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Discovery and structure–activity relationship of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines as a new series of potent apoptosis inducers

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Abstract—We report the discovery and SAR study of a series of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines as potent inducers of apoptosis. *N*-(3-Acetylphenyl)-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-amine (**2**) was discovered through our cell- and caspase-based HTS assays as an inducer of apoptosis. Compound **2** is active against cancer cells derived from several human solid tumors, with EC_{50} values ranging from 400 to 700 nM. SAR study of hit **2** led to the discovery of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines as a novel series of potent apoptosis inducers, with 1,3-dimethyl-*N*-(4-propionylphenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-amine (**6b**) having EC_{50} values ranging from 30 to 70 nM in cancer cells. These compounds also demonstrated potent activity in the cell growth inhibition assay, with GI_{50} values of 16–42 nM for compound **6b**.

1. Introduction

Apoptosis is a highly regulated process of cellular suicide, and it plays a crucial role in normal cell development and tissue homeostasis.[1] Apoptosis enables organisms to control their cell numbers and to eliminate unneeded cells that may threaten their survival.[2] It is known that inappropriate apoptosis induction results in excessive cell death, and could be the cause of degenerative diseases.[3] Inadequate apoptosis, however, leads to over proliferation of cells, and could result in cancer.[4] It has been reported that the anti-tumor efficacy of several chemotherapeutical agents is correlated to their apoptosis inducing ability.[5] Therefore, the identification of apoptosis inducers represents an attractive approach for the discovery and development of potential anticancer agents.

The mechanism of apoptosis involves a caspase cascade that is activated sequentially. Caspases are a family of cysteine proteases that require an asparatic acid residue at the P_1 position of substrate for cleavage. Within the caspase family, caspase-3 has been identified as one of the key effector caspases that cleave multiple protein substrates in cells, leading to irreversible cell death.[6] In our effort to discover and develop apoptosis inducers as new anticancer agents, we have developed a cell-based, high throughput screening technology, Apoptosis Screening and AntiCancer Platform (ASAP), to identify apoptosis inducers by measuring the activation of caspase-3.[7] One of the major advantages of this technology is that by monitoring the activation of downstream caspase-3 in a cell-based assay, we can discover compounds that initiate the apoptosis signal pathway through a known mechanism or known molecular targets, as well as by novel mechanism or molecular targets.[8]

Applying this high throughput assay, we have discovered and optimized several classes of small molecules as novel apoptosis inducers. *N*-Phenyl nicotinamides,[9] represented by 6-methyl-*N*-(4-ethoxy-2-nitro-phenyl)-pyridine-3-carboxamide (**1a**), were discovered as a series of potent apoptosis inducers that interact with tubulin. 4-Aryl-4*H*-chromenes, exemplified by 2-amino-3-cyano-7-dimethylamino-4-(3-bromo-4,5-dimethoxyphenyl)-4*H*-chromene (**1b**),[10] showed potent apoptosis inducing activity and several of them demonstrated vascular

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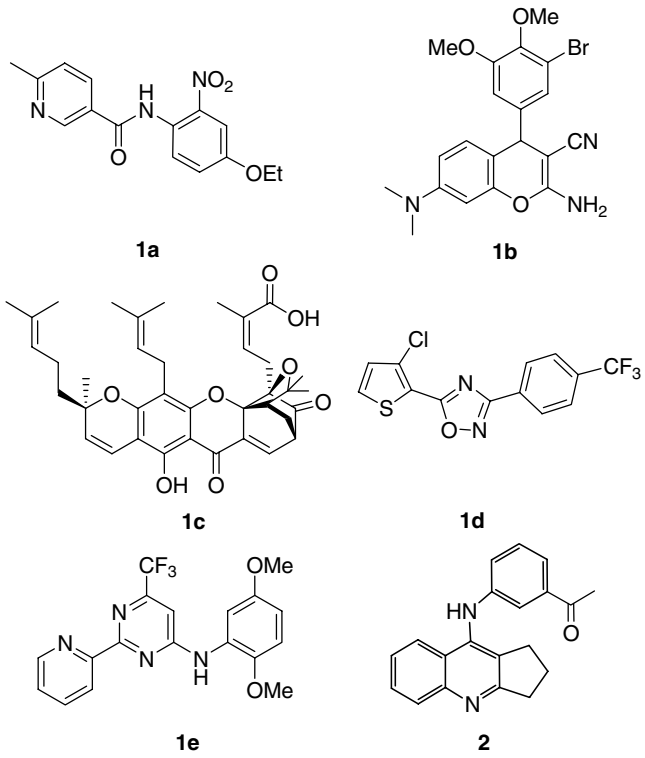


Chart 1.

disrupting activity (VDA) with anti-tumor efficacy in several animal models.[11] Gambogic acid (**1c**), isolated from gamboge resin, was discovered as a fast apoptosis inducer.[12] Derivatives of gambogic acid with good in vivo activity have been discovered and its molecular target has been identified as transferrin receptor.[13] 3-Aryl-5-aryl-1,2,4-oxadiazoles, exemplified by 5-(3-chlorothiophen-2-yl)-3-(4-trifluoromethylphenyl)-1,2,4-oxadiazole (**1d**), were found to induce apoptosis selectively in certain tumor types,[14] and TIP47, an insulin growth factor II (IGF II) receptor binding protein, has been identified as its molecular target.[15] In addition, 4-anilino-2-(2-pyridyl)pyrimidines such as **1e** were reported recently as a series of novel apoptosis inducers that arrest cells at G_2/M.[16] Herein, we report the identification of *N*-(4-acetylphenyl)-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-amine (**2**, Chart 1) as a potent apoptosis inducer, and SAR study of **2** leading to the discovery of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines as a series of potent apoptosis inducers.

