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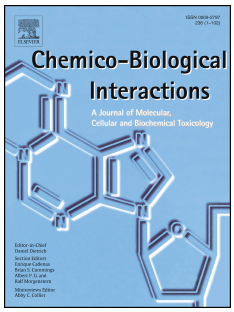


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# $NO_2$ functionalized coumarin derivatives suppress cancer progression and facilitate apoptotic cell death in KRAS mutant colon cancer

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## Keywords

Coumarin derivatives; apoptosis; cancer inhibition; KRAS mutant colon cancer.

## Abstract

Colon cancer is one of the most lethal cancers worldwide even with the significant progress made in screening techniques and therapeutic agents. Genetic mutations in tumors complicated the treatments, and the survival rate remains low for patients at late or metastatic stages. KRAS gene mutation which leads to failure of the EGFR targeted therapies stands for an example of the challenges in clinical sites. Therefore, development of novel agents for colon cancer treatment is in need. Natural and synthetic coumarin derivatives have been suggested with various biological activities with pharmacologic potential including anti-cancer capacity. Here in this study, five coumarin derivatives, include trifluoromethyl-, dimethoxy-, and/or nitro- substitutions at different positions, were synthesized. Their cancer inhibition potential was investigated in various cancer cell lines. Our data demonstrated that one nitro-coumarin derivate, 5,7-Dimethoxy-4-methyl-6-nitro-chromen-2-one, exhibits cytotoxicity specifically towards colon cancer cells under competitive $EC_{50}$. Our results showed that this compound can effectively suppress colon cancer cells harboring either wild type or mutant KRAS genes, and that it could inhibit short-term proliferation, long term proliferation, and migration capacities of cancer cells. Finally, we demonstrated that this coumarin derivate facilitates cancer cell death through activation of apoptosis pathway. Our results suggest that this coumarin derivate is a promising lead drug worth further investigation and development for future cancer treatment.

## Keywords

Coumarin derivatives; apoptosis; cancer inhibition; KRAS mutant colon cancer.

## Abbreviations

EGFR: epidermal growth factor receptor; KRAS: Kirsten rat sarcoma 2 viral oncogene homolog; NMR: Nuclear magnetic resonance; FT: fourier-transform; IR: Infrared; HRMS: High Resolution Mass Spectrometry; TLC: Thin layer chromatography; PI: Propidium Iodide; ECL: Enhanced chemiluminescence; PARP: Poly (ADP-ribose) polymerase; BAK: Bcl-2 homologous antagonist/killer protein; BAX: BCL2 associated X protein; PUMA: p53 upregulated modulator of apoptosis.

## Introduction

Cancer is the second leading death cause in the world, and among all the cancer types, colon cancer is one of the most lethal cancers which resulted in estimated over 50 thousand deaths in 2018 in the US according to the American Cancer Society report. Even though significant advances have been made in screening techniques and therapeutic agents for colon cancer, the treatment outcome remains poor for late stages or metastatic patients. [1, 2] Moreover, genetic mutations aggravate the challenge for colon cancer clinical treatment. [3-7] For example, approximately 30%-45% of colorectal cancer patients were reported to carry Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) mutant (Mut) oncogenes which would lead to failure of the epidermal growth factor receptor (EGFR) targeted therapies. [3, 8] Therefore, identification of biomarkers and development of novel agents for colon cancer suppression are in need.

Coumarins, natural secondary metabolites containing phenolic substances originally discovered in various plant families, are consisted of a fused benzene with an alpha-pyrone ring. [9] These compounds were firstly found in tonka bean and then found exist in different kinds of plants such as vanilla and woodruff, in human dietary components such as citrus fruits, green tea, and cinnamon products, or in essential oils such as cinnamon bark and lavender oils, etc. [9-11] Literatures suggested that natural coumarins and synthetic derivatives exhibit a variety of biological activities with pharmacologic potential, include anti-bacterial, anti-inflammatory, anti-oxidant, and anti-tumor capacities, etc. [12-17] Various coumarin derivatives with pharmacological activities have been successfully applied in the clinical sites. [17-20]

Coumarin-based pharmacophoric substituents were reported under development for anti-cancer agents by medicinal chemists.[15, 20-22] Among the numerous bioactive coumarin derivatives, it was demonstrated that coumarin, 7-hydroxycoumarin, and 6-nitro-7-hydroxycoumarin exhibit potent anti-proliferation capacity towards different cancer cells.[17, 23, 24] Also, 6-methoxy-7-hydroxycoumarin could induce apoptosis pathway to suppress leukemic cell growth.[25] In this study, five coumarin derivatives were synthesized, and the anti-cancer potential of these compounds was examined in human cell lines, include colon cancer cells, breast cancer cells, lung cancer cells, and non-cancerous cells, and then, molecular regulations and tumor inhibition mechanisms of the coumarin compounds were investigated.

## Material and Method

**Chemical synthesis of coumarin compounds: general experimental conditions**

Melting points were determined in a capillary tube using a MEL-TEMP II melting point apparatus by Laboratory Devices. Nuclear magnetic resonance (NMR) spectroscopy was recorded on Bruker DMX-500 fourier-transform (FT)-NMR spectrometers; chemical shifts were recorded in parts per million downfield from Me4Si. Infrared (IR) spectroscopy was determined with a Perkin-Elmer 1760-X FT-IR spectrometer. Mass spectra was recorded on Jeol JMS-D300 and FINNIGAN TSQ-46C mass spectrometers; High Resolution Mass Spectrometry (HRMS) was obtained with a Jeol JMSHX110 spectrometer. Thin layer chromatography (TLC) was performed on Merck (Art. 5715) silica gel plates and visualized under UV light (254 nm), upon treatment with iodine vapor, or upon heating after treatment with 5% phosphomolybdic acid in ethanol. Flash chromatography was performed with Merck (Art. 9385) 40–63 lm silica gel 60. Reverse-phase high performance liquid chromatography (RP-HPLC) analysis of the compounds was conducted as the following method: Column: ACE 5 C18 4.6 x 250 mm, 5 µm; wavelength: 254 nm; flow: 1.0 mL/min; mobile phase: $CH_3OH$: 1% TFA.

**5,7-Dihydroxy-4-methyl-chromen-2-one (9)**

Concentrated $H_2SO_4$ (0.2 mL) was added to a mixture of phloroglucinol (**6**, 1.0 g, 7.9 mmol) and ethyl acetoacetate (**7**, 1.9 mL, 15.3 mmol) in glacial HOAc (6 mL) and stirred for 2 h at room temperature. The precipitate was filtered, washed with water, and crystallized with MeOH to give the title product. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$2.48 (s, 3H), 5.83 (s, 1H), 6.15 (d, J = 2.0 Hz, 1H), 6.24 (d, J = 2.0 Hz, 1H), 10.28 (s, 1H), 10.51 (s, 1H).

