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2-Aroylquinoline-5,8-diones as potent anticancer agents displaying tubulin and heat shock protein 90 (HSP90) inhibition†

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This study reports the synthesis of a series of 2-aroylquinoline-5,8-diones (**11–23**) on the basis of scaffold hopping. The presence of a methoxy group at C6 assists the highly regioselective incorporation with various amines, and simplifies the structural identification process. Among the synthetic compounds, 6-dimethylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (**12**) and 7-pyrrolidin-1-yl-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (**23**) exhibit remarkable anti-proliferative activity against the cancer cell lines tested with mean IC_{50} values of 0.14 and 0.27 μM, respectively. Compound **23** showed moderate inhibitory activity against tubulin polymerization with an IC_{50} value of 5.9 μM. In a western blot analysis, **23** caused induction of HSP70 and degradation of Akt, revealing that it possesses HSP90 inhibitory activity.

Introduction

Natural sources have been widely exploited in medicinal chemistry, and natural products provide diverse templates for further structural modification. Streptonigrin (**1**) was isolated from *Streptomyces flocculus*,[1] and recognized in 1973 as a potent antitumor and antiviral agent.[2] Lavendamycin (**2**), which has the same quinoline-5,8-dione core as streptonigrin, and is derived from *Streptomyces lavendulae* has been found to exhibit antimicrobial and antitumor properties.[3] The quinoline-5,8-dione skeleton in streptonigrin (**1**) and lavendamycin (**2**) has triggered much research centered on quinoline-5,8-dione as a component of potentially bioactive agents. 7-Chloro-6-(2-morpholin-4-ylethylamino)quinoline-5,8-dione (DA 3003-2, **3**) for example, was identified as a potential inhibitor of cdc25 phosphatases, which are crucial to the cell cycle progression.[4] 7-Chloro-6-piperidin-1-yl-quinoline-5,8-dione (PT-262, **4**) was found as a new ROCK inhibitor[5] and other quinoline-5,8-diones **5**[6] and **6**[7] have also shown antiproliferative activity (Fig. 1).

Geldanamycin (GA, **7**) is a benzoquinone ansamycin antibiotic isolated from *Streptomyces hygroscopicus*.[8] It was identi-

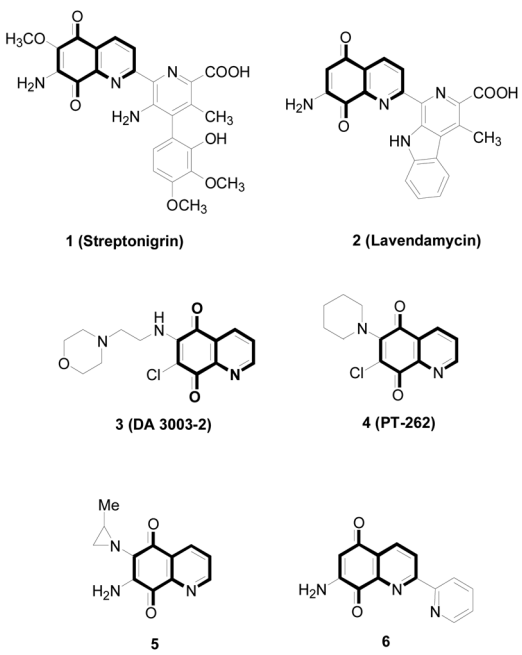

Fig. 1 Natural and synthetic quinonin-5,8-diones.

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fied as an inhibitor of the heat shock protein (HSP90) and entered clinical trials as an anticancer agent in 1994,[9] but the clinical evaluations were hampered by its serious hepatotoxicity.[10] Replacement of the methoxy group in geldanamycin with an allylamino group afforded 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG, **8**) which also displayed HSP90 inhibitory activity[11] and was less toxic.[12] In 2003, Skibo *et al.* modified the 2-aminobenzoquinone moiety of geldanamycin, using the concept of ring expansion to afford a series of quinoline-5,8-diones[13] and continued work demonstrated that compound **9** exhibited an HSP90 response.[14] This result attracted our attention due to the presence in **9** of a 3,4,5-trimethoxybenzene moiety which had also been used in our previous work on 2-aroylquinolines.[15] The continuing studies on compound **10** demonstrated that the carbonyl linkage can be replaced with various groups such as sulfone or sulfide without affecting the compound's cellular activity and pharmacological function.[16] In an attempt to widen the biological window of this series of compounds, the present study explores scaffold hopping in this case, changing the central quinoline to a quinoline-5,8-dione. A series of 2-aroylquinoline-5,8-diones (**11–23**) were synthesized and the effect of substitution on biological activity is examined in this study. Additionally, we provide an efficient synthetic methodology to overcome the regioselectivity of amination of target compounds, without a requirement for advanced instrumental analytical techniques such as COSY and HMBC (Fig. 2).

