Full Paper

Synthesis and Cytotoxicity Studies of Novel 2-Hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-ones

Guogang Zhang, Yajing Liu, Shuobing Wang, Chuan Zhou, Qingchang Huang, and Ping Gong

Key Laboratory of Original New Drugs Design and Discovery of Ministry of Education,
School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang, P. R. China

In an attempt to develop potent antitumor agents, a series of novel 2-hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-one derivatives were designed and synthesized. All the prepared compounds were screened for their cytotoxic activities against A549, MDA-MB-231 and HT-29 cell lines *in vitro*. Pharmacological data indicated that five of the target compounds showed cytotoxicity against A549 cell line below a concentration of 1 μM. Compound **15g** was the most potent one with IC_{50} values of 0.19, 2.11 and 2.15 μM against A549, MDA-MB-231 and HT29 cell lines, respectively.

Keywords: Anticancer activity / Apoptosis / 2-Hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-ones

Received: March 24, 2011; Revised: May 17, 2011; Accepted: May 18, 2011

DOI 10.1002/ardp.201100103

Introduction

Apoptosis plays a vital role in the control of cell numbers and defective apoptosis represents a major causative factor in the development and progression of cancer [1]. The ability of tumor cells to evade apoptosis led to their resistance to conventional therapeutic regimens [2]. Therefore, development of apoptosis inducers as new anticancer agents is a promising approach and has been a focus of research recently [3–6].

Calpains and caspases are families of cysteine proteases and there is a level of collaboration between them in the induction of apoptosis [7–9]. Calpains are ubiquitously expressed in many tissues and implicated in numerous calcium-regulated cellular processes, such as signal transduction, cell proliferation, differentiation and apoptosis [9–11]. Inhibitors of two major isoforms of calpain (μ-calpain and *m*-calpain) were selectively cytotoxic to various human tumor cells in a dose-dependent manner, and were not cytotoxic to normal cells and tissues [12]. SJA7029 (Fig. 1), a 3,4-dihydroquinoxalin-2(1*H*)-one derivative, was identified as a new potent inhibitor of calpains with IC_{50} values of 0.12 μM (μ-calpain) and 0.17 μM (*m*-calpain), respectively [13].

Caspase-3 is an important effector protease in the caspase family, which can catalyze the hydrolysis of a multitude of protein substrates within the cells and induce apoptosis in cancer cells selectively [14]. Putt et al. reported that PAC-1 (Fig. 1) induced death in cancer cells through direct activation of procaspase-3 via the sequestration of inhibitory zinc ions. The phenolic hydroxyl group is the essential PAC-1 functional group responsible for zinc chelation [15].

Inspired by SJA-7029 and PAC-1, we designed and synthesized a series of 2-hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-one derivatives. Various substituted *ortho*-hydroxyl hydrazones were incorporated into the C-2 position to investigate the influence of substituted position and spatial effects of arylidene motif. Furthermore, phenyl, 4-methoxyphenyl and 4-(trifluoromethoxy)phenyl groups were introduced into the N-4 position of skeleton, in order to develop novel analogues and to investigate the cytotoxic activities against human cancer cell lines *in vitro*.

Results and discussion

Chemistry

The synthetic route of the target compounds **5a–5h**, **10a–10h** and **15a–15h** is illustrated in Scheme 1. The commercially available 2-chloro-3-nitropyridine and aniline were treated with *N*,*N*-diisopropylethylamine in isopropanol to give the compound **1**, which was reduced and cyclized with oxalic

Correspondence: Ping Gong, Key Laboratory of Original New Drugs Design and Discovery of Ministry of Education, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenhe District, 110016 Shenyang, Liaoning, P. R. China.
E-mail: gongpinggp@126.com
Fax: +86 24 2398-6429

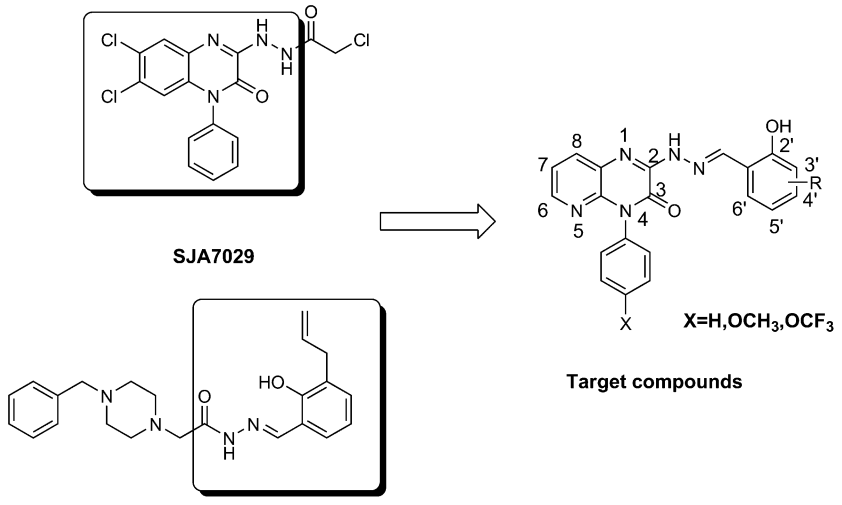
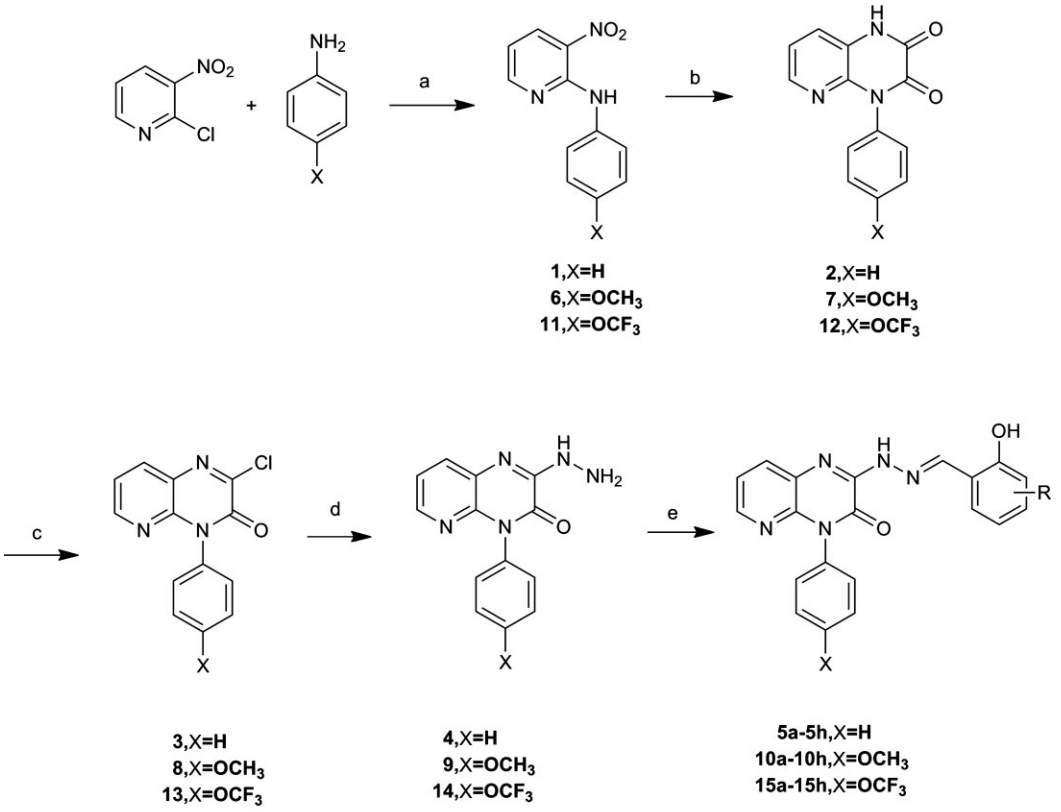


