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4-取代-1-(2-甲基-6-(吡啶-3-基)-烟酰)氨基脲类衍生物合成与生物活性研究

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摘要 经分子杂交技术合成了一系列 4-取代-1-(2-甲基-6-(吡啶-3-基)-烟酰)氨基脲类衍生物. 采用噻唑蓝(MTT)比色法研究了目标化合物对人肝癌细胞(QGY-7703)、人肺癌细胞(NCl-H460)和乳腺癌细胞(MCF-7)的体外抗肿瘤活性. 1-(2-甲基-6-(吡啶-3-基)烟酰基)-4-(2,4,6-三氯苯基)氨基脲(**4n**)显示了最优的活性, 其半数抑制浓度(IC_{50})为 8.89～11.45 μmol/L. 细胞体内的生物研究显示, **4n** 药物处理能明显增加细胞体内 PARP 切割水平以及诱导 QGY-7703 肿瘤细胞的凋亡.

关键词 氨基脲; 联吡啶; 分子杂交; 生物活性

Synthesis and Biological Activity Research of 4-Substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) Semicarbazides

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Abstract A series of 4-substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) semicarbazides were synthesized via molecular hybridization strategy. The synthesized compounds were screened for their anticancer potential against different cancer cells viz human hepatocelular carcinoma (QGY-7703), non-small cell lung (NCl-H460) and human breast (MCF-7) cancer cell lines by methyl thiazolyl tetrazolium (MTT) assay. 1-(2-Methyl-6-(pyridin-3-yl)nicotinoyl)-4-(2,4,6-trichlorophenyl)semicarbazide (**4n**) showed significant anticancer activity in these cancer cell lines with a range of IC_{50} values from 8.89 μmol/L to 11.45 μmol/L. Further biology studies showed that **4n** treatment obviously increased the level of cleaved PARP and induced the apoptosis in QGY-7703 cells.

Keywords semicarbazides; bipyridine; molecular hybridization; biological activity

1 Introduction

Cancer is the second most lethal disease on the earth, 15% the world's deaths are caused by cancer in 2010.[1] Although there are some traditional types of cancer treatments such as surgery, chemotherapy and radiation therapy, the successful treatment of cancer still remains a huge challenge mainly due to poor curative effect, toxic side effect, unpleasant experience and so on. With the progress of molecular biology, targeted tumor therapy has become a hot research topic in cancer treatment recently, a number of targeted drugs were approved for clinical application.[2]

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It is well known that a wide range of heterocyclic molecules have shown different pharmacological properties such as antitubercular, anticonvulsant, antimalarial and antibacterial activities.[3~6] Furthermore, some heterocyclic molecules such as bipyridine, pyrrole, benzimidazole, indole, benzotriazole and thienopyrimidine derivatives have shown good antitumor activities.[7~12]

Molecular hybridization is a new valuable structure modification method in drug design and development, based on the combination of pharmacophoric moieties of different bioactive substances to generate a new hybrid compound with improved efficacy, low toxicity and undesired side effects.[13,14] Bipyridine[6] and semicarbazides[15] have already been demonstrated antitumor activity, with the aim of developing antitumor candidates with improved properties, a series of 4-substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) semicarbazides **4a**～**4r** (Figure 1) were designed and synthesized by the molecular hybridization strategy. The cytotoxicity *in vitro* against QGY7703, NCl-H460, and MCF7 cell lines was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Most of such compounds have the ability to inhibit cancer cells proliferation and one compound **4n** is the best one for QGY7703 cells inhibition with the IC_{50} 8.89 μmol/L. Apoptosis is an important kind of cancer cell deaths, and the cleaved PARP is the marker of it. Further cell biology studies showed that **4n** treatment dramatically increases the cleaved PARP and induces the apoptosis in QGY7703 cells. So such kind of compounds can be a potential direction for anti-cancer drug development.

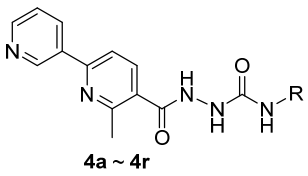
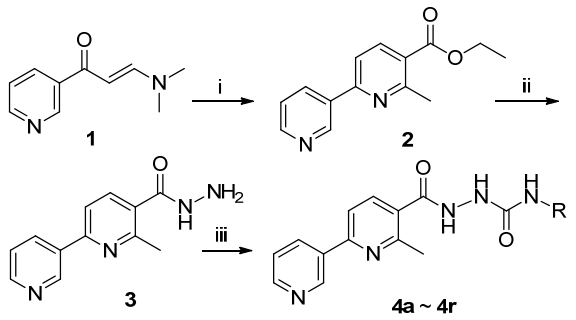


