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Synthesis and evaluation of novel 7-azaindazolyl-indolyl-maleimide derivatives as antitumor agents and protein kinase C inhibitors

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ABSTRACT

A series of novel 7-azaindazolyl-indolyl-maleimides were synthesized and evaluated for their antiproliferative activity in vitro against various human cancer cell lines and protein kinase C inhibitory activity. Compounds **8a–c, 8e** and **14a** were the most promising compounds against K562, A549, ECA-109, KB and SMMC-7721 cell lines in vitro. Compounds **9a**–**j** showed moderate PKC inhibition. Further mechanism of action studies revealed that the antiproliferative activity of compound **8b** in KB cells might involve the mitochondria-mediated apoptosis pathway.

1. Introduction

Cancer is the second leading cause of death in the world. Currently, there are many different targets in cancer chemotherapy. Among them, protein kinase C (PKC) is a suitable target in anticancer drug design because its function is altered in some neoplasias, and this dysfunction is related to uncontrolled proliferation. PKC inhibitors such as UCN-01, CGP 41251 and Enzastaurin are in clinical trials for their potential role in cancer therapy (Fig. 1).[1–6] Bisindolylmaleimides (e.g., Ro 31-6233), were reported to exhibit remarkable PKC inhibition.[7–10] We reasoned that by introducing nitrogen atoms into the indole ring, one might be able to enhance the binding of the compounds to PKC. On the other hand, SAR studies on bisindolylmaleimides indicated that attaching hydrophilic substituent(s) on the appropriate position of the compounds could enhance their inhibitory potency toward PKC.[11,12] This prompted us to design a series of novel bisindolylmaleimide analogues in an attempt to improve potency against PKC and to find more potent anticancer compounds. In this paper, we described the synthesis of the 7-azaindazolyl-indolyl-maleimides with a hydrophilic chain at N^1-position of the 7-azaindazole ring, the evaluation of these compounds as anticancer agents against various human cancer cell lines in vitro, and their PKC inhibitory potency.

2. Results and discussion

2.1. Chemistry

7-Azaindazolyl-indolyl-maleimides **9a**–**j**, **10** and **14a**–**d** were prepared as shown in Scheme 1. Compound **3** was synthesized following the method described in the literature with minor modification.[13,14] Treatment of **3** with iodine in DMF afforded compound **4**,[15] which was subsequently reacted with ethyl magnesium bromide and tributyltin chloride to generate **5**.[15] Stille coupling of **5** with **6**[16,17] in the presence of $Pd(Ph_3)_2Cl_2$ succeeded to obtain **7**.[15,18] Alkylation of **7** with different alkyl halides resulted in **8a**–**j**.[15] Compounds **9a**–**j** and **10** were prepared by treatment of **8a**–**j** or **7** with NH_4OAc, respectively.[19,20]

Reaction of **7** with methylamine afforded **11**, which was reacted with 5 mol/L KOH in ethanol to generate **12**.[19–21] Compounds **13a–d** were obtained by treating **12** with different aryl amines in the presence of catalytic amount of *p*-methylphenylsulfonic acid in enthanol.[22] Alkylation of **13a–d** with 3-piperidinopropyl chloride afforded **14a–d**.[15]

2.2. Biological activity and molecular modeling

The prepared compounds and reference compound (Ro 31-6233)[10] were tested for their antiproliferative activity against K562, A549, ECA-109, KB and SMMC-7721 cell lines by MTT assay in vitro[23] and PKC inhibitory activity using PepTag® Non-radioactive Protein Kinase C Kit. The results are listed in Table 1.

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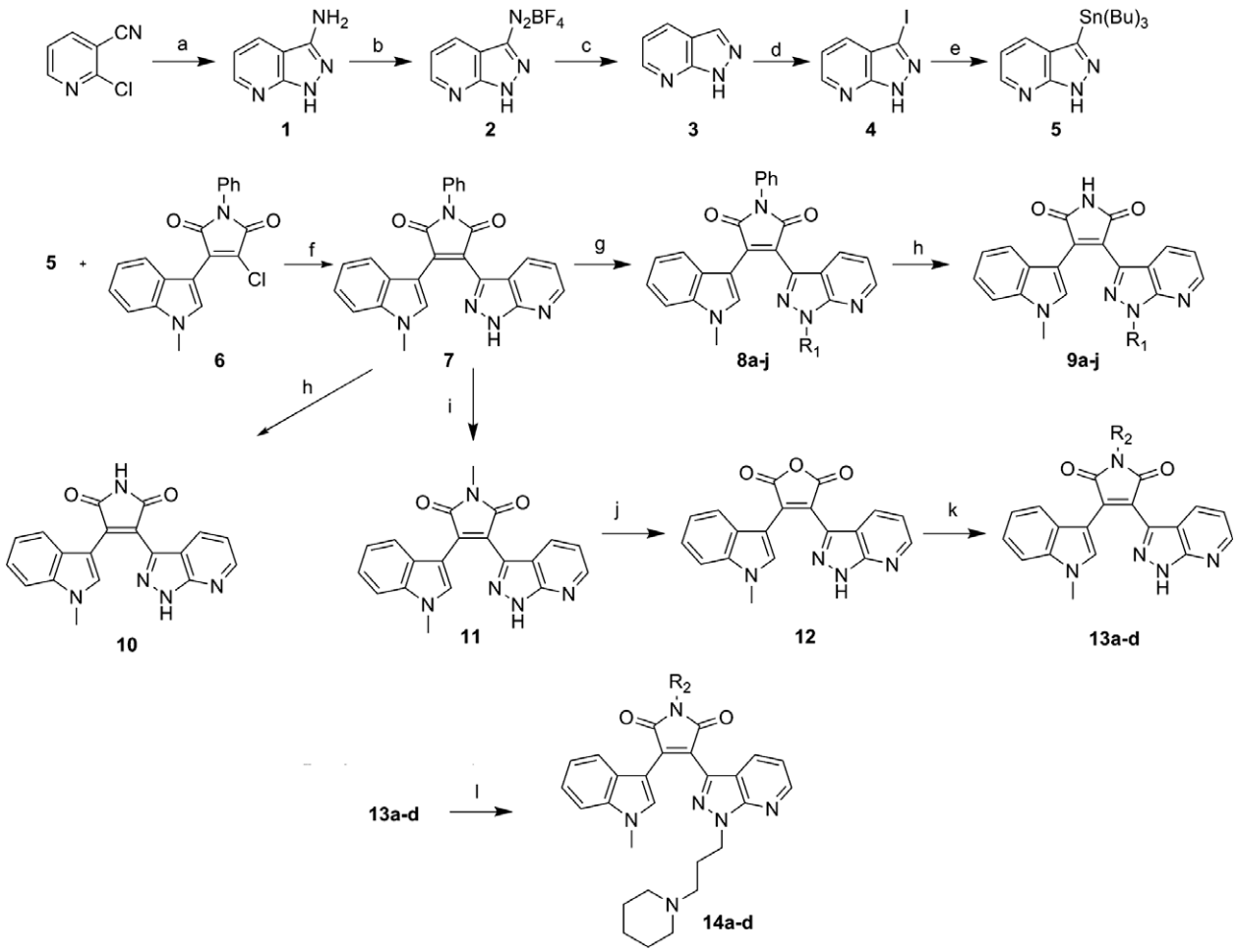


Scheme 1. Synthetic route to compounds **9a–j**, **10** and **14a–d**. Reagents and conditions: (a) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (b) $CHMe_2CH_2CH_2NO_2$, HBF_4, CH_3OH, 0–5 °C; (c) HPO_2, H_2O, rt; (d) I_2, KOH, DMF, rt; (e) EtMgBr, $(Bu)_3SnCl$, THF, −5 to 0 °C; (f) $Pd(Ph_3)_2Cl_2$, LiCl, toluene; (g) alkyl halides, K_2CO_3, DMF, 65–75 °C; (h) NH_4OAc, 140 °C; (i) CH_3NH_2, CH_3OH, rt; (j) 5 mol/L KOH, EtOH, 30–35 °C; (k) aryl amines, PTS, EtOH; (l) 3-piperidinopropylchloride, K_2CO_3, DMF, 65–75 °C.