Figure 1. Structures of SJA7029, PAC-1 and 2-hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-ones.



Reagents and conditions: a) TEA/ IPA, rt., 1h, r.f., 8 h; b) zinc powder/NH_4Cl/EtOH, r.f., 5 h; oxalic acid/4 N HCl, r.f., 15 h; c) $POCl_3$, CH_3CN, r.f., 3 h; d) 80% $NH_2NH_2 \cdot H_2O$, 60°C, 1 h; e) EtOH, 60°C, 5 h.

Scheme 1. Synthesis of target compounds.

acid to afford compound **2**. Subsequent treatment of **2** with phosphorus oxychloride afforded chloro-derivative **3.** Compound **3** was reacted with an excess of 80% hydrazine hydrate in ethanol to furnish the key intermediate **4**. Another two important intermediates **9** and **14** were obtained according to the same method as described for compound **4** when aniline was replaced by 4-methoxyaniline and 4-(trifluoromethoxy)aniline, respectively. Finally, the target compounds **5a–5h**, **10a–10h** and **15a–15h** were successfully obtained *via* the reaction of intermediate **4**, **9** and **14** with different aromatic aldehydes in the refluxing ethanol, respectively. The products were purified by column chromatography on 200–300 mesh silica gel.

Biological evaluation

The newly synthesized twenty-four compounds (**5a–5h**, **10a–10h** and **15a–15h**) were performed by MTT assay using three human cancer cell lines, A549, MDA-MB-231 and HT29, with PAC-1 as a positive control. The results of the assay are summarized in Table 1. Among them, compounds **10d**, **15a**, **15b**, **15d** and **15g** showed cytotoxicity against A549 cell line below a concentration of 1 μM. Compound **15g** was the most active compound on A549, MDA-MB-231 and HT-29 with IC_{50} value of 0.19, 2.11 and 2.15 μM, respectively.

The preliminary structure–activity relationships (SARs) suggested that compounds with 4-methoxyphenyl or 4-(trifluoromethoxy)phenyl groups on the N-4 position were generally more potent than with unsubstituted phenyl group. The pharmacological data indicated that 4-methoxyphenyl analogue **10d** showed a dramatic 7-fold improvement in cytotoxicity against A549 cell lines (IC_{50} = 0.95 μM) compared with phenyl analogue **5c** (IC_{50} = 6.94 μM). It could be observed that 4-methoxyphenyl group was generally preferred and that 4-(trifluoromethoxy)phenyl group was also well tolerated. Further data indicated that 4-(trifluoromethoxy)phenyl analogue **15b** was nearly 5-fold more active than phenyl analogue **5b** against A549 cell line.

The pharmacological results indicated that the substituents on 4′-postion of the 2′-hydroxybenzylidene were benefit to activity. It was obvious that introducing a hydroxyl group to the 4′-postion of 2′-hydroxybenzylidene of compound (**15g**) improved its potency against all three cell lines, other substitution such as chloro (**15d**) and benzyloxy (**15h**) group at the same position were well tolerated but not significantly better than hydroxyl group.

Interestingly, the volume of groups on 3′-position and 5′-position of the 2′-hydroxybenzylidene would influence antitumor activity. Generally, introducing the bulky groups,

Table 1. The substituents. Cytotoxicity of the tested compounds against A549, MDA-MB-231 and HT-29 cell lines.