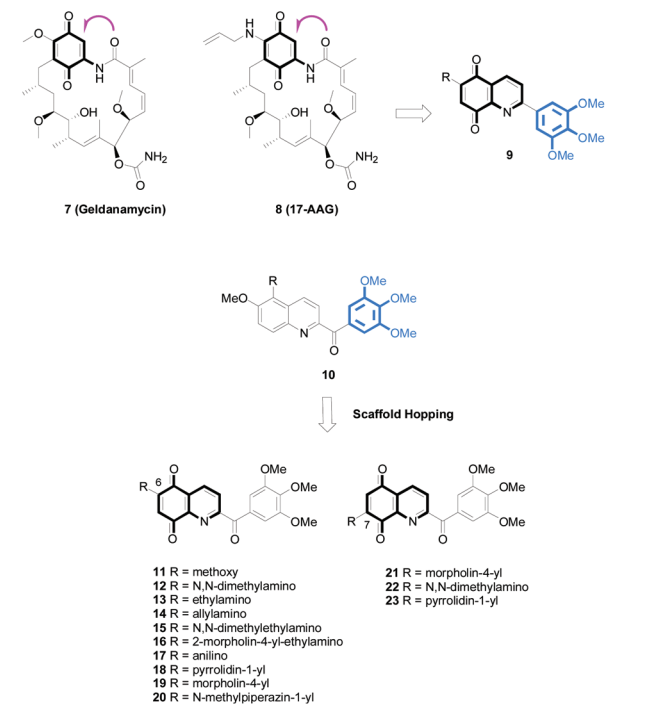


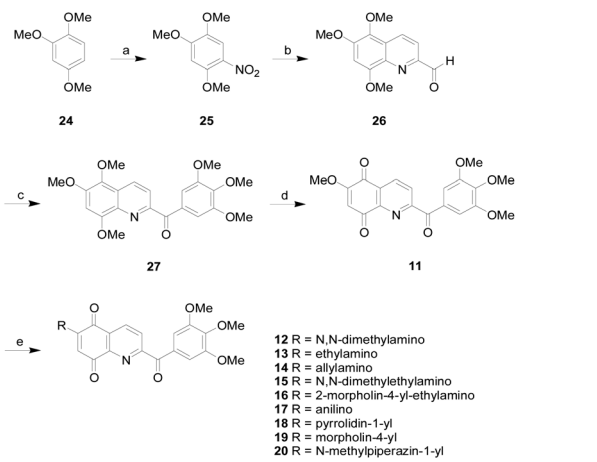
Fig. 2 The diagram of the design of 2-aroylquinoline-5,8-diones (**11–23**).

Results and discussion

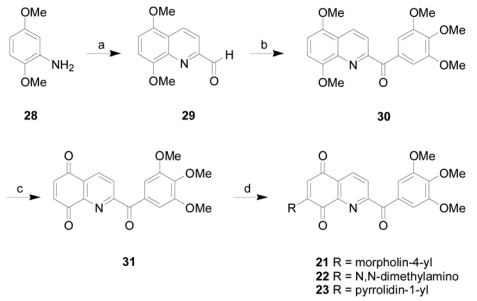
Chemistry

The synthetic routes to 6-substituted 2-aroylquinoline-5,8-diones (**11–20**) are shown in Scheme 1. The commercially available 1,2,4-trimethoxybenzene (**24**) underwent nitration to produce **25** whose nitro group was reduced by Pd/C and H_2. The resulting amine was reacted with crotonaldehyde in the presence of boiling concentrated HCl and then subjected to benzylic oxidation by selenium dioxide to give **26**. The reaction of **26** with 3,4,5-trimethoxyphenylmagnesium bromide followed by the oxidation with pyridinium dichromate (PDC) yielded compound **27**. This was subsequently oxidized by ceric (IV) ammonium nitrate (CAN) to furnish 6-methoxyquinoline-5,8-dione (**11**) which, when subjected to substitution reactions with various amines,[17] yielded compounds **12–20**. The presence of a methoxy group at C6 allows amination specifically at the C6 position without advanced instrumental analysis to confirm the structures of the products.

Scheme 2 describes the synthesis of 7-substituted quinoline-5,8-diones (**21–23**). The starting material, 2,5-dimethoxyaniline (**28**) was reacted with crotonaldehyde and subsequently, selenium dioxide to afford the corresponding aldehyde **29**. This aldehyde was reacted with 3,4,5-trimethoxyphenylmagnesium bromide and then oxidized with pyridinium dichromate (PDC) to furnish **30**. Treatment of **30** with ceric(IV) ammonium nitrate (CAN) provided the quinoline-5,8-dione (**31**) which upon amination, gave compounds **21–23**. Unlike the methodology illustrated in Scheme 1, the absence of C6-OMe resulted in the generation of C6- and C7-substituted regio-isomers. The C7-substituted regio-isomers (**21–23**) were readily purified by column chromatography after the C6-substituted isomers (**12**, **18**, and **19**) were identified.



Scheme 1 Reagents and conditions: (a) HNO_3, AcOH, rt, 86.8%; (b) i. Pd/C, H_2, MeOH, rt; ii. crotonaldehyde, 6 N HCl, toluene, 100–110 °C; iii. SeO_2, *p*-xylene, 100–110 °C, 23.7%; (c) i. 3,4,5-trimethoxyphenylmagnesium bromide, THF, rt; ii. PDC, MS, DCM, rt, 75.9%; (d) CAN, CH_3CN, H_2O, 0 °C, 21.2%; (e) amines, DMF in air, 70–80 °C, 19–57%.



