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DESIGN, SYNTHESIS, *IN VITRO* ANTIPROLIFERATIVE ACTIVITY EVALUATION OF 2-ALKANOYLAMIDOTHIOPHENE-3-CARBOXAMIDE DERIVATIVES

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Abstract – A series of 2-alkanoylamidothiophene-3-carboxamide derivatives were synthesized based on the hit compound **1**. The anti-proliferative activity of all the compounds *in vitro* against MGC-803 (stomach) and HCT-116 (colon) cancer cell lines using SRB assays were tested. Several compounds showed improved anti-proliferative activity against MGC-803 and HCT-116. SAR study revealed that chlorine substituent in the 2-acetylamino part was important for anti-proliferative activity. **5a**, **11b**, **11c** and **11d** were the most potent compounds against MGC-803 (IC_{50}s = 2.32-2.95 μM), and **5a** and **11c** also showed good anti-proliferative activity against HCT-116 cells (IC_{50}s = 3.41-3.75 μM). In addition, the anti-proliferative activity of **11b** and **11d** could be attributed to the apoptosis in HCT116 cells via caspase 3 activation, confirmed by flow cytometry assay and western blot analysis. Meanwhile, **11b** and **11d** decreased the mitochondrial membrane potential (MMP) in HCT116 cells.

INTRODUCTION

The 2-acylaminothiophene-3-carboxamide derivatives have gained much interest because of their wide range of biological activities, such as anti-diabetic,[1] anti-virus (compound **I**, Figure 1),[2] anti-bacterial,[3]

anti-inflammatory,[4] anti-neurodegenerative (compound **II**, Figure 1),[5] analgesic,[6] cystic fibrosis treatment[7] and aspartic protease inhibiting activities[8] which have been extensively reported in literature. In addition, studies focus on their potentials as anticancer agents have also been performed. Recently, Cao *et al*. developed a multi-target inhibitor (compound **III**, Figure 1) bearing a 2-acylaminothiophene-3-carboxamide scaffold against ABL kinases and tubulin. Compound **III** showed significant inhibition against both ABLs driven cell lines in the dose-response fashion while only moderate effect on the parental cells.[9] Compound **IV** (Figure 1) as the enzyme Fms-like tyrosine kinase-3(FLT3) inhibitor (IC_{50} = 0.027 μM) blocked the proliferation of MV4-11 cells (IC_{50} = 0.41 μM).[10] In addition, kinesin spindle protein (KSP) inhibitors which subsequently induced apoptosis (compound **V**, Figure 1)[11] were also reported and compound **V** showed inhibition against A-549 cells with the EC_{50} = 2.0 ± 0.2 μM.

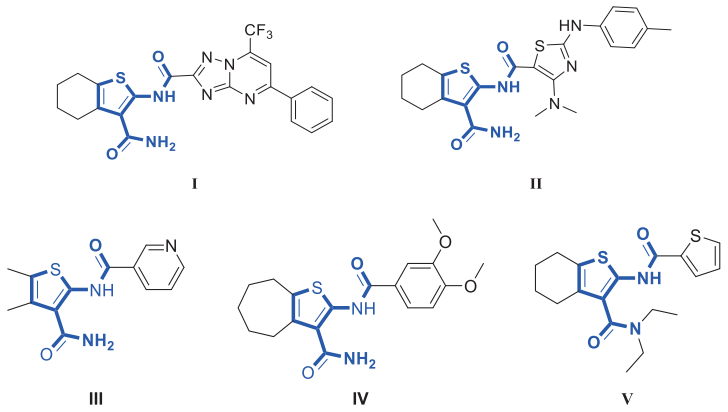


Figure 1. Biological active 2-acylaminothiophene-3-carboxamide derivatives

We have synthesized series of 2-(substituted acetamino)thiophene-3-carboxamides in our lab in recent years. Through an anti-proliferative activity screening, compound **1** was identified as an anti-proliferative agent with modest potency against MGC-803 and HCT-116 (IC_{50} = 5.96 ± 0.08 and 19.80 ± 0.65 μM,

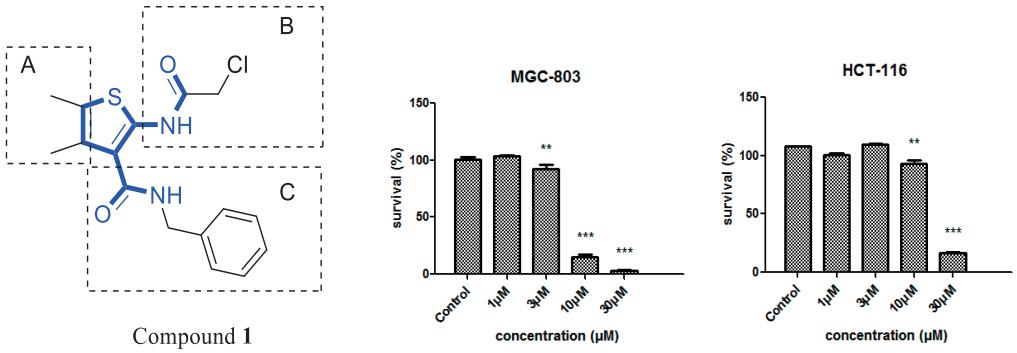


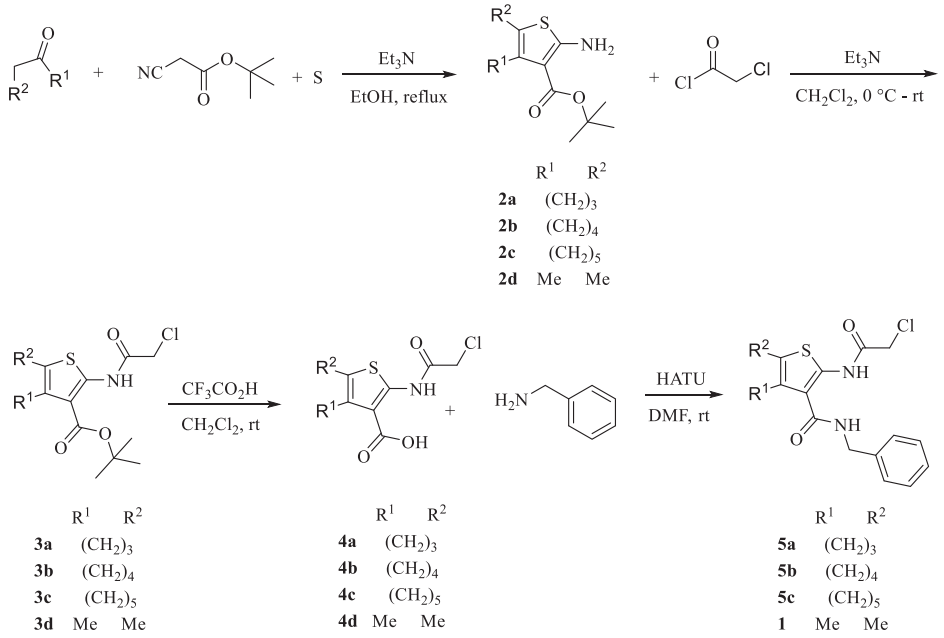
Figure 2. Structure of compound **1** and antiproliferative activity of compound **1** in MGC-803 and HCT-116 at different concentrations (Values are mean ± SD, n=2, *p<0.05, **p<0.01 and ***p<0.001 compared with DMSO (1‰) group. Differences between individual groups are analyzed by T-test).

respectively) (Figure 2; entry 1 in Table 1). Song *et al*. suggested that halogen substituted acetylamino chains at the 3-position of the benzoylurea derivatives played a significant role in regulating the anti-proliferative activities. Their anti-proliferative activity in tumor cells was ranked in an order of the nature of halogens: I > Br > Cl > F.[12] Herein, we describe the synthesis of series novel 2-(chloroacetamido)thiophene-3-carboxamides or terminal chloro-subsituted alkanoylamidothiophene-3-carboxamides based on hit compound **1**, and *in vitro* evaluation of the anti-proliferative activity against MGC-803 and HCT-116 cells is also discussed.

