Accepted Manuscript

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PII: S0960-894X(18)30612-7
DOI: https://doi.org/10.1016/j.bmcl.2018.07.032
Reference: BMCL 25963

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 14 May 2018
Revised Date: 1 July 2018
Accepted Date: 18 July 2018

Please cite this article as: Lu, S., Obianom, O.N., Ai, Y., Novel cinnamaldehyde-based aspirin derivatives for the treatment of colorectal cancer, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.07.032

Novel cinnamaldehyde-based aspirin derivatives for the treatment of colorectal cancer

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Abstract

Colorectal cancer (CRC) is a leading cause of mortality worldwide. Current treatments of CRC involve anti-cancer agents with relatively good efficacy but unselectively target both cancer and non-cancer cells. Thus, there is a need to discover and develop novel CRC therapeutics that have potent anti-cancer effects, but show reduced off-target cell effects. Here, a novel series of cinnamaldehyde-based aspirin derivatives were designed and synthesized. Biological evaluation indicated that the most active compound **1f** exhibited more than 10-fold increase in the anti-proliferation efficacy in HCT-8 cells compared to the parent compounds. Its effects were similarly reproduced in another CRC cell line, DLD-1, but with 7- to 11-fold less inhibitory activity in non-tumorigenic colon cells. Flow cytometry analysis showed that **1f** induced cell cycle arrest and apoptosis, which was further validated with immunoblot analysis of the relative protein levels of cleaved caspase 3 and PARP as well as the ROS production in CRC cells. More so, **1f** significantly inhibited the growth of implanted CRC *in vivo* in mouse xenograft model. Taken together, our results show that cinnamaldehyde-based aspirin derivatives such as **1f** show promise as novel anti-CRC agent for further pharmaceutical development.

Keywords: aspirin, cinnamaldehyde, colorectal cancer, anti-proliferative activity, apoptosis

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related death worldwide [1, 2]. Chemotherapeutic drugs like 5-Fluorouracil (5-FU), Capecitabine (Xeloda), Irinotecan (Camptosar), Oxaliplatin (Eloxatin), and Trifluridine and tipiracil (Lonsurf, a combination drug in pill form) are widely used for the treatment of CRC. However, in many cases, they produce mild to grave side effects such as hair loss, mouth sores, nausea, diarrhea, immunosuppression, bleeding, and fatigue [3-7]. In recent years, with various novel targets and therapeutic strategies discovered in targeting CRC, a number of groups have developed newer drugs to specifically alter these new targets. For example, the rapid increase in development of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) inhibitors [8]. Nonetheless, these new treatments still pose serious side effects that include blood clotting, severe bleeding, colonic perforations, slow wound healing, cardiomyopathy and nephropathy. Therefore, novel therapeutics for CRC with reduced side effects is still urgently needed.

Aspirin (ASA, **2**, Fig. 1), a widely used nonsteroidal anti-inflammatory drug (NSAID), has been shown to be effective for the prevention and remission of cancers, especially in CRC [9-13]. In fact, the Colorectal Adenoma/Carcinoma Prevention Program (CAPP2) randomized clinical trial in carriers of hereditary forms of CRC, lynch syndrome, demonstrated substantial protection by ASA in these patients [14]. They found that long-term treatment with 600 mg of ASA per day substantially reduced cancer incidence in lynch syndrome patients in a span of 55.7 months [14]. As a result, many synthetic ASA derivatives have now been prepared (Fig. 1), including NO- and HNO-releasing ASA (**3** and **4**) [15-19], HS-releasing ASA (**5**) [20-22], dual nitric oxide- and hydrogen sulfide-releasing ASA (**6**) [23, 24], phospho-ASA (**7**) [25], 6-gingerol-based ASA prodrug (**8**) [26], resveratrol-based ASA prodrug (**9**) [27], and Se-ASA (**10**) [28]. These synthetic ASA derivatives

possess stronger cytotoxicity and chemo-preventive effects especially against CRC cells than ASA. Another ASA derivative extensively reported in the literature is the Asplatin (**11**), a Pt (IV) prodrug of cisplatin containing the ASA moiety, which exhibits significantly higher cytotoxicity than cisplatin towards tumor cells and almost fully overcomes drug resistance in cisplatin resistant cells [29, 30]. In the same way, further discovery and development of novel ASA derivatives with improved anti-cancer selectivity, but lower off-target cellular effects is warranted. One means to achieve this objective is by hybrid drug approach as demonstrated with Asplatin.

Cinnamaldehyde (CA, **12**, Fig. 1) is an important bioactive ingredient found in the cinnamon [31]. It is widely used in food and beverages and has been known to exert anticancer activity through reactive oxygen species (ROS) generation [32, 33]. Although CA contains active Michael acceptor pharmacophore, it is not genotoxic or carcinogenic *in vivo* [34]. Furthermore, it is considered generally recognized as safe (GRAS) and widely used in foodstuffs in the United States and European countries [35]. More importantly, CA has been reported to induce ROS generation mainly in the mitochondria and inhibit growth of human cancer cells, with minimal cytotoxicity to normal cells [36, 37].