Compd.	X	R	IC_{50} (μM)		
			A549	MDA-MB-221	HT-29
5a	H	2,3,4-trihydroxy	13.35 ± 1.58	16.82 ± 1.84	4.23 ± 0.45
5b	H	3-allyl-2-hydroxy	4.75 ± 0.67	3.63 ± 0.48	4.67 ± 0.43
5c	H	3-allyl-2-hydroxy-5-methyl	6.94 ± 0.78	12.42 ± 1.53	5.26 ± 0.49
5d	H	3-allyl-2-hydroxy-5-isopropyl	9.04 ± 0.83	15.87 ± 1.68	13.61 ± 1.12
5e	H	3-allyl-5-*tert*-butyl-2-hydroxy	13.54 ± 1.47	48.66 ± 3.66	52.98 ± 4.84
5f	H	2-hydroxy-3-(2-methylallyl)	NA[a]	18.54 ± 1.23	2.78 ± 0.46
5g	H	3,5-di-*tert*-butyl-2-hydroxy	NA[a]	NA[a]	NA[a]
5h	H	4-(4-chlorobenzyloxy)-2-hydroxy	2.82 ± 0.09	6.04 ± 0.14	12.03 ± 1.08
10a	$-OCH_3$	2-hydroxy	6.53 ± 0.22	28.16 ± 2.34	12.24 ± 0.48
10b	$-OCH_3$	4-chloro-2-hydroxy	4.82 ± 0.59	8.54 ± 0.94	6.23 ± 0.48
10c	$-OCH_3$	3-allyl-2-hydroxy	1.24 ± 0.15	11.83 ± 0.88	2.58 ± 0.37
10d	$-OCH_3$	3-allyl-2-hydroxy-5-methyl	0.95 ± 0.15	11.63 ± 1.19	2.43 ± 0.33
10e	$-OCH_3$	3-allyl-5-*tert*-butyl-2-hydroxy	11.8 ± 0.18	13.83 ± 1.76	26.25 ± 2.41
10f	$-OCH_3$	2-hydroxy-3-(2-methylallyl)	NA[a]	19.63 ± 2.49	12.61 ± 1.17
10g	$-OCH_3$	2,4-dihydroxy	3.84 ± 0.29	10.86 ±1.67	10.94 ± 1.98
10h	$-OCH_3$	2,3,4-trihydroxy	3.83 ± 0.43	8.57 ± 0.68	2.38 ± 0.39
15a	$-OCF_3$	3-allyl-5-*tert*-butyl-2-hydroxy	0.83 ± 0.13	28.57 ± 3.31	48.38 ± 3.69
15b	$-OCF_3$	3-allyl-2-hydroxy	0.94 ± 0.13	4.47 ± 0.36	1.61 ± 0.27
15c	$-OCF_3$	2-hydroxy-3-(2-methylallyl)	NA[a]	12.85 ± 1.87	2.41 ± 0.38
15d	$-OCF_3$	4-chloro-2-hydroxy	0.81 ± 0.07	3.85 ± 0.23	4.61 ± 0.68
15e	$-OCF_3$	3-allyl-2-hydroxy-5-methyl	1.49 ± 0.25	6.91 ± 0.85	1.67 ± 0.36
15f	$-OCF_3$	3,5-di-*tert*-butyl-2-hydroxy	NA[a]	NA[a]	NA[a]
15g	$-OCF_3$	2,4-dihydroxy	0.19 ± 0.05	2.11 ± 0.25	2.15 ± 0.39
15h	$-OCF_3$	4-(benzyloxy)-2-hydroxy	1.09 ± 0.06	3.47 ± 0.53	5.72 ± 0.47
PAC-1			0.66 ± 0.08	6.67 ± 0.58	1.62 ± 0.13

[a] NA means no activity.

such as *tert*-butyl, isopropyl and 2-methylallyl resulted in dramatic decrease or abolishment in antitumor potency. A case in point was incorporation of allyl group at 3′-position appeared to be beneficial, with compounds **5b**, **10c** and **15b** displaying potent activities. However, incorporation of 2-methylallyl group at the same position exhibited a reduction of potency against MDA-MB-231 cell line and no cytotoxicity against A549 cell line. In addition, substitution to the 5′-position of the 3′-allyl-2′-hydroxybenzylidene with a more bulky aliphatic group resulted in declined antitumor activity (methyl **5c** > isopropyl **5d** > *tert*-butyl **5e**). Significantly, introduction of 3,5-di-*tert*-butyl group into the 2′-hydroxybenzylidene (**5g** and **15f**) led to no antitumor activity against all three cell lines.

Experimental protocols

Chemistry

All melting points were obtained on a Büchi Melting Point B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were uncorrected. Mass spectra (MS) were taken in ESI mode on Agilent 1100 LC-MS (Agilent, Palo Alto, CA, USA). Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) was performed using Bruker ARX-400, 400-MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with TMS as an internal standard. Elemental analyses were performed with a Carlo-Erba 1106 analyzer. Unless otherwise noted, all the materials were obtained from commercially available sources and were used without further purification.

*2-Anilino-3-nitropyridine (**1**)*

To a stirred solution of aniline 111.6 g (1.2 mol) and triethylamine 187.6 mL (1.3 mol) in isopropanol (1500 mL) 2-chloro-3-nitropyridine 124.0 g (1.0 mol) was added slowly at 25°C, and then the reaction mixture was stirred at room temperature for 1 h and heated to reflux for 8 h. The mixture was cooled, separated by filtration and washed with EtOH and water to give off an orange crystal (114.0 g, 53%). M.p.: 72–73°C. ESI-MS *m/z*: 216.1 $(M+H)^+$. $C_{11}H_9N_3O_2$ (215.07).

*1,4-Dihydro-4-phenylpyrido[2,3-b]pyrazine-2,3-dione (**2**)*

To a stirred solution of **1** 114 g (0.53 mol) in 95% EtOH (1000 mL) a solution of zinc powder 175 g (2.7 mol) and NH_4Cl 14.4 g (0.27 mol) was portionwise added, then the reaction mixture was stirred at room temperature for 1 h and heated to reflux. After 5 h later, the mixture was separated by filtration at hot and washed with hot EtOH. The filter liquor was concentrated under reduced pressure and the residue was cooled to room temperature. Oxalate 47.7 g (0.53 mol) and 4 N HCl (900 mL) were added after which the mixture was refluxed for additional 15 h. At last the mixture was cooled, separated by filtration and washed with a mixture of water and EtOH to afford a black solid (42.3 g, 33%). M.p.: >300°C. ESI-MS *m/z*: 239.1 $(M+H)^+$. $C_{13}H_9N_3O_2$ (239.07).

*2-Chloro-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**3**)*

To a stirred solution of $POCl_3$ (210 mL) and CH_3CN (420 mL) 42.3 g **2** (0.17 mol) were added at room temperature, and then 3 drops of DMF were added to the mixture. The reaction mixture was heated to 80°C for 3 h. The mixture was concentrated under reduced pressure. The residue was poured into ice water (1000 mL), separated by filtration to give off a grey solid (34.2 g, 71.6%). M.p.: 180–181°C. ESI-MS *m/z*: 257.1, 259.1 $(M+H)^+$. $C_{13}H_8ClN_3O$ (257.68).

