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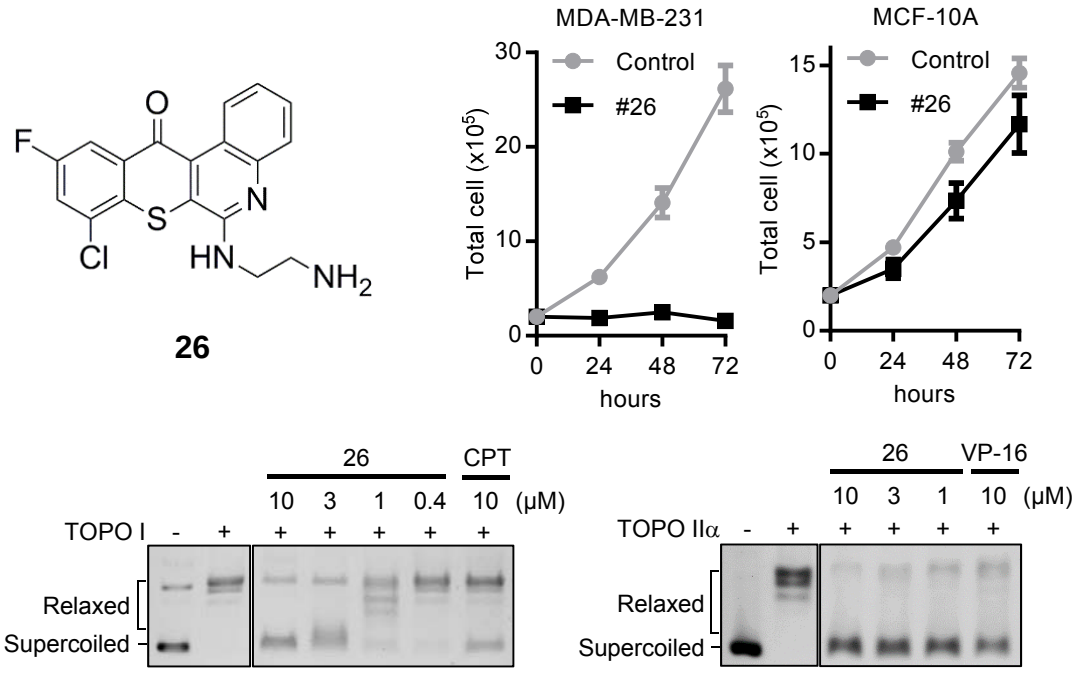
Graphic Abstract



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Design, synthesis, and biological evaluation of heterotetracyclic quinolinone derivatives as anticancer agents targeting topoisomerases

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Abstract

A series of thiochromeno[2,3-*c*]quinolin-12-one derivatives with various substitutions were synthesized and evaluated as topoisomerase (Topo) inhibitors. Six (**8**, **10**, **12**, **14**, **19**, and **26**) of 23 compounds showed strong inhibitory activities against Topo-mediated DNA relaxation and proliferation of five human cell lines including breast (MDA-MB-231, MDA-MB-468 and MCF7), colorectal (HCT116) and non-small cell lung (H1299) cancers. Among these, compounds **14** and **26** exhibited full inhibitory activities against Topo I at 3 μM and Topo IIα at 1 μM. Cancer cells treated with **26** accumulated DNA damage and were arrested at the G_2/M phase. With time, cells proceeded to apoptosis, as revealed by increased amounts of cells with fragmented DNA and cleavage of caspase-8 and -9. In contrast, normal breast epithelial cells showed low sensitivity to **26**. Taken together, our study identifies **26** as a potent Topo dual-inhibitor with low toxicity to normal cells, and elucidates that the terminal amino group of *N*-2-aminoethylamino or *N*-3-aminopropylamino at the 6^{th} position and 8,10-di-halogen substituents on thiochromeno[2,3-*c*]quinolin-12-one are critical for the Topo-inhibiting and cancer-killing activities.

Keywords: thiochromeno[2,3-*c*]quinolin-12-one, topoisomerase, dual inhibitor

1. Introduction

Cancer is defined as malignant cells that divide out of control and can invade and spread to other tissues. Cancer cells thus possess high levels of stress generated from various cellular metabolic pathways that are essential for cell growth. To bypass these impediments for survival, cancer cells highly rely on mechanisms that can overcome such problems [1]. During genome replication, segregation, transcription and recombination, the super-helical doubled-stranded DNA needs to be unwound for factor accessibility. This in turn triggers over-winding of downstream DNA that prevents the metabolic event from moving forward [2]. Topoisomerases (Topos) are the key factors that resolve the topologic stresses. In mammals, Topo I and II respectively create single- and double-strand DNA breaks, which allows the passage or rotation of the unbroken DNA through sites of breakage and thus relieves the stresses due to DNA hyper-winding. Through transesterification, Topo transiently forms a covalent binary complex with one broken end of DNA. After supercoil disengagement, another broken end of DNA nucleophilically attacks the protein-DNA bond, followed by re-ligation of the two DNA ends and release of Topo [2, 3].

Given that suppressing Topo activity can hinder genome functions, jeopardize chromosome stability, and therefore trigger deleterious cytotoxicity, several agents targeting Topos have been developed and used in clinic as cancer therapeutics [4, 5].

Among these agents, Topo poisons disturb Topo functions by binding and stabilizing the covalently linked enzyme-DNA complex to limit DNA re-ligation. This generates toxic DNA lesions in collision with replication/transcription machineries or when chromosomes are segregating, ultimately leading to cell apoptosis ([3]and references therein). Many Topo poisons, such as camptothecin (CPT), podophyllotoxin and anthracycline, are derived from natural products. CPT is a well-known Topo I-specific poison with a planar pentacyclic structure. However, its insolubility and instability have limited its clinical evaluation [6, 7]. Subsequently synthesized water-soluble analogs irinotecan and topotecan were approved by the US Food and Drug Administration (FDA), but the dose-dependent toxicities like neutropenia and diarrhea are still observed [7]. FDA-approved Topo II poisons include etoposide/teniposide and doxorubicin/daunorubicin, which respectively are podophyllotoxin and anthracycline drugs [7]. Although these drugs have been in long-term use in cancer chemotherapeutics, severe cardiac toxicity and secondary malignancies, possibly resulting from inhibition of the Topo IIβ isoform and generation of reactive oxygen species, are the main challenges in their use as medications [8-10]. The emerging Topo II inhibitor pixantrone, an anthracenedione agent structurally closed to mitoxantrone, exhibits a higher inhibitory activity toward Topo IIα than IIβ and a reduced iron-binding ability [11, 12], potentially providing a

safer way to avoid side effects that result from anthracycline treatment.

Several studies have made great efforts to discover agents with dual Topo-inhibitory activities [13-16]. Topopyrones, the anthraquinone polyphenols initially identified from fungal culture broths, possess poisonous activity toward Topo I and IIα, which relies on the orientation of the 1,4-pyrone ring moiety and the chloro substituent at C7 [14, 17, 18]. In addition, the naturally occurring quinazoline-carboline alkaloid evodiamine and its derivative, thio-evodiamine, were shown to interfere with relaxation of supercoiled DNA by both Topos probably through a non-poison mechanism [19, 20]. Recently, Chen et al. synthesized and evaluated a series of tetracyclic heterocyclic azathioxanthone compounds [13, 21-23]. Most of these compounds, unfortunately, exhibited weak Topo-inhibitory activities and no or low toxicity in over 60 tested cell lines. Only 10-chloro-6-(piperazin-1-yl)-12*H*-thiochromeno[2,3-*c*]quinolin-12-one (CPTQ) showed a significant cytotoxicity, with IC_{50} values of 5.01 ± 1.68 μM in DU-145 cells and 2.84 ± 0.64 μM in PC-3 cells. CPTQ displayed dual inhibitory activities at 10 and 25 μM to Topo I and IIα, respectively [13]. According to clues, further structural optimization is thus required for new drug development.

In this study, we based on the scaffold structure of CPTQ to design and synthesize a series of thiochromeno[2,3-*c*]quinolin-12-one derivatives with various

substituents. A variety of substituents are summarized in Figure 1 and Chart 1. Our work reveals that the amino end (-NH_2) of the ethyl/propyl chain substituted at the 6^{th} position together with 8,10-dual halogen substituents contribute to the high cytotoxicity and dual Topo inhibition. This study also discoveries **26** as a potent anticancer agent with low toxicity to normal cells.

2. Chemistry

The synthesis processes of target compounds **5**~**27** were shown in Scheme 1. The starting compound, 2- or 4-substituted thiophenol, was reacted with chloroacetic acid in NaOH (aq) to give substituted phenylthioacetic acids **2a**~**2i**. The phenylthioacetic acid compounds were treated with isatin in the presence of sodium acetate in the process of the Pfitzinger reaction while being heated to 150 °C for 1~5 h to obtain intermediate quinoline-4-carboxylic acids **3a**~**3i**. These intermediates, thiochromeno[2,3-*c*]quinolin-12-ones **4a**~**4i**, were produced from cyclization and chlorination of quinolone-4-carboxylic acids reacted with $POCl_3$. The title compounds **5** and **7**~**27** were afforded *via* substitution of compounds **4a**~**4i** with various appropriate amines in dimethyl sulfoxide. Compound **6** was yielded from compound **5** via debenzylation by trifluoroacetic acid (TFA), followed by an amide coupling reaction. Analytical purification of each product was achieved by silica gel

chromatography. All new compounds were characterized and determined by nuclear magnetic resonance (NMR), High-resolution mass spectrometry (HRMS), and high pressure liquid chromatography (HPLC). The yields of title compounds **5**~**27** from compounds **4a**~**4i** are listed in Chart 1. We observed that compounds **26** and **27** had low yields, which may be because of the presence of dual halogens, F and Cl, on C8 and C10, respectively. Given that these atoms belong to deactivating groups, the reactivity is typically lower than other substituents, such as methyl and methoxy. This probably makes it difficult to produce compounds possessing such substituents, especially ones possessing both, like **26** and **27**. Moreover, to obtain purified products, we needed to repeat the purification process several times, which further lowered the yields of these compounds.

3. Biological evaluation

3.1. Quick compound selection

Our synthesized thiochromeno[2,3-c]quinolin-12-one derivatives were previously found to suppress FMS-like tyrosine kinase 3 (FLT-3), a receptor tyrosine kinase [24]. However, their inhibitory activities toward FLT-3 in vitro did not correlate with the cytotoxicities ([24] and unpublished data), suggesting that FLT-3 is not the main target of these compounds in cells. CPTQ, the compound with a

similar scaffold structure as our synthesized compounds, was reported to inhibit Topo I at 10 μM in vitro [13]. We thus evaluated our compounds by determining the efficiency of in vitro Topo I-mediated relaxation of the supercoiled plasmid DNA at 10 μM. CPT was used as a positive control in this assay. As shown in Figure 2A, six (**8**, **10**, **12**, **14**, **19**, and **26**) of our designed compounds almost completely suppressed Topo I activity, whereas CPT exhibited only a partial inhibitory activity against Topo I at 10 μM.

We then assayed the in vitro cytotoxicity of our compounds. We primarily focused on triple-negative breast cancers (TNBCs) as they are often highly malignant and lack efficient therapeutics. MDA-MB-231 cells were treated with 10 μM of each compound, followed by an analysis of cell survival through measuring the cellular NAD(P)H-dependent oxidoreductase activity. As shown in Figure 2B, about three quarters of these thiochromeno[2,3-*c*]quinolin-12-one derivatives exhibited moderate cytotoxic activities. Seven (**6**, **11**, **13**, **15**, **16**, **22**, and **23**) were able to reduce <20% of cell viability, whereas ten (**5**, **7**, **9**, **17, 18**, **20**, **21**, **24**, **25**, and **27**) were able to reduce 21~40% of cell viability. We found that alkylamino and benzylamino groups at the C6 are more potent than picolinamido group (**7**≒**5**>**6**). In addition, chloro group on position 10 of thiochromeno[2,3-*c*]quinolin-12-one had higher impact on cytotoxicity than positions 8 (R^1), 9 (R^2) and 11 (R^4) (**7**>**15**≒**16**≒**13**). Moreover, substituents of

electron-attracting halogens at the C10 were more toxic than electron-donating methyl and methoxy groups, with the following sequence: Cl ≒ F > CH_3 > OCH_3 (**7**≒**9**>**20**>**11**). In contrast, substitution at the C8 by either OCH_3 or Cl behaved more active than F (**24**≒**27**>**22**).

