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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis and anticancer activity of functionalized spiro-quinolines with barbituric and thiobarbituric acids

Ravi Kiran Bhaskarachar[1] · Vijayakumar G. Revanasiddappa[1] · Subramanya Hegde[2] · Janardhana P. Balakrishna[3] · Suman Y. Reddy[4]

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Abstract A new series of spiro-quinoline compounds have been accomplished by the reaction of barbituric acid or thiobarbituric acid with derivatives of benzisoxazole-5-carbaldehyde or 2-substituted benzaldehyde. These compounds were evaluated for their in vitro cytotoxicity on two mammalian cancer cell lines MCF-7 and KB. The compounds exhibit cytotoxicity against these cell lines in micromolar range. Among the series of compounds, **11**(**a**–**j**) particularly **11b** and **11e** showed relatively good activity against both the tested cell lines. Compound **11b** was found to exhibit the highest cytotoxic activity with IC_{50} value 90.2 μM for MCF-7 and 49.8 μM for KB cell line. Flow cytometric analysis study confirmed that these molecules induced cytotoxicity via apoptosis.

Keywords Spiro-quinoline · Barbituric acid · Benzisoxazole · [1–5] Shift · MTT assay · Flow cytometry

Introduction

Cancer has taken a leading place among all the life-threatening diseases, and a large group of scientific community is still working for the elimination of this disease. Chemotherapy has been proved to be one of the promising cancer treatment methods. Finding new drugs for the better treatment of this life-threatening disease is always challenging for the chemists and has been well documented in recent years (Mona and Naida, 2014; Eric *et al.*, 2014).

Tetrahydroquinoline is a privileged heterocyclic ring system which featured in a large number of natural products and their analogues (Michael, 2004, 2005, 2007, 2008). Moreover, its skeleton is present in bioactive alkaloids, pharmaceuticals (Kim *et al.*, 1996a, b) and medicinally relevant compounds (Rakotoson *et al.*, 1998; Mimi *et al.*, 2014; Atul *et al.*, 2011). Importance of tetrahyrdroquinolines as potential antiviral, antibacterial, antimalarial and antifungal agents has been well known in the recent literature (Brendan *et al.*, 2015; Jon *et al.*, 2014; Romain *et al.*, 2014; Weixing *et al.*, 2014). Several of the tetrahydroquinoline derivatives have also been used as pharmacological relevant agents viz. chemotherapeutic targets and pharmacodynamic targets. Further, its application has extended in the preparation of coordination ligands (Wang and Ding, 2009; Kaiser *et al.*, 2006; Pullmann *et al.*, 2010) and dyes (Yamashita *et al.*, 2008; Agbo *et al.*, 2000; Hallas and Zhai, 1996). Likewise, spiro-tetrahydroquinolines are also important structural motifs and find wide applications in medicinal chemistry field. Furthermore, spiro fusion with tetrahydroquinoline moiety at the C3-position was documented as possessing significant biological activities (Kouznetsov *et al.*, 2010; Patel *et al.*, 2008). Spiro compounds with tetrahydroquinoline can be obtained for many molecules, and one such conversion

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✉ Vijayakumar G. Revanasiddappa
vijaykumargr18@yahoo.co.in

[1] Department of Chemistry, UCS, Tumkur University, Tumakuru 572 103, India

[2] Department of Chemistry, Government Science College, Nrupathunga Road, Bangalore, India

[3] Stellixir Biotech Pvt Ltd., Bangalore 560057, India

[4] Department of Chemistry, Indian Institute of Technology, Kanpur 208 016, India

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could be using biologically demanding pyrimidine derivatives viz. barbituric and thiobarbituric acids (Ruble *et al.*, 2009). Wide range of pharmacological activities can be accounted for barbituric acid derivatives (Sweidan *et al.*, 2011; Schwarz *et al.*, 2013; Bassin and Bleck, 2008; Humar *et al.*, 2004; Singh *et al.*, 2009), which reveal the importance of its structural modification. Synthetic strategy for the six-membered spiro-quinoline compounds using simple aldehydes has been revealed in earlier reports (Ruble *et al.*, 2009; Krasnov and Kartsev, 2005). However, the scope of the reaction is not extended for aldehydes comprising benzisoxazole, or any other heterocyclic moieties such as benzofuran or benzothiazole.

It would thus be interesting to synthesize molecules possessing tetrahydroquinoline moieties fused with pyrimidines at C3-position and evaluate their biological properties. So an attempt to design, synthesize and evaluation of in vitro cytotoxic activity of novel spiro-quinoline compounds containing pyrimidinetrione or thioxopyrimidinediones is made. We describe herein a synthetic method for the preparation of benzisoxazole and benzene carbaldehydes having substituted secondary amine group followed by six-membered spiro-quinolines with barbituric acid or thiobarbituric acid. Synthesized spiro-quinoline compounds were screened for their in vitro anticancer activity against two mammalian cell lines viz MCF-7 and KB by using MTT assay, and the cell death mechanism has been studied by flow cytometric analysis.

Experimental

Materials and methods

Melting points were determined using open capillary, and values given are uncorrected. IR spectra were recorded by using JASCO FTIR-4100 Spectrophotometer by KBr pellet method. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on JEOL-400 MHz NMR instrument using $CDCl_3$/DMSO-d_6 as solvent. Chemical shift values were expressed in δ (ppm) relative to tetramethylsilane (TMS) as an internal reference standard. Mass spectra of the compounds were recorded on Shimadzu LC-2010EV with ESI probe. High-resolution mass spectroscopy of the final compounds was conducted with electron spray ionization method and a time of flight analyser using Waters Micromass Q-top instrument. The reaction progress was monitored by thin layer chromatography using TLC Silica gel 60 F_{254} (Merck), and spots were visualized by using ultraviolet light of 254 nm. All the solvents employed were of analytical grade and distilled once before use. Starting materials and reagents were purchased from Sigma Chemical Co. (Saint Louis, USA).

*3,4-Difluoro-2-hydroxybenzoic acid (**1**)*

Solid sodium hydroxide (4.52 g, 113 mmol) was added in portions to an ice-cooled and stirred solution of 2,3,4-trifluorobenzoic acid (5.0 g, 28 mmol) in dimethylimidazolidinone (10 mL), and the mixture was heated to 120 °C for 2 h during which TLC analysis showed the disappearance of 2,3,4-trifluorobenzoic acid. Reaction mixture was cooled to room temperature and acidified (pH 5–6) with 2 N hydrochloric acid (7.5 mL). The white solid separated out was filtered, washed with excess of water and dried. White solid (3.6 g, 73.4 %); mp: 174–177 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 6.94–7.01 (m, 1H, aromatic), 7.64–7.68 (m, 1H,). IR (KBr, cm^{-1}) 3208.9 (OH), 1654.1 (C=O), 1625.7 (C=C) (aromatic), 1315.2 (C–O).

*Methyl 3,4-difluoro-2-hydroxybenzoate (**2**)*

To an ice-cooled and stirred solution of 3,4-difluoro-2-hydroxybenzoic acid (**1**) (1 g, 5 mmol) in dry dichloromethane (5.7 mL), oxalyl chloride (1.45 g, 11 mmol) and *N*,*N*-dimethylformamide (0.25 mL) were added at 0 °C and the mixture was stirred at room temperature for 3 h. Reaction mixture was cooled to 0 °C, and methanol (1 mL) was added dropwise over a period of 10 min and stirred at room temperature for 12 h. DCM was removed under vacuum, and the solution was poured into the ice-cold water to get white solid. The wet solid was dissolved in diethyl ether and dried over sodium sulphate. Removal of solvent under vacuum afforded the title compound as white solid. White solid (1.07 g, 99.0 %); mp: 42–45 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 3.97 (s, 3H), 6.67–6.73 (m, 1H), 7.59–7.63 (m, 1H), 11.01 (s, 1H). IR (KBr, cm^{-1}) 3357.5 (OH), 2961.2 (CH_3), 1786.7 (C=O), 1631.5 (C=C, aromatic), 1340.4 (C–O).

*3,4-Difluoro-N,2-dihydroxybenzamide (**3**)*

To a solution of **2** (1 g, 5 mmol) in methanol (9 mL), hydroxylamine hydrochloride (0.74 g, 10 mmol) and potassium hydroxide pellets (1.19 g, 21 mmol) were added. The mixture was stirred for 4 h at room temperature, temperature was gradually increased to 60 °C, and stirring was continued for further 2 h. The reaction mixture was cooled to 10 °C, and pH of the solution was adjusted to 2.0 using 1.5 N HCl to afford the title compound as white solid. The solid was washed with water and dried. White solid (0.87 g, 87.0 %); mp: 165–168 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 6.92–6.99 (m, 1H), 7.51–7.55 (m, 1H), 9.54 (s, 1H), 11.68 (s, 1H), 13.12 (s, 1H). IR (KBr, cm^{-1}) 3316.9 (NH), 3136.6 (OH), 1730.8 (C=O, amide), 1503.2 (C–C, aromatic), 850.4 (C–H, aromatic).

*6,7-Difluorobenzo[d]isoxazol-3(2H)-one (**4**)*

CDI (5.15 g, 31 mmol) in dry THF (18.1 mL) was added dropwise at 65 °C to a stirring solution of **3** (3.3 g, 17 mmol) in dry THF (26.4 mL), and stirring was continued at the same temperature for further 1 h. Solvents were removed under vacuum and the semi-solid obtained was stirred vigorously with 1.5 N HCl for 10 min. The white solid obtained was washed with water and dried. White solid (2.03 g, 67.6 %); mp 147–150 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 7.39–7.46 (m, 1H), 7.59–7.62 (m, 1H), 12.90 (s, 1H). IR (KBr, cm^{-1}) 1645.9 (C=O), 3015.1 (N–H), 1477.2 (C=C, aromatic). ESI MS (m/z) 170.2, calculated mass: 171.1.

