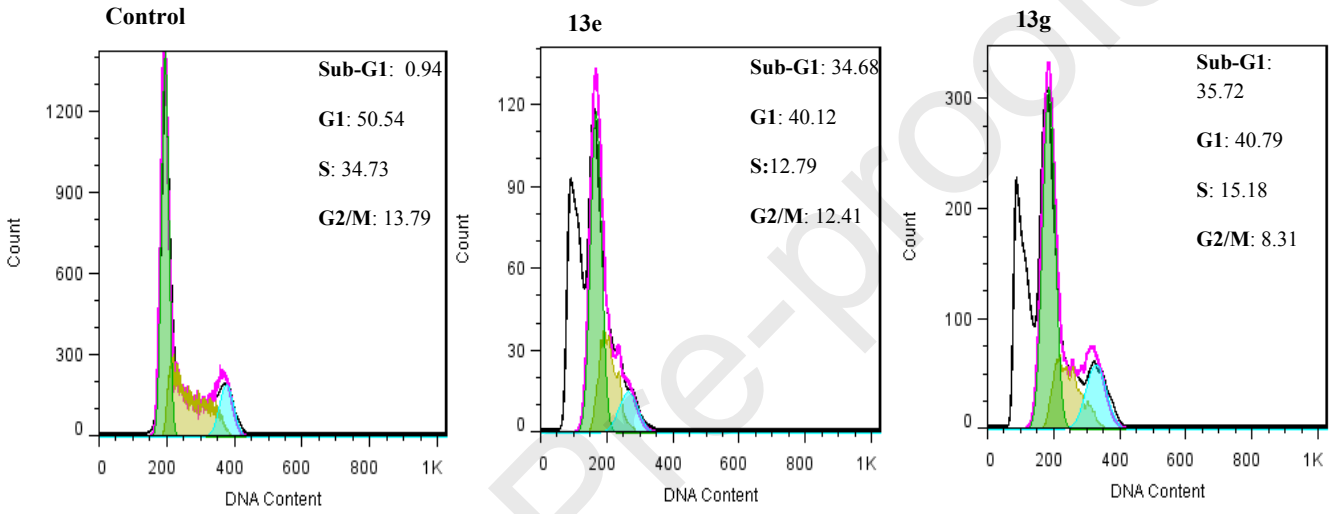


Figure 5. The cell cycle analyzes of MCF-7 cells after treating with compounds **13e** and **13g** (IC_{50} concentration, 48 h). The untreated cells were as control group.

*2.5. Evaluation of compounds **13e** and **13g** for DNA damaging by comet assay*

The most important cytotoxic mechanisms of quinolones could be the conversion of transient DNA double-strand breaks into permanent lesions followed by cell-cycle arrest and apoptosis in cancer cells [24]. The comet assay is known as a facile and delicate in which single cell gel electrophoresis is used to detecting DNA damage of individual cells [25].

The possible ability of compounds **13e**, **13g** and CF (as parent drug**)** on the DNA damage was investigated by the alkaline comet assay in cancer cell MCF-7. Doxorubicin was also included as standard drug with well-known interfering action on the DNA function and structure. Precisely, compounds **13e**, **13g** and CF were tested at the concentrations of 1, 5 and 10 µM and compared to

doxorubicin (1, 5 and 10 μM) as positive control. Different parameters including tail length, % DNA in tail and tail moment was used to scoring of DNA damage as shown in Fig. 6. Doxorubicin at the lower concentration of 1 μM showed higher potentials of DNA damage in MCF-7 cell. Also both compounds **13e** and **13g** even at low concentration of 1 μM displayed a significant increase in the DNA damage in comparison with control group. Furthermore, all parameters including tail length, % DNA in tail and tail moment, in compounds **13e**- or **13g**-treated cells were significantly higher than those of parent quinolone CF. However, the effect of both compounds was less than that of doxorubicin as compared by all parameters.