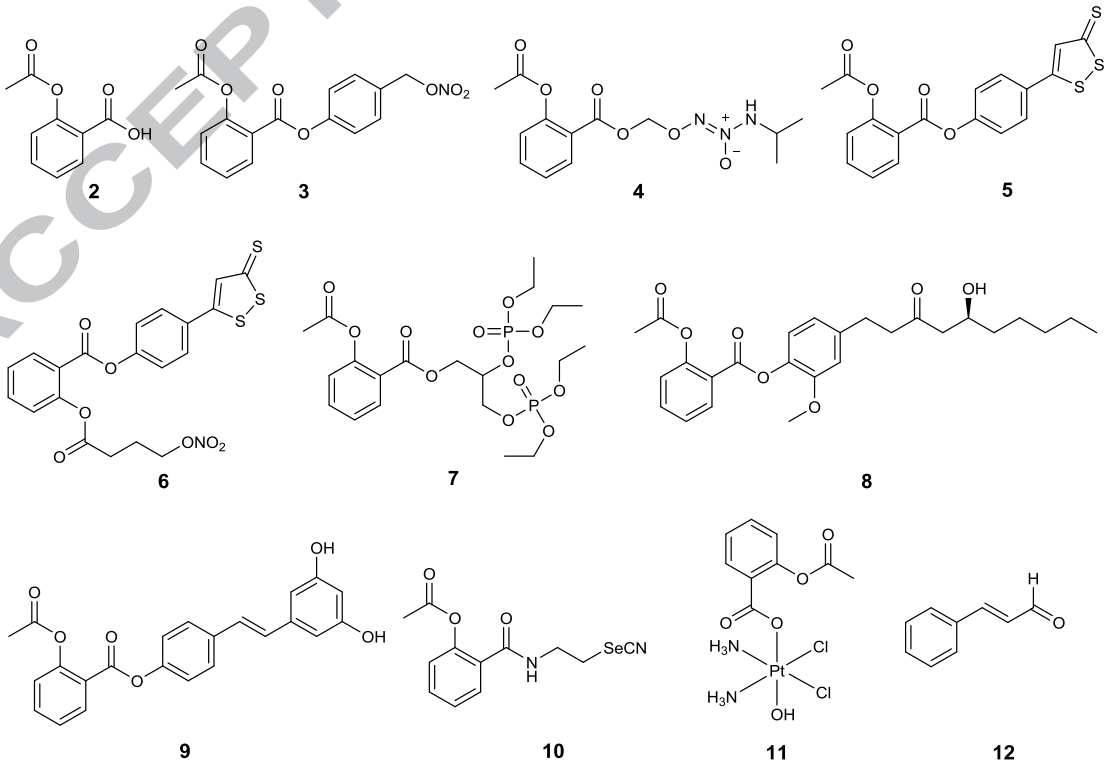
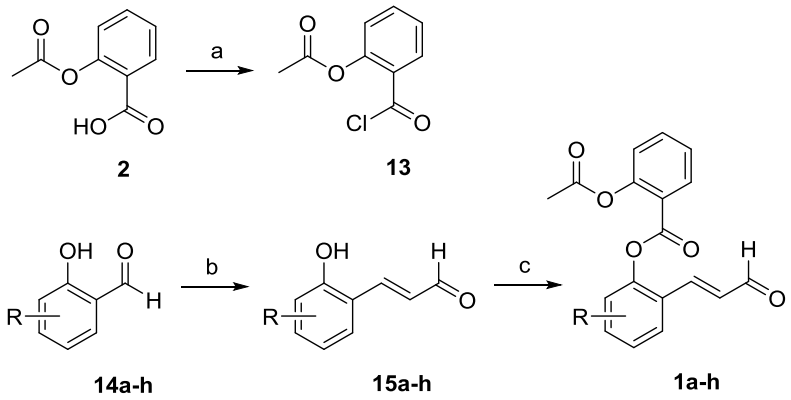


Fig. 1. Chemical structures of ASA, representative synthetic ASA derivatives and CA.

Considering the aforementioned chemo-preventive effects of ASA and its derivatives and the biological effects of CA, along with the previous reports that support the modification of ASA to obtain more potent and selective antitumor agents, we designed novel hybrid CA-ASA derivatives. We hypothesized that these hybrids will exert potent cytotoxicity against CRC cells with good selectively compared to normal cells. Accordingly, we synthesized this novel class of CA-based ASA derivatives (**1a-h**, Scheme 1), and evaluated their bioactivity *in vitro* and *in vivo*.