*3-Chloro-6,7-difluorobenzo[d]isoxazole (**5**)*

To an ice-cooled mixture of **4** (0.25 g, 1 mmol), phosphorous oxy chloride (0.67 g, 4 mmol) and triethylamine (0.14 g, 1 mmol) were added and the mixture was heated at 140 °C in a sealed tube for 2 h. The reaction mass was cooled to room temperature and then poured into crushed ice slowly with vigorous stirring. The solid thus obtained was filtered and washed with ice-cold water. The wet solid was then dissolved in diethyl ether and dried over anhydrous sodium sulphate. Removal of solvent under vacuum afforded the title compound as pale brown solid. Brown solid (0.17 g, 61.59 %); mp 35–38 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 7.25–7.31 (m, 1H), 7.41–7.45 (m, 1H). IR (KBr, cm^{-1}) 757.8 (C–Cl), 1645.9 (C=C, aromatic), 3095.1 (C–H, aromatic).

*3-Chloro-6,7-difluorobenzo[d]isoxazole-5-carbaldehyde (**6**)*

To a stirred solution of diisopropylamine (0.162 g, 1 mmol) in dry THF (1 mL), *n*-butyl lithium (0.112 g, 1 mmol) was added dropwise at −10 °C and stirring was continued for 30 min at the same temperature. The reaction mixture was then cooled to −78 °C, and a solution of **5** (0.25 g, 1 mmol) in dry THF (1 mL) was slowly added over a period of 30 min followed by *N*,*N*-dimethylformamide (0.195 g, 2 mmol). Stirring was continued at the same temperature for another 30 min. The reaction was quenched by the addition of saturated solution of ammonium chloride and then extracted two times (100 mL × 2) with MTBE (methyl tertiary butyl ether). The combined organic layer was washed with water followed by brine and then dried over anhydrous sodium sulphate. Removal of solvent under vacuum afforded the crude material, which was purified by silica gel (230–400 mesh) column chromatography using 2 % MTBE in pet ether. Pale yellow solid (0.140 g, 48.9 %); mp: low-melting solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.08 (s, 1H), 10.38 (s, 1H). IR (KBr, cm^{-1}) 3064.0 (C–H, aromatic), 2902.1 (C–H, alkane), 1687.8 (C=O, aldehyde), 731.11 (C–Cl).

*General method for the preparation of compounds 7(**a**–**e**)*

To an ice-cooled and stirred solution of **6** (0.250 g, 1 mmol) in acetonitrile (2.5 mL), DIPEA (0.178 g, 1 mmol) was added followed by morpholine (for **7a**) (0.122 g, 1 mmol) and heated at 85 °C for 4 h. It was cooled to room temperature and then concentrated. The residue was dissolved in ethyl acetate, washed with water followed by brine and then dried over anhydrous sodium sulphate. The solvent was removed under vacuum to get crude compound. The obtained crude material was purified over silica gel column (60–120 mesh) using gradient ethyl acetate/pet ether to afford title compound as pale yellow solid. The compounds **7**(**b**–**e**) were similarly synthesized.

3-Chloro-7-fluoro-6-morpholinobenzo [d]isoxazole-5-carbaldehyde (**7a**) Pale yellow solid (0.29 g, 88.6 %); mp: 101–103 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 3.37 (dd, J = 2.0, 3.8 Hz, 4H), 3.89 (t, J = 9.2 Hz, 4H), 8.01 (s, 1H), 10.39 (s, 1H). IR (KBr, cm^{-1}) 3051.8 (C–H, aromatic), 2863.7 (C–H, alkane), 1693.1 (C=O, aldehyde), 851.4 (C–Cl).

3-Chloro-7-fluoro-6-thiomorpholinobenzo[d]isoxazole-5-carbaldehyde (**7b**) The title compound was prepared according to the general procedure as described in **7a**. Pale yellow solid (0.28 g, 81.1 %); mp: 96–98 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 2.83 (t, J = 8.00 Hz, 4H), 3.57–3.60 (m, 4H), 8.00 (s, 1H), 10.36 (s, 1H). IR (KBr, cm^{-1}) 3051.8 (C–H, aromatic), 2863.7 (C–H, alkane), 1693.1 (C=O, aldehyde), 851.4 (C–Cl).

3-Chloro-7-fluoro-6-(4-methylpiperazin-1-yl)benzo[d]isoxazole-5-carbaldehyde (**7c**) The title compound was prepared according to the general procedure as described in **7a**. Lemon yellow solid (0.17 g, 62.3 %); mp: 98–101 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 2.40 (s, 3H), 2.63 (s, 4H), 3.41 (s, 4H), 7.98 (s, 1H), 10.34 (s, 1H). IR (KBr, cm^{-1}) 2925.4 (C–H, aromatic), 2853.1 (C–H, alkane), 1693.1 (C=O, aldehyde), 877.4 (C–Cl).

3-Chloro-6-(4-ethylpiperazin-1-yl)-7-fluorobenzo[d] isoxazole-5-carbaldehyde (**7d**) The title compound was prepared according to the general procedure as described in **7a**. Pale yellow solid (0.21 g, 73.4 %); mp: 115–117 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 1.07 (t, J = 16.00 Hz, 3H), 2.45 (q, J = 24.00 Hz, 2H), 2.57 (s, 4H), 3.34 (s, 4H), 7.90 (s, 1H), 10.26 (s, 1H). IR (KBr, cm^{-1}) 3063.7 (C–H, aromatic), 2853.1 (C–H, alkane), 1693.1 (C=O, aldehyde), 876.4 (C–Cl).

3-Chloro-7-fluoro-6-(4-phenylpiperazin-1-yl)benzo[d]isoxazole-5-carbaldehyde (**7e**) The title compound was prepared according to the general procedure as described in **7a**. Brown solid (0.31 g, 93.9 %); mp: 164–167 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 3.38 (s, 4H), 3.54 (s, 4H), 6.93–7.01 (m, 3H), 7.31 (t, $J = 13.60$ Hz, 2H), 8.01 (s, 1H), 10.41 (s, 1H). IR (KBr, cm^{-1}) 3061.4 (C–H, aromatic), 2857.0 (C–H, alkane), 1690.3 (C=O, aldehyde), 877.4 (C–Cl).

*General method for the preparation of **10(a–e)***

To an ice-cooled and stirred solution of **9** (500 mg, 1 mmol) in acetonitrile (2.5 mL), DIPEA (1 mmol) was added followed by morpholine (for **10a**) (1 mmol) and heated at 85 °C for 4 h. It was cooled to room temperature and then concentrated. The residue was dissolved in ethyl acetate, washed with water followed by brine and then dried over anhydrous sodium sulphate. The solvent was removed under vacuum to get crude compound. The obtained crude material was purified over silica gel column (60–120 mesh) using gradient ethyl acetate/pet ether to give title compound as pale yellow solid. Compounds **10**(**b**–**e**) were similarly synthesized.

2-Morpholinobenzaldehyde (**10a**) Pale yellow solid (0.39 g, 50.6 %); mp: 84–86 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.85 (t, $J = 10.00$ Hz, 4H), 3.35 (t, $J = 10.00$ Hz, 4H), 7.12–7.17 (m, 2H), 7.52-7.56 (m, 1H), 7.80–7.83 (m, 1H), 10.33 (s, 1H). IR (KBr, cm^{-1}) 3067.2 (C–H, aromatic), 2960.2 (C–H, alkane), 2827.1 (C=O, aldehyde), 1332.6 (C–N, aromatic).

2-Thiomorpholinobenzaldehyde (**10b**) The title compound was prepared according to the general procedure as described in **10a**. Pale yellow solid (0.43 g, 51.4 %); mp: 78–80 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.10 (t, $J = 4.80$ Hz, 4H), 3.90 (t, $J = 9.20$ Hz, 4H), 7.11–7.17 (m, 2H), 7.53–7.57 (m, 1H), 7.81–7.83 (m, 1H), 10.35 (s, 1H). IR (KBr, cm^{-1}) 3065.3 (C–H, aromatic), 2910.1 (C–H, alkane), 2826.2 (C=O, aldehyde), 1284.4 (C–N, aromatic).

2-(4-Methylpiperazin-1-yl)benzaldehyde (**10c**) The title compound was prepared according to the general procedure as described in **10a**. Pale yellow solid (0.45 g, 54.8 %); mp: 82–84 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.39 (s, 3H), 2.64 (s, 4H), 3.14 (t, $J = 9.60$ Hz, 4H), 7.10–7.14 (m, 2H), 7.51–7.55 (m, 1H), 7.79–7.82 (m, 1H), 10.33 (s, 1H). IR (KBr, cm^{-1}) 3065.3 (C–H, aromatic), 2926.4 (C–H, alkane), 2797.2 (C=O, aldehyde), 1289.2 (C–N, aromatic).

2-(4-Ethylpiperazin-1-yl)benzaldehyde (**10d**) The title compound was prepared according to the general procedure as described in **10a**. Pale yellow solid (0.40 g, 45.9 %); mp: 95–97 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.14 (t, $J = 14.80$ Hz, 3H), 2.52 (q, $J = 21.60$ Hz, 2H), 2.67 (m, 4H), 3.15 (t, $J = 10.00$ Hz, 4H), 7.10–7.14 (m, 2H), 7.51–7.55 (m, 1H), 7.79–7.82 (m, 1H), 10.33 (s, 1H). IR (KBr, cm^{-1}) 3065.3 (C–H, aromatic), 2925.5 (C–H, alkane), 2820.4 (C=O, aldehyde), 1284.4 (C–N, aromatic).