Figure 1 Structures of target compounds **4a**～**4r**

2 Results and discussion

2.1 Synthetic chemistry

The synthetic strategy to prepare target compounds is outlined in Scheme 1. The intermediate 6-methyl-(2,3'-bipyridine)-5-carbohydrazide (**3**) was synthesized by condensation of 3-(dimethylamino)-1-(pyridin-3-yl)prop-2-en-1-one (**1**) and ethyl acetoacetate with 73.5% yield, followed by reaction with hydrazine hydrate with 79.4% yield after the reaction condition optimization. The intermediate **3** was further reacted with isocyanates to form a series of novel 4-substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) semicarbazides **4a**～**4r** with yields range from 43.3% to 82.0%. Ethanol and toluene were investigated as solvents in the synthesis of **4a**～**4r**. The yield was better and melting point range of compounds was shorter when ethanol was used as solvent. The structures of target compounds **4a**～**4r** were characterized by ^{1}H NMR, ^{13}C NMR and HRMS.



Reagent and conditions: (i) ethyl acetoacetate, acetic acid, ammonium acetate, reflux, 5 h; (ii) hydrazine hydrate, ethanol, reflux, 5 h (iii) isocyanates, ethanol, 5 h

Scheme 1 Synthesis of 4-substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) semicarbazides

2.2 Antitumor activities

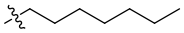
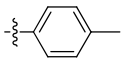
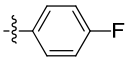
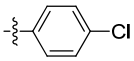
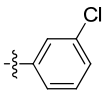
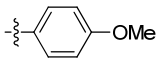
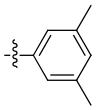
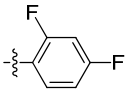
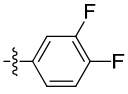
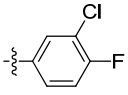
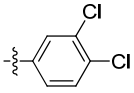
The *in vitro* cytotoxic activity of target compounds **4a**～**4r** was evaluated in human cancer lines including QGY-7703, NCl-H460 and MCF-7 by the MTT assay, and imatinib was used as the positive control. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and summarized in Table 1. The IC_{50} values of compounds **4a** and **4b** were over 100 mol/L for all tested human cancer cell lines, indicating that the introduction of a *n*-alkyl group that had more than three carbon atoms to the pharmaceutical core was unfavorable for anti-proliferative properties. Among the other synthesized compounds (**4c**～**4r**), the cytotoxic activity order is as following: compounds **4n**～**4r** bearing multi fluorine or chlorine-substituted phenyl group＞compounds **4e**～**4l** bearing mono-substituted phenyl group＞compounds **4c**～**4d** bearing cyclic aliphatic group. Compound **4n** with the substitution of 2,4-difluorophenyl displayed the most potential cytotoxicity with IC_{50} value of 8.89 μmol/L against QGY-7703 line.

In order to test the effect of compound **4n** on normal cells, MCR-5 cell line (human normal lung cell line) and LO2 cell line (the normal hepatocytes cell line) were used. The results showed that compound **4n** had IC_{50} values for the LO2 cells was about 77.65 μmol/L and the IC_{50} values for the MRC-5 cells was about 21.32 μmol/L. The results indicated that the normal lung cells (MRC-5 cells) and normal hepatocytes cells (LO2 cells) were more resistant to the toxic activity of compound **4n** than H460 cells and QGY7703 cells.

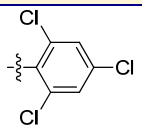
2.3 Effect of 4n on apoptosis in QGY7703 cells

According to the MTT assay of different compounds, we found that compound **4n** most effectively inhibited cell viability in QGY7703 cells, so **4n** was selected for further cellular studies. MTT result showed that **4n** had the anti-proliferative activity in QGY7703 cells with the IC_{50} value 8.89 μmol/L, so firstly, the QGY7703 cells were treated with **4n** for 24 h with different drug concentrations. After **4n** treatment, the cleaved PARP (the mark of apoptosis) was tested. The Western blot result showed that after **4n** treatment, the cleaved PARP could be observed obviously even

Table 1 Cytotoxicity of target compounds **4a**～**4r** against QGY-7703, NCl-H460 and MCF-7 cancer cell lines

Compound	R	IC_{50}±S.D./(μmol•L^{-1})		
		QGY7703	H460	MCF7
4a		＞100	＞100	＞100
4b		＞100	＞100	＞100
4c		55.89±0.98	67.89±0.78	54.78±0.09
4d		45.80±0.18	64.89±0.20	55.78±0.34
4e		33.78±0.10	43.89±0.13	34.90±0.20
4f		33.48±0.40	33.44±0.20	23.96±0.11
4g		22.45±0.11	31.89±0.34	26.90±0.10
4h		22.45±0.19	31.89±0.30	26.90±0.19
4i		23.48±0.11	23.44±0.89	23.80±0.45
4j		22.40±0.34	15.89±0.19	16.90±0.45
4k		22.40±0.32	21.89±0.78	16.50±0.45
4l		23.89±0.30	21.90±0.34	27.09±0.44
4m		24.89±0.13	27.80±0.16	24.10±0.45
4n		8.89±0.20	10.90±0.32	11.45±0.44
4o		12.80±0.22	12.78±0.12	14.40±0.13
4p		13.89±0.10	15.60±0.33	15.09±0.20
4q		14.89±0.23	17.60±0.10	18.10±0.40

Continued

Compound	R	IC_{50}±S.D./(μmol•L^{-1})		
		QGY7703	H460	MCF7
4r		10.60±0.20	11.80±0.30	12.40±0.22
Imatinib		8.54±0.16	10.78±0.23	9.45±0.22

at the low drug concentration (10 μmol/L) (Figure 2).