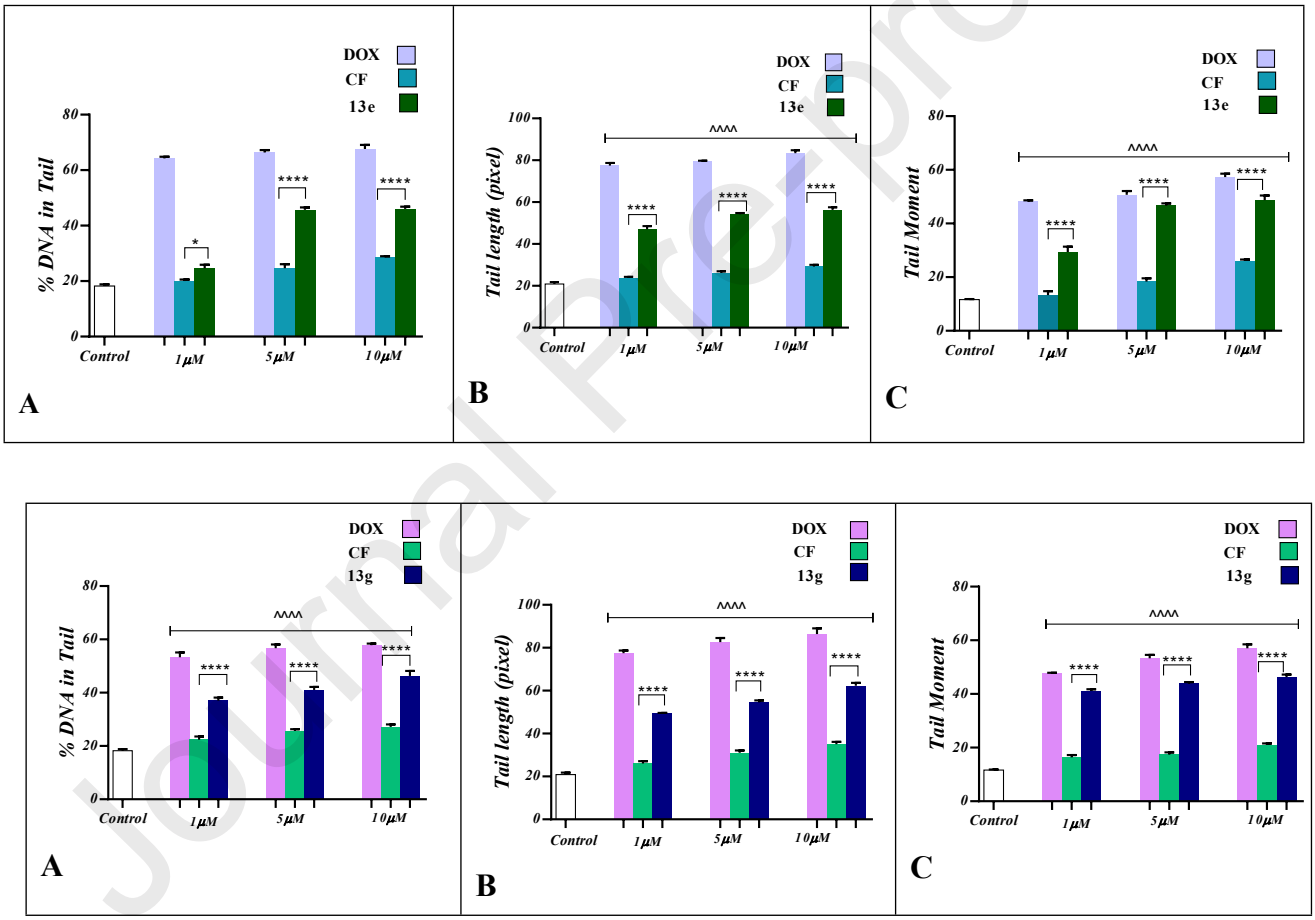


Figure 6. DNA damage in MCF-7 cells evaluated by the comet assay and scored as tail parameters: (A) Tail length; (B) % DNA in tail and (C) tail moment. The cells were treated with the compounds **13e** and **13g** (1, 5 and 10 μM) and compared to doxorubicin (Dox) and ciprofloxacin (CF) (same concentrations), and negative control (untreated).

3. Conclusion

We have synthesized a series of C-3 modified ciprofloxacin derivatives containing *N*-(5-(benzylthio)-1,3,4-thiadiazol-2-yl)-carboxamide moiety as novel anticancer agents. The MTT assay of synthesized compounds against three different cancer cells including MCF-7, A549 and SKOV-3 indicated that most of them have remarkable activity (IC_{50} values ≤ 26.28 μM), in contrast to ciprofloxacin with no anticancer activity (IC_{50} values > 100 μM). Certainly, the inhibitory activity of compounds **13a-e** and **13g** against MCF-7 cell line (IC_{50}s = 3.31-3.90 μM) was comparable to that of standard drug doxorubicin. Although the IC_{50} values of compounds **13a-e** and **13g** against other cells (A549 and SKOV-3) was ≤ 10.53 μM, but the 4-fluorobenzyl derivatives **13h** and **14b** with IC_{50} values of 3.58 and 2.79 μM displayed superior activity on SKOV-3 and A549 cells, being as potent as doxorubicin. The flow cytometric analysis of two representative compounds **13e** and **13g** in MCF-7 cells demonstrated that both compounds could significantly induce apoptosis in MCF-7 cells, cell cycle arrest and increment of cells in the sub-G1 phase, which further confirmed by comet assay, indicating the potential of compounds in DNA damage and oligonucleosomal DNA fragmentation in MCF-7 cells. The results of this study revealed the C-3 conjugated quinolone antibacterial drug ciprofloxacin with 5-(benzylthio)-1,3,4-thiadiazol-2-amine derivatives can be considered as new lead for development of anticancer agents.

4. Experimental

4.1. General chemistry

All solvents and reagents were obtained from Merck Company and used without further purification. The required benzyl halides were also commercially available (Merck or Sigma-Aldrich). Ciprofloxacin hydrochloride was production of Temad Co. (Tehran, Iran). The intermediate compound **8** was prepared from thiosemicarbazide and CS_2 based on the reported method [26].

*4.2. General procedure for the preparation of S-benzyl derivatives **9a-j***

A solution of 5-amino-1,3,4-thiadiazole-2-thiol (**8**, 5 mmol), in ethanol 80% (75 mL) was stirred at room temperature. Then, a 0.1 N NaOH solution (25 mL) was added dropwise and the stirring was continued. After 0.5 h, the appropriate benzyl halide (5 mmol) was added, and the reaction mixture was stirred at r.t. for 24 h. The resulting white precipitate was filtered and washed with water to give corresponding product **9** which was used for next step without further purification.

*4.3. Synthesis of N-Boc-CF (**10**)*

To a mixture of CF (**1a**, 5 mmol) in THF-H_2O (1:1) was added $NaHCO_3$ (5 mmol) and stirred for 30 min. Then, the reaction mixture was cooled down to 0-5 ºC and di-*tert*-butyl dicarbonate (5 mmol) was added over 10 min and subsequently stirred at ambient temperature overnight. The reaction progression was monitored by TLC using $CHCl_3$-CH_3OH (9:1) as mobile phase. Upon completion of the reaction, ice-cold water was poured onto the reaction mixture and the white participated solid was filtered, washed with water and dried to give compound **10**. Yield 93%, m.p. 240-245 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.10-1.40 (m, 4H, *c*-Pr), 1.46 (s, 9H, *t*-Bu), 2.10-2.67 (m, 4H, Pip), 3.5-3.58 (m, 4H, Pip), 3.7-3.9 (m, 1H, *c*-Pr), 7.61 (d, 1H, J = 7.2 Hz, H-8 Quin), 7.95 (d, 1H, J = 13.2 Hz, H-5 Quin), 8.69 (s, 1H, H-2 Quin), 15.21 (s, 1H, OH). Anal. Calcd for $C_{22}H_{26}FN_3O_5$: C, 61.24; H, 6.07; N, 9.74. Found: C, 61.20; H, 6.21; N, 9.76.