2-(4-Phenylpiperazin-1-yl)benzaldehyde (**10e**) The title compound was prepared according to the general procedure as described in **10a**. Pale yellow solid (0.51 g, 47.6 %); mp: 110–112 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.25–3.27 (m, 4H), 3.38–3.40 (m, 4H), 6.89–7.85 (m, 9H), 10.39 (s, 1H). IR (KBr, cm^{-1}) 3066.3 (C–H, aromatic), 2923.6 (C–H, alkane), 2831.0 (C=O, aldehyde), 1326.8 (C–N, aromatic).

*General procedure for the preparation of compounds **8(a–j), 11(a–j)***

Barbituric acid/thiobarbituric acid (1 eqiv) was added to a stirred solution of **7**(**a**–**e**) or **10**(**a**–**e**) (l eqiv) in IPA (isopropyl alcohol) (2 mL) and refluxed for 8 h. The reaction mass was cooled to room temperature and then poured into crushed ice slowly with vigorous stirring. The solid thus obtained was dissolved in ethyl acetate. The combined organic layer was washed with water followed by brine and then dried over anhydrous sodium sulphate. Removal of solvent under vacuum afforded the title compound. The solid compound was purified by triturating with nonpolar pet ether solvent.

8-Chloro-11-fluoro-2,4,4a,6-tetrahydro-1H,1′H-spiro[isoxazolo[4,5-g][1,4]oxazino[4,3-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**8a**) Brown solid (0.11 g, 79.7 %); mp: 178–180 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 3.23 (s, 2H), 3.35–3.53 (m, 3H), 3.63 (t, $J = 19.20$ Hz, 1H), 3.76–3.89 (m, 3H), 7.28 (s, 1H), 11.31 (s, 1H), 11.61 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 36.44, 49.75, 49.90, 60.16, 65.98, 66.13, 111.58, 114.08, 114.11, 125.23, 132.86, 148.57, 148.60, 168.51, 170.50.IR (KBr, cm^{-1}) 3321.7 (N–H) 2924.5 (C–H, aromatic), 2853.1 (C–H, alkane), 1732.7 (C=O), 760.7 (C–Cl). ESI MS (m/z) 392.9, Calcd. mass: 394.7.HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd. for $C_{16}H_{12}ClFN_4O_5Na$ 417.0378; Found 417.0399.

8-Chloro-11-fluoro-2,4,4a,6-tetrahydro-1H,1′H-spiro[isoxazolo[4,5-g][1,4]thiazino[4,3-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**8b**) Pink solid (0.12 g, 89.7 %); mp: 190–192 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 2.31 (d, $J = 12.8$ Hz, 1H), 2.56 (d, $J = 13.2$ Hz, 1H), 2.94 (t, $J = 23.6$ Hz, 1H), 3.13 (t, $J = 22.4$ Hz, 1H), 3.28 (d, $J = 15.2$ Hz, 1H), 3.42 (d, $J = 15.6$ Hz, 1H), 3.66 (t,

$J = 24.8$ Hz, 1H), 4.31 (d, $J = 10.0$ Hz, 1H), 4.46 (d, $J = 14.4$ Hz, 1H), 7.01 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 26.44, 36.21, 54.41, 54.69, 54.79, 59.69, 62.76, 111.43, 113.80, 125.42, 132.74, 148.55, 149.78, 152.17, 152.34, 168.19, 170.27. IR (KBr, cm^{-1}) 3219.5 (N–H) 2924.5 (C–H, aromatic), 2853.1 (C–H, alkane), 1730.8 (C=O), 742.4 (C–Cl). ESI MS (m/z) 410.8, Calcd. mass: 410.8. HRMS (ESI-TOF)*m/z*:$[M+Na]^+$ Calcd. for $C_{16}H_{12}ClFN_4O_4SNa$ 433.0150; Found 433.0151.

8-Chloro-11-fluoro-3-methyl-1,2,3,4,4a,6-hexahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino [1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**8c**) Brown solid (0.09 g, 66.1 %); mp: 248–250 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 2.19 (s, 4H), 2.72 (dd, $J = 10.4$, 30.4 Hz, 2H), 3.28 (s, 3H), 3.49 (d, $J = 15.60$ Hz, 1H), 3.79 (d, $J = 8.00$ Hz, 1H), 4.14 (d, $J = 12.40$ Hz, 1H), 7.23 (s, 1H), 11.36 (s, 2H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 35.72, 45.46, 49.41, 49.54, 54.06, 54.99, 60.43, 111.27, 113.81, 125.61, 132.72, 148.52, 149.78, 152.00, 152.14, 168.49, 170.81. IR (KBr, cm^{-1}) 3443.2 (N–H) 2924.5 (C–H, aromatic), 2853.1 (C–H, alkane), 1730.8 (C=O), 742.4 (C–Cl). ESI MS (m/z) 407.9, Calcd. mass: 407.8. HRMS (ESI-TOF)*m/z*:$[M+H]^+$ Calcd. for $C_{17}H_{15}ClFN_5O_4H$ 408.0875; Found 408.0880.

8-Chloro-3-ethyl-11-fluoro-1,2,3,4,4a,6-hexahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino [1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**8d**) Brown solid (0.06 g, 76.5 %); mp: 256–259 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 1.54 (d, $J = 8.00$ Hz, 2H), 2.26 (d, $J = 59.60$ Hz, 6H), 2.87 (d, $J = 7.60$ Hz, 3H), 3.51 (d, $J = 16.00$ Hz, 1H), 3.76 (d, $J = 14.40$ Hz, 1H), 4.12 (d, $J = 12.00$ Hz, 1H), 7.27 (s, 1H), 11.32 (s, 1H), 11.62 (s, 1H). IR (KBr, cm^{-1}) 3410.0 (N–H) 2927.4 (C–H, aromatic), 2856.8 (C–H, alkane), 1707.0 (C=O), 800.8 (C–Cl). ESI MS (m/z) 421.9, Calcd. mass: 421.8.HRMS (ESI-TOF)*m/z*:$[M+H]^+$ Calcd. for $C_{18}H_{17}ClFN_5O_4H$ 422.1031; Found 422.1035.

8-Chloro-11-fluoro-3-phenyl-1,2,3,4,4a,6-hexahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino [1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**8e**) Dark brown solid (0.11 g, 84.6 %); mp: 278–281 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 2.75 (q, $J = 22.00$ Hz, 1H), 2.92 (q, $J = 20.00$ Hz, 1H), 3.24 (d, $J = 15.60$ Hz, 1H), 3.46–3.64 (m, 3H), 3.73 (d, $J = 11.60$ Hz, 1H), 3.99–4.09 (m, 1H), 4.26 (d, $J = 12.80$ Hz, 1H), 6.80 (t, $J = 14.40$ Hz, 1H), 6.93 (d, $J = 8.00$ Hz, 2H), 7.19–7.27 (m, 3H), 11.30 (s, 1H), 11.63 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 37.22, 48.46, 49.31, 50.85, 59.47, 59.69, 111.18, 114.05, 115.49, 115.81, 119.22, 124.95, 128.94, 132.60, 149.86, 150.29, 152.14, 152.28, 168.30, 170.73. IR (KBr, cm^{-1}) 3208.4 (N–H) 2924.1 (C–H, aromatic), 2850.5 (C–H, alkane), 1695.1 (C=O), 760.5 (C–Cl). ESI MS (m/z) 469.9, Calcd. mass: 469.8.HRMS (ESI-TOF)*m/z*:$[M+Na]^+$ Calcd. for $C_{22}H_{17}ClFN_5O_4Na$ 492.0851; Found 492.0851.

8-Chloro-11-fluoro-2′-thioxo-2,2′,3′,4,4a,6-hexahydro-1H,1′H-spiro[isoxazolo[4,5-g] [1,4]oxazino[4,3-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**8f**) Brown solid (0.10 g, 86.9 %); mp: 162–164 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 3.27 (s, 1H), 3.39–3.52 (m, 3H), 3.64 (t, $J = 18.80$ Hz, 1H), 3.79–3.87 (m, 3H), 4.03 (t, $J = 13.60$ Hz, 1H), 7.30 (s, 1H), 12.40 (s, 1H), 12.64 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 36.01, 50.58, 59.69, 60.25, 65.94, 66.06, 111.86, 114.32, 125.12, 133.01, 148.63, 148.66, 151.84, 166.62, 168.47, 178.61. IR (KBr, cm^{-1}) 3196.6 (N–H) 2924.7 (C–H, aromatic), 2853.2 (C–H, alkane), 1701.2 (C=O), 887.4 (C–Cl). ESI MS (m/z) 409.0, Calcd. mass: 410.8.

8-Chloro-11-fluoro-2′-thioxo-2,2′,3′,4,4a,6-hexahydro-1H,1′H-spiro[isoxazolo[4,5-g][1,4]thiazino[4,3-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**8g**) Dark pink solid (0.11 g, 78.0 %); mp: 188–190 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 1.98 (s, 1H), 2.60 (s, 1H), 2.74 (s, 1H), 2.90 (s, 1H), 3.16 (d, $J = 13.20$ Hz, 1H), 3.53 (t, $J = 40.40$ Hz, 2H), 4.26 (d, $J = 42.00$ Hz, 2H), 7.33 (s, 1H), 12.41 (s, 1H), 12.65 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 25.83, 26.26, 35.56, 54.59, 55.01, 62.63, 111.59, 114.05, 125.08, 132.78, 148.61, 152.13, 152.27, 166.14, 168.13, 178.62. IR (KBr, cm^{-1}) 3280.5 (N–H) 2907.8 (C–H, aromatic), 2853.6 (C–H, alkane), 1698.5 (C=O), 871.4 (C–Cl). ESI MS (m/z) 424.9, Calcd. mass: 426.9. HRMS (ESI-TOF)*m/z*:$[M+Na]^+$ Calcd. for $C_{16}H_{12}ClFN_4O_3S_2Na$ 448.9921; Found 448.9920.

