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Novel hybrids derived from aspirin and chalcones potently suppress colorectal cancer *in vitro* and *in vivo*[†]

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Colorectal cancer (CRC) remains the fourth leading cause of cancer deaths around the world despite the availability of many approved small molecules for treatment. The issues lie in the potency, selectivity and targeting of these compounds. Therefore, new strategies and targets are needed to optimize and develop novel treatments for CRC. Here, a group of novel hybrids derived from aspirin and chalcones were designed and synthesized based on recent reports of their individual benefits to CRC targeting and selectivity. The most active compound **7h** inhibited proliferation of CRC cell lines with better potency compared to 5-fluorouracil, a currently used therapeutic agent for CRC. Importantly, **7h** had 8-fold less inhibitory activity against non-cancer CCD841 cells. In addition, **7h** inhibited CRC growth *via* the inhibition of the cell cycle in the G1 phase. Furthermore, **7h** induced apoptosis by activating caspase 3 and PARP cleavage, as well as increasing ROS in CRC cells. Finally, **7h** significantly retarded the CRC cell growth in a mouse xenograft model. These findings suggest that **7h** may have potential to treat CRC.

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Introduction

Colorectal cancer (CRC) originates from the inner walls of the colon and the rectum. It is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths worldwide.^{1,2} Common drugs for CRC treatment include 5-fluorouracil (5-FU), capecitabine, irinotecan, and oxaliplatin. The metastasis of CRC has fueled the discovery of many novel targets in recent years, and a number of groups have developed newer drugs to specifically alter these new targets, for example, the development of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) inhibitors.³ However, in many cases, there have been reports on mild to grave side effects encountered with these new drugs due to their non-selectivity towards cancer cells.^{4–8} For this reason, novel therapeutics for CRC with reduced side effects are still urgently needed.

Acetylsalicylic acid, commonly known as aspirin (ASA, **1**, Fig. 1), is a widely used non-steroidal anti-inflammatory drug (NSAID) that has been shown to be effective for the prevention and remission of cancers, especially in CRC.^{9–14} To improve its relatively low anticancer potency, many novel synthetic ASA derivatives have been developed including HS-releasing ASA,^{15–17} HNO- and NO-releasing ASA,^{18–22} dual

nitric oxide- and hydrogen sulfide-releasing ASA,^{23,24} phospho-ASA,²⁵ 6-gingerol-based ASA²⁶ and resveratrol-based ASA prodrugs,²⁷ Se-ASA,²⁸ and asplatin.^{29,30} These ASA derivatives are more cytotoxic and chemo-preventive against CRC cells than ASA, hence, the advancement of new ASA derivatives with more favorable and safer profiles continues to be a welcome advancement.

Natural products and their derivatives provide a diverse source of biologically active lead compounds for drug discovery and development.^{31–34} For example, chalcones (**2**, Fig. 1) are naturally occurring compounds, which bear an α,β -unsaturated ketone moiety and can act as Michael acceptors. Chalcones exert anticancer activity through a number of mechanisms including inhibition of thioredoxin reductases (TrxRs),^{35–38} nuclear factor κ B (NF- κ B),^{39–47} and tubulin polymerization,^{48–60} as well as inhibition of receptor tyrosine kinases such as EGFRs⁶¹ and vascular endothelial growth factor receptor 2 (VEGFR-2).⁶² Moreover, chalcones are recognized as a privileged scaffold for the incorporation of different molecules or pharmacophores using linkers such as amide, diol, and esters or using triazoles *via* click chemistry.^{63–78} These hybrids typically retain or enhance the biological potency and selectivity of the parent chalcones.

The aforementioned investigations led us to hypothesize that combination of ASA and chalcones can be an attractive strategy to selectively treat CRC. Accordingly, we synthesized a novel class of hybrids derived from aspirin and chalcones (**7a–p**, Fig. 1) and evaluated their bioactivity *in vitro* and *in vivo* against CRC cells.

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Fig. 1 Chemical structures of ASA, chalcones and hybrids 7a-p.

Scheme 1 Synthesis of hybrids 7a-p. Reagents and conditions: (a) SOCl₂, anhydrous CH₂Cl₂, reflux, 2 h; (b) i) PPh₃, anhydrous THF, rt, 16 h; ii) KOH, MeOH, rt, 12 h; (c) aldehydes, anhydrous toluene, 60 °C, 8 h; (d) TEA, anhydrous CH₂Cl₂, 12 h.

Results and discussion

Chemistry

The target compounds 7a-p were synthesized as depicted in Scheme 1. Initially, aspirin 1 was converted to the acid chloride 3 upon treatment with thionyl chloride. Then the corresponding chalcones 6a-p were prepared by the Wittig reaction starting from aldehydes 4a-f in moderate to high yields. Next, the reaction of acid chloride 3 with 6a-l in dry CH₂Cl₂, in the presence of the non-nucleophilic base trimethylamine (TEA), furnished

the respective target compounds 7a-p in good yields. The structures of all hybrids were fully characterized by ¹H and ¹³C NMR spectroscopy as well as by mass spectral analysis.

