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Preliminary communication

Design and synthesis of novel 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives as potential anticancer agents

Wenlin Xie [a,b,c,*], Shimin Xie [d], Ying Zhou [a,b,c], Xufu Tang [a,b,c], Jian Liu [e], Wenqian Yang [a,b,c], Minghua Qiu [a,b,c]

[a] School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan 411201, China
[b] Key Laboratory of Theoretical Chemistry and Molecular Simulation of Ministry of Education, Hunan University of Science and Technology, China
[c] Hunan Provincial University Key Laboratory of QSAR/QSPR, China
[d] Hunan University of Humanities, Science and Technology, Loudi 417000, China
[e] Shenzhen Hanyu Pharmaceutical Co., Ltd., Shenzhen 518057, China

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ABSTRACT

A series of 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives(2a-2n) and 5,6-disubstituted pyridine-2,3-dione S-benzyl-3-thiosemicarbazones(3a-3g) were synthesized starting from 2,3-dihydroxypyridine via oxidation-Michael additions, condensations and nucleophilic substitutions. The structures of the compounds were established by IR, ^{1}H NMR, ^{13}C NMR, and HRMS. All newly synthesized compounds were screened for their anticancer activity against Breast cancer (MCF-7), Colon cancer (HCT-116) and hepatocellular cancer (BEL7402) cell lines. Bioassay results indicated that most of the prepared compounds exhibited cytotoxicity against various cancer cells in vitro. Some of the compounds exhibited promising antiproliferative activity, which were comparable to the positive control (5-fluorouracil). The structure–activity relationship was discussed.

1. Introduction

Following cardiovascular disease, cancer is the second leading cause of death in both developed and developing countries [1,2], the treatment of cancer still remains an important and challenging problem. For the majority of cancers, chemotherapy has become one of the methods that are being adopted to treat cancer. Many compounds have been synthesized with this aim, but their clinical use has been limited by their relatively high risk of toxicity, because they lack specificity and produce adverse effects related to the impact on rapidly dividing noncancerous cells [2,3]. Therefore, to improve efficacy and decrease the adverse effect potential is one of the goals in developing new anticancer drugs. Another major goal for developing new anticancer agents is to overcome cancer resistance to drug treatment that has made many of the currently available chemotherapeutic agents ineffective [4]. Nitrogen-containing heterocycles have always constituted a subject of great interest due to their ubiquity in nature and extensive presence as part of the skeletal backbone of many therapeutic agents. Of these heterocycles, pyridinone derivatives possess important biological activity such as anti-tumor activity [5], leukemia activity [6], anti-inflammatory activity [7], antimicrobial activity [8], HIV Reverse Transcriptase inhibitor [9] and antimalarial activity [10], pyridinone also is found applications in the preparation of deferiprone [11]. pyridinone-fused heterocycles including 4-(phenylthio)-2(1*H*)-pyridinones [12], [2R-trans,5Z(E,E)]-1,4-dihydroxy-5-phenyl-3-[tetrahydro-3-methyl-5-(6-methyl-2,4-octadienylidene)-2H-pyran-2-yl]-2(1H)-pyridinone(TMC-69) [13], and 3-amino-4-(pyridine-4-yl)-2(1*H*)- pyridinone (Amrinone) [14](Fig. 1) have attracted considerable attention.

On the other hand, thiosemicarbazide also exhibits various biological activities and have attracted considerable pharmaceutical interest [15]. They have been evaluated as antiviral [16], antibacterial [17], anti-inflammatory [18], antimalarial [19], anti-leukemic [20] and anticancer [21–23] activities, therefore, the thiosemicarbazide is a highly efficient pharmacophore in drug molecular design. Recently, several kinds of thiosemicarbazone derivatives have been synthesized and their antitumor activities were also reported [15]. In order to find novel potent pyridinone antitumor agents, we focused on designing and synthesizing some new pyridinone derivatives, wherein the active pharmacophores

* Corresponding author. School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan 411201, China.
E-mail address: xwl2000zsu@163.com (W. Xie).

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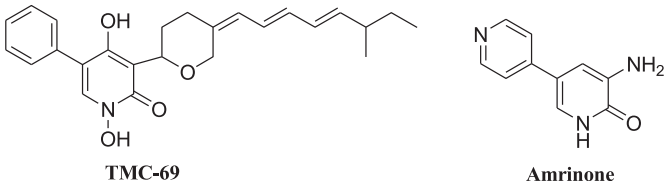


Fig. 1. Chemical structure of compound TMC-69 and Amrinone.

thiosemicarbazide has been attached at the 3-position of the pyridine ring, and the antitumor activity of these novel pyridinone derivatives was screened in vitro in order to get more potent and selective anticancer agents.