*2-Hydrazinyl-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**4**)*

A solution of 80% hydrazine hydrate ($NH_2NH_2 \cdot H_2O$) 170 mL (4.9 mol) and 34.2 g **3** (0.13 mol) was stirred at 60°C for 1 h. The mixture was cooled, separated by filtration and washed with EtOH to give off a light yellow crystal (34.9 g, 75%). M.p.: 210–211°C. ESI-MS *m/z*: 254.1 $(M+H)^+$. $C_{13}H_{11}N_5O$ (253.26).

*General procedure for preparation of 4-phenyl-(2-arylmethylenehydrazinyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**5a–5h**)*

To a stirred solution of aromatic aldehydes (1.8 mmol) in anhydrous ethanol 0.3 g (1.2 mmol) was added **4** at room temperature. The mixture was heated to 60°C for 5 h. The mixture was cooled, separated by filtration and washed with EtOH to give off a light yellow crystal and purified by chromatography on silica gel using MeOH/CH_2Cl_2 to obtain **5a–5h** as light yellow crystals.

*4-Phenyl-2-(2-(2,3,4-trihydroxybenzylidene)hydrazinyl)-pyrido[2,3-b]pyrazin-3(4H)-one (**5a**)*

Yield: 56%. M.p.: 207–208°C. ESI-MS *m/z*: 390.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 13.13 (s, 1H), 11.85 (s, 1H), 9.75 (s, 1H), 8.15 (m, 2H), 7.95 (m, 3H), 7.56 (d, J = 7.7 Hz, 2H), 7.41–7.36 (m, 3H), 7.36–7.32 (m, 2H), 7.26 (m, 2H); anal. calcd. for $C_{20}H_{15}N_5O_4$ (%): C 61.69, H 3.88, N 17.99, O 16.44; found C 62.02, H 4.63, O 16.38. $C_{20}H_{15}N_5O_4$ (389.36).

*2-(2-(3-Allyl-2-hydroxybenzylidene)hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5b**)*

Yield: 53%. M.p.:169–170°C. ESI-MS *m/z*: 398.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.14 (s, 1H), 11.86 (s, 1H), 8.78 (s, 1H), 8.12 (d, J = 4.2 Hz, 1H), 7.95 (d, J = 7.7 Hz, 1H), 7.59 (t, J = 7.7 Hz, 2H), 7.47 (t, J = 7.3 Hz, 1H), 7.41 (d, J = 7.1 Hz, 2H), 7.31 (dd, J = 7.7, 4.7 Hz, 1H), 7.25 (d, J = 7.5 Hz, 1H), 7.20 (d, J = 7.2 Hz, 1H), 6.90 (t, J = 7.5 Hz, 1H), 6.04 (m, 1H), 5.08 (t, J = 13.6 Hz, 2H), 3.44 (d, J = 6.4 Hz, 2H); anal. calcd. for $C_{23}H_{19}N_5O_2$ (%): C 69.51, H 4.82, N 17.62, O 8.05; found C 69.32, H 4.62, O 17.73. $C_{23}H_{19}N_5O_2$ (397.43).

*2-(2-(3-Allyl-2-hydroxy-5-methylbenzylidene)hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5c**)*
Yield: 46%. M.p.: 185–186°C. ESI-MS *m/z*: 442.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.99 (s, 1H), 11.94 (s, 1H), 8.73 (s, 1H), 8.12 (s, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.57 (t, J = 7.7 Hz, 2H), 7.47 (t, J = 7.3 Hz, 1H), 7.41 (d, J = 7.1 Hz, 2H), 7.33 (dd, J = 7.9, 4.6 Hz, 1H), 7.05 (s, 1H), 7.02 (s, 1H), 6.03 (m, J = 17.0, 10.2 Hz, 1H), 5.08 (t, J = 12.3 Hz, 2H), 3.41 (d, J = 6.6 Hz, 2H), 2.25 (s, 3H); anal. calcd. for $C_{24}H_{21}N_5O_2$ (%): C 70.06, H 5.14, N 17.02, O 7.78; found C 69.98, H 5.23, O 7.89. $C_{24}H_{21}N_5O_2$ (411.46).

*2-(2-(3-Allyl-2-hydroxy-5-isopropylbenzylidene)-hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5d**)*
Yield: 54%. M.p.: 192–193°C. ESI-MS *m/z*: 440.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.99 (s, 1H), 11.90 (s, 1H), 8.68 (s, 1H), 8.05 (dd, J = 4.6, 1.5 Hz, 1H), 7.89 (dd, J = 7.9, 1.4 Hz, 1H), 7.51 (t, J = 7.4 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.2 Hz, 2H), 7.24 (dd, J = 7.9, 4.7 Hz, 1H), 7.01 (s, 2H), 5.98 (m, 1H), 5.03 (m, 2H), 3.36 (d, J = 6.5 Hz, 2H), 2.79 (m, 1H), 1.14 (s, 3H), 1.12 (s, 3H); anal. calcd. for $C_{26}H_{25}N_5O_2$ (%): C 71.05, H 5.73, N 15.93, O 7.28; found C 71.23, H 5.90, O 7.33. $C_{26}H_{25}N_5O_2$ (439.51).

*2-(2-(3-Allyl-5-tert-butyl-2-hydroxybenzylidene)-hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5e**)*
Yield: 44%. M.p.: 189–190°C. ESI-MS *m/z*: 454.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.02 (s, 1H), 11.89 (s, 1H), 8.69 (s, 1H), 8.05 (dd, J = 4.6, 1.5 Hz, 1H), 7.89 (dd, J = 7.9, 1.5 Hz, 1H), 7.51 (t, J = 7.4 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.2 Hz, 2H), 7.24 (dd, J = 7.9, 4.7 Hz, 1H), 7.18 (d, J = 2.1 Hz, 1H), 7.11 (d, J = 2.3 Hz, 1H), 5.98 (m, J = 16.7, 10.0, 6.6 Hz, 1H), 5.02 (m, 2H), 3.37 (d, J = 6.5 Hz, 2H), 1.22 (s, 9H); anal. calcd. for $C_{27}H_{27}N_5O_2$ (%): C 71.50, H 6.00, N 15.44, O 7.06; found C 71.56, H 6.10, O 7.03. $C_{27}H_{27}N_5O_2$ (453.54).