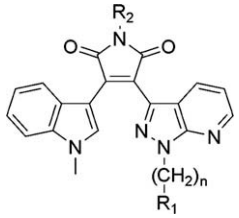
The data in Table 1 indicated that some compounds (i.e., **8a–e**, **9c**, **9e**, **14a**, **14d**) exhibited moderate to higher antiproliferative activity against tested cancer cells than the reference compound. Among them, **8a–e** and **14a** exhibited more potent antiproliferative activity than **9a–j**, suggesting that the presence of an aryl group on the nitrogen of the maleimide ring was essential for antiproliferative activity. Moreover, when the aryl was unsubstituted phenyl or 1-naphthyl, compounds (i.e., **8b**, **14a**) exerted better activity against most tested cancer cells as compared to **14b–c**. The introduction of different hydrophilic side chains at the N^1-position of the 7-azaindazole ring affected antiproliferative activity remarkably. Compared with compound **7**, the activity of **8a–c** (R_1 was dimethylamino, piperidinyl and imidazolyl, respectively) was greatly improved while the activity of **8f** and **8g** (R_1 was Pyrrolyl and 1*H*-1,2,4-triazole-1-yl, respectively) was reduced. Introduction of a 3-(piperidin-1-yl)propyl side chain at N^1-position of 7-azaindazole ring increased the antiproliferative activity markedly as evidenced by comparing compounds **13a** and **14a**. The result indicated that the alkaline-moieties at the end of the chain was important for the activity. The length of alkyl linker also affected the antiproliferative activity significantly. Comparison of the activity of **8c** and **8e** with **8d** showed that compounds with a longer (3 or 4 carbon atoms) alkyl chain were more potent than compounds with shorter alkyl chains. The same conclusion could be drawn from the results of **9c–e**.

Compared with the reference compound, compounds **9a–j** exhibited moderate inhibitory activity toward PKC, while **8a–g** and **14a–d** showed less activity than **9a–j**. This fact indicated that a hydrogen bond donor in the maleimide ring was critical for maintaining PKC inhibitory activity. Comparison of **10** with **9a–j** showed that compounds **9a–j** with hydrophilic chain at 1-position of 7-azaindazole ring possessed more potent inhibition. Compounds **9c** and **9e** were about 10-fold more potent than **9b** in inhibiting PKC. It suggested that the length of the hydrophilic chain attached to N^1-position of the 7-azaindazole ring was a key feature for enhancing inhibitory potency. However, the best inhibitory activity, for the same length of alkyl chain, depended on the hydrophilic group at the end of the chain (i.e., **9a–c**). The result was not consistent with the rank of their antiproliferative activity against human cancer cell lines. This indicated that the tested compounds might have other mechanism against human cancer cell lines besides PKC inhibition.

The results prompted us to study other mechanism of the tested compounds in detail. The antiproliferative mechanism of compound **8b**, a representative compound of this class, was further explored in KB cells.

KB cells were incubated with 10 μM and 20 μM **8b** for 48 h, and the effects of **8b** on the morphology of KB cells were observed with light microscope. As shown in Figure 2A, compound **8b** exhibited great antiproliferatvie activity against KB cells. In addition, the apoptosis of KB cells was determined by Caspase-Glo 3/7 Kit and Electrophoretic Analysis of DNA Fragmentation (Fig. 2B and Fig. 2C).[24] We found that compared with control cells, cells exposed to **8b** slightly increased caspase-3/7 activity, and resulted

Table 1
Antiproliferative and PKC inhibitory activity of the compounds **7**, **8a–g**, **9a–j**, **10**, **13a** and **14a–d**[a]



Compound	R_1	R_2	*n*	IC_{50}[b,c] (μM)					IC_{50}[d] (μM)
				K562	A549	ECA-109	KB	SMMC-7721	PKC
Ro 31-6233				17.46	56.61	83.55	53.78	>100	0.35 (0.55[e])
7	H	Ph	0	3.26	>100	>100	>100	>100	>10
8a	Me_2N	Ph	3	1.70	13.01	5.84	14.11	31.2	>10
8b	1-Pipe	Ph	3	3.79	20.55	12.31	5.6	3.05	>10
8c	1-Imid	Ph	3	5.51	1.56	1.44	6.8	34.77	>10
8d	1-Imid	Ph	2	26.43	43.06	8.77	>100	>100	>10
8e	1-Imid	Ph	4	5.03	21.65	7.54	47.30	1.76	>10
8f	1-Pyrr	Ph	3	36.56	>100	>100	>100	>100	>10
8g	Tria	Ph	3	>100	>100	>100	>100	>100	>10
9a	Me_2N	H	3	50.4	>100	>100	>100	>100	0.72
9b	1-Pipe	H	3	5.96	>100	42.34	97.98	7.67	0.65
9c	1-Imid	H	3	13.76	>100	>100	>100	>100	0.90
9d	1-Imid	H	2	>100	>100	>100	>100	>100	9.10
9e	1-Imid	H	4	31.75	2.38	23.48	>100	29.16	0.86
9f	1-Pyrr	H	3	21.56	>100	>100	>100	>100	1.10
9g	Tria	H	3	>100	>100	>100	>100	>100	2.20
9h	1-Mor	H	3	>100	>100	>100	>100	>100	5.95
9i	MePip	H	3	46.73	>100	>100	>100	>100	1.86
9j	OH	H	3	85.78	>100	>100	>100	>100	3.15
10	H	H	0	>100	>100	46.11	62.02	>100	>10
13a	H	1-Nap	0	>100	>100	>100	>100	>100	>10
14a	1-Pipe	1-Nap	3	3.18	2.82	1.83	13.44	0.22	>10
14b	1-Pipe	ChlPh	3	1.80	28.68	>100	>100	>100	>10
14c	1-Pipe	2-Py	3	>100	>100	7.30	>100	>100	>10
14d	1-Pipe	MetPh	3	>100	30.28	3.34	43.95	1.74	>10

[a] Abbreviations for R_1 and R_2: Imid, imidazolyl; Pipe, piperidinyl; Pyrr, pyrrolyl; Tria, 1*H*-1,2,4-triazole-1-yl; Mor, morpholinyl; MePip, 4-methyl-1-piperazinyl; Ph, phenyl; Nap, naphthyl; ChlPh, 4-chlorophenyl; Py, pyridyl; MetPh, 4-methoxyphenyl.
[b] The IC_{50} values represent the concentration which results in a 50% decrease in cell growth after 48 h incubation.
[c,d] The IC_{50} values were the mean values of three repeated experiments.
[e] From the literature.[10]

in a characteristic fragmentation of DNA, a common feature of apoptotic cell death. DNA Fragmentation was later conducted in apoptotic cells, compared with increasing caspase-3/7 activity. These theoretical events may explain that treating with 20 μM **8b**, a severely high dose, could induce KB cells DNA Fragmentation.

In order to approach signal transduction inducing apoptosis, we further detected JNK, Bax, Bcl-2, procaspase 9 and procaspase 3 protein expressions with Western Blotting methods (Fig. 3A). The results suggested that compound **8b** induced an increase of JNK protein expression and an obvious decrease of Bcl-2, procaspase 9 and procaspase 3 protein expressions, but failed to exhibit any effect on Bax protein expression. The increase of Bax/Bcl-2 ratio, which could induce the release of cytochrome *c*,[25] was also observed after treating with **8b** in KB cells (Fig. 3B). Taken together, all results suggested that mitochondria-mediated apoptosis pathway was involved in **8b** induced antiproliferative activity in KB cells.

3. Conclusion

In conclusion, a series of novel 7-azaindazolyl-indolyl-maleimides were prepared. Some compounds showed moderate antiproliferative activity against human cancer cell lines including K562, A549, ECA-109, KB and SMMC-7721. Among them, the *N*-aryl group containing compounds **8a–c, 8e** and **14a** were the most promising compounds against the tested cancer cell lines. Compounds **9a–j** showed moderate PKC inhibition. Representative compound **8b** was used to investigate other antiproliferative mechanism besides PKC inhibition in KB cells. Apoptosis pathway contributed to the antiproliferative activity of compound **8b**. The results provided valuable information for the design of anticancer agents and PKC inhibitors. Future progress on related series will be reported in due course.