RESULTS AND DISCUSSION

CHEMISTRY

Although some aroyl substituted aminothiophenes at ortho-position have been developed (Figure 1),[2d, 6, 13] alkanoyl substituted corresponding compound has rarely been reported. We designed and synthesized seventeen 2-alkanoylamidothiophene-3-carboxamide derivatives and the synthetic routes were outlined in Schemes 1-4. With commercially available ketones as starting materials, **2a**-**2d** were synthesized via Gewald cyclization.[14] Acylation of the 2-amino group of **2a**-**2d** afforded **3a**-**3d** which subsequently gave **4a**-**4d** after removing the *tert*-butyl group.[15] HATU-facilitated condensations of **4a**-**4d** with benzylamine produced **5a**-**5c** and compound **1**[16] (Scheme 1).



	R^1	R^2
2a		$(CH_2)_3$
2b		$(CH_2)_4$
2c		$(CH_2)_5$
2d	Me	Me

	R^1	R^2
3a		$(CH_2)_3$
3b		$(CH_2)_4$
3c		$(CH_2)_5$
3d	Me	Me

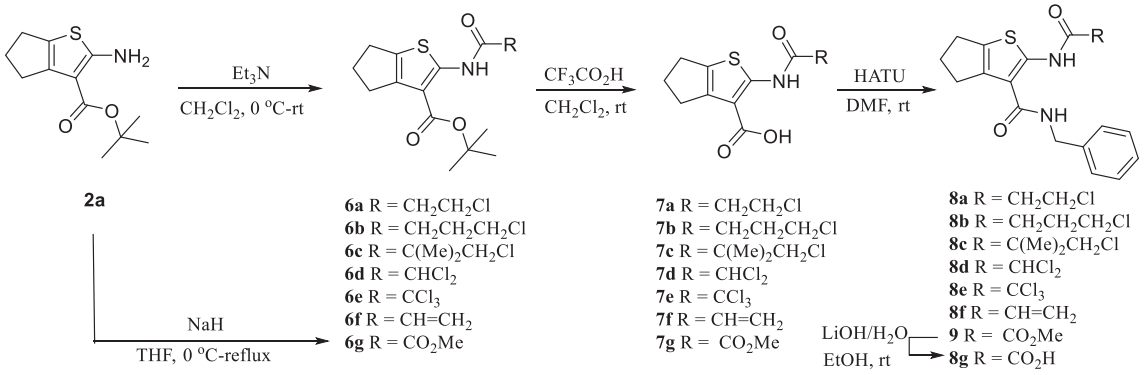
	R^1	R^2
4a		$(CH_2)_3$
4b		$(CH_2)_4$
4c		$(CH_2)_5$
4d	Me	Me

	R^1	R^2
5a		$(CH_2)_3$
5b		$(CH_2)_4$
5c		$(CH_2)_5$
1	Me	Me

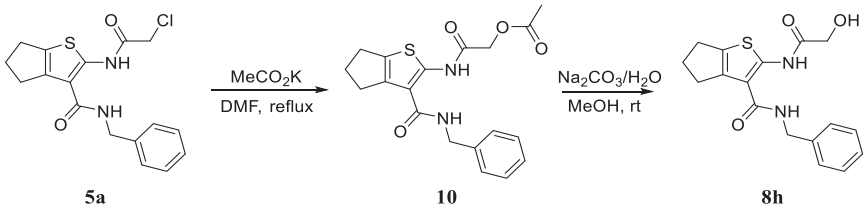
Scheme 1

Acylation of the 2-amino group of **2a** with corresponding acid chlorides in the presence of Et_3N afforded **6a**-**6f**. In addition, **6g** was prepared by condensation of **2a** with dimethyl oxalate in the presence of NaH.[17] Subsequent deprotection of the *tert*-butyl group in **6a**-**6g** afforded **7a**-**7g** which underwent the

HATU-facilitated condensations with benzylamine, giving **8a-8f** and **9**. Hydrolysis of **9** with $LiOH/H_2O$[18] afforded product **8g** (Scheme 2). Nucleophilic substitution of **5a** with potassium acetate[19] followed by deacetylation provided **8h** (Scheme 3).

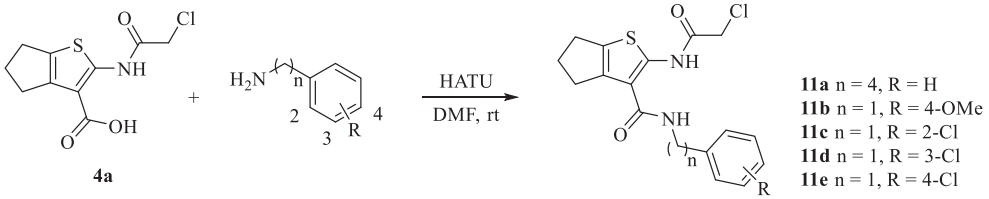


Scheme 2



Scheme 3

HATU-facilitated coupling reactions of **4a** with corresponding amines produced **11a-11e** (Scheme 4).



Scheme 4

Biological study

In vitro anti-proliferative activities and SAR

All the final products were tested for the anti-proliferative activity *in vitro* against MGC-803 and HCT-116 for 72 h using SRB assays with cisplatin as positive control.[20] As depicted in Table 1, 9 compounds (**5a**, **5c**, **8d**, **8f**, **11a**, **11b**, **11c**, **11d**, **11e**) showed anti-proliferative activity against MGC-803 ($IC_{50} < 10$ μM) and 6 compounds (**5a, 11a**, **11b**, **11c**, **11d**, **11e**) showed anti-proliferative activity against HCT-116 ($IC_{50} < 10$ μM).

Compared to the hit compound **1** with two methyl groups at 4- and 5- positions, introduction of hindered aliphatie cycles to the thiophene ring had different performances. In detail, the cyclopentyl compound **5a** exhibited increase of anti-proliferative activities on MGC-803 and HCT-116 with IC_{50} values of 2.53 ± 0.11 and 3.41 ± 1.13 μM, respectively. However, the anti-proliferative activities decreased with the expansion of ring size (**5b** showed a decreased activity on MGC-803 and **5c** showed a decreased activity on HCT-116). It is indicated that a five-member ring substituted at 4- and 5- positions of the thiophene ring was beneficial to the activity potency.

When the cyclopentyl group was determinated to be the better fusing aliphatic ring of thiophene, we focused our attention on the α-chloro at 2-acetylamino group which had the tendency of being nucleophilically substituted. At first, terminal chlorinated alkyl chains with different lengths were introduced, affording compounds **8a** and **8b** with lower nucleophilic substitution reaction activity. Comparing with **5a** (IC_{50} = 2.53 ± 0.11 μM), IC_{50} of **8a** in which the chain was extended with one carbon was increasing to 14.63 ± 5.18 μM against MGC-803. And **8b** with further extended chain length showed no anti-proliferative activity (IC_{50} > 30 μM). This tendency matched well with their anti-proliferative activities against HCT-116. On the other hand, **8c** with increased steric hindrance at β-carbon of terminal chlorine, which decreased the tendency of being nucleophilically substituted, also showed no anti-proliferative activity against both MGC-803 (IC_{50} > 30 μM) and HCT-116 (IC_{50} > 30 μM). Compounds bearing one more terminal chlorine atom, such as **8d**, although not as active as **5a**, showed considerable anti-proliferative activity against MGC-803 (IC_{50} = 5.75 ± 0.32 μM). However, no anti-proliferative activity against MGC-803 (IC_{50} > 30 μM) and HCT-116 (IC_{50} > 30 μM) was observed when the terminal carbon was substituted with three chlorine atoms (**8e**). Similarly, **8g** and **8h** in which the 2-acetylamino position was substituted with 3-oxopropanoic acid or 2-hydroxyacetamido group, also showed no anti-proliferative activity against both MGC-803 (IC_{50} > 30 μM) and HCT-116 (IC_{50} > 30 μM). While the anti-proliferative activity was maintained when the 2-acetylamino group was substituted with electrophilic vinyl group (**8f**, IC_{50} = 7.88 ± 0.54 μM against MGC-803). These results were consistent to the suggestion that halogen substituted acetylamino chains at the 3-position in the benzoylurea derivatives play a significant role in regulating the anti-proliferative activities.[12] Thus, it is believed that the electrophilicity of the 2-acetylamino moiety in the molecule would be in favor of its anti-proliferative activity, and the chlorine substituent in the 2-acetylaminopart may play a key role as a leaving group.