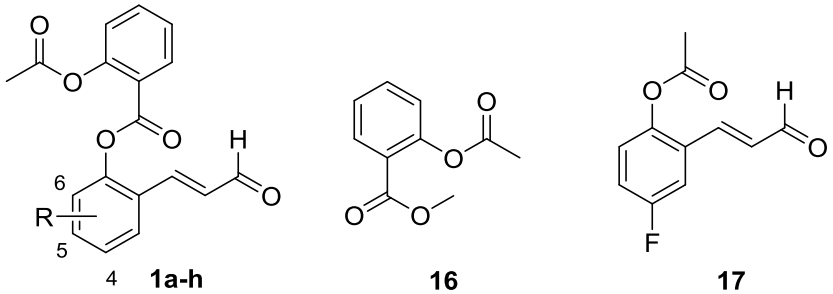
The target compounds **1a-h** were synthesized as depicted in Scheme 1. Initially, aspirin **2** was converted to the acid chloride **13** upon treatment with thionyl chloride. Then the corresponding cinnamaldehyde **15a-h** were prepared by the Wittig reaction [38-41] starting from aldehydes **14a-h** in higher yields (55-79%). Next, reaction of acid chloride **13** with **15a-h** in dry CH_2Cl_2, in the presence of the non-nucleophilic base triethylamine (TEA), furnished the respective target compounds **1a-h** in good yields (75-89%). The structures of all hybrids were fully characterized by their ^1H and ^{13}C NMR spectroscopic data as well as by mass spectral analysis (data in Supplementary Materials).



Scheme 1. Synthesis of cinnamaldehyde-based aspirin derivatives **1a-h**. Reagents and conditions: a) $SOCl_2$, anhydrous CH_2Cl_2, reflux, 2h; b) Ph_3P=CHCHO, PhMe, 60°C, 8h, N_2; c) **3**, TEA, anhydrous CH_2Cl_2, rt, 12h.

Compounds **1a-h** were screened for their inhibitory activity against HCT-8 by measuring cell proliferation using MTT assay (Supplementary Materials) with 5-Fluorouracil (5-FU) as a positive control. As shown in Table 1, compounds **1b** and **1e-h** exhibited more potent cell growth inhibitory activity (IC_{50} = 2.6-9.3 μM) against HCT-8 cells compared to 5-FU (IC_{50} = 28.9 μM), ASA (IC_{50} > 100 μM) and CA (IC_{50} = 29.4 μM), and even the combination of ASA and CA (IC_{50} = 15.6 μM, physical mixture, mole ratio, 1:1). Among them, compounds **1f** exhibited the most potent inhibitory activity (IC_{50} = 2.6 μM), which was 11, 38, 4, and 2.5 fold higher than that of the positive control 5-FU, two individual moieties **16** (IC_{50} > 100 μM, methyl ester of aspirin) and **17** (IC_{50} = 10.5 μM, O-acetylated cinnamaldehyde derivative, Supplementary Materials), and the physical combination of **16** and **17** (IC_{50} = 6.6 μM), respectively. These results suggest that the antitumor activity of **1f** may be most likely attributed to the synergic effects derived from both the cinnamaldehyde and aspirin moieties. It was obvious that R substitution on the benzene ring of the CA as in **1b-h** was crucial for their anticancer activity. Generally, the compounds with substituents like methoxy, or methyl, or halogen (**1b-h**) displayed better inhibitory activity than that of **1a** without any substitution at the R position. Substitutions on the carbon 4 (**1b**) led to preferable anticancer activity than others (**1c** and **1d**). More distinctly, the compounds with an electron-withdrawing group such as halogens (**1f-h**) on the benzene ring showed more potent inhibitory activity than those bearing an electron-donating group such as methoxy, or methyl (**1b** and **1e**).

Table 1. The anti-proliferative activity of compounds **1a-h**.



1a-h **16** **17**

Compounds	R	IC_{50} (μM)[a]
		HCT-8
1a	H	20.3 ± 1.7
1b	4-OMe	9.3 ± 0.7
1c	5-OMe	15.8 ± 1.1
1d	6-OMe	17.6 ± 1.0
1e	4-Me	8.5 ± 0.4
1f	4-F	2.6 ± 0.6
1g	4-Cl	4.3 ± 0.9
1h	4-Br	5.6 ± 0.7
ASA		>100
CA		29.4 ± 1.3
ASA+CA		15.6 ± 1.6
16		>100
17		10.5 ± 1.3
16+**17**		6.6 ± 0.9
5-FU		28.9 ± 2.7

[a]Data are expressed as mean IC_{50} (μM) ± SD of each compound from three independent experiments. IC_{50} is the drug concentration inhibiting 50% of the cell proliferation.

Subsequently, the most potent compound **1f** was further investigated for its antitumor efficacy and selectivity in other cell lines by MTT assay (Table 2). We found that **1f** displayed more potent activity (IC_{50} = 4.3 μM) than 5-FU (IC_{50} = 33.0 μM) against a human colorectal adenocarcinoma cell line, DLD-1. While 5-FU had similar inhibitory activity in both CRC and nontumor CCD841 cells, **1f** exhibited a 7- to 11-fold less anti-proliferative activity against CCD841 cells (IC_{50} = 35.7 μM). This suggests that **1f** may have anti-proliferative activity selectively against CRC cells relative to normal cells.