8-Chloro-11-fluoro-3-methyl-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**8h**) Brown solid (0.12 g, 84.5 %); mp: 267–269 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 1.22–1.36 (m, 7H), 1.50–1.57 (m, 2H), 3.07–3.19 (m, 1H), 3.38–3.55 (m, 2H), 7.38 (s, 1H), 11.64 (s, 1H), 12.43 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm)38.87, 46.44, 71.09, 95.24, 114.21, 126.55, 128.61, 131.51, 172.85, 178.63. IR (KBr, cm^{-1}) 3194.2 (N–H) 2929.0 (C–H, aromatic), 2853.9 (C–H, alkane), 1704.8 (C=O), 745.7 (C–Cl). ESI MS (m/z) 423.8, Calcd. mass: 423.8.HRMS (ESI-TOF)*m/z*:$[M+H]^+$ Calcd. for $C_{17}H_{15}ClFN_5O_3SH$ 424.0646; Found 424.0643.

8-Chloro-3-ethyl-11-fluoro-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**8i**) Brown solid (0.11 g, 78.5 %); mp: 270–272 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 0.88 (t, $J = 14.80$ Hz, 4H), 1.27–1.34 (m, 4H), 1.53–1.61 (m, 3H), 2.83 (t, $J = 14.80$ Hz, 3H), 7.35 (s, 1H), 11.62 (s, 1H). IR (KBr, cm^{-1}) 3189.1 (N–H) 2960.9 (C–H, aromatic), 2865.3 (C–H, alkane), 1711.7

(C=O), 877.3 (C–Cl). ESI MS (m/z) 437.8, Calcd. mass: 437.9. HRMS (ESI-TOF)*m/z*:$[M+H]^+$ Calcd. for $C_{18}H_{17}ClFN_5O_3SH$ 438.0803; Found 438.0807.

8-Chloro-11-fluoro-3-phenyl-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[isoxazolo[4,5-g]pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**8j**) Dark brown solid (0.11 g, 90.9 %); mp: 292–295 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 2.92 (t, J = 20.80 Hz, 1H), 3.26 (s, 2H), 3.44–3.57 (m, 3H), 3.69 (dd, J = 12.0, 19.6 Hz, 2H), 4.08 (d, J = 7.60 Hz, 1H), 6.80 (t, J = 14.40 Hz, 1H), 7.15–7.23 (m, 4H), 7.35 (s, 1H), 12.38 (s, 1H), 12.67 (s, 1H).^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 36.90, 48.43, 49.29, 49.41, 51.70, 59.46, 114.28, 115.52, 124.17, 124.65, 125.25, 128.14, 128.92, 132.68, 148.58, 150.30, 152.09, 152.23, 167.62, 168.63, 178.68. IR (KBr, cm^{-1}) 3196.4 (N–H) 2924.1 (C–H, aromatic), 2855.1 (C–H, alkane), 1695.1 (C=O), 746.7 (C–Cl). ESI MS (m/z) 485.8, Calcd. mass: 485.9.HRMS (ESI-TOF)*m/z*:$[M+Na]^+$ Calcd. for $C_{22}H_{17}ClFN_5O_3SNa$ 508.0622; Found 508.0622.

2,4,4a,6-Tetrahydro-1H,1′H-spiro[[1,4]oxazino[4,3-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**11a**) Cream solid (0.15 g, 95.5 %); mp: 167–169 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.84–2.91 (m, 1H), 3.10-3.30 (m, 2H), 3.38 (d, J = 2.40 Hz, 2H), 3.46–3.53 (m, 1H), 3.70 (dd, J = 2.4, 10.6 Hz, 1H), 3.85 (t, J = 21.20 Hz, 2H), 6.68 (t, J = 14.00 Hz, 1H), 6.93 (dd, J = 8.4, 26.6 Hz, 2H), 7.06 (t, J = 12.00 Hz, 1H), 11.29 (s, 1H), 11.50 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 33.19, 46.70, 48.89, 58.64, 65.75, 66.02, 112.67, 118.04, 121.39, 126.47, 128.36, 144.28, 149.92, 168.66, 171.28. IR (KBr, cm^{-1}) 3323.7 (N–H) 3092.3 (C–H, aromatic), 2987.2 (C–H, alkane), 1752.0 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:$[M+Na]^+$ Calcd. for $C_{15}H_{15}N_3O_4Na$ 324.0960; Found 324.0960.

2,4,4a,6-Tetrahydro-1H,1′H-spiro[[1,4]thiazino[4,3-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**11b**) Orange solid (0.11 g, 71.8 %); mp: 180–182 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.57 (d, J = 13.20 Hz, 2H), 2.82 (t, J = 17.60 Hz, 2H), 3.29–3.46 (m, 3H), 4.00 (d, J = 10.40 Hz, 1H), 4.39 (d, J = 14.80 Hz, 1H), 6.68 (t, J = 14.40 Hz, 1H), 6.82 (d, J = 8.40 Hz, 1H), 7.02–7.12 (m, 2H), 11.12 (s, 1H), 11.32 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 25.42, 25.57, 50.33, 53.64, 61.94, 62.45, 113.28, 117.57, 122.21, 126.53, 128.91, 141.04, 167.66, 169.59, 169.99. IR (KBr, cm^{-1}) 3443.3 (N–H) 3090.4 (C–H, aromatic), 2924.5 (C–H, alkane), 1726.0 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:$[M+Na]^+$ Calcd. for $C_{15}H_{15}N_3O_3SNa$ 340.0732; Found 340.0730.

3-Methyl-1,2,3,4,4a,6-hexahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**11c**) Brown solid (0.14 g, 92.1 %); mp: 208–211 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 1.86 (s, 1H), 2.03 (d, J = 3.20 Hz, 1H), 2.21 (s, 3H), 2.65 (d, J = 12.80 Hz, 1H), 2.83 (t, J = 21.60 Hz, 2H), 3.15 (d, J = 4.40 Hz, 2H), 3.46 (s, 1H), 3.95 (d, J = 13.20 Hz, 1H), 6.66 (t, J = 14.00 Hz, 1H), 6.89-6.96 (m, 2H), 7.03 (t, J = 15.60 Hz, 1H), 11.28 (s, 1H), 11.45 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 32.20, 45.50, 46.49, 50.00, 53.13, 54.88, 112.87, 117.82, 121.58, 126.41, 128.33, 150.05, 168.88, 171.48. IR (KBr, cm^{-1}) 3433.6 (N–H) 3092.3 (C–H, aromatic), 2923.6 (C–H, alkane), 1726.9 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:$[M+H]^+$ Calcd. for $C_{16}H_{18}N_4O_3H$ 315.1457; Found 315.1455.

3-Ethyl-1,2,3,4,4a,6-hexahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**11d**) Pale pink solid (0.13 g, 88.6 %); mp: 224–226 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 1.02 (t, J = 19.60 Hz, 3H), 1.27 (q, J = 25.20 Hz, 4H), 2.03 (d, J = 3.20 Hz, 1H), 2.33 (s, 2H), 2.69 (q, J = 18.80 Hz, 1H), 2.84 (q, J = 38.80 Hz, 2H), 3.15 (dd, J = 16.8, 29.2 Hz, 2H), 3.94 (d, J = 11.60 Hz, 1H), 6.67 (t, J = 14.40 Hz, 1H), 6.93 (dd, J = 8.0, 20.6 Hz, 2H), 7.04 (t, J = 23.60 Hz, 1H), 11.27 (s, 1H), 11.47 (s, 1H).^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 11.65, 32.51, 46.61, 49.96, 50.93, 51.47, 52.90, 112.96, 117.82, 121.62, 126.36, 128.31, 150.06, 168.99. IR (KBr, cm^{-1}) 3419.2 (N–H) 3091.3 (C–H, aromatic), 2978.5 (C–H, alkane), 1723.1 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:$[M+H]^+$ Calcd. for $C_{17}H_{20}N_4O_3H$ 329.1614; Found 329.1614.

3-Phenyl-1,2,3,4,4a,6-hexahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-2′,4′,6′(3′H)-trione (**11e**) Buff solid (0.15 g, 84.1 %); mp: 258–261 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.07 (t, J = 20.80 Hz, 2H), 3.17 (d, J = 16.80 Hz, 2H), 3.44 (d, J = 15.20 Hz, 2H), 3.60–3.67 (m, 2H), 4.08 (d, J = 12.40 Hz, 1H), 6.67 (t, J = 14.40 Hz, 1H), 6.82 (t, J = 14.40 Hz, 1H), 6.89 (d, J = 7.60 Hz, 2H), 6.97 (t, J = 14.00 Hz, 2H), 7.06 (t, J = 15.60 Hz, 1H), 7.24 (t, J = 16.00 Hz, 2H), 11.30 (s, 1H), 11.49 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 33.01, 46.55, 47.49, 48.69, 50.09, 58.42, 112.99, 115.66, 117.74, 119.52, 121.18, 126.64, 128.44, 129.09, 143.92, 150.05, 150.31, 167.70, 168.83, 171.52. IR (KBr, cm^{-1}) 3317.0 (N–H) 3093.3 (C–H, aromatic), 2955.4 (C–H, alkane), 1683.6 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:$[M+Na]^+$ Calcd. for $C_{21}H_{20}N_4O_3Na$ 399.1433; Found 399.1437.