At last, we tried to modify the chain length and the substituents on the aromatic ring as well. Compound **11a** in which the chain length was prolonged to four carbons maintained anti-proliferative activity against both MGC-803 (IC_{50} = 3.89 ± 0.30 μM) and HCT-116 (IC_{50} = 8.03 ± 0.63 μM). While **11b** having a 4-methyoxy group on the aromatic ring showed an increased anti-proliferative activity against both

MGC-803 (IC_{50} = 2.32 ± 0.39 μM) and HCT-116 (IC_{50} = 5.89 ± 0.63 μM) compared with compound **1**. Meanwhile, 2-chloro (**11c**), 3-chloro (**11d**) and 4-chloro (**11e**) substituted on the aromatic ring also increased the anti-proliferative activities against both MGC-803 and HCT-116. The results suggested that both electron-donating groups and electron-withdrawing groups on the aromatic ring increase the anti-proliferative activity. Thus, both the chain length of the 2-acylamino moiety and the substituents on the aromatic ring play important roles for the anti-proliferative activity.

Table 1. Anti-proliferative activities of 2-alkanoylamidothiophene-3-carboxamide derivatives against MGC-803 and HCT-116 cells

Entry	Compound	IC_{50} (μM)	
		MGC-803	HCT-116
1	**1**	5.96 ± 0.08	19.80 ± 0.65
2	**5a**	2.53 ± 0.11	3.41 ± 1.13
3	**5b**	10.90 ± 2.78	20.91 ± 0.17
4	**5c**	4.83 ± 0.85	> 30
5	**8a**	14.63 ± 5.18	15.33 ± 0.03
6	**8b**	> 30	> 30
7	**8c**	> 30	> 30
8	**8d**	5.75 ± 0.32	16.75 ± 0.45
9	**8e**	> 30	> 30
10	**8f**	7.88 ± 0.54	16.12 ± 0.30
11	**8g**	> 30	> 30
12	**8h**	> 30	> 30
13	**11a**	3.89 ± 0.30	8.03 ± 0.63
14	**11b**	2.32 ± 0.39	5.89 ± 0.63
15	**11c**	2.95 ± 0.05	3.75 ± 0.68
16	**11d**	2.76 ± 0.45	6.23 ± 3.04
17	**11e**	3.01 ± 0.09	7.71 ± 2.69
18	Cisplatin	6.04 ± 0.20	3.57 ± 0.23

Compounds 11b and 11d induce apoptotic cell death

Apoptosis is a programmed cell death process by which the body eliminates damaged or unnecessary cells and is playing a vital role in cancer development and tumor cell survival.[21] To characterize whether the anti-proliferative activity was accomplished by inducing cell apoptosis, HCT116 cells were treated with vehicle alone or with selected compound **11b** or **11d** for 48 h and then stained with FITC-annexin V and propidium iodide (PI)[22] (Figure 3). The results showed that compounds **11b** and **11d** induced 57.93% and 42.58% apoptosis, respectively, comparing to 9.77% in the control group after treated for 48 h (10 μmol/L dose). Therefore, it is evident that the anti-proliferative activities of these compounds are related to inducing apoptosis in HCT-116 cell lines.

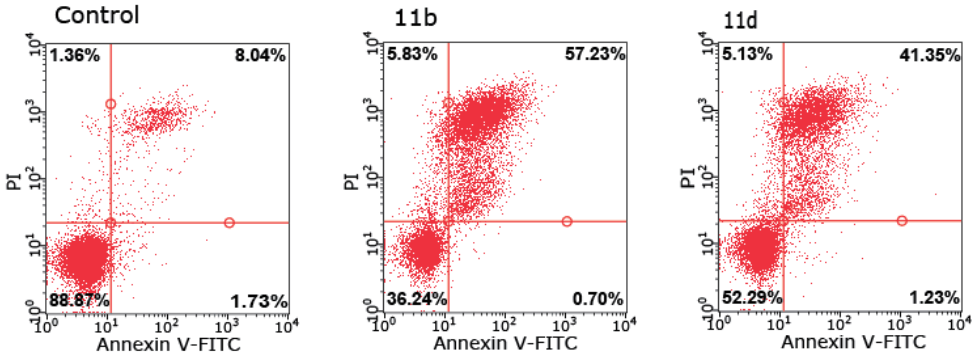


Figure 3. 11b and **11d** induced apoptosis of HCT-116 cells. Cells were stained with Annexin V-FITC/PI and quantitated by flow cytometry. The cells were treated with control (1‰ DMSO) or 10 μM compound **11b** and **11d** for 48 h.

Activation of caspase 3 was involved in the apoptosis induced by 11b and 11d

Caspase 3 is one of the key executioners of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase 1 (PARP1).[23] To investigate the molecular mechanisms involved in the observed apoptosis, we measured the expression of cleaved-caspase 3 and PARP1 in HCT-116 cell line treated with **11b** and **11d** by Western blotting. HCT-116 was treated with 15 μM of **11b** and **11d** for 24 h and 48 h, then all cells were harvested and assayed for cleaved-caspase 3 and PARP1 using GAPDH as a loading control (Figure 4). Comparing to the control, the relative activities of cleaved-caspase 3 were increased in HCT-116 cell line after treated with **11b** and **11d** for 48 h, while we didn't observe the activation of cleaved-caspase 3 after treating for 24 h. In addition, it was found that **11b** and **11d** significantly increased the cleavage of PARP1 in HCT-116 cell line after 24 h and 48 h treatment. Together, these findings revealed that compound **11b** and **11d** induced HCT-116 cells apoptosis through activation of caspase 3.

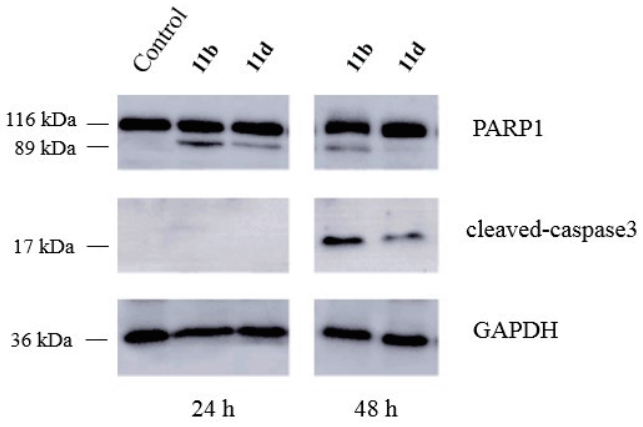


Figure 4. Effects of **11b** and **11d** on activation of caspase 3. HCT-116 was treated with 15 μM of **11b** and **11d** for 24 h and 48 h and then the cells were harvested and assayed for caspase 3 activation and PARP1 cleavage using GAPDH as a loading control.

Compounds 11b and 11d decrease the mitochondrial membrane potential (MMP)

Decreasing of MMP during apoptosis has been reported in a number of studies, leading to the general notion that depolarization of mitochondria is one of the first events to occur during apoptosis.[24] The effect of **11b** and **11d** on the MMP in HCT-116 cells was investigated with the fluorescent probe JC-1, a mitochondrion-specific and voltage-dependent dye.[25] Treatment of HCT-116 cells with **11b** and **11d** at 10 μM for 24 h resulted in the percentage of cells with depolarized MMP from 2.85% of control cells to 37.28% and 16.54%, respectively (Figure 5).