Scheme 2 Reagents and conditions: (a) i. crotonaldehyde, 6 N HCl, toluene, 100–110 °C; ii. SeO_2, *p*-xylene, 100–110 °C, 21.2%; (b) i. 3,4,5-trimethoxyphenylmagnesium bromide, THF, rt; ii. PDC, MS, DCM, rt, 80.1%; (c) CAN, CH_3CN, H_2O, 0 °C, 46.5%; (d) amines, DMF in air, 70–80 °C, 23–87%.

Biological evaluation

***In vitro* cell growth inhibitory activity.** In an attempt to evaluate the effect of various 2-aroylquinoline-5,8-diones on cancer cell inhibition, all the synthetic compounds (**11–23**) and the reference compound 17-AAG (**8**), were evaluated for the antiproliferative activities of three human cancer cell lines: nasopharyngeal carcinoma HONE-1 cells, colon carcinoma HCT116 cells, and cervical carcinoma KB cells. The results are shown in Table 1. Compound **11** shows inhibitory activity comparable to that of 17-AAG, with IC_{50} values of 0.30, 0.19, and 0.36 μM against HONE-1, HCT116, and KB cell lines, respectively. The replacement of –OMe by $–NMe_2$ (**12**) contributes to an improvement of the inhibitory activity in the HCT116 and KB cells. Compound **12** exhibits 2- to 4-fold more cytotoxicity than 17-AAG in inhibiting HONE-1, HCT116, and KB cells' growth with IC_{50} values of 0.3, 0.06, and 0.05 μM, respectively. The linear amines at C6 in **14** and **16** cause a dramatic loss of cellular potency. The 6-NHEt of compound **13** leads to a 6-fold enhancement of inhibitory potency toward HCT116 cells with an IC_{50} value of 0.03 μM, but a 6-fold decrease of inhibitory activity of KB cells as compared with 17-AAG. Compound **15**, with a *N*-(*N*,*N*-dimethylaminoethyl)amino group at C6 exhibits moderate inhibitory activity towards the cancer cells tested with a mean IC_{50} value of 0.67 μM. The cyclic amino groups in compounds **18–20** fail to produce significant improvement of cytotoxicity; compound **19**, with a 6-morpholino group displays moderate inhibitory activity toward HONE-1 and HCT116 cells, with IC_{50} values of 0.30 and 0.45 μM, respectively. The shift of $–NMe_2$ from C6 (**12**) to C7 (**22**) leads to a minor decrease in activity and the 7-morpholino group in **21** causes a 1- to 6-fold decrease of antiproliferative activity as compared with compound **19**. In contrast, the pyrrolidin-1-yl group in compound **23** causes a 6- to 10-fold increase in the inhibition of the growth of HCT116 and KB cells with IC_{50} values of 0.07 and 0.18 μM, respectively.

Inhibition of tubulin polymerization and colchicine binding activity. To investigate the relationship of the synthetic products in the current study with the microtubule system, compounds **12**, **15**, **22**, **23**, and reference compounds colchicine and CA4 were evaluated for tubulin polymerization inhibitory activity, producing the results shown in Table 2. Among the compounds tested, **23** shows the greatest activity in inhibiting tubulin polymerization (IC_{50} = 5.9 μM), but it is less potent than colchicine and CA4. As shown in Fig. 3, compound **23** inhibits polymerization of pure MAP-rich tubulins in a concentration-dependent manner and disrupts tubulin assembly with an IC_{50} value of 5.9 μM.

Effect on HSP90 client proteins. Inhibition of HSP90 activity results in the induction of HSP70 and degradation of Akt.[19,20] Six compounds (compounds **11**, **12**, **13**, **15**, **19**, and **23**) and reference compound **8** were tested for their interaction with these two client proteins of HSP90 (Fig. 4). Fig. 4A shows that compound **23** causes dose-independent induction of HSP70 but the other compounds have no significant influence. Cell treatment with compound **23** also leads to the degradation of Akt in a concentration-dependent manner (Fig. 4B). Thus compound **23** appears to be able to inhibit the function of HSP90.

Table 1 Antiproliferative activity of compounds **11–23**

	IC_{50} ± SD[a] (μM)		
Compd	HONE-1	HCT116	KB
11	0.30 ± 0.10	0.19 ± 0.02	0.36 ± 0.04
12	0.30 ± 0.14	0.06 ± 0.03	0.05 ± 0.03
13	0.39 ± 0.18	0.03 ± 0.02	1.19 ± 1.6
14	1.04 ± 0.01	2.13 ± 0.66	1.31 ± 0.22
15	0.53 ± 0.09	0.86 ± 0.08	0.62 ± 0.07
16	7.27 ± 2.6	1.91 ± 1.28	1.89 ± 0.14
17	>50	>50	43.6 ± 8.93
18	12.2 ± 3.2	12.4 ± 2.12	19.2 ± 0.52
19	0.30 ± 0.00	0.45 ± 0.23	1.74 ± 0.01
20	37.3 ± 2.0	33.5 ± 4.12	35.7 ± 6.93
21	1.85 ± 0.01	0.81 ± 0.14	1.82 ± 0.05
22	1.42 ± 0.13	0.05 ± 0.01	0.18 ± 0.01
23	0.55 ± 0.07	0.07 ± 0.03	0.18 ± 0.01
17-AAG (**8**)	0.76 ± 0.21	0.20 ± 0.04	0.20 ± 0.16

[a] SD: standard deviation, all experiments were independently performed at least three times.