2′-Thioxo-2,2′,3′,4,4a,6-hexahydro-1H,1′H-spiro[[1,4]oxazino[4,3-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**11f**) Greenish yellow solid (0.15 g, 95.1 %); mp: 122–124 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 2.87-2.93 (m, 1H), 3.17 (t, J = 32.40 Hz, 2H), 3.29 (t, J = 21.20 Hz, 2H), 3.49 (q, J = 20.40 Hz, 1H), 3.69 (d, J = 8.80 Hz, 1H), 3.84 (d,

J = 12.00 Hz, 2H), 6.70 (t, J = 14.40 Hz, 1H), 6.90 (d, J = 8.00 Hz, 1H), 6.99 (d, J = 7.60 Hz, 1H), 7.06 (t, J = 14.00 Hz, 1H), 12.40 (s, 1H), 12.54 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 32.59, 46.84, 49.96, 59.18, 65.61, 65.68, 112.89, 118.26, 121.49, 126.51, 128.42, 144.20, 166.71, 169.16, 178.69. IR (KBr, cm^{-1}) 3304.4 (N–H) 3113.5 (C–H, aromatic), 2921.6 (C–H, alkane), 1735.6 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:[M+Na]$^+$ Calcd. for $C_{15}H_{15}N_3O_3SNa$ 340.0732; Found 340.0735.

2′-Thioxo-2,2′,3′,4,4a,6-hexahydro-1H,1′H-spiro[[1,4]thiazino[4,3-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**11g**) Brown solid (0.15 g, 95.0 %); mp: 156–158 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 1.98 (d, J = 12.80 Hz, 1H), 2.15 (d, J = 13.60 Hz, 1H), 2.57 (t, J = 28.40 Hz, 1H), 2.83 (q, J = 37.60 Hz, 2H), 3.36 (q, J = 49.20 Hz, 2H), 3.89 (d, J = 10.40 Hz, 1H), 4.41 (d, J = 14.80 Hz, 1H), 6.70 (t, J = 14.40 Hz, 1H), 6.83 (d, J = 8.00 Hz, 1H), 7.06 (q, J = 25.60 Hz, 2H), 12.20 (s, 1H), 12.38 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 20.02, 23.07, 25.07, 25.47, 54.41, 61.92, 113.39, 117.78, 122.11, 126.65, 129.03, 140.89, 167.73, 167.83, 179.07. IR (KBr, cm^{-1}) 3445.2 (N–H) 3092.3 (C–H, aromatic), 2921.6 (C–H, alkane), 1735.6 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:[M+Na]$^+$ Calcd. for $C_{15}H_{15}N_3O_2S_2Na$ 356.0503; Found 356.0505.

3-Methyl-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**11h**) Pale yellow solid (0.15 g, 98.7 %); mp: 210–212 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 2.86-3.00 (m, 7H), 3.23 (s, 3H), 3.45 (s, 2H), 6.98-7.17 (m, 4H), 11.48 (s, 1H), 11.57 (s, 1H), ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 28.13, 28.97, 31.13, 42.72, 49.54, 53.10, 61.99, 121.16, 124.25, 126.39, 129.28, 140.08, 149.44, 162.35, 163.54, 172.51. IR (KBr, cm^{-1}) 3419.2 (N–H) 3100.0 (C–H, aromatic), 2923.6 (C–H, alkane), 1621.8 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:[M+H]$^+$ Calcd. for $C_{16}H_{18}N_4O_2SH$ 331.1229; Found 331.1231.

3-Ethyl-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione (**11i**) Light purple solid (0.13 g, 82.8 %); mp: 243–246 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 0.98 (t, J = 14.00 Hz, 3H), 1.27 (t, J = 25.20 Hz, 2H), 2.33 (s, 2H), 2.71 (d, J = 10.80 Hz, 1H), 2.79-2.89 (m, 2H), 3.15 (q, J = 46.80 Hz, 3H), 3.94 (d, J = 12.00 Hz, 1H), 6.67 (t, J = 14.40 Hz, 1H), 6.93 (dd, J = 8.0, 20.6 Hz, 2H), 7.03 (t, J = 15.60 Hz, 1H), 11.11 (s, 1H), 11.47 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 8.92, 28.06, 28.93, 29.79, 31.11, 49.57, 50.78, 95.96, 121.03, 124.21, 126.38, 129.30, 140.12, 149.44, 163.62, 172.49. IR (KBr, cm^{-1}) 3420.1 (N–H) 3095.2 (C–H, aromatic), 2926.4 (C–H, alkane), 1718.3 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:[M+H]$^+$ Calcd. for $C_{17}H_{20}N_4O_2SH$ 345.1385; Found 345.1384.

3-Phenyl-2′-thioxo-1,2,2′,3,3′,4,4a,6-octahydro-1′H-spiro[pyrazino[1,2-a]quinoline-5,5′-pyrimidine]-4′,6′-dione ***(11j)*** Greenish buff solid (0.16 g, 83.7 %); mp: 272–275 °C. ^{1}H-NMR(400 MHz, DMSO-d_6): δ 3.22 (q, J = 41.20 Hz, 4H), 3.43 (d, J = 12.00 Hz, 4H), 4.09 (d, J = 12.40 Hz, 1H), 6.69 (t, J = 14.40 Hz, 1H), 6.81–6.89 (m, 3H), 6.96–7.09 (m, 3H), 7.24 (t, J = 16.00 Hz, 2H), 12.40 (s, 1H), 12.53 (s, 1H). ^{13}C-NMR (250 MHz, DMSO-d_6): δ (ppm) 30.65, 51.13, 58.76, 113.18, 115.69, 119.62, 121.25, 128.52, 129.09, 143.75, 150.30, 166.91, 169.32, 178.80. IR (KBr, cm^{-1}) 3419.2 (N–H) 2953.4 (C–H, aromatic), 2851.2 (C–H, alkane), 1699.0 (C=O general carbonyl). HRMS (ESI-TOF) *m/z*:[M+Na]$^+$ Calcd. for $C_{21}H_{20}N_4O_2SNa$ 415.1205; Found 415.1201.

In vitro cytotoxicity assay (MTT Assay)

In vitro cytotoxicity study was carried out on MCF-7 (human breast cancer cell line) and KB (human nasopharyngeal carcinoma cell line) (Both from National Centre for Cell Science, Pune, India). The cells were cultured in DMEM supplemented with 10 % heat-inactivated FBS, 2 % penicillin–streptomycin and 2.5 µg/mL amphotericin B solution (All from Hi-Media Labs, Mumbai, India); cell lines were incubated at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2. Following 24–48 h of incubation period, the adherent cells were detached using trypsin–EDTA solution 1X/0.25 % (HiMedia Labs, Mumbai, India). Cell count was carried out using the Luna automated cell counter (Logos Biosystems, India) based on trypan blue dye exclusion method.

Cytotoxicity of the synthesised spiro-quinolines **8**(**a**–**j**) and **11**(**a**–**j**) on the cancer cells lines was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A total of 200 µL cell suspension of each cell line was seeded in 96-well microplates (Corning, USA) at a density of 25,000 cells/well and incubated for 24 h, after which the cells were exposed to an increasing concentration of synthesized spiro-quinoline compounds (100–500 µM for MCF-7 and 25–125 µM for KB cell line) for 24 h. All cells were seeded in duplicates and incubated in a CO_2 incubator (atmospheric with 5 % CO_2 and 37 °C temperature). Treated cells were thereafter incubated with 10 % of MTT in DMEM (HiMedia Labs, Mumbai, India) for 3 h. The culture medium was aspirated, and 100µL dimethyl sulphoxide (DMSO; Sigma-Aldrich, India) was added to each well. The spiro-quinolines-untreated cells were used as controls. Cell viability was determined by measuring the absorbance on a microplate reader

(SPECTRO star Nano, BMG LABTECH, Germany) at 570 nm. Viability was calculated as a percentage of viable cells at different test concentrations relative to the control (untreated) cells (% cell viability = (A570 of treated cells/A570 of control cells) × 100). The spiro-quinolines **8**(**a**–**j**) and **11**(**a**–**j**) concentration that resulted in 50 % inhibition of cell growth was calculated as the half maximal inhibitory concentration (IC_{50}) by constructing a dose–response curve.

IC_{50} value determination

The IC_{50} for each cell line was determined by using the spectrophotometric results of five different concentrations (100–500 μM for MCF-7 and 25–125 μM for KB cell line) for which a linear curve fit was generated. Cell viability percentages (*y*-axis) were plotted against increasing concentrations of tested compounds on the *x*-axis. IC_{50} value was estimated by using the linear equation $y = mx + c$ where 50 was substituted for *y*, yielding *x* as the IC_{50} value.