**5,7-Dimethoxy-4-methyl-chromen-2-one (10)**

Methyl iodide (0.54 mL, 8.7 mmol) was added to a mixture of **9** (0.5 g, 2.9 mmol) and potassium carbonate (0.8 g, 5.8 mmol) in anhydrous acetone (20 mL). Then reflux the mixture for 2 h. The mixture was cooled at room temperature and added dist. water, and filtration, washed with water. The crude product was crystallized with MeOH to give the product **10**. $^1$H NMR (500 MHz, $CD_3OD$) $\delta$2.56 (d, J = 1.0 Hz, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 5.96 (d, J = 1.0 Hz, 1H), 6.26 (d, J = 2.0 Hz, 1H), 6.51 (d, J = 2.0 Hz, 1H).

**5,7-Dihydroxy-4-trifluormethyl-chromen-2-one (1)**

Concentrated $H_2SO_4$ (0.5 mL) was added to a mixture of phloroglucinol (**6**, 5.0 g, 39.6 mmol) and ethyl trifluoroacetoacetate (**8**, 8.6 mL, 47.5 mmol) in glacial HOAc

(20 mL) and heated at 100°C for 2 h. The reaction mixture was cooled at room temperature and diluted with water. The precipitate was filtered, washed with water, and crystallized with MeOH to give the title product. 95.0% purity (mobile phase: $CH_3OH$: 1% TFA = 1:1, injection: 20 μL, $t_R$: 15.0 min). $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$6.28 (d, J = 2.5 Hz, 1H), 6.32 (d, J = 2.5 Hz, 1H), 6.53 (s, 1H), 10.66 (s, 1H), 10.91 (s, 1H).

**5,7-Dimethoxy-4-trifluoromethyl-chromen-2-one (2)**

The title compound was obtained from compound **1** in a similar way as described compound **10**. 99.3% purity (mobile phase: $CH_3OH$: 1% TFA = 7:3, injection: 20 μL, $t_R$: 8.9 min). $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$3.89 (s, 3H), 3.89 (s, 3H), 6.62 (d, J = 2.5 Hz, 1H), 6.72 (d, J = 2.5 Hz, 1H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$ 56.20, 56.70, 94.55, 96.37, 98.37, 113.10 (q, J = 32.3 Hz), 121.75 (q, J = 1090.7 Hz), 138.77 (q, J = 133.9 Hz), 156.73, 156.91, 158.69, 163.87.

**5,7-Dimethoxy-4-methyl-3-nitro-chromen-2-one (3) and**
**5,7-Dimethoxy-4-methyl-6-nitro-chromen-2-one (4)**

To a mixture of 5,7-Dimethoxy-4-methyl-chromen-2-one (**10**, 0.1 g, 0.45 mmol) and $Cr(NO_3)_2{\cdot}9H_2O$ (0.22 g, 0.5 mmol) in acetic anhydride (5 mL) was stirred at room temperature for 2 h. After the completion of the reaction, as monitored on TLC, the reaction mixture was filtered and the filtrate was diluted with water. The contents were extracted with ethyl acetate and dried over anhydrous $MgSO_4$ and filtration. The solvent was removed by distillation under reduced pressure and the resulting crude product was subjected to column chromatography (EtOAc/n-hexane = 1/10) to give **3** and **4**.

**3**: 98.1% purity (mobile phase: $CH_3OH$: 1% TFA = 7:3, injection: 20 μL, $t_R$: 6.7 min). $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$2.50 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H), 6.63 (d, J = 2.0 Hz, 1H), 6.74 (d, J = 2.0 Hz, 1H); $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$18.16, 56.33, 56.74, 94.19, 96.84, 101.60, 134.70, 147.30, 153.17, 155.06, 160.70, 164.76; MS (EI, 70 eV) m/z 266 ($M+H^+$), 157 (base peak); HRMS (EI) calculated for $C_{12}H_{12}NO_6^+$: 266.0659, found: 266.0660.

**4**: 96.4% purity (mobile phase: $CH_3OH$: 1% TFA = 6:4, injection: 10 μL, $t_R$: 6.3 min). $^1H$ NMR (500 MHz, $CDCl_3$/DMSO-$d_6$) $\delta$2.51 (s, 3H), 4.00 (s, 3H), 4.03 (s, 3H), 6.11 (s, 1H), 6.75 (s, 1H); $^{13}C$ NMR (125 MHz, $CDCl_3$/DMSO-$d_6$) $\delta$23.66, 56.89, 57.28, 92.56, 103.33, 111.44, 123.54, 147.03, 153.80, 154.02, 157.29, 160.08; MS (EI, 70 eV) m/z 266 ($M+H^+$), 157 (base peak); HRMS (EI) calculated for $C_{12}H_{12}NO_6^+$: 266.0659, found: 266.0660.

**6-amino-5,7-Dimethoxy-4-methyl-chromen-2-one (5)**

To a mixture of **4** (0.12 g, 0.45 mmol), Fe (0.14 g, 2.5 mmol), and $NH_4Cl$ (0.13 g, 2.5 mmol) in 75% EtOH (10 mL) was refluxed for 1 h. The reaction mixture was filtrated by celite-aided, then evaporated to remove EtOH. The residue was diluted with water, basified with 10% NaOH(aq) to pH 10-11, and extracted with ethyl acetate. The organic layer was dried (anhydrous $MgSO_4$) and filtered. The solvent was removed by distillation under reduced pressure and the resulting crude product was subjected to column chromatography (MeOH/$CH_2Cl_2$ = 1/10) to give **5**. 99.3% purity (mobile phase: $CH_3OH$: 1% TFA = 1:1, injection: 20 μL, $t_R$: 4.3 min). $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$2.51 (s, 3H), 3.84 (s, 3H), 3.92 (s, 3H), 5.98 (s, 1H), 6.64 (s, 1H); $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$23.69, 56.11, 56.60, 93.36, 103.89, 110.40, 118.27, 141.42, 149.12, 149.57, 154.53, 159.55; MS (EI, 70 eV) m/z 236 ($M+H^+$), 157 (base peak); HRMS (EI) calculated for $C_{12}H_{14}NO_4^+$: 236.0917, found: 236.0917.