Table 2 Inhibition of tubulin polymerization by compounds **12**, **15**, **22**, **23**, and CA4

Compd	Tubulin[a] IC_{50} ± SD (μM)
12	8.9
15	>10
22	6.2
23	5.9
Colchicine[b]	4.2
CA4[c]	1.9

[a] Inhibition of tubulin polymerization. [b] Data from ref. 18. [c] Data from ref. 16.

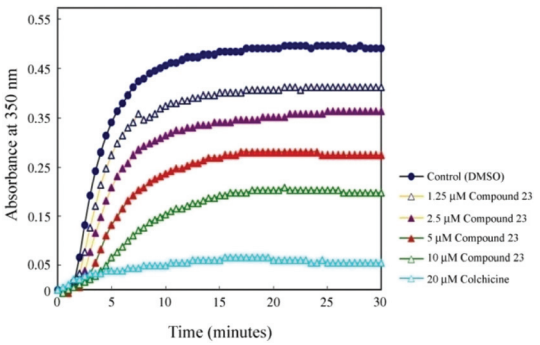


Fig. 3 Effect of compound **23** on *in vitro* tubulin polymerization. MAP-rich tubulins were incubated at 37 °C in the absence [dimethyl sulfoxide (DMSO) control] or presence of drugs (colchicine or serial concentrations of compound **23**). The absorbance at 350 nm was measured every 30 s for 30 min and is presented as the increased polymerized microtubule.

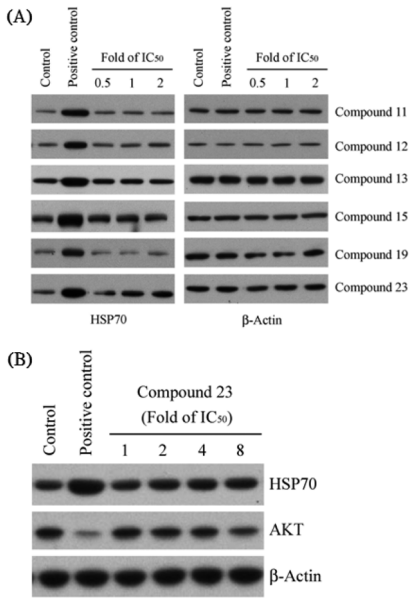


Fig. 4 Investigation of the effect of tested compounds on the expression level of HSP70 and AKT proteins in human nasopharyngeal carcinoma HONE-1 cells. The HSP90 inhibitory activity of test compounds was assessed by HSP70 induction (A) and HSP90 client protein AKT degradation (B) in HONE-1 cells. Cells were treated with various concentrations of compounds **11**, **12**, **13**, **15**, **19**, or **23** for 24 hours. Cell lysates were prepared after the treatment and subjected to western blot analysis for assessment of HSP70 and AKT protein levels. β-Actin was used as a loading control. Cells treated with 1 μM of compound **8** (17-AAG) was used as a positive control in this study.

Conclusions

We have synthesized a series of 2-aroylquinoline-5,8-diones (**11–23**). The highly regioselective incorporation of compound **11** with various amines assists in the straightforward generation of the designed compounds (**12–20**). In antiproliferation inhibition assays, compounds **12** and **23** are more potent than 17-AAG. Compounds **12** and **23** inhibit the growth of cancer cells tested with mean IC_{50} values of 0.14 and 0.27 μM, respectively. Compound **23** inhibits tubulin polymerization with an IC_{50} value of 5.9 μM. Compound **23** also induces the expression of HSP70 and triggers the degradation of Akt in a dose-dependent manner. These results indicate that these 2-aroylquinoline-5,8-diones are potential anticancer agents with novel promising pharmacological mechanisms.

Experimental section

Chemistry

Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were obtained with a Bruker Fourier 300 and DRX-500 spectrometers, with chemical shifts in parts per million (ppm, δ) downfield from TMS as an internal standard. High-resolution mass spectra (HRMS) were recorded with a FINNIGAN MAT 95S mass spectrometer. Purity of the final compounds was determined using a Hitachi 2000 series HPLC system using a C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm, 4.6 mm × 150 mm) with the solvent system (elution conditions: mobile phase A consisting of MeCN; mobile phase B consisting of H_2O containing 0.1% formic acid + 10 mmol NH_4OAc) and was found to be ≥95%. Flash column chromatography was performed using silica gel (Merck Kieselgel 60, no. 9385, 230–400 mesh ASTM). All reactions were carried out under an atmosphere of dry nitrogen.