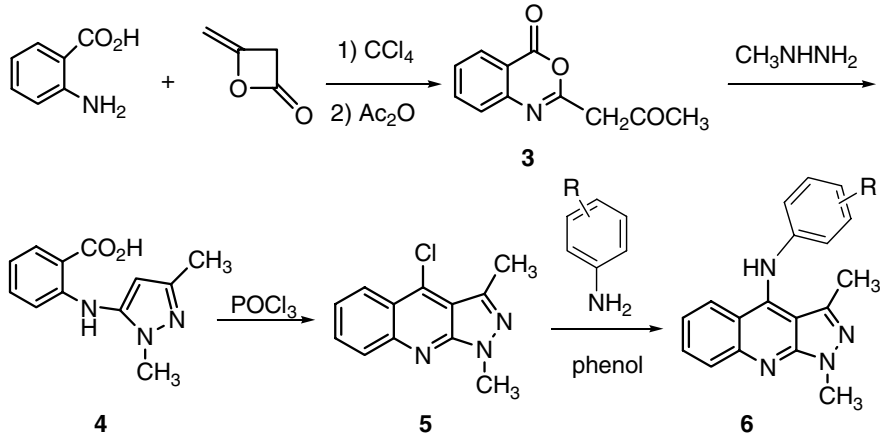
2. Results and discussion

2.1. Chemistry

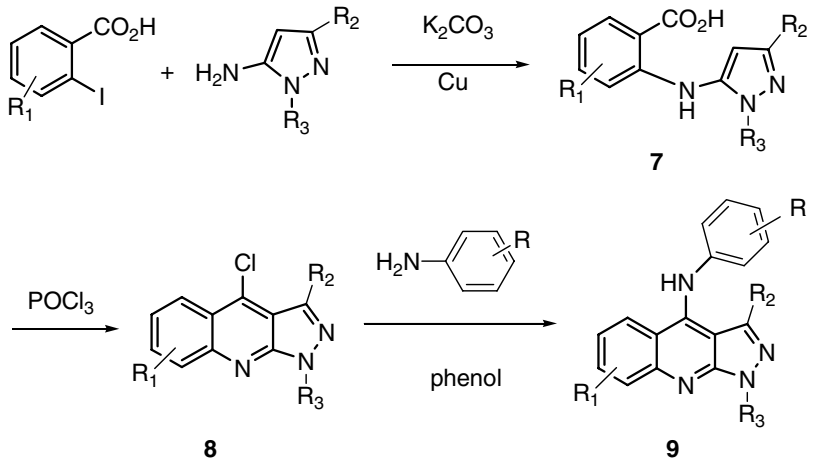
Compounds **2**, **6a**, **6c**, and **9a** were purchased from a commercial library. Compounds **6b**, **6d–6g** were prepared as shown in Scheme 1. The key intermediate, 4-chloro-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoline (**5**), was prepared according to literature procedure.[17] Reaction of anthranilic acid with 4-methyleneoxetan-2-one in CCl_4, followed by treatment with acetic anhydride, produced 2-(2-oxopropyl)-4*H*-benzo[*d*][1,3]oxazin-4-one (**3**), which was reacted with methylhydrazine to produce 2-(1,3-dimethyl-1*H*-pyrazol-5-ylamino)benzoic acid (**4**). Cyclization of **4** via treatment with $POCl_3$ produced **5**. Reaction of **5** with a substituted aniline, such as 4-propionylbenzenamine, produced 1,3-dimethyl-*N*-(4-propionylphenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (**6b**). Acid **6h** was prepared through hydrolysis of the corresponding methyl ester **6f**. Reduction of compound **6b** using sodium borohydride in methanol produced compound **6i**.

Other substituted 4-chloro-1*H*-pyrazolo[3,4-*b*]quinolines (**8**) were prepared following literature procedures[18] as shown in Scheme 2. For example, reaction of 2-iodobenzoic acid with 1-methyl-1*H*-pyrazol-5-amine produced 2-(1-methyl-1*H*-pyrazol-5-ylamino)benzoic acid (**7c**), which was cyclized by treatment with $POCl_3$ to produce 4-chloro-1-methyl-1*H*-pyrazolo[3,4-*b*]quinoline (**8c**). Reaction of **8c** with 4-acetylbenzenamine produced *N*-(4-acetylphenyl)-1*H*-methyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (**9c**).

4-(4-Acetylphenoxy)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]-quinoline (**10**) was prepared via reaction of **5** with 4′-hydroxyacetophenone. Similarly, reaction of **5** with



Scheme 1.

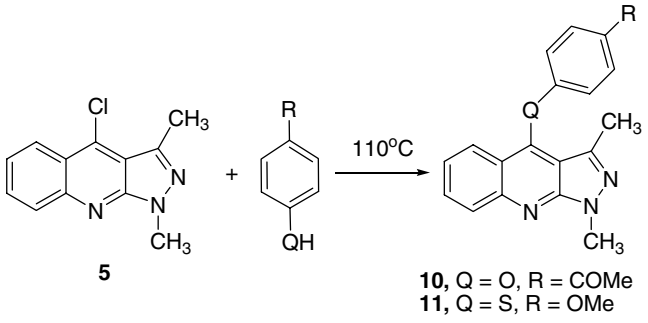


Scheme 2.

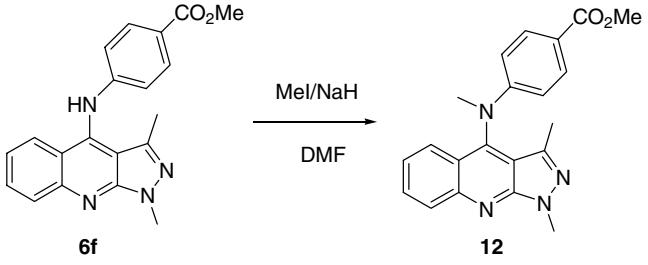
4-methoxybenzenethiol produced 4-(4-methoxyphenylthio)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoline **11** (Scheme 3). *N*-(4-Methoxycarbonylphenyl)-*N*,1,3-trimethyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (**12**) was prepared via reaction of **6f** with MeI in DMF with sodium hydride (Scheme 4).

2.2. HTS assay

N-(3-Acetylphenyl)-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-amine (**2**) was identified as an inducer of apoptosis using our cell- and caspase 3-based assays as described previously.[9] Briefly, human breast cancer T47D cells, in a 384-well microtiter plate containing various concentrations of testing compounds, were incubated for 24 h to induce apoptosis. To the treated cells was then added the fluorogenic caspase-3 substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110[7] in a caspase buffer and the sample was incubated at room temperature for 3 h. Using a fluorescent plate reader, employing excitation at 485 nm and emission at 530 nm, the fluorescence of cleaved product ethoxycarbonyl-R110 was measured, thereby determining the level of caspase activation. Compounds that induce apoptosis and activate caspases produced a fluorescent signal that is higher than the background (signal/background ratio). Compounds found to give a ratio of >3 are considered active and retested in triplet for confirmation. Compounds confirmed to be active are then tested at several concentrations to give a dose–response and the caspase activation activity EC_{50} is calculated.



Scheme 3.



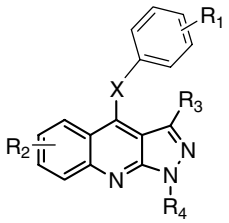
Scheme 4.

Compound **2** was found to have an EC_{50} of 450 nM in T47 breast cancer cell. It also demonstrated potent activity on HCT116 colon cancer cell lines and SNU398 liver cancer cell lines (Table 1). Encouraged by these potent activities, a series of structurally related compounds was purchased from commercial libraries and tested, including 1,3-dimethyl-*N*-(4-acetylphenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (**6c**) which was found to have activity similar to that of compound **2.** Since replacement of the tricyclic cyclopenta[*b*]quinoline by a pyrazolo[3,4-*b*]quinoline reduces Clog P by about 1 unit and introduces a basic pyrazolo ring, which could improve solubility profile, we selected the pyrazolo[3,4-*b*]quinoline system as the core scaffold to explore the SAR including modification of various rings and the linker between top ring and the pyrazole[3,4-*b*]quinoline.