**Cell culture**

Human cell lines were from ATCC. Colon cancer cell lines, DLD-1 and HCT116 were sustained in RPMI medium (Gibco). Colon cancer HT29 cells, breast cancer cell lines, MDA231 and MCF7, lung cancer cell lines, A549 and H1299, and IMR90 lung cells were cultured in DMEM medium (Gibco). Cell culture medium was supplemented with 5-10% Fetal bovine serum (Gibco) as well as 1% antibiotic (Gibco) and incubated at 37℃ with 5% $CO_2$.

**Colony formation assay**

Cells were seeded in the 6 well plates with 100 cells in each well, and the next day, different treatments were applied. 14 days later, colonies were examined by fixing and staining the cells with 0.5% crystal violet in acetic acid/ methanol solution. After the staining, plates were rinse with water and dried for colony counting. The experiment was performed in triplicate in each group. [26]

**Wound healing assay**

Cells were seed in 6 or 12 well plates for next day over 90% cell confluence. Then, treatments were done, scratches (wounds) were made, and cell movement capacity was monitored under the microscope at indicated time points. Migration rate of the cells was examined and determined as (distance at 0hr – distance at each time point) / distance at 0hr.

**Live and dead cell assay**

Calcein-AM (Cayman) and Propidium Iodide (PI, Omics Bio) were dissolved in

DMSO and diluted in PBS. Cells were seeded in the 96 well plates, and then the treatments were applied on the next day. 48hr later, the medium in each well was removed, and 6μM Calcein-AM and 6μM PI were added as 1:1 ratio into each well for cell staining. At the end, cells were monitored under the immunofluorescence microscope at 490nm.

**Western blot assay**

DLD-1 cells were treated with coumarin compound #4 and harvested at indicated time points. Samples were analyzed by western blot assay as described.[26, 27] In brief, samples were lysed, and proteins were extracted, separated by SDS polyacrylamide gel electrophoresis, and transferred to PVDF membrane. Primary antibodies (GeneTex) probing for specific proteins were used as indicated. Signals were detected by Enhanced chemiluminescence (ECL) and analysis. Expression of proteins relative to control was analyzed by image J software.

**MTT cell proliferation assay**

Cells were seeded in 96 well plates, and treatments were applied on the next day. Cell viability was then analyzed at indicated time points by MTT((3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay (Goldbio) as described.[28] $EC_{50}$ was analyzed and determined by SigmaPlot software. The experiment was performed in at least triplicate in each group.

**Statistical analysis**

Representative data were shown, and all the experiments were repeated in at least triplicate in each condition. Data were analyzed by one-way ANOVA or student t-test and showed as mean ± SD. P value <0.05 was taken as significant data and marked as "*."

## Results

**Synthetic scheme of coumarin derivatives**

The coumarins **1-5** were synthesized as in Figure 1. Phloroglucinol (**6**) treated with ethyl acetoacetate/trifluoroacetoacetate (**7/8**) under glacial acetic acid and catalyzed by conc. $H_2SO_4$ to give 5,7-dihydroxy-4-methylcoumarin **9** and 5,7-dihydroxy-4-trifluoromethylcoumarin **1**. Methylation of **9** and **1** yielded 5,7-dimethoxycoumarin **10** and **2**, and then nitration of **10** obtained 6-nitro-5,7-dimethoxy-4-methylcoumarin **4** and 3-nitrocoumarin **3**. Reduction of nitro-coumarin **4** yielded amino-coumarin **5** (Figure 1). The purity of compounds was then determined by HPLC. The purities of all five compounds were higher than 95%,

and the data were shown in Supplementary Figure 1. These five coumarin compounds were named by order (Figure 1a) as #1 to #5 in the following experiments.

**The coumarin derivatives showed specific cytotoxicity towards colon cancer cells**

As mentioned, anti-cancer potential of synthetic coumarin derivatives has been suggested in cancer cells before. Therefore, we firstly examined our five compounds in different cancer cell types, to investigate their cancer inhibition capacity in various cancers. Colon, breast, and lung cancer cell lines were treated with 1, 10, or 100μM of these compounds, and then cell viability was examined 72 hours later by MTT assay. The results showed that among these five compounds, compound #4 exhibited the strongest and specific anti-cancer capacity towards colon cancer cells, in both DLD-1 and HT29 cells (Figure 2a and 2b), rather than in other cancer types (Figure 2c-2f) or in non-cancerous IMR90 cells (Figure 2g). Even though not as powerful as #4, compound #3 also exhibited certain anti-cancer effectiveness in breast cancer cells (Figure 2c and 2d). Also, generally speaking, compound #3, #4, and #5 exhibited better dose dependent cytotoxicity towards colon cancer cells then in breast or lung cancer cells (Figure 2a-2f). Our results suggest that compound #4 could inhibit colon cancer cells with high specificity (Figure 2).

**Coumarin derivatives can serve as proliferation inhibitors to KRAS mutant colon cancer cells**

Among the above two different colon cancer cells, DLD-1 cells carry Mut KRAS gene while as HT29 cells carry wild type (WT) KRAS gene. In order to examine and confirm whether our coumarin compound #4 could suppress Mut KRAS colon cancer, we applied HCT116, another Mut KRAS colon cancer cell line, in the study. Next, we had a closer cancer inhibition investigation towards compounds #3, #4, and #5 in DLD-1 and HCT116 cells. Cells were treated with different concentrations of compounds, from 0, 5, 10, 20, to 40μM, and then cell viability was examined at 48hr and 72hr time points by MTT assay. The results showed that among these three compounds, compound #4 exhibited best cancer inhibition capacity in both DLD-1 (Figure 3a-3c) and HCT116 cells (Figure 3d-3f), and therefore compound #4 was further investigated in the following experiments. Cell morphology was monitored and recorded (Figure 3c and 3f).

**Compound #4 suppressed short term and long term proliferation of colon cancer cells**

To better understand how compound #4 inhibits colon cancer cells, we examined it anti-cancer effect in short term MTT proliferation assay, identified the $EC_{50}$ (or $IC_{50}$)

concentration of compound #4, and then in colony formation assay for investigation of their long term effect. Cells were treated with 0, 2.5, 5, 7.5, or 10μM of compound #4, and then examined by MTT assay at 48hr time point. The results showed that compound #4 exhibited dose dependent cancer inhibition effect in both DLD-1 and HCT116 cells (Figure 4a and 4c). $EC_{50}$ of compound #4 was also examined for these two cell lines as about 6.5μM for DLD-1 cells and nearly 7.7μM for HCT116 cells (Figure 4b and 4d). Next, colony formation assay was carried out under the treatments with 0, 5, 10, and 20μM of compound #4 (Figure 4e). Numbers of colonies were organized and normalized to untreated control group in Figure 4f as a bar chart. The results indicate that compound #4 could suppress short term and long term colon cancer proliferation. Also, DLD-1 cells seemed to be more sensitive to the coumarin compound #4 compared to HCT116 cells (Figure 3 and 4), and therefore DLD-1 cells were applied for the following assays.