Strikingly, six compounds (**8**, **10**, **12**, **14**, **19**, and **26**) with strong Topo I-suppressing activities (Figure 2A) displayed a very high cytotoxicity, reducing more than 95% viability at 10 μM. The latter six compounds possess a terminal amino group on propylamino or ethylamino side chain substituted on C6 (R^5) of thiochromeno[2,3-c]quinolin-12-one. These results indicate that this amino group is essential for Topo inhibition and cytotoxicity, suggesting that the side chain may contribute to a special interaction with the Topo I.

3.2. *In vitro* anti-proliferation activities

Next, we assessed the anticancer potency of the selected compounds in five human cell lines from breast cancers (MDA-MB-231, MDA-MB-468 and MCF7), colorectal cancer (HCT116) and non-small cell lung carcinoma (H1299) by measuring the values of the half-maximal inhibitory concentration (IC_{50}). As shown in Table 1, these six compounds exhibited strong anti-proliferation activities, and two TNBC lines (MDA-MB-231 and MDA-MB-468) were more susceptible to

these compounds compared to MCF7, HCT116 and H1299 cells. This suggests that different cell types or expression levels of receptors for estrogen or progesterone may contribute to the compound sensitivity of cells. Consistent with previous results (Figure 2B), substituents of electron-attracting halogens at C10 produced better cytotoxicity than the electron-donating methyl and methoxy groups (**8**, **10** >**12**, **19**) in all test cell lines. It is worthy to note that compound **14** showed a greater activity than compound **8**, perhaps because of the chlorine group existed at C8. Accordingly, compound **26** with 8,10-di electron-attracting halogens exhibited the strongest and non-differential toxicities in all tested cell lines, with IC_{50} values of 1.14±0.04, 0.22±0.04, 0.80±0.21, 0.53±0.31 and 0.53±0.08 μM in MDA-MB-231, MDA-MB-468, MCF7, HCT116 and H1299 cells, respectively. The inhibition of DNA relaxation catalyzed by Topo I and IIα by these six potent compounds were further evaluated in detail, as both enzymes were found to be oncogenes in several cancers, and Topo IIα is generally highly expressed in proliferating and tumor cells [25-27].

3.3. Topo inhibition

We determined the lowest concentration of selected compounds inhibiting Topo-mediated DNA relaxation. We showed that compounds **8**, **10**, **12**, and **19** fully

suppressed Topo I at 10 μM and displayed a partial inhibitory activity at 3 μM (Figure 3A). Importantly, compounds **14** and **26**, the two most toxic derivatives, had at least 3-fold higher activity of Topo inhibition compared to the other selected compounds, sharply and partially inhibiting Topo I at 3 and 1 μM, respectively (Figure 3A). Similar results were obtained from assays of Topo IIα inhibitory activity. All six selected compounds strongly reduced DNA relaxation by Topo IIα at 3 μM, and compounds **14** and **26** exhibited full inhibitory activity against Topo IIα even at 1 μM (Figure 3B). These results indicate that the Topo-suppressive activities of these compounds are highly correlated with their cytotoxicity. These structure-activity relationship (SAR) results were summarized in Figure 3C.

To determine if the new compounds are Topo poisons that stabilize the Topo-DNA complex, we p

relaxation, leading to accumulation of supercoiled DNA. The mobility of the accumulated DNA was reduced, as shown by band up-shifting, when Topo I remained during gel electrophoresis. Increasing the amount of **26** further restricted DNA mobility and gave rise to DNA stuck in wells (Figure 4A). Moreover, we observed that levels of Topo I and IIα loading on chromatin significantly increased when cells were treated with **26** (Figure 4B). These findings strongly suggest that **26** functions as a poison that traps topoisomerases on DNA, perhaps stabilizing the cleavage intermediates and preventing DNA re-ligation.

3.4. Anti-proliferation activities in MDA-MB-231 and MCF-10A cells

Both growth efficiency and cell death can contribute to cell viability. We thus measured DNA content using propidium iodide (PI) staining to monitor the status of MDA-MB-231 cells treated with the most potent compounds, **14** and **26**, followed by flow cytometric analysis. Figure 5A shows that, 24 hours after compound treatment, a large portion of cells treated with **26** at 4 μM accumulated in the sub-G_1 phase with fragmented DNA, indicative of cell death. This phenotype was also observed when cells were treated with 20 μM of **14 (**Figure 5A). We further examined the cleaved caspases, the active forms in mammalian apoptosis pathways, and found that treatment of **26** clearly triggered cleavages of caspase-8 and -9 (Figure 5B).

To compare the toxicities of **26** in normal and cancer cells, we examined proliferation of MDA-MB-231 breast cancer cells and the normal MCF-10A epithelial cells in the absence and presence of **26**. As shown in Figure 5C, treatment with **26** completely stopped proliferation of MDA-MB-231 cells, whereas MCF-10A cells displayed only a weak response to **26**, showing a slight reduction in the growth rate (Figure 5C). Consistently, FACS analysis showed that treatment with **26** increased the sub-G_1 population in MDA-MB-231 but not MCF-10A cells (Figure 5D). These results indicate that **26** is a potent cancer-killing tetracyclic heterocyclic

azathioxanthone with low toxicity to normal cells.

3.5. Mitotic arrest and DNA damage

Topo inhibition was found to interfere with chromosome segregation in mitosis due to accumulation of DNA lesions [28, 29]. To specifically assess cell cycle progression, we synchronized cells in the early-S phase with a high concentration of thymidine and then treated cells with **26** two hours after release from thymidine blockage (T2). The untreated cells reached the G_2/M phase and the subsequent G1 phase 8 and 12 h after release (T8 and T12), respectively (Figure 6A). In contrast, upon treatment with **26**, most cells failed to exit mitosis, remaining in the G_2/M phase at T12. Consistent with the observations from asynchronized cells (Figure 5A), the sub-G_1 population largely increased after long-term treatment with **26** (T24; Figure 6A). In addition, we detected the phosphorylation of Ser 139 of histone H2AX (γH2AX), a marker of DNA damage, in **26**-treated cells (Figure 6B). These results indicate that **26** triggers DNA damage and impairs mitosis, further supporting that **26** behaves as a Topo inhibitor in cells.

4. Conclusions

We designed and synthesized a series of tetracyclic azathioxanthones targeting

both Topo I and IIα. Six compounds were found to effectively suppress proliferation of cancer cells and activity of Topos, indicating that the terminal amino group of 6-*N*-2-aminoethylamino or 6-*N*-3-aminopropylamino is important for topo targeting. In addition, **14** and **26** exhibited strong inhibitory activities against Topos and **26** showed the highest cytotoxicity. These reveal that dual modifications of electron-attracting halogens at C8 and C10 (8-chloro and 10-fluoro) can significantly enhance anti-cancer effect of thiochromeno[2,3-*c*]quinolin-12-ones.Production of these desired compounds, however, is in a very lower yield, which remains to be resolved in the future. Our data show that **26** is a potent anticancer agent with low toxicity to normal cells. Treatment with **26** in cancer cells triggered DNA damage and impaired mitosis, ultimately leading to apoptosis. Further detailed molecular pharmacological studies examining derivative **26** are currently underway.

5. Materials and methods

5.1. Chemistry

All materials were commercially available and purchased from Sigma-Aldrich and Merck without further purification. The reaction was monitored using thin layer chromatography (TLC) as performed with Merck silica gel 60 coated with the F254 fluorescent indicator. Nuclear magnetic resonance (NMR) spectra were recorded on

Agilent 400 MR DD2 (400 MHz), Bruker: Avance DRX (500 MHz), and Agilent (600 MHz DD2 NMR) using DMSO-d_6, $CDCl_3$, and CF_3COOD as deuterated solvents. Multiplicities were recorded as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin), sextet, and double doublet (dd). Coupling constants (*J*) are indicated in Hz. Liquid chromatography (LC)-MS analysis of compounds was conducted as per one of the following methods: Column: Symmetry- C18 4.6 x 75 mm, 3.5 µm; wavelength: 254 nm; flow: 0.8 mL/min; run time: 12 min; time (in min) and mobile phase-gradient/B (%): 0/50, 3/95, 9/95, 10/50, 12/50 [B: Acetonitrile; A: Ammonium formate (20 mmol in water)]; LC-Agilent technologies- 1260 Infinity II Series; MASS: Agilent technologies- 6120 Quadrupole LC/MS-API-ESI. Spectral data of MS are recorded as *m/z* values. High-resolution mass spectra (HRMS) were recorded with a JEOL (JMS-700) electron impact (EI) mass spectrometer.

*5.2.1. Synthesis of compounds **3a**~3**i***

Step 1: An aqueous solution of chloroacetic acid (1.1 eq.) and aq. NaOH (3.0 eq. dissolved in ~2 vol. of water) were simultaneously added to a solution of corresponding substituted thiophenol (1.0 eq.) in water, and the mixture was heated to 50 °C for 4~5 hours. After completing the reaction, as monitored by TLC, the reaction mixture was cooled to room temperature and slowly diluted with 5 N HCl (pH 4~5).

The resulting suspension was filtered, washed with dilute acidic water, and vacuum-dried to afford compounds **2a**~**2i** as off-white solids.

Step 2: A mixture of isatin (0.9 eq.), **2a~2i** (1.0 eq.), and sodium acetate (0.2 eq.) in acetic acid (4~5 vol.) was heated at 150 °C for 24 hours. After cooling, the solid formed was suspended in acetic acid (10 vol.), filtered and washed with acetic acid/water (1:9) to remove unreacted isatin. The remaining solid was again washed with water and dried under a vacuum to afford compounds **3a**~3**i**.

*5.2.1.1. 3-((4-Chlorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3a**).* Physical data of compound **3a** matched those of a previous report [13].

*5.2.1.2. 3-((4-Fluorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3b**).* ^{1}H NMR (500 MHz, $DMSO_6$) δ 12.16 (s, 1H), 7.59 (t, J = 8.0 Hz, 1H), 7.44 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 9.0 Hz, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.27 (t, J = 8.0 Hz, 1H), 7.11 (t, J = 9.0 Hz, 1H).

*5.2.1.3. 2-Hydroxy-3-((4-methoxyphenyl)thio)quinoline-4-carboxylic acid (**3c**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.08 (s, 1H), 7.57 (t, J = 7.5 Hz, 1H), 7.43 (d, J = 7.5 Hz, 1H), 7.36 (d, J = 8.5 Hz, 1H), 7.31 (d, J = 9.0 Hz, 2H), 7.26 (t, J = 7.5 Hz, 1H),

6.86 (d, *J* = 9.0 Hz, 2H), 3.69 (s, 3H).

*5.2.1.4. 3-((2-Chlorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3d**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.49~7.44 (m, 3H), 7.30 (t, *J* = 8.0 Hz, 1H), 7.21~7.16 (m, 2H), 6.90~6.88 (m, 1H).

*5.2.1.5. 3-((3-Chlorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3e**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.26 (s, 1H), 7.63 (td, *J* = 8.0, 1.0 Hz, 1H), 7.48 (d, *J* = 7.5 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 1H), 7.32~7.21 (m, 4H), 7.20 (d, *J* = 7.5 Hz, 1H).

*5.2.1.6. 2-Hydroxy-3-(p-tolylthio)quinoline-4-carboxylic acid (**3f**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.14 (s, 1H), 7.59 (t, *J* = 7.5 Hz, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.27 (t, *J* = 7.5 Hz, 1H), 7.18 (d, *J* = 8.0 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H), 2.23 (s, 3H).

*5.2.1.7. 3-((2-Fluorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3g**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.24 (s, 1H), 7.62 (t, *J* = 8.0 Hz, 1H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.29 (t, *J* = 7.5 Hz, 1H), 7.25~7.19 (m, 2H), 7.09~7.06 (m, 2H).