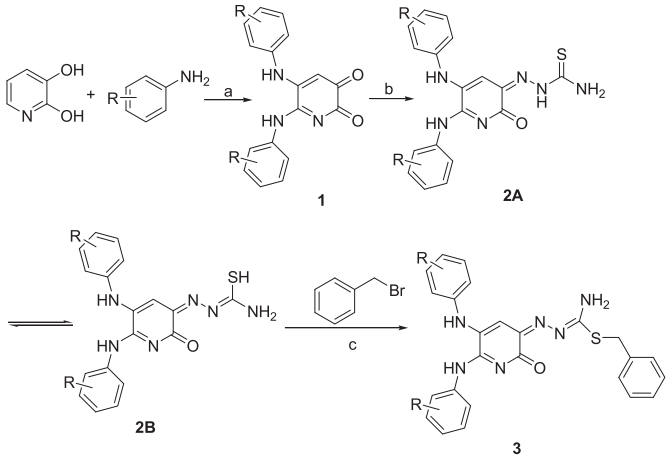
2. Results and discussion

2.1. Chemistry

The synthetic route for 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives **2,3** is represented in Scheme 1. Commercially available 2,3-dihydroxypyridine reacting with aromatic amine in the presence of oxidant $NaIO_3$ in one pot via oxidation-Michael additions gave compound **1**. The compounds **1** were characterized by using IR, NMR, and MS techniques. Their analytical and spectroscopic data agreed with those reported in the literature [24] for the same compounds. Subsequently, the condensation of the obtained 5,6-disubstituted pyridine-2,3-diones **1** with thiosemicarbazide afforded compounds **2a**–**n**, and then the compounds **2** reacted with benzyl bromide in ethanol medium in the presence of Et_3N by nucleophilic substitution reaction to give **3a**–**g** with good yields. The structures of all the newly synthesized compounds **2a**–**n** and **3a**–**g** were confirmed by ^1H NMR, ^{13}C NMR, IR and HRMS. At the same time, we also observed that the thiosemicarbazones **2** existed thione and thioltautomeric from 2A to 2B, because the ^1H NMR spectra of thiosemicarbazones **2** exhibit two sets peaks (integral ratio is 1: 0.3). Further evidence for the existence of thione and thioltautomeric in thiosemicarbazones **2** was obtained from their ^{13}C NMR spectra, which showed a signal at 178.07–179.86 ppm assigned to (C=S), after the thiosemicarbazones **2** reacted with benzyl bromide converted to compound **3**, the signals at 178.07–179.86 ppm disappeared.

2.2. Biological activity

The in vitro antitumor activity of the newly synthesized compounds **2a**–**n** and **3a**–**g** was evaluated against a panel of three human cancer cell lines, including MCF-7 (breast adenocarcinoma cell), HCT116 (colon carcinoma cell) and BEL-7402 (hepatoma carcinoma cell) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method [25]. The inhibitory activities (IC_{50}) are summarized in Table 1 and the well-known anticancer drug 5-fluorouracil was used as positive control [26,27]. From the screening results in Table 1, it was observed that most of the synthesized compounds exhibited more potent cytotoxic activities ($IC_{50} < 7.0$ μM) against the three human cancer cell lines in comparison with 5-FU. Special compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, **2k** and **2l** displayed higher cytotoxicity activity than 5-FU against all three human cancer cell lines, the compound **2f** showed promising antiproliferative activity with IC_{50} values in the range of 0.19–1.37 μM. Although most compounds showed potent antitumor activity ($IC_{50} < 7.0$ μM), some compounds exhibited selectivity between the three human cancer cell lines. For MCF-7 cell line, the compounds **2a**, **2b**, **2e**, **2f** and **2k**, showed the more potent inhibitory activity with IC_{50} 0.58 ± 0.06 μM, 0.15 ± 0.02 μM, 0.634 ± 0.06 μM, 1.37 ± 0.04 μM and 1.12 ± 0.22 μM, respectively. For HCT-116 cell line, the compounds **2a** and **2f** displayed the best inhibitory activity with IC_{50} 1.52 ± 0.18 μM and 0.19 ± 0.02 μM respectively. For BEL-7402 cell line, the compounds **2a** and **2f** exhibited more potent inhibitory activity with corresponding IC_{50} 1.71 ± 0.26 μM and 0.82 ± 0.03 μM respectively.



Scheme 1. Synthesis of the 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives(**2,3**). Reagent and conditions: (a) $NaIO_3$, rt; (b) $NH_2NHCSNH_2$, EtOH, H_2SO_4, reflux; (c) EtOH, Et_3N, reflux.

From the antitumor activity against three human cancer cell lines, preliminary structure–activity relationships of the synthesized compounds were achieved. In general, compared with compounds 2a-2n, substituent on 5, 6-position benzene ring of the pyridine ring had great effect on the cytotoxicity, for example, the compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, **2k** and **2l** displayed the more

Table 1
In vitro antiproliferative activity of 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives.

Compound	R	IC_{50}[a] (μM)		
		MCF-7[b]	HCT-116[b]	BEL-7402[b]
2a	o-CH_3	0.58 ± 0.06	1.52 ± 0.18	1.71 ± 0.26
2b	m-CH_3	0.15 ± 0.02	8.65 ± 0.98	2.73 ± 0.26
2c	p-CH_3	3.06 ± 0.29	7.04 ± 0.44	4.33 ± 0.37
2d	o-Cl	3.49 ± 0.11	3.54 ± 0.25	3.42 ± 0.23
2e	m-Cl	0.63 ± 0.06	3.38 ± 0.97	2.19 ± 0.18
2f	p-Cl	1.37 ± 0.04	0.19 ± 0.02	0.82 ± 0.03
2g	o-CH_3O	10.16 ± 0.98	20.76 ± 0.85	13.39 ± 0.96
2h	m-CH_3O	>50	NT[d]	NT[d]
2i	p-CH_3O	10.37 ± 0.85	22.37 ± 0.37	17.76 ± 1.76
2j	p-CH_3CH_2O	13.69 ± 0.95	21.32 ± 0.89	31.01 ± 0.87
2k	p-F	1.12 ± 0.22	3.72 ± 0.34	3.66 ± 0.12
2l	p-Br	2.58 ± 0.22	2.15 ± 0.029	5.84 ± 0.31
2m	m-NO_2	11.11 ± 0.02	19.37 ± 0.88	8.11 ± 0.99
2n	H	7.11 ± 0.11	24.65 ± 1.71	11.87 ± 1.70
3a	o-CH_3	16.33 ± 0.94	13.80 ± 1.26	17.09 ± 0.91
3b	m-CH_3	9.34 ± 0.98	12.85 ± 0.96	8.72 ± 0.97
3c	p-CH_3	13.42 ± 0.94	8.27 ± 0.98	7.68 ± 0.98
3d	m-Cl	15.16 ± 0.91	11.69 ± 0.93	7.16 ± 0.98
3e	p-Cl	7.72 ± 0.97	9.60 ± 0.97	17.30 ± 0.74
3f	p-F	7.58 ± 0.97	11.45 ± 0.96	7.69 ± 0.98
3g	o-CH_3O	8.34 ± 0.96	14.47 ± 0.83	13.13 ± 0.95
5-FU		7.08 ± 0.92	10.54 ± 1.06	11.12 ± 0.72