*4.4. General procedure for the synthesis of ciprofloxacin amides (**12** and **13**)*

A mixture of *N-Boc*-CF (**10,** 1 mmol) and triethylamine (2 mmol) in dry CH_2Cl_2 (5 mL) was stirred at room temperature for 30 min. Then the mixture was cooled to 0ºC, ethyl chloroformate (1 mmol) was added dropwise and stirring was continued for 1 h while the temperature reached to the room temperature gradually. After getting a solution, the appropriate thiadiazole-amine **9** (1 mmol) was added and the reaction mixture was stirred overnight. Finally, the obtained mixture was concentrated under reduced pressure and the residue was washed with ethyl acetate (for removing the unreacted intermediate **9**) to give crude products **12a-j**. In the case of **12b** and **12h**, crude product was purified by flash column chromatography eluting with chloroform-methanol (98:2) to afford pure compounds.

For deprotection of **12a-j**, a mixture of crude product *N-Boc*-ciprofloxacin amide **12a-j** in dichloromethane (DCM) was treated with trifluoroacetic acid (TFA) in an ice bath. After 0.5 h stirring, the mixture was stirred at room temperature for 4 h. At the end, the obtained solution was concentrated under reduced pressure and the residue was participated between CH_2Cl_2 and saturated $NaHCO_3$ solution in water. The separated organic layer was washed with water, dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give target compounds **13a-j**.

*4.4.1. tert-Butyl 4-(3-((5-((3-chlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)carbamoyl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)piperazine-1-carboxylate (**12b**)*

Yield 51%, m.p. 227-230 °C, IR (KBr, cm^{-1}): 3435, 2918, 2849, 1696, 1676, 1614, 1520, 1482, 1379, 1243, 1164. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.15-1.40 (m, 4H, *c*-Pr), 1.45 (s, 9H, 3×CH_3), 3.27-3.34 (m, 4H, Pip), 3.53-3.59 (m, 4H, Pip), 3.78-3.90 (m, 1H, *c*-Pr), 4.54 (s, 2H, CH_2), 7.30-7.46 (m, 3H, H-4, H-5, H-6 Ph), 7.54 (s, 1H, H-2 Ph), 7.59 (d, 1H, *J*= 8.0 Hz, H-8 Quin), 7.99 (d, 1H, *J*=13.2 Hz, H-5 Quin) and 8.78 (s, 1H, H-2 Quin), 14.01 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 8.04, 28.52, 36.07, 37.09, 49.79, 79.65, 107.17, 111.98, 120.55, 127.99, 128.21, 129.30, 130.83, 133.46, 138.98, 140.03, 148.54, 153.52 (d, *J*= 247.5 Hz), 154.18, 158.53, 162.46, 174.99. MS (m/z, %): 670 (M^+ <1), 429 (2), 382 (3), 355 (5), 282 (20), 266 (38), 207 (100), 191 (11), 149 (11), 125 (34), 91 (28). Anal. Calcd for $C_{31}H_{32}ClFN_6O_4S_2$: C, 55.47; H, 4.81; N, 12.52. Found: C, 55.56; H, 4.80; N, 12.33.

*4.4.2. tert-Butyl 4-(1-cyclopropyl-6-fluoro-3-((5-((4-fluorobenzyl)thio)-1,3,4-thiadiazol-2-yl)carbamoyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazine-1-carboxylate (**12h**)*

Yield 56%, m.p. 227-230 °C, IR (KBr, cm^{-1}): 3434, 3073, 2921, 2853, 1690, 1669, 1607, 1575, 1506, 1479, 1420, 1389, 1339, 1334, 1245, 1214, 1156, 1129, 1033, 1001, 948, 897, 847, 801, 761, 730. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.23-1.40 (m, 4H, *c*-Pr), 1.46 (s, 9H, 3×CH_3), 3.27-3.48 (m, 6H, Pip), 3.54-3.62 (m, 2H, Pip), 3.79-3.86 (m, 1H, *c*-Pr), 4.52 (s, 2H, CH_2), 7.19 (t, 2H, *J*= 8.8 Hz, H-3, H-5 Ph), 7.49 (dd, 2H, *J*= 8.4 and 5.6 Hz, H-2 and H-6 Ph), 7.59 (d, 1H, *J*= 8.0 Hz, H-8 Quin), 7.98 (d, 1H, *J*=13.2 Hz, H-5 Quin) and 8.77 (s, 1H, H-2 Quin), 13.99 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 8.03, 28.51, 29.79, 36.27, 37.12, 49.77, 79.65, 107.94, 111.99,

115.82 (d, *J*= 21.4 Hz), 120.59, 131.56 (d, *J*= 8.2 Hz), 133.57, 138.97, 145.18, 148.43, 153.70 (d, *J*= 247.3 Hz), 154.18, 158.47, 158.71, 162.16 (d, *J*= 245.0 Hz), 162.54, 174.98. MS (m/z, %): 654 (M^+, 2), 362 (2), 308 (3), 265 (3), 203 (4), 191 (5), 148 (22), 137 (7), 123 (9), 111 (12), 97 (19), 85 (35), 69 (76), 57 (76), 43 (100). Anal. Calcd for $C_{31}H_{32}F_2N_6O_4S_2$: 56.87; H, 4.93; N, 12.84. Found: 57.01; H, 4.99; N, 12.81.