6-Methoxy-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (11). A solution of **27** (0.2 g, 0.48 mmol) in ACN (15 mL) was placed in a round-bottomed flask which was equipped with an addition funnel. A solution of CAN (1.0 g, 1.8 mmol) in H_2O (10 mL) was added to the funnel. The solution was added slowly into the round-bottomed flask at room temperature, and then the resulting solution was stirred for 1 h. The reaction was quenched with H_2O (30 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The organic layer was collected and purified by column chromatography (EtOAc/*n*-hexane = 2 : 1) to afford **11** (0.1 g, 48.4%) as a red solid. mp = 174–175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.96 (s, 6H), 3.97 (s, 3H), 3.98 (s, 3H), 6.43 (s, 1H), 7.86 (s, 2H), 8.41 (d, J = 8.5 Hz, 1H), 8.65 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.45, 56.95, 61.14, 109.60, 111.31, 128.10, 128.85, 130.17, 136.27, 143.58, 146.32, 152.99, 159.33, 160.55, 179.19, 182.25, 188.92. HRMS (ESI) for $C_{20}H_{17}NNaO_7$ ($M + Na^+$) calcd 406.0903, found 406.0906. HPLC purity of 100.0% (retention time = 32.31).

6-Dimethylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (12). A solution of **11** (0.04 g, 0.1 mmol), *N*,*N*-dimethylamine (40% w/v, 0.015 mL, 0.13 mmol), and DMF (1 mL) was stirred at 70 °C for 1 h. The reaction was quenched with H_2O (20 mL) and extracted with EtOAc (30 mL × 3). The organic layer was collected and purified by column chromatography (EtOAc/*n*-hexane = 2 : 1) to afford **12** (0.02 g, 48.4%) as a red solid. mp = 202–203 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.98–3.00 (m, 6H), 3.96 (s, 9H), 5.98 (s, 1H), 7.89 (s, 2H), 8.31 (d, J = 8.1 Hz, 1H), 8.57 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$)

δ 29.55, 29.93, 56.58, 61.21, 102.63, 109.75, 127.13, 128.38, 130.46, 135.73, 143.55, 148.14, 148.90, 153.06, 159.48, 180.43, 181.27, 189.25. HRMS (ESI) for $C_{21}H_{20}N_2NaO_6$ (M + Na^+) calcd 419.1219, found 419.1222. HPLC purity of 100.0% (retention time = 31.23).

6-Ethylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (13). The title compound was obtained in 57.1% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 180.9–181.7 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.38 (t, J = 7.5 Hz, 3H), 3.27–3.33 (m, 2H), 3.96 (s, 3H), 3.97 (s, 6H), 5.86–5.90 (m, 1H), 5.99 (s, 1H), 7.91 (s, 2H), 8.32 (d, J = 8.0 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.60, 37.77, 56.43, 61.13, 100.97, 109.57, 129.24, 130.38, 131.94, 135.83, 143.37, 145.27, 148.58, 152.92, 157.61, 179.64, 180.82, 189.09. HRMS (ESI) for $C_{21}H_{19}N_2O_6$ (M − H) calcd 395.1243, found 395.1253. HPLC purity of 99.33% (retention time = 29.83).

6-Allylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (14). The title compound was obtained in 56.3% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 167.5–168.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (t, J = 5.7 Hz, 2H), 3.96 (s, 9H), 5.30–5.38 (m, 2H), 5.83–5.97 (m, 1H), 6.01 (s, 1H), 6.06 (t, J = 5.7 Hz), 7.89 (s, 2H), 8.32 (d, J = 8.1 Hz, 1H), 8.56 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 45.09, 56.35, 60.97, 103.21, 109.53, 118.65, 126.92, 128.19, 130.22, 131.23, 135.50, 143.29, 147.56, 147.74, 152.80, 159.18, 180.33, 181.04, 188.94. HRMS (ESI) for $C_{22}H_{20}N_2NaO_6$ (M + Na^+) calcd 431.1219, found 431.1227. HPLC purity of 97.40% (retention time = 30.48).

6-(3-Dimethylamino-propylamino)-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (15). The title compound was obtained in 29.6% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 151.2–151.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (t, J = 9.5 Hz, 2H), 2.55 (t, J = 9.5 Hz, 2H), 3.36 (q, J = 9.0 Hz, 2H), 3.99 (s, 3H), 4.00 (s, 6H), 5.98 (s, 1H), 7.94 (s, 2H), 8.00–8.10 (m, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.16, 56.19, 57.12, 60.99, 61.61, 98.60, 109.81, 121.88, 125.37, 130.86, 131.31, 133.70, 135.34, 142.53, 150.68, 151.50, 152.62, 153.80, 190.24. HRMS (ESI) for $C_{24}H_{28}N_3O_6$ (M + H^+) calcd 454.1978, found 454.1973. HPLC purity of 95.23% (retention time = 17.73).

6-(2-Morpholin-4-yl-ethylamino)-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (16). The title compound was obtained in 19.9% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 167.5–168.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.50–2.54 (m, 4H), 2.72 (t, J = 6.0 Hz, 2H), 3.27 (q, J = 6.0 Hz, 2H), 3.74–3.78 (m, 4H), 3.96–3.97 (m, 10H), 5.97 (s, 1H), 7.90 (s, 2H), 8.33 (d, J = 8.1 Hz, 1H), 8.56 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 30.01, 38.76, 53.49, 55.74, 56.67, 61.31, 67.19, 103.06, 109.86, 127.22, 128.55, 130.56, 135.81, 148.03, 153.17, 159.56, 161.08, 180.59, 181.36, 189.31. HRMS (ESI) for $C_{25}H_{28}N_3O_7$ (M + H^+) calcd 482.1927, found 482.1924. HPLC purity of 95.42% (retention time = 17.80).