*5.2.1.8. 2-Hydroxy-3-((2-methoxyphenyl)thio)quinoline-4-carboxylic acid (**3h**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 14.18 (brs, 1H), 12.22 (s, 1H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.44 (d, *J* = 8.0 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 1H), 7.28 (t, *J* = 7.5 Hz, 1H), 7.12 (td, *J* = 8.5, 1.5 Hz, 1H), 6.99 (d, *J* = 8.0 Hz, 1H), 6.80 (t, *J* = 7.5 Hz, 1H), 6.71 (dd, *J* = 7.5, 1.0 Hz, 1H), 3.84 (s, 3H).

*5.2.1.9. 3-((2-Chloro-4-fluorophenyl)thio)-2-hydroxyquinoline-4-carboxylic acid (**3i**).* ^{1}H NMR (500 MHz, DMSO-d_6) δ 14.40 (brs, 1H), 12.31 (s, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.30 (t, *J* = 8.0 Hz, 1H), 7.12 (td, *J* = 8.5, 2.5 Hz, 1H), 7.00 (dd, *J* = 9.0, 5.5 Hz, 1H).

*5.2.2. Synthesis of compounds **4a**~4**i***

A solution of **3a**~3**i** in $POCl_3$ (3~4 vol.) was heated to 160 °C for 48 h. After cooling, the mixture was carefully poured into ice at 0 °C. The resulting precipitate that separated out was collected by filtration. The filtered cake was suspended in a 10% $NaHCO_3$ solution and stirred vigorously for 1 h. The resulting precipitate was collected and washed with H_2O. The crude solid was washed with an EtOAc/THF mixture (1:1) and vacuum-dried to afford the following scaffold compounds **4a**~4**i**.

*5.2.2.1. 6,10-Dichloro-12H-thiochromeno[2,3-c]quinolin-12-one (**4a**).* Physical data of compound **4a** is matched with previously report [13].

*5.2.2.2. 6-Chloro-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one (**4b**).* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.52 (d, J = 8.8 Hz, 1H), 8.04 (d, J = 2.8 Hz, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.61 (t, J = 8.0 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 7.45 (t, J = 8.0 Hz, 1H), 7.31 (dd, J = 8.8, 2.8 Hz, 1H).

*5.2.2.3. 6-Chloro-10-methoxy-12H-thiochromeno[2,3-c]quinolin-12-one (**4c**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76~9.74 (m, 1H), 8.09~8.07 (m, 1H), 8.03 (d, J = 3.0 Hz, 1H), 7.78~7.74 (m, 2H), 7.63 (d, J = 9.0 Hz, 1H), 7.34 (dd, J = 9.0, 3.0 Hz, 1H), 3.97 (s, 3H).

*5.2.2.4. 6,8-Dichloro-12H-thiochromeno[2,3-c]quinolin-12-one (**4d**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.67 (dd, J = 9.5, 2.0 Hz, 1H), 8.55 (dd, J = 7.5, 1.0 Hz, 1H), 8.11 (dd, J = 7.5, 1.0 Hz, 1H), 7.83~7.77 (m, 3H), 7.58 (t, J = 7.5 Hz, 1H).

*5.2.2.5. 6,9-Dichloro-12H-thiochromeno[2,3-c]quinolin-12-one (**4e1**) and*

*6,11-Dichloro-12H-thiochromeno[2,3-c]quinolin-12-one (**4e2**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.67 (dd, J = 8.5, 1.0 Hz, 1H), 8.97 (d, J = 9.0 Hz, 1H), 8.51 (d, J = 8.5 Hz, 1H), 8.08 (dd, J = 9.0, 1.5 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.81~7.75 (m, 3H), 7.73~7.70 (m, 2H), 7.62~7.57 (m, 2H), 7.55~7.51 (m, 2H).

*5.2.2.6. 6-Chloro-10-methyl-12H-thiochromeno[2,3-c]quinolin-12-one (**4f**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (dd, J = 8.5, 2.0 Hz, 1H), 8.38 (s, 1H), 8.07 (dd, J = 7.5, 2.0 Hz, 1H), 7.78~7.73 (m, 2H), 7.61 (d, J = 8.5 Hz, 1H), 7.53 (dd, J = 8.0, 1.5 Hz, 1H), 2.52 (s, 3H).

*5.2.2.7. 6-Chloro-8-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one (**4g**).* ^{1}H NMR (500 MHz, CF_3COOD) δ 9.75~9.78 (m, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.34~8.36 (m, 1H), 8.15~8.20 (m, 2H), 7.82~7.86 (m, 1H), 7.71~7.74 (m, 1H); ^{13}C NMR (125 MHz, CF_3COOD) δ 119.83 (d, J = 19.3 Hz), 121.15, 123.27 (d, J = 17.4 Hz), 125.36 (d, J = 3.2 Hz), 125.83, 126.82, 129.91 (d, J = 7.8 Hz), 131.38, 132.88, 133.80, 134.19, 135.46, 137.65, 147.79, 157.75 (d, J = 248.9 Hz), 182.0.

*5.2.2.8. 6-Chloro-8-methoxy-12H-thiochromeno[2,3-c]quinolin-12-one (**4h**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.70 (dd, J = 8.0, 1.5 Hz, 1H), 8.17 (d, J = 8.5 Hz, 1H), 8.07 (dd,

J = 7.5, 1.5 Hz, 1H), 7.75 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 7.52 (t, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 8.0 Hz, 1H), 4.06 (s, 3H).

5.2.2.9. 6,8-Dichloro-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one ***(4i)***. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.65 (d, *J* = 8.0 Hz, 1H), 8.24 (dd, *J* = 9.0, 2.5 Hz, 1H), 8.10 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.83~7.76 (m, 2H), 7.60 (dd, *J* = 7.5, 2.5 Hz, 1H).

5.2.3. Synthesis of compounds ***5~27***

5.2.3.1.

10-Chloro-6-((4-methoxybenzyl)amino)-12H-thiochromeno[2,3-c]quinolin-12-one ***(5)***.

A suspension of **4a** (1.0 eq.) in DMSO (4~5 vol.) was added drop-wise to a stirred solution of (4-methoxyphenyl)methanamine (5.0 eq.) in DMSO (~ 5 vol.) at 120 °C, and the mixture was maintained at the same temperature for 2~3 hours. After completion of the reaction, as monitored by TLC, the reaction mixture was cooled to room temperature (RT) and quenched in water. The resulting precipitate was diluted with a 10% MeOH/CH_2Cl_2 mixture and extracted. The aqueous layer was separated, and then extracted with 10% MeOH/CH_2Cl_2. The combined organic extract was washed with brine solution, dried over anhydrous Na_2SO_4, and then concentrated to give the crude compounds. The crude mixtures were purified by flash column

chromatography (FCC) using MeOH/CH_2Cl_2/aq. NH_3 mixtures to afford target compounds as pale-yellow to yellow solids. Yield: 300 mg (92%). ES-MS $[M+1]^+$: 432.9; t_R: 6.31 min (purity: 94.5%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.44 (d, J = 8.5 Hz, 1H), 8.55 (d, J = 2.5 Hz, 1H), 7.85 (d, J = 8.5 Hz, 1H), 7.86~7.59 ((m, 2H), 7.55 (d, J = 8.5 Hz, 1H), 7.45 (td, J = 8.0, 1.0 Hz, 1H), 7.39, 7.40 (d, J = 8.5 Hz, 2H), 6.91 (dd, J = 9.0, 2.0 Hz, 2H), 5.05 (t, J = 5.0 Hz, 1H), 4.83 (d, J = 5.0 Hz, 1H), 3.80 (s, 3H);^{13}C NMR (125 MHz, $CDCl_3$): δ 46.02, 55.33, 114.17, 120.95, 123.54, 124.81, 125.88, 127.24, 127.46, 129.40, 129.54, 129.61, 129.79, 130.84, 131.05, 132.52, 132.55, 134.15, 145.53, 150.33, 159.18, 180.95. HRMS (ESI) for $C_{24}H_{18}N_2O_2SCl$ ($[M+1]^+$): calcd., 433.0778; found, 433.0778.

*5.2.3.2. N-(10-Chloro-12-oxo-12H-thiochromeno[2,3-c]quinolin-6-yl)picolinamide (**6**).* A mixture of TFA (~10 vol.) and **5** (1.0 eq.) was stirred at RT for 4~5 hours. After completion of the reaction, as monitored by TLC, the reaction mixture was concentrated on a rotary evaporator. The resulting residue was quenched in ice-cold water, and the resulting solid was then filtered. The filtered cake was suspended in sat. $NaHCO_{3(aq)}$ (pH ~8) and stirred for 30 min. The suspension was filtered; washed with water and vacuum-dried to afford intermediate 6-amino-12H-thiochromeno[2,3-*c*]quinolin-12-one as a pale-yellow to yellow solid in

an almost pure form. 6-Amino-12H-thiochromeno[2,3-*c*]quinolin-12-one (1.0 eq.) was added at once to a stirred mixture of 2-picolinic acid (1.3 eq.) and CDI (1.9 eq.) in DMSO (4~5 vol.) at 70 °C, and stirring continued at the same temperature for 30 min. The temperature was raised to 140 °C, and the reaction mixture was further stirred at same temperature for 16 hours. After completion of the reaction, as monitored by TLC, the reaction mixture was cooled and quenched in water. The resulting precipitate was diluted with a 10% MeOH/CH_2Cl_2 mixture and extracted. The aqueous layer was separately extracted with 10% MeOH/CH_2Cl_2. The combined organic extract was washed with brine solution, dried over anhydrous Na_2SO_4, and then concentrated to give the crude compound **6**. The crude mixture was purified by FCC using a MeOH/CH_2Cl_2/$NH_{3(aq)}$ mixture to afford the target compound as a yellow solid. Yield: 120 mg (36%). ES-MS $[M+1]^+$: 417.9; t_R: 4.77 min (purity: 95.3%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.56 (s, 1H), 9.69 (dd, J = 9.0, 1.0 Hz, 1H), 8.73 (d, J = 4.5 Hz, 1H), 8.58 (d, J = 1.0 Hz, 1H), 8.36 (d, J = 7.5 Hz, 1H), 8.15 (dd, J = 9.0, 1.5 Hz, 1H), 7.97 (td, J = 8.0, 1.0 Hz, 1H), 7.80~7.74 (m, 2H), 7.64~7.57 (m, 3H);; ^{13}C NMR (125 MHz, $CDCl_3$): δ 123.09, 124.56, 125.93, 127.26, 127.68, 129.31, 129.35, 129.54, 129.85, 130.54, 130.71, 132.30, 132.70, 132.75, 134.05, 137.83, 144.97, 145.51, 148.42, 148.80, 163.45, 180.92. HRMS (ESI) for $C_{22}H_{13}N_3O_2SCl$ ($[M+1]^+$): calcd., 418.0417; found, 418.0418.

*5.2.3.3. 10-Chloro-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one (**7**).*

The title compound was synthesized from *n*-propylamine in a similar manner as described for **5**. Yield: 120 mg (75%). ES-MS $[M+1]^+$: 355.0; t_R: 6.84 min (purity: 95.2%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.42 (d, J = 8.5 Hz, 1H), 8.54 (d, J = 2.0 Hz, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.62~7.56 (m, 3H), 7.42 (t, J = 7.5 Hz, 1H), 4.85 (s, 1H), 3.67 (q, J = 7.0 Hz, 2H), 1.79 (sextet, J = 7.0 Hz, 2H), 1.08 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.67, 22.65, 44.14, 120.69, 123.52, 124.53, 125.84, 127.17, 127.46, 129.39, 129.45, 129.68, 131.06, 132.48, 132.54, 134.12, 145.68, 150.64, 181.00. HRMS (ESI) for $C_{19}H_{16}N_2OSCl$ ($[M+1]^+$): calcd., 355.0672; found, 355.0676.

5.2.3.4.