2.3. Structure–activity relationship (SAR) studies

The cell based caspase activation assay was used for the testing of all the compounds related with **2** for SAR studies. The caspase activation activity (EC_{50}) of these compounds in three cell lines, T47D breast cancer cells, HCT116 colorectal cancer cells, and SNU398 liver cancer cells is summarized in Table 1. The SAR of substitution at the top phenyl ring was explored first. The *para*-position was found to tolerate a wide variety of groups,

Table 1. Caspase activation activity of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines



Compound	R_1	X	R_2	R_3	R_4	EC_{50}[a] (μM)		
						T47D	HCT116	SNU398
2						0.45 ± 0.023	0.66 ± 0.020	0.44 ± 0.06
6a	4-MeO	NH	H	Me	Me	1.01 ± 0.058	1.25 ± 0.020	0.48 ± 0.069
6b	4-EtOC	NH	H	Me	Me	0.071 ± 0.007	0.064 ± 0.010	0.034 ± 0.006
6c	4-MeOC	NH	H	Me	Me	0.52 ± 0.017	0.66 ± 0.087	0.27 ± 0.033
6d	4-*i*-PrOC	NH	H	Me	Me	0.44 ± 0.037	0.39 ± 0.083	0.24 ± 0.071
6e	4-NH_2OC	NH	H	Me	Me	>10	>10	>10
6f	4-MeO_2C	NH	H	Me	Me	0.54 ± 0.017	0.67 ± 0.074	0.27 ± 0.033
6g	3-MeO	NH	H	Me	Me	7.26 ± 0.47	>10	>10
6h	4-HO_2C	NH	H	Me	Me	>10	>10	>10
6i	4-(1-HOPr)	NH	H	Me	Me	0.14 ± 0.010	0.33 ± 0.083	0.053 ± 0.012
9a	4-MeO	NH	H	Me	H	>10	>10	>10
9b	4-EtOC	NH	H	Me	Et	0.21 ± 0.018	0.19 ± 0043	0.15 ± 0.019
9c	4-MeOC	NH	H	H	Me	1.15 ± 0.046	1.36 ± 0.047	0.73 ± 0.073
9d	4-MeOC	NH	H	*i*-Pr	Me	>10	>10	>10
9e	4-MeOC	NH	6-Me	Me	Me	>10	>10	>10
9f	4-MeOC	NH	8-Me	Me	Me	1.11 ± 0.21	0.94 ± 0.37	0.66 ± 0.040
9g	4-EtOC	NH	8-NO_2	Me	Me	0.51 ± 0.072	0.48 ± 0.081	0.27 ± 0.069
10	4-MeOC	O	H	Me	Me	>10	>10	>10
11	4-MeO	S	H	Me	Me	5.47 ± 0.19	6.67 ± 0.18	4.99 ± 0.073
12	4-MeO_2C	NMe	H	Me	Me	5.21 ± 0.63	6.08 ± 0.91	1.33 ± 0.015

[a] Data are mean of three or more experiments and are reported as means ± standard error of the mean (SEM).

such as acetyl, propionyl, methoxy carbonyl, and methoxy. Compound **6b** was found to be the most potent compound in this series, which is about 7-fold more potent than the original hit **2** in T47D cells. It is also interesting to note that compound **6i** with a 1-hydroxypropyl at the *para*-position also showed potent activity. Introduction of polar groups at the *para*-position of the phenyl ring results in completely inactive compounds (**6e** and **6h**), suggesting a hydrophobic binding pocket at this position. Compound **6g** was about 7-fold less active than **6a**, suggesting substitution at the *para*-position is preferred over *meta*-position.

We then explored substitution at the 1- and 3-positions of the pyrazolo ring. Compound **9a** was inactive up to 10 μM, which was at least 10-fold less active than **6a**, indicating that an alkyl group at the 1-position of pyrazole ring is critical for activity. Compound **9b** was 3-fold less active than **6b**, indicating that a small group such as Me is preferred at the 1-position. Compound **9c** was 2-fold less active than the 3-Me analog **6c**, and compound **9d** with a 3-isopropyl group is inactive up to 10 μM, indicating that a small substituent at the 3-position of pyrazole is important for activity and a large group at the 3-position is not tolerated.

Substitutions at the 6- and 8-positions of the pyrazolo[3,4-*b*]quinoline also were explored. Compound **9f** with a Me group at the 8-position was about 2-fold less active than **6c**. The 8-nitro analog **9g** was about 7-fold less active than **6b**. These data indicate that substitution at the 8-position is relatively well tolerated. In comparison, the 6-Me analog **9e** was not active up to 10 μM, indicating that substitution at 6-position is not tolerated.

We also explored the replacement of the NH linker between the pyrazolo[3,4-*b*]quinoline scaffold and top phenyl ring by other groups. Compound **10** with an O linker was found to be inactive up to 10 μM, which is about 20-fold less active than the corresponding NH analog **6c**. Compound **11**, with a S linker, was 5-fold less active than the corresponding NH analog **6a**. The *N*-Me compound **12** was 10-fold less active than the corresponding NH analog **6f**. Overall these data indicate that an NH linker is highly preferred.

The activities of these *N*-phenyl-1*H*-pyrazolo[3,4-b]-quinolin-4-amines toward the human colorectal cancer cells HCT116 and liver cancer cells SNU398 were roughly parallel to their activity toward T47D cells. SNU398 cells were slightly more sensitive than T47D cells to these compounds as indicated by the about 2-fold difference in EC_{50} values. HCT116 cells were about as sensitive as T47D cells with similar EC_{50} values for most of these compounds.