4. Experimental

4.1. Chemistry

All reactions were monitored by thin-layer chromatography (TLC). All reagents were obtained from commercial sources and used without further purification unless stated. Et_2O, THF and benzene were distilled from sodium–benzophenone. DMF was distilled from calcium hydride. Melting points were determined with a BÜCHI Melting Point B-450 apparatus (Büchi Labortechnik, Flawil, Switzerland). The ^1H NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ on Bruker Avance DMX 400 at 400 MHz (chemical shifts are expressed as δ values relative to TMS as internal standard). ESI-MS were obtained on an Esquire-LC-00075 mass spec-

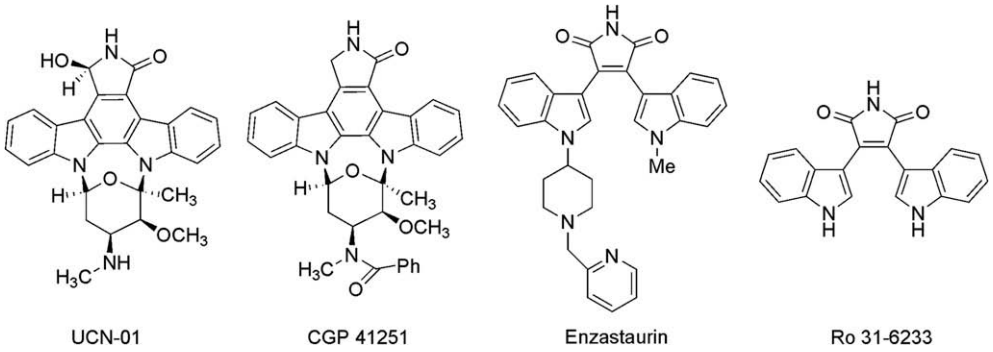


Figure 1. Structure of UCN 01, CGP 41251, Enzastaurin and Ro 31-6233.

trometer (Bruker, USA). Elemental analyses were performed by ERBA-1110 analyzer (Carlo, Italy).

4.2. 3-Iodo-1*H*-pyrazolo[3,4-*b*]pyridine (4)

Iodine (18.7 g, 73.6 mmol) was added to the solution of **3** (3.5 g, 29.4 mmol) in DMF (50 mL), followed by KOH (6.6 g, 118.0 mmol). The reaction mixture was stirred at room temperature for 2 h. After that, it was poured into brine (500 mL), and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with saturated $NaHSO_3$ (50 mL) and brine (3 × 300 mL), dried over Na_2SO_4 and concentrated in vacuum to afford compound **4** (6.3 g, 87.5%) as a white solid, mp: 187–189 °C (Lit.[26] 188–190 °C).

4.3. 3-(Tributylstannyl)-1*H*-pyrazolo[3,4-*b*]pyridine (5)

Under the protection of N_2, compound **4** (8.0 g, 32.6 mmol) was dissolved in THF (200 mL) and the solution was cooled down to 0 °C. Then ethyl magnesium bromide in Et_2O (50 mL, 1.3 M) was added dropwise. The mixture was stirred at 0 °C for 30 min, and then a solution of $(n\text{-Bu})_3SnCl$ (26.5 g, 82.2 mmol) in THF (20 mL) was added dropwise. The mixture was stirred at 0 °C for 1 h then

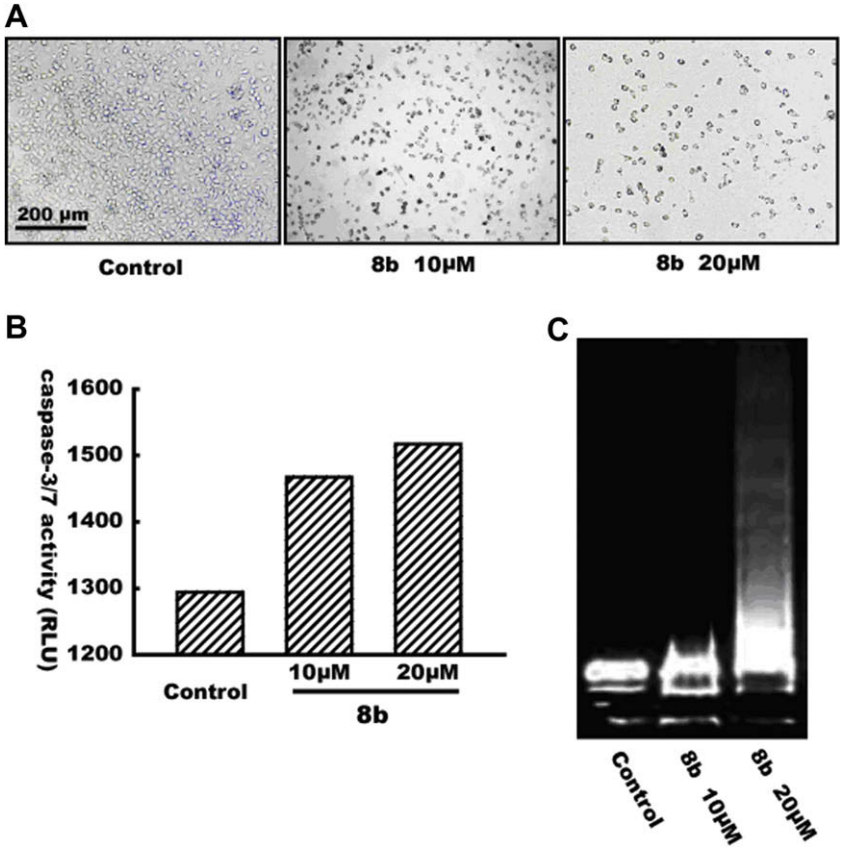


Figure 2. The antiproliferative activity of compound **8b** in KB cells: (A) compound **8b** exhibited great antiproliferatvie activity against KB cells. KB cells were cultured with 10 μM and 20 μM compound **8b**, respectively for 48 h, and then photographed by Leica DMI400B microscope; (B) compound **8b** increased the activity of caspase-3/7. KB cells were cultured with 10 μM and 20 μM compound **8b**, respectively for 48 h, then followed the protocol of caspase-3/7 activity Kit; (C) compound **8b** could obviously induced DNA fragmentation in KB cells. KB cells were cultured with 10 μM and 20 μM compound **8b**, respectively for 48 h, DNA in KB cells were extracted and loaded on agrose gel, after electrophoresis, DNA ladder was photographed by Bio-Rad GD2000.

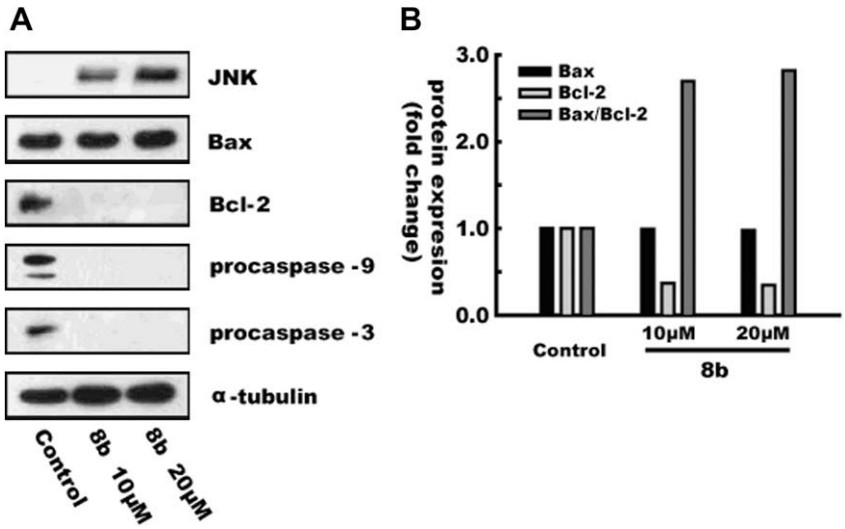


Figure 3. The apoptosis associated protein expression of KB cells treated with various concentrations of **8b**: (A) the protein expression change in KB cells treated with **8b** for 48 h. KB cells were cultured with 10 μM and 20 μM compound **8b**, respectively for 48 h, then were lysed and subjected to Western Blotting using anti-JNK, anti-Bax, anti-Bcl-2, anti-procaspase-9, anti-procaspase-3,and anti-tubulin antibodies; (B) compound **8b** induced the decrease of Bcl-2 protein expression, and, the strong increase of Bax/Bcl-2 ratio in KB cells.

at room temperature for 1 h. After that, it was poured into brine (400 mL), and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with brine (3 × 100 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (6:1, v/v) as eluent to afford compound **5** (8.1 g, 60.9%) as a light yellow liquid, ^{1}H NMR ($CDCl_3$, δ): 0.89 (9H, t, J = 7.2 Hz), 1.23–1.28 (6H, t, J = 7.2 Hz), 1.35–1.39 (6H, m), 1.60–1.64 (6H, m), 7.12–7.15 (1H, m), 8.07 (1H, dd, J = 1.6, 8.0 Hz), 8.62 (1H, dd, J = 1.6, 4.8 Hz), 12.71 (1H, br s). ESI-MS: m/z = 409 $[M+H]^+$. Anal. Calcd for $C_{18}H_{31}N_3Sn$: C, 52.97; H, 7.66; N, 10.29. Found: C, 52.83; H, 7.64; N, 10.43.