[a] IC50 is the concentration of compound required to inhibit the cell growth by 50% compared to an untreated control.
[b] MCF-7 cells were the human breast adenocarcinoma cells, HCT-116 cells were the human colon carcinoma cells and BEL-7402 cells were the human hepatoma carcinoma cells.
[d] NT means not tested.

potent antitumor activity than the compound with no substituent (**2n**) against the three human cancer cell lines. Among these compounds, compounds with methyl group substituent (**2a**, **2b** and **2c**) and halogen substituent (**2d**, **2e**, **2f**, **2k** and **2l**) on the benzene ring showed stronger inhibitory activity than the compound with methoxyl, ethyoxyl and nitryl substituent on the benzene ring (**2g**, **2h**, **2i**, **2j** and **2m**). In comparison to compounds **2a**, **2b** and **2c**, the compound **2g**, **2h**, **2i** and **2j** (R = p-CH_3CH_2O), with bulky group on the benzene ring, exhibited greater reduction in the inhibitory activity. On the other hand, the introduction of benzyl group on sulfur atom of thiosemicarbazones (**3a**–**3g**) also lowered the inhibitory activity. We found that most of the compounds **2** without substituent on sulfur atom of thiosemicarbazone have higher cytotoxicity than the compounds **3** with benzyl groups. Furthermore, from the above-mentioned analysis, it can be concluded that the benzylation of the thiosemicarbazones reduced the biological activity, and methyl or chloro or fluoro or bromo substituted benzene ring in compounds 2 were found to be the most favorable for the antitumor activity.

3. Conclusions

In summary, a series of novel 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives (**2a**–**2n**; **3a**–**3g**) were synthesized and screened for antiproliferative activity against MCF-7, HCT-116 and BEL-7402 three human cancer cell lines. The preliminary investigation showed most of them displayed higher activity against all three human cancer cell lines than 5-FU. The results of the structure–activity relationships indicated that the benzylation of the thiosemicarbazones reduced the biological activity, and methyl or chloro or fluoro or bromo substituted benzene ring in compounds 2 were found to be the most favorable for the antitumor activity, which will encourage us to further design more potent antitumor agents. These findings also have conducted us to further investigate their detailed pharmacological mechanisms.

4. Experimental section

4.1. General

Melting points were determined in open capillaries and were uncorrected. IR spectra were recorded on a PE-2000 spectrometer in KBr pellets and are reported in cm^{-1}. All NMR spectra were recorded on a Bruker AV-II 500 MHz NMR spectrometer, operating at 500 MHz for 1H, and 125 MHz for ^{13}C, TMS was used as an internal reference for ^{1}H and ^{13}C chemical shifts and $CDCl_3$ and DMSO were used as solvent. Mass spectra were collected on a Waters Xevo Q-TOF HRMS instrument. 2,3-dihydroxypyridine was purchased from Aldrich; other commercially available materials were purchased from Aladdin-Reagent and Sinopharm Chemical Reagent, and were used without further purification.

4.2. Synthesis of 5,6-disubstituted pyridine-2,3-dione (*1*)

According to the literature method [24], sodium iodate (1.38 g, 7.0 mmol) was added to a solution of 2,3-dihydroxypyridine (0.70 g, 6.3 mmol) in 0.5 mol/L phosphate-buffered saline (35.0 ml, pH 6.0) and 125 ml methanol. After stirring for 15 min, the appropriate aromatic amine (12.6 mmol) was added and the resulting mixture was stirred at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, 150 ml water was added, and the precipitate formed was isolated by filtration. The resulting crude product was purified by column chromatography on silica gel to afford the corresponding **1**.

4.3. General procedure for the synthesis of 5,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone derivatives (*2*)

To a solution of 5,6-disubstituted pyridine-2,3-diones **1** (4 mmol) in anhydrous ethanol (60 ml) were added thiosemicarbazide (4.2 mmol) and sulfuric acid (0.04 mmol). The reaction mixture was refluxed and the reaction monitored by TLC, after the completion of the reaction, 200 ml water was added, and the precipitate formed was isolated by filtration. The resulting crude product was purified by recrystallization with ethanol to afford the desired compounds **2**.