6-Phenylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (17). The title compound was obtained in 25.9% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.96 (s, 6H), 3.96 (s, 3H), 6.65 (s, 1H), 7.27–7.57 (m, 5H), 7.89 (s, 2H), 8.37 (d, J = 8.0 Hz, 1H), 8.64 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.03, 56.61, 94.28, 106.79, 112.34, 116.74, 121.78, 123.96, 127.25, 129.67, 131.27, 136.05, 136.22, 138.34, 141.38, 142.09, 153.28, 157.99, 171.03, 182.42, 190.30. HRMS (ESI) for $C_{25}H_{20}N_2NaO_6$ (M + Na^+) calcd 467.1219, found 464.1216. HPLC purity of 97.91% (retention time = 33.77).

6-Pyrrolidin-1-yl-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (18). The title compound was obtained in 36.3% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 178.8–179.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.50–1.57 (m, 4H), 2.00–2.10 (m, 4H), 3.97 (4, 9H), 6.02 (s, 1H), 7.91 (s, 2H), 8.31 (d, J = 8.0 Hz, 1H), 8.53 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.62, 56.64, 61.26, 106.82, 109.83, 126.90, 129.43, 130.65, 136.00, 143.51, 147.77, 148.74, 153.08, 159.10, 179.93, 182.50, 189.52. HRMS (ESI) for $C_{23}H_{22}N_2NaO_6$ (M + Na^+) calcd 445.1376, found 445.1372. HPLC purity of 97.99% (retention time = 30.67).

6-Morpholin-4-yl-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (19). The title compound was obtained in 30.6% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp = 172–173 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.58–3.61 (m, 4H), 3.88–3.91 (m, 4H), 3.95 (s, 6H), 3.96 (s, 3H), 6.26 (s, 1H), 7.86 (s, 1H), 8.34 (d, J = 8.0 Hz, 1H), 8.53 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 49.30, 56.45, 61.12, 66.50, 109.61, 112.65, 127.48, 130.29, 136.27, 143.48, 146.55, 152.89, 152.96, 159.05, 181.21, 182.04, 189.20. HRMS (ESI) for $C_{23}H_{22}N_2NaO_7$ (M + Na^+) calcd 461.1325, found 461.1340. HPLC purity of 100.0% (retention time = 31.29).

6-(4-Methyl-piperazin-1-yl)-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (20). The title compound was obtained in 21.2% overall yield from compound **11** in a manner similar to that described for the preparation of **12**: mp 171–172 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.36 (s, 3H), 2.59–2.62 (m, 4H), 3.61–3.64 (m, 4H), 3.95 (s, 6H), 3.96 (s, 3H), 6.27 (s, 1H), 7.86 (s, 2H), 8.33 (d, J = 8.0 Hz, 1H), 8.52 (d, J = 8.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 46.02, 48.97, 54.65, 56.45, 61.10, 109.61, 112.50, 127.36, 130.34, 136.22, 143.43, 146.67, 152.95, 158.96, 181.12, 182.17, 189.27. HRMS (ESI) for $C_{24}H_{25}N_3NaO_6$ (M + Na^+) calcd 474.1641, found 474.1644. HPLC purity of 96.03% (retention time = 18.93).

7-Morpholin-4-yl-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (21). The title compound was obtained in 24.2% overall yield from compound **31** in a manner similar to that described for the preparation of **12**: mp = 171–172 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.62–3.66 (m, 4H), 3.90–3.95 (m, 4H), 4.01 (s, 9H), 6.17 (s, 1H), 7.84 (s, 2H), 8.42 (d, J = 8.1 Hz, 1H), 8.60 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 47.94, 55.15, 59.79, 65.17, 108.20, 109.26, 127.42, 128.73, 128.94, 134.04, 138.54, 142.09, 145.63, 151.63, 152.81, 179.30, 180.15, 188.05. HRMS (ESI) for $C_{23}H_{22}N_2NaO_7$ (M + Na^+) calcd 461.1325,

found 461.1313. HPLC purity of 100.0% (retention time = 31.29).

7-Dimethylamino-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (22). The title compound was obtained in 35.7% overall yield from compound **31** in a manner similar to that described for the preparation of **12**: mp = 212–213 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.29 (s, 6H), 3.97 (s, 9H), 5.97 (s, 1H), 7.80 (s, 2H), 8.36 (d, J = 8.0 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 40.07, 53.64, 58.30, 103.63, 106.72, 125.87, 127.59, 127.83, 132.38, 140.48, 143.88, 150.12, 150.38, 154.70, 177.56, 178.30, 186.64. HRMS (ESI) for $C_{21}H_{20}N_2NaO_6$ ($M + Na^+$) calcd 419.1219, found 419.1224. HPLC purity of 100.0% (retention time = 31.13).