**Compound #4 inhibited the migration of colon cancer cells**

Metastasis is one of the major concerns for clinical colorectal cancer treatment, and high migration capacity of colon cancer cells could at the end result in lung or liver metastasis [29]. Therefore, we next investigated whether our coumarin compound can impact on the migration ability of colon cancer cells. Wound healing assay was performed in cells to examine the migration inhibition potential of compound #4 at 0, 5, 10, or 50μM concentrations. The results showed that compared to untreated control group, compound #4 could significantly slow down the migration of DLD-1 cells at 10μM and 50μM (Figure 5a). The wound recovery rates or migration rates of different groups were organized as a bar chart (Figure 5b). When incubated with 50 μM compound #4, DLD-1 cells did not really move during the whole experimental period of time. The data suggest that compound #4 exhibits the migration inhibition capacity of colon cancer cells.

**Compound #4 induced apoptotic cell death of colon cancer cells**

Our data suggested that compound #4 exhibits specific cytotoxicity effect towards colon cancer cells (Figure 2). In order to better understand the molecular mechanism of cell death caused by this coumarin compound, we continued to investigate whether compound #4 induces colon cancer death through apoptosis pathway. First, live and dead assay was carried out by applying Calcein-AM and PI staining as described in materials and methods. After 48hr treatments with different concentrations of compound #4, cells were stained, and then live cells with green fluorescence emission, or dead cells with red emission, were monitored under the fluorescence microscope (Figure 6a). The intensity of red fluorescence increased along with the increase of

compound #4, confirming that compound #4 kills colon cancer cells with a dose dependent fashion (Figure 6a). According to the previous study, coumarin derivatives could exhibit anti-tumor effect and induce apoptosis in cancer cells [30]. Therefore, we continued to examine whether compound #4 induce cytotoxicity of colon cancer cells through upregulating the apoptosis pathway. Cells were treated with compound #4 at 0, 5, or 10 μM concentrations, and then harvested at 24hr, 48hr, or 72hr time points for western blot analysis. Regulation of apoptotic markers include Poly (ADP-ribose) polymerase (PARP), Bcl-2 homologous antagonist/killer protein (BAK), BCL2 associated X protein (BAX), and p53 upregulated modulator of apoptosis (PUMA), were examined. The results showed that treatment of compound #4 resulted in PAPR cleavage and induction of BAK and PUMA proteins (Figure 6b). The expression of apoptotic proteins relative to controls was analyzed as a bar chart (Figure 6c). The induction of BAK can be observed in different time points; the pick expression of cleaved PARP fell in the 10μM 48hr time point; the pick expression of PUMA fell in the 10μM 24hr time point; slight elevation of BAX was observed after the treatments (Figure 6b). The data suggest that compound #4 carried out its anti-tumor capacity through activation of apoptosis signaling pathway.

## Summary and Discussion

Colon cancer was targeted by other coumarin derivatives previously. Osthol, one of the coumarin derivatives with methoxy on the C7, was reported as a strong anti-tumor compound. Among several different types of tumors, Osthol seemed to exhibit tissue specific effect towards colon cancer cells (HCT116 and SW480 cells)[31], of which the dose of Osthol applied was much lower than in other cancer cells, such as prostate and breast cancer cells. Compatibly in our study, colon cancer cells were generally more sensitive to our coumarin compounds, which have the methoxy instead of hydroxyl group, compared to other cancer types (Figure 2).

According to our data, among the five coumarin derivatives examined in this study, compounds #3 and #4 contain nitro group and methoxy group instead of trifluoromethyl group (in compound #1 and #2), another frequently used backbone for anti-tumor effect in coumarin derivatives studies [32, 33], were more effective in colon cancer suppression (Figure 1 and 2). It suggested that nitro- substitution, compared to trifluoromethyl- substitution, could lead to a better cancer inhibition performance. Moreover, the anti-cancer potential of nitro- and/or hydroxyl-coumarin derivatives, such as 6-nitro-7-hydroxycoumarin, 7,8-dihydroxycoumarin, 3,6,8-nitro-7-hydroxycoumarin, and 8-nitro-7-hydroxycoumarin, were previously investigated in kidney, skin, and lung cancer cells, respectively. [34-36] The

comparison of 3- or 6-substitution position of $NO_2$ on the compound (compound #3 or #4) also suggested that 6-$NO_2$ substitution coumarin could result in higher cytotoxicity efficiency, and that 6-amino- substitution does not seem to be a promising anti-cancer option (Figure 1 and 3) for colon cancer treatment. Also, between compound #3, #4, and #5, the structure of compounds #5 is similar to the other two but contain $NH_2$ rather than $NO_2$ functional group (Figure 1). The cancer cell inhibition data suggested that $NO_2$ functional group is more powerful than $NH_2$ on a cumoarin derivative for producing anti-cancer capacity (Figure 3).

Coumarin derivatives were reported to induce anti-proliferative effect and cell apoptosis toward cancer cells previously, and one of the regulated apoptotic pathways was mediated through mitochondria. [5] Compatibly, in this study, we discovered that our coumarin compound #4 induced cell death in a dose dependent manner (Figure 6a). The data also suggest that compound #4 induced cytotoxicity of colon cancer cells through facilitating the intrinsic and extrinsic apoptosis pathways with activation or elevation of several apoptotic markers, include PARP, BAK, BAX, and PUMA (Figure 6b).

As mentioned, one of the famous mutations in tumors, KRAS mutation, could lead to inefficiency of colon cancer treatment in patients under EGFR monoclonal antibody targeted therapies.[8] Coumarin derivatives were reported to suppress lung cancer cells harboring Mut KRAS gene before.[37] Here, in this study, we applied three colon cancer cells lines for demonstrating the cytotoxicity effect of coumarin compound #4, and among them, HT29 cells contain Wt KRAS gene, while as DLD-1 and HCT116 cells harbor mutated KRAS genes, suggested that colon cancers with either Wt or Mut KRAS genes can potentially be treated by our coumarin compound #4. Moreover, in terms of the tissue specific effect of coumarin compound #4 and whether coumarin compound #4 could suppress other cancer types with mutated KRAS gene, our data also provided some suggestions. In Figure 2, we applied cancer cell lines harboring Wt or Mut KRAS. Colon cancer cells DLD1 (Mut), and HT29 (Wt); breast cancer cells MDA231 (Mut) and MCF7 (Wt); lung cancer cells A549 (Mut) and H1299 (Wt). Our data suggest that coumarin derivate #4 targeted with high specificity towards colon cancer carrying either Wt or Mut KRAS gene, rather than towards other cancer cells.