4.4. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (7)

Under the protection of N_2, a mixture of compound **5** (2.0 g, 4.9 mmol), LiCl (0.48 g, 11.4 mmol), $Pd(Ph_3)_2Cl_2$ (0.48 g, 0.75 mmol) and compound **6** (1.73 g, 5.14 mmol) in anhydrous toluene (70 mL) was stirred at 100 °C for 10 h. After cooling, the mixture was poured into water (500 mL) and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with brine (3 × 100 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol (30:1, v/v) as eluent to afford compound **7** (1.13 g, 55.0%) as a red solid, mp: >250 °C. ^{1}H NMR (DMSO-d_6, δ): 3.92 (3H, s), 6.33 (1H, d, J = 8.0 Hz), 6.72 (1H, t, J = 8.0 Hz), 7.11 (1H, t, J = 8.0 Hz), 7.18–7.22 (1H, m), 7.42–7.44 (1H, m), 7.49 (1H, d, J = 8.0 Hz), 7.51–7.57 (4H, m), 8.18 (1H, dd, J = 1.6, 8.4 Hz), 8.25 (1H, s), 8.54 (1H, dd, J = 1.6, 4.4 Hz), 14.02 (1H, s). ESI-MS: m/z = 420 $[M+H]^+$. Anal. Calcd for $C_{25}H_{17}N_5O_2$: C, 71.59; H, 4.09; N, 16.70. Found: C, 71.33; H, 4.14; N, 16.79.

4.5. General procedure for the synthesis of 8a–i

A mixture of compound **7** (0.24 mmol), K_2CO_3 (0.48 mmol) and alkyl halides (0.36 mmol) in DMF (10 mL) was stirred at 65–75 °C for 8 h. After cooling, the mixture was poured into water (200 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined, washed with brine (3 × 100 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol/ triethylamine (120:4:1, v/v/v) as eluent to afford **8a–i**.

4.5.1. 3-(1-(3-(Dimethylamino)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-4-(1-methyl-1*H*-indol-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8a)

According to the general method, the reaction of compound **7** with 3-chloro-*N*,*N*-dimethylpropan-1-amine afforded compound **8a** in 75.6% yield as a red solid, mp: 149–151 °C. ^{1}H NMR ($CDCl_3$, δ): 1.84–1.87 (2H, m), 2.14 (6H, s), 2.22 (2H, t, J = 7.2 Hz), 3.89 (3H, s), 4.48 (2H, t, J = 7.2 Hz), 6.33 (1H, d, J = 8.0 Hz), 6.78 (1H, t, J = 8.0 Hz), 7.14–7.16 (2H, m), 7.31(1H, d, J = 8.0 Hz), 7.38–7.40 (1H, m), 7.49–7.53 (4H, m), 8.18 (1H, s), 8.25 (1H, d, J = 8.0 Hz), 8.56 (1H, d, J = 4.4 Hz). ESI-MS: m/z = 505 $[M+H]^+$. Anal. Calcd for $C_{30}H_{28}N_6O_2$: C, 71.41; H, 5.59; N, 16.66. Found: C, 71.23; H, 5.44; N, 16.91.

4.5.2. 3-(1-Methyl-1*H*-indol-3-yl)-1-phenyl-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (8b)

According to the general method, the reaction of compound **7** with 1-(3-chloropropyl)piperidine afforded compound **8b** in 73.6% yield as a red solid, mp: 158–161 °C. ^{1}H NMR ($CDCl_3$, δ): 1.37–1.42 (2H, m), 1.51–1.55 (4H, m), 1.85–1.89 (2H, m), 2.23–2.27 (6H, m), 3.89 (3H, s), 4.45 (2H, t, J = 7.2 Hz), 6.35 (1H, d, J = 8.0 Hz), 6.78 (1H, t, J = 8.0 Hz), 7.11–7.18 (2H, m), 7.31 (1H, d, J = 8.4 Hz), 7.38–7.42 (1H, m), 7.50–7.54 (4H, m), 8.19 (1H, s), 8.25 (1H, dd, J = 1.6, 8.0 Hz), 8.55 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 545 $[M+H]^+$. Anal. Calcd for $C_{33}H_{32}N_6O_2$: C, 72.77; H, 5.92; N, 15.43. Found: C, 72.83; H, 5.96; N, 15.69.

4.5.3. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(1*H*-imidazol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8c)

According to the general method, the reaction of compound **7** with 1-(3-chloropropyl)-1*H*-imidazole afforded compound **8c** in 73.0% yield as a red solid, mp: 94–96 °C. ^{1}H NMR ($CDCl_3$, δ): 2.04–2.07 (2H, m), 3.55 (2H, t, J = 6.8 Hz), 3.88 (3H, s), 4.37 (2H, t, J = 6.4 Hz), 6.33 (1H, d, J = 8.0 Hz), 6.70–6.74 (2H, m), 6.98 (1H,

br s), 7.12 (1H, t, *J* = 8.4 Hz), 7.18–7.22 (1H, m), 7.24 (1H, br s), 7.30 (1H, d, *J* = 8.0 Hz), 7.39–7.41 (1H, m), 7.50–7.54 (4H, m), 8.12 (1H, s), 8.38 (1H, dd, *J* = 1.6, 8.0 Hz), 8.59 (1H, dd, *J* = 1.6, 4.4 Hz). ESI-MS: *m/z* = 528 $[M+H]^+$. Anal. Calcd for $C_{31}H_{25}N_7O_2$: C, 70.57; H, 4.78; N, 18.58. Found: C, 70.33; H, 4.74; N, 18.79.

4.5.4. 3-(1-(2-(1*H*-Imidazol-1-yl)ethyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-4-(1-methyl-1*H*-indol-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8d)

According to the general method, the reaction of compound **7** with 1-(2-chloroethyl)-1*H*-imidazole afforded compound **8d** in 71.2% yield as a red solid, mp: 145–148 °C. ^{1}H NMR ($CDCl_3$, δ): 3.96 (3H, s), 4.55 (2H, t, *J* = 6.8 Hz), 4.89 (2H, t, *J* = 6.8 Hz), 6.32 (1H, d, *J* = 8.0 Hz), 6.73–6.75 (2H, m), 7.02 (1H, br s), 7.09–7.11 (1H, m), 7.16 (1H, t, *J* = 8.4 Hz), 7.36 (1H, d, *J* = 8.0 Hz), 7.41–7.43 (1H, m), 7.50–7.54 (4H, m), 8.15 (1H, dd, *J* = 1.6, 8.0 Hz), 8.18 (1H, s), 8.46 (1H, dd, *J* = 1.6, 4.4 Hz), 8.60 (1H, br s). ESI-MS: *m/z* = 514 $[M+H]^+$. Anal. Calcd for $C_{30}H_{23}N_7O_2$: C, 70.16; H, 4.51; N, 19.09. Found: C, 70.33; H, 4.44; N, 19.19.

4.5.5. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(4-(1*H*-imidazol-1-yl)butyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8e)

According to the general method, the reaction of compound **7** with 1-(4-chlorobutyl)-1*H*-imidazole afforded compound **8e** in 68.0% yield as a red solid, mp: 114–117 °C. ^{1}H NMR ($CDCl_3$, δ): 1.63–1.65 (4H, m), 3.79 (2H, t, *J* = 6.4 Hz), 3.88 (3H, s), 4.44 (2H, t, *J* = 6.4 Hz), 6.27 (1H, d, *J* = 8.0 Hz), 6.70 (1H, t, *J* = 8.0 Hz), 6.79 (1H, br s), 7.01 (1H, br s), 7.14–7.17 (2H, m), 7.32 (1H, d, *J* = 8.0 Hz), 7.39–7.41 (2H, m), 7.50–7.54 (4H, m), 8.16 (1H, s), 8.30 (1H, d, *J* = 8.0 Hz), 8.57 (1H, d, *J* = 4.4 Hz). ESI-MS: *m/z* = 542 $[M+H]^+$. Anal. Calcd for $C_{32}H_{27}N_7O_2$: C, 70.96; H, 5.02; N, 18.10. Found: C, 70.83; H, 5.14; N, 18.29.

4.5.6. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(1*H*-pyrrol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8f)

According to the general method, the reaction of compound **7** with 1-(3-chloropropyl)-1*H*-pyrrole afforded compound **8f** in 77.0% yield as a red solid, mp: 85–88 °C. ^{1}H NMR ($CDCl_3$, δ): 2.09 (2H, m), 3.60 (2H, t, *J* = 7.2 Hz), 3.89 (3H, s), 4.39 (2H, t, *J* = 6.8 Hz), 6.07–6.09 (2H, m), 6.37 (1H, d, *J* = 8.0 Hz), 6.46–6.48 (2H, m), 6.74 (1H, t, *J* = 8.0 Hz), 7.14 (1H, t, *J* = 8.0 Hz), 7.18–7.20 (1H, m), 7.30 (1H, d, *J* = 8.0 Hz), 7.40–7.42 (1H, m), 7.50–7.54 (4H, m), 8.15 (1H, s), 8.35 (1H, dd, *J* = 1.6, 8.0 Hz), 8.58 (1H, dd, *J* = 1.6, 4.4 Hz). ESI-MS: *m/z* = 527 $[M+H]^+$. Anal. Calcd for $C_{32}H_{26}N_6O_2$: C, 72.99; H, 4.98; N, 15.96. Found: C, 72.83; H, 4.94; N, 15.89.