*4.4.3. N-(5-(Benzylthio)-1,3,4-thiadiazol-2-yl)-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13a**)*

Yield 33%; m.p. 176-179 °C; IR (KBr, cm^{-1}): 3436, 2917, 2849, 1670, 1610, 1523, 1483, 1337, 1271, 1257, 1185, 799, 720. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.10-1.43 (m, 4H, *c*-Pr), 3.08-3.17 (m, 4H, Pip), 3.27-3.36 (m, 4H, Pip), 3.53-3.59 (m, 1H, *c*-Pr), 4.52 (s, 2H, CH_2 benzyl), 7.25-7.38 (m, 5H, Ph), 7.43 (d, 1H, *J*= 6.8 Hz, H-8 Quin), 8.08 (d, 1H, *J*= 13.2 Hz, H-5 Quin), 8.84 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 8.02, 36.17, 37.98, 44.65, 49.34, 106.85, 107.18, 111.87 (d, *J*= 22.5 Hz), 120.48, 128.08, 129.03, 129.50, 137.15, 138.98, 145.11 (d, *J*= 10.1 Hz), 148.37, 153.32 (d, *J*= 247.3 Hz), 158.38, 158.94, 162.49, 174.92. MS (m/z, %): 536 (M^+, <1), 524 (4), 495 (2), 368 (13), 313 (6), 239 (7), 95 (16), 69 (62), 43 (100). Anal. Calcd for $C_{26}H_{25}FN_6O_2S_2$: C, 58.19; H, 4.70; N, 15.66. Found: C, 58.33; H, 4.62; N, 15.63.

*4.4.4. N-(5-((3-Chlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13b**)*

Yield 36%; m.p. 187-191°C; IR (KBr, cm^{-1}): 3435, 2921, 2848, 1674, 1625, 1612, 1518, 1481, 1336, 1257, 798, 679.^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.20-1.48 (m, 4H, *c*-Pr), 2.00 (brs, 1H, N-H Pip), 3.01-3.20 (m, 4H, Pip), 3.20-3.38 (m, 4H, Pip) 3.51-3.62 (m, 1H, *c*-Pr), 4.47 (s, 2H, CH_2 benzyl), 7.21-7.37 (m, 4H, H-8 Quin, H-4, H-5 and H-6 Ph), 7.43 (s, 1H, H-2 Ph), 8.00 (d, 1H, *J*= 13.2 Hz, H-5 Quin), 8.79 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 8.21, 35.25, 37.58, 45.86, 50.96, 104.76, 108.23, 112.72, 112.96, 127.38, 127.95, 129.19, 129.91, 134.41, 138.48, 145.88, 147.35, 149.1, 152.5, 155.1, 158.62, 158.85, 162.72, 170.42, 175.08. MS (m/z, %): 570 (M^+, <1), 329 (2), 314 (2), 257 (4), 155 (3), 125 (100), 102 (12), 89 (42), 41 (28). Anal. Calcd for $C_{26}H_{24}ClFN_6O_2S_2$: C, 54.68; H, 4.24; N, 14.72. Found: C, 54.44; H, 4.32; N, 14.71.

*4.4.5. N-(5-((4-Chlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13c**)*

Yield 34%; m.p. 116-119°C; IR (KBr, cm^{-1}): 3436, 2919, 2849, 1671, 1624, 1611, 1522, 1482, 1337, 1256, 798, 761. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.20-1.44 (m, 4H, *c*-Pr), 1.73 (brs, 1H, N-H Pip), 3.07-3.15 (m, 4H, Pip), 3.26-3.34 (m, 4H, Pip) 3.52-3.60 (m, 1H, *c*-Pr), 4.47 (s, 2H, CH_2 benzyl), 7.29 (d, 2H, *J*= 8.4 Hz, H-2 and H-6 Ph), 7.33 (d, 1H, *J*= 7.2 Hz, H-8 Quin), 7.37 (d, 2H, *J*= 8.4 Hz, H-3 and H-5 Ph), 8.04 (d, 1H, *J*= 13.2 Hz, H-5 Quin), 8.82 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 7.98, 36.09, 37.11, 45.81, 51.13, 106.39, 107.06, 111.70 (d, *J*= 23.0 Hz), 120.03 (d, *J*= 7.5 Hz), 128.96, 131.35, 132.63, 136.49, 138.98, 145.78 (d, *J*= 9.7 Hz), 148.18, 153.36 (d, *J*= 247.4 Hz), 158.47, 158.53, 162.49, 174.85. MS (m/z, %): 570 (M^+, <1), 287 (4), 245 (5), 161 (7), 125 (100), 89 (52), 57 (36), 43 (53). Anal. Calcd for $C_{26}H_{24}ClFN_6O_2S_2$: C, 54.68; H, 4.24; N, 14.72. Found: C, 54.89; H, 4.20; N, 14.83.