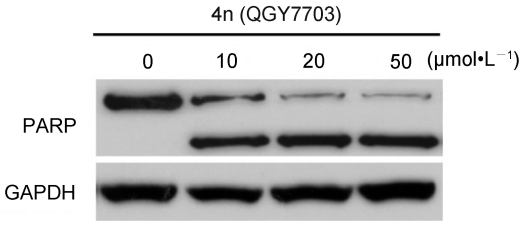


Figure 2 Western blot analysis to test the cleaved PARP after **4n** treatment in QQY7703 cells and GAPDH was used as internal control

In order to conform that **4n** can promote apoptosis in QGY7703 cells, after the **4n** treatment for 24 h, the QGY7703 cells were doubly stained with annexin V/PI (propidium iodide). As the FACS results shown in Figure 3, compound **4n** can effectively induce apoptosis in QGY7703 cells in does-dependent manner (Figure 3).

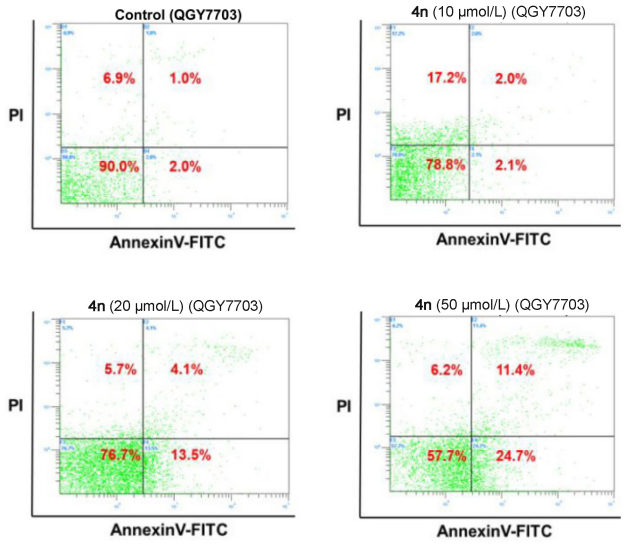


Figure 3 QGY7703 cells were treated with **4n** at the indicated concentrations for 24 h, and stained annexin V/PI. The apoptosis was detected by FACS

3 Conclusions

A series of 4-substitued-1-(2-methyl-6-(-3-pyridy)-nicotinoyl) semicarbazides were designed and synthesized via molecular hybridization strategy. The reaction condition was optimized and eighteen target compounds **4a**～**4r** were prepared under best condition. The structures of target compounds **4a**～**4r** were characterized by ^{1}H NMR, ^{13}C NMR and HRMS. The cytotoxic activity *in vitro* of compounds **4a**～**4r** against QGY-7703, NCl-H460, and MCF7 cell lines was evaluated by the MTT assay, and compound **4n** displayed the most potential cytotoxic activity with IC_{50} value of 8.89, 10.90, and 11.45 μmol/L against QGY-7703, NCl-H460, and MCF7, respectively. The effect of **4n** on apoptosis in QGY-7703 cells was detected by Western blot and annexin V/PI staining (FACS), and **4n** treatment can induce the increase of cleaved PARP and late stage apoptosis.

4 Experimental section

4.1 Materials and instruments

All chemicals were analytically pure and purchased from commercial sources. All melting points were determined on a WRS-1B Digital Melting Point Apparatus (uncorrected). All new compounds were characterized by ^{1}H NMR, ^{13}C NMR and HRMS. NMR spectra were recorded in DMSO-d_6 on a Bruker AV 600 MHz instrument. HRMS spectra were obtained on a Agilent 6230 mass spectrometer. Human breast cell line MCF-7, human hepatocelular carcinoma cell line QGY-7703 and non-small cell lung cancer cell line NCl-H460 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences.