4.5.7. 3-(1-Methyl-1*H*-indol-3-yl)-1-phenyl-4-(1-(3-(1*H*-1,2,4-triazol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (8g)

According to the general method, the reaction of compound **7** with 1-(3-chloropropyl)-1*H*-1,2,4-triazole afforded compound **8g** in 69.4% yield as a red solid, mp: 163–165 °C. ^{1}H NMR ($CDCl_3$, δ): 2.14–2.16 (2H, m), 3.87 (3H, s), 3.99 (2H, t, *J* = 6.8 Hz), 4.38 (2H, t, *J* = 6.4 Hz), 6.39 (1H, d, *J* = 8.0 Hz), 6.74 (1H, t, *J* = 8.0 Hz), 7.06 (1H, t, *J* = 8.0 Hz), 7.26–7.28 (1H, m), 7.42–7.45 (2H, m), 7.53–7.57 (4H, m), 7.97 (1H, s), 8.23–8.26 (2H, m), 8.29 (1H, br s), 8.59 (1H, dd, *J* = 1.6, 4.4 Hz). ESI-MS: *m/z* = 529 $[M+H]^+$. Anal. Calcd for $C_{30}H_{24}N_8O_2$: C, 68.17; H, 4.58; N, 21.20. Found: C, 68.33; H, 4.44; N, 21.06.

4.5.8. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-morpholinopropyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (8h)

According to the general method, the reaction of compound **7** with 4-(3-chloropropyl)morpholine afforded compound **8h** in 68.7% yield as a red solid, mp: 152–154 °C. ^{1}H NMR ($CDCl_3$, δ): 1.88–1.90 (2H, m), 2.27–2.31 (6H, m), 3.62–3.66 (4H, m), 3.91 (3H, s), 4.48 (2H, t, *J* = 7.2 Hz), 6.32 (1H, d, *J* = 8.0 Hz), 6.77 (1H, t, *J* = 8.0 Hz), 7.13–7.17 (2H, m), 7.32 (1H, d, *J* = 8.0 Hz), 7.38–7.40 (1H, m), 7.50–7.54 (4H, m), 8.18 (1H, s), 8.26 (1H, d, *J* = 8.4 Hz), 8.55 (1H, d, *J* = 4.4 Hz). ESI-MS: *m/z* = 547 $[M+H]^+$. Anal. Calcd for $C_{32}H_{30}N_6O_3$: C, 70.31; H, 5.53; N, 15.37. Found: C, 70.33; H, 5.44; N, 15.59.

4.5.9. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(4-methylpiperazin-1-yl)propyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (8i)

According to the general method, the reaction of compound **14** with 1-(3-chloropropyl)-4-methylpiperazine afforded compound **8i** in 70.5% yield as a red solid, mp: 195–198 °C. ^{1}H NMR ($CDCl_3$, δ): 1.82–1.85 (2H, m), 2.15–2.40 (13H, m), 3.90 (3H, s), 4.47 (2H, t, *J* = 7.2 Hz), 6.33 (1H, d, *J* = 8.0 Hz), 6.77 (1H, t, *J* = 8.0 Hz), 7.13–7.16 (2H, m), 7.31 (1H, d, *J* = 7.6 Hz), 7.38–7.39 (1H, m), 7.50–7.52 (4H, m), 8.17 (1H, s), 8.27 (1H, d, *J* = 8.4 Hz), 8.50 (1H, d, *J* = 4.4 Hz). ESI-MS: *m/z* = 560 $[M+H]^+$. Anal. Calcd for $C_{33}H_{33}N_7O_2$: C, 70.82; H, 5.94; N, 17.52. Found: C, 71.03; H, 5.84; N, 17.50.

4.5.10. 3-(1-Methyl-1*H*-indol-3-yl)-1-phenyl-4-(1-(3-(*tert*-butyldimethylsilyloxy)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (8j)

A mixture of compound **7** (100.0 mg, 0.24 mmol), K_2CO_3 (86.2 mg, 0.48 mmol), (3-bromopropoxy) (*tert*-butyl)dimethylsilane (91.1 mg, 0.36 mmol) in DMF (10 mL) was stirred at 60 °C for 2 h. After cooling, the mixture was poured into water (200 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined and washed with brine (3 × 100 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol (60:1, v/v) as eluent to afford **8j** (97.5 mg, 68.6%) as a red solid, mp: 110–113 °C. ^{1}H NMR ($CDCl_3$, δ): 0.01 (6H, s), 0.88 (9H, s), 1.87–1.90 (2H, m), 3.52 (2H, t, *J* = 6.8 Hz), 3.92 (3H, s), 4.54 (2H, t, *J* = 7.2 Hz), 6.39 (1H, d, *J* = 8.0 Hz), 6.79 (1H, t, *J* = 7.6 Hz), 7.16–7.18 (2H, m), 7.33 (1H, d, *J* = 8.0 Hz), 7.41–7.42 (1H, m), 7.51–7.55 (4H, m), 8.21(1H, s), 8.30 (1H, dd, *J* = 1.6, 8.0 Hz), 8.56 (1H, dd, *J* = 1.6, 4.4 Hz). ESI-MS: *m/z* = 592 $[M+H]^+$. Anal. Calcd for $C_{34}H_{37}N_5O_3Si$: C, 69.01; H, 6.30; N, 11.83. Found: c, 69.23; H, 6.17; N, 11.72.

4.6. General procedure for the synthesis of 9a–j and 10

Under the protection of N_2, compound **8a–j** or **7** (0.057 mmol) was heated with NH_4OAc (57.0 mmol) for 6 h at 140 °C. The mixture was cooled, poured into water (20 mL), adjusted to weak alkalinity with Na_2CO_3 and extracted with ethyl acetate (3 × 20 mL). The organic layer was washed with brine (3 × 50 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol/ triethylamine (120:4:1, v/v/v) as eluent to afford **9a–j** and **10**.

4.6.1. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(dimethylamino)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9a)

According to the general method, the reaction of compound **8a** with NH_4OAc afforded compound **9a** in 90.2% yield as a red solid, mp: 192–194 °C. ^{1}H NMR ($CDCl_3$, δ): 2.04–2.06 (2H, m), 2.30 (6H, s), 2.49 (2H, t, *J* = 7.6 Hz), 3.87 (3H, s), 4.52 (2H, t, *J* = 6.8 Hz), 6.28 (1H, d, *J* = 8.4 Hz), 6.75 (1H, t, *J* = 8.0 Hz), 7.14–7.16 (2H, m), 7.30 (1H, d, *J* = 8.0 Hz), 8.11 (1H, s), 8.14 (1H, dd, *J* = 1.6, 8.0 Hz), 8.55 (1H, dd, *J* = 1.6, 4.0 Hz). ESI-MS: *m/z* = 429 $[M+H]^+$. Anal. Calcd for

$C_{24}H_{24}N_6O_2$: C, 67.27; H, 5.65; N, 19.61. Found: C, 67.36; H, 5.60; N, 19.73.

4.6.2. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9b)

According to the general method, the reaction of compound **8b** with NH_4OAc afforded compound **9b** in 89.4% yield as a red solid, mp: 108–111 °C. ^{1}H NMR ($CDCl_3$, δ): 1.37–1.39 (2H, m), 1.52–1.56 (4H, m), 1.90–1.93 (2H, m), 2.24–2.30 (6H, m), 3.86 (3H, s), 4.46 (2H, t, J = 7.2 Hz), 6.33 (1H, d, J = 8.0 Hz), 6.76 (1H, t, J = 8.0 Hz), 7.12 (2H, m), 7.28 (1H, d, J = 8.0 Hz), 8.11 (1H, s), 8.15 (1H, dd, J =1.6, 8.4 Hz), 8.53 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 469 $[M+H]^+$. Anal. Calcd for $C_{27}H_{28}N_6O_2$: C, 69.21; H, 6.02; N, 17.94. Found: C, 69.03; H, 5.98; N, 18.03.