*2-(2-(2-Hydroxy-3-(2-methylallyl)benzylidene)hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5f**)*
Yield: 46%. M.p.: 185–186°C. ESI-MS *m/z*: 412.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.12 (s, 1H), 11.88 (s, 1H), 8.72 (s, 1H), 8.05 (dd, J = 4.6, 1.6 Hz, 1H), 7.89 (dd, J = 7.9, 1.6 Hz, 1H), 7.51 (t, J = 7.4 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.2 Hz, 2H), 7.24 (dd, J = 7.9, 4.7 Hz, 1H), 7.19 (dd, J = 7.7, 1.4 Hz, 1H), 7.11 (d, J = 7.4 Hz, 1H), 6.85 (t, J = 7.5 Hz, 1H), 4.74 (s, 1H), 4.59 (s, 1H), 3.32 (s, 2H), 1.67 (s, 3H); anal. calcd. for $C_{24}H_{21}N_5O_2$ (%): C 70.06, H 5.14, N 17.02, O 7.78; found C 70.15, H 5.14, O 7.67. $C_{24}H_{21}N_5O_2$ (411.46).

*2-(2-(3,5-Di-tert-butyl-2-hydroxybenzylidene)hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5g**)*
Yield: 57%. M.p.: 174–175°C. ESI-MS *m/z*: 470.23 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.32 (s, 1H), 11.82 (s, 1H), 8.70 (s, 1H), 8.05 (dd, J = 4.6, 1.2 Hz, 1H), 7.90 (dd, J = 7.8, 1.2 Hz, 1H), 7.51 (t, J = 7.5 Hz, 2H), 7.44 (t, J = 7.3 Hz, 1H), 7.35 (d, J = 7.4 Hz, 2H), 7.24 (m, 2H), 7.08 (d, J = 2.0 Hz, 1H), 1.39 (s, 9H), 1.20 (s, 9H); anal. calcd. for $C_{28}H_{31}N_5O_2$ (%): C 71.62, H 6.65, N 14.91, O 6.81; found C 72.45, H 7.36, O 7.29. $C_{28}H_{31}N_5O_2$ (469.58).

*2-(2-(4-(4-Chlorobenzyloxy)-2-hydroxybenzylidene)-hydrazinyl)-4-phenylpyrido[2,3-b]pyrazin-3(4H)-one (**5h**)*
Yield: 53%. M.p.: 158–159°C. ESI-MS *m/z*: 498.1, 500.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.51 (s, 1H), 8.96 (s, 1H), 8.06 (d, J = 3.3 Hz, 1H), 7.93 (m, 2H), 7.63–7.45 (m, 10H), 7.39 (d, J = 7.1 Hz, 2H), 7.27 (m, J = 7.8, 4.7 Hz, 1H), 6.87 (d, J = 1.9 Hz, 1H), 6.78 (d, J = 8.7 Hz, 1H); anal. calcd. for $C_{27}H_{20}ClN_5O_3$ (%): C 65.13, H 4.05, Cl 7.12, N 14.06, O 9.64; found C 65.08, H 4.09, O 9.58. $C_{27}H_{20}ClN_5O_3$ (497.93).

*2-(4-Methoxyphenyl)-3-nitropyridine (**6**)*
It was prepared in a similar procedure as described for **1**, from 2-chloro-3-nitropyridine 79 g (0.5 mol) and 4-methoxyaniline 92.2 g (0.75 mol) to give off a dark brown solid (78 g, 64.3%). M.p.: 147–148°C. ESI-MS *m/z*: 216.0 $(M+H)^+$. $C_{15}H_{10}O_2$ (215.21).

*1,4-Dihydro-4-(4-methoxyphenyl)pyrido[2,3-b]-pyrazine-2,3-dione (**7**)*
It was prepared in a similar procedure as described for **2**, from 78 g **6** (0.32 mol) to give off a black crystal (30.2 g, 35%). M.p.: >300°C. ESI-MS *m/z*: 270.1 $(M+H)^+$. $C_{14}H_{11}N_3O_3$ (269.26).

*2-Chloro-4-(4-methoxyphenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**8**)*
It was prepared in a similar procedure as described for **4**, from 30 g **7** (0.11 mol) to give off a gray solid (24.0 g, 76%). M.p.: 145–146°C. ESI-MS *m/z*: 288.0, 290.0 $(M+H)^+$. $C_{14}H_{11}N_3O_3$ (287.71).

*2-Hydrazinyl-4-(4-methoxyphenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**9**)*
It was prepared in a similar procedure as described for **5**, from 24.0 g **8** (83.6 mmol) to give off a light yellow solid (14.4 g, 61%). M.p.: 177–178°C. ESI-MS *m/z*: 284.1 $(M+H)^+$. $C_{14}H_{13}N_5O_2$ (283.29).

*General procedure for preparation of compound (**10a–10h**)*
They were prepared in a similar procedure as described for **5a–5h**.

*2-(2-(2-Hydroxybenzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10a**)*
Yield: 64%. M.p.: 173–175°C. ESI-MS *m/z*: 388.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.93 (s, 1H), 11.58 (s, 1H),

8.72 (s, 1H), 8.12 (s, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.35 (t, J = 6.8 Hz, 1H), 7.25–7.29 (m, 4H), 7.09 (d, J = 8.9 Hz, 2H), 7.07 (d, J = 8.0 Hz, 1H), 6.95 (d, J = 6.8 Hz, 1H), 3.85 (s, 3H); anal. calcd. for $C_{21}H_{17}N_5O_3$ (%): C 65.11, H 4.42, N 18.08, O 12.39; found C 65.06, H 4.46, O 12.47. $C_{21}H_{17}N_5O_3$ (387.39).

*2-(2-(4-Chloro-2-hydroxybenzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10b**)*
Yield: 61%. M.p.: 177–179°C. ESI-MS m/z: 422.1, 424.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.98 (s, 1H), 11.92 (s, 1H), 8.75 (s, 1H), 8.11 (d, J = 4.6 Hz, 1H), 7.93 (d, J = 7.3 Hz, 1H), 7.56 (d, J = 8.2 Hz, 1H), 7.32–7.29 (m, 3H), 7.10 (d, J = 8.8 Hz, 2H), 7.05 (s, 1H), 6.99 (d, J = 8.0 Hz, 1H), 3.84 (s, 3H); anal. calcd. for $C_{21}H_{16}ClN_5O_3$ (%): C 59.79, H 3.82, Cl 8.40, N 16.60, O 11.38; found C 59.83, H 3.89, O 11.37. $C_{21}H_{16}ClN_5O_3$ (421.84).