Table 2. The anti-proliferative activity of compound **1f**.

Compounds	IC_{50} (μM) [a]	
	DLD-1	CCD841
1f	4.3 ± 0.5	30.7 ± 2.9
5-FU	33.0 ± 2.1	35.7 ± 3.2

[a]Data are expressed as mean IC_{50} (μM) ± SD of each compound from three independent experiments. IC_{50} is the drug concentration inhibiting 50% of the cell proliferation.

To evaluate the in vivo anticancer activity of **1f**, BALB/c nude mice were inoculated subcutaneously with HCT-8 cells. After the establishment of solid tumor, the mice were randomly treated intraperitoneally with **1f** (60 mg/kg) or the vehicle consisting of Tween 80/PBS (5:95) daily for 32 consecutive days, respectively. Treatment with 60 mg/kg of **1f** significantly reduced the volume of implanted colon tumors ($P < 0.001$ vs. the vehicle-treated control, Figs. 3A and 3B). More so, the tumor weights in the mice treated with **1f** at 60 mg/kg were 47% (w/w) less than that in the vehicle-treated controls (1.95 ± 0.42 g versus 1.03 ± 0.18 g, $P < 0.001$, Fig. 3C) while the body weights of the mice were not significantly changed by treatment with **1f** (Fig. 3D). Together, our data clearly demonstrated that **1f** inhibited the growth of implanted tumors in vivo with little off-target cell toxicity.

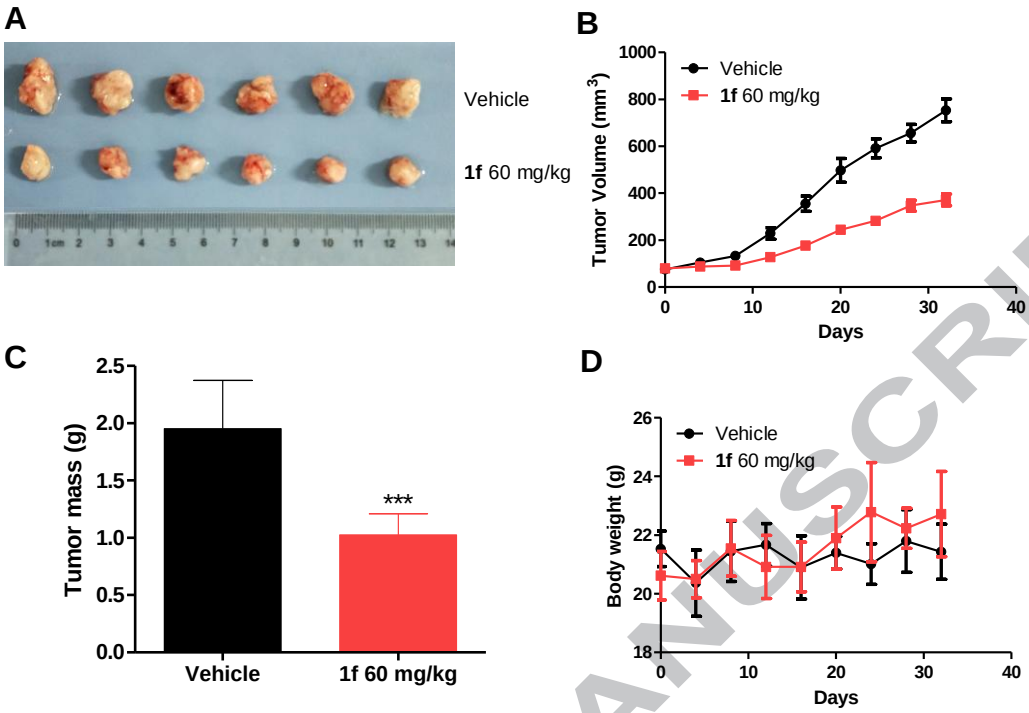


Fig. 3. Anticancer effects of **1f** in mice inoculated with HCT-8 cells. (A) Images of the tumors. The effect of intraperitoneal **1f** (60 mg/kg) on tumor volumes (B), tumor weight (C) and body weight (D) in a HCT-8 xenograft model. Data are expressed as the mean ± SD from each group of mice (n = 6 per group). (***) $P < 0.001$ vs the vehicle-treated.

To understand the mechanisms underlying the action of **1f**, the effects of **1f** on CRC cell cycle were examined. HCT-8 cells were treated with varying concentrations (0, 3, 6, and 12 μM) of **1f** for 24 h and then stained with propidium iodide (PI), followed by flow cytometry analysis. Figure 4 shows that treatment with **1f** induced HCT-8 cell cycle arrest at G_1 in a concentration dependent manner – 40.05% (0 μM), 43.17% (3 μM), 48.66% (6 μM), and 51.62% (12 μM) respectively, which were significantly higher than that of vehicle-treated control. As a result, the fraction of CRC cells at the G2 phase was significantly lower in compound treated than vehicle-treated control. This suggests that the anti-cancer mechanisms of **1f** may include its ability to induce cycle arrest in the cells.