4.2 Synthesis

4.2.1 Synthesis of ethyl (2-methyl-6-(-3-pyridy))nicotinate (**2**)

3-(Dimethylamino)-1-(pyridin-3-yl)prop-2-en-1-one (**1**) (5 mmol, 0.88 g), ethyl acetoacetate (5.5 mmol, 0.75 g) and ammonium acetate (40 mmol, 0.31 g) were dissolved in acetic acid (15 mL), and the reaction mixture was allowed to reflux for 5 h. After completion of reaction, the reaction mixture was cooled down to room temperature. To the reaction mixture was added ice cold water (100 mL), the aqueous phase was extracted with ethyl acetate (30 mL×3), and the combined organic phase was dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford a residue which was purified by column [*V*(ethyl acetate) ∶ *V*(petroleum ether)=1 ∶ 4] to afford the product **2** (0.89 g, 73.5%) as yellow solid. m.p. 160.2～161.0 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.32 (d, J=2.02 Hz, 1H), 8.68 (dd, J=1.65, 4.77 Hz, 1H), 8.53～8.44 (m, 1H), 8.28 (d, J=8.25 Hz, 1H), 8.03 (d, J=8.25 Hz, 1H), 7.55 (dd, J=4.77, 8.07 Hz, 1H), 4.34 (q, J=7.09 Hz, 2H), 2.80 (s, 3H), 1.35 (t, J=7.06 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 166.2, 159.3, 155.9, 151.0, 148.6, 139.8, 134.8, 133.4,

124.6, 124.2, 118.3, 61.5, 25.1, 14.5; HRMS calcd for $C_{14}H_{15}N_2O_2$ $[M+H]^+$ 243.1134, found 243.1132.

4.2.2 Synthesis of 6-methyl-[2,3'-bipyridine]-5-carbohydrazide (**3**)

Compound **2** (5 mmol, 0.61 g) was dissolved in ethanol (5 mL). To the mixture was added hydrazine hydrate (2 mL). The reaction mixture was heated under reflux for 5 h. After completion of the reaction, the reaction mixture was cooled down to room temperature and precipitated. The solid was filtered off and washed with ethanol, to afford product **3** (0.91g, 79.4%) as white solid. m.p. 174.2～174.3 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.65 (s, 1H), 9.28 (d, J=1.83 Hz, 1H), 8.65 (dd, J=1.47, 4.77 Hz, 1H), 8.45 (dt, J=1.79, 7.98 Hz, 1H), 7.94 (d, J=8.07 Hz, 1H), 7.82 (d, J=7.89 Hz, 1H), 7.53 (dd, J=4.77, 7.89 Hz, 1H), 4.56 (brs, 2H), 2.62 (s, 3H); ^{13}C NMR (150MHz, DMSO-d_6) δ: 167.5, 156.5, 154.1, 150.6, 148.4, 137.0, 134.5, 133.9, 130.3, 124.3, 118.0, 23.4; HRMS calcd for $C_{12}H_{13}N_4O$ $[M+H]^+$ 229.1089, found 229.1088.

4.2.3 General procedure for the synthesis of 4-substituted-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl)semcarbazides (**4a**～**4r**)

Compound **3** (5 mmol, 1.14 g) was dissolved in ethanol (10 mL). To the mixture was added different isocyanate (5.5 mmol). The reaction mixture was heated under reflux for 3 h. After completion of the reaction, the reaction mixture was filtered off and washed with ethanol to afford products **4a**～**4r**.

N-Butyl-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4a**): White solid, yield 80.5%. m.p. 163.6～165.9 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.04 (brs, 1H), 9.30 (brs, 1H), 8.66 (brs, 1H), 8.48 (d, J=7.15 Hz, 1H), 7.99 (brs, 2H), 7.95 (brs, 1H), 7.58～7.46 (m, 1H), 6.51 (brs, 1H), 3.17～3.00 (m, 2H), 2.66 (s, 3H), 1.44～1.36 (m, 2H), 1.34～1.23 (m, 2H), 0.88 (t, J=7.15 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.1, 158.5, 156.9, 154.4, 150.6, 148.4, 137.4, 134.6, 133.9, 129.6, 124.3, 117.9, 39.4, 32.5, 23.5, 19.9, 14.2; HRMS calcd for $C_{17}H_{22}N_5O_2$ $[M+H]^+$ 328.1768, found 328.1767.

N-Heptyl-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)-hydrazine-1-carboxamide (**4b**): White solid, yield 76.5%. m.p. 161.8～162.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.03 (d, J=1.83 Hz, 1H), 9.31 (d, J=1.65 Hz, 1H), 8.66 (dd, J=1.38, 4.68 Hz, 1H), 8.48 (dt, J=1.95, 8.02 Hz, 1H), 8.01～7.98 (m, 2H), 7.94 (s, 1H), 7.61～7.45 (m, 1H), 6.49 (brs, 1H), 3.06 (q, J=6.72 Hz, 2H), 2.67 (s, 3H), 1.50～1.38 (m, 2H), 1.34～1.21 (m, 9H), 0.88～0.85 (m, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.1, 158.6, 156.9, 154.4, 150.7, 148.4, 137.4, 134.6, 133.9, 129.6, 124.3, 117.8, 39.7, 31.8, 30.4, 29.0, 26.8, 23.5, 22.5, 14.4; HRMS calcd for $C_{20}H_{28}N_5O_2$ $[M+H]^+$ 370.2238, found 370.2240.