Flow cytometric analysis

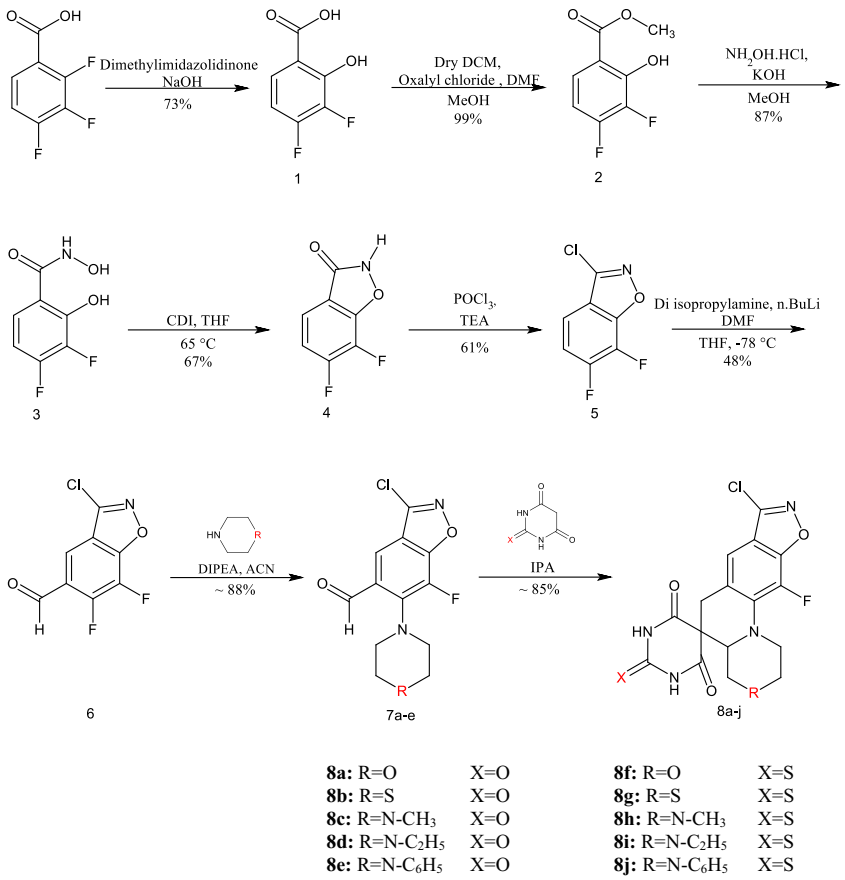
The apoptotic effect of **11b**, **11c**, **11e** and **11g** on KB cell line was assessed using flow cytometry using 6MP (6-mercapto purine) as reference standard. The KB cells were treated with 75 μM of each drug and incubated in CO_2 incubator (Thermo scientific) at 37 °C. The floated cells were discarded and adherent cells were trypsinized and washed with PBS and annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) by centrifugation at 2000 rpm for 5 min. A total of 5 μL solution of annexin V-FITC (Life Technologies) was added to 100 μl of cell suspension and incubated at room temperature for 15 min in dark. The cells were washed with annexin V binding buffer at 2000 rpm for 5 min, and ice-cold 70 % ethanol was added dropwise by gentle vortexing and incubated at 4 °C for overnight. Cells were washed with annexin V binding buffer at 2500 rpm for 5 min and incubated with 0.5 mg/mL of RNase in PBS at 37 °C for 20 min. Finally, propidium iodide was added to the cell suspension at the final concentration of 50 μg/mL and incubated for 10 min in dark. Flow cytometry was performed by using MACS Quant analyser (Miltenyi Biotec, Germany), and the DNA histograms were further analysed using MACSQuantify software.

Results and Discussion

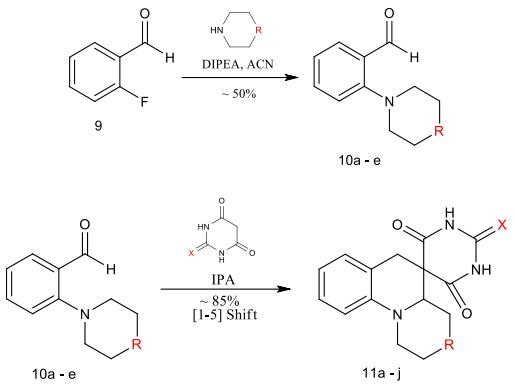
Chemistry

The synthesis of spiro-quinolines with barbituric acid and thiobarbituric acid has been specified in Scheme 1 and Scheme 2. The benzisoxazole ring with F groups at sixth and seventh positions was constructed by using starting material 2,3,4-trifluorobenzoic acid (Scheme 1). The compound 2,3,4-trifluorobenzoic acid was first converted into 3,4-difluro-2-hydroxybenzoic acid (**1**) in the presence of dimethylimidazolidinone with NaOH as reagent. The reaction which is highly regio-selective with respect to the replacement of F with OH group (Kazuto *et al.*, 2003) and yielded **1**. The formation of **1** was confirmed from the XRD data (Ravi Kiran *et al.*, 2014). The desired compound **1** was converted into its ester methyl 3,4-difluoro-2-hydroxybenzoate (**2**) by using oxalyl chloride and methanol. Conversion of F to OH was confirmed by ^{1}H-NMR spectrum of methyl 3,4-difluoro-2-hydroxybenzoate (**2**) by the presence of singlet signal at δ (ppm) 11.01. However, the same signal was not found in the ^{1}H-NMR spectrum of 3,4-difluro-2-hydroxybenzoic acid (**1**) due to the existence of hydrogen bonding between carboxylic acid and OH group. Compound **2** was further treated with hydroxylamine hydrochloride to get 3,4-difluoro-*N*,2-dihydroxybenzamide (**3**) (Massaro *et al.*, 2007; Riva *et al.*, 2009) which was cyclized using CDI to afford 6,7-difluorobenzo[d]isoxazol-3(2H)-one (**4**) (de Figueiredo *et al.*, 2006) in good yield. The obtained compound **4** was subjected to chlorination using $POCl_3$ and TEA in sealed tube conditions at 140 °C to give chlorinated benzisoxazole (**5**). In ^{1}H-NMR spectrum of cyclized product **4**, a singlet signal at δ (ppm) 12.90 is due to the presence of N–H proton. Further disappearance of N–H signal in (**5**) confirmed the conversion of C=O to C–Cl. An aldehyde group was generated at C5 position of compound (**5**) using *n*-BuLi and DMF at −78 °C to afford (**6**) with moderate yield (∼48 %). ^{1}H-NMR peak at δ (ppm) 10.38 of (**6**) indicated the presence of an aldehyde proton. The C6 fluorine of compound (**6**) was selectively replaced and coupled with morpholine to give (**7a**). Similarly, thiomorpholine and piperazine derivatives were employed to get compounds **7**(**b**–**e**). The same reaction did not crop up with the chlorine group in place of fluorine when it was attempted for 2-chlorobenzaldehyde for the synthesis of spiro-quinolines **11**(**a**–**j**). The final barbiturates and thiobarbiturates **8**(**a**–**j**) and **11**(**a**–**j**) were obtained by the Knoevenagel condensation reaction between aldehyde functional group of **7**(**a**–**e**) and **10**(**a**–**e**) with active methylene group of barbituric acid or thiobarbituric acid followed by [1–5] hydride shift (Ruble *et al.*, 2009). Small traces of impurities present in the final compounds were purified by trituration with nonpolar solvent petroleum ether. A detailed procedure including purification and spectral characterization for the preparation of compounds is given in experimental section. Synthesized compounds were characterized by using IR, ^{1}H-NMR, ^{13}C-NMR and HRMS spectral analysis.

Scheme 1 Preparation of functionalized spiro-quinolines **8**(**a**–**j**) with barbituric acid/ thiobarbituric acid



8a: R=O X=O
8b: R=S X=O
8c: R=N-CH_3 X=O
8d: R=N-C_2H_5 X=O
8e: R=N-C_6H_5 X=O
8f: R=O X=S
8g: R=S X=S
8h: R=N-CH_3 X=S
8i: R=N-C_2H_5 X=S
8j: R=N-C_6H_5 X=S



11a: R=O X=O
11b: R=S X=O
11c: R=N-CH_3 X=O
11d: R=N-C_2H_5 X=O
11e: R=N-C_6H_5 X=O
11f: R=O X=S
11g: R=S X=S
11h: R=N-CH_3 X=S
11i: R=N-C_2H_5 X=S
11j: R=N-C_6H_5 X=S

Scheme 2 Synthesis of spiro-quinolines **11**(**a**–**j**)

Cytotoxic activity study

Cell viability assay is one of the vital steps in analysing the cellular response to toxic compounds, playing fundamental roles in determining the cell death and survival rates and assessing the metabolic activity. In our study, the in vitro cytotoxicity of synthesized spiro-quinolines **8**(**a**–**j**) and **11**(**a**–**j**) in MCF-7 and KB cells was evaluated by measuring the cellular reduction of the tetrazolium salt, MTT, to a purple coloured formazan product. The MTT assay yielded concentration-dependent curves in all cell lines, indicating the impact of **8**(**a**–**j**) and **11**(**a**–**j**) in inducing increased cytotoxicity at higher concentrations. Results revealed that **11**(**a**–**j**) compounds, particularly **11b** and **11e**, exhibited significant activity against both the tested cell lines. IC_{50} values found for **11**(**a**–**j**) compounds were less than 150 µM (except **11a** and **11 h**) for MCF-7 and <100 µM (except **11a**) for KB (Fig. 1). Compound **11b** having R=S (sulphur group) and X=O (oxygen) was found to exhibit highest cytotoxic activity with IC_{50} value 90.2 µM for MCF-7 and 49.8 µM for KB cell line. 5-Fluorouracil (IC_{50} = 10.8 µM for MCF and 11.2 µM for KB) was used as reference standard for cytotoxic activity study. Compounds **8**(**a**–**j**) showed progressive activity against KB cell line having IC_{50} values less than 150 µM (except **8a** and **8d**), whereas for MCF-7 the values are in the range of 242.1–447.3 µM (Fig. 1).

The effects of substituents on the spiro-quinolines with barbituric acid and thiobarbituric acid were investigated.