4.3.1. (Z)-5,6-di(o-methylanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2a***

Yellow solid; yield 76.3%; mp. 208–209 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.14 (s, 3H, $-CH_3$), 2.25 (s, 3H, $-CH_3$), 6.86 (d, J = 8.0 Hz, 1H, Ar–H), 7.04–7.33(m, 8H, Ar–H, pyridine-H), 7.95 (s, 1H, $-NH_2$), 8.30 (s, 1H, $-NH_2$), 8.65 (s, 1H, –NH–), 10.65 (s, 1H, –NH–), 12.86 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 17.96, 18.19, 101.35, 120.22, 124.65, 125.70, 125.84, 127.12, 127.30, 129.50, 130.93, 131.48, 133.65, 137.06, 138.45, 141.43, 145.31, 159.70, 160.44, 178.14; IR (KBr) v: 3321, 2971, 2924, 2857, 1695, 1648, 1613, 1596, 1517, 1484, 1461, 1387, 1317, 1245, 1156, 1107, 1045 cm^{-1}; MS (ESI, m/z): 393 $(M + H)^+$. HRMS (ESI, m/z) calcd for $[C_{20}H_{21}N_6OS]^+$ $(M + H)^+$ 393.1492, found 393.1501.

4.3.2. (Z)-5,6-di(m-methylanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2b***

Orange solid; yield 78.6%; mp. 145–146 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.32 (s, 3H, $-CH_3$), 2.33 (s, 3H, $-CH_3$), 6.46 (s, 1H, Ar–H), 6.76 (d, J = 8.0 Hz, 2H, Ar–H), 6.90–7.29 (m, 6H, Ar–H, pyridine-H), 8.05 (s, 1H, $-NH_2$), 8.29 (s, 1H, $-NH_2$), 8.67 (s, 1H, –NH–), 10.39 (s, 1H, –NH–), 12.87 (s, 1H, =N–NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 21.56, 21.60, 94.43, 118.48, 122.90, 123.04, 124.47, 124.89, 125.47, 126.29, 129.55, 129.68, 130.58, 135.65, 136.77, 139.17, 145.19, 156.09, 161.45, 179.02; IR (KBr) v: 3301, 3256, 3172, 3026, 2921, 1660, 1643, 1620, 1602, 1588, 1522, 1459, 1333, 1328, 1281, 1258, 1237, 1119 cm^{-1}; MS (ESI, m/z): 393 $(M + H)^+$. HRMS (ESI, m/z) calcd for $[C_{20}H_{21}N_6OS]^+$ $(M + H)^+$ 393.1492, found 393.1510.

4.3.3. (Z)-5,6-di(p-methylanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2c***

Chinese red solid; yield 85.6%; mp. 168–169 °C; ^{1}H NMR(DMSO-d_6, 500 MHz) δ: 2.32 (s, 6H, $-CH_3$), 6.36 (s, 1H, Ar–H), 6.87–7.32 (m, 8H, Ar–H, pyridine-H), 8.12 (s, 1H, $-NH_2$), 8.32 (s, 1H, $-NH_2$), 8.69 (s, 1H, –NH–), 10.47 (s, 1H, –NH–), 12.87 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 20.94, 21.10, 93.60, 102.10, 121.35 (2C), 122.87 (2C), 130.20 (2C), 133.35 (2C), 133.65, 134.56, 136.97, 143.09, 143.86, 159.59, 160.22, 179.86; IR (KBr) v: 3235, 3393, 3235, 3154, 2920, 2861, 1667, 1634, 1606, 1520, 1467, 1394, 1325, 1308, 1239, 1114 cm^{-1}; MS (ESI, m/z): 393 $(M + H)^+$. HRMS (ESI, m/z) calcd for $[C_{20}H_{21}N_6OS]^+$ $(M + H)^+$ 393.1492, found 393.1499.

4.3.4. (Z)-5,6-di(o-chloroanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2d***

Red solid; yield 80.1%; mp. 247–248 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.34 (s, 1H, Ar–H), 7.06 (d, J = 8.0 Hz, 1H, Ar–H), 7.34–7.63 (m, 7H, Ar–H, pyridine-H), 8.09 (s, 1H, $-NH_2$), 8.30 (s, 1H, $-NH_2$), 8.51 (s, 1H, –NH–), 11.32 (s, 1H, –NH–), 12.84 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 95.89, 115.36, 122.72, 124.95, 125.37, 126.73, 127.21, 130.10, 130.92, 131.12, 132.47, 132.81, 134.73, 141.75, 149.54, 157.38, 160.27, 178.07; IR (KBr) v: 3333, 3275, 3170, 1602, 1591, 1533, 1468, 1448, 1323, 1286, 1236, 1120 cm^{-1}; MS (ESI, m/z): 433 $(M + H)^+$. HRMS (ESI, m/z) calcd for $[C_{18}H_{15}Cl_2N_6OS]^+$ $(M + H)^+$ 433.0400, found 433.0408.

4.3.5. (Z)-5,6-di(m-chloroanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2e***

Yellow solid; yield 75.8%; mp. 249–250 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.55 (s, 1H, Ar–H), 7.01–7.39 (m, 8H, Ar–H, pyridine-H), 8.37 (s, 1H, $-NH_2$), 8.44 (s, 1H, $-NH_2$), 8.81 (s, 1H, –NH–), 11.04 (s, 1H, –NH–), 12.92 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 96.21, 105.27, 120.00, 120.31, 120.65, 121.27, 121.79, 122.87, 124.30, 130.17, 131.25, 131.34, 134.01, 134.07, 142.38, 148.45, 160.44, 178.46; IR (KBr) *v*: 3324, 3297, 3253, 3170, 3024, 2824, 1664, 1620, 1589, 1513, 1463, 1395, 1327, 1271, 1239, 1123 cm^{-1}; MS (ESI, *m/z*): 433 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{15}Cl_2N_6OS]^+$ $(M + H)^+$ 433.0400, found 433.0405.