*6-((3-Aminopropyl)amino)-10-chloro-12H-thiochromeno[2,3-c]quinolin-12-one (**8**).*

The title compound was synthesized from 1,3-diaminopropane in a similar manner as described for **5**. Yield: 295 mg (88%). ES-MS $[M+1]^+$: 370.0; t_R: 2.52 min (purity: 97.3%); ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.37 (d, J = 9.0 Hz, 1H), 8.43 (d, J = 1.2 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.92 (dd, J = 8.4, 2.4 Hz, 1H), 7.72 (d, J = 8.4 Hz, 1H), 7.63 (td, J = 8.4, 1.2 Hz, 1H), 7.35 (td, J = 8.0, 1.5 Hz, 1H), 6.65 (brs, 2H), 3.68

(t, *J* = 6.6 Hz, 2H), 2.86 (t, *J* = 6.6 Hz, 2H), 1.94 (quin, *J* = 6.6 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 28.1, 37.8, 40.0, 119.8, 123.7, 124.8, 125.4, 126.5, 128.0, 128.5, 128.9, 129.3, 131.7, 131.9, 132.7, 132.9, 145.0, 150.9, 180.2. HRMS (ESI) for $C_{19}H_{17}N_3OSCl$ ($[M+1]^+$): calcd., 370.0781; found, 370.0786.

5.2.3.5. 10-Fluoro-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one ***(9)***. The title compound was synthesized from **4b** and *n*-propylamine in a similar manner as described for **5**. Yield: 190 mg (71%). ES-MS $[M+1]^+$: 339.0; t_R: 5.87 min (purity: 95.8%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.46 (dd, *J* = 8.5, 1.0 Hz, 1H), 8.26 (dd, *J* = 9.5, 2.5 Hz, 1H), 7.82 (d, *J* = 7.5, 1.0 Hz, 1H), 7.65 (dd, *J* = 9.0, 5.0 Hz, 1H), 7.60 (td, *J* = 8.5, 1.0 Hz, 1H), 7.44~7.41 (m, 2H), 4.88 (s, 1H), 3.70~3.66 (m, 2H), 1.79 (sextet, *J* = 7.5 Hz, 2H), 1.08 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.63, 22.61, 44.10, 115.46 (*J* = 23.6 Hz), 120.77 (J = 25.0 Hz), 121.07, 123.81, 124.47, 125.84, 127.11, 128.10 (*J* = 7.9 Hz), 128.23, 128.99, 129.34, 133.25 (*J* = 6.8 Hz), 145.63, 150.62, 162.06 (*J* = 247.3 Hz), 181.19. HRMS (ESI) for $C_{19}H_{16}N_2OSF$ ($[M+1]^+$): calcd., 339.0967; found, 339.0968.

5.2.3.6. 6-(3-Aminopropylamino)-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one ***(10)***. The title compound was synthesized from 1,3-diaminopropane in a similar

manner as described for **9**.Yield: 210 mg (75%). ES-MS $[M+1]^+$: 354.0; t_R: 2.35 min (purity: 96.7%); ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.39 (d, J = 9.0 Hz, 1H), 8.18 (dd, J = 9.6, 2.8 Hz, 1H), 8.05 (dd, J = 8.4, 4.8 Hz, 1H), 7.80 (td, J = 8.4, 2.8 Hz, 1H), 7.71 (d, J = 7.8 Hz, 1H), 7.62 (t, J = 7.5 Hz, 1H), 7.39 (t, J = 7.2 Hz, 1H), 3.68 (t, J = 6.6 Hz, 2H), 2.82 (t, J = 6.6 Hz, 2H), 1.86 (quin, J = 6.6 Hz, 2H);^{13}C NMR (150 MHz, DMSO-d_6): δ 29.1, 38.3, 40.0, 114.2 (J = 23.0 Hz), 119.8, 121.6 (J = 24.2 Hz), 123.6, 125.1, 125.4, 126.5, 127.8, 129.0 (J = 2.3 Hz), 129.2, 129.5 (J = 8.1 Hz), 132.2 (J = 6.9 Hz), 145.1, 151.0, 161.8 (J = 245.0 Hz), 180.5. HRMS (ESI) for $C_{19}H_{17}N_3OSF$ ($[M+1]^+$): calcd., 354.1076; found, 354.1077.

*5.2.3.7. 10-Methoxy-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one (**11**).*

The title compound was synthesized from **4c** and *n*-propylamine in a similar manner as described for **5**. Yield: 120 mg (45%). ES-MS $[M+1]^+$: 351.0; t_R: 5.71 min (purity: 98.5%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.52 (d, J = 8.5, 1H), 8.04 (d, J = 2.5 Hz, 1H), 7.82 (dd, J = 8.5, 1.0 Hz, 1H), 7.59 (td, J = 8.0, 1.0 Hz, 1H), 7.55 (d, J = 9.0 Hz, 1H), 7.42 (td, J = 8.0, 1.0 Hz, 1H), 7.28 (dd, J = 8.5, 2.5 Hz, 1H), 4.89 (t, J = 4.5 Hz, 1H), 3.96 (s, 3H), 3.70~3.66 (m, 2H), 1.79 (sextet, J = 7.5 Hz, 2H), 1.08 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.67, 22.67, 44.08, 55.75, 110.29, 121.02, 122.65, 124.13, 124.26, 124.73, 125.94, 127.08, 127.34, 129.02, 129.07, 132.76, 145.51,

150.80, 159.47, 181.76. HRMS (ESI) for $C_{20}H_{19}N_2O_2S$ ($[M+1]^+$): calcd., 351.1167; found, 351.1167.

5.2.3.8.

6-(3-Aminopropylamino)-10-methoxy-12H-thiochromeno[2,3-c]quinolin-12-one ***(12)****.* The title compound was synthesized from **4c** and 1,3-diaminopropane in a similar manner as described for **5**. Yield: 130 mg (46%). ES-MS $[M+1]^+$: 366.0; t_R: 2.12 min (purity: 99.0%); ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.44 (dd, J = 8.4, 1.2 Hz, 1H), 7.95 (d, J = 2.4 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.71 (d, J = 7.8 Hz, 1H), 7.61 (td, J = 8.4, 1.2 Hz, 1H), 7.51 (dd, J = 8.4, 2.4 Hz, 1H), 7.36 (td, J = 8.4, 1.2 Hz, 1H), 3.95 (s, 3H), 3.69 (t, J = 6.6 Hz, 2H), 2.82 (t, J = 6.6 Hz, 2H), 1.89 (quin, J = 6.6 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 28.97, 38.28, 40.05, 55.64,110.14, 120.06, 122.49, 123.49, 124.89, 125.03, 125.43, 126.48, 127.83, 128.21, 128.96, 131.83, 144.95, 151.08, 159.13, 180.85. HRMS (ESI) for $C_{20}H_{20}N_3O_2S$ ($[M+1]^+$): calcd., 366.1276; found, 366.1276.

5.2.3.9. 8-Chloro-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one ***(13)****.* The title compound was synthesized from **4d** and *n*-propylamine in a similar manner as described for **5**. Yield: 630 mg (84%). ES-MS $[M+1]^+$: 355.0; t_R: 6.57 min (purity:

99.2%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.41 (dd, J = 8.5, 0.5 Hz, 1H), 8.51 (d, J = 8.5 Hz, 1H), 7.81 (d, J = 8.5 Hz, 1H), 7.72 (dd, J = 8.0, 1.0 Hz, 1H), 7.60 (td, J = 8.5, 1.0 Hz, 1H), 7.52 (t, J = 7.5 Hz, 1H), 7.341 (td, J = 7.0, 1.0 Hz, 1H), 5.05 (t, J = 4.5 Hz, 1H), 3.70 (quin, J = 5.5 Hz, 2H), 1.82 (sextet, J = 7.5 Hz, 2H), 1.09 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.69, 22.67, 44.17, 120.40, 123.09, 124.44, 125.79, 127.13, 127.59, 128.35, 129.34, 129.51, 130.44, 132.35, 132.43, 133.27, 145.80, 150.84, 181.90. HRMS (ESI) for $C_{19}H_{16}N_2OSCl$ ($[M+1]^+$): calcd., 355.0672; found, 355.0672.

5.2.3.10.

6-(3-Aminopropylamino)-8-chloro-12H-thiochromeno[2,3-c]quinolin-12-one ***(14)****.*

The title compound was synthesized from **4d** and 1,3-diaminopropane in a similar manner as described for **5**. Yield: 400 mg (47%). ES-MS $[M+1]^+$: 370.0; t_R: 2.34 min (purity: 99.1%); ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.33 (d, J = 8.4 Hz, 1H), 8.46 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 7.8 Hz, 1H), 7.73 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 1H), 7.62 (td, J = 7.8, 0.6 Hz, 1H), 7.38 (t, J = 7.2 Hz, 1H), 3.69 (t, J = 6.6 Hz, 2H), 2.76 (t, J = 6.6 Hz, 2H), 1.84 (quin, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 30.9, 39.1, 40.0, 119.4, 123.5, 123.8, 125.3, 126.5, 128.0, 128.3, 128.35, 129.4, 129.5, 132.4, 132.5, 132.9, 145.4, 151.1, 181.2. HRMS (ESI) for

$C_{19}H_{17}N_3OSCl$ ($[M+1]^+$): calcd., 370.0781; found, 370.0784.

*5.2.3.11. 9-Chloro-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one (**15**) and 11-chloro-6-(propylamino)-12H-thiochromeno[2,3-c]quinolin-12-one (**16**).* The title compound was synthesized from a **4e1**/**4e2** mixture and *n*-propylamine in a similar manner as described for **5**. The non-polar product of the two isomers present in the crude mixture was isolated by FCC and confirmed as **15** and **16**. **15**: Yield: 195 mg. ES-MS $[M+1]^+$: 355.0; t_R: 6.60 min (purity: 98.2%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.42 (d, J = 8.5 Hz, 1H), 8.49 (d, J = 9.0 Hz, 1H), 7.80 (d, J = 8.5 Hz, 1H), 7.62~7.58 (m, 2H), 7.50 (dd, J = 8.5, 2.0 Hz, 1H), 7.41 (td, J = 8.5, 1.0 Hz, 1H), 4.83 (s, 1H), 3.66 (q, J = 6.0 Hz, 2H), 1.79 (sextet, J = 7.0 Hz, 2H), 1.08 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.63, 22.62, 44.11, 120.66, 123.12, 124.47, 125.24, 125.86, 127.14, 128.25, 129.44, 129.89, 130.01, 131.40, 134.28, 138.86, 145.74, 150.59, 181.35. HRMS (ESI) for $C_{19}H_{16}N_2OSCl$ ($[M+1]^+$): calcd., 355.0672; found, 355.0670. **16**: Yield: 176 mg. ES-MS $[M+1]^+$: 312.9; t_R: 3.24 min (purity: 99.70%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.73 (d, J = 8.0 Hz, 1H), 7.78 (d, J = 8.0 Hz, 1H), 7.57 (td, J = 8.0, 0.5 Hz, 1H), 7.54~7.51 (m, 2H), 7.44 (t, J = 8.0 Hz, 1H), 7.36 (td, J = 8.0, 0.5 Hz, 1H), 4.81 (t, J = 5.0 Hz, 1H), 3.65 (q, J = 7.0 Hz, 2H), 1.77 (sextet, J = 7.5 Hz, 2H), 1.06 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.63, 22.66, 43.92,

119.51, 120.15, 124.02, 124.72, 124.80, 127.05, 129.15, 129.53, 130.53, 131.42, 133.72, 135.00, 135.35, 146.01, 150.31, 182.48. HRMS (ESI) for $C_{19}H_{16}N_2OSCl$ ($[M+1]^+$): calcd., 355.0672; found, 355.0672.

5.2.3.12.

10-Fluoro-6-(pyridin-3-ylmethylamino)-12H-thiochromeno[2,3-c]quinolin-12-one ***(17)****.* The title compound was synthesized from **4b** and 3-(aminomethyl)pyridine in a similar manner as described for **5**. Yield: 200 mg (54%). ES-MS $[M+1]^+$: 387.9; t_R: 4.33 min (purity: 95.5%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.48 (d, J = 8.0 Hz, 1H), 8.76 (s, 1H), 8.54 (d, J = 3.5 Hz, 1H), 8.26 (dd, J = 9.0, 2.5 Hz, 1H), 7.83 (d, J = 8.0 Hz, 2H), 7.63~7.61 (m, 2H), 7.46 (t, J = 8.0 Hz, 1H), 7.41 (td, J = 9.0, 3.0 Hz, 1H), 7.29~7.26 (m, 1H), 5.24 (s, 1H), 4.93 (d, J = 5.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 43.76, 115.53 (J = 23.0 Hz), 121.11 (J = 24.5 Hz), 121.14, 123.56, 123.70, 125.14, 125.91, 127.29, 128.02, 128.15 (J = 7.8 Hz), 129.21, 129.52, 133.24 (J = 7.1 Hz), 134.60, 135.94, 145.20, 148.87, 149.73, 150.01, 162.12 (J = 248.1 Hz), 181.02. HRMS (ESI) for $C_{22}H_{15}N_3OSF$ ($[M+1]^+$): calcd., 388.0920; found, 38.0921.