Selected compounds were also tested by the traditional inhibition of cell proliferation (GI_{50}) assay to confirm

Table 2. Inhibition of cell proliferation activity of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines

Compound	GI_{50}[a] (μM)		
	T47D	HCT116	SNU398
2	0.56 ± 0.042	0.50 ± 0.022	0.17 ± 0.001
6b	0.026 ± 0.004	0.042 ± 0.004	0.016 ± 0.001
9a	>10	>10	>10
6i	0.59 ± 0.122	0.93 ± 0.033	0.14 ± 0.013

[a] Data are mean of three or more experiments and are reported as means ± standard error of the mean (SEM).

that the active compounds can inhibit tumor cell growth. The growth inhibition assays in T47D, HCT116, and SNU398 cells are run in a 96-well microtiter plate as described previously.[9] The GI_{50} values were summarized in Table 2. Compound **6b** was highly active with GI_{50} values of 16–42 nM in the three cell lines tested. Compound **9a**, which was inactive in the caspase activation assay, also was inactive in the growth inhibition assay, confirming a good correlation between our caspase activation assay and the traditional growth inhibition assay.[9]

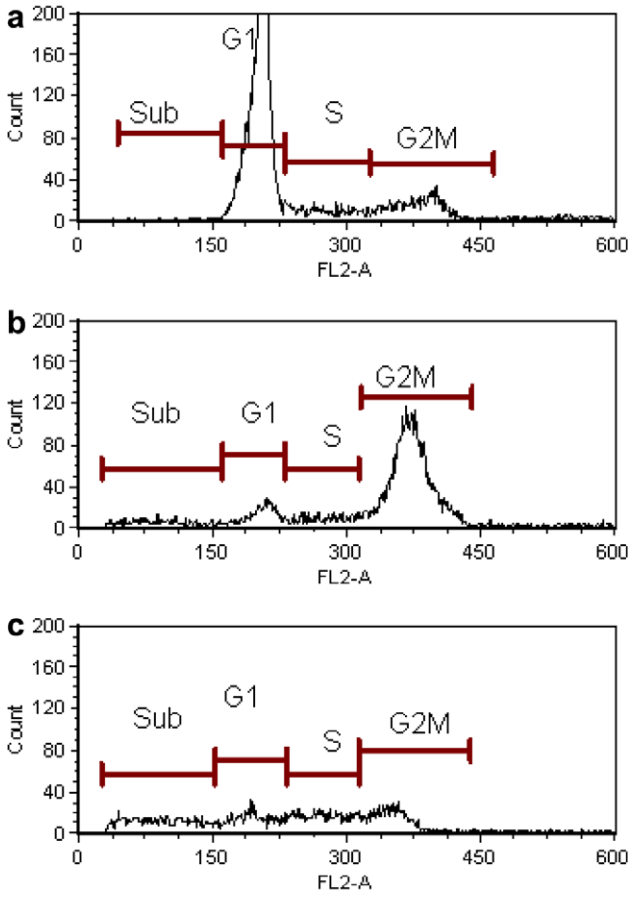
2.4. Additional characterization of compound 6b

The apoptosis-inducing activities of compound **6b** were further characterized by flow cytometry. T47D cells were treated with 50 nM of compound **6b** for 24 or 48 h, then stained with propidium iodide and analyzed by flow cytometry. As shown in Figure 1a, control cells have a normal cell cycle profile with most cells in the G_1 phase. Cells treated with compound **6b** for 24 h show a shift of the population into G_2/M phase (Fig. 1b), followed by induction of apoptosis after 48-h treatment (Fig. 1c). Similar results were found in several other cell lines, including MDA MB435 (breast cancer), MX-1 (breast cancer), Jurkat (T-cell leukemia), and SNU398 (liver cancer) (data not shown).

Since **6b** arrested cells in G_2/M followed by induction of apoptosis, which is similar to many tubulin interacting agents such as vinblastine and colchicine, we suspected that **6b** might interact with tubulin. In addition, a structurally related *N*-(4-acetylphenyl)furo[2,3-*b*]quinoline-4-amine (**13**, CIL-102, Chart 2) has been reported to bind to tubulin and disrupt microtubule organization.[19] Interestingly, when **6b** was tested in a tubulin polymerization assay,[16] no effects on tubulin polymerization were observed up to 50 μM. Therefore, the molecular target of **6b** and related analogs remains to be identified.

3. Conclusion

In conclusion, *N*-(3-acetylphenyl)-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-amine (**2**) was identified as a potent apoptosis inducer through our cell and caspase based HTS assay. SAR study of **2** led to the identification of a series of *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines as potent apoptosis inducers. The top *N*-phenyl group was found to tolerate various substitu-



Entry	Sub	G1	S	G_2M
1a	0.9	67.8	13.6	17.3
1b	4.3	43.5	7.6	44.0
1c	12.0	11.0	18.8	57.0

Figure 1. Drug-induced apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (a) Control cells showing most of the cells in G_1 phase of the cell cycle. (b) Cells treated with 50 nM of compound **6b** for 24 h showing most of the cells arrested in G_2/M phase and reduction of cells in G_1 and S phases. (c) Cells treated with 50 nM of compound **6b** for 48 h showing a progression from G_2/M to cells with sub-diploid DNA content, which are apoptotic cells with fragmented nuclei.

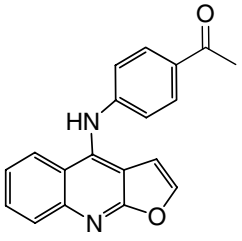


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Chart 2.

tions, especially at the *para*-position. The NH linker between the top phenyl ring and pyrazolo[3,4-*b*]quinoline was found to be important for activity, and its replacement by NMe, O or S all led to decreased activity. A Me group at the 1-position and a Me group at the 3-position were found to be important for activity. Substitution at the 8-position was found to be tolerated while substitution at the 6-position led to inactive compound. Through SAR study, compound **6b** was identified as a potent apoptosis inducer with an EC_{50} of 71 nM in T47D cells, which was about 7-fold more potent than hit **2**. Compound **6b** also is a potent inhibitor of cell proliferation with a GI_{50} of 26 nM in T47D cells, which was >10-fold more potent than **2**. The ability of compound **6b** to induce apoptosis was confirmed in a flow cytometry assay in T47D cells, which showed that treatment of cells with **6b** resulted in G_2/M arrest, followed by apoptosis. However, unlike some of the classical G_2/M arresting anticancer agents such as vinblastine which inhibit tubulin polymerization, compound **6b** was found to have no effect in the tubulin polymerization assay, suggesting that **6b** induces apoptosis through a different mechanism. Additional studies to identify the mechanism of action of these compounds are in progress.

4. Experimental

4.1. General methods and materials

Commercial-grade reagents and solvents were obtained from Acros, Aldrich, or Lancaster and were used without further purification except as indicated. All reaction were stirred magnetically; moisture-sensitive reactions were performed under argon in flame-dried glassware. Thin-layer chromatography (TLC), usually using ethyl acetate/hexane as the solvent system, was used to monitor reactions. Solvents were removed by rotary evaporation under reduced pressure; where appropriate, the compounds were further dried using a vacuum pump. The ^{1}H NMR spectra were recorded at 300 MHz. All samples were prepared as dilute solutions in deuteriochloroform ($CDCl_3$) with v/v 0.05% tetramethylsilane (TMS). Chemical shifts are reported in parts per million (ppm) downfield from TMS (0.00 ppm) and *J* coupling constants are reported in hertz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Human breast cancer cells T47D and other cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Compounds **2**, **6a**, **6c**, and **9a** were purchased from a commercial library (Aldrich Rare chemicals) and the structures were confirmed by ^{1}H NMR and MS.