4.3.6. (Z)-5,6-di(p-chloroanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2f***

Yellow solid; yield 86.4%; mp. 226–227 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.47 (s, 1H, Ar–H), 6.98–7.41 (m, 8H, Ar–H, pyridine-H), 8.29 (s, 1H, $-NH_2$), 8.32 (s, 1H, $-NH_2$), 8.74 (s, 1H, –NH–), 10.85 (s, 1H, –NH–), 12.91 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 95.03, 123.27 (2C), 123.80 (2C), 124.34 (2C), 126.57, 127.18, 128.78, 129.56 (2C), 130.32, 135.31, 139.63, 157.17, 159.75, 178.55; IR (KBr) *v*: 3372, 3302, 3233, 3046, 2830, 1668, 1655, 1607, 1539, 1504, 1489, 1345, 1392, 1356, 1301, 1282, 1235, 1118, 1091, 1011 cm^{-1}; MS (ESI, *m/z*): 433 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{15}Cl_2N_6OS]^+$ $(M + H)^+$ 433.0400, found 433.0406.

4.3.7. (Z)-5,6-di(o-methoxyanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2g***

Yellow solid; yield 83.4%; mp. 234–235 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.76 (s, 3H, $-CH_3$), 3.85 (s, 3H, $-CH_3$), 6.49 (s, 1H, Ar–H), 6.89–7.69 (m, 8H, Ar–H, pyridine-H), 8.00 (s, 1H, $-NH_2$), 8.40 (s, 1H, $-NH_2$), 8.77 (s, 1H, –NH–), 10.75 (s, 1H, –NH–), 12.90 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 56.23, 56.29, 94.31, 111.70, 112.01, 119.79, 121.20, 122.55, 123.93, 124.11, 125.84, 128.23, 128.91, 130.36, 133.94, 142.46, 150.29, 150.63, 160.05, 178.32; IR (KBr) *v*: 3318, 3233, 2964, 2923, 2853, 1680, 1656, 1625, 1598, 1524, 1491, 1460, 1349, 1322, 1299, 1251, 1219, 1206 cm^{-1}; MS (ESI, *m/z*): 425 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{20}H_{21}N_6O_3S]^+$ $(M + H)^+$ 425.1390, found 425.1392.

4.3.8. (Z)-5,6-di(m-methoxyanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2h***

Orange solid; yield 80.4%; mp. 234–235 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.75 (s, 3H, $-CH_3$), 3.87 (s, 3H, $-CH_3$), 6.52 (s, 1H, Ar–H), 6.71–7.33 (m, 8H, Ar–H, pyridine-H), 8.05 (s, 1H, $-NH_2$), 8.21 (s, 1H, $-NH_2$), 8.75 (s, 1H, –NH–), 10.61 (s, 1H, –NH–), 12.90 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 55.53, 55.54, 95.68, 107.80, 109.04, 110.51, 110.56, 113.53, 114.05, 114.33, 130.34, 130.47, 130.67, 133.23, 135.27, 143.33, 147.83, 159.53, 160.38, 178.29; IR (KBr) *v*: 3255, 3339, 3571, 3339, 3255, 3166, 2921, 2853, 1681, 1635, 1608, 1590, 1519, 1473, 1333, 1321, 1265, 1218, 1172, 1150, 1107 cm^{-1}; MS (ESI, *m/z*): 425 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{20}H_{21}N_6O_3S]^+$ $(M + H)^+$ 425.1390, found 425.1396.

4.3.9. (Z)-5,6-di(p-methoxyanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2i***

Yellow solid; yield 85.8%; mp. 204–205 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.75 (s, 3H, $-CH_3$), 3.78 (s, 3H, $-CH_3$), 6.16 (s, 1H, Ar–H), 6.92–7.03 (m, 5H, Ar–H), 7.24 (d, J = 8.5 Hz, 2H, Ar–H), 7.34 (d, J = 8.5 Hz, 1H, pyridine-H), 8.07 (s, 1H, $-NH_2$), 8.28 (s, 1H, $-NH_2$), 8.64 (s, 1H, –NH–), 10.53 (s, 1H, –NH–), 12.85 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 55.67, 55.75, 94.55, 122.76 (2C), 125.06 (2C), 125.45 (2C), 130.98 (2C), 132.33, 132.95, 138.14, 139.08, 156.24, 156.50, 159.84, 160.31, 178.08; IR (KBr) *v*: 3331, 3264, 3045, 2993, 2931, 2831, 1667, 1655, 1605, 1560, 1537, 1506, 1466, 1354, 1390, 1356, 1291, 1245, 1182, 1106 cm^{-1}; MS (ESI, *m/z*): 425 $^{(M + H)+}$. HRMS (ESI, *m/z*) calcd for $[C_{20}H_{21}N_6O_3S]^+$ $(M + H)^+$ 425.1390, found 425.1398.