N-Cyclopentyl-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4c**): White solid, yield 78.5%. m.p. 203.3～203.9 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.01 (s, 1H), 9.30 (d, J=1.83 Hz, 1H), 8.66 (dd, J=1.65, 4.77 Hz, 1H), 8.47 (dt, J=1.86, 8.21 Hz, 1H), 8.00～7.96 (m, 1H), 7.95～7.92 (m, 1H), 7.84 (s, 1H), 7.55 (ddd, J=0.73, 4.77, 7.89 Hz, 1H), 6.41 (d, J=7.52 Hz, 1H), 3.94 (sxt, J=6.93 Hz, 1H), 2.67 (s, 3H), 1.90～1.76 (m, 2H), 1.69～1.60 (m, 2H), 1.55～1.46 (m, 2H), 1.44～1.36 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.1, 158.1, 156.8, 154.4, 150.7, 148.4, 137.3, 134.6, 134.5, 133.9, 129.6, 124.3, 117.9, 51.6, 33.1, 23.7, 23.5; HRMS calcd for $C_{18}H_{22}N_5O_2$ $[M+H]^+$ 340.1768, found 340.1770.

N-Cyclohexyl-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4d**): White solid, yield 82.0%. m.p. 216.8～217.1 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.02 (s, 1H), 9.30 (d, J=1.65 Hz, 1H), 8.66 (dd, J=1.56, 4.68 Hz, 1H), 8.47 (dt, J=1.86, 8.21 Hz, 1H), 8.01～7.96 (m, 1H), 7.95～7.91 (m, 1H), 7.89～7.86 (m, 1H), 7.56～7.53(m, 1H), 6.32 (d, J=8.07 Hz, 1H), 3.49～3.41 (m, 1H), 2.67 (s, 3H), 1.83～1.74 (m, 2H), 1.73～1.64 (m, 2H), 1.62～1.48 (m, 1H), 1.32～1.11 (m, 5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.1, 157.7, 156.8, 154.4, 150.7, 148.4, 137.3, 134.6, 133.9, 129.7, 124.3, 117.9, 48.6, 33.5, 25.7, 25.1, 23.5; HRMS calcd for $C_{19}H_{24}N_5O_2$ $[M+H]^+$ 354.1925, found 354.1930.

N-(*m*-Tolyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)-hydrazine-1-carboxamide (**4e**): White solid, yield 46.6%. m.p. 217.5～217.6 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.22 (s, 1H), 9.31 (d, J=1.65 Hz, 1H), 8.84 (brs, 1H), 8.67 (dd, J=1.10, 4.58 Hz, 1H), 8.55～8.42 (m, 1H), 8.27 (s, 1H), 8.05～7.95 (m, 2H), 7.55 (dd, J=4.77, 7.89 Hz, 1H), 7.34 (s, 1H), 7.28 (d, J=8.07 Hz, 1H), 7.16 (t, J=7.79 Hz, 1H), 6.80 (d, J=7.34 Hz, 1H), 2.70 (s, 3H), 2.28 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.8, 155.8, 154.5, 150.7, 148.4, 140.0, 138.3, 137.4, 134.6, 133.9, 129.5, 129.0, 124.3, 123.2, 119.5, 118.0, 116.1, 23.5, 21.7; HRMS calcd for $C_{20}H_{20}N_5O_2$ $[M+H]^+$ 362.1612, found 362.1611.

N-(*p*-Tolyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)-hydrazine-1-carboxamide (**4f**): White solid, yield 50.5%. m.p. 200.5～202.9 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.23 (s, 1H), 9.32 (d, J=1.47 Hz, 1H), 8.83 (s, 1H), 8.67 (dd, J=1.28, 4.59 Hz, 1H), 8.48 (dt, J=1.86, 8.02 Hz, 1H), 8.26 (s, 1H), 8.06～7.94 (m, 2H), 7.55 (dd, J=4.77, 7.52 Hz, 1H), 7.39 (d, J=8.25 Hz, 2H), 7.09 (d, J=8.25 Hz, 2H), 2.71 (s, 3H), 2.25 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.3, 156.9, 155.9, 154.5, 150.7, 148.4, 137.5, 137.3, 134.6, 133.9, 131.2, 129.6, 129.5, 124.3, 119.1, 117.9, 23.5, 20.8. HRMS calcd for $C_{20}H_{20}N_5O_2$ $[M+H]^+$ 362.1612, found 362.16416.