4.1.1. 2-(2-Oxopropyl)-4*H*-benzo[*d*][1,3]oxazin-4-one (3). To a solution of anthranilic acid (8.77 g, 64 mmol) in CCl_4 (100 mL) was added 4-methyleneoxetan-2-one (5.38 g, 64 mmol) at 70 °C, then the mixture was heated at reflux for 20 min. To the mixture was added acetic anhydride (6.53 g, 64 mmol) and the mixture was stirred at 105 °C for 1 h. The mixture was cooled and the product was isolated by filtration to give 11.5 g (89%) of **3** as solids which was used for the next reaction without further purification.

4.1.2. 2-(1,3-Dimethyl-1*H*-pyrazol-5-ylamino)benzoic acid (4). To a solution of methylhydrazine (1.38 g, 30 mmol) in water (6 mL) was added **3** (5.07 g, 25 mmol) with stirring, then was added saturated aqueous sodium carbonate (25 mL). The solution was stirred at room temperature for 6 h. It was neutralized with 2 N HCl to pH 5. The precipitates were filtered, washed with water, and dried to give 4.7 g (79%) of **4** as solids which was used for the next reaction without further purification.

4.1.3. 4-Chloro-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoline (5). A mixture of **4** (2.37 g, 10 mmol) and $POCl_3$ (6 ml) was stirred at room temperature for 30 min and then was heated to 100 °C and stirred for 2 h. The solution was cooled, poured into 100 mL of ice water, and adjusted to pH 4–5 with 2 N aqueous sodium hydroxide. The precipitates were filtered, washed with water, dried, and purified by flash chromatography (Hexane/ EtOAc 3:1) to give 1.8 g (78%) of **5** as solids. ^{1}H NMR ($CDCl_3$): 8.37 (d, J = 9.3 Hz, 1H), 8.07 (d, J = 9.3 Hz, 1H), 7.77 (t, J = 7.5 Hz, 1H), 7.51 (t, J = 7.5 Hz, 1H), 4.15 (s, 3H), 2.87 (s, 3H).

4.1.4. 1,3-Dimethyl-*N*-(4-(methoxycarbonyl)phenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (6f). A mixture of **5** (0.23 g, 1.0 mmol), methyl 4-aminobenzonate (0.30 g, 2.0 mmol), and phenol (1.5 g, 16 mmol) was stirred at 100 °C for 4 h. To the mixture was added 2 N sodium carbonate and the mixture was extracted with ethyl acetate (3× 30 mL). The combined organic extracts were washed with saline, dried, and concentrated to dryness, and the residue was purified by column chromatography (Hexane/EtOAc 3:1) to give 0.24 g (68%) of **6f** as solids. ^{1}H NMR ($CDCl_3$): 8.08 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8.7 Hz, 1H), 7.92 (d, J = 8.7 Hz, 2H), 7.73 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.2 Hz, 1H), 6.86 (d, J = 8.7 Hz, 2H), 6.70 (bs, 1H), 4.16 (s, 3H), 3.88 (s, 3H), 2.46 (s, 3H). Anal. Calcd for $C_{20}H_{18}N_4O_2 \cdot 0.5\ H_2O$: C, 69.35; H, 5.24; N, 16.17. Found: C, 69.46; H, 5.64; N, 16.30.

The following compounds were prepared from the corresponding substituted aniline and **5** by a procedure similar to that described for the preparation of **6f.**

4.1.5. 1,3-Dimethyl-*N*-(4-propionylphenyl)-1*H*-pyrazolo-[3,4-*b*]quinolin-4-amine (6b). Yield: 35%, white solids. ^{1}H NMR ($CDCl_3$): 8.10 (d, J = 8.7 Hz, 1H), 7.92 (d, J = 8.7 Hz, 1H), 7.85 (d, J = 7.8 Hz, 2H), 7.71 (t, J = 7.2 Hz, 1H), 7.30 (t, J = 8.7 Hz, 1H), 6.85 (d, J = 8.1 Hz, 2H), 6.69 (s, 1H), 4.15 (s, 3H), 2.95 (q, J = 7.8 Hz, 2H), 2.45 (s, 3H), 1.25 (t, J = 7.8 Hz, 3H). Anal. Calcd for $C_{21}H_{20}N_4O$: C, 73.23; H: 5.85; N, 16.27. Found: C, 72.85; H, 5.84; N, 16.05.

4.1.6. 1,3-Dimethyl-*N*-(4-isobutyrylphenyl)-1*H*-pyrazolo-[3,4-*b*]quinolin-4-amine (6d). Yield: 60%, white solids. ^{1}H NMR ($CDCl_3$): 8.11 (d, J = 8.7 Hz, 1H), 7.97 (d, J = 8.7 Hz, 1H), 7.88 (d, J = 8.7 Hz, 2H), 7.72 (m, 1H), 7.35 (m, 1H), 6.87 (d, J = 8.7 Hz, 2H), 6.73 (s,

1H), 4.16 (s, 3H), 3.45 (q, J = 6.6 Hz, 2H), 2.48 (s, 3H), 1.19 (d, J = 6.6 Hz, 6H). Anal. Calcd for $C_{22}H_{22}N_4O$: C, 73.72; H, 6.19; N, 15.63. Found: C, 73.09; H, 6.55; N, 15.16.

4.1.7. *N*-(4-Carbamoylphenyl)-1,3-dimethyl-1*H*-pyrazolo-[3,4-*b*]quinolin-4-amine (6e). Yield: 71%, white solids. ^{1}H NMR ($CDCl_3$): 8.09 (d, J = 8.7 Hz, 1H), 7.97 (d, J = 8.7 Hz, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.33 (t, J = 8.4 Hz, 1H), 6.90 (t, J = 8.4 Hz, 2H), 6.69 (s, 1H), 5.69 (bs, 2H), 4.16 (s, 3H), 2.48 (s, 3H). Anal. Calcd for $C_{19}H_{17}N_5O$: C, 65.32; H, 5.48; N, 20.04. Found: C, 65.60; H, 6.04; N, 19.74.

4.1.8. *N*-(3-Methoxyphenyl)-1,3-dimethyl-1*H*-pyrazolo-[3,4-*b*]quinolin-4-amine (6g). Yield 69%, white solids. ^{1}H NMR ($CDCl_3$): 8.02 (d, J = 8.7 Hz, 1H), 7.95 (d, J = 8.7 Hz, 1H), 7.70 (t, J = 8.4 Hz, 1H), 7.26 (t, J = 8.4 Hz, 1H), 7.16 (t, J = 8.4 Hz, 1H), 6.65 (s, 1H), 6.55–6.45 (m, 3H), 4.12 (s, 3H), 3.71 (s, 3H), 2.43 (s, 3H). Anal. Calcd for $C_{19}H_{18}N_4O$): C, 71.68; H, 5.70; N, 17.60. Found: C, 70.95; H, 5.97; N, 17.42.