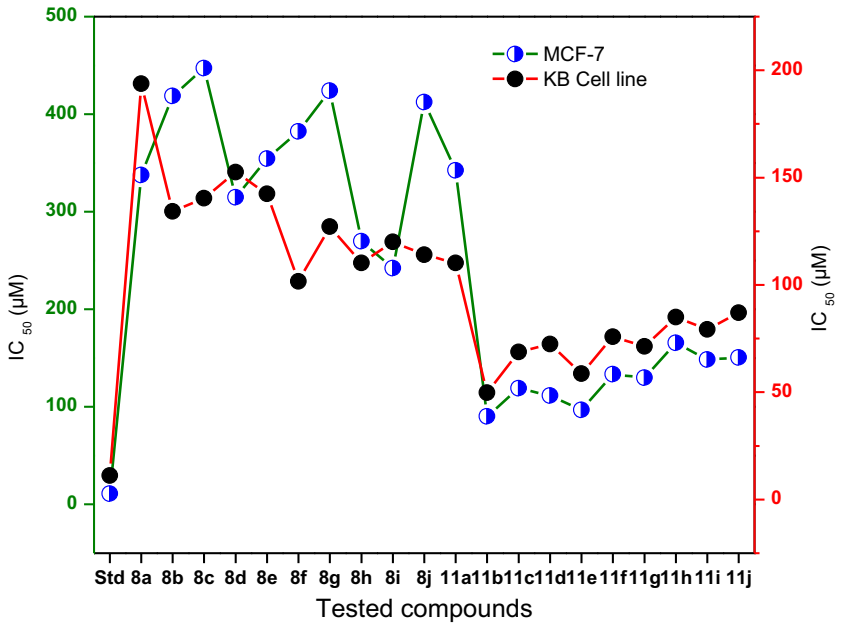


Fig. 1 Cytotoxicity of **8(a–j) and 11(a–j)** compounds against MCF-7, human breast cancer and KB, human nasopharyngeal carcinoma cell lines. Std: reference standard 5-fluorouracil

Presence of isoxazole ring with chloro group and fluoro functional group (both are electron withdrawing groups) (**8a–j**) on the spiro-quinoline system generally reduced the potency of the compounds. However, the potency was increased to two- to threefold, for the compounds **11(a–j)** with no isoxazole ring and fluoro functional group. The compound **11b** exhibited high potency against both the tested cell lines, and this can be attributed due to the presence of barbituric acid group and donor atom **S** at **R**. In exchange of donor atom **S** with N-alkyl and aryl group and X=**O**, potency was found to decrease in case of **11(c–j)**. The potency was completely lost in **11a** with **X=O** and **R=O.**

Apoptosis/necrosis analysis

The translocation of phosphatidylserine (PS) molecules to the outer surface of the cell membrane due to the disruption of phospholipid asymmetry was evidenced as an indicator of apoptosis (Vermes *et al.*, 1995). Since the early apoptotic cells with intact cell membrane exclude the DNA-binding dyes, whereas the necrotic cells with disrupted membrane allows the dyes to bind with the DNA, annexin V-FITC and propidium iodide double staining was used to determine the cell death mechanism. Apoptosis and necrosis induced by the drugs **11b**, **11c**, **11e** and **11g** in KB cell line were examined by MACS Quant analyser, and MACS Quantify software was used to analyse the date.

The percentage of apoptotic and necrotic cells was expressed in flow cytometric histograms and is as follows: cells in the lower left quadrant (LL1) represented live cells, negative for annexin V and PI; cells in the lower right quadrant (LR1) represented early apoptotic cells; cells in the upper right quadrant (UR1) represented late apoptotic cells; and the cells in upper left quadrant (UL1) represent necrotic cells (Fig. 2a). The addition of both early (LR1 region) and late apoptotic cells (UR1) which are annexin V-FITC-positives was expressed as the total percentage value of apoptotic cells.

Since **11b**, **11c**, **11e** and **11g** showed significant IC_{50} values against KB cell line, we have used these drugs for further analysis of their mode of action, at the concentration of 75 μM for 24 h. Herein, for flow cytometric analysis, 6MP has been used as reference standard. The flow cytometric data indicated that **11b**, **11c**, **11e** and **11g** were able to induce apoptosis in KB cells.

The lipophilicity of the barbituric acid-containing molecules increased with the presence S group and also by elongation of aliphatic group (spiro-quinoline moiety) at the active methylene group (Kepczynska *et al.*, 2007). Earlier reports demonstrated that drug with more lipophilicity induces more necrosis and drug with low lipophilicity induces apoptosis (Onizuka *et al.*, 2011). Flow cytometric analysis studies confirmed cytotoxicity of these molecules via apoptosis, and therefore, these are not enough lipophilic to induce the necrosis. Further lipophilicity nature is varied between these molecules, which indicated from the relative percentage of apoptotic and necrotic cells (Fig. 2b). The percentage of apoptotic cells is more in **11e** and **11c** which have absence of sulphur group and is less in **11b** and **11g** which have presence of sulphur group.

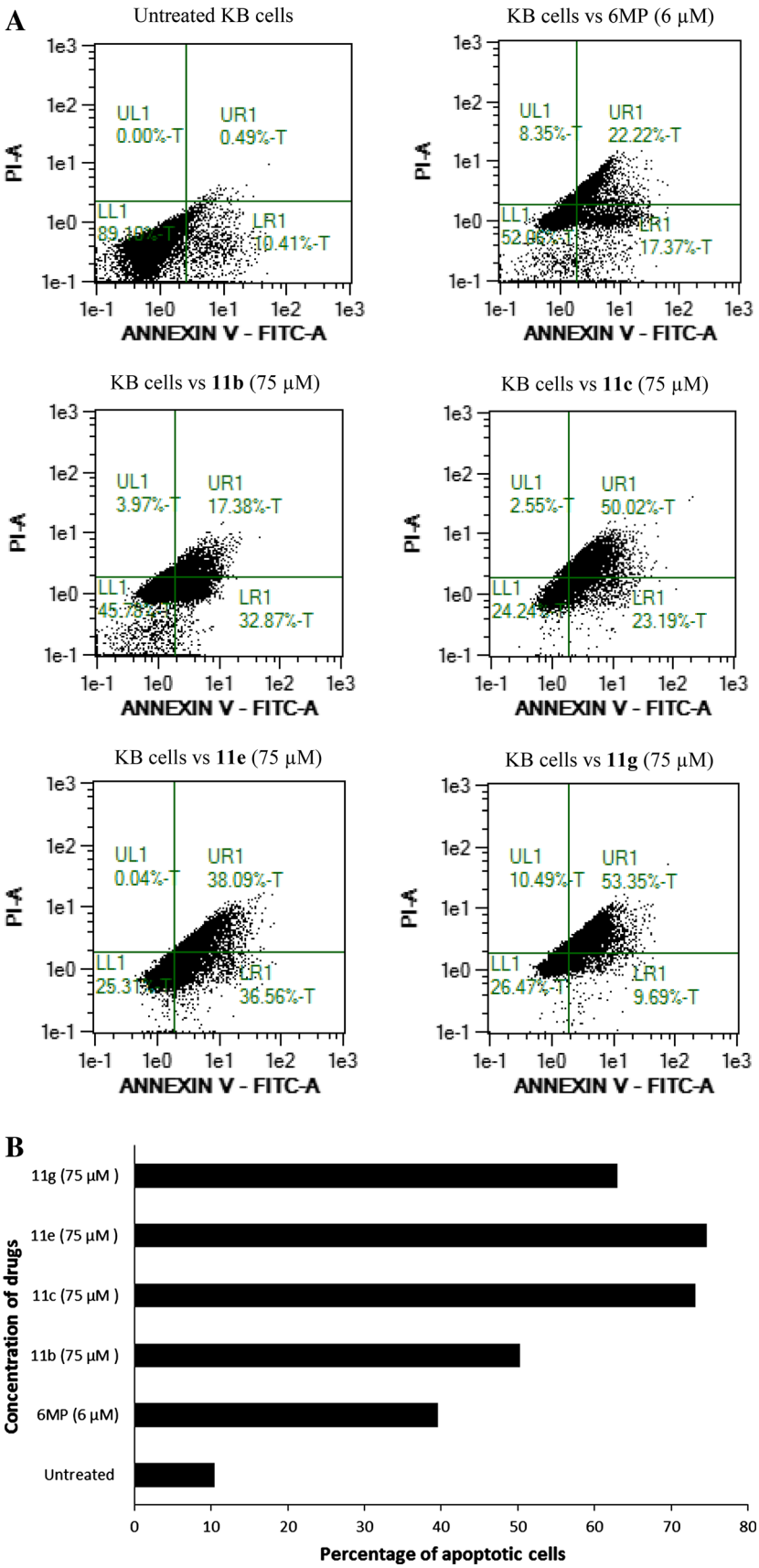


Fig. 2 **a** Flow cytometric analysis of apoptosis/necrosis by using annexin V and PI. LL1—% of live cells; LR1—% of early apoptotic cells; UR1—% of late apoptotic/necrotic cells; UL1—% of necrotic cells. **b** Percentage of apoptotic cells stained with annexin V-FITC

Conclusion

In summary, we have reported the synthetic route for the preparation of new class of functionalized six-membered spiro-quinolines with barbituric and thiobarbituric acids. The desired analogues of spiro-quinolines were prepared in moderate-to-very good yield and selectivity by the reaction of 6-substituted benzisoxazole-5-carbaldehydes **7**(**a**–**e**), 2-substituted benzaldehydes **10**(**a**–**e**) with active methylene group of barbituric acid or thiobarbituric acid. Additionally all the synthesized compounds showed broad spectrum of anticancer activities against MCF-7 and KB cell lines. The activity (IC_{50} value) is related to the unresolved enantiomers since the product obtained was the racemic mixture. Among the screened compounds, **11b** exhibited highest activity against both MCF-7 and KB cell lines. Flow cytometric analysis study confirmed that **11b**, **11c**, **11e** and **11g** induced cytotoxicity via apoptosis. It is concluded that the title compounds may become prominent anticancer drug molecules and preparation of more such compounds with functional group modification to deliver the compounds with high potency having activity in nanomolar range is currently in progress.