5.2.3.13. 6-(Benzylamino)-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one ***(18)****.* The title compound was synthesized from **4b** and benzylamine in a similar manner as

described for **5**. Yield: 127 mg (41.5%). ES-MS $[M+1]^+$: 386.9; t_R: 5.72 min (purity: 97.9%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.48 (d, J = 9.5 Hz, 1H), 8.26 (dd, J = 9.5, 1.5 Hz, 1H), 7.85 (d, J = 8.5 Hz, 1H), 7.64~7.60 (m, 2H), 7.49~7.36 (m, 6H), 7.31 (t, J = 7.0 Hz, 1H), 5.14 (t, J = 5.0 Hz, 1H), 4.92 (d, J = 5.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 46.47, 115.50 (J = 23.5 Hz), 121.01, 121.05 (J = 24.1 Hz), 123.84, 124.84, 125.91, 127.25, 127.60, 128.12, 128.18, 128.22, 128.77, 129.14, 129.46, 133.26 (J = 6.7 Hz), 138.87, 145.46, 150.33, 162.10 (J = 247.7 Hz), 181.14. HRMS (ESI) for $C_{23}H_{16}N_2OSF$ ($[M+1]^+$): calcd., 387.0967; found, 387.0966.

5.2.3.14.

6-(3-Aminopropylamino)-10-methyl-12H-thiochromeno[2,3-c]quinolin-12-one ***(19)****.*

The title compound was synthesized from **4f** and 1,3-diaminopropane in a similar manner as described for **5**. Yield: 130 mg (63%). ES-MS $[M+1]^+$: 350.0; t_R: 2.28 min (purity: 99.1%), ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.38 (d, J = 8.4 Hz, 1H), 8.30 (s, 1H), 8.00 (brs, 3H), 7.85 (d, J = 7.8 Hz, 1H), 7.73 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.62 (td, J = 7.8, 0.6 Hz, 1H), 7.40 (td, J = 8.4, 1.2 Hz, 1H), 7.34 (t, J = 6.0 Hz, 1H), 3.70 (q, J = 6.6 Hz, 2H), 2.91 (brs, 2H), 2.52 (s, 3H), 2.03 (quin, J = 7.2 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 20.9, 26.6, 36.9, 38.4, 120.0, 123.6, 124.8, 125.4, 126.5, 126.5, 128.6, 128.8, 129.0, 130.0, 130.4, 134.1, 137.9, 144.9,

151.0, 181.3. HRMS (ESI) for $C_{20}H_{20}N_3OS$ ($[M+1]^+$): calcd., 350.1327; found, 350.1328.

*5.2.3.15. 6-(Ethylamino)-10-methyl-12H-thiochromeno[2,3-c]quinolin-12-one (**20**),* The title compound was synthesized from ethylamine in a similar manner as described for **19**. Yield: 95 mg (30%). ES-MS $[M+1]^+$: 321.0; t_R: 6.10 min (purity: 96.9%), ^1H NMR (500 MHz, $CDCl_3$): δ 9.46 (d, J = 8.0 Hz, 1H), 8.38 (s, 1H), 7.82 (d, J = 8.5 Hz, 1H), 7.59 (td, J = 8.5, 1.0 Hz, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.47 (dd, J = 8.5, 1.5 Hz, 1H), 7.43 (td, J = 8.0, 1.5 Hz, 1H), 4.83 (s, 1H), 3.78~3.72 (m, 2H), 2.51 (s, 3H), 1.39 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.81, 21.38, 37.21, 120.95, 123.84, 124.29, 125.86, 125.92, 127.07, 129.11, 129.52, 129.77, 129.94, 131.46, 133.51, 137.89, 145.55, 150.75, 182.27. HRMS (ESI) for $C_{19}H_{17}N_2OS$ ($[M+1]^+$): calcd., 321.1062; found, 321.1064.

*5.2.3.16. 6-(Benzylamino)-10-methyl-12H-thiochromeno[2,3-c]quinolin-12-one (**21**).* The title compound was synthesized from benzylamine in a similar manner as described for **19**. Yield: 178 mg (55%). ES-MS $[M+1]^+$: 382.9; t_R: 8.41 min (purity: 98.8%), ^1H NMR (500 MHz, $CDCl_3$): δ 9.47 (d, J = 8.5 Hz, 1H), 8.38 (s, 1H), 7.84 (dd, J = 8.0, 0.5 Hz, 1H), 7.60 (td, J = 8.5, 1.0 Hz, 1H), 7.52~7.42 (m, 5H), 7.37 (t, J

= 7.5 Hz, 2H), 7.30 (t, *J* = 7.5 Hz, 1H), 5.16 (t, *J* = 5.0 Hz, 1H), 4.92 (d, *J* = 5.0 Hz, 2H), 2.50 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.38, 46.41, 121.24, 123.85, 124.58, 125.86, 125.95, 127.16, 127.54, 128.20, 128.74, 129.19, 129.53, 129.70, 130.08, 131.44, 133.55, 137.94, 139.00, 145.36, 150.52, 182.20. HRMS (ESI) for $C_{24}H_{19}N_2OS$ ($[M+1]^+$): calcd., 383.1218; found, 383.1219.

*5.2.3.17. 6-(Ethylamino)-8-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one(**22**).* The title compound was synthesized from **4g** and ethylamine in a similar manner as described for **5**. Yield: 164 mg (53%). ES-MS $[M+1]^+$: 324.9; t_R: 5.97 min (purity: 99.0%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.42 (dd, *J* = 9.0, 1.0 Hz, 1H), 8.38 (d, *J* = 8.0 Hz, 1H), 7.81 (dd, *J* = 8.5, 0.5 Hz, 1H), 7. 60 (td, *J* = 8.0, 1.5 Hz, 1H), 7.56~7.52 (m, 1H), 7.45~7.41 (m, 2H), 4.92 (s, 1H), 3.79~3.73 (m, 2H), 1.40 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.78, 37.31, 117.50 (*J* = 19.3 Hz), 120.60, 121.74 (*J* = 17.6 Hz), 122.40, 124.52, 125.28 (*J* = 3.1 Hz), 125.90, 127.17, 127.68 (*J* = 7.4 Hz), 129.50, 129.87, 132.97, 145.78, 150.76, 157.39 (*J* = 246.3 Hz), 181.32. HRMS (ESI) for $C_{18}H_{14}N_2OSF$ ($[M+1]^+$): calcd., 325.0811; found, 325.0811.

*5.2.3.18. 6-(Benzylamino)-8-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one (**23**).* The title compound was synthesized from **4g** and benzylamine in a similar manner as

described for **5**. Yield: 255 mg (69%). ES-MS $[M+1]^+$: 386.9; t_R: 6.41 min (purity: 99.0%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.44 (d, J = 8.5 Hz, 1H), 8.37 (d, J = 7.5 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.62 (td, J = 8.5, 1.0 Hz, 1H), 7.56~7.51 (m, 1H), 7.49 (d, J = 7.5 Hz, 2H), 7.46~7.38 (m, 4H), 7.32 (t, J = 7.5 Hz, 1H), 5.25 (t, J = 5.0 Hz, 1H), 4.93 (d, J = 5.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 46.49, 117.55 (J = 19.0 Hz), 120.89, 121.68 (J = 17.1 Hz), 122.43, 124.82, 125.29 (J = 2.7 Hz), 125.93, 127.27, 127.60, 127.73 (J = 7.3 Hz), 128.25, 128.77, 129.58, 130.00, 132.94, 138.82, 145.58, 150.52, 157.38 (J = 246.6 Hz), 181.25. HRMS (ESI) for $C_{23}H_{16}N_2OSF$ ($[M+1]^+$): calcd., 387.0967; found, 387.0967.

*5.2.3.19. 6-(Ethylamino)-8-methoxy-12H-thiochromeno[2,3-c]quinolin-12-one (**24**).* The title compound was synthesized from **4h** and ethylamine in a similar manner as described for **5**. Yield: 196 mg (63%); ES-MS $[M+1]^+$: 336.9; t_R: 7.04 min (purity: 94.0%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.48 (dd, J = 8.5, 1.0 Hz, 1H), 8.19 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.59 (td, J = 8.5, 1.5 Hz, 1H), 7.50 (d, J = 8.0, Hz, 1H), 7.40 (td, J = 8.5, 1.0 Hz, 1H), 7.13 (d, J = 8.0 Hz, 1H), 5.01 (brs, 1H), 4.04 (s, 3H), 3.78~3.73 (m, 2H), 1.40 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.81, 37.22, 56.54, 111.91, 120.77, 121.47, 123.17, 123.79, 124.24, 126.00, 127.00,

127.44, 129.15, 129.56, 132.54, 145.58, 151.07, 154.36, 182.21. HRMS (ESI) for $C_{19}H_{17}N_2O_2S$ ($[M+1]^+$): calcd., 337.1011; found, 337.1014.

*5.2.3.20. 6-(Benzylamino)-8-methoxy-12H-thiochromeno[2,3-c]quinolin-12-one (**25**).* The title compound was synthesized from **4h** and benzylamine in a similar manner as described for **24**. Yield: 144 mg (39%); ES-MS $[M+1]^+$: 399.0; t_R: 7.76 min (purity: 98.9%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.50 (dd, J = 8.5, 1.0 Hz, 1H), 8.20 (dd, J = 8.0, 0.5 Hz, 1H), 7.84 (dd, J = 8.0, 1.0 Hz, 1H), 7.60 (td, J = 8.5, 1.0 Hz, 1H), 7.60~7.49 (m, 3H), 7.43 (td, J = 7.5, 1.0 Hz, 1H), 7.38 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 1H), 7.13 (d, J = 8.0 Hz, 1H), 5.35 (t, J = 5.5 Hz, 1H), 4.93 (d, J = 5.5 Hz, 2H), 4.02 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 46.44, 56.51, 111.95, 121.06, 121.48, 123.12, 123.81, 124.54, 126.03, 127.11, 127.50, 128.34, 128.71, 129.23, 129.72, 132.55, 139.03, 145.39, 150.85, 154.38, 182.17. HRMS (ESI) for $C_{24}H_{19}N_2O_2S$ ($[M+1]^+$): calcd., 399.1167; found, 399.1168.

5.2.3.21.

*6-(2-Aminoethylamino)-8-chloro-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one (**26**).* The title compound was synthesized from **4i** and ethylenediamine in a similar manner as described for **5**. Yield: 100 mg (11%); ES-MS $[M+1]^+$: 374.0; t_R: 4.38 min

(purity: 94.5%), ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.32 (d, J = 8.4 Hz, 1H), 8.17 (d, J = 7.8 Hz, 2H), 7.70 (d, J = 8.4 Hz, 1H), 7.63 (td, J = 7.8, 1.2 Hz, 1H), 7.40 (td, J = 7.8, 1.2 Hz, 1H), 3.65 (t, J = 6.0 Hz, 2H), 2.94 (t, J = 6.0 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 40.2, 44.2, 79.2, 113.9 (J = 19.1 Hz), 119.5, 122.0 (J = 22.0 Hz), 123.9, 124.3, 125.4, 126.6, 127.5, 128.8, 129.5, 131.2 (J = 8.6 Hz), 145.3, 151.2, 160.7 (J = 205.9 Hz), 180.3. HRMS (ESI) for $C_{18}H_{14}N_3OSClF$ ($[M+1]^+$): calcd., 374.0530; found, 374.0534.

5.2.3.22.