4.6.3. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(1*H*-imidazol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9c)

According to the general method, the reaction of compound **8c** with NH_4OAc afforded compound **9c** in 92.2% yield as a red solid, mp: 106–108 °C. 1H NMR ($CDCl_3$, δ): 2.02–2.04 (2H, m), 3.52 (2H, t, J = 6.8 Hz), 3.82 (3H, s), 4.34 (2H, t, J = 6.4 Hz), 6.25 (1H, d, J = 8.0 Hz), 6.66–6.68 (2H, m), 6.98 (1H, br s), 7.06 (1H, t, J = 8.0 Hz), 7.17–7.19 (1H, m), 7.23–7.25 (2H, m), 8.04 (1H, s), 8.29 (1H, dd, J = 1.6, 8.0 Hz), 8.58 (1H, dd, J = 1.6, 4.4 Hz), 8.96 (1H, br s). ESI-MS: m/z = 452 $[M+H]^+$. Anal. Calcd for $C_{25}H_{21}N_7O_2$: C, 66.51; H, 4.69; N, 21.72. Found: C, 66.36; H, 4.75; N, 21.53.

4.6.4. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(2-(1*H*-imidazol-1-yl)ethyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9d)

According to the general method, the reaction of compound **8d** with NH_4OAc afforded compound **9d** in 88.3% yield as a red solid, mp: 231–234 °C. ^{1}H NMR ($CDCl_3$, δ): 3.91 (3H, s), 4.23 (2H, t, J = 6.8 Hz), 4.76 (2H, t, J = 6.8 Hz), 6.29 (1H, d, J = 8.0 Hz), 6.76–6.78 (2H, m), 6.95 (1H, br s), 7.10–7.11 (1H, m), 7.15 (1H, t, J = 8.0 Hz), 7.29 (1H, br s), 7.33 (1H, d, J = 8.0 Hz), 8.07 (1H, s), 8.11–8.13 (2H, m), 8.50 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 438 $[M+H]^+$. Anal. Calcd for $C_{24}H_{19}N_7O_2$: C, 65.89.32; H, 4.38; N, 22.41. Found: C, 65.66; H, 4.32; N, 22.58.

4.6.5. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(4-(1*H*-imidazol-1-yl)butyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9e)

According to the general method, the reaction of compound **8e** with NH_4OAc afforded compound **9e** in 87.0% as a red solid, mp: 93–95 °C. ^{1}H NMR ($CDCl_3$, δ): 1.62–1.66 (4H, m), 3.78 (2H, t, J = 6.4 Hz), 3.88 (3H, s), 4.44 (2H, t, J = 6.4 Hz), 6.21 (1H, d, J = 8.0 Hz), 6.67 (1H, t, J = 7.6 Hz), 6.78 (1H, br s), 7.03 (1H, br s), 7.13–7.15 (2H, m), 7.30 (1H, d, J = 8.4 Hz), 7.37 (1H, br s), 8.10 (1H, s), 8.22 (1H, dd, J = 1.6, 8.0 Hz), 8.30 (1H, br s), 8.57 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 466 $[M+H]^+$. Anal. Calcd for $C_{26}H_{23}N_7O_2$: C, 67.08; H, 4.98; N, 21.06. Found: C, 67.19; H, 5.02; N, 20.93.

4.6.6. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(1*H*-pyrrol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9f)

According to the general method, the reaction of compound **9f** with NH_4OAc afforded compound **9f** in 85.9% yield as a red solid, mp: 195–198 °C. 1H NMR ($CDCl_3$, δ): 2.18–2.20 (2H, m), 3.62 (2H, t, J = 7.2 Hz), 3.89 (3H, s), 4.40 (2H, t, J = 6.8 Hz), 6.07–6.09 (2H, m), 6.30 (1H, d, J = 8.0 Hz), 6.49–6.51 (2H, m), 6.73 (1H, t, J = 8.0 Hz), 7.12 (1H, t, J = 8.0 Hz), 7.20–7.21 (1H, m), 7.30 (1H, d, J = 8.0 Hz), 7.69 (1H, br s), 8.10 (1H, s), 8.27 (1H, dd, J = 1.6, 8.0 Hz), 8.58 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 451 $[M+H]^+$. Anal. Calcd for $C_{26}H_{22}N_6O_2$: C, 69.32; H, 4.92; N, 18.66. Found: C, 69.46; H, 4.82; N, 18.49.

4.6.7. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(1*H*-1,2,4-triazol-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9g)

According to the general method, the reaction of compound **8g** with NH_4OAc afforded compound **9g** in 88.1% yield as a red solid, mp: 237–239 °C. 1H NMR($CDCl_3$ + DMSO-d_6, δ): 2.08–2.10 (2H, m), 3.63 (2H, t, J = 6.8 Hz), 3.77 (3H, s), 4.21 (2H, t, J = 6.4 Hz), 6.22 (1H, d, J = 8.0 Hz), 6.60 (1H, t, J = 8.0 Hz), 6.98 (1H, t, J = 8.0 Hz), 7.07–7.08 (1H, m), 7.18 (1H, d, J = 8.0 Hz), 7.68 (1H, s), 7.78 (1H, s), 7.95 (1H, s), 8.16 (1H, dd, J = 1.6, 8.0 Hz), 8.46 (1H, dd, J = 1.6, 4.4 Hz), 10.1 (1H, br s). ESI-MS: m/z = 453 $[M+H]^+$. Anal. Calcd for $C_{24}H_{20}N_8O_2$: C, 63.71; H, 4.46; N, 24.76. Found: C, 63.91; H, 4.55; N, 24.59.

4.6.8. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-morpholinopropyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9h)

According to the general method, the reaction of compound **8h** with NH_4OAc afforded compound **9h** in 93.6% yield as a red solid, mp: 93–95 °C. 1H NMR ($CDCl_3$, δ): 1.86–1.88 (2H, m), 2.25–2.28 (6H, m), 3.62–3.65 (4H, m), 3.89 (3H, s), 4.49 (2H, t, J = 7.2 Hz), 6.27 (1H, d, J = 8.0 Hz), 6.74 (1H, t, J = 7.6 Hz), 7.12–7.14 (2H, m), 7.30 (1H, d, J = 8.4 Hz), 7.90 (1H, br s), 8.11 (1H, s), 8.16 (1H, dd, J = 1.6, 8.0 Hz), 8.55 (1H, dd, J = 1.6, 4.0 Hz). ESI-MS: m/z = 471 $[M+H]^+$. Anal. Calcd for $C_{26}H_{26}N_6O_3$: C, 66.37; H, 5.57; N, 17.86. Found: C, 66.49; H, 5.51; N, 17.69.

4.6.9. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(4-methylpiperazin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (9i)

According to the general method, the reaction of compound **8i** with NH_4OAc afforded compound **9i** in 91.7% yield as a saffron yellow solid, mp: 102–104 °C. 1H NMR ($CDCl_3$, δ): 1.87–1.89 (2H, m), 2.25–2.60 (13H, m), 3.86 (3H, s), 4.47 (2H, t, J = 7.2 Hz), 6.30 (1H, d, J = 7.6 Hz), 6.74 (1H, t, J = 8.0 Hz), 7.12–7.15 (2H, m), 7.29 (1H, d, J = 8.0 Hz), 8.09 (1H, s), 8.16 (1H, dd, J = 1.6, 8.0 Hz), 8.53 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 484 $[M+H]^+$. Anal. Calcd for $C_{27}H_{29}N_7O_2$: C, 67.06; H, 6.04; N, 20.28. Found: C, 67.26; H, 5.98; N, 20.03.

4.6.10. 3-(1-(3-Hydroxypropyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (9j)

According to the general method, the reaction of compound **8j** with NH_4OAc afforded compound **9j** in 49.0% as a red solid, mp: 119–121 °C. ^{1}H NMR ($CDCl_3$, δ): 1.80–1.83 (2H, m), 3.32–3.35 (2H, m), 3.91 (3H, s), 4.46 (2H, t, J = 6.8 Hz), 4.53 (1H, t, J = 5.2 Hz), 6.28 (1H, d, J = 8.0 Hz), 6.70 (1H, t, J = 8.0 Hz), 7.10 (1H, t, J = 8.4 Hz), 7.18–7.20 (1H, m), 7.46 (1H, d, J = 8.0 Hz), 8.11 (1H, s), 8.22 (1H, dd, J = 1.6, 8.0 Hz), 8.55 (1H, dd, J = 1.6, 4.0 Hz), 11.20 (1H, s). ESI-MS: m/z = 402 $[M+H]^+$. Anal. Calcd for $C_{22}H_{19}N_5O_3$: C, 65.83; H, 4.77; N, 17.45. Found: C, 65.69; H, 4.81; N, 17.69.