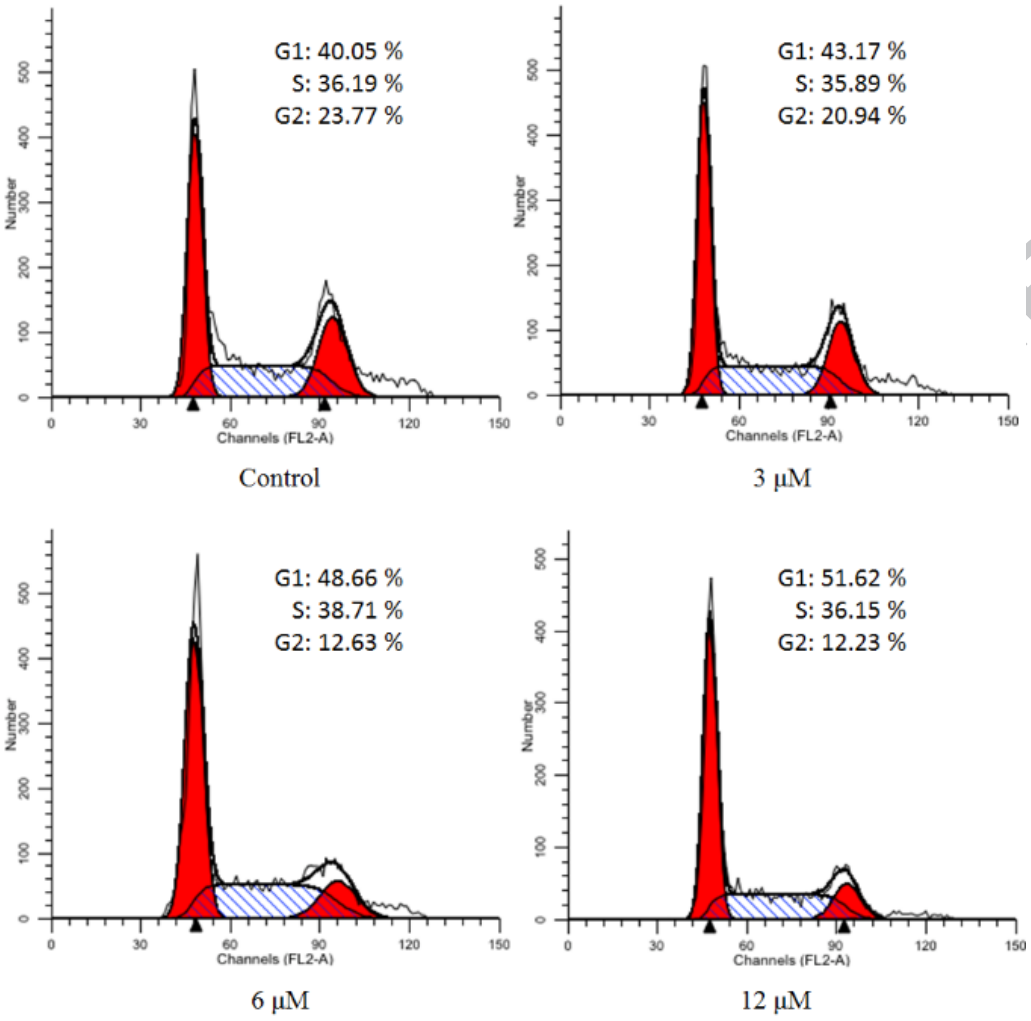


Fig. 4. Effects of **1f** on cell cycle distribution in HCT-8 cells. Cells were treated with the indicated concentrations of **1f** for 24 h and stained with PI, followed by flow cytometry analysis.

To determine whether the inhibitory effects of **1f** on CRC cell proliferation are accompanied by enhanced apoptosis, we next carried out Annexin V-FITC and PI staining on **1f** treated cells. HCT-8 cells were incubated with vehicle, or **1f** (3, 6, and 12 μM) for 24 h. Then flow cytometry analysis was used to determine the apoptotic status of the cells. We observed that treatment with **1f** induced apoptosis in HCT-8 cells in a concentration dependent manner (Fig. 5). Increasing concentration of **1f** specifically increased the fraction of cells undergoing early apoptosis as indicated by the lower right quadrant. There was not no significant difference in the early apoptosis of the cells between 6 and 12 μM. However, in the upper right quadrant indicating cells in the late apoptotic state, increase from 0 to 3 to 6 to 12 μM **1f** led to an

increase from 2.40 to 5.42 to 17.03 to 27.71 % of cells in this quadrant. This suggests that **1f** treatment significantly increases apoptosis in cancer cells as a result of its anti-cancer effects. Furthermore, apoptosis rate was significantly decreased in N-acetyl-L-cysteine (NAC, ROS scavenger) co-treated cells (12.98%) compared to **1f** (6 μM)-treated cells (32.59%). These data demonstrate that apoptosis resulting from **1f** treatment is due to its induction of intracellular ROS in HCT-8 cells.