*8-Chloro-6-(ethylamino)-10-fluoro-12H-thiochromeno[2,3-c]quinolin-12-one(**27**).*

The title compound was synthesized from **4i** and ethylamine in a similar manner as described for **5**. Yield: 87 mg (18%). ES-MS $[M+1]^+$: 358.8;t_R: 6.88 min (purity: 97.3%), ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.41 (d, J = 8.5 Hz, 1H), 8.22 (dd, J =9.0, 3.0 Hz , 1H), 7.81 (d, J = 9.0 Hz, 1H), 7.61 (td, J = 8.0, 1.0 Hz, 1H), 7.54 (dd, J = 7.5, 2.5 Hz, 1H), 7.43 (td, J = 7.0, 1.0 Hz, 1H), 4.95 (s, 1H), 3.79~3.74 (m, 2H), 1.41 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.76, 37.31, 114.57 (J = 22.9 Hz), 120.28, 121.38 (J = 26.9 Hz), 123.18, 124.65, 125.74, 127.18, 128.23 (J = 3.4 Hz), 128.40, 129.62, 131.63 (J = 9.2 Hz), 134.15 (J = 6.9 Hz), 145.80, 150.58, 161.20 (J =

250.1 Hz), 180.80. HRMS (ESI) for $C_{18}H_{13}N_2OSClF$ ($[M+1]^+$): calcd., 359.0421; found, 359.0421.

5.3. Bioassay

5.3.1. DNA Topo I assay

DNA Topo I activity was measured based on its ability to resolve the supercoiled form of pHOT-1 plasmid DNA. Assays were performed using Topoisomerase I Assay Kit (TopoGEN) according to the manufacturer's instructions. Briefly, 250 ng of the pHOT-1 plasmid was incubated with DMSO, CPT or synthesized compounds in the reaction buffer (10 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine and 5% glycerol), followed by the addition of 2 U of recombinant human Topo I purified from baculovirus-infected insect cells. After a 30-min incubation at 37 °C, the reaction was stopped by adding 1% sodium dodecylsulfate and 50 mg/mL of proteinase K. Samples were resolved by electrophoresis with 1% agarose gel in TAE buffer at 50 V for 2 hours. Gels were stained with ethidium bromide, and DNA was visualized using BioradVersaDoc.

5.3.2. Cell culture

The human MDA-MB-231 and MDA-MB-468 breast cancer cell lines were cultured in L15 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine (PSG) at 37 °C in a humidified atmosphere without CO_2. The human MCF-7 breast cancer cell line, HCT116 colorectal cancer cell line, and H1299 non-small cell lung carcinoma cell line were cultured in alpha-minimum essential medium (αMEM), McCoy's 5A and RPMI, respectively, with 10% FBS and 1% PSG at 37 °C in a humidified atmosphere with 5% CO_2. The human MCF-10A normal breast epithelial cell line was cultured in DMEM/F12 medium containing 5% horse serum (HS), 20 μg/L EGF, 10 mg/L insulin, 500 μg/L hydrocortisone, 100 μg/L cholera toxin, 1% PSG and 1% non-essential amino acids at 37 °C in a humidified atmosphere with 5% CO_2. All reagents for cell culture were purchased from Invitrogen. Vi-CELL XR cell counter (Beckman Coulter) was used for cell counting in all cell-based assays.

5.3.3. MTT assays

Four thousand cells/well on 96-well plate were treated with the indicated compounds for 72 hours (MDA-MB-231, MDA-MB-468, HCT116 and H1299) or 5 days (MCF-7), followed by incubation with 1 mg/mL3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma)

at 37 °C for 4 hours. Formazan crystals were dissolved in DMSO (Sigma) and the absorbance at 490 nm was measured with Perkin Elmer Victor^3 1420 Multilabel Counter. The half-maximal inhibitory concentration (IC_{50}) was calculated by CompuSyn software.

5.3.4. DNA Topo IIα assay

DNA Topo IIα activity was measured using Topoisomerase IIα Assay Kit (TopoGEN) according to the manufacturer's instructions. Briefly, 250 ng of the pHOT-1 plasmid was incubated with DMSO, VP16 (etoposide) or synthesized compounds in the reaction buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol and 2 mM ATP), followed by addition of 8 U of recombinant human Topo IIα purified from baculovirus-infected insect cells. After a 45-min incubation at 37 °C, the reaction was stopped by adding 1% SDS and 50 mg/mL of proteinase K. Samples were resolved by electrophoresis on a 1% agarose gel in TAE buffer at 50 V for 2 hours. Gels were then stained with ethidium bromide and DNA was visualized using BioradVersaDoc.

5.3.5. Single thymidine synchronization

In total, 1.6×10^5 cells/well on 6-well plate were incubated with culture medium

containing 4 mM thymidine (Sigma) for 24 hours to accumulate cells in the early-S phase. Cells were then thoroughly washed and incubated with culture medium to relieve the thymidine blockage. Two hours after release, cells were treated with or without compound **26** and collected at the indicated time for cell cycle analysis.

5.3.6. Flow cytometry

Cells were fixed with 70% ethanol and incubated with phosphate-buffered saline (PBS) containing 0.05 mg/mL propidium iodide (PI) and 0.25 mg/mL RNase A for 30 min at 37 °C. DNA content was measured using BD FACS Calibur and the cell-cycle distribution was plotted and analyzed using FlowJo software.

5.3.7. Western blot analysis

Cells were directly lysed in Laemmli sample buffer [LSB; 60 mM Tris-HCl (pH 6.8), 2% SDS and 10% glycerol)]. To obtain the chromatin-bound fractions, cells were pre-extracted with RIPA buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP40, 0.1% SDS, 0.5% Sodium deoxycholate and protease inhibitors) to remove the soluble proteins, and the pellets were then dissolved in LSB. Protein concentrations were determined by using BCA Protein Assay Kit (Thermo Scientific). Extracts were fractionated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and

immobilized on a nitrocellulose membrane. After blocking, membranes with 5% nonfat milk in PBST (PBS with 0.1% Tween 20) and targeted proteins were probed with specific primary antibodies, followed by incubation with species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was developed using Clarity™ Western ECL Substrate kit (Bio-Rad) and analyzed by ImageQuant LAS 4000. Primary antibodies used in the Western blot analysis were caspase-8 (Cell Signaling Technology), caspase-9 (Cell Signaling Technology), α-tubulin (Sigma), γH2AX (Millipore) and histone H2A (Abcam). HRP-conjugated goat anti-mouse and -rabbit antibodies were purchased from GeneTex.

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Figure legends

Figure 1. Structures of CPTQ and the designed thiochromenoquinolin-12-one derivatives.

Figure 2. Quick selection of compounds. (A) In vitro analysis of compound activity of inhibiting topoisomerase I-mediated DNA relaxation at a concentration of 10 μM. One representative result out of two biological replicates is shown. (B) Screening for compound cytotoxicity. MDA-MB-231 cells were treated with 10 μM of each compound for 72 hours and cell viability was determined by an MTT assay. Results are displayed as the mean ± SD from three independent experiments (*n*=9).

Figure 3. In vitro analysis of compound activity on inhibiting DNA decatenation catalyzed by topoisomerase I (A) or IIα (B). One representative result out of two biological replicates is shown. (C) SARs of chromeno[2,3-c]quinolin-12-one derivatives **5**~**27**

Figure 4. Compound **26** exhibits a topoisomerase poison. (A) In vitro topoisomerase I assay. One representative result out of two biological replicates is shown. (B) Western blot analysis of chromatin-bound fractions of MDA-MB-231 cells treated with compound **26** or VP16 for 30 minutes. One representative result out of two biological replicates is shown.

Figure 5. Compounds **14** and **26** trigger apoptosis in MDA-MB-231 cells. (A) Cell-cycle distribution of MDA-MB-231 cells treated with compound **14** or **26** for 24 hours. One representative result out of two biological replicates is shown. (B) Western blot analysis of apoptotic signaling in MDA-MB-231 cells treated with compound **26** for 24 hours. Staurosporine was used as a positive control. One representative result out of two biological replicates is shown. (C) Cell proliferation assay of MDA-MB-231 (left) and MCF-10A (right) cells treated with 2.5 μM of compound **26**. Results are displayed as the mean ± SD from two independent experiments (*n*=4). (D) Cell-cycle distribution of MDA-MB-231 and MCF-10A cells treated with 2.5 μM of compound **26** for 72 hours. One representative result out of two biological replicates is shown.

Figure 6. Compound **26** triggers cell-cycle arrest at the G_2/M phase and DNA damage. (A) Cell-cycle progression of MDA-MB-231 cells treated with compound **26**. Cells were synchronized in the early-S phase by thymidine blockage and treated with

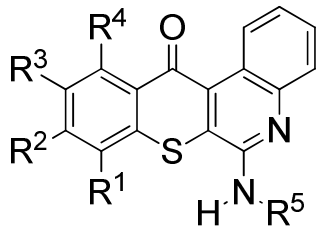
compound **26** 2-hours after release. DNA content was analyzed and histograms were plotted using FlowJo software. Experimental scheme (upper) and one representative result out of two biological replicates (lower) are shown. (B) Western blot analysis of the DNA damage response in MDA-MB-231 cells treated with compound **26** for 24 hours. Staurosporine was used as a positive control. One representative result out of two biological replicates is shown.

Table 1. IC_{50} values for 6 selected compounds.

				IC_{50} (μM)				
Cpd. no.	R^1	R^3	n	MDA-MB-231	MDA-MB-468	MCF7	HCT116	H1299
8	H	Cl	3	3.39±0.20	1.09±0.21	4.36±1.41	3.54±0.41	4.73±0.52
10	H	F	3	2.90±0.11	1.24±0.17	3.98±0.72	3.05±0.30	3.02±0.43
12	H	OMe	3	4.50±0.19	3.04±0.32	>5.00	>5.00	>5.00
14	Cl	H	3	2.19±0.10	0.27±0.18	4.49±1.72	2.51±0.42	3.69±0.54
19	H	Me	3	3.69±0.20	2.89±0.24	>5.00	4.22±0.41	>5.00
26	Cl	F	2	1.14±0.04	0.22±0.04	0.80±0.21	0.53±0.31	0.53±0.08

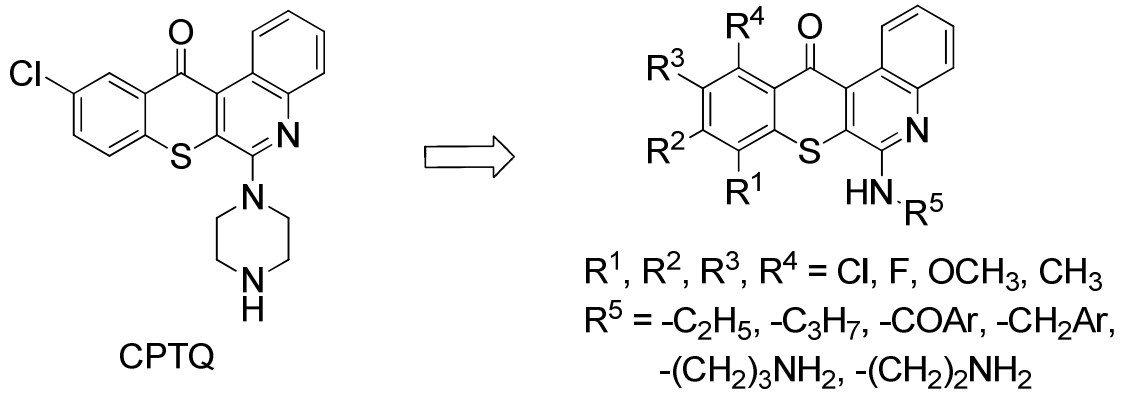
Five cancer cell lines were treated with a series of concentration of the indicated compounds and cell viability was determined by MTT assay. The concentration (μM) inhibiting 50% of cell growth (IC_{50}) was calculated by CompuSyn software and displayed as mean ± S.D. from three independent experiments (n=9).