7-Pyrrolidin-1-yl-2-(3,4,5-trimethoxybenzoyl)-quinoline-5,8-dione (23). The title compound was obtained in 33.5% overall yield from compound **31** in a manner similar to that described for the preparation of **12**: mp = 183.2–183.9 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58–1.59 (m, 4H), 2.03–2.07 (m, 4H), 3.96 (s, 6H), 3.97 (s, 3H), 5.88 (s, 1H), 7.81 (s, 2H), 8.37 (d, J = 8.0 Hz, 1H), 8.59 (d, J = 8.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.82, 31.05, 56.43, 61.10, 104.92, 109.53, 128.78, 130.48, 131.13, 135.24, 143.27, 145.98, 149.74, 152.92, 157.32, 180.01, 180.84, 189.54. HRMS (ESI) for $C_{23}H_{22}N_2NaO_6$ ($M + Na^+$) calcd 445.1376, found 445.1377. HPLC purity of 97.64% (retention time = 30.70).

1,2,4-Trimethoxy-5-nitrobenzene (25). A mixture of 1,2,4-trimethoxybenzene (5.0 g, 29.7 mmol), AcOH (150 mL), and HNO_3 (2.0 mL, 47.9 mmol) was stirred for 12 h. The reaction was quenched with H_2O (500 mL), and the resulting product was collected by filtration and washed with a large amount of H_2O to afford **25** as a yellow solid (5.5 g, 86.8%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89 (s, 3H), 3.97 (s, 3H), 3.98 (s, 3H), 6.56 (s, 1H), 7.58 (s, 1H).

5,6,8-Trimethoxy-quinoline-2-carbaldehyde (26). A mixture of compound **25** (4.0 g, 18.8 mmol), Pd/C (0.5 g) and MeOH (100 mL) was stirred under hydrogen for 3 h. The Pd/C was filtered off and the filtrate was concentrated *in vacuo*. The resulting residue was dissolved in 6 N HCl (60 mL) and heated to reflux for 10 min. To the mixture was added a solution of crotonaldehyde (4.0 mL, 48.5 mmol) in toluene (10 mL) and then heated at 110 °C for an additional 6 h. After cooling, the mixture was basified with concentrated NaOH solution in an ice-bath followed by extraction with EtOAc (3 × 300 mL). The organic layers were collected and purified by flash column chromatography over silica gel (EtOAc/*n*-hexane = 1 : 1) to give a crude product. To the product was added SeO_2 (2.8 g, 25.2 mmol) and *p*-xylene (50 mL) and the mixture was then allowed to reflux for 2 h. The mixture was cooled and filtered with a pad of celite. The filtrate was purified by flash column chromatography over silica gel (EtOAc/*n*-hexane = 1 : 1) to give **26** as a yellow solid. (1.1 g, 23.7%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.94 (s, 3H), 4.08 (s, 3H), 4.15 (s, 3H), 6.94 (s, 1H), 8.04 (d, J = 9.0 Hz, 1H), 8.52 (d, J = 8.5 Hz, 1H), 10.25 (s, 1H).

(3,4,5-Trimethoxy-phenyl)-(5,6,8-trimethoxy-quinolin-2-yl)-methanone (27). 3,4,5-Trimethoxyphenylmagnesium bromide (0.5 M in THF, 25 mmol) was added to a solution of **26** (2.6 g, 10.5 mmol) in THF (60 mL) and the mixture was stirred for 3 h. The mixture was then filtered and the filtrate was concentrated *in vacuo* to afford an oily adduct. PDC (5.0 g, 10.3 mmol), molecular sieves (5.0 g), and DCM (50 mL) were added to this residue and then the mixture was stirred at room temperature for 1 h. The mixture was filtered and the filtrate was purified by flash column chromatography over silica gel (EtOAc/*n*-hexane = 1 : 1) to yield **27** (3.3 g, 75.9%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95–4.05 (m, 12H), 4.10 (s, 3H), 4.12 (s, 3H), 6.95 (s, 1H), 8.02 (s, 2H), 8.27 (d, J = 9.0 Hz, 1H), 8.59 (d, J = 8.7 Hz, 1H).

5,8-Dimethoxy-quinoline-2-carbaldehyde (29). A mixture of 2,5-dimethoxyaniline (3.0 g, 19.6 mmol), crotonaldehyde (4.25 mL, 51.5 mmol), 6 N HCl (80 mL), and toluene (10 mL) was heated to reflux for 2 h. After cooling down, the resulting mixture was basified with concentrated NaOH solution followed by the extraction of EtOAc (3 × 300 mL). The organic layers were collected and purified by flash column chromatography to give a crude product. The residue was further reacted with SeO_2 (3.0 g, 27.0 mmol) in the presence of refluxing *p*-xylene (30 mL) for 6 h. The mixture was cooled down and filtered with a pad of celite. The filtrate was purified by flash column chromatography over silica gel (EtOAc/*n*-hexane = 1 : 1) to give **29** (0.9 g, 21.2%) as a yellow solid: mp = 188–191 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.99 (s, 3H), 4.11 (s, 3H), 6.91 (d, J = 8.5 Hz, 1H), 7.04 (d, J = 8.5 Hz, 1H), 8.06 (d, J = 8.5 Hz, 1H), 8.71 (d, J = 8.5 Hz, 1H), 10.31 (s, 1H).