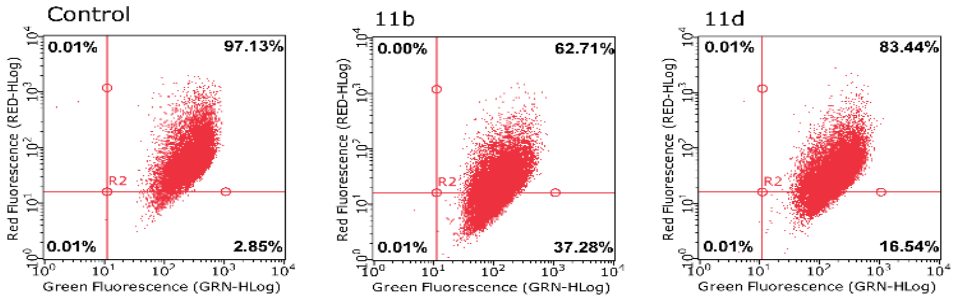


Figure 5. Effects of **11b** and **11d** on mitochondrial membrane potential. Incubation with **11b**, **11d** at 10 μM for 24 h, the mitochondrial membrane potential (MMP) was analyzed by JC-1 fluorescence with flow cytometry analysis.

CONCLUSIONS

In summary, a series of 2-alkanoylamidothiophene-3-carboxamide derivatives were synthesized based on the hit compound **1**. The anti-proliferative activity of all the compounds against MGC-803 and HCT-116 cell lines was tested using SRB assays. Nine compounds (**5a**, **5c**, **8d**, **8f**, **11a**, **11b**, **11c**, **11d**, **11e**) showed anti-proliferative activity against MGC-803 (IC_{50} < 10 μM) and 6 compounds (**5a,11a**, **11b**, **11c**, **11d**, **11e**) showed anti-proliferative activity against HCT-116 (IC_{50} < 10 μM). The SAR study revealed that terminal chloro-substituent at ortho position of alkanoylamidothiophene was important for anti-proliferative activity. **5a**, **11b**, **11c** and **11d** were the most potent compounds against MGC-803 and HCT-116 cell lines (IC_{50} = 2.32-6.23 μM). Annexin V-FITC/Propidium iodide (PI) double staining assay in HCT-116 cells suggested that the anti-proliferative activity of compound **11b** and **11d** occurred via apoptosis. Western blot analysis showed that the anti-proliferative activity of **11b** and **11d** could be attributed to the apoptosis in HCT116 cells via caspase 3 activation. In addition, **11b** and **11d** depolarized the mitochondrial membrane in HCT116 cells. Further studies on improving the anti-proliferative activity and exploring the action mechanism are ongoing.

EXPERIMENTAL

General comments

Starting materials, reagents and chemicals were purchased from commercial suppliers and used without further purification unless otherwise stated. The progress of reactions was monitored by silica gel thin layer chromatography (TLC) plates, visualized under UV. Flash column chromatography was performed using Qingdao Haiyang silica gel (200 - 300) with distilled solvents. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker DRX-400 Fourier transform spectrometer. ^{1}H chemical shifts are reported in δ (ppm) using the δ 7.26 signal of $CDCl_3$ or the δ 2.50 signal of DMSO-d_6 as internal standards. ^{13}C chemical shifts are reported in δ (ppm) using the δ 77.23 signal of $CDCl_3$ as internal standard or the δ 39.52 signal of DMSO-d_6 as internal standards. High-resolution mass data were obtained on a MicrOTOF II spectrometer. All melting points were obtained on a Laboratory Devices MEL-TEMP II melting apparatus and are uncorrected.

Chemical Synthesis

General procedure for preparation of compounds 2a–2d

To a mixture of ketone (1.0 mmol), *tert*-butyl cyanoacetate (1.0 mmol), and sulfur (1.1 mmol) in EtOH (20 mL) was added triethylamine (2 mmol) and the reaction mixture was refluxed for 16 h. Afterwards the reaction mixture was concentrated and the residue was partitioned between water and EtOAc. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and EtOAc (20:1) as the eluent.

***tert*-Butyl 2-amino-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate** (**2a**). Prepared from cyclopentanone, petroleum ether:EtOAc = 20:1, to give white solid (0.17 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.79 (s, 2H), 2.78-2.81 (m, 2H), 2.69-2.72 (m, 2H), 2.26-2.33 (m, 2H), 1.53 (s, 9H).

***tert*-Butyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate** (**2b**). Prepared from cyclohexanone, petroleum ether:EtOAc = 20:1, to give white solid (0.13 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.87 (s, 2H), 2.67 (t, J = 5.6 Hz, 2H), 2.49 (t, J = 5.6 Hz, 2H), 1.72-1.78 (m, 4H), 1.54 (s, 9H).

***tert*-Butyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate** (**2c**). Prepared from cycloheptanone and the base is morpholine, petroleum ether:EtOAc = 20:1, to give white solid (0.17 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.67 (s, 2H), 2.93-2.96 (m, 2H), 2.56-2.58 (m, 2H), 1.77-1.83 (m, 2H), 1.59-1.66 (m, 4H), 1.55 (s, 9H).

***tert*-Butyl 2-amino-4,5-dimethylthiophene-3-carboxylate** (**2d**). Prepared from butan-2-one, petroleum ether:EtOAc = 20:1, to give white solid (0.13 g, 56%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.83 (s, 2H), 2.14 (s, 6H), 1.55 (s, 9H).

General procedure for preparation of compounds 3a–3d

tert-Butyl 2-aminothiophene-3-carboxylate (1.0 mmol) was dissolved in CH_2Cl_2 (15.0 mL) and treated

with triethylamine (3.0 mmol). To this mixture, the solution of acid chloride (1.2 mmol) in CH_2Cl_2 (5 mL) was added dropwise under 0 °C. This mixture was stirred for 0.5 h at room temperature. The mixture was diluted by CH_2Cl_2, and washed with water. The organic phase was dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and EtOAc (20:1) as the eluent.

***tert*-Butyl 2-(2-chloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (3a)**. Prepared from **2a** and chloroacetyl chloride, petroleum ether:EtOAc = 20:1, to give pale yellow solid (0.29 g, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.90 (s, 1H), 4.24 (s, 2H), 2.83-2.90 (m, 4H), 2.34-2.41 (m, 2H), 1.58 (s, 9H).

***tert*-Butyl 2-(2-chloroacetamino)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (3b)**. Prepared from **2b**, petroleum ether:EtOAc = 20:1, to give white solid (0.33 g, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.17 (s, 1H), 4.24 (s, 2H), 2.75 (t, J = 5.2 Hz, 2H), 2.65 (t, J = 5.2 Hz, 2H), 1.75-1.79 (m, 4H), 1.59 (s, 9H).

***tert*-Butyl 2-(2-chloroacetamino)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate (3c)**. Prepared from **2c**, petroleum ether:EtOAc = 20:1, to give white solid (0.33 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.97 (s, 1H), 4.22 (s, 2H), 3.00-3.03 (m 2H), 2.72-2.74 (m, 2H), 1.82-1.88 (m, 2H), 1.64-1.67 (m, 2H), 1.58-1.61(m, 11H).

***tert*-Butyl 2-(2-chloroacetamino)-4,5-dimethylthiophene-3-carboxylate (3d)**. Prepared from **2d**, petroleum ether:EtOAc = 20:1, to give white solid (0.25 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.19 (s, 1H), 4.24 (s, 2H), 2.27 (s, 3H), 2.23 (s, 3H), 1.60 (s, 9H).

General procedure for preparation of compounds 4a–4d

tert-Butyl 2-acylaminothiophene-3-carboxylate was dissolved in 20% of trifluoroacetic acid in CH_2Cl_2. This mixture was stirred for 2-3 h at room temperature and diluted by CH_2Cl_2, and washed with water. The organic phase was dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of CH_2Cl_2 and MeOH (20:1) as the eluent.