Chart 1. Structure of chromeno[2,3-*c*]quinolin-12-one derivatives **5-27**



Cpd. no.	R^1	R^2	R^3	R^4	R^5	yield (%)
5	-H	-H	-Cl	-H	$-CH_2C_6H_4OCH_3$(*p*-)	92
6	-H	-H	-Cl	-H	-CO(2-pyridinyl)	36
7	-H	-H	-Cl	-H	$-CH_2CH_2CH_3$	75
8	-H	-H	-Cl	-H	$-CH_2CH_2CH_2NH_2$	88
9	-H	-H	-F	-H	$-CH_2CH_2CH_3$	71
10	-H	-H	-F	-H	$-CH_2CH_2CH_2NH_2$	75
11	-H	-H	$-OCH_3$	-H	$-CH_2CH_2CH_3$	45
12	-H	-H	$-OCH_3$	-H	$-CH_2CH_2CH_2NH_2$	46
13	-Cl	-H	-H	-H	$-CH_2CH_2CH_3$	84
14	-Cl	-H	-H	-H	$-CH_2CH_2CH_2NH_2$	47
15	-H	-Cl	-H	-H	$-CH_2CH_2CH_3$	52
16	-H	-H	-H	-Cl	$-CH_2CH_2CH_3$	47
17	-H	-H	-F	-H	$-CH_2$(3-pyridinyl)	54
18	-H	-H	-F	-H	$-CH_2C_6H_5$	42
19	-H	-H	$-CH_3$	-H	$-CH_2CH_2CH_2NH_2$	63
20	-H	-H	$-CH_3$	-H	$-CH_2CH_3$	30
21	-H	-H	$-CH_3$	-H	$-CH_2C_6H_5$	55
22	-F	-H	-H	-H	$-CH_2CH_3$	53
23	-F	-H	-H	-H	$-CH_2C_6H_5$	69
24	$-OCH_3$	-H	-H	-H	$-CH_2CH_3$	63
25	$-OCH_3$	-H	-H	-H	$-CH_2C_6H_5$	39
26	-Cl	-H	-F	-H	$-CH_2CH_2NH_2$	11
27	-Cl	-H	-F	-H	$-CH_2CH_3$	18

Figure 1



CPTQ

R^1, R^2, R^3, R^4 = Cl, F, OCH_3, CH_3

R^5 = $-C_2H_5$, $-C_3H_7$, -COAr, $-CH_2Ar$, $-(CH_2)_3NH_2$, $-(CH_2)_2NH_2$

Figure 2

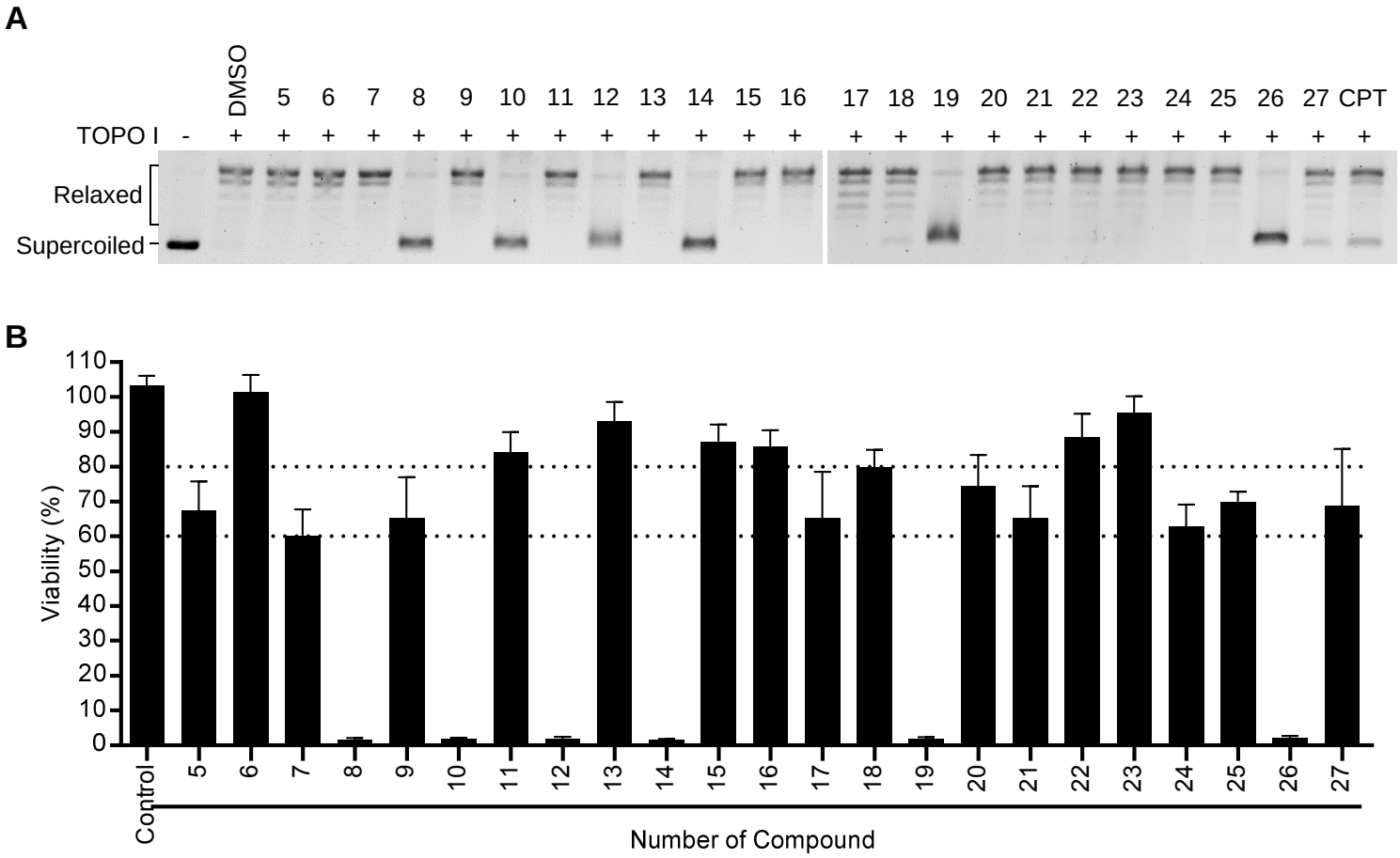
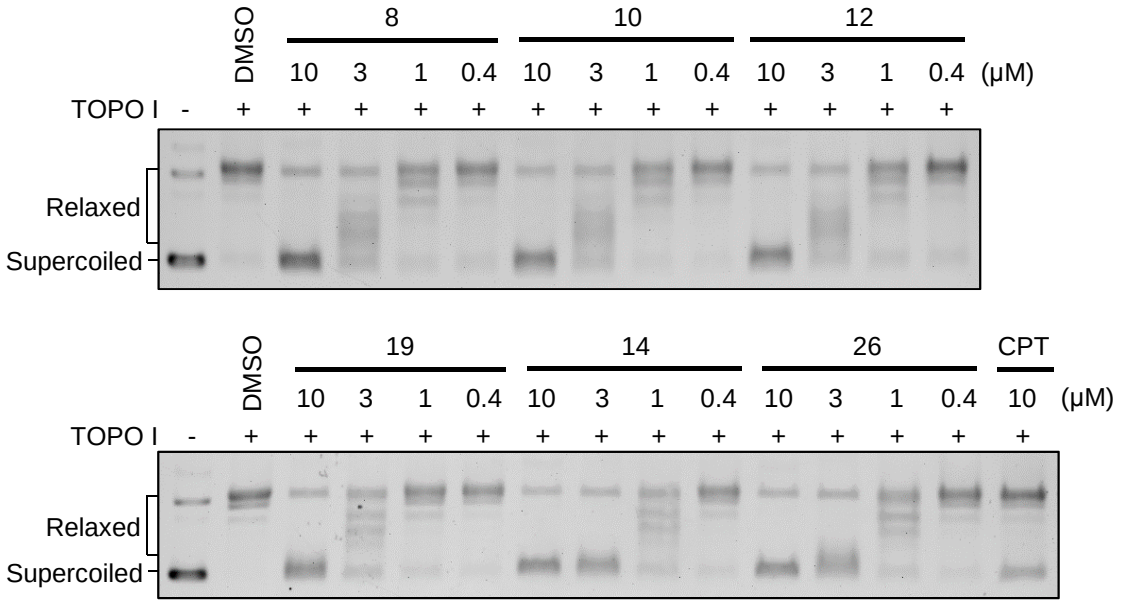
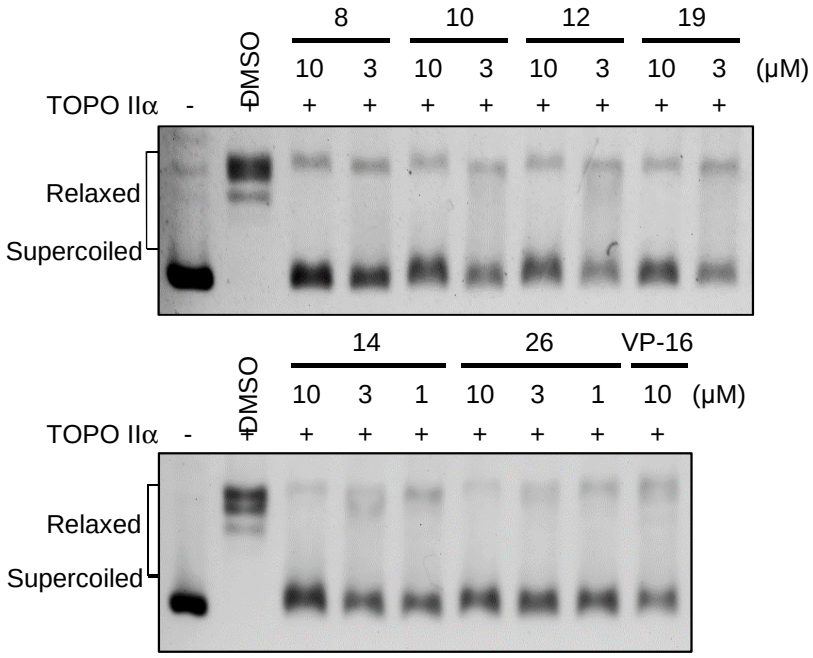

A
DMSO
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 CPT
TOPO I - +
Relaxed
Supercoiled
B
Viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
Control
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Number of Compound

Figure 3

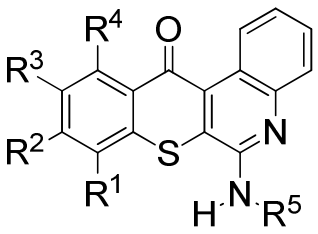
A



B



C



R^1: Replacement of OCH_3 or Cl increases cytotoxicity and topo inhibitory activity.
R^2: Not available.
R^3: Replacement of electron-attracting halogens (Cl or F) increases cytotoxicity.
R^4: Not available.
R^5: Replacement of 2-aminoethyl or 3-aminopropyl group strongly enhances cytotoxicity and topo inhibitory activity.

Figure 4.