N-(3-Fluorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4g**): Yellow solid, yield 61.2%. m.p. 224.5～225.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ:10.29 (s, 1H), 9.33 (d, J=1.65 Hz, 1H), 9.21 (brs, 1H), 8.68 (dd, J=1.47, 4.58 Hz, 1H), 8.53～8.42 (m, 2H), 7.97～8.07 (m, 2H), 7.62～7.48 (m, 2H), 7.35～7.29 (m, 1H), 7.25 (d, J=7.52 Hz, 1H), 6.81 (dt, J=1.83, 8.44 Hz, 1H), 2.72 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 162.8 (d, J=240.0 Hz), 162.0, 156.9, 155.7, 154.6, 150.7, 148.4, 142.0 (d, J=2.3 Hz), 137.3, 134.6, 133.9, 130.7 (d, J=2.3 Hz), 129.4, 124.3, 118.0, 114.7, 108.7 (d,

J=10.5 Hz), 105.6 (d, *J*=14.3.0 Hz), 23.5; HRMS calcd for $C_{19}H_{17}FN_5O_2$ $[M+H]^+$ 366.1361, found 366.1370.

N-(4-Fluorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4h**): Yellow solid, yield 64.5%. m.p. 232.2～232.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.26 (s, 1H), 9.33 (d, *J*=1.83 Hz, 1H), 9.01 (brs, 1H), 8.68 (dd, *J*=1.47, 4.77 Hz, 1H), 8.49 (dt, *J*=1.81, 8.11 Hz, 1H), 8.37 (s, 1H), 8.02 (s, 2H), 7.59～7.50 (m, 3H), 7.19～7.09 (m, 2H), 2.72 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.3, 157.3 (d, *J*=202.0 Hz),157.1, 156.9, 157.3, 154.5, 150.7, 148.4, 137.3, 136.4 (d, *J*=2.2 Hz), 134.6, 133.9, 129.4, 124.3, 120.8, 117.9, 115.6 (d, *J*=21.0 Hz), 23.5; HRMS calcd for $C_{19}H_{17}FN_5O_2$ $[M+H]^+$ 366.1361, found 366.1370.

N-(4-Chlorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4i**): White solid, yield 56.2%. m.p. 229.6 ～ 230.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.25 (s, 1H), 9.32 (d, *J*=1.65 Hz, 1H), 9.10 (brs, 1H), 8.67 (dd, *J*=1.56, 4.68 Hz, 1H), 8.49 (dt, *J*=1.90, 8.12 Hz, 1H), 8.41 (s, 1H), 8.04～7.98 (m, 2H), 7.58～7.53 (m, 3H), 7.36～7.31 (m, 2H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.9, 155.8, 154.5, 150.7, 148.4, 139.1, 137.3, 134.6, 133.9, 129.4, 129.0, 126.0, 124.3, 120.5, 118.0, 23.5; HRMS calcd for $C_{19}H_{17}ClN_5O_2$ $[M+H]^+$ 382.1065, found 382.1069.

N-(3-Chlorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4j**): White solid, yield 58.4%. m.p. 230.2 ～ 230.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.27 (s, 1H), 9.32 (d, *J*=1.83 Hz, 1H), 9.17 (brs, 1H), 8.68 (dd, *J*=1.47, 4.77 Hz, 1H), 8.52～8.44 (m, 2H), 8.05～7.98 (m, 2H), 7.75 (t, *J*=1.93 Hz, 1H), 7.57～7.54 (m, 1H), 7.39 (d, *J*=7.15 Hz, 1H), 7.31 (t, *J*=8.07 Hz, 1H), 7.08～6.98 (m, 1H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.9, 155.7, 154.6, 150.7, 148.4, 141.7, 137.4, 134.6, 133.8, 133.6, 130.8, 129.4, 124.3, 122.0, 118.3, 118.0, 117.4, 23.5; HRMS calcd for $C_{19}H_{17}ClN_5O_2$ $[M+H]^+$ 382.1065, found 382.1070.

N-(2-Chlorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4k**): White solid, yield 58.9%. m.p. 227.8～228.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.40 (s, 1H), 9.32 (d, *J*=1.65 Hz, 1H), 9.06 (brs, 1H), 8.68 (dd, *J*=1.47, 4.77 Hz, 1H), 8.49 (dt, *J*=1.88, 8.16 Hz, 1H), 8.40 (brs, 1H), 8.14 (d, *J*=8.07 Hz, 1H), 8.04～8.01 (m, 1H), 8.00～7.97 (m, 1H), 7.57～7.54 (m, 1H), 7.48 (dd, *J*=1.38, 7.98 Hz, 1H), 7.35～7.30 (m, 1H), 7.06 (dt, *J*=1.38, 7.66 Hz, 1H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.7, 155.4, 154.6, 150.7, 148.4, 137.3, 136.2, 134.6, 133.8, 129.7, 129.3, 128.1, 124.3, 124.1, 121.9, 118.0, 23.4; HRMS calcd for $C_{19}H_{17}ClN_5O_2$ $[M+H]^+$ 382.1065, found 382.1070.