*4.4.6. 1-Cyclopropyl-N-(5-((2,4-dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13d**)*

Yield 41%; m.p 185-188 °C; IR (KBr, cm^{-1}): 3435, 2922, 2850, 1674, 1624, 1613, 1517, 1481, 1337, 1256, 798, 761. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.11-1.38 (m, 4H, *c*-Pr), 2.86-2.93 (m, 4H, Pip), 3.15-3.21 (m, 4H, Pip), 3.37 (brs, 1H, NH), 3.61-3.83 (m, 1H, *c*-Pr), 4.54 (s, 2H, CH_2 benzyl), 7.40 (dd, 1H, *J*= 8.2 and 2.0 Hz, H-5 Ph), 7.44 (d, 1H, *J*= 7.6 Hz, H-6 Ph), 7.53 (d, 1H, *J*= 8.4 Hz, H-8 Quin) 7.66 (d, 1H, *J*= 2.4 Hz, H-3 Ph), 7.78 (d, 1H, *J*=13.2 Hz, H-5 Quin), 8.65 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 7.98, 35.58, 36.15, 45.79, 51.13, 106.42, 107.03, 111.70 (d, *J*= 23.2 Hz), 120.03 (d, *J*= 7.2 Hz), 128.00, 129.52, 133.12, 133.73, 133.98, 134.76, 138.98, 145.78 (d, *J*= 9.9 Hz), 148.19, 153.36 (d, *J*= 247.3 Hz), 157.73, 158.86, 162.53, 174.86. MS (m/z, %): 604 (M^+, 10), 515 (4), 401 (9), 347 (5), 302 (5), 245 (9), 217 (8), 180 (10), 160 (53), 144 (10), 125 (100), 108 (12), 89 (57), 76 (58), 59 (39), 44 (66). Anal. Calcd for $C_{26}H_{23}Cl_2FN_6O_2S_2$: C, 51.57; H, 3.83; N, 13.88. Found: C, 51.55; H, 3.71; N, 14.01.

*4.4.7. 1-Cyclopropyl-N-(5-((3,4-dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13e**)*

Yield 31%; m.p. 104-107°C; IR (KBr, cm^{-1}): 3421, 2918, 2849, 1671, 1624, 1611, 1522, 1482, 1338, 1256, 798. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.08-1.43 (m, 4H, *c*-Pr), 2.80-3.89 (m, 9H, Pip and *c*-Pr), 4.52 (s, 2H, CH_2 benzyl), 7.43 (d, 1H, *J*= 7.5 Hz, H-6 Ph), 7.48 (d, 1H, *J*= 7.2 Hz, H-5 Ph), 7.61 (d, 1H, *J*=8.1 Hz, H-8 Quin), 7.72 (s, 1H, H-2 Ph), 7.82 (d, 1H, *J*= 13.5 Hz, H-5 Quin), 8.67 (s, 1H, H-2 Quin). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 8.03, 36.19, 36.50, 45.06, 49.94, 106.67, 107.13, 111.82 (d, *J*= 22.7 Hz), 120.39, 129.84, 130.60, 131.11, 131.44, 138.92, 138.98, 145.24, 148.26, 153.30 (d, *J*= 250 Hz), 158.25, 158.61, 162.48, 174.86 (d, *J*= 2.3 Hz). MS (m/z, %): 604 (M^+, 2), 532 (2), 451 (4), 377 (5), 281 (6), 239 (6), 224 (5), 209 (2), 193 (2), 162 (5), 149 (12), 137 (8), 124 (8), 112 (9), 101 (9), 83 (20), 69 (26), 57 (41), 44 (100). Anal. Calcd for $C_{26}H_{23}Cl_2FN_6O_2S_2$: C, 51.57; H, 3.83; N, 13.88. Found: C, 51.65; H, 3.70; N, 13.73.

*4.4.8. 1-Cyclopropyl-N-(5-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13f**)*

Yield 35%; m.p. 240-244°C; IR (KBr, cm^{-1}): 3436, 2920, 2841, 1677, 1626, 1508, 1480, 1335, 1285, 1258, 796, 761. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.20-1.29 (m, 2H, *c*-Pr), 1.37-1.45 (m, 2H, *c*-Pr), 1.71 (brs, 1H, NH Pip), 3.09-3.17 (m, 4H, Pip), 3.27-3.35 (m, 4H, Pip), 3.53-3.61 (m, 1H, *c*-Pr), 4.82 (s, 2H, CH_2), 7.19 (t, 1H, *J*= 8.0 Hz, H-4 Ph), 7.31-7.38 (m, 3H, H-3, H-5 Ph, H-8 Quin), 8.07 (d, 1H, *J*=13.2 Hz, H-5 Quin), 8.84 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 8.21, 34.96, 45.92, 51.06, 104.69, 108.40, 112.97 (d, *J*= 23.5 Hz), 121.12, 128.45, 129.48, 132.52, 134.19, 136.14, 138.50, 140.72, 145.83, 147.35, 152.45, 153.8 (d, *J*= 235 Hz), 158.43, 159.29, 162.84, 175.17. MS (m/z, %): 604 (M^+, <1), 488 (2), 416 (4), 352 (71), 330 (9), 314 (9), 288 (11), 256 (5), 192 (8), 159 (100), 125 (53), 104 (12), 89 (33), 63 (23), 44 (12). Anal. Calcd for $C_{26}H_{23}Cl_2FN_6O_2S_2$: C, 51.57; H, 3.83; N, 13.88. Found: C, 51.66; H, 3.89; N, 13.80.

*4.4.9. 1-Cyclopropyl-6-fluoro-N-(5-((2-fluorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13g**)*

Yield 38%; m.p. 201-203 °C; IR (KBr, cm^{-1}): 3412, 2919, 2848, 1669, 1624, 1611, 1523, 1482, 1338, 1256, 798, 760. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.19-1.45 (m, 4H, *c*-Pr), 1.79 (brs, 1H, N-H, Pip), 3.08-3.18 (m, 4H, Pip), 3.25-3.39 (m, 4H, Pip), 3.45-3.61 (m, 1H, *c*-Pr), 4.55 (s, 2H, CH_2 benzyl), 7.03-7.14 (m, 2H, H-5, H-6 Ph), 7.23-7.31 (m, 1H, H-3 Ph), 7.34 (d, 1H, *J*= 6.8 Hz, H-8