4.6.11. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (10)

According to the general method, the reaction of compound **7** with NH_4OAc produced compound **10** in 93.0% yield as a red solid, mp: >250 °C. 1H NMR (DMSO-d_6, δ): 3.89 (3H, s), 6.25 (1H, d, J = 8.0 Hz), 6.68 (1H, t, J = 8.0 Hz), 7.08 (1H, t, J = 8.0 Hz), 7.14–7.17 (1H, m), 7.45 (1H, d, J = 8.4 Hz), 8.08 (1H, d, J = 8.0 Hz), 8.17 (1H, s), 8.52 (1H, d, J = 4.4 Hz), 11.20 (1H, s), 13.94 (1H, s). ESI-MS: m/z = 334 $[M+H]^+$. Anal. Calcd for $C_{19}H_{13}N_5O_2$: C, 66.47; H, 3.82; N, 20.40. Found: C, 66.23; H, 3. 38; N, 20.52.

4.7. 1-Methyl-3-(1-methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-1*H*-pyrrole-2,5-dione (11)

32.0% Methylamine in methanol (16 mL) was added to compound **7** (0.80 g, 1.9 mmol) and the mixture was stirred at room temperature for 2 h. The solvent was removed in vacuum and the residue was purified by flash column chromatography on silica gel using dichloromethane/methanol (30:1, v/v) as eluent to afford compound **11** (0.58 g, 86.0%) as a red solid, mp: >250 °C. ^{1}H NMR (DMSO-d_6 , δ): 3.07 (3H, s), 3.90 (3H, s), 6.29 (1H, d, J = 8.0 Hz), 6.69 (1H, t, J = 7.6 Hz), 7.08 (1H, t, J = 8.0 Hz), 7.13–7.16 (1H, m), 7.46 (1H, d, J = 8.0 Hz), 8.06 (1H, d, J = 8.0 Hz), 8.20 (1H, s), 8.52 (1H, dd, J = 1.2, 4.4 Hz), 13.97 (1H, s). ESI-MS: m/z = 358 $[M+H]^+$. Anal. Calcd for $C_{20}H_{15}N_5O_2$: C, 67.22; H, 4.23; N, 19.60. Found: C, 67.43; H, 4.38; N, 19.52.

4.8. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)furan-2,5-dione (12)

To a solution of compound **11** (0.62 g, 1.7 mmol) in ethanol (50 mL), 5 M aqueous KOH (25 mL) was added. The mixture was stirred at 25–30 °C for 5 h. Water (400 mL) was added to the solution and the mixture was acidified with 2 M HCl and extracted with ethyl acetate (3 × 80 mL). The combined organic layer was washed with brine (3 × 240 mL), dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (8:1, v/v) as eluent to afford compound **12** (0.44 g, 72.6%) as a saffron yellow solid, mp: >250 °C. ^{1}H NMR (DMSO-d_6, δ): 3.93 (3H, s), 6.34 (1H, d, J = 8.0 Hz), 6.77 (1H, t, J = 7.6 Hz), 7.14 (1H, t, J = 8.0 Hz), 7.18–7.19 (1H, m), 7.51 (1H, d, J = 8.0 Hz), 8.15 (1H, dd, J = 1.6, 8.0 Hz), 8.34 (1H, s), 8.56 (1H, dd, J = 1.6, 4.0 Hz), 14.20 (1H, s). ESI-MS: m/z = 345 $[M+H]^+$. Anal. Calcd for $C_{19}H_{12}N_4O_3$: C, 66.28; H, 3.51; N, 16.27. Found: C, 66.43; H, 3.48; N, 16.39.

4.9. General procedure for the synthesis of 13a–d

A mixture of **12** (80.0 mg, 0.23 mmol), arylamines (2.3 mmol) and 4-methylbenzenesulfonic acid (8.0 mg, 0.046 mmol) in ethanol (30 mL) was refluxed for 12 h. After that, the solvent was removed in vacuum and the residue was purified by flash column chromatography on silica gel using dichloromethane/methanol (30:1, v/v) as eluent to afford **13a–d**.

4.9.1. 3-(1-Methyl-1*H*-indol-3-yl)-1-(naphthalen-1-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (13a)

According to the general method, the reaction of **12** with naphthalen-1-amine afforded compound **13a** in 78.0% yield as a yellow solid, mp: >250 °C. ^{1}H NMR (DMSO-d_6, δ): 3.93 (3H, s), 6.40 (1H, d, J = 8.0 Hz), 6.74 (1H, t, J = 8.4 Hz), 7.12 (1H, t, J = 8.0 Hz), 7.15–7.18 (1H, m), 7.50 (1H, d, J = 8.0 Hz), 7.60–7.64 (2H, m), 7.67–7.70 (2H, m), 7.92 (1H, dd, J = 2.0, 8.0 Hz), 8.09–8.11 (2H, m), 8.16 (1H, dd, J = 1.6, 8.0 Hz), 8.27 (1H, s), 8.54 (1H, dd, J = 1.6, 4.0 Hz), 14.20 (1H, br s). ESI-MS: m/z = 470 $[M+H]^+$. Anal. Calcd for $C_{29}H_{19}N_5O_2$: C, 74.19; H, 4.08; N, 14.92. Found: C, 74.33; H, 4.18; N, 15.02.

4.9.2. 1-(4-Chlorophenyl)-3-(1-methyl-1*H*-indol-3-yl)-4-(1H-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (13b)

According to the general method, the reaction of **12** with 4-chloroaniline afforded compound **13b** in 71.4% yield as a red solid, mp: >250 °C. 1H NMR (DMSO-d_6, δ): 3.92 (3H, s), 6.36 (1H, d, J = 7.6 Hz), 6.73 (1H, t, J = 8.0 Hz), 7.11 (1H, t, J = 8.0 Hz), 7.15–7.18 (1H, m), 7.48 (1H, d, J = 8.4 Hz), 7.60–7.64 (4H, m), 8.17 (1H, d, J = 8.4 Hz), 8.25 (1H, s), 8.44 (1H, d, J = 4.0 Hz), 14.01 (1H, s). ESI-MS: m/z = 455 $[M+H]^+$. Anal. Calcd for $C_{25}H_{16}ClN_5O_2$: C, 66.16; H, 3.55; Cl, 7.81; N, 15.43. Found: C, 66.23; H, 3.48; Cl, 7.61; N, 15.52.

4.9.3. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-(pyridin-2-yl)-1*H*-pyrrole-2,5-dione (13c)

According to the general method, the reaction of **12** with pyridin-2-amine afforded compound **13c** in 68.6% yield as a red solid, mp: 233–235 °C. 1H NMR (DMSO-d_6, δ): 3.92 (3H, s), 6.35 (1H, d, J = 8.0 Hz), 6.73 (1H, t, J = 8.0 Hz), 7.11 (1H, t, J = 8.0 Hz,), 7.15–7.18 (1H, m), 7.49 (1H, d, J = 8.0 Hz), 7.52–7.54 (1H, m), 7.64 (1H, t, J = 8.4 Hz), 8.06 (1H, td, J = 1.6, 8.0 Hz), 8.12 (1H, dd, J = 1.6, 8.0 Hz), 8.25 (1H, s), 8.54 (1H, dd, J = 1.6, 4.4 Hz), 8.66 (1H, dd, J = 1.6, 4.8 Hz), 13.99 (1H, s). ESI-MS: m/z = 421 $[M+H]^+$. Anal. Calcd for $C_{24}H_{16}N_6O_2$: C, 68.56; H, 3.84; N, 19.99. Found: C, 68.73; H, 3.88; N, 19.82.

4.9.4. 1-(4-Methoxyphenyl)-3-(1-methyl-1*H*-indol-3-yl)-4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (13d)

According to the general method, the reaction of **12** with 4-methoxyaniline afforded compound **13d** in 71.3% yield as a red solid, mp: >250 °C. ^{1}H NMR (DMSO-d_6, δ): 3.82 (3H, s), 3.92 (3H, s), 6.36 (1H, d, J = 8.0 Hz), 6.72 (1H, t, J = 8.0 Hz), 7.08–7.11 (3H, m), 7.15–7.17 (1H, m), 7.43–7.46 (2H, m), 7.48 (1H, d, J = 8.0 Hz), 8.15 (1H, dd, J = 1.6, 8.0 Hz), 8.23 (1H, s), 8.44 (1H, dd, J = 1.6, 4.4 Hz), 13.99 (1H, s). ESI-MS: m/z = 450 $[M+H]^+$. Anal. Calcd for $C_{26}H_{19}N_5O_3$: C, 69.48; H, 4.26; N, 15.58. Found: C, 69.23; H, 4.38; N, 15.42.