Finally, our data showed efficient anti-cancer effect of compound #4 towards these cells with low $EC_{50}$ (Figure 4), indicating that compound #4 is a promising lead drug for colon cancer inhibition. Further *in vivo* investigation in the future would be helpful

for confirming the anti-tumor drug development potential of compound #4.

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## Conflict of interest

None.

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## Figure Legends

**Figure 1. Coumarin derivatives and synthetic scheme.** Coumarin derivatives **1-5** (a) and synthetic scheme of coumarins **3-5** (b) are shown here. Reagents and conditions: a: glacial HOAc, $H_2SO_4$, RT, 90%; b: $K_2CO_3$, $CH_3I$, anhydrous Acetone, reflux, 87.5%; c: $Cr(NO_3)_3{\cdot}9H_2O$, anhydrous acetic anhydride, RT, 59% (**3**: 26%, **4**: 33%); d: Fe, $NH_4Cl$, 75% EtOH, reflux, 75%.

**Figure 2. Examination of the cytotoxicity of coumarin derivatives compounds towards different cancers.** Different cancer cells were applied treated with 0, 1, 10,100 μM of coumarin derivatives compounds for 72hr, and then cell viability was examined by MTT assay. Colon cancer cells (a) DLD-1 and (b) HT29, breast cancer cells, (c) MDA231 and (d) MCF, lung cancer cells, (e) A549 and (f) H1299, or non-cancerous human cells IMR90, were treated with compound #1-#5 in this experiment. n=3.

**Figure 3. Coumarin derivatives served as proliferation inhibitor to KRAS mutant colon cancer cells.** Coumarin compounds #3-5 were applied for treating DLD-1 cells (a)-(c) or HCT116 cells (d)-(f). n=2 for (a)(d), and n=3 for (b)(e). Cell viability was examined at indicated time points, and cells were also monitored under the bright field microscope at 72hr time point (c) and (f).

**Figure 4. Compound #4 suppressed short term and long term proliferation of colon cancer cells.** DLD-1 cells (a)-(b) and HCT116 cells (c)-(d) were treated with indicated concentrations of compound #4, cell viability was analyzed at 48hr time point by MTT assay, and $EC_{50}$ was determined by SigmaPlot software (b) and (d). n=3 for (a)-(d). (e) DLD-1 cells were applied in colony formation assay under the treatment of compound #4 with indicated concentrations. Colony numbers were organized in (f) as a bar chart. n=2.

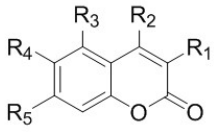
**Figure 5. Compound #4 inhibited the migration of colon cancer cells.** Wound healing assay was perform in DLD-1 cells under compound #4 treatment at indicated concentrations. After the scratches were made, cell migration was monitored at 0hr, 4hr, 8hr, 24hr time points under the microscope (a) and the migration rate (wound recovery) was organized in (b) as a bar chart. n=2.

**Figure 6. Compound #4 induced apoptotic cell death of colon cancer cells.** (a) DLD-1 cells were treated with different concentrations of compound #4, and cells were analyzed by live and dead assay and then examined by fluorescence microscope. (b) DLD-1 cells treated with different concentrations of compound #4 were harvested at indicated time points. Specific protein expression of these samples was analyzed by western blot. GAPDH and vinculin were used as loading control. (c) Expression of proteins relative to control was analyzed, and cleaved PARP was analyzed here. n=2.

**Supplementary Figure 1. Determined the purity of compounds 1-5 by HPLC.** The conditions applied were shown as below: Hitach L2420; UV-Vis detector: 254 nm; Column: ACE 5 C18, 4.6 x 250 mm, 5 µm; Concentration: 1 mg/mL; Flow rate: 1 mL/min; Mobile phase: $CH_3OH$: 1% TFA. (a) Compound 1. (b) Compound 2. (c) Compound 3. (d) Compound 4. (e) Compound 5.

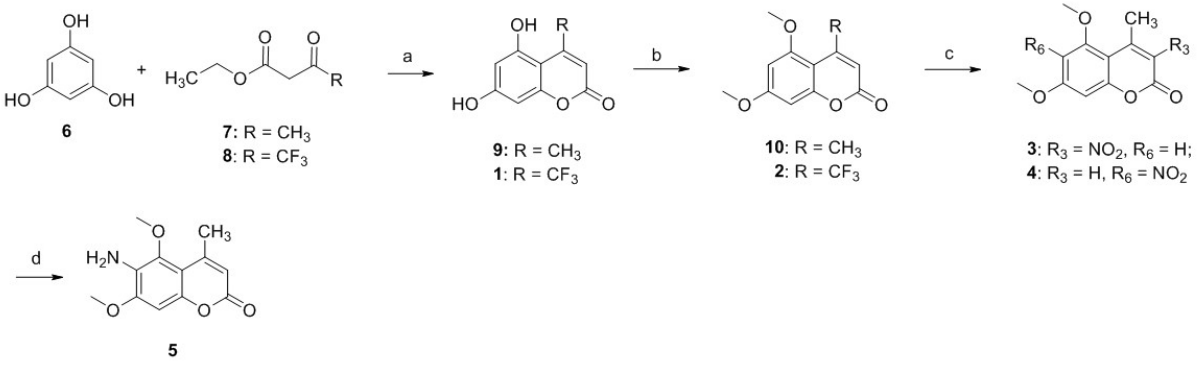
# Figure 1

a)




**1**: $R_1 = R_4 = H$, $R_2 = CF_3$, $R_3 = R_5 = OH$;
**2**: $R_1 = R_4 = H$, $R_2 = CF_3$, $R_3 = R_5 = OCH_3$;
**3**: $R_1 = NO_2$, $R_4 = H$, $R_2 = CH_3$, $R_3 = R_5 = OCH_3$;
**4**: $R_1 = H$, $R_4 = NO_2$, $R_2 = CH_3$, $R_3 = R_5 = OCH_3$;
**5**: $R_1 = H$, $R_4 = NH_2$, $R_2 = CH_3$, $R_3 = R_5 = OCH_3$

b)