N-(4-Methoxyphenyl)-2-(6-methyl-[2,3'-bipyrdine]-5-carbonyl)hydrazine-1-carboxamide (**4l**): White solid, yield 49.0%. m.p. 222.6～223.2 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.20 (d, *J*=0.92 Hz, 1H), 9.32 (d, *J*=1.65 Hz, 1H), 8.75 (s, 1H), 8.67 (dd, *J*=1.38, 4.68 Hz, 1H), 8.52～8.43 (m, 1H), 8.22 (s, 1H), 8.04～7.94 (m, 2H), 7.55 (dd, *J*=4.77, 7.89 Hz, 1H), 7.40 (d, *J*=8.99 Hz, 2H), 6.88 (d, *J*=9.17 Hz, 2H), 3.72 (s, 3H), 2.70 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.3, 156.9, 156.1, 155.0, 154.5, 150.7, 148.4, 137.4, 134.6, 133.9, 133.1, 129.5, 124.3, 120.8, 117.9, 114.3, 55.6, 23.5; HRMS calcd for $C_{20}H_{20}N_5O_3$ $[M+H]^+$ 378.1561, found 378.1565.

N-(3,5-Dimethylphenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4m**): White solid, yield 66.5%. m.p. 229.6～230.0 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.22 (s, 1H), 9.32 (d, *J*=1.7 Hz, 1 H), 8.76 (brs, 1H), 8.67 (dd, *J*=4.6, 1.3 Hz, 1H), 8.54～8.41 (m, 1H), 8.25 (s, 1H), 8.04～7.95 (m, 2H), 7.55 (dd, *J*=7.7, 4.8 Hz, 1H), 7.13 (s, 2H), 6.62 (s, 1H), 2.71 (s, 3H), 2.24 (s, 6H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.8, 155.8, 154.5, 150.7, 148.4, 139.9, 138.1, 137.4, 134.6, 133.9, 129.5, 124.3, 124.0, 118.0, 116.7, 23.5, 21.6; HRMS calcd for $C_{21}H_{22}N_5O_2$ $[M+H]^+$ 376.1768, found 376.1769.

N-(2,4-Difluorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4n**): White solid, yield 66.7%. m.p. 291.9 ～ 293.6 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.35 (s, 1H), 9.32 (d, *J*=1.83 Hz, 1H), 8.72 (brs, 1H), 8.68 (dd, *J*=1.47, 4.77 Hz, 1H), 8.60 (s, 1H), 8.49 (dt, *J*=1.90, 8.12 Hz, 1H), 8.05～7.96 (m, 3H), 7.57～7.54 (m, 1H), 7.35～7.31 (m, 1H), 7.11～7.02 (m, 1H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 158.5 (d, *J*=256.5 Hz), 156.8, 155.7, 154.6, 150.7, 148.4, 137.3, 134.6, 133.8, 129.4, 124.3, 118.0, 111.6, 111.4, 104.5, 104.3 (d, *J* = 2.2 Hz), 104.2, 23.4; HRMS calcd for $C_{19}H_{16}F_2N_5O_2$ $[M+H]^+$ 384.1267; found 384.1270.

N-(3,4-Difluorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4o**): White solid, yield 60.0%. m.p. 224.0 ～ 224.3 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.28 (s, 1H), 9.33 (d, *J*=1.47 Hz, 1H), 9.21 (brs, 1H), 8.68 (dd, *J*=1.19, 4.68 Hz, 1H), 8.54～8.46 (m, 2H), 8.06～7.99 (m, 2H), 7.74～7.70 (m, 1H), 7.56 (dd, *J*=4.77, 7.89 Hz, 1H), 7.39～7.32 (m, 1H), 7.27 (brs, 1H), 2.72 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.3, 156.9 (d, *J*=256.5 Hz), 155.8, 154.6, 150.7, 149.5 (dd, *J*=13.3, 229.5 Hz), 148.4, 145.0 (dd, *J*=13.3, 232.5 Hz), 134.6, 134.2, 133.8, 129.4, 124.3, 117.9, 117.8, 117.7, 115.1, 107.9, 23.5; HRMS calcd for $C_{19}H_{16}F_2N_5O_2$ $[M+H]^+$ 384.1267, found 384.1271.

N-(3-Chloro-4-fluorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4p**): White solid, yield 43.3%. m.p. 238.3～238.6 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.27 (s, 1H), 9.33 (d, *J*=1.65 Hz, 1H), 9.18 (brs, 1H), 8.68 (dd, *J*=1.56, 4.68 Hz, 1H), 8.52 (s, 1H), 8.49 (dt, *J*=1.88, 8.16 Hz, 1H), 8.12～7.97 (m, 2H), 7.85 (dd, *J*=2.57, 6.79 Hz, 1H), 7.57～7.54 (m, 1H), 7.43 (brs, 1H), 7.39～7.30 (m, 1H), 2.72 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.3, 156.4 (d, *J*=220.0 Hz), 154.6, 153.7, 152.1, 150.7, 148.4, 137.5, 137.4 (d, *J*=3.0 Hz), 134.6, 133.8, 129.4, 124.3, 120.3, 119.6, 119.4, 119.3, 117.9, 117.2 (d, *J* = 10.5 Hz), 23.5; HRMS calcd for $C_{19}H_{16}ClFN_5O_2$ $[M+H]^+$ 400.0971, found 400.0973.