(5,8-Dimethoxyquinolin-2-yl)-(3,4,5-trimethoxyphenyl)-methanone (30). The title compound was obtained in 80.1% overall yield from compound **17** in a manner similar to that described for the preparation of **18**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.96 (s, 6H), 4.00 (s, 3H), 4.02 (s, 3H), 6.99 (d, J = 8.7 Hz, 1H), 7.01 (d, J = 8.7 Hz, 1H), 7.99 (s, 2H), 8.21 (d, J = 8.7 Hz, 1H), 8.73 (d, J = 8.7 Hz, 1H).

2-(3,4,5-Trimethoxybenzoyl)-quinoline-5,8-dione (31). The title compound was obtained in 46.5% overall yield from compound **17** in a manner similar to that described for the preparation of **18**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (d, J = 8.1 Hz, 1H), 8.49 (d, J = 8.1 Hz, 1H), 7.90 (s, 2H), 7.25 (s, 1H), 7.20 (s, 1H), 4.01 (s, 3H), 4.00 (s, 6H).

Biology

Reagents for cell culture were obtained from Gibco-BRL Life Technologies (Gaitherburg, MD). Microtubule-associated protein (MAP)-rich tubulin was purchased from Cytoskeleton, Inc. (Denver, CO). [^{3}H]Colchicine (specific activity, 60–87 Ci mmol^{-1}) was purchased from PerkinElmer Life Sciences (Boston, MA).

Cell growth inhibitory assay. Human nasopharyngeal carcinoma HONE-1, cervical carcinoma KB, and colorectal carcinoma HCT116 cells were grown in Dulbecco's modified Eagle's medium, minimal essential medium, or RPMI 1640 medium. All cell cultures were supplemented with 10% fetal bovine serum, 2 μM glutamine, 100 units per mL penicillin, and 100 μg mL^{-1} streptomycin and incubated under a humidified atmosphere (95% air and 5% CO_2) at 37 °C. *In vitro*

growth inhibition was assessed with the methylene blue assay.[21] Exponentially growing cells were seeded into 24-well culture plates at a density of 8000–20000 cells per mL per well (depending on the doubling time of the cell line) and allowed to adhere overnight. The cells were incubated with various concentrations of drugs for 72 h. Then the A595 of the resulting solution from 1% *N*-lauroylsarcosine extraction was measured. The IC_{50} was calculated on the basis of the A595 of untreated cells (taken as 100%). The values shown are the means and standard errors of at least three independent experiments performed in duplicate.

Tubulin polymerization *in vitro* assay.[22,23] Turbidimetric assays of microtubules were performed as described by Bollag *et al.*[24] In brief, microtubule-associated protein (MAP)-rich tubulin (from bovine brain, Cytoskeleton, Inc.) was dissolved in reaction buffer [100 mM PIPES (pH 6.9), 2 mM $MgCl_2$, and 1 mM GTP] to prepare a 4 mg mL^{-1} tubulin solution. The tubulin solution (240 μg of MAP-rich tubulin per well) was placed in a 96-well microtiter plate in the presence of test compounds or 2% (v/v) DMSO as a vehicle control. The increase in absorbance at 350 nm was measured in a PowerWave X microplate reader (BIO-TEK Instruments, Winooski, VT) at 37 °C and recorded every 30 s for 30 min. The area under the curve (AUC) was used to determine IC_{50}. The AUCs of the untreated control and 10 μM colchicine were set to 100 and 0% polymerization, respectively, and the IC_{50} was calculated by nonlinear regression in at least three experiments.

Tubulin competition binding scintillation proximity assay.[25–27] This assay was performed in a 96-well plate. Briefly, 0.08 μM [^{3}H]colchicine was mixed with the test compound and 0.5 μg of special long-chain biotin-labeled tubulin (0.5 μg) and then incubated in 100 μL of reaction buffer [80 mM PIPES (pH 6.8), 1 mM EGTA, 10% glycerol, 1 mM $MgCl_2$, and 1 mM GTP] for 2 h at 37 °C. Then 80 μg of streptavidin-labelled SPA beads was added to each reaction mixture. The radioactive counts were then directly measured with a scintillation counter and the inhibition constant (K_i) was calculated using the Cheng–Prusoff equation.[28]

Western blot analysis.[22,23] HONE-1 cells were initially seeded at a density of 1×10^6 in 100 mm^2 dishes. After overnight culture, the cells were treated with equal potent concentrations, indicated as 0.5, 1, and 2 fold of IC_{50}, of tested compounds for 24 h. Adherent cells were collected and lysed with the CelLytic™ M cell lysis reagent (Sigma-Aldrich, St Louis, MO) containing 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor (Roche, Indianapolis, IN) for whole cell lysate preparation. Equal amounts of lysate (on a protein basis) were separated by SDS-PAGE, blotted on polyvinylidene difluoride membranes, conjugated with various specific primary antibodies, and then probed with appropriate secondary antibodies. The immunoreactive bands were detected using the enhanced chemiluminescence method and visualized on a Kodak Bio MAX MR film. Primary antibodies against heat shock protein 70 (HSP70) and AKT proteins were purchased from Cell Signalling Technology (Danvers, MA, USA). Primary antibody against β-actin protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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Notes and references

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