# Figure 2

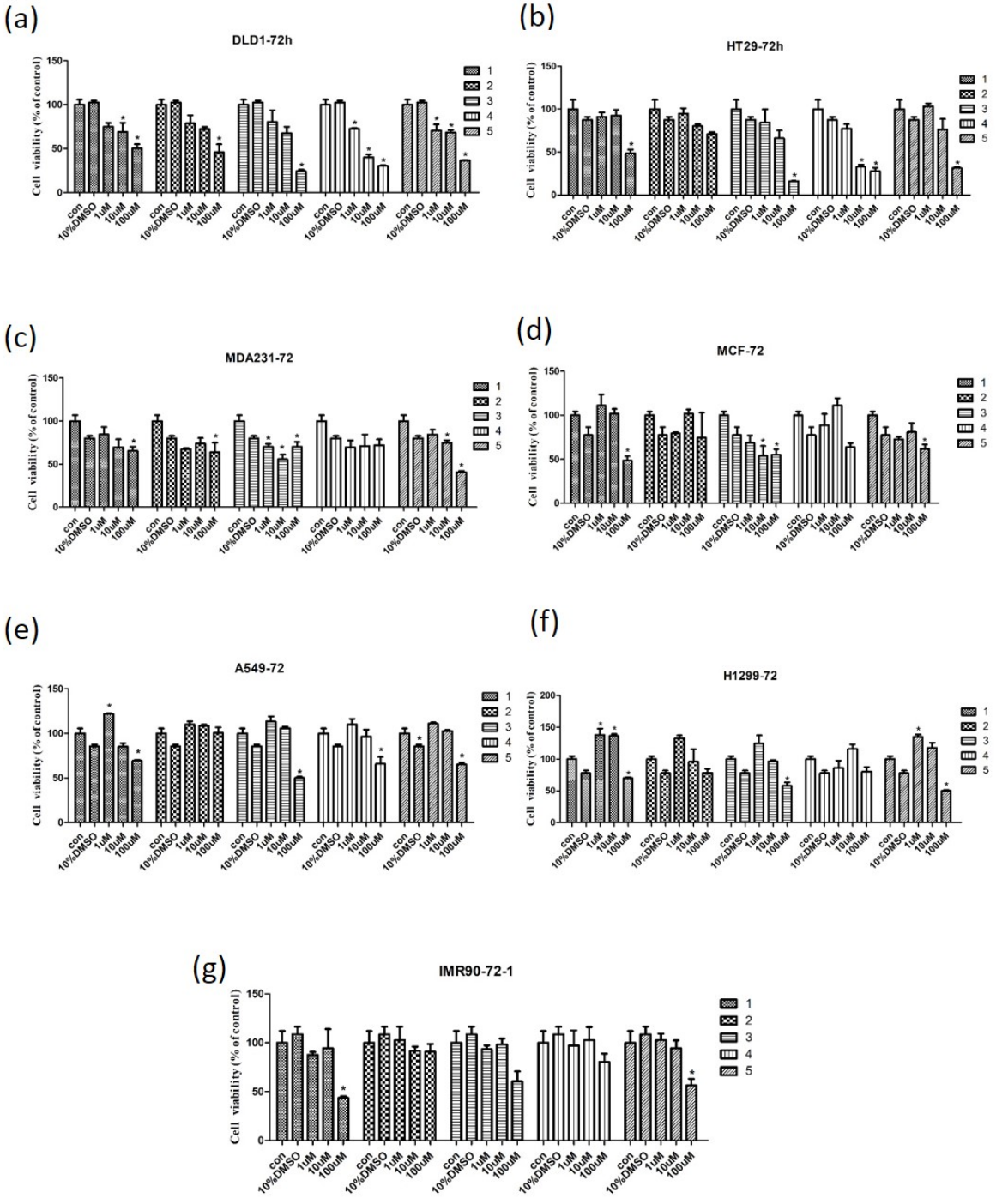
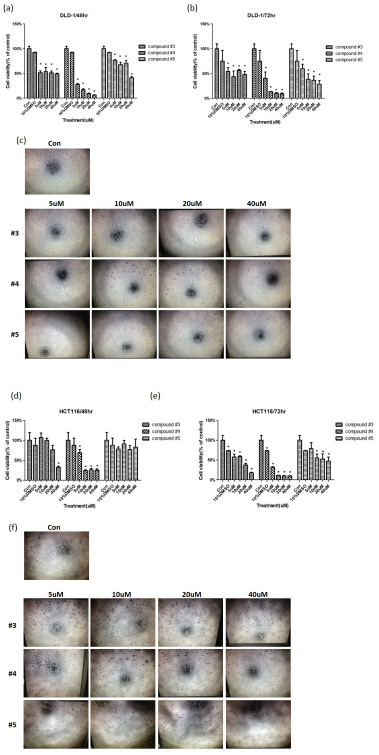
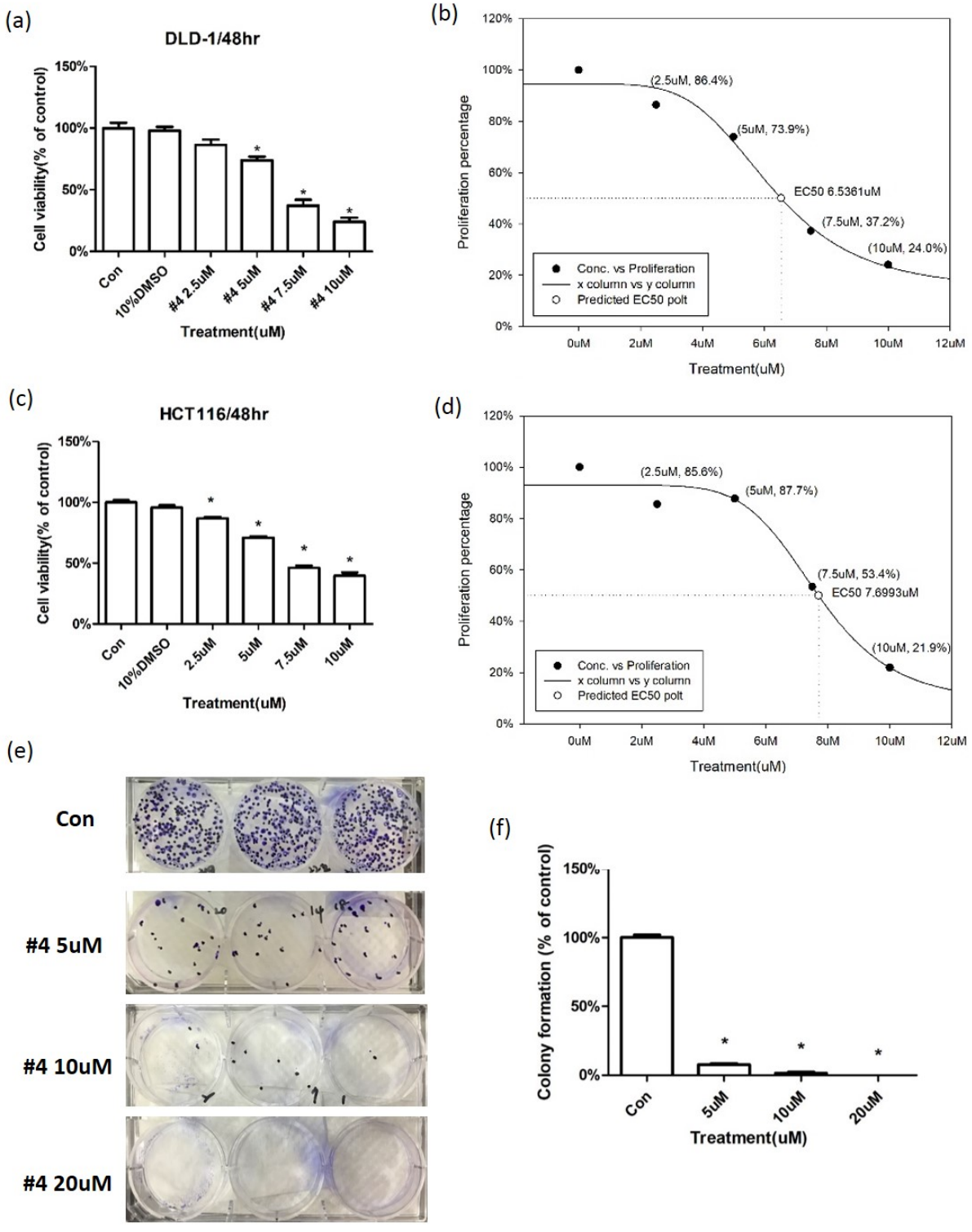