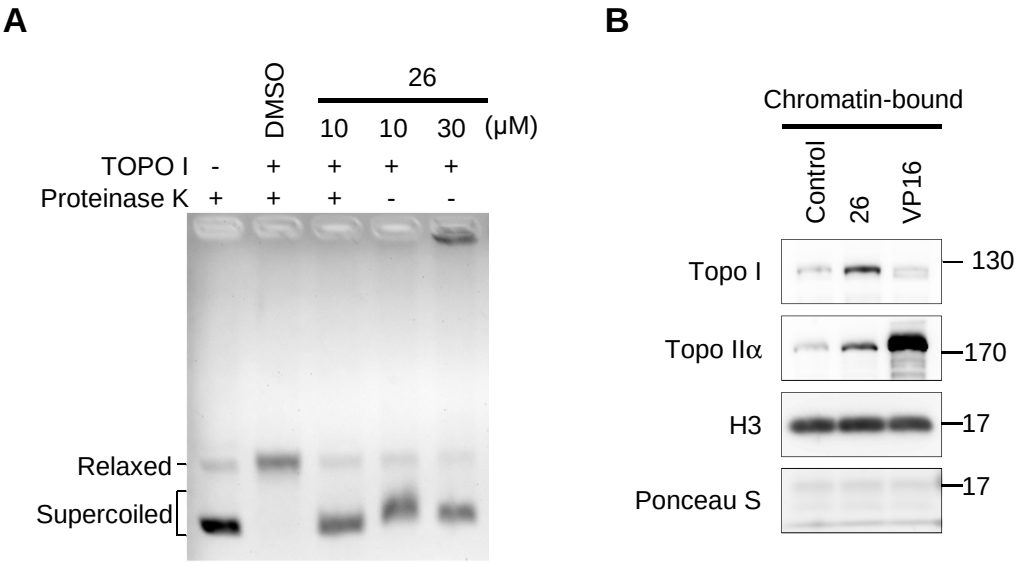

A
DMSO
26
10
10
30
(μM)
TOPO I - + + + +
Proteinase K + + + - -
Relaxed
Supercoiled
B
Chromatin-bound
Control
26
VP16
Topo I
130
Topo IIα
170
H3
17
Ponceau S
17

Figure 5

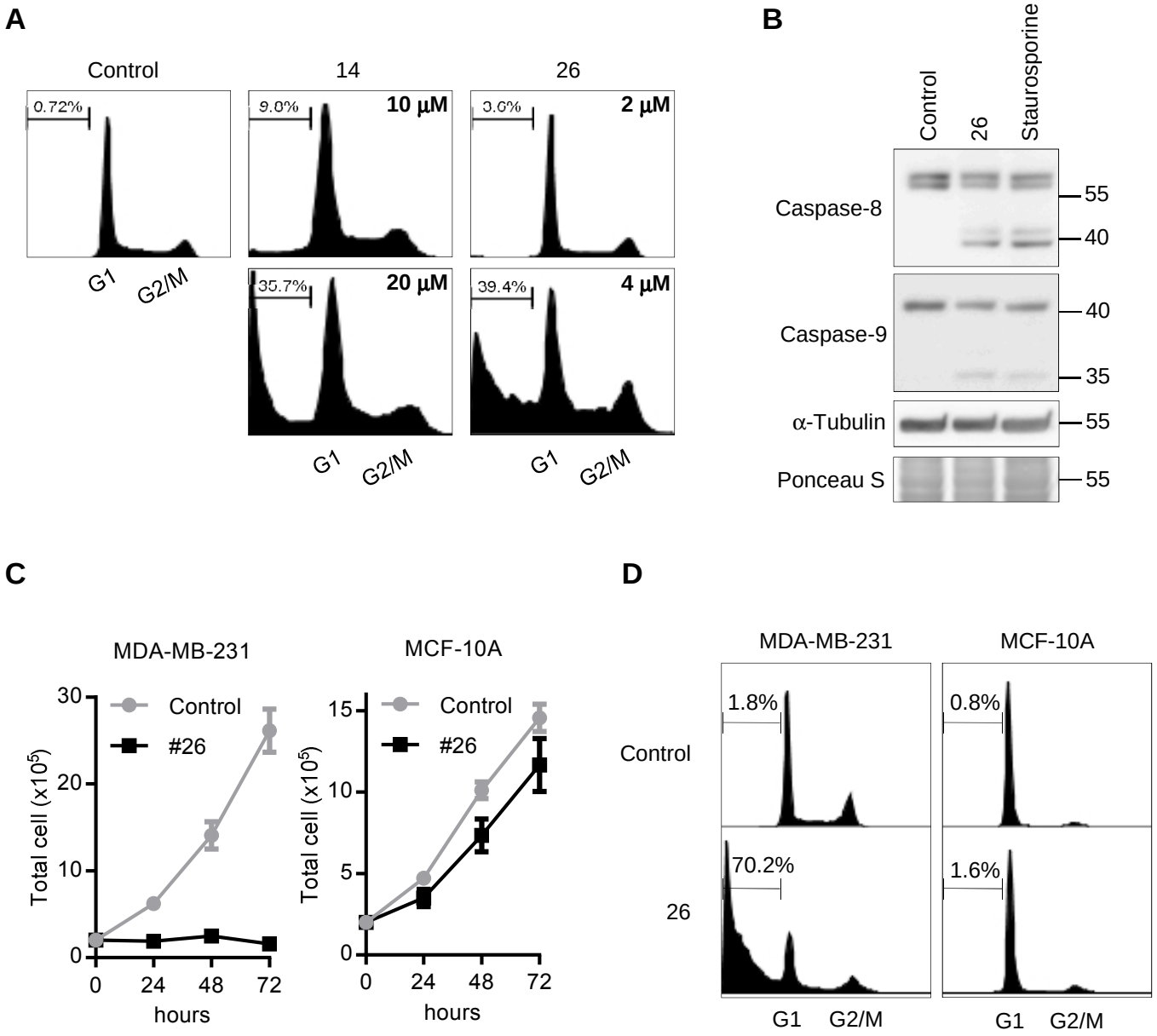
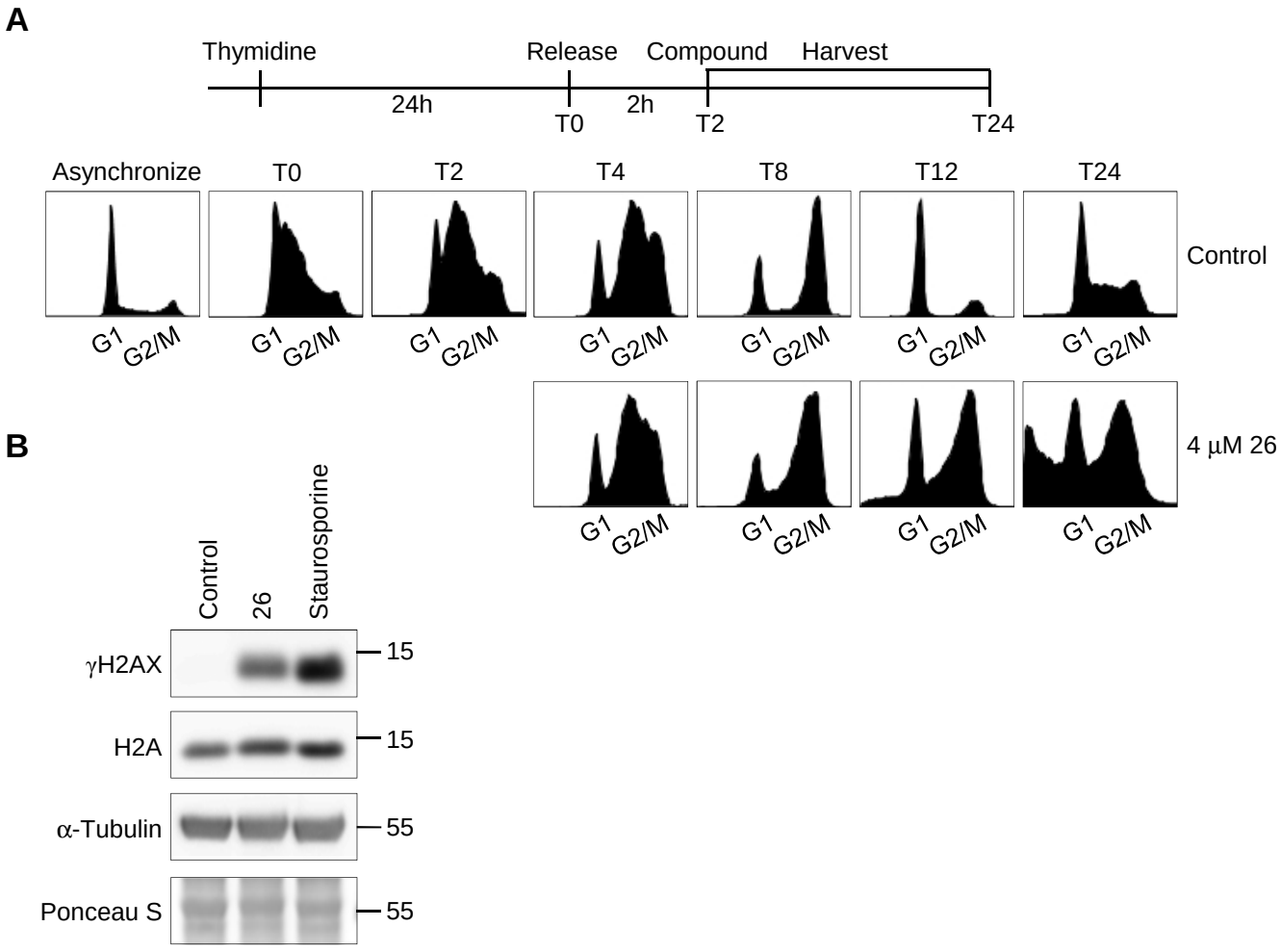
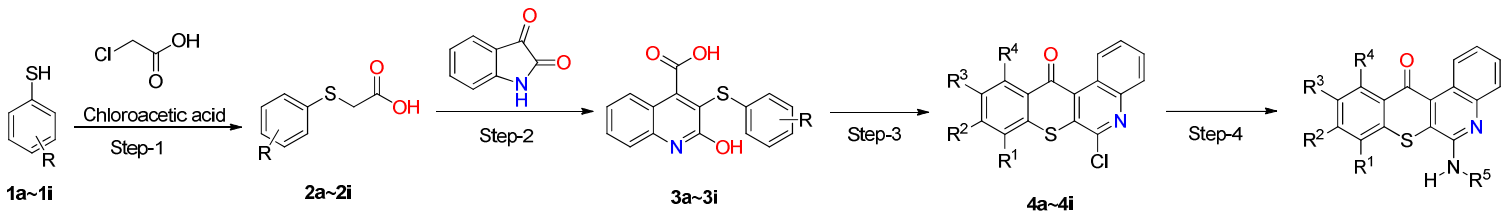

A
Control
14
26
0.72%
9.0%
10 µM
3.6%
2 µM
35.7%
20 µM
39.4%
4 µM
G1
G2/M
B
Control
26
Staurosporine
Caspase-8
Caspase-9
α-Tubulin
Ponceau S
55
40
40
35
55
55
C
MDA-MB-231
MCF-10A
Control
#26
Total cell (x10^5)
hours
D
MDA-MB-231
MCF-10A
Control
26
1.8%
0.8%
70.2%
1.6%
G1
G2/M

Figure 6


A
Thymidine
Release
Compound
Harvest
24h
2h
T0
T2
T24
Asynchronize
T0
T2
T4
T8
T12
T24
Control
4 μM 26
G1
G2/M
B
Control
26
Staurosporine
γH2AX
H2A
α-Tubulin
Ponceau S
15
15
55
55

Scheme 1. Synthesis of compounds **5-27**



a: R = 4-Cl;
b: R = 4-F;
c: R = 4-OCH_3;
d: R = 2-Cl;
e: R = 3-Cl;
f: R = 4-CH_3;
g: R = 2-F;
h: R = 2-OCH_3;
i: R = 2-Cl, 4-F.

a: $R^1 = R^2 = R^4 = -H$, $R^3 = -Cl$;
b: $R^1 = R^2 = R^4 = -H$, $R^3 = -F$;
c: $R^1 = R^2 = R^4 = -H$, $R^3 = -OCH_3$;
d: $R^1 = -Cl$, $R^2 = R^3 = R^4 = -H$;
e1: $R^1 = R^3 = R^4 = -H$, $R^2 = -Cl$;
e2: $R^1 = R^2 = R^3 = -H$, $R^4 = -Cl$;
f: $R^1 = R^2 = R^4 = -H$, $R^3 = -CH_3$;
g: $R^1 = -F$, $R^2 = R^3 = R^4 = -H$;
h: $R^1 = -OCH_3$, $R^2 = R^3 = R^4 = -H$;
i: $R^1 = -Cl$, $R^2 = R^4 = -H$, $R^3 = -F$.

Reagents and conditions: (step 1) chloroacetic acid, NaOH (2.6 eq.), H_2O, reflux, 3-12 h; (step 2) isatin (0.9 eq.), NaOAc (0.2 eq.), 150 °C, 1~5 h; (step 3) $POCl_3$, 150 °C, 48 h; (step 4) 1° , 2° amine, 120 °C, DMSO, 2~3 h.

Research Highlights

- Six compounds identified as dual Topo inhibitors.
- **26** exhibits the highest toxicity in five cancer cell lines but is less toxic in normal breast epithelial cells.
- **26** traps Topo I and IIα on chromatin and suppresses their activities.
- **26** triggers DNA damage, cell-cycle arrest at the G_2/M phase, followed by apoptosis.