4.1.9. *N*-(4-Carboxyphenyl)-1,3-dimethyl-1*H*-pyrazolo-[3,4-*b*]quinolin-4-amine (6h). To a mixture of **6f** (80 mg, 0.23 mmol) in methanol (15 mL) was added 2 N NaOH (1 mL). The mixture was refluxed for 4 h, then it was concentrated. The residue was diluted with water (5 mL), and the solution was washed with ethyl acetate (10 mL). The aqueous solution was acidified to pH 5, and it was extracted by ethyl acetate (3× 10 mL). The extracts were dried, concentrated to give 45 mg (59%) of **6 h** as solids. ^{1}H NMR (CD_3OD): 8.20 (d, J = 9.0 Hz, 1H), 8.08 (d, J = 9.0 Hz, 1H), 7.88 (d, J = 9.0 Hz, 2H), 7.79–7.74 (m, 1H), 7.42– 7.36 (m, 1H), 6.93 (d, J = 9.0 Hz, 2H), 4.08 (s, 3H), 2.15(s, 3H). Anal. Calcd for $C_{19}H_{16}N_4O_2 \cdot 0.3\ H_2O$: C, 67.56; H, 4.95; N, 15.63. Found: C, 67.65; H, 5.41; N, 16.44.

4.1.10. *N*-(4-(1-Hydroxypropyl)phenyl)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (6i). To a solution of **6b** (103 mg, 0.30 mmol) in methanol (15 mL) was added sodium borohydrate (38 mg, 1.0 mmol). It was stirred at room temperature for 2 h, diluted with water (5 mL), quenched with 2 N HCl (3 mL), and then neutralized to pH 8 by aqueous $NaHCO_3$. The mixture was extracted with EtOAc (3× 10 mL) and the extracts were dried and concentrated to give **6i** (100 mg, 96%) as white solids. ^{1}H NMR ($CDCl_3$): 8.04 (d, J = 8.7 Hz, 1H), 7.93 (d, J = 8.7 Hz, 1H), 7.68 (t, J = 7.6 Hz, 1H), 7.28–7.22 (m, 3H), 6.96 (d, J = 8.4 Hz, 2H), 6.69 (s, 1H), 4.57 (m, 1H), 4.12 (s, 3H), 2.38 (s, 3H), 1.81 (m, 2H), 0.91 (t, J = 7.2 Hz, 3H). Anal. Calcd for $C_{21}H_{22}N_4O$: C, 72.81; H, 6.40; N, 15.63. Found: C, 73.51; H, 6.75; N, 15.78.

4.1.11. 2-(1,3-Dimethyl-1*H*-pyrazol-5-ylamino)-3-methylbenzoic acid (7f). A mixture of 2-iodo-3-methylbenzoic acid (2.62 g, 10 mmol), 5-amino-1,3-dimethylpyrazole (1.11 g, 10 mmol), potassium carbonate (1.38 g, 10 mmol), and Cu powder (0.32 g, 5.0 mmol) in water (20 mL) was refluxed for 24 h and the solid was removed by filtration. The aqueous solution was extracted with ethyl acetate (50 mL). The extract was dried over sodium sulfate and concentrated in vacuo to give 1.6 g (66%) of crude **7f** which was used for the next reaction without further purification.

4.1.12. 4-Chloro-1,3,8-trimethyl-1*H*-pyrazolo[3,4-*b*]quinoline (8f). A mixture of **7f** (1.4 g, 5.7 mmol) and $POCl_3$ (5 ml) was refluxed for 4 h. The solution was cooled and diluted with ethyl acetate (40 mL), quenched with 20 mL of ice water, and neutralized with saturated potassium carbonate. The organic layer was separated, dried, and evaporated, and the residue was purified by flash chromatography (Hexane/EtOAc 3:1) to give 0.51 g (37%) of **8f** as solids. ^{1}H NMR ($CDCl_3$): 8.20 (d, J = 9.0 Hz, 1H), 7.61 (d, J = 6.9 Hz, 1H), 7.26 (m, 1H), 4.15 (s, 3H), 2.86 (s, 3H), 2.83 (s, 3H).

4.1.13. *N*-(4-Acetylphenyl)-1,3,8-trimethyl-1*H*-pyrazolo[3, 4-*b*]quinolin-4-amine (9f). A mixture of **8f** (0.16 g, 0.65 mmol), 1-(4-aminophenyl)ethanone (0.10 g, 0.72 mmol), and phenol (0.31 g, 3.3 mmol) was stirred at 100 °C for 4 h. To the mixture was added 2 N sodium carbonate and the mixture was extracted with ethyl acetate (3× 30 mL). The combined extracts were washed with saline, dried, and concentrated to dryness, and the residue was purified by column chromatography (Hexane/EtOAc 3:1) to give 0.035 g (16%) of **9f** as orange solids. ^{1}H NMR ($CDCl_3$): 7.85 (d, J = 8.7 Hz, 2H), 7.84 (m, 1H), 7.58 (d, J = 6.9 Hz, 1H), 7.23 (m, 1H), 6.81 (d, J = 8.7 Hz, 2H), 6.65 (bs, 1H), 4.17 (s, 3H), 2.86 (s, 3H), 2.53 (s, 3H), 2.46 (s, 3H). Anal. Calcd for $C_{21}H_{20}N_4O{\cdot}1.2\ H_2O$): C, 72.06; H, 6.45; N, 16.01. Found: C, 72.38; H, 5.99; N, 15.89.

The following compounds **9b–9e** and **9g** were synthesized from the corresponding substituted 2-iodobenzoic acid, substituted 5-aminopyrazole and substituted aniline using procedures similar to that described for the preparation of compounds **7f**, **8f**, and **9f**.

4.1.14. 2-(1-Ethyl-3-methylpyrazol-5-ylamino)benzoic acid (7b). Yield: 97%.

4.1.15. 4-Chloro-1-ethyl-3-methyl-1*H*-pyrazolo[3,4-*b*]quinoline (8b). Yield: 41%, solids. ^{1}H NMR ($CDCl_3$): 8.36 (d, J = 9.0 Hz, 1H), 8.06 (d, J = 9.0 Hz, 1H), 7.76 (m, 1H), 7.50 (m, 1H), 4.60 (q, J = 6.9 Hz, 2H), 2.88 (s, 3H), 1.55 (t, J = 6.9 Hz, 3H).