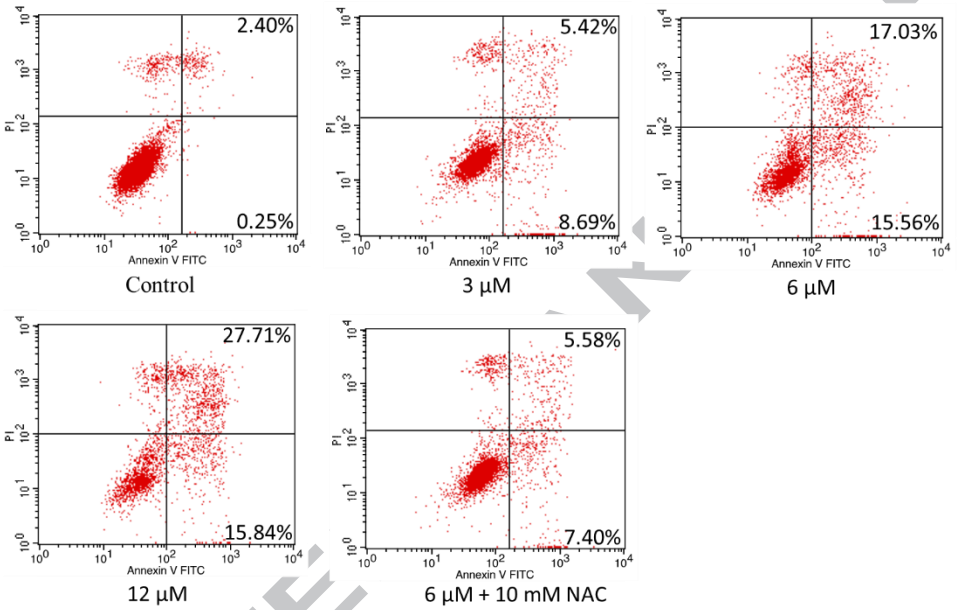


Fig. **5**. Effects of **1f** on the induction of apoptosis. HCT-8 cells were treated with the indicated concentrations of **1f** or vehicle (control) for 24 h. The cells were stained with Annexin V-FITC and propidium iodide (PI), followed by flow cytometry analysis.

To understand the mechanism of **1f**-induced apoptosis, we further examined the changes of the intracellular proteins related to apoptosis such as caspase-3, and PARP in HCT-8 cells treated with **1f** (0, 3, 6, and 12 μM). As shown in Figure 6, compound **1f** significantly induced the relative levels of cleaved caspase 3 and poly(ADP-ribose)polymerase (PARP) in a dose-dependent manner in HCT-8 cells. Overall, our results confirm that compound **1f** inhibits the growth of CRC cells by inducing apoptosis in addition to its effects on cell cycle.

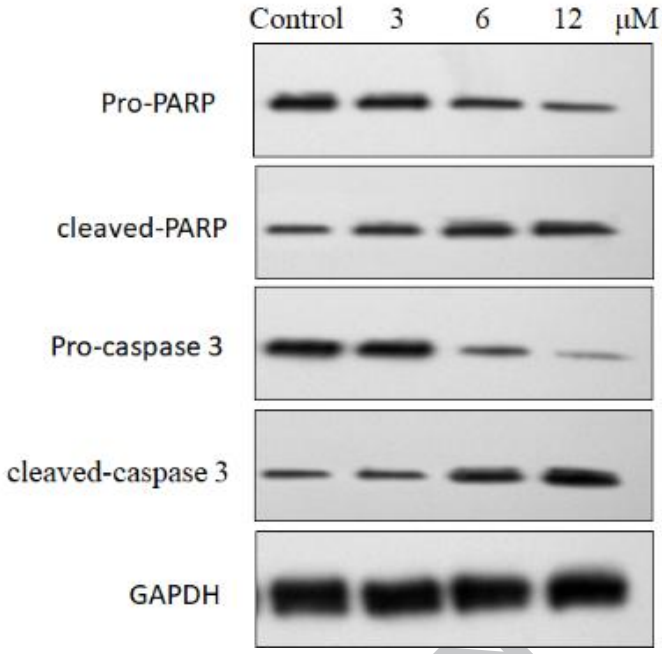


Fig. 6. Effect of **1f** on the expression of apoptotic markers. The relative protein expression levels of PARP and Caspase 3 were measured following **1f** treatment in HCT-8 cells. Cells were treated with increasing concentrations of **1f** or control (0.1% DMSO) for 24 h and immunoblotting of the whole cell lysate was carried out to determine the protein levels.