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Conflict of interest None.

References

Agbo SI, Hallas G, Towns AD (2000) Properties of some novel monoazo disperse dyes derived from ester-substituted tetrahydroquinoline and indoline coupling components. Dyes Pigm 47:33–43

Atul K, Suman S, Garima G, Vinita C, Sudhir S, Srivastava R (2011) Natural product inspired diversity oriented synthesis of tetrahydroquinoline scaffolds as antitubercular agent. ACS Comb Sci 13:65–71

Bassin SL, Bleck TP (2008) Barbiturates for the treatment of intracranial hypertension after traumatic brain injury. Crit Care 12:185

Brendan K, Michela M, Carolina M, Jorge EO, Javier MJ, Luis FC, Isabel R (2015) α2-Adrenoceptor antagonists: synthesis, pharmacological evaluation, and molecular modeling investigation of pyridinoguanidine, pyridino-2-aminoimidazoline and their derivatives. J Med Chem 58:963–977

de Figueiredo RM, Fröhlich R, Christmann M (2006) *N,N′*-Carbonyldiimidazole-mediated cyclization of amino alcohols to substituted azetidines and other N-heterocycles. J Org Chem 71:4147–4154

Eric BL, Monique AB, Thatyana RAV, Manuel OM, Raquel CM, Jullianne DY, Katia ZL (2014) Synthesis and anticancer activity of (E)-2-benzothiazole hydrazones. Eur J Med Chem 86:12–16

Hallas G, Zhai KY (1996) Synthesis and electronic spectra of some azo disperse dyes derived from *N*-alkyl-1,2,3,4-tetrahydroquinoline. Dyes Pigm 32:187–192

Humar M, Andriopoulos N, Pischke SE, Loop T, Schmidt R, Hoetzel A, Roesslein M, Pahl HL, Geiger KK, Pannen BH (2004) Inhibition of activator protein 1 by barbiturates is mediated by differential effects on mitogen-activated protein kinases and the small g proteins Ras and Rac-1. J Pharmacol Exp Ther 311:1232–1240

Jon JH, Todd JF, Karen AR, Joanne MS, Cailan W, Zhong S, James RC, Tianan F, Pancras CW, Alan RR, Frank AB, Jeffrey MB, Joseph ML, Carol AW, Ge Z, Anzhi W, Vidhyashankar R, Paul EM, Gregory SB, Srinath S, Piramanayagam A, Arvind M, Dietmar AS, Ruth RW, Mimi LQ (2014) Phenylimidazoles as potent and selective inhibitors of coagulation factor XIa with in vivo antithrombotic activity. J Med Chem 57:9915–9932

Kaiser S, Smidt SP, Pfaltz A (2006) Iridium catalysts with bicyclic Pyridine–Phosphinite Ligands: asymmetric hydrogenation of olefins and furan derivatives. Angew Chem Int Ed 45:5194–5197

Kazuto U, Fumiya T, Yoshikazu K (2003) Regio-selective hydroxysubstitution of fluorobenzoic acid derivatives: facile synthesis of fluorosalicylic acid derivatives. J Fluorine Chem 121:97–99

Kepczynska E, Obloza E, Stasiewicz-Urban A, Bojarski J, Pyka A (2007) Lipophilicity of thiobarbiturates determined by TLC. Acta Pol Pharm 64:295–302

Kim WG, Kim JP, Kim CJ, Lee KH, Yoo ID (1996a) Benzastatins A, B, C, and D: new free radical scavengers from Streptomyces nitrosporeus 30643. I. Taxonomy, fermentation, isolation, physico–chemical properties and biological activities. J Antibiot 49:20–25

Kim WG, Kim JP, Yoo ID (1996b) Benzastatins A, B, C, and D: new free radical scavengers from Streptomyces nitrosporeus 30643. II. Structure determination. J Antibiot 49:26–30

Kouznetsov VV, Arenas DRM, Arvelo F, Forero JSB, Sojo F, Muñoz A (2010) 4-Hydroxy-3-methoxyphenyl substituted 3-methyltetrahydroquinoline derivatives obtained through imino Diels–Alder reactions as potential antitumoral agents. Lett Drug Des Discov 7:632–639

Krasnov KA, Kartsev VG (2005) Synthesis of spiro heterocyclic systems from barbituric acids and *N,N*-disubstituted o-amino benzaldehydes. Russ J Org Chem 41:901–906

Massaro A, Mordini A, Reginato G, Russo F, Taddei M (2007) Microwave-assisted transformation of esters into hydroxamic acids. Synthesis. doi:10.1055/s-2007-990803.

Michael JP (2004) Quinoline, quinazoline and acridone alkaloids. Nat Prod Rep 21:650–668

Michael JP (2005) Quinoline, quinazoline and acridone alkaloids. Nat Prod Rep 22:627–646

Michael JP (2007) Quinoline, quinazoline and acridone alkaloids. Nat Prod Rep 24:223–246

Michael JP (2008) Quinoline, quinazoline and acridone alkaloids. Nat Prod Rep 25:166–187

Mimi LQ, Pancras CW, Cailan W, Francis W, Joanne MS, Frank AB, Jeffrey MB, Randi LB, Mark RH, Joseph ML, Paul EM, Tara P, Vidhyashankar R, Alan RR, Karen AR, Carol AW, Anzhi W, Ge Z, Dietmar S, Ruth RW (2014) Tetrahydroquinoline derivatives as potent and selective factor XIa inhibitors. J Med Chem 57:955–969

Mona MK, Naida YMA (2014) Synthesis of novel 1,2,4-triazoles, triazolothiadiazines and triazolothiadiazoles as potential anticancer agents. Eur J Med Chem 86:75–80

Onizuka S, Yonaha T, Tsuneyoshi I (2011) Local anesthetics with high lipophilicity are toxic, while local anesthetics with low pka induce more apoptosis in human leukemia cells. J Anesth Clin Res 2(1):1–5

Patel DV, Gless RDJ, Webb H, Heather K, Anandan SK, Aavula BR (2008) Soluble epoxide hydrolase inhibitors. Int Patent WO 2008/073623 A2

Pullmann T, Engendahl B, Zhang Z, Hölscher M, Zanotti-Gerosa A, Dyke A, Francio G, Leitner W (2010) Quinaphos and dihydroquinaphos phosphine–phosphoramidite ligands for asymmetric hydrogenation. Chem Eur J 16:7517–7526

Rakotoson JH, Fabre N, Jacquemond CI, Hannedouche S, Fouraste I, Moulis C (1998) Alkaloids from Galipea officinalis. Planta Med 64:762–763

Ravi Kiran B, Palakshamurthy BS, Vijayakumar GR, Bharath HS (2014) 3,4-Difluoro-2-hydroxybenzoic acid. Acta Cryst E 70:0519. doi:10.1107/S1600536814007211

Riva E, Gagliardi S, Mazzoni C, Passarella D, Rencurosi A, Vigo D, Martinelli M (2009) Efficient continuous flow synthesis of hydroxamic acids and suberoylanilide hydroxamic acid preparation. J Org Chem 74:3540–3543

Romain G, Van LN, Jérôme T, Christophe S, Jean-Marie GB, Gael K, Olivier M, Alizon MR, Eric VB, Lionel T, Paul B, Hugh C, C C, Leanne C, Emmanuel HD, Rejbinder K, Antonia JL, Mark BS, Peter ES, Simon T, Ann LW, Walker MD, Rab KP, Edwige N (2014) The discovery of I-BET726 (GSK1324726A), a potent tetrahydroquinoline ApoA1 up-regulator and selective BET bromodomain inhibitor. J Med Chem 57:8111–8131

Ruble CJ, Hurd AR, Johnson TA, Sherry DA, Barbachyn MR, Toogood PL, Bundy GL, Graber DR, Kamilar GM (2009) Synthesis of (−)-pnu-286607 by asymmetric cyclization of alkylidene barbiturates. J Am Chem Soc 131:3991–3997

Schwarz M, Bonhotal J, Bischoff K, EbelJr JG (2013) Fate of barbiturates and non-steroidal anti-inflammatory drugs during carcass composting trends. Anim Vet Sci 4:1–12

Singh P, Kaur M, Verma P (2009) Design, synthesis and anticancer activities of hybrids of indole and barbituric acids-identification of highly promising leads. Bioorg Med Chem Lett 19:3054–3058

Sweidan K, Engelmann J, Joshi R, Mubarak MS, El-Abadelah MM (2011) Synthesis of some cyclic methylene 1,3-diaza barbiturates derivatives. Lett Org Chem 8:603–605

Vermes I, Haanen C, Steffens NH, Reutelingsperger C (1995) A novel assay for apoptosis flow cytometric detection of phosphatidyl serine early apoptotic cells using fluorescein labelled expression on Annexin V. J Immunol Methods 184:39–51

Wang D, Ding K (2009) 2-Pyridinyl β-ketones as new ligands for room-temperature CuI-catalysed C–N coupling reactions. Chem Commun 14:1891–1893

Weixing Z, Qingzhong H, Nina H, Chris JVK, Rolf WH (2014) Potent 11β-hydroxylase inhibitors with inverse metabolic stability in human plasma and hepatic S9 fractions to promote wound healing. J Med Chem 57:7811–7817

Yamashita K, Imahashi S, Ito S (2008) Synthesis of benzylideneketone dyes and their photochemical properties as a sensitizer for alkali-developable photopolymerization systems. Dyes Pigm 76:748–753