Quin), 7.47 (t, 1H, *J*= 7.6 Hz, H-4 Ph), 8.05 (d, 1H, *J*= 13.2 Hz, H-5 Quin), 8.82 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 8.21, 29.71, 31.51, 35.22, 45.78, 50.86, 104.78, 108.36, 112.90 (d, *J*= 22.8 Hz), 115.55 (d, *J*= 20.8 Hz), 121.14, 124.22, 129.61, 129.69, 131.40, 138.49, 145.84, 147.36, 153.80 (d, *J*= 249.0 Hz), 158.71, 159.07, 160.05 (d, *J*= 220.0 Hz), 162.75, 175.16. MS (m/z, %): 554 (M^+, <1), 438 (2), 349 (6), 288 (3), 240 (3), 142 (4), 109 (100), 83 (20), 57 (8), 45 (8). Anal. Calcd for $C_{26}H_{24}F_2N_6O_2S_2$: C, 56.30; H, 4.36; N, 15.15. Found: C, 56.36; H, 4.27; N, 15.18.

*4.4.10. 1-Cyclopropyl-6-fluoro-N-(5-((4-fluorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13h**)*

Yield 33%; m.p 189-191 °C; IR (KBr, cm^{-1}): 3434, 2921, 2849, 1675, 1625, 1610, 1508, 1481, 1258, 841, 798, 761. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.21-1.28 (m, 2H, *c*-Pr), 1.35-1.43 (m, 2H, *c*-Pr), 3.06-3.14 (m, 4H, Pip), 3.22-3.29 (m, 4H, Pip), 3.52-3.59 (m, 1H, *c*-Pr), 4.45 (s, 2H, CH_2 benzyl), 7.00 (t, 2H, *J*= 8.4 Hz, H-3, H-5 Ph), 7.29 (d, 1H, *J*=7.2 Hz, H-8 Quin), 7.39 (dd, 2H, *J*= 8.8 Hz, *J*= 5.6 Hz, H-2, H-6 Ph), 7.92 (d, 1H, *J*=13.2 Hz, H-5 Quin), 8.75 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 8.20, 35.25, 37.59, 45.85, 50.92, 104.76, 108.10, 112.68 (d, *J*= 23.3 Hz), 115.58 (d, *J*= 21.4 Hz), 120.89 (d, *J*= 7.4 Hz), 130.85 (d, *J*= 8.2 Hz), 132.16 (d, *J*= 2.9 Hz), 138.45, 145.78 (d, *J*= 10.4 Hz), 147.35, 153.57 (d, *J*= 249.3 Hz), 158.55, 159.05, 162.26 (d, *J*= 245.0 Hz), 162.61, 174.99. MS (m/z, %): 554 (M^+, <1), 438 (2), 349 (3), 245 (2), 164 (2), 142 (7), 109 (100), 83 (10), 59 (6), 42 (6). Anal. Calcd for $C_{26}H_{24}F_2N_6O_2S_2$: C, 56.30; H, 4.36; N, 15.15. Found: C, 56.48; H, 4.35; N, 15.06.

*4.4.11. N-(5-((4-Bromobenzyl)thio)-1,3,4-thiadiazol-2-yl)-1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13i**)*

Yield 21%; m.p. 123-125°C; IR (KBr, cm^{-1}): 3425, 2919, 2848, 1671, 1624, 1611, 1522, 1482, 1337, 1256, 798, 761. ^{1}H NMR (500 MHz, DMSO-d_6) *δ*: 1.16-1.50 (m, 4H, *c*-Pr), 2.92 (m, 4H, Pip), 3.23 (m, 4H, Pip), 3.33 (m, 1H, *c*-Pr), 4.48 (s, 2H, CH_2 benzyl), 7.35-7.58 (m, 5H, Ph, and H-8 Quin), 7.89 (d, 1H, *J*= 13.0 Hz, H-5 Quin), 8.72 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 8.01, 36.15, 37.16, 44.86, 49.65, 106.71, 107.14, 111.85 (d, *J*= 22.6 Hz), 120.38, 121.180, 131.68, 131.89, 136.93, 138.97, 145.23 (d, *J*= 10.0 Hz), 148.33, 153.33 (d, *J*= 247.5 Hz),

158.47, 158.58, 162.49, 174.88. MS (m/z, %): 614 (M^+, <1), 471 (4), 204 (8), 184 (4), 171 (100), 121 (5), 106 (11), 91 (62), 76 (13), 63 (31), 45 (13). Anal. Calcd for $C_{26}H_{24}BrFN_6O_2S_2$: C, 50.73; H, 3.93; N, 13.65. Found: C, 50.61; H, 4.09; N, 13.88.

*4.4.12. 1-Cyclopropyl-6-fluoro-N-(5-((4-nitrobenzyl)thio)-1,3,4-thiadiazol-2-yl)-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (**13j**)*

Yield 28%; m.p. 193-196°C; IR (KBr, cm^{-1}): 3435, 2917, 2849, 1668, 1625, 1611, 1515, 1482, 1351, 1257, 798, 761. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.16-1.37 (m, 4H, *c*-Pr), 2.88-2.98 (m, 4H, Pip), 3.20-3.27 (m, 4H, Pip), 3.78-3.88 (m, 1H, *c*-Pr), 4.65 (s, 2H, CH_2 benzyl), 7.50 (d, 1H, *J*=7.6 Hz, H-8 Quin), 7.70 (d, 2H, *J*= 8.4 Hz, H-2, H-6 Ph), 7.89 (d, 2H, *J*=13.6 Hz, H-5 Quin) 8.20 (d, 2H, *J*=8.8 Hz, H-3, H-5, Ph), 8.72 (s, 1H, H-2 Quin). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 7.99, 36.17, 36.96, 45.65, 50.93, 106.63, 107.11, 111.82 (d, *J*= 23.5 Hz), 120.16, 124.10, 130.76, 139.09, 145.78, 147.21, 148.38, 152.20, 153.8 (d, *J*= 245.0 Hz), 158.12, 158.72, 162.67, 175.01. MS (m/z, %): 581 (M^+, 6), 513 (5), 366 (6), 356 (4), 314 (9), 287 (17), 269 (9), 245 (22), 202 (25), 186 (10), 168 (11), 157 (10), 137 (72), 118 (48), 106 (45), 91 (46), 77 (59), 65 (65), 44 (100). Anal. Calcd for $C_{26}H_{24}FN_7O_4S_2$: C, 53.69; H, 4.16; N, 16.86. Found: C, 53.70; H, 4.29; N, 16.82.