2-(2-Chloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (4a). Prepared from **3a,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.24 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.69 (s, 1H), 4.58 (s, 2H), 2.79-2.85 (m, 4H), 2.28-2.35 (m, 2H).

2-(2-Chloroacetamino)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (4b). Prepared from **3b,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.27 g, 99%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.25 (br, 1H), 11.92 (s, 1H), 4.57 (s, 2H), 2.73 (t, J = 5.6 Hz, 2H), 2.61 (t, J = 5.6 Hz, 2H), 1.71-1.73 (m, 4H).

2-(2-Chloroacetamino)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic acid (4c). Prepared from **3c,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.27 g, 93%). ^{1}H NMR (400 MHz,

DMSO-d_6): δ 13.45 (br, 1H), 11.78 (s, 1H), 4.55 (s, 2H), 3.02-3.05 (m, 2H), 2.70-2.72 (m, 2H), 1.79-1.80 (m, 2H), 1.53-1.59 (m, 4H).

2-(2-Chloroacetamino)-4,5-dimethylthiophene-3-carboxylic acid (**4d**). Prepared from **3d,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.23 g, 92%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.38 (br, 1H), 11.93 (s, 1H), 4.58 (s, 2H), 2.24 (s, 3H), 2.21 (s, 3H).

General procedure for preparation of compounds 5a–5c and 1.

2-Acylaminothiophene-3-carboxylic acid (1.0 mmol) was dissolved in DMF (20 mL) and HATU (1.5 mmol) was added. After stirring at room temperature for 10 min, amine (1.5 mmol) was added. The mixture was stirred at room temperature for another 2-3 h. Then the reaction was quenched with water and the aqueous solution was extracted with EtOAc (20 mL×3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and EtOAc (7:1-2:1) as the eluent.

***N*-Benzyl-2-(2-chloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide** (**5a**). Prepared from **4a**, petroleum ether:EtOAc = 5:1-2:1, to white solid (0.1 g, yield 29%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.41 (s, 1H), 7.74 (t, J = 5.9 Hz, 1H), 7.31-7.36 (m, 4H), 7.22-7.27 (m, 1H), 4.52 (s, 2H), 4.51 (d, J = 5.9 Hz, 2H), 2.99 (t, J = 7.0 Hz, 2H), 2.82 (t, J = 7.0 Hz, 2H), 2.35-2.42 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.95, 163.48, 146.88, 139.37, 138.78, 132.69, 128.34, 126.98 (2C), 126.76 (2C), 112.00, 42.49, 42.35, 29.25, 28.30, 27.77. ESI-HRMS (*m/z*): $[M-H]^-$ calcd. for $C_{17}H_{16}ClN_2O_2S$, 347.0626; found 347.0627. mp 195.0-195.7 °C.

***N*-Benzyl-2-(2-chloroacetamino)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide** (**5b**). Prepared from **4b**, petroleum ether:EtOAc = 3:1-2:1, to give white solid (0.23 g, yield 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.96 (s, 1H), 7.33-7.37 (m, 5H), 6.22 (br, 1H), 4.65 (d, J = 5.0 Hz, 2H), 4.23 (s, 2H), 2.67-2.69 (m, 4H), 1.82 (s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.95, 163.48, 139.99, 139.27, 128.95, 128.28 (2C), 127.14 (2C), 127.05, 126.73, 117.89, 42.54, 42.47, 25.05, 23.79, 22.45, 22.29, ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{18}H_{19}ClN_2NaO_2S$, 385.0748; found 385.0756. mp 196.4-197.2 °C.

***N*-Benzyl-2-(2-chloroacetamino)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide** (**5c**). Prepared from **4c**, petroleum ether:EtOAc = 7:1-3:1, to give white solid (0.09 g, yield 23%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.87 (s, 1H), 8.53 (t, J = 5.8 Hz, 1H), 7.32-7.35 (m, 4H), 7.22-7.27 (m, 1H), 4.45 (d, J = 5.8 Hz, 2H), 4.40 (s, 2H), 2.65-2.71 (m, 4H), 1.76-1.83 (m, 2H), 1.54-1.60 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.80, 163.49, 139.22, 135.01, 133.66, 131.30, 128.24 (2C), 127.27 (2C), 126.76, 123.57, 42.66, 42.35, 31.61, 28.29, 28.02, 27.67, 27.09. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{19}H_{21}ClN_2NaO_2S$, 399.0904; found 399.0904. m. p. 216.0-216.5 °C.

***N*-Benzyl-2-(2-chloroacetamino)-4,5-dimethylthiophene-3-carboxamide** (**1**). Prepared from **4d**,

petroleum ether:EtOAc = 7:1-3:1, to give white solid (0.27 g, yield 81%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.47 (s, 1H), 8.32 (t, J = 5.6 Hz, 1H), 7.33-7.34 (m, 4H), 7.23-7.28 (m, 1H), 4.49 (d, J = 5.6 Hz, 2H), 4.46 (s, 2H), 2.24 (s, 3H), 2.17 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.98, 163.36, 139.16, 137.50, 128.19 (2C), 127.11 (2C), 127.05, 126.67, 124.08, 120.51, 42.50, 42.34, 13.08, 12.07. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{16}H_{17}ClN_2NaO_2S$, 359.0591; found 359.0622. mp 160.2-161.5 °C.

General procedure for preparation of compounds 6a–6f

tert-Butyl 2-aminothiophene-3-carboxylate (**2a**) (1.0 mmol) was dissolved in CH_2Cl_2 (15 mL) and treated with triethylamine (3 mmol). To this mixture, the solution of acid chloride (1.2 mmol) in CH_2Cl_2 (5 mL) was added dropwise under 0 °C. This mixture was stirred for 0.5 h at room temperature. The mixture was diluted by CH_2Cl_2 (20 mL), and washed with water. The organic phase was dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and EtOAc (5:1-2:1) as the eluent.

***tert*-Butyl 2-(3-chloropropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (6a)**. Prepared from **2a** and 3-chloropropionyl chloride, triethylamine (3 mmol) was replaced by pyridine (1.5 mmol), petroleum ether:EtOAc = 2:1, to give pale yellow solid 0.267 g. Yield 81%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.16 (s, 1H), 3.88 (t, J = 6.64 Hz, 2H), 2.93 (t, J = 6.64 Hz, 2H), 2.82-2.88 (m, 4H), 2.33-2.40 (m, 2H), 1.57 (s, 9H).

***tert*-Butyl 2-(4-chlorobutanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (6b)**. Prepared from **2a** and 4-chlorobutyryl chloride, petroleum ether:EtOAc = 2:1, to give pale yellow solid (0.30 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.08 (s, 1H), 3.66 (t, J = 6.12 Hz, 2H), 2.82-2.88 (m, 4H), 2.67 (t, J = 6.12 Hz, 2H), 2.33-2.40 (m, 2H), 2.19-2.26 (m, 2H), 1.57 (s, 9H).

***tert*-Butyl 2-(3-chloro-2,2-dimethylpropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (6c)**. Prepared from **2a** and 3-chloropivaloyl chloride, petroleum ether:EtOAc = 2:1, to give pale yellow solid (0.31 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.55 (s, 1H), 3.71 (s, 2H), 2.82-2.88 (m, 4H), 2.34-2.40 (m, 2H), 1.57 (s, 9H), 1.45 (s, 6H).

***tert*-Butyl 2-(2,2-dichloroacetamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (6d)**. Prepared from **2a** and dichloroacetyl chloride, petroleum ether:EtOAc = 5:1, to give pale yellow solid (0.28 g, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.08 (s, 1H), 6.13 (s, 1H), 2.84-2.91 (m, 4H), 2.34-2.42 (m, 2H), 1.58 (s, 9H).

***tert*-Butyl 2-(2,2,2-trichloroacetamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (6e)**. Prepared from **2a** and trichloroacetyl chloride, petroleum ether:EtOAc = 5:1, to give pale yellow solid (0.32 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.45 (s, 1H), 2.88-2.91 (m, 4H), 2.36-2.43 (m, 2H), 1.58 (s, 9H).