Studies have shown that the anticancer effect of CA is associated with increase in ROS levels [32, 37, 42]. To investigate whether induced cytotoxic effect of **1f** is related to ROS production, we measured ROS levels in **1f**-treated CRC cells by flow cytometry analysis. Again, we treated HCT-8 with different concentrations (0, 3, 6, and 12 μM) of **1f**, and measured the ROS levels. As shown in Figure 7, a dramatic increase (from 3.67 to 47.83) in the levels of ROS generation in HCT-8 cells was observed after treatment with **1f**. These results suggest that the induction of cell death by **1f** may be due to ROS production.

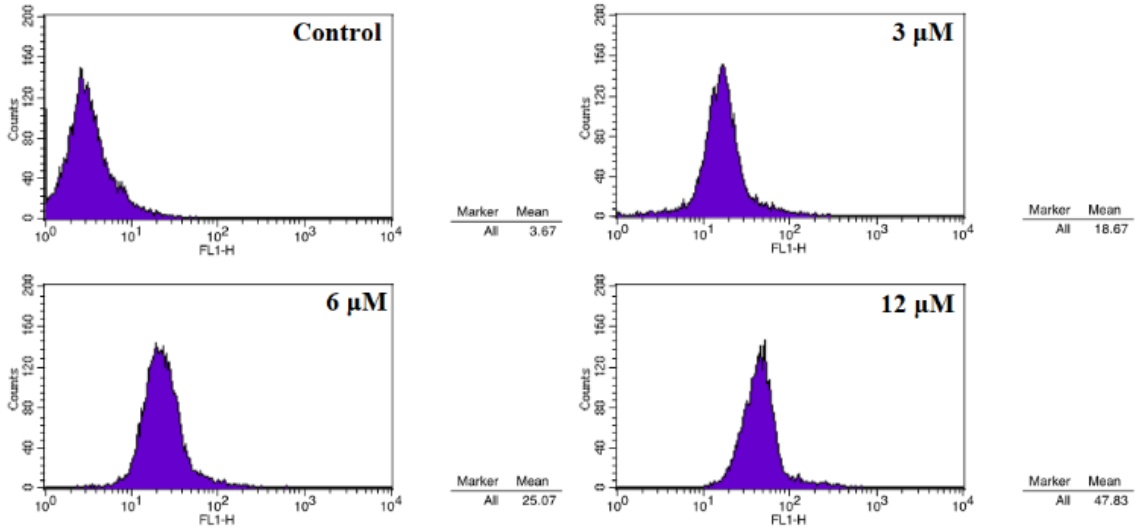


Fig. 7. Effect of compound **1f** on ROS levels in CRC cells. Flow cytometry analysis of HCT-8 cells treated with the indicated concentrations of **1f** for 24 h and stained with the fluorogenic dye 2', 7'-dichlorofluorescin diacetate (DCFH-DA).

In summary, we have designed and synthesized a novel group of CA-based ASA derivatives **1a-h**. We found that compound **1f** had potent and selective anti-proliferative activity against CRC cells relative to normal cells in vitro. Furthermore, treatment with **1f** significantly inhibited the growth of implanted CRC cancer in mice. In addition, treatment with **1f** induced CRC cell cycle arrest at G1 phase and apoptosis in vitro, increased the relative levels of cleaved caspase 3 and PARP as well as the ROS in HCT-8 cells. These findings may provide a proof of principle that **1f** may be potential chemotherapeutic agents for the intervention of CRC.

Acknowledgments

This work was supported by grants from Qing Miao Plan of Hubei University of Chinese Medicine (2016ZZX013).

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, CA Cancer J. Clin. 66 (2016) 7-30.

[2] M. Arnold, M.S. Sierra, M. Laversanne, et al. Gut 66 (2016) 683-691.

[3] D.B. Longley, D.P. Harkin, P.G. Johnston, Nat. Rev. Cancer. 3 (2003) 330-338.

[4] B.R. Hirsch, S.Y. Zafar, Cancer Manag. Res. 3 (2011) 79-89.

[5] K. Fujita, Y. Kubota, H. Ishida, et al. World J. Gastroenterol. 21 (2015) 12234-12248.

[6] P. Comella, R. Casaretti, C. Sandomenico, et al. Ther. Clin. Risk Manag. 5 (2009) 229-238.

[7] T. Kish, P. Uppal, P T. 41 (2016) 314-317, 325.

[8] K. Jin, H. Lan, F. Cao, et al Int. J. Oncol. 41 (2012) 583-588.

[9] C. Sostres, C.J. Gargallo, A. Lanas, World J. Gastrointest. Pharmacol. Ther. 5 (2014) 40-49.

[10] G.J. Tsioulias, M.F. Go, B. Rigas, Curr. Pharmacol. Rep. 1 (2015) 295-301.

[11] P.C. Elwood, A.M. Gallagher, G.G. Duthie, et al. Lancet 373 (2009) 1301-1309.

[12] M.A. Thorat, J. Cuzick, Curr. Oncol. Rep. 15 (2013) 533-540.

[13] M.J. Thun, E.J. Jacobs, C. Patrono, Nat. Rev. Clin. Oncol. 9 (2012), 259-267.

[14] J. Burn, A.M. Gerdes, F. Macrae, et al. Lancet 378 (2011) 2081-2087.