(a)
DLD1-72h
(b)
HT29-72h
(c)
MDA231-72
(d)
MCF-72
(e)
A549-72
(f)
H1299-72
(g)
IMR90-72-1
Cell viability (% of control)
con
10%DMSO
1uM
10uM
100uM
1
2
3
4
5

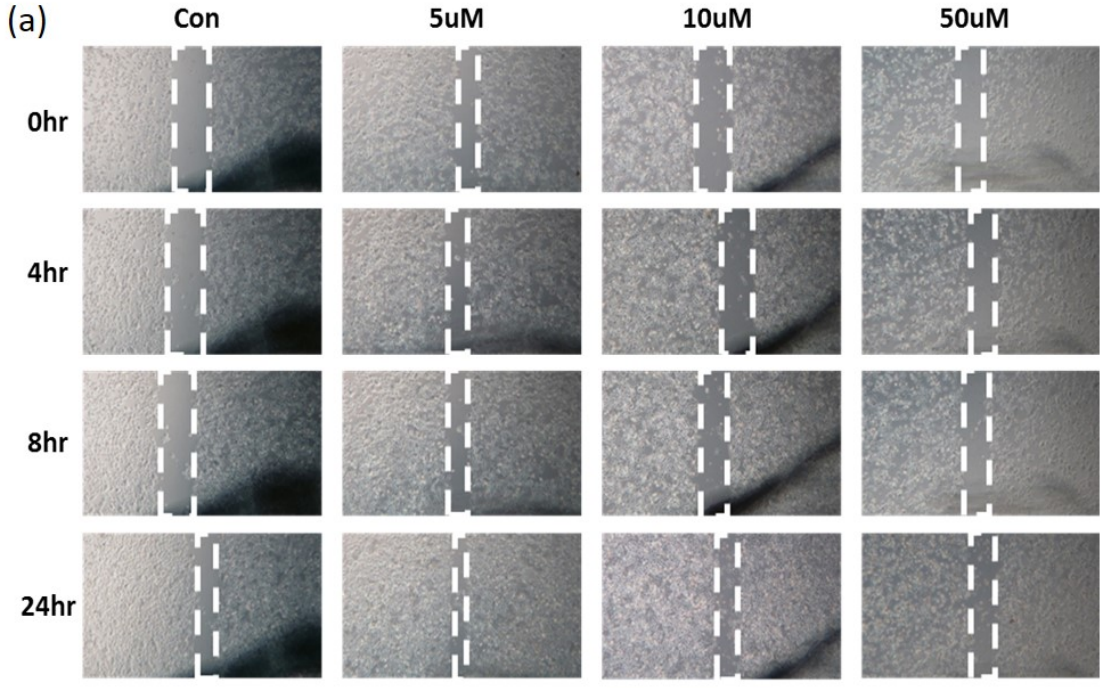
**Figure 3**

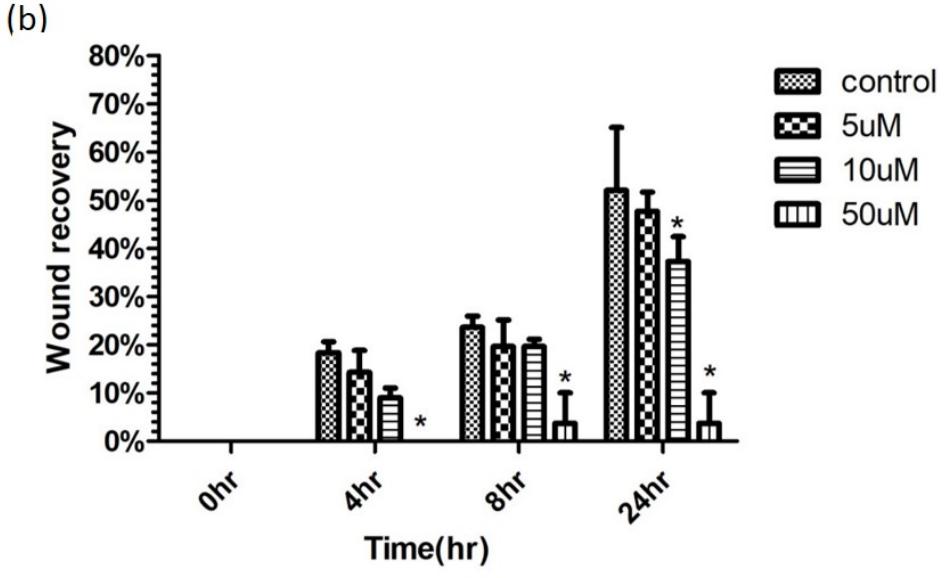


# Figure 4

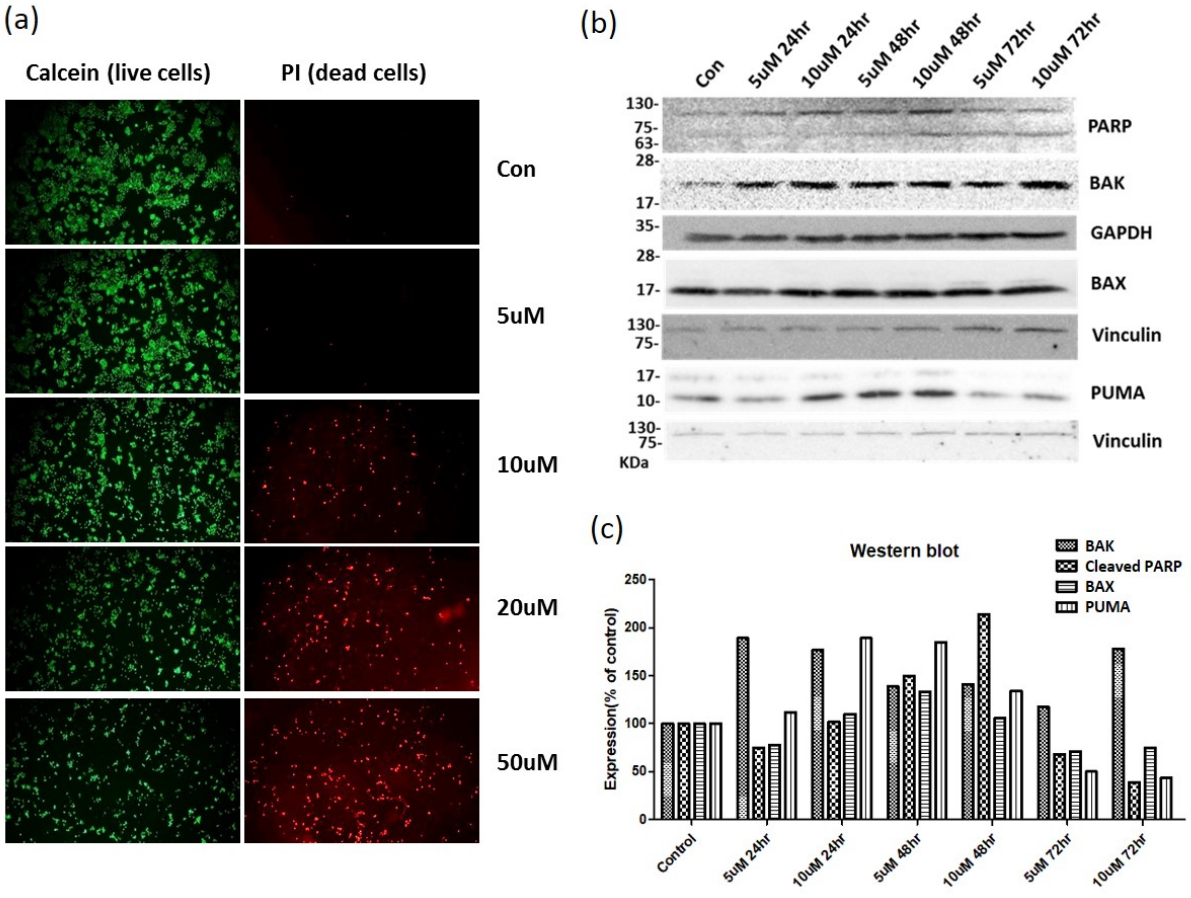

(a)
DLD-1/48hr
Cell viability(% of control)
150%
100%
50%
0%
Con
10%DMSO
#4 2.5uM
#4 5uM
#4 7.5uM
#4 10uM
Treatment(uM)
(b)
Proliferation percentage
120%
100%
80%
60%
40%
20%
0%
(2.5uM, 86.4%)
(5uM, 73.9%)
EC50 6.5361uM
(7.5uM, 37.2%)
(10uM, 24.0%)
Conc. vs Proliferation
x column vs y column
Predicted EC50 polt
0uM
2uM
4uM
6uM
8uM
10uM
12uM
Treatment(uM)
(c)
HCT116/48hr
Cell viability(% of control)
150%
100%
50%
0%
Con
10%DMSO
2.5uM
5uM
7.5uM
10uM
Treatment(uM)
(d)
Proliferation percentage
120%
100%
80%
60%
40%
20%
0%
(2.5uM, 85.6%)
(5uM, 87.7%)
(7.5uM, 53.4%)
EC50 7.6993uM
(10uM, 21.9%)
Conc. vs Proliferation
x column vs y column
Predicted EC50 polt
0uM
2uM
4uM
6uM
8uM
10uM
12uM
Treatment(uM)
(e)
Con
#4 5uM
#4 10uM
#4 20uM