4.3.10. (Z)-5,6-di(p-ethoxyanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2j***

Red solid; yield 80.1%; mp. 124–125 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.24 (t, J = 8.0 Hz, 3H, $-CH_3$), 1.27 (t, J = 8.0 Hz, 3H, $-CH_3$), 3.99 (q, J = 8.5 Hz, 2H, $-CH_2-$), 4.07 (q, J = 8.5 Hz, 2H, $-CH_2-$), 6.23 (s, 1H, Ar–H), 7.14–7.33 (m, 8H, Ar–H, pyridine-H), 8.69 (s, 1H, $-NH_2$), 8.84 (s, 1H, $-NH_2$), 9.43 (s, 1H, –NH–), 11.03 (s, 1H, –NH–), 12.80 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 21.21, 21.50, 32.25, 33.73, 94.98, 115.45(2C), 122.72(2C), 122.84(2C), 125.34, 126.70, 127.76(2C), 130.21, 131.48, 133.09, 136.57, 143.17, 160.27, 178.48; IR (KBr) *v*: 3334, 3247, 2979, 2809, 1700, 1600, 1510, 1474, 1393, 1301, 1243, 1114, 1045 cm^{-1}; MS (ESI, *m/z*): 453 $^{(M + H)+}$. HRMS (ESI, *m/z*) calcd for $[C_{22}H_{25}N_6O_3S]^+$ $(M + H)^+$ 453.1703, found 453.1710.

4.3.11. (Z)-5,6-di(p-fluoroanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2k***

Yellow solid; yield 86.7%; mp. 205–206 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.32 (s, 1H, Ar–H), 7.20 (d, J = 8.0 Hz, 2H, Ar–H), 7.26–7.67 (m, 6H, Ar–H, pyridine-H), 8.17 (s, 1H, $-NH_2$), 8.27 (s, 1H, $-NH_2$), 8.68 (s, 1H, –NH–), 10.89 (s, 1H, –NH–), 12.81 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 93.83, 100.94, 116.47(2C), 123.08(2C), 125.00(2C), 127.16(2C), 130.54, 134.24, 136.97, 137.54, 142.61, 158.16, 158.50, 160.35, 160.77, 178.90; IR (KBr) *v* : 3404, 3247, 2979, 2809, 1700, 1600, 1510, 1474, 1393, 1301, 1243, 1114, 1045 cm^{-1}; MS (ESI, *m/z*): 401 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{15}F_2N_6OS]^+$ $(M + H)^+$ 401.0991, found 401.0996.

4.3.12. (Z)-5,6-di(p-bromoanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2l***

Yellow solid; yield 80.1%; mp. 191–192 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.47 (s, 1H, Ar–H), 6.91 (d, J = 8.0 Hz, 2H, Ar–H), 7.15–7.56 (m, 6H, Ar–H, pyridine-H), 8.27 (s, 2H, $-NH_2$), 8.66 (s, 1H, –NH–), 10.71 (s, 1H, –NH–), 13.02 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 95.17, 114.95, 116.78, 123.70(2C), 124.02(2C), 124.70, 130.63, 132.48(2C), 135.35(2C), 139.22, 140.13, 146.18, 161.01, 178.47; IR (KBr) *v*: 3372, 3300, 3235, 3049, 2925, 1668, 1654, 1606, 1583, 1537, 1504, 1488, 1345, 1390, 1357, 1302, 1283, 1262, 1233, 1177, 1118, 1071 cm^{-1}; MS (ESI, *m/z*): 522 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{15}Br_2N_6OS]^+$ $(M + H)^+$ 520.9389, found 522.9395.

4.3.13. (Z)-5,6-di(m-nitroanilino) pyridine-2,3-dione-3-thiosemicarbazone ***2m***

Red solid; yield 80.1%; mp. 273–274 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.63 (s, 1H, Ar–H), 7.39–7.97 (m, 8H, Ar–H, pyridine-H), 8.09 (s, 1H, $-NH_2$), 8.48 (s, 1H, $-NH_2$), 8.85 (s, 1H, –NH–), 11.27 (s, 1H, –NH–), 12.90 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 95.57, 115.44, 117.11, 119.16, 127.03, 128.15, 129.87, 130.33, 130.98, 133.35, 134.46, 135.36, 143.67, 148.93, 149.09, 157.79, 160.49, 178.50; IR (KBr) *v* : 3366, 3333, 1698, 1608, 1522, 1393, 1351, 1298, 1122 cm^{-1}; MS (ESI, *m/z*): 455 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{15}N_8O_5S]^+$ $(M + H)^+$ 455.0881, found 455.0888.

4.3.14. (Z)-5,6-dianilino pyridine-2,3-dione-3-thiosemicarbazone ***2n***

Chinese red solid; yield 83.4%; mp. 162–163 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.47 (s, 1H, Ar–H), 6.97(d, J = 7.5 Hz, 2H, Ar–H), 7.14–7.47 (m, 8H, Ar–H, pyridine-H), 8.24 (s, 1H, $-NH_2$), 8.37 (s, 1H, $-NH_2$), 8.73 (s, 1H, –NH–), 10.64 (s, 1H, –NH–), 12.88 (s, 1H, =N–NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 95.92, 121.44(2C), 122.35(2C), 129.70(2C), 129.83(2C), 130.59, 133.37, 135.73, 136.70,

139.70, 143.18, 158.77, 160.32, 178.24; IR (KBr) *v*: 3378, 3310, 3209, 3150, 2973, 2926, 1623, 1594, 1527, 1486, 1454, 1396, 1333, 1310, 1236, 1210, 1091, 1049 cm^{-1}; MS (ESI, *m/z*): 365 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{18}H_{17}N_6OS]^+$ $(M + H)^+$ 365.1179, found 365.1183.