[15] D. Basudhar, G. Bharadwaj, R.Y. Cheng, et al. J. Med. Chem. 56 (2013) 7804-7820.

[16] V. Kozoni, T. Rosenberg, B. Rigas, Acta Pharmacol. Sin. 28 (2007) 1429-1433.

[17] C.V. Rao, B.S. Reddy, V.E. Steele, et al. Mol. Cancer Ther. 5 (2006) 1530-1538.

[18] J.L. Williams, S. Borgo, I. Hasan, et al. Cancer Res. 61(2001) 3285-3289.

[19] J.L. Williams, N. Nath, J. Chen, et al. Cancer Res. 63 (2003) 7613-7618.

[20] M. Chattopadhyay, R. Kodela, N. Nath, et al. Biochem. Pharmacol. 83 (2012) 723-732.

[21] M. Chattopadhyay, R. Kodela, N. Nath, et al. Biochem. Pharmacol. 83 (2012) 715-722.

[22] M. Chattopadhyay, R. Kodela, N. Nath, et al. Biochem. Pharmacol. 83(2012) 733 -740.

[23] M. Chattopadhyay, R. Kodela, K.R. Olson, et al. Biochem. Biophys. Res. Commun. 419 (2012) 523-528.

[24] R. Kodela, M. Chattopadhyay, K. Kashfi, ACS Med. Chem. Lett. 3 (2012)

257-262.

[25] L. Huang, G.G. Mackenzie, Y. Sun, et al. Cancer Res. 71 (2011) 7617 -7627.

[26] Y. Zhu, F. Wang, Y. Zhao, et al. Sci. Rep. 7 (2017) 40119.

[27] Y. Zhu, J. Fu, K.L. et al. J. Med. Chem. 58 (2015) 6494-6506.

[28] D. Plano, D.N. Karelia, M.K. Pandey, et al. J. Med. Chem. 59(2016) 1946-1959.

[29] Q. Cheng, H. Shi, H. Wang, et al. Chem. Commun. 50 (2014) 7427-7430.

[30] Q. Cheng, H. Shi, H. Wang, et al. Metallomics 8 (2016) 672-678.

[31] M. Friedman, J. Agric. Food Chem. 65 (2017) 10406-10423.

[32] H. Ka, H.J. Park, H.J. Jung, et al. Cancer Lett. 196 (2003) 143-152.

[33] C.M. Cabello, W.B. 3rd Bair, S.D. Lamore, et al. Free Radic. Biol. Med. 46(2009) 220-231.

[34] R. Kiwamoto, D. Ploeg, I.M. Rietjens, et al. Toxicol. In Vitro 31(2016) 114-125.

[35] J. Cocchiara, C.S. Letizia, J. Lalko, et al. Food Chem. Toxicol. 43 (2005) 867-923.

[36] B. Kim, E. Lee, Y. Kim, et al. Adv. Funct. Mater. 23 (2013) 5091-5097.

[37] D.C. Han, M.Y. Lee, K.D. Shin, et al. J. Biol. Chem. 279 (2004) 6911-6920.

[38] L. Lyu, M.Y. Jin, Q. He, et al. Org. Biomol. Chem. 14 (2016) 8088-8091.

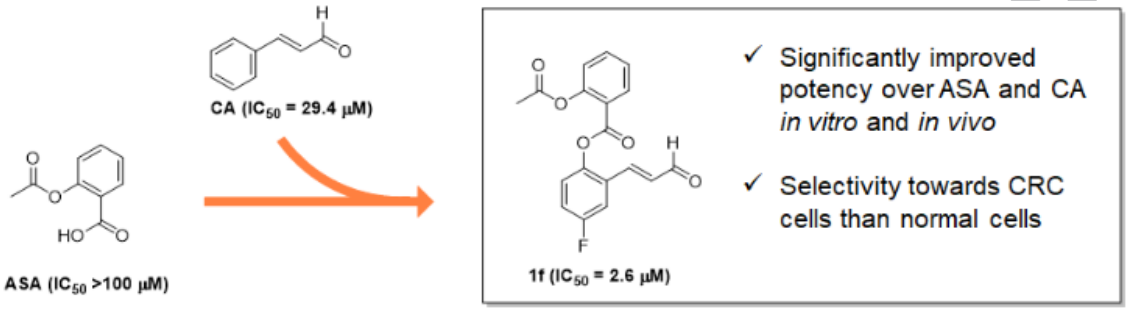
[39] R. Maity, S.C. Pan, Org. Biomol. Chem. 16 (2018) 1598-1608.

[40] X.L. Sun, Y.H. Chen, D.Y. Zhu, et al. Org. Lett. 18 (2016) 864-867.

[41] H. Liu, Y. Wang, X. Guo, et al. Org. Lett. 20 (2018) 546-549.

[42] J. Noh, B. Kwon, E. Han, et al. Nat. Commun. 6 (2015) 6907.

Graphical Abstract



Highlights

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