N-(3,4-Dichlorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4q**): White solid, yield 49.9%. m.p. 204.0～204.9 ℃; ^{1}H NMR (600 MHz, DMSO-

d_6) δ: 10.28 (s, 1H), 9.32 (d, J=1.65 Hz, 1H), 9.29 (d, J=1.65 Hz, 1H), 8.68 (dd, J=1.65, 4.77 Hz, 1H), 8.58 (s, 1H), 8.49 (dt, J=1.79, 8.16 Hz, 1H), 8.06～7.98 (m, 2H), 7.94～7.90 (m, 1H), 7.57～7.54 (m, 1H), 7.54～7.52 (m, 1H), 7.47 (d, J=6.79 Hz, 1H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.2, 156.9, 155.7, 154.6, 150.7, 148.4, 140.4, 137.4, 134.6, 133.8, 131.4, 131.0, 129.3, 124.3, 123.7, 120.1, 119.1, 118.0, 23.5; HRMS calcd for $C_{19}H_{16}Cl_2N_5O_2$ [M+H]$^+$ 416.0603, found 416.0606.

N-(2,4,6-Trichlorophenyl)-2-(6-methyl-[2,3'-bipyridine]-5-carbonyl)hydrazine-1-carboxamide (**4r**): White solid, yield 45%. m.p. 230.0～231.5 ℃; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.30 (brs, 1H), 9.31 (d, J=1.83 Hz, 1H), 8.69～8.63 (m, 2H), 8.60 (brs, 1H), 8.48 (dt, J=1.90, 8.12 Hz, 1H), 8.00 (d, J=7.70 Hz, 1H), 7.76～7.71 (m, 2H), 7.55～7.53 (m, 1H), 2.67 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 168.0, 157.0, 154.5, 150.7, 150.5, 148.4, 148.3, 137.4, 135.7, 134.6, 134.5, 133.8, 133.5, 132.1, 129.4, 128.6, 128.5, 124.3, 117.9, 23.5; HRMS calcd for $C_{19}H_{15}Cl_3N_5O_2$ [M+H]$^+$ 450.0286, found 450.0284.

4.3 MTT toxicity assay

The *in vitro* antiproliferative activity of target compounds **4a**～**4r** against three human cancer lines including QGY-7703, NCl-H460 and MCF-7 cancer cell lines was measured by the MTT colorimetric method.[16~18] In all experiments, cells in good condition and logarithmic growth phase were taken and digested by 0.25% trypsin. The adherent cells were detached and counted, a (2～4)×10^4 cells/mL suspension was prepared and inoculated into 96-well cell culture plate with 180 μL a well, placed in constant temperature carbon dioxide incubator and cultured for 24 h. Then the medium was replaced with the dimethyl sulfoxide (DMSO) solution of target compound (20 μL) a well, and added 10 vol% serum culture medium (80 μL), and cultured for 48 h. MTT (20 μL) was added to each well and reacted for 4 h. The supernatant was removed and DMSO (150 μL per well) was added to dissolve MTT. The plate was shaken on platform rocker for 5 min, and the optical density (OD) of each well was measured on enzyme-linked immunosorbent assay (ELISA) meter at the wavelength of 570 nm. The inhibition activity of cell proliferation was determined. Dissolvent control group was used as negative control group, and 4-substitued-1-(2-methyl-6-(pyridin-3-yl)-nicotinoyl) semicarbazides was used as positive control group. The inhibition rate of target compounds on tumor cell growth was calculated according to the following formula:

Inhibition rate(%)=[OD(negative control)−OD(tested compound)/OD(negative control)×100%

The IC_{50} value of target compounds was calculated based on the inhibition rates of different concentrations.

4.4 Annexin V/PI staining

Cells were cultured in six-well with about 1×10^5 cells in each well and treated with varying concentrations of **4n** for 24 h. Then the apoptosis analysis was performed following the protocols (BD Bioscience). After stained with FITC annexin V/PI, the apoptosis status was analyzed using a Beckman Epics Altra Culter and data were analyzed with EXP032 software. The status and percentage of cells undergoing apoptosis was defined as early apoptosis (annexin V-positive and PI-negative) and late apoptosis (annexin V-positive and PI-positive).

4.5 Western blot analysis

To check the level of cleaved PARP1 after **4n** treatment, the Western blots were performed using PARP1 antibody (9542, CST, Shanghai, China), meanwhile the GAPDH antibody (G8795, Sigma) was used as control. After **4n** treatment for 24 h, the whole cells lysates were used for Western blot analysis. Both anti-rabbit and anti-mouse IgG were purchased from Sigma.

References

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