4.10. General procedure for the synthesis of 14a–d

A mixture of compounds **13a–d** (0.1 mmol), K_2CO_3 (27.6 mg, 0.2 mmol), 1-(3-chloropropyl)piperidine (58.1 mg, 0.36 mmol) in DMF (15 mL) was stirred at 70 °C for 8 h. After cooling, the mixture was poured into water (300 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined and washed with brine (3 × 150 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol/triethylamine (120:4:1, v/v/v) as eluent to afford **14a–d**.

4.10.1. 3-(1-Methyl-1*H*-indol-3-yl)-1-(naphthalen-1-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (14a)

According to the general method, the reaction of **13a** with 1-(3-chloropropyl)piperidine afforded compound **14a** in 81.2% yield as a red solid, mp: 161–163 °C. ^{1}H NMR ($CDCl_3$, δ): 1.49–1.50 (2H, m), 1.83–1.87 (4H, m), 2.25–2.27 (2H, m), 2.63–2.67 (6H, m), 3.93 (3H, s), 4.54 (2H, t, J = 7.2 Hz), 6.39 (1H, d, J = 8.0 Hz), 6.85 (1H, t, J = 8.0 Hz), 7.14–7.16 (1H, m), 7.21 (1H, t, J = 8.0 Hz), 7.37 (1H, d, J = 8.0 Hz), 7.55–7.58 (3H, m), 7.63 (1H, t, J = 8.0 Hz), 7.79–7.81 (1H, m), 7.96–7.99 (2H, m), 8.24 (1H, s), 8.28 (1H, dd, J = 1.2, 8.0 Hz), 8.58 (1H, dd, J = 1.2, 4.4 Hz). ESI-MS: m/z = 595 $[M+H]^+$. Anal. Calcd for $C_{37}H_{34}N_6O_2$: C, 74.73; H, 5.76; N, 14.13. Found: C, 74.93; H, 5.68; N, 14.32.

4.10.2. 1-(4-Chlorophenyl)-3-(1-methyl-1*H*-indol-3-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (14b)

According to the general method, the reaction of **13b** with 1-(3-chloropropyl)piperidine afforded compound **14b** in 65.4% yield as a red solid, mp: 135–137 °C. ^{1}H NMR ($CDCl_3$, δ): 1.40–1.42 (2H, m), 1.50–1.53 (4H, m), 1.86–1.88 (2H, m), 2.23–2.27 (6H, m), 3.90 (3H, s), 4.46 (2H, t, J = 7.2 Hz), 6.34 (1H, d, J = 8.0 Hz), 6.77 (1H, t, J = 8.0 Hz), 7.13–7.15 (2H, m), 7.32 (1H, d, J = 8.0 Hz), 7.46–7.49 (4H, m), 8.17 (1H, s), 8.21 (1H, dd, J = 1.6, 8.0 Hz), 8.55 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 580 $[M+H]^+$. Anal. Calcd for

$C_{33}H_{31}ClN_6O_2$: C, 68.44; H, 5.40; Cl, 6.12; N, 14.51. Found: C, 68.23; H, 5.48; Cl, 6.21; N, 14.32.

4.10.3. 3-(1-Methyl-1*H*-indol-3-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-(pyridin-2-yl)-1*H*-pyrrole-2,5-dione (14c)

According to the general method, the reaction of **13c** with 1-(3-chloropropyl)piperidine afforded compound **14c** in 66.0% yield as a red solid, mp: 138–141 °C. ^{1}H NMR ($CDCl_3$, δ): 1.43–1.45 (2H, m), 1.65–1.69 (4H, m), 2.03–2.05 (2H, m), 2.40–2.44 (6H, m), 3.90 (3H, s), 4.48 (2H, t, J = 6.8 Hz), 6.35 (1H, d, J = 8.0 Hz), 6.78 (1H, t, J = 8.0 Hz), 7.13–7.15 (2H, m), 7.34–7.35 (2H, m), 7.52 (1H, d, J = 8.0 Hz), 7.90 (1H, t, J = 7.6 Hz), 8.21 (1H, s), 8.25 (1H, d, J = 8.4 Hz), 8.54 (1H, d, J = 4.4 Hz), 8.71 (1H, d, J = 4.8 Hz). ESI-MS: m/z = 546 $[M+H]^+$. Anal. Calcd for $C_{32}H_{31}N_7O_2$: C, 70.44; H, 5.73; N, 17.97. Found: C, 70.63; H, 5.68; N, 17.82.

4.10.4. 1-(4-Methoxyphenyl)-3-(1-methyl-1*H*-indol-3-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1*H*-pyrrole-2,5-dione (14d)

According to the general method, the reaction of **13d** with 1-(3-chloropropyl)piperidine afforded compound **14d** in 68.7% yield as a red solid, mp: 165–168 °C. ^{1}H NMR ($CDCl_3$, δ): 1.51–1.54 (2H, m), 1.81–1.84 (4H, m), 2.21–2.23 (2H, m), 2.56–2.60 (6H, m), 3.87 (3H, s), 3.90 (3H, s), 4.52 (2H, t, J = 6.4 Hz), 6.34 (1H, d, J = 8.0 Hz), 6.80 (1H, t, J = 8.0 Hz), 7.03–7.05 (2H, m), 7.15–7.18 (2H, m), 7.34 (1H, d, J = 8.0 Hz), 7.38–7.39 (2H, m), 8.19 (1H, s), 8.24 (1H, dd, J = 1.6, 8.0 Hz), 8.57 (1H, dd, J = 1.6, 4.4 Hz). ESI-MS: m/z = 575 $[M+H]^+$. Anal. Calcd for $C_{34}H_{34}N_6O_3$: C, 71.06; H, 5.96; N, 14.62. Found: C, 71.23; H, 5.88; N, 14.52.

4.11. Pharmacology

4.11.1. Antiproliferative activity assay

The tumor cell lines (K562, A549, ECA-109, KB, SMMC-7721) were obtained from Shanghai Institute of Pharmaceutical Industry.

The antiproliferative activity in vitro was measured using the MTT assay. After treatment in 96-well plates, MTT solution (5.0 mg/mL in RPIM-1640, Sigma, St Louis, MO, USA) was added (10.0 μL/well), and plates were incubated for a further 4 h at 37 °C. The purple formazan crystals were dissolved in 100.0 μl DMSO. After 5 min, the plates were read on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. Assays were performed in triplicate in three independent experiments. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated using the software 'Dose-Effect Analysis with Microcomputers'. The tumor cell line panel consisted of K562, A549, ECA-109, KB and SMMC-7721. In all of these experiments, three replicate wells were used to determine each point.

4.11.2. Western blotting

After treatment, cell pellets were collected and lysed in a lysis buffer [150 mM NaCl, 50 mM Tris–HCl pH 8.0, 2 mM ethylene glycol-bis (β-aminoethyl ether), 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride]. Total protein concentrations of whole cell lysis were determined using BioRad BCA method (PIERCE, Rockford, IL). Equal amounts of protein sampled from whole cell lysis were subjected to electrophoresis on 10–12% Tris-Glycine pre-cast gels (Novex, San Diego, CA) and electroblotted onto Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, Massachusetts), and probed with primary antibodies and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (ECL) Western Blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

4.11.3. Electrophoretic analysis of DNA fragmentation

KB cells were lysed in 200.0 mL lysis buffer (10.0 mM EDTA; 50.0 mM Tris–HCl, pH 8.0; 0.5% sodium lauryl sulfate; 100.0 mg/mL proteinase K) at 37 °C for 12 h, then incubated with RNase (50.0 mg/ml) at 37 °C for an additional 1 h. After incubation, DNA in the lysate was extracted with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), then with chloroform. DNA was precipitated with two volumes of ethanol in the presence of 0.3 M sodium acetate. After centrifugation at 12,000*g* for 15 min, the DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 20.0 mL TE (10.0 mM Tris–HCl and 1.0 mM EDTA, pH 8.0). DNA was separated on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed by Bio-Rad GD2000 (Bio-Rad, Hercules, CA, USA).

4.11.4. PepTag assay for nonradioactive detection of PKC activity

The PepTag assay utilizes a brightly colored, florescent peptide substrate that is highly specific to PKC (according to the manufacturer's instructions (Promega). Phosphorylation by PKC changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated versions of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates toward the positive electrode, while the nonphosphorylated substrate migrates toward the negative electrode. The phosphorylated peptide in the band can then be visualized under UV light. PKC recombination kinases were incubated with PKC reaction mixture (25 μL) according to the manufacturer's (Promega) protocol at 30 °C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath. After adding 80% glycerol (1 μL), the samples were loaded onto an agarose gel (0.8% agarose in 50 mM Tris–HCl, pH 8.0). The samples were separated on the agarose gel in the same buffer at 100 V for 15 min, and the bands were visualized under UV light.

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