*4.5. Preparation of enrofloxacin derivatives **14a** and **14b***

To a mixture of enrofloxacin (**1b**, 1.0 mmol) in dry CH_2Cl_2 (3 mL) was added triethylamine (2 mmol), and stirred at room temperature for 30 min. Then, ethyl chloroformate (1mmol) was added dropwise at 0 ºC and the stirring was continued for 1 h and allowed the reaction mixture to reach room temperature to get a solution. After addition of the corresponding thiadizole-amine **9**, the reaction was stirred overnight. Finally, the precipitated solid was filtered and washed carefully with cold dichloromethane to obtain desired amide **14**.

*4.5.1. N-(5-(Benzylthio)-1,3,4-thiadiazol-2-yl)-1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamide (**14a**)*

Yield 35%; m.p. 236-240°C; IR (KBr, cm^{-1}): 3467, 2925, 2821, 1674, 1626, 1610, 1523, 1481, 1380, 1334, 1253, 1126, 1049, 1024, 762, 677. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.07 (t, 3H,

CH_3), 1.12-1.43 (m, 4H, *c*-Pr) 2.61 (m, 4H, Pip), 3.20-3.73 (m, 7H, Pip and NCH_2Me), 3.80-3.95 (m, 1H, *c*-Pr), 4.52 (s, 2H, CH_2 benzyl), 7.30 (t, 1H, *J*= 8.0 Hz, H-4 Ph), 7.36 (t, 2H, *J*= 7.6 Hz, H-3, H-5 Ph), 7.44 (d, 2H, *J*= 7.2 Hz, H-2, H-6 Ph), 7.55 (d, 1H, *J*= 7.2 Hz, H-8 Quin), 7.93 (d, 1H, *J*= 12.0 Hz, H-5 Quin), 8.75 (s, 1H, H-2 Quin), 13.97 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 7.99, 12.39, 36.13, 37.97, 49.82, 51.98, 52.42, 55.39, 106.56, 107.09, 111.72 (d, *J*= 23.6 Hz), 120.18 (d, *J*= 7.2 Hz), 128.08, 129.03, 129.50, 137.11, 138.92, 145.21 (d, *J*= 10.1 Hz), 148.21, 153.29 (d, *J*= 247.4 Hz), 158.32, 158.89, 162.42, 174.83. MS (m/z, %): 564 (M^+, 6), 383 (4), 368 (8), 342 (27), 286 (6), 257 (8), 121 (4), 91 (100), 70 (8), 57 (33), 42 (36). Anal. Calcd for $C_{28}H_{29}FN_6O_2S_2$: C, 59.56; H, 5.18; N, 14.88. Found: C, 59.71; H, 5.32; N, 14.69.

*4.5.2. 1-Cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-N-(5-((4-fluorobenzyl)thio)-1,3,4-thiadiazol-2-yl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (**14b**)*

Yield 33%; m.p. > 300°C; IR (KBr, cm^{-1}): 3469, 3401, 2918, 1667, 1627, 1608, 1504, 1482, 1278, 1217, 1031, 921, 779, 676. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 1.15-1.40 (m, 7H, *c*-Pr and CH_3), 3.10-3.50 (m, 8H, Pip), 3.55-3.67 (m, 2H, NCH_2Me), 3.81-3.93 (m, 1H, *c*-Pr), 4.50 (s, 2H, CH_2 benzyl), 7.16 (t, 2H, *J*= 8.5 Hz, H-3, H-5 Ph), 7.42-7.50 (m, 2H, H-2, H-6 Ph), 7.59 (d, 1H, *J*= 6.5 Hz, H-8 Quin), 7.98 (d, 1H, *J*= 12.5 Hz, H-5 Quin), 8.76 (s, 1H, H-2 Quin), 13.90 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 8.06, 12.29, 36.15, 37.12, 46.77, 48.99, 50.36, 51.16, 107.33, 111.89 (d, *J*= 23.0 Hz), 115.82 (d, *J*= 20.7 Hz), 127.18, 131.57 (d, *J*= 7.9 Hz), 138.97, 138.45, 143.94, 148.87, 153.39 (d, *J*= 247.8 Hz), 158.67, 158.78, 162.12 (d, *J*= 245.5 Hz), 162.57, 174.84. MS (m/z, %): 582 (M^+, 4), 368 (3), 342 (14), 257 (3), 139 (5), 109 (100), 83 (14), 70 (5), 57 (21), 42 (20). Anal. Calcd for $C_{28}H_{28}F_2N_6O_2S_2$: C, 57.72; H, 4.84; N, 14.42. Found: C, 57.65; H, 4.82; N, 14.49.

4.6. MTT assay

The MTT (3-(4,5-dimethyl thiadiazole-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay was performed to evaluate the cytotoxic effect of synthesized compounds (**12b**, **12h**, **13a-j**, **14a** and **14b**) against three different cancerous cell lines MCF-7, A549 and SKOV-3. The cell lines were obtained from Pasture Institute, Iran. The procedures used for cell culture, treatment and viability

assay were based on reported methods in literatures [27,28]. The reference drug doxorubicin and DMSO 1% was used as positive and negative controls, respectively. The quinolone drug CF was included in the cell viability assay as parent compound for more comparisons. The IC_{50} values were determined from curves constructed by plotting cell viability (%) versus concentration, using Prism 6, GraphPad Software.