*2-(2-(3-Allyl-2-hydroxybenzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10c**)*
Yield: 53%. M.p.: 177–179°C. ESI-MS m/z: 428.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.10 (s, 1H), 11.79 (s, 1H), 8.81 (s, 1H), 8.14 (dd, J = 4.2, 1.4 Hz, 1H), 7.96 (dd, J = 7.7, 1.4 Hz, 1H), 7.32 (dd, J = 7.7, 4.7 Hz, 1H), 7.28 (m, 3H), 7.22 (d, J = 7.2 Hz, 1H), 7.11 (d, J = 9.0 Hz, 2H), 6.92 (t, J = 7.7 Hz, 1H), 6.06 (m, 1H), 5.09 (t, J = 13.6 Hz, 2H), 3.83 (s, 3H), 3.38 (d, J = 6.9 Hz, 2H); anal. calcd. for $C_{24}H_{21}N_5O_3$ (%): C 67.44, H 4.95, N 16.38, O 11.23; found C 67.40, H 4.99, O 11.29. $C_{24}H_{21}N_5O_3$ (427.46).

*2-(2-(3-Allyl-2-hydroxy-5-methylbenzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10d**)*
Yield: 53%. M.p.: 191–192°C. ESI-MS m/z: 442.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.15 (s, 1H), 11.83 (s, 1H), 8.72 (s, 1H), 8.12 (d, J = 4.7 Hz, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.34 (dd, J = 7.9, 4.7 Hz, 1H), 7.30 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.9 Hz, 2H), 7.04 (s, 1H), 7.01 (s, 1H), 6.02 (m, 1H), 5.10 (t, J = 12.4 Hz, 2H), 3.83 (s, 3H), 3.39 (d, J = 6.7 Hz, 2H), 2.24 (s, 3H); anal. calcd. for $C_{25}H_{23}N_5O_3$ (%): C 68.01, H 5.25, N 15.86, O 10.87; found C 67.94, H 5.26, O 10.92. $C_{25}H_{23}N_5O_3$ (441.48).

*2-(2-(3-Allyl-5-tert-butyl-2-hydroxybenzylidene)-hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10e**)*
Yield: 44%. M.p.: 168–169°C. ESI-MS m/z: 484.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.90 (s, 1H), 11.66 (s, 1H), 8.77 (s, 1H), 8.12 (d, J = 4.6 Hz, 1H), 7.90 (dd, J = 7.9, 1.5 Hz, 1H), 7.31 (m, J = 8.7 Hz, 3H), 7.10 (d, J = 8.9 Hz, 2H), 6.92 (s, 1H), 6.90 (s, 1H), 6.05 (dd, J = 17.0, 10.1 Hz, 1H), 5.07 (t, J = 12.4 Hz, 2H), 3.82 (s, 3H), 3.45 (d, J = 6.7 Hz, 2H), 1.29 (s, 9H); anal. calcd. for $C_{28}H_{29}N_5O_3$ (%): C 69.55, H 6.04, N 14.48, O 9.93; found C 69.42, H 6.11, O 10.02. $C_{28}H_{29}N_5O_3$ (483.56).

*2-(2-(2-Hydroxy-3-(2-methylallyl)benzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10f**)*
Yield: 62%. M.p.: 80–81°C. ESI-MS m/z: 442.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.13 (s, 1H), 11.92 (s, 1H), 8.76 (s, 1H), 8.07 (dd, J = 4.6, 1.5 Hz, 1H), 7.96 (dd, J = 7.9, 1.5 Hz, 1H), 7.34–7.28 (m, 3H), 7.27 (d, J = 7.9 Hz, 1H), 7.19 (d, J = 7.5 Hz, 1H), 7.10 (d, J = 8.9 Hz, 2H), 6.88 (t, J = 7.5 Hz, 1H), 4.76 (s, 1H), 4.63 (s, 1H), 3.83 (s, 3H), 3.37 (s, 2H), 1.70 (s, 3H); anal. calcd. for $C_{25}H_{23}N_5O_3$ (%): C 68.01, H 5.25, N 15.86, O 10.87; found C 68.06, H 5.28, O 10.84. $C_{25}H_{23}N_5O_3$ (441.48).

*2-(2-(2,4-Dihydroxybenzylidene)hydrazinyl)-4-(4-methoxyphenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10g**)*
Yield: 52%. M.p.: 177–178°C. ESI-MS m/z: 404.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.90 (s, 1H), 11.76 (s, 1H), 10.12 (s, 1H), 8.71(s, 1H), 8.12 (s, 1H), 7.91 (d, J = 7.2 Hz, 1H), 7.31 (m, 3H), 7.23 (d, J = 8.3 Hz, 1H), 7.10 (d, J = 8.9 Hz, 2H), 6.34 (m, 2H), 3.83 (s, 3H); anal. calcd. for $C_{21}H_{17}N_5O_4$ (%): C 62.53, H 4.25, N 17.36, O 15.86; found C 62.52, H 4.30, O 15.79. $C_{21}H_{17}N_5O_4$ (403.39).

*4-(4-Methoxyphenyl)-2-(2-(2,3,4-trihydroxybenzylidene)-hydrazinyl)pyrido[2,3-b]pyrazin-3(4H)-one (**10h**)*
Yield: 51%. M.p.: 176–178°C. ESI-MS m/z: 420.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 8.62 (s, 1H), 8.14 (s, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 1.6 Hz, 1H), 7.34 (dd, J = 7.9, 4.7 Hz, 1H), 7.28 (d, J = 8.8 Hz, 2H), 7.22 (d, J = 7.2 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 7.04 (d, J = 8.4 Hz, 1H), 3.84 (s, 3H); anal. calcd. for $C_{21}H_{17}N_5O_5$ (%): C 60.14, H 4.09, N 16.70, O 19.07; found C 60.24, H 4.04, O 18.96. $C_{21}H_{17}N_5O_5$ (419.39).