***tert*-Butyl 2-acrylamino-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate** (**6f**). Prepared from **2a** and 3-chloropropanoyl chloride, petroleum ether:EtOAc = 2:1, to give pale yellow solid (0.27 g, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.22 (s, 1H), 6.48 (d, J = 16.96 Hz, 1H), 6.34 (dd, J = 16.96, 10.20 Hz, 1H), 5.84 (d, J = 10.20 Hz, 1H), 2.83-2.89 (m, 4H), 2.33-2.41 (m, 2H), 1.57 (s, 9H).

***tert*-Butyl 2-(2-methoxy-2-oxoacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate** (**6g**). A mixture of **2a** (1.0 g, 4.17 mmol) and dimethyl oxalate (1.97 g, 16.7mmol) in THF (30 mL) was cooled to 0 °C and NaH (0.15 g, 6.25mmol) was added. The mixture was refluxed for 8 h. The reaction was quenched with water. THF was removed in vacuo. The crude product was diluted with EtOAc (100 mL) and water (20 mL) and washed with a saturated aqueous NaCl solution (15 mL), dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether:EtOAc = 5:1, to give white solid (1.07g, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.25 (s, 1H), 3.99 (s, 3H), 2.85-2.92 (m, 4H), 2.35-2.42 (m, 2H), 1.59 (s, 9H).

General procedure for preparation of compounds 7a–7g

tert-Butyl 2-acylaminothiophene-3-carboxylate (1.0 mmol) was dissolved in 20% of trifluoroacetic acid (5 mL) in CH_2Cl_2 (20 mL). This mixture was stirred for 2-3 h at room temperature and diluted by CH_2Cl_2, and washed with water. The organic phase was dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of CH_2Cl_2 and MeOH (20:1) as the eluent.

2-(3-Chloropropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7a**). Prepared from **6a,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.26 g, 95%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.99 (br, 1H), 11.06 (s, 1H), 3.88 (t, J = 6.20 Hz, 2H), 3.03 (t, J = 6.20 Hz, 2H), 2.77-2.83 (m, 4H), 2.27-2.34 (m, 2H).

2-(4-Chlorobutanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7b**). Prepared from **6b,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.24 g, 85%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.97 (br, 1H), 11.01 (s, 1H), 3.69 (t, J = 6.60 Hz, 2H), 2.76-2.83 (m, 4H), 2.65 (t, J = 7.24 Hz, 2H), 2.27-2.34 (m, 2H), 2.02-2.09 (m, 2H).

2-(3-Chloro-2,2-dimethylpropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7c**). Prepared from **6c,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.26 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.26 (s, 1H), 3.71 (s, 2H), 2.96 (t, J = 6.88 Hz, 2H), 2.87 (t, J = 6.88 Hz, 2H), 2.38-2.45 (m, 2H), 1.45 (s, 6H).

2-(2,2-Dichloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7d**). Prepared from **6d,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.20 g, 79%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.38 (br, 1H), 11.94 (s, 1H), 7.20 (s, 1H), 2.83-2.84 (m, 4H), 2.29-2.36 (m, 2H).

2-(2,2,2-Trichloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7e**). Prepared from **6e,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.30 g, 91%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.66 (br, 1H), 12.66 (s, 1H), 2.84-2.88 (m, 4H), 2.31-2.38 (m, 2H).

2-Acrylamino-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7f**). Prepared from **6f,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.21 g, 89%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.02 (br, 1H), 11.18 (s, 1H), 6.62 (dd, J = 16.96, 10.32 Hz, 1H), 6.30 (d, J = 16.96 Hz, 1H), 5.88 (d, J = 10.32 Hz, 1H), 2.79-2.84 (m, 4H), 2.28-2.35 (m, 2H).

2-(2-Methoxy-2-oxoacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic acid (**7g**). Prepared from **6g,** CH_2Cl_2:MeOH = 20:1, to give white solid (0.25 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.34 (br, 1H), 12.17 (s, 1H), 3.87 (s, 3H), 2.81-2.86 (m, 4H), 2.29-2.37 (m, 2H).

General procedure for preparation of compounds 8a-8f and 9

2-Acylaminothiophene-3-carboxylic acid (1.0 mmol) was dissolved in DMF (20 mL) and HATU (1.5 mmol) was added. After stirring at room temperature for 10 min, amine (1.5 mmol) was added. The mixture was stirred at room temperature for another 2-3 h. Then the reaction was quenched with water and the aqueous solution was extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and ethyl acetate (7:1-2:1) as the eluent.

***N*-Benzyl-2-(3-chloropropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide** (**8a**). Prepared from **7a**, petroleum ether:EtOAc = 5:1-2:1, to give white solid (0.21 g, yield 59%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.74 (s, 1H), 7.73 (t, J = 5.2 Hz, 1H), 7.32-7.35 (m, 4H), 7.24-7.25 (m, 1H), 4.49 (d, J = 5.2 Hz, 2H), 3.86 (t, J = 6.08 Hz, 2H), 2.94-2.97 (m, 4H), 2.81 (t, J = 6.1 Hz, 2H), 2.33-2.41 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.46, 165.90, 149.70, 138.04, 137.14, 133.89, 129.12 (2C), 127.92, 127.60 (2C), 110.38, 43.55, 39.88, 39.45, 30.28, 28.73, 28.43. ESI-HRMS (*m/z*): $[M-H]^-$ calcd. for $C_{18}H_{18}ClN_2O_2S$, 361.0783; found 361.0791. mp 134.8-138.1 °C.

***N*-Benzyl-2-(4-chlorobutanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide** (**8b**). Prepared from **7b**, petroleum ether:EtOAc = 2:1, to give white solid (0.30 g, yield 80%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.72 (s, 1H), 7.69 (t, J = 5.7 Hz, 1H), 7.32-7.36 (m, 4H), 7.24-7.26 (m, 1H), 4.49 (d, J = 5.7 Hz, 2H), 3.67 (t, J = 6.6 Hz, 2H), 2.96 (t, J = 6.8 Hz, 2H), 2.80 (t, J = 7.0 Hz, 2H), 2.58 (t, J = 7.3 Hz, 2H), 2.33-2.40 (m, 2H), 2.00-2.06 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.41, 165.02, 147.62, 139.41, 138.53, 131.80, 128.25 (2C), 126.98 (2C), 126.68, 110.99, 44.56, 42.33, 32.86, 29.21, 28.22, 27.72, 27.70. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{19}H_{21}ClN_2NaO_2S$, 399.0904; found 399. 0930. mp 83.1-86.4 °C.

***N*-Benzyl-2-(3-chloro-2,2-dimethylpropanamido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide** (**8c**). Prepared from **7c**, petroleum ether:EtOAc = 2:1, to give white solid (0.30 g, yield 76%). ^{1}H

NMR (400 MHz, DMSO-d_6): δ 12.36 (s, 1H), 7.67 (t, J = 5.6 Hz, 1H), 7.32-7.34 (m, 4H), 7.24-7.26 (m, 1H), 4.52 (d, J = 5.6 Hz, 2H), 3.75 (s, 2H), 2.98 (t, J = 6.7 Hz, 2H), 2.81 (t, J = 6.7 Hz, 2H), 2.36-2.40 (m, 2H), 1.32 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ171.23, 165.41, 148.04, 139.27, 138.51, 132.16, 128.29 (2C), 126.91 (2C), 126.72, 111.07, 52.36, 44.22, 42.26, 29.19, 28.21, 27.74, 22.61 (2C). ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{20}H_{23}ClN_2NaO_2S$, 413.1061; found 413.1027. mp 149.6-150.0 °C.