(f)
Colony formation (% of control)
150%
100%
50%
0%
Con
5uM
10uM
20uM
Treatment(uM)

# Figure 5


(a)
Con
5uM
10uM
50uM
0hr
4hr
8hr
24hr


(b)
Wound recovery
80%
70%
60%
50%
40%
30%
20%
10%
0%
0hr
4hr
8hr
24hr
Time(hr)
control
5uM
10uM
50uM
*
*
*
*

# Figure 6


(a)
Calcein (live cells)
PI (dead cells)
Con
5uM
10uM
20uM
50uM
(b)
Con
5uM 24hr
10uM 24hr
5uM 48hr
10uM 48hr
5uM 72hr
10uM 72hr
130-
75-
63-
PARP
28-
17-
BAK
35-
28-
GAPDH
17-
BAX
130-
75-
Vinculin
17-
10-
PUMA
130-
75-
Vinculin
KDa
(c)
Western blot
BAK
Cleaved PARP
BAX
PUMA
Expression(% of control)
250
200
150
100
50
0
Control
5uM 24hr
10uM 24hr
5uM 48hr
10uM 48hr
5uM 72hr
10uM 72hr

## Highlights

- Coumarin derivatives were synthesized and examined in different human cancer cells.
- A novel compound exhibited cytotoxicity specifically towards colon cancer cells.
- It can inhibit short-term and long-term cancer cell proliferation.
- It can suppress migration capacity of colon cancer cells.
- It can facilitate cancer cell death through activation of apoptosis pathway.