*3-Nitro-N-(4-(trifluoromethoxy)phenyl)pyridin-2-amine (**11**)*
It was prepared in a similar procedure as described for **1**, from 2-chloro-3-nitropyridine, 79 g (0.50 mol), and 4-(trifluoromethoxy)aniline, 133 g (0.75 mol), to give off an orange solid (65.4 g, 43.6%). M.p.: 157–159°C. ESI-MS m/z: 300.3 $(M+H)^+$. $C_{12}H_8F_3N_3O_3$ (299.21).

*1,4-Dihydro-4-(4-(trifluoromethoxy)phenyl)pyrido-[2,3-b]pyrazine-2,3-dione (**12**)*
It was prepared in a similar procedure as described for **2**, from 65 g **11** (0.22 mol) to give off a black crystal (28.4 g, 40%). M.p.: >300°C. ESI-MS m/z: 270.3 $(M+H)^+$. $C_{15}H_9F_3N_2O_3$ (322.24).

*2-Chloro-4-(4-(trifluoromethoxy)phenyl)pyrido-[2,3-b]pyrazin-3(4H)-one (**13**)*
It was prepared in a similar procedure as described for **4**, from 28.4 g **12** (88 mmol) to give off a gray solid (22.4 g, 74.7%). M.p.: 157–158°C. ESI-MS m/z: 340.8, 342.8 $(M+H)^+$. $C_{15}H_8ClF_3N_2O_2$ (340.68).

*2-Hydrazinyl-4-(4-(trifluoromethoxy)phenyl)pyrido-[2,3-b]pyrazin-3(4H)-one (**14**)*

It was prepared in a similar procedure as described for **5**, from 22.4 g **13** (66 mmol) to give off a light yellow solid (13.2 g, 59%). M.p.: 185–186°C. ESI-MS *m/z*: 337.3 $(M+H)^+$. $C_{15}H_{11}F_3N_4O_2$ (336.27).

*General procedure for preparation of compound (**15a–15h**)*

They were prepared in a similar procedure as described for **5a–5h**.

*2-(2-(3-Allyl-5-tert-butyl-2-hydroxybenzylidene)-hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)-pyrido[2,3-b]pyrazin-3 (4H)-one (**15a**)*

Yield: 64%. M.p.: 180–181°C. ESI-MS *m/z*: 538.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.96 (s, 1H), 11.29 (s, 1H), 8.79 (s, 1H), 8.14 (dd, $J = 4.6$, 1.5 Hz, 1H), 7.95 (d, $J = 7.6$ Hz, 1H), 7.57 (s, 4H), 7.36 (d, $J = 8.0$ Hz, 1H), 6.92 (s, 1H), 6.90 (s, 1H), 6.05 (m, 1H), 5.07 (t, $J = 12.4$ Hz, 2H), 3.45 (d, $J = 6.7$ Hz, 2H), 1.29 (s, 9H); anal. calcd. for $C_{28}H_{26}F_3N_5O_3$ (%): C 62.56, H 4.88, F 10.60, N 13.03, O 8.93; found C 62.60, H 4.82, O 9.04. $C_{28}H_{26}F_3N_5O_3$ (537.53).

*2-(2-(3-Allyl-2-hydroxybenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15b**)*

Yield: 61%. M.p.: 192–193°C. ESI-MS *m/z*: 482.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.96 (s, 1H), 11.83 (s, 1H), 8.80 (s, 1H), 8.15 (dd, $J = 4.2$, 1.3 Hz, 1H), 7.97 (dd, $J = 7.7$, 1.2 Hz, 1H), 7.61 (s, 4H), 7.33 (dd, $J = 7.8$, 4.7 Hz, 1H), 7.27 (d, $J = 7.5$ Hz, 1H), 7.22 (d, $J = 7.2$ Hz, 1H), 6.92 (t, $J = 7.5$ Hz, 1H), 6.06 (m, $J = 16.9$, 6.6, 3.4 Hz, 1H), 5.10 (t, $J = 13.6$ Hz, 2H), 3.46 (d, $J = 6.4$ Hz, 2H); anal. calcd. for $C_{24}H_{18}F_3N_5O_3$ (%): C 59.88, H 3.77, F 11.84, N 14.55, O 9.97; found C 60.09, H 3.84, O 9.96. $C_{24}H_{18}F_3N_5O_3$ (481.43).

*2-(2-(2-Hydroxy-3-(2-methylallyl)benzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]pyrazin-3(4H)-one (**15c**)*

Yield: 55%. M.p.: 170–171°C. ESI-MS *m/z*: 496.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.14 (s, 1H), 11.97 (s, 1H), 8.79 (s, 1H), 8.12 (dd, $J = 7.9$, 1.5 Hz, 1H), 7.96 (dd, $J = 7.9$, 1.5 Hz, 1H), 7.59 (s, 4H), 7.31 (dd, $J = 7.9$, 4.7 Hz, 1H), 7.27 (d, $J = 7.9$ Hz, 1H), 7.19 (d, $J = 7.5$ Hz, 1H), 6.92 (t, $J = 7.5$ Hz, 1H), 4.80 (s, 1H), 4.66 (s, 1H), 3.39 (s, 2H), 1.73 (s, 3H); anal. calcd. for $C_{25}H_{20}F_3N_5O_3$ (%): C 60.60, H 4.07, F 11.50, N 14.14, O 9.97; found C 60.71, H 4.12, O 9.84. $C_{25}H_{20}F_3N_5O_3$ (495.45).

*2-(2-(4-Chloro-2-hydroxybenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15d**)*

Yield: 57%. M.p.: 167–169°C. ESI-MS *m/z*: 476.1, 478.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.94 (br, 2H), 8.79 (s, 1H), 8.11 (s, 1H), 7.93 (d, $J = 7.3$ Hz, 1H), 7.57–7.52 (m, 5H), 7.31 (s, 1H), 7.05 (s, 1H), 6.99 (d, $J = 8.0$ Hz, 1H); anal. calcd. for $C_{21}H_{13}ClF_3N_5O_3$ (%): C 53.01, H 2.75, Cl 7.45, F 11.98, N 14.72, O, 10.09; found C 52.83, H 2.84, O 10.26. $C_{21}H_{13}ClF_3N_5O_3$ (475.81).