***N*-Benzyl-2-(2,2-dichloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (8d)**. Prepared from **7d**, petroleum ether:EtOAc = 2:1, to give white solid (0.31 g, yield 82%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.72 (s, 1H), 7.86 (t, J = 6.0 Hz, 1H), 7.31-7.39 (m, 4H), 7.24-7.27 (m, 1H), 7.14 (s, 1H), 4.52 (d, J = 6.0 Hz, 2H), 3.00 (t, J = 7.1 Hz, 2H), 2.85 (t, J = 7.1 Hz, 2H), 2.37-2.41 (m, 2H); ^{13}C NMR(100 MHz DMSO-d_6): δ164.84, 160.50, 145.91, 139.15 (2C), 133.71, 128.29 (2C), 126.94 (2C), 126.74, 113.20, 66.40, 42.39, 29.15, 28.31, 27.71. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{16}Cl_2N_2NaO_2S$, 405.0202; found 405.0227. mp 142.4-143.2 °C.

***N*-Benzyl-2-(2,2,2-trichloroacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (8e)**. Prepared from **7e**, petroleum ether:EtOAc = 7:1-3:1, to give white solid (0.31 g, yield 73%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.61 (s, 1H), 7.91 (t, J = 5.3 Hz, 1H), 7.31-7.36 (m, 4H), 7.23-7.27 (m, 1H), 4.54 (d, J = 5.3 Hz, 2H), 3.03 (t, J = 6.8Hz, 2H), 2.88 (t, J = 7.0 Hz, 2H), 2.37-2.44 (m, 2H); ^{13}C NMR (100 MHz DMSO-d_6): δ 165.11, 157.90, 145.82, 139.47, 138.97, 134.57, 128.36 (2C), 126.94 (2C), 126.82, 113.71, 91.10, 42.42, 29.15, 28.40, 27.73. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{15}Cl_3N_2NaO_2S$, 438.9812; found 438.9824. mp 149.6-150.3 °C.

2-Acrylamino-*N*-benzyl-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (8f). Prepared from **7f**, petroleum ether:EtOAc = 2:1, to give white solid 0.23 g, yield 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.22 (s, 1H), 7.33-7.37 (m, 5H), 6.47 (d, J = 16.9 Hz, 1H), 6.33 (dd, J = 16.9, 10.2 Hz, 1H), 6.22 (br, 1H), 5.82 (d, J = 10.2 Hz, 1H), 4.62 (d, J = 5.2 Hz, 2H), 2.86-2.88 (m, 4H), 2.46-2.48 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.98, 161.40, 147.27, 139.36, 138.86, 132.74, 130.45, 128.31, 128.27(2C), 126.97(2C), 126.69, 111.83, 42.36, 29.20, 28.28, 27.73. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{18}H_{18}N_2NaO_2S$, 349.0981; found 349.0961. mp 97.7-98.1 °C.

Methyl 2-((3-(benzylcarbamoyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophen-2-yl)amino)-2-oxoacetate (9). Prepared from **7g**, petroleum ether:EtOAc = 5:1-2:1, to give white solid (0.26 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.93 (s, 1H), 7.78 (t, J = 5.64 Hz, 1H), 7.32-7.34 (m, 4H), 7.24-7.26 (m, 1H), 4.52 (d, J = 5.64 Hz, 2H), 3.85(s, 3H), 3.01 (t, J = 6.68 Hz, 2H), 2.85 (t, J = 6.80 Hz, 2H), 2.38-2.43 (m, 2H).

2-((3-(Benzylcarbamoyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophen-2-yl)amino)-2-oxoacetic acid (8g). To a solution of **9** (0.28 g, 0.78 mmol) in 50 mL of MeOH was added a solution of LiOH (56 mg, 2.34 mmol) in water. After stirring the mixture at room temperature for 8 h, the solvent was evaporated and adjusted the water pH to 3-4. Extracting with EtOAc, and concentrated the organic layer, the residue was

purified by flash column chromatography using a mixture of CH_2Cl_2: MeOH = 20:1, to give white solid (0.22 g), yield 82%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.88 (s, 1H), 7.77 (t, J = 5.8 Hz, 1H), 7.32-7.33 (m, 4H), 7.24-7.25 (m, 1H), 4.51 (d, J = 5.8 Hz, 2H), 3.00 (t, J = 6.3 Hz, 2H), 2.85 (t, J = 6.7 Hz, 2H), 2.38-2.41 (m, 2H); ^{13}C NMR (100 MHz DMSO-d_6): δ164.73, 160.33, 153.99, 146.19, 139.30, 139.19, 133.61, 128.29 (2C), 127.01 (2C), 126.72, 112.80, 42.37, 29.16, 28.31, 27.75. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{16}N_2NaO_4S$, 367.0723; found 367.0730. mp 196.2-196.8 °C.

2-((3-(Benzylcarbamoyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophen-2-yl)amino)-2-oxoethyl acetate (10). To a solution of **5a** (0.5 g, 1.43 mmol) in 20 mL of DMF was added potassium acetate (0.21 g, 2.14 mmol). Following reflux of the mixture for 3 h, the solution was evaporated, the mixture taken up in 70 ml of CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was separated and dried over anhydrous Na_2SO_4, and then evaporated in vacuo. The crude product was purified by flash column chromatography using a mixture of petroleum ether: EtOAc = 2:1 to give white solid (0.48 g, 90%).^{1}H NMR (400 MHz, DMSO-d_6): δ 12.23 (s, 1H), 7.73 (t, J = 5.76 Hz, 1H), 7.32-7.34 (m, 4H), 7.23-7.27 (m, 1H), 4.75 (s, 2H), 4.49 (d, J = 5.76 Hz, 2H), 2.97 (t, J = 6.96 Hz, 2H), 2.81 (t, J = 7.00 Hz, 2H), 2.34-2.41 (m, 2H), 2.15 (s, 3H).

***N*-Benzyl-2-(2-hydroxyacetamino)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (8h)**. To a solution of **10** (0.3 g, 0.81 mmol) in 20 mL of MeOH was added a solution of Na_2CO_3 (0.13 g, 1.21 mmol). After stirring the mixture at room temperature for 3 h, the solvent was evaporated and the residue was purified by flash column chromatography using a mixture of petroleum ether: EtOAc = 1:1 to give white solid (0.21 g, 78%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.30 (s, 1H), 7.65 (t, J = 5.9 Hz, 1H), 7.32-7.33 (m, 4H), 7.22-7.26 (m, 1H), 6.08 (t, J = 5.8 Hz, 1H), 4.49 (d, J = 6.0 Hz, 2H), 4.05 (d, J = 5.8 Hz, 2H), 2.98 (t, J = 7.1 Hz, 2H), 2.81 (t, J = 7.0 Hz, 2H), 2.36-2.41 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.64, 164.79, 147.16, 139.51, 138.54, 131.86, 128.26 (2C), 126.97 (2C), 126.66, 111.29, 61.04, 42.28, 29.26, 28.24, 27.74. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{18}N_2NaO_3S$, 353.0930; found 353.0944. mp 170.6-171.2 °C.

General procedure for preparation of compounds 11a-11e

2-Acylaminothiophene-3-carboxylic acid (1.0 mmol) was dissolved in DMF (20 mL) and HATU (1.5 mmol) was added. After stirring at room temperature for 10 min, amine (1.5 mmol) was added. The mixture was stirred at room temperature for another 2-3 h. Then the reaction was quenched with water and the aqueous solution was extracted with EtOAc (20 mL×3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography using a mixture of petroleum ether and EtOAc (7:1-3:1) as the eluent.

2-(2-Chloroacetamino)-*N*-(4-phenylbutyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (11a). Prepared from **4a**, petroleum ether: EtOAc = 7:1-3:1, to give white solid (0.29 g, yield 73%). ^{1}H

NMR (400 MHz, DMSO-d_6): δ 12.46 (s, 1H), 7.25-7.29 (m, 2H), 7.15-7.21 (m, 4H), 4.53 (s, 2H), 3.27-3.32 (m, 2H), 2.91 (t, J = 6.8 Hz, 2H), 2.81 (t, J = 6.96 Hz, 2H), 2.60 (t, J = 6.9 Hz, 2H), 2.33-2.38 (m, 2H), 1.52-1.63 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.82, 163.42, 146.37, 142.16, 138.78, 132.60, 128.35 (2C), 128.28 (2C), 125.70, 112.36, 42.48, 38.67, 34.84, 29.10, 28.77, 28.46, 28.29, 27.75. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{20}H_{23}ClN_2O_2S$, 413.1061; found 413.1030. mp 107.3-108.4 °C.