4.4. General procedure for the synthesis of 5,6-disubstituted pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone derivatives (**3**)

To a solution of **5**,6-disubstituted pyridine-2,3-dione-3-thiosemicarbazone 2 (1.0 mmol) in ethanol (30 ml) were added triethylamine (0.1 ml). The reaction mixture was refluxed for 30 min at 60 °C, followed by immediate addition of benzyl bromide(1.1 mmol). The reaction mixture was continue to refluxing for 4h. TLC monitor the reaction process, after the completion of the reaction, concentrated under vacuum, the precipitate was redissolved in ethyl acetate, and The solution was successively washed with aqueous sodium hydroxide, saturated salt water, dried over anhydrous sodium sulfate, and evaporated. The resulting crude product was purified by recrystallization with ethyl acetate to afford the desired compounds **3**.

4.4.1. (3Z, 2′E)-5,6-di(o-methylanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3a***

Yellow solid; yield 58.7%; mp. 122–123 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 2.18 (s, 3H, $-CH_3$), 2.32 (s, 3H, $-CH_3$), 4.29 (s, 2H, $-CH_2-$), 5.89 (brs, 2H, $-NH_2$), 6.85 (d, $J = 7.5$ Hz, 1H, Ar–H), 7.00–7.14 (m, 3H, Ar–H), 7.22–7.30 (m, 9H, Ar–H), 7.43 (s, 1H, –NH–), 7.52 (s, 1H, pyridine-H), 7.94 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 17.82, 17.87, 33.98, 95.59, 119.66, 122.83, 124.94, 125.35, 126.74, 127.34, 127.40, 128.58 (2C), 128.82 (2C), 129.20, 129.50, 131.17, 131.45, 131.48, 135.10, 137.27, 142.87, 143.85, 144.76, 160.81, 164.00; IR (KBr) *v*: 3269, 2921, 2851, 1687, 1584, 1506, 1348, 1393, 1332, 1302, 1145 cm^{-1}; MS (ESI, *m/z*): 483 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{27}H_{27}N_6OS]^+$ $(M + H)^+$ 483.1962, found 483.1972.

4.4.2. (3Z, 2′E)-5,6-di(m-methylanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3b***

Yellow solid; yield 81.9%; mp. 143–144 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 2.24 (s, 3H, $-CH_3$), 2.38 (s, 3H, $-CH_3$), 4.38 (s, 2H, $-CH_2-$), 5.94 (brs, 2H, $-NH_2$), 6.75–6.82 (m, 3H, Ar–H), 7.01–7.13 (m, 4H, Ar–H), 7.25–7.34 (m, 6H, Ar–H), 7.61 (s, 2H, –NH–, pyridine-H), 8.04 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 21.42, 21.53, 34.16, 96.18, 117.67, 118.39, 121.21, 121.87, 124.76, 126.11, 127.53, 128.67, 128.97 (2C), 129.27 (2C), 130.00, 134.49, 137.10, 139.15, 139.44, 140.20, 143.33, 144.63, 145.40, 160.79, 164.28; IR (KBr) *v*: 3269, 2974, 2923, 1683, 1598, 1461, 1396, 1311, 1202 cm^{-1}; MS (ESI, *m/z*): 483 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{27}H_{27}N_6OS]^+$ $(M + H)^+$ 483.1962, found 483.1970.

4.4.3. (3Z, 2′E)-5,6-di(p-methylanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3c***

Yellow solid; yield 83.2%; mp. 169–170 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 2.24 (s, 3H, $-CH_3$), 2.37 (s, 3H, $-CH_3$), 4.36 (s, 2H, $-CH_2-$), 5.89 (brs, 2H, $-NH_2$), 6.84 (d, $J = 8.0$ Hz, 2H, Ar–H), 7.03 (d, $J = 8.5$, 2H, Ar–H), 7.17 (d, $J = 8.5$, 2H, Ar–H), 7.22–7.35 (m, 7H, Ar–H), 7.49 (s, 1H, –NH–), 7.55 (s, 1H, pyridine-H), 8.06 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 20.79, 20.89, 33.98, 95.56, 120.51 (2C), 121.68 (2C), 127.37, 128.54 (2C), 128.81 (2C), 129.88 (2C), 130.62 (2C), 133.71, 133.87, 133.94, 135.63, 136.33, 137.27, 143.28, 144.74, 160.70, 163.85; IR (KBr) *v*: 3296, 3227, 2921, 2855, 1702, 1581, 1519, 1506, 1470, 1348, 1326, 1308, 1238, 1102 cm^{-1}; MS (ESI, *m/z*): 483 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{27}H_{27}N_6OS]^+$ $(M + H)^+$ 483.1962, found 483.1971.

4.4.4. (3Z, 2′E)-5,6-di(m-chloroanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3d***

Yellow solid; yield 72.7%; mp. 156–157 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 4.42 (s, 2H, $-CH_2-$), 6.05 (brs, 2H, $-NH_2$), 6.83 (d, $J = 7.5$ Hz, 1H, Ar–H), 6.97–7.00 (m, 2H, Ar–H), 7.13–7.20 (m, 3H, Ar–H), 7.26–7.38 (m, 7H, Ar–H), 7.58 (s, 1H, –NH–), 7.67 (s, 1H, pyridine-H), 8.00 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 34.37, 97.54, 118.97, 119.14, 120.78, 121.18, 123.80, 125.51, 127.60, 128.70 (2C), 129.02 (2C), 130.54, 131.27, 133.30, 135.20, 135.87, 136.84, 140.47, 143.79, 143.87, 146.61, 160.48, 165.48; IR (KBr) *v*: 3301, 2924, 1684, 1518, 1468, 1358, 1386, 1319, 1092 cm^{-1}; MS (ESI, *m/z*): 523 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{25}H_{21}Cl_2N_6OS]^+$ $(M + H)^+$ 523.0869, found 523.0879.