4.7. Cell cycle analysis

The cell cycle analysis was performed in MCF-7 cells, treated with compounds **13e** and **13g**, using flow cytometric method. Briefly, MCF-7 cells (3×10^5 cells/well) were seeded in 6-well plate and incubated with IC_{50} concentration of **13e** and **13g** for 48 h. Subsequently, both treated and untreated cells were collected and washed with PBS (phosphate buffered saline) twice, and fixed with 70% ethanol for 3 hours. Then, RNase A (5 µL, 10 mg/mL concentration) was added to each sample and after 1 h at r.t, mixed with 10 µL of PI (50 µg/mL final concentration). At the end, 30 min after addition of PI, the cell distribution was determined by FACS Calibur flow cytometer and utilizing FlowJo software ver. X.0.7 [28].

4.8. Annexin-V/PI double staining assay

For analyzing apoptotic and necrotic cells after treatment with the selected compounds **13e** and **13g**, annexin-V/PI double staining method was carried out followed by flow cytometry [29]. Briefly, MCF-7 cells with optimum density (3×10^5) were cultured in 6-well plates and after 24 h of incubation; the cells were treated with IC_{50} concentration of **13e** and **13g**. After 48 h, the treated and untreated cells were harvested and washed twice with cold PBS. Then, the cells were resuspended in binding buffer (500 µL) containing 10 µL of Annexin V-fluorescein isothiocyanate (FITC). Subsequently, propidium iodide (PI) solution (10 µL) was added to each sample and the stained cells were kept in the dark at r.t for 15 min and subjected to flow cytometry. For determining the percentage of apoptotic cells in each quadrant, output data were analyzed using FlowJo software ver. X.0.7.

4.8. Comet assay

The DNA damage in MCF-7 cells, exposed to **13e** and **13g** was measured by alkaline single cell gel electrophoresis (SCGE) method as reported previously [30]. The MCF-7 cells were treated with the compounds **13e** and **13g** at the concentrations of 1, 5 and 10 μM for 1 h. Doxorubicin and CF were included for comparison. The untreated cells were used as negative control. The DNA damage parameters were estimated by using Comet Score software project (CASP).

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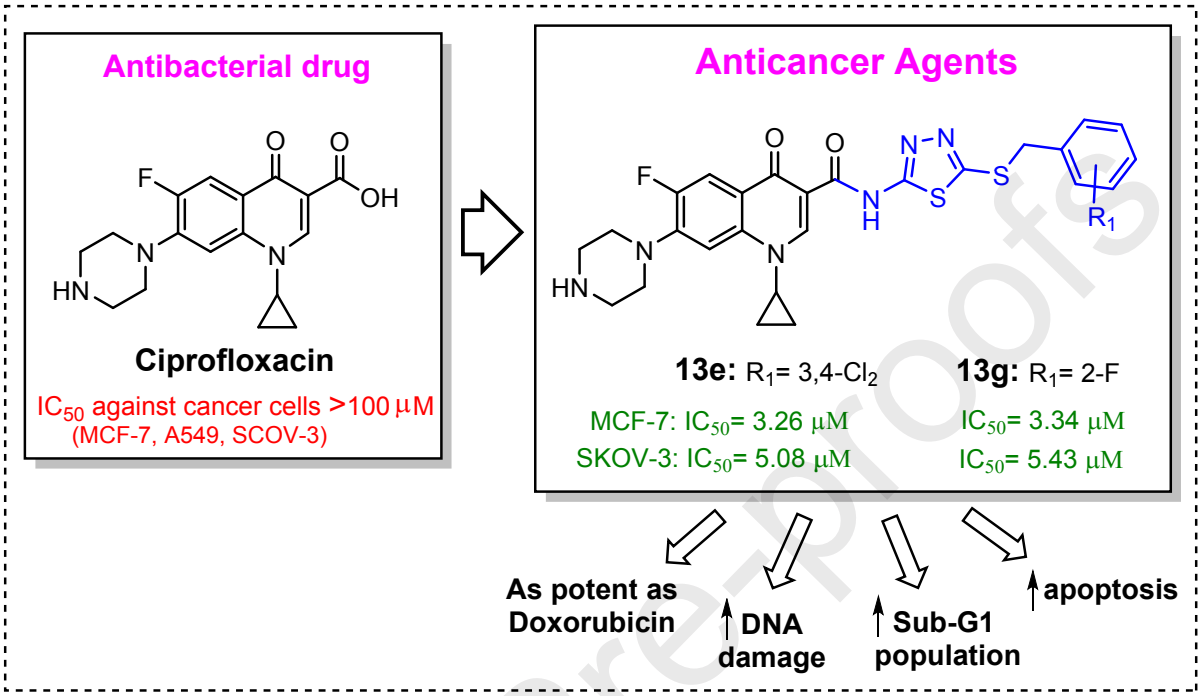
Graphical Abstract



Research Highlights

- C-3 modified ciprofloxacin analogs bearing thiadiazole-carboxamide were synthesized as anticancer agents.
- MTT assay showed that most of them have significant activity against MCF-7, A549 and SKOV-3.
- Compounds **13a-e** and **13g** were as potent as doxorubicin against MCF-7 cell line (IC_{50}s = 3.26-3.90 μM).
- Promising compounds **13e** and **13g** induced apoptosis and increased sub-G1 cell population in MCF-7 cells.
- Comet assay demonstrated the potential of compounds in DNA damage and fragmentation.