*2-(2-(3-Allyl-2-hydroxy-5-methylbenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15e**)*

Yield: 64%. M.p.: 167–169°C. ESI-MS *m/z*: 496.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.88 (s, 1H), 11.72 (s, 1H), 8.75 (s, 1H), 8.14 (d, $J = 4.6$ Hz, 1H), 7.97 (d, $J = 8.0$ Hz, 1H), 7.59 (s, 4H), 7.36 (d, $J = 8.0$ Hz, 1H), 7.06 (s, 1H), 7.03 (s, 1H), 6.04 (m, 1H), 5.12 (t, $J = 12.6$ Hz, 2H), 3.85 (s, 3H), 3.41 (d, $J = 6.8$ Hz, 2H), 2.26 (s, 3H); anal. calcd. for $C_{25}H_{20}F_3N_5O_3$ (%): C 60.60, H 4.07, F 11.50, N 14.14, O 9.97; found C 62.32, H 4.40, O 10.59. $C_{25}H_{20}F_3N_5O_3$ (495.45).

*2-(2-(3,5-Di-tert-butyl-2-hydroxybenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15f**)*

Yield: 60%. M.p.: 144–145°C. ESI-MS *m/z*: 534.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 12.42 (s, 1H), 11.93 (s, 1H), 8.73 (s, 1H), 8.08 (dd, $J = 4.6$, 1.2 Hz, 1H), 7.93 (dd, $J = 7.9$, 1.2 Hz, 1H), 7.58 (s, 4H), 7.46 (t, $J = 7.3$ Hz, 1H), 7.26 (s, 1H), 7.13 (s, 1H), 1.40 (s, 9H), 1.22 (s, 9H); anal. calcd. for $C_{29}H_{30}F_3N_5O_3$ (%): C 62.92, H 5.46, F 10.30, N 12.65, O 8.67; found C 62.85, H 5.42, O 8.74. $C_{29}H_{30}F_3N_5O_3$ (553.58).

*2-(2-(2,4-Dihydroxybenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15g**)*

Yield: 63%. M.p.: 158–160°C. ESI-MS *m/z*: 458.1 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.88 (s, 1H), 11.72 (s, 1H), 9.99 (s, 1H), 8.66 (s, 1H), 8.08 (s, 1H), 7.88 (d, $J = 7.2$ Hz, 1H), 7.57 (s, 4H), 7.29 (s, 1H), 7.24 (d, $J = 8.3$ Hz, 1H), 6.47–6.16 (m, 2H); anal. calcd. for $C_{21}H_{14}F_3N_5O_4$ (%): C 55.15, H 3.09, F 12.46, N 15.31, O 13.99; found C 55.20, H 3.40, O 14.03. $C_{21}H_{14}F_3N_5O_4$ (457.36).

*2-(2-(4-(Benzyloxy)-2-hydroxybenzylidene)hydrazinyl)-4-(4-(trifluoromethoxy)phenyl)pyrido[2,3-b]-pyrazin-3(4H)-one (**15h**)*

Yield: 64%. M.p.: 180–181°C. ESI-MS *m/z*: 548.2 $(M+H)^+$; ^{1}H-NMR (400 MHz, DMSO) δ: 11.92 (s, 1H), 11.19 (s, 1H), 8.75 (s, 1H), 8.12 (d, $J = 4.7$ Hz, 1H), 7.93 (d, $J = 7.9$ Hz,

1H), 7.58 (s, 4H), 7.46 (d, $J = 7.1$ Hz, 2H), 7.40 (t, $J = 7.4$ Hz, 2H), 7.35–7.30 (m, 2H), 7.15 (d, $J = 3.0$ Hz, 1H), 7.01 (d, $J = 8.9$ Hz, 1H), 6.89 (d, $J = 8.9$ Hz, 1H), 5.08 (s, 2H); anal. calcd. for $C_{28}H_{20}F_3N_5O_4$ (%): C 61.43, H 3.68, F 10.41, N 12.79, O 11.69; found C 62.05, H 3.46, O 11.53. $C_{28}H_{20}F_3N_5O_4$ (547.48).

Cytotoxicity assay *in vitro*

The cytotoxic activities of compounds **5a–5h**, **10a–10h** and **15a–15h** were evaluated with A549 (human lung adenocarcinoma epithelial cell line), MDA-MB-231 (human breast cancer cell line) and HT-29 (human colon adenocarcinoma cell line) by the standard MTT assay *in vitro* [16], with PAC-1 as the positive control. The cancer cell lines were cultured in minimum essential medium (MEM) supplement with 10% fetal bovine serum (FBS).

Approximately 4×10^3 cells, suspended in MEM medium, were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37°C for 24 h. The test compounds at indicated final concentrations were added to the culture medium and the cell cultures were continued for 72 h. Fresh MTT was added to each well at a terminal concentration of 5 μg/mL and incubated with cells at 37°C for 4 h. The formazan crystals were dissolved in 100 μL DMSO per well, and the absorbency at 492 nm (for absorbance of MTT formazan) and 630 nm (for the reference wavelength) was measured with the ELISA reader. All of the compounds were tested twice in each of the cell lines. The results expressed as IC_{50} (inhibitory concentration 50%) were the averages of two determinations and calculated by using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software.

Conclusion

In summary, we have designed and synthesized twenty-four 2-hydrazonylpyrido[2,3-*b*]pyrazin-3(4*H*)-one derivatives, and evaluated their cytotoxic activities against three cancer cell lines (A549, MDA-MB-231 and HT-29). Some compounds displayed potential inhibition in the low micromolar or submicromolar range. The preliminary SARs showed that introduction of 4-methoxylphenyl or 4-(trifluoromethoxy)phenyl group into the N-4 position resulted in more antitumor potency than unsubstituted phenyl group. Furthermore, substituents, especially hydroxyl, on the 4′-position of 2′-hydroxybenzylidene led to the greatly enhanced activity. Significantly, bulky groups on 3'-position or 5'-position resulted in a decrease in cytotoxicity, dramatically even vanished.

This work was supported by a grant from the National S & T Major Project (No.2009ZX09301-012).

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