4.4.5. (3Z, 2′E)-5,6-di(p-chloroanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3e***

Yellow solid; yield 85.2%; mp. 165–166 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 4.48 (s, 2H, $-CH_2-$), 6.15 (brs, 2H, $-NH_2$), 6.92 (d, $J = 8.5$ Hz, 2H, Ar–H), 7.15 (s, 1H, Ar–H), 7.22–7.32 (m, 6H, Ar–H), 7.38–7.43 (m, 4H, Ar–H), 7.44 (s, 1H, –NH–), 7.79 (s, 1H, pyridine-H), 8.23 (s, 1H, –NH–); ^{13}C NMR (DMSO-d_6, 125 MHz) *δ*: 35.06, 114.22, 122.52 (2C), 125.00 (2C), 127.21, 128.22, 129.44 (2C), 129.60 (2C), 130.14 (2C), 130.46 (2C), 134.44, 138.39, 138.43, 141.30, 142.00, 147.74, 155.49, 168.88, 171.35; IR (KBr) *v*: 3231, 3062, 1604, 1589, 1560, 1491, 1335, 1385, 1251 cm^{-1}; MS (ESI, *m/z*): 523 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{25}H_{21}Cl_2N_6OS]^+$ $(M + H)^+$ 523.0869, found 523.0876.

4.4.6. (3Z, 2′E)-5,6-di(p-fluoroanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3f***

Yellow solid; yield 78.9%; mp. 155–156 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 4.49 (s, 2H, $-CH_2-$), 5.98 (brs, 2H, $-NH_2$), 6.94–7.05 (m, 3H, Ar–H), 7.13–7.18 (m, 4H, Ar–H), 7.24–7.33 (m, 5H, Ar–H),7.41 (s, 1H, Ar–H), 7.43 (s, 1H, –NH–), 7.71 (s, 1H, pyridine-H), 8.22 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 34.01, 95.65, 116.19, 119.70, 122.33, 123.77, 123.84, 127.43 (2C), 127.57 (2C), 128.62 (2C), 128.68 (2C), 131.48, 134.98, 137.33, 142.91, 143.73, 143.90, 144.21, 144.75, 164.10, 164.78; IR (KBr) *v*: 3302, 1690, 1639, 1587, 1518, 1469, 1359, 1385, 1319 cm^{-1}; MS (ESI, *m/z*): 491 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{25}H_{21}F_2N_6OS]^+$ $(M + H)^+$ 491.1460, found 491.1462.

4.4.7. (3Z, 2′E)-5,6-di(o-methoxyanilino) pyridine-2,3-dione S-benzyl-3-isothiosemicarbazone ***3g***

Yellow solid; yield 62.5%; mp. 168–169 °C; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 3.82 (s, 3H, $-OCH_3$), 3.86 (s, 3H, $-OCH_3$), 4.39 (s, 2H, $-CH_2-$), 6.06 (brs, 2H, $-NH_2$), 6.71 (t, 1H, $J = 7.5$ Hz, Ar–H), 6.88–7.01 (m, 5H, Ar–H), 7.15–7.38 (m, 6H, Ar–H), 7.50 (d, $J = 7.5$ Hz, 1H, Ar–H), 7.63 (s, 1H, –NH–), 7.92 (s, 1H, pyridine-H), 8.08 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$, 125 MHz) *δ*: 34.10, 34.12, 55.74, 96.33, 110.88, 112.17, 119.35, 120.62, 121.63, 122.82, 123.40, 126.24, 127.48, 128.67 (2C), 128.84, 128.92 (2C), 133.95, 134.04, 137.41, 143.75, 144.84, 149.59, 150.46, 160.89, 164.21; IR (KBr) *v*: 3283, 2936, 2838, 1692, 1646, 1591, 1532, 1466, 1331, 1323, 1246, 1120 cm^{-1}; MS (ESI, *m/z*): 515 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{27}H_{27}N_6O_3S]^+$ $(M + H)^+$ 515.1860, found 515.1868.

4.5. Anticancer activity

The anticancer activities of the prepared compounds against MCF-7, HCT-116 and BEL-7402 cells were evaluated as described elsewhere with some modifications [25]. Target tumor cell lines were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were harvested during logarithmic growth phase and seeded in 96 well plates at a density of

2×10^4 cells/ml, and incubated at 37 °C in a 5% CO_2 incubator. After 24 h incubation at 37 °C, 10 μl tested compounds was added to 96-well plates and cultured at 37 °C for 72 h. 20 μl of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. Discarded the suspension and added 150 ml of dimethyl sulfoxide (DMSO) to each well and shook the plates to dissolve the dark blue crystals (formazan); the absorbance was measured using a microplate reader at a wavelength of 570 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC_{50}) was determined from the dose–response curves according to the inhibition ratio for each concentration. There was a good reproducibility between replicate wells with standard errors below 10%. The results were summarized in Table 1.

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