4.1.16. 1-Ethyl-3-methyl-*N*-(4-propionylphenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (9b). Yield: 67%, orange solids. ^{1}H NMR ($CDCl_3$): 8.07 (d, J = 7.8 Hz, 1H), 7.96 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 8.7 Hz, 2H), 7.72 (m, 1H), 7.33 (m, 1H), 6.87 (d, J = 8.7 Hz, 2H), 6.70 (s, 1H), 4.60 (q, J = 7.5 Hz, 1H), 2.93 (q, J = 7.5 Hz, 1H), 1.58 (t, J = 7.8 Hz, 1H), 1.21 (t, J = 7.8 Hz, 1H). Anal. Calcd for $C_{22}H_{22}N_4O{\cdot}H_2O$: C, 70.19; H, 6.43; N, 14.88. Found: C, 70.70; H, 6.59; N, 14.06.

4.1.17. 2-(1-Methylpyrazol-5-ylamino)benzoic acid (7c). Yield: 83%.

4.1.18. 4-Chloro-1-methyl-1*H*-pyrazolo[3,4-*b*]quinoline (8c). Yield: 49%, solids. ^{1}H NMR ($CDCl_3$): 8.35 (d,

J = 8.7 Hz, 1H), 8.32 (s, 1H), 8.12 (d, J = 8.7 Hz, 1H), 7.80 (t, J = 8.4 Hz, 1H), 7.55 (t, J = 8.4 Hz, 1H), 4.22 (s, 3H).

4.1.19. 1-Methyl-*N*-(4-acetylphenyl)-1*H*-pyrazolo[3,4-*b*]-quinolin-4-amine (9c). Yield: 41%, white solids. ^{1}H NMR (CDCl3): 8.90 (d, J = 9.3 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.75 (t, J = 7.2 Hz, 1H), 7.53 (s, 1H), 7.40–7.30 (m, 3H), 4.17 (s, 3H), 2.63 (s, 3H). Anal. Calcd for $C_{19}H_{16}N_4O$: C, 72.14; H, 5.10; N, 17.71. Found: C, 71.75; H, 5.26; N, 17.47.

4.1.20. 2-(1-Methyl-3-isopropylpyrazol-5-ylamino)benzoic acid (7d). Yield: 97%.

4.1.21. 4-Chloro-1-methyl-3-isopropyl-1*H*-pyrazolo[3,4-*b*] quinoline (8d). Yield: 56%.

4.1.22. *N*-(4-Acetylphenyl)-1-methyl-3-isopropyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (9d). Yield: 32%, orange solids. ^{1}H NMR ($CDCl_3$): 8.10 (d, J = 8.7 Hz, 1H), 7.92 (d, J = 8.7 Hz, 1H), 7.85 (d, J = 7.8 Hz, 2H), 7.71 (t, J = 7.2 Hz, 1H), 7.30 (t, J = 8.7 Hz, 1H), 6.80 (d, J = 8.1 Hz, 2H), 6.69 (s, 1H), 4.18 (s, 3H), 3.30 (m, 1H), 2.55 (s, 3H), 1.58 (s, 3H), 1.35 (d, J = 7.2 Hz, 6H). Anal. Calcd for $C_{22}H_{22}N_4O$: C, 73.72; H, 6.19; N, 15.63. Found: C, 73.71; H, 5.97; N, 15.58.

4.1.23. 2-(1,3-Dimethyl-1*H*-pyrazol-5-ylamino)-5-methylbenzoic acid (7e). Yield: 60%.

4.1.24. 4-Chloro-1,3,6-trimethyl-1*H*-pyrazolo[3,4-*b*]quinoline (8e). Yield: 35%, solids. ^{1}H NMR ($CDCl_3$): 8.10 (s, 1H), 7.96 (d, J = 9.0 Hz, 1H), 7.60 (d, J = 9.0 Hz, 1H), 4.13 (s, 3H), 2.86 (s, 3H), 2.59 (s, 3H).

4.1.25. *N*-(4-Acetylphenyl)-1,3,6-trimethyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (9e). Yield: 53%, white solids. ^{1}H NMR ($CDCl_3$): 8.02 (d, J = 8.4 Hz, 1H), 7.87 (d, J = 8.7 Hz, 2H), 7.73 (s, 1H), 7.57 (d, J = 8.4 Hz, 1H), 6.84 (d, J = 8.7 Hz, 2H), 6.62 (s, 1H), 4.15 (s, 3H), 2.54 (s, 3H), 2.48 (s, 3H), 2.44 (s, 3H). Anal. Calcd for $C_{21}H_{20}N_4O{\cdot}1.3H_2O$: C, 71.69; H, 6.47; N, 15.92. Found: C, 71.81; H, 6.04; N, 15.64.

4.1.26. 2-(1,3-Dimethyl-1*H*-pyrazol-5-ylamino)-3-nitrobenzoic acid (7g). Yield: 54%.

4.1.27. 4-Chloro-1,3-dimethyl-8-nitro-1*H*-pyrazolo[3,4-*b*]quinoline (8g). Yield: 52%, solids. ^{1}H NMR ($CDCl_3$): 8.59 (dd, J_1 = 8.7 Hz, J_2 = 1.2 Hz, 1H), 8.10 (dd, J_1 = 8.7 Hz, J_2 = 1.2 Hz, 1H), 7.54 (m, 1H), 4.13 (s, 3H), 2.89 (s, 3H).

4.1.28. 1,3-Dimethyl-8-nitro-*N*-(4-propionylphenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (9g). Yield: 57%, brown solids. ^{1}H NMR ($CDCl_3$): 8.05 (dd, J_1 = 8.7 Hz, J_2 = 1.5 Hz, 1H), 7.99 (dd, J_1 = 8.7 Hz, J_2 = 1.5 Hz, 1H), 7.89 (d, J = 9.0 Hz, 2H), 7.23 (m, 1H), 6.99 (s, 1H), 6.88 (d, J = 9.0 Hz, 2H), 4.08 (s, 3H), 2.93 (q, J = 7.8 Hz, 2H), 2.48 (s, 3H), 1.25 (t, J = 7.8 Hz, 3H). Anal. Calcd for $C_{21}H_{19}N_5O_3$: C, 64.77; H, 4.92; N, 17.98. Found: C, 65.26; H, 5.25; N, 17.30.

4.1.29. 4-(4-Acetylphenoxy)-1,3-dimethyl-1*H*-pyrazolo-[3,4-*b*]quinoline (10). A mixture of **5** (0.12 g, 0.50 mmol) and 4′-hydroxyacetophenone (0.68 g, 5.0 mmol) was heated at 110 °C for 3 h. It was cooled to room temperature and the mixture was purified by column chromatography (Hexane/EtOAc 3:1) to give 0.095 g (57%) of **10** as white solids. ^{1}H NMR ($CDCl_3$): 8.11 (d, J = 8.7 Hz, 1H), 8.01 (d, J = 9.0 Hz, 1H), 7.92 (m, 2H), 7.43 (t, J = 8.7 Hz, 1H), 7.35 (d, J = 8.1 Hz, 1H), 6.93 (m, 2H), 4.17 (s, 3H), 2.55 (s, 3H), 2.39 (s, 3H). Anal. Calcd for $C_{20}H_{17}N_3O_2$: C, 71.33; H, 5.27; N, 12.48. Found: C, 71.61; H, 5.58; N, 12.70.