2-(2-Chloroacetamino)-*N*-(4-methoxybenzyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (11b). Prepared from **4a**, petroleum ether: EtOAc = 7:1-3:1, to give white solid (0.30 g, yield 79%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.41 (s, 1H), 7.65 (t, J = 5.9 Hz, 1H), 7.24 (d, J = 8.5 Hz, 2H), 6.89 (d, J = 8.5 Hz, 2H), 4.52 (s, 2H), 4.43 (d, J = 6.0 Hz, 2H), 3.73 (s, 3H), 2.95 (t, J = 6.9 Hz, 2H), 2.82 (t, J = 7.0 Hz, 2H), 2.35-2.39 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.82, 163.47, 158.21, 146.78, 138.76, 132.68, 131.27, 128.42 (2C), 113.73 (2C), 112.08, 55.06, 42.49, 41.81, 29.23, 28.29, 27.76. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{18}H_{19}ClN_2NaO_3S$, 401.0697; found 401.0670. mp 148.7-149.2 °C.

2-(2-Chloroacetamino)-*N*-(2-chlorobenzyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (11c). Prepared from **4a**, petroleum ether: EtOAc = 7:1-3:1, to give white solid (0.25 g, yield 64%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.35 (s, 1H), 7.76 (t, J = 5.8 Hz, 1H), 7.47 (d, J = 7.32 Hz, 1H), 7.29-7.34 (m, 3H), 4.56 (d, J = 5.8 Hz, 2H), 4.53 (s, 2H), 3.02 (t, J = 6.9 Hz, 2H), 2.84 (t, J = 7.0 Hz, 2H), 2.38-2.42 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.09, 163.55, 147.11, 138.79, 136.11, 132.80, 131.79, 129.16, 128.59, 128.31, 127.27, 111.79, 42.48, 40.49, 29.24, 28.32, 27.78. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for$C_{17}H_{16}Cl_2N_2NaO_2S$, 405.0202; found 405.0211. mp 183.1-183.6 °C.

2-(2-Chloroacetamino)-*N*-(3-chlorobenzyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (11d). Prepared from **4a**, petroleum ether: EtOAc = 7:1-3:1, to give white solid (0.30 g, yield 78%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.37 (s, 1H), 7.81 (t, J = 6.0 Hz, 1H), 7.35-7.39 (m, 2H), 7.28-7.32 (m, 2H), 4.53 (s, 2H), 4.50 (d, J = 6.0 Hz, 1H), 2.99 (t, J = 7.0 Hz, 2H), 2.83 (t, J = 7.0 Hz, 2H), 2.35-2.42 (m, 2H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ165.02, 163.52, 147.02, 142.07, 138.77, 132.97, 132.74, 130.23, 126.91, 126.73, 125.73, 111.84, 42.49, 41.96, 29.25, 28.31, 27.75. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{16}Cl_2N_2NaO_2S$, 405.0202; found 405.0227. mp 141.6-142.1 °C.

2-(2-Chloroacetamino)-*N*-(4-chlorobenzyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (11e). Prepared from **4a**, petroleum ether: EtOAc = 7:1-3:1, to give white solid (0.35 g, yield 92%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.37 (s, 1H), 7.78 (t, J = 5.7 Hz, 1H), 7.39 (d, J = 8.2 Hz, 2H), 7.34 (d, J = 8.2 Hz, 2H), 4.52 (s, 2H), 4.48 (d, J = 6.0 Hz, 2H), 2.98 (d, J = 6.3 Hz, 2H), 2.82 (t, J = 6.7 Hz, 2H), 2.37-2.40 (m, 2H); ^{13}C NMR (100 MHz DMSO-d_6): δ 164.99, 163.51, 146.98, 138.76, 138.47, 132.73, 131.30, 128.93 (2C), 128.27 (2C), 111.87, 42.49, 41.80, 29.25, 28.31, 27.76. ESI-HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{16}Cl_2N_2NaO_2S$, 405.0202; found 405.0198. mp 156.6-158.0 °C.

Cell viability assay

Cell viability was measured by the SRB assay. MGC-803 cells (1500 cells/well) and HCT-116 (1800 cells/well) of 180 μL medium were seeded in 96-well plates and incubated overnight for cell adhering before treatments. Cells were then treated with each compound at increasing concentrations ranging from 0.03-30 μM giving the final volume of 200 μL each well and incubated for 72 h. Remove the cell culture medium, gently, add 100 μL cold 10% TCA (20 g TCA in 200 mL ddH_2O) to each well, and incubate the plates at 4 °C for 1 h. Washing the plates five times with ddH_2O, then allow them to air-dry at room temperature. Adding 100 μL 0.4% SRB solution (0.8 g SRB in 200 mL 1% acetic acid) to each well, drying at room temperature for 15 min and then quickly rinse the plates five times with 1% acetic acid (5ml acetic acid in 500ml ddH_2O) to remove unbound dye then allow them to air-dry at room temperature. Adding 150 μL 10 mM Tris base solution (0.6057 g Tris in 500 mL ddH_2O) to each well to solubilize the protein-bound dye. Absorbance was read at 570 nm with Spectra Max M 5 microplate spectrophotometer. The IC_{50} value was defined as the concentration of drug that inhibits 50% cell growth compared with the cisplatin (positive control).

Annexin V-FITC and propidium iodide (PI) double staining

HCT-116 (250000 cells/dish) of 5 mL medium were seeded in 60 mm dishes and incubated overnight for cell adhering before treatments. Cells were then treated with **11b**, **11d** of 10 μM each dish and incubated for 48 h, the control treated with DMSO. After 48 h, an Annexin V-FITC/PI binding assay was conducted using a purchased kit and the manufacturer's protocol (Dojindo, Japan). HCT-116 cells were collected, washed twice with PBS and resuspended in Annexin V-FITC binding solution for 1×10^6 cells/ml, and incubated with 5 μL Annexin V-FITC in the dark for 15 min at room temperature. Then added 400 μL Annexin V-FITC binding solution and 5 μL propidium iodide (PI) and kept in the dark at room temperature. Flow analysis was immediately performed using a flow cytometer (Guava easy Cyte 6HT-2L, Merck Millipore).

General method for western blot analysis

Cells were treated with compounds (10 μM) for 48 h, after treatment, the both floating and adherent cells were collected, washed twice with PBS (pH 7.4) and suspended in RIPA lysis buffer containing 1X protease inhibitor for 30 min on ice. Samples were centrifuged at 12000 rpm for 30 min at 4 °C and supernatant was collected as total protein lysate. Protein concentrations were quantified with the DC Protein Assay Kit and bovine serum albumin as standard, total protein was divided into the gel-loading concentration of proteins (40 μg) and heated for 5 min at 100 °C. Equal amount of protein was loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane. After blocking with 5% skim milk at room temperature for 1 h, membranes were

washed with TPBS and membrane was incubated with the desired antibody for 2 h either at room temperature or at 4 °C overnight. The membrane was then incubated with corresponding peroxidase-conjugated secondary antibody for 2 h (cleaved-caspase 3 1:1000, PARP1 1:500) at room temperature, and protein expression was detected by ECL kit and visualized using a chemiluminescence detection system (Amersham Imager 600, GE).

General method for evaluating MMP

HCT-116 cells were treated with compounds (10 μM) for 24 h, after treatment, the both floating and adherent cells were harvested, and washed twice with PBS; 106 cells were incubated in 0.5 mL PBS containing 10 μg JC-1 for 15 min at 37 °C in the dark. Stained cells were washed twice with PBS, resuspended in 500 μL PBS, and used immediately with flow cytometer (Guava easy Cyte 6HT-2L, Merck Millipore).

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