4.1.30. 4-(4-Methoxyphenylthio)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoline (11). A mixture of **5** (0.12 g, 0.50 mmol) and 4-methoxybenzenethiol (0.42 g, 1.5 mmol) was heated at 110 °C for 3 h. It was cooled to room temperature and the mixture was purified by column chromatography (Hexane/EtOAc 3:1) to give 0.068 g (41%) of **11** as solids. ^{1}H NMR ($CDCl_3$): 8.60 (d, J = 8.4 Hz, 1H), 8.09 (d, J = 7.8 Hz, 1H), 7.72 (t, J = 8.1 Hz, 1H), 7.41 (t, J = 8.1 Hz, 1H), 7.04 (d, J = 8.4 Hz, 2H), 6.76 (d, J = 8.4 Hz, 2H), 4.17 (s, 3H), 3.71 (s, 3H), 2.81 (s, 3H). Anal. Calcd for $C_{19}H_{17}N_3OS$: C, 68.03; H, 5.11; N, 12.53. Found: C, 68.30; H, 5.44; N, 12.56.

4.1.31. *N*-1,3-Trimethyl-*N*-(4-(methoxycarbonyl)phenyl)-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (12). To a solution of **6f** (40 mg, 0.12 mmol) in DMF (2 mL) kept at 0 °C was added sodium hydride (4.0 mg, 0.17 mmol), followed by dropwise addition of methyl iodide (98 mg, 0.69 mmol). It was stirred at room temperature for 0.5 h and was diluted with ethyl ester (30 mL). The solution was washed with water (3× 10 mL), dried, and concentrated to dryness, and the residue was purified by column chromatography (Hexane/EtOAc 3:1) to give 33 mg (80%) of **12** as solids. ^{1}H NMR ($CDCl_3$): 8.18 (d, J = 1.8 Hz, 1H), 8.05 (bs, 1H), 7.90 (d, J = 1.8 Hz, 1H), 7.73 (t, J = 8.1 Hz, 1H), 7.70 (bs, 1H), 7.38 (t, J = 7.2 Hz, 1H), 7.05 (bs, 1H), 6.05 (bs, 1H), 4.20 (s, 3H), 3.85 (s, 3H), 3.55 (s, 3H), 2.31 (s, 3H). Anal. Calcd for $C_{21}H_{20}N_4O_2$: C, 69.98; H, 5.59; N, 15.55. Found: C, 69.65; H, 5.56; N, 15.20.

4.2. Caspase activation assay (EC_{50})

T47D human breast cancer cells, HCT116 colon cancer cells, and SNU398 liver cancer cells were grown according to media component mixtures designated by American Type Culture Collection in RPMI-1640 +10% FCS in a 5% CO_2–95% humidity incubator at 37 °C. Cells were harvested using trypsin and washed at 600 g and resuspended at 0.65×10^6 cells/mL into RPMI media +10% FCS. An aliquot of 22.5 μL of cells was added to a well of a 384-well microtiter plate containing 2.5 μL of 0.05–100 μM of test compound in RPMI-1640 containing 25 mM HEPES media solution with 10% DMSO (0.005–10 μM final). An aliquot of 22.5 μL of cells was added to a well of a 384-well microtiter plate containing 2.5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample. The samples were then incubated at

37°C for 24 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of a solution containing 14 μM of *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110 fluorogenic substrate in caspase buffer was added. The samples were incubated at 37 °C. Using a fluorescent plate reader (Model Spectrafour Plus Tecan), an initial reading ($T = 0$) was made approximately 1–2 min after addition of the substrate solution employing excitation at 485 nm and emission at 525 nm, to determine the background fluorescence of the control sample. After the 3-h incubation, the samples were read for fluorescence as above ($T = 3$ h).

4.2.1. Calculation. The relative fluorescence unit values (RFU) were used to calculate the sample readings as follows. The activity of caspase activation was determined by the ratio of the net RFU value for the test compound to that of control samples. The EC_{50} (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The caspase activation activity (EC_{50}) in the three cancer cell lines, T47D, HCT116, and SNU398 is summarized in Table 1.

4.3. Cell growth inhibition assay (GI_{50})

Cells were grown and harvested as described above. An aliquot of 45 μL of cells (4.4×10^4 cells/mL) was added to a well of a 96-well microtiter plate, then 5 μL of 0.01–100 μM of test compound (0.001–10 μM final concentration) in RPMI-1640 media solution with 10% DMSO was added. An aliquot of 45 μL of cells was added to a well of a 96-well microtiter plate containing 5 μL of RPMI-1640 media solution with 10% DMSO and without test compound as the control sample for maximal cell proliferation (L_{max}). The samples were then incubated at 37 °C for 48 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of CellTiter-Glo reagent (Promega) was added. The samples were mixed by agitation and incubated at room temperature for 10–15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument) to give L_{test} value.

Baseline for GI_{50} (dose for 50% inhibition of cell proliferation) of initial cell numbers was determined by adding an aliquot of 45 μL of cells and 5 μL of RPMI-1640 media solution with 10% DMSO to wells of a 96-well microtiter plate. The samples were then incubated at 37 °C for 0.5 h in a 5% CO_2–95% humidity incubator. After incubation, the samples were removed from the incubator and 25 μL of CellTiter-Glo reagent (Promega) was added. The samples were mixed by agitation and incubated at room temperature for 10–15 min. Luminescence was read as above to give L_{start} value, defining luminescence for initial cell number used as baseline in GI_{50} determinations.

4.3.1. Calculation. GI_{50} (dose for 50% inhibition of cell proliferation) is the concentration where $[(L_{test} - L_{start})/(L_{max} - L_{start})] = 0.5$. The GI_{50} (μM) values for T47D, HCT116, and SNU398 cells are summarized in Table 2.

4.4. Measurement of apoptosis by flow cytometry

Briefly, T47D cells were treated with 50 nM of compound **6b** and incubated for 24 h or 48 h at 37 °C. Control cells were treated with the solvent (DMSO). After the 24-h or 48-h incubation, cells were treated with propidium iodide and analyzed on a flow cytometer. All flow cytometry analyses were performed on FACScalibur (Becton–Dickinson) using Cell Quest analysis software. Fluorescence intensity is plotted on the *x*-axis and the number of cells with that fluorescence intensity is plotted on the *y*-axis. The sub-diploid amount of DNA is indicative of apoptotic cells that have undergone DNA degradation or fragmentation.

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