Biological evaluation

Assessment of *in vitro* anti-proliferative activity

The anti-proliferative activity of compounds 7a-p against HCT-8 and DLD-1 cells was initially evaluated using MTT assays with 5-FU as a positive control. As shown in Fig. 2,

Fig. 2 Inhibition of CRC cell proliferation. Cells were treated with, or without, the indicated compounds at 10 μM for 72 h, and the cell proliferation was measured. The inhibition (%) of each compound was determined. Data are presented as the mean (%) ± SD of each compound from three independent experiments.

Table 1 The anti-proliferative activity of compounds **7h** and **7i**

Compounds	IC ₅₀ ^a (μM)		
	HCT-8	DLD-1	CCD841
7h	2.4 ± 0.1	2.7 ± 0.2	19.4 ± 0.9
7i	2.7 ± 0.5	3.5 ± 0.4	ND
6h	19.7 ± 2.5	14.7 ± 1.8	ND
6h + ASA	6.3 ± 0.9	6.9 ± 0.9	ND
6i	25.6 ± 3.2	13.2 ± 1.4	ND
6i + ASA	9.3 ± 1.0	8.0 ± 0.6	ND
ASA	>100	>100	ND
5-FU	28.9 ± 2.7	33.0 ± 2.1	35.7 ± 3.2

^a Data are expressed as mean IC₅₀ (μM) ± SD of each compound from three independent experiments. IC₅₀ is the drug concentration inhibiting 50% of the cell proliferation. ND, not determined.

compounds **7c**, **7f**, **7h**, and **7i** at 10 μM displayed potent anti-proliferative activities (>50%) against both HCT-8 and DLD-1 cells, which were more potent than that of 5-FU. It was obvious that the R₁ and R₂ substituents on the benzene ring of the hybrids may be crucial for their anticancer activity. In gen-

eral, the compounds with a Cl or NO₂ group on the left benzene ring (**7c** and **7f**) showed more potent inhibitory activity than those bearing an Me, OMe, Br, or CN group (**7a**, **7b**, **7d**, or **7e**). Moreover, the compounds with an OMe or F group at the 5-position of the beta benzene ring (**7h** and **7i**) generally showed more potent inhibitory activity than those bearing a 3-OMe, 4-OMe, 5-Cl, 5-Br, or 5-Me group (**7l**, **7g**, **7j**, **7k**, or **7m**).

The active compounds **7h** and **7i** were further assayed for their anti-proliferative activity against two CRC cell lines (Table 1). The IC₅₀ values of **7h** are 2.4 and 2.7 μM against HCT-8 and DLD-1 cells, respectively, which are superior to those of 5-FU, individual drugs **6h** (IC₅₀ = 19.7, 14.7 μM) and ASA (IC₅₀ > 100 μM), and even the combination of **6h** and ASA (IC₅₀ = 6.3, 6.9 μM, physical mixture, mole ratio, 1:1). Similarly, compound **7i** displays a more potent anticancer activity than its individual moieties **6i** (IC₅₀ = 25.6, 13.2 μM) and ASA (IC₅₀ > 100 μM) and the combination of **6i** and ASA (IC₅₀ = 9.3, 8.0 μM). These results confirm that the antitumor activity of **7h** and **7i** may be most likely attributed to the synergic effects derived from their chalcone and ASA moieties.

Fig. 3 Effects of **7h** on the cell cycle distribution in HCT-8 cells. Cells were treated with the indicated concentrations of **7h** for 24 h and stained with PI, followed by flow cytometry analysis.

Given the improved potency of **6h** + ASA and **6i** + ASA, it is probable that **7h** and **7i** may act as prodrugs and this will be tested in future studies. In non-tumorigenic cells, CCD841, **7h** had 8-fold less inhibitory activity ($IC_{50} = 19.4 \mu M$) than that against the cancer cells tested. Hence, **7h** preferably inhibited the proliferation of CRC cells and was selected for further investigation.

Effect of **7h** on cell cycle arrest in CRC cells

Since the anticancer agents can prevent the proliferation of cancer cells by blockade of the cell cycle at specific checkpoints, we performed cell cycle analysis after treatment of HCT-8 cells with compound **7h**. In short, HCT-8 cells were treated with varying concentrations (0, 2.5, 5, and 10 μM) of **7h** for 24 h and then stained with propidium iodide (PI), followed by flow cytometry analysis. Fig. 3 shows that treatment with **7h** induced HCT-8 cell cycle arrest at G_0/G_1 in a concentration dependent manner – 48.08% (2.5 μM), 51.03% (5 μM), and 56.46% (10 μM), higher than that of the vehicle-treated control. As a result, the fraction of CRC cells in the

G2 phase was significantly lower in **7h**-treated cells than the vehicle-treated control. This suggests that the anti-cancer mechanisms of **7h** may include its ability to induce cycle arrest in the CRC cells.

Effect of **7h** on apoptosis in CRC cells

To determine whether the inhibitory effects of **7h** on CRC cell proliferation are accompanied by enhanced apoptosis, we next carried out annexin V-FITC and PI staining on **7h** treated cells. HCT-8 cells were incubated with the vehicle or **7h** (2.5, 5, and 10 μM) for 24 h. Then flow cytometry analysis was used to determine the apoptotic status of the cells. We observed that the treatment with **7h** induced apoptosis in HCT-8 cells in a concentration dependent manner (Fig. 4). This suggests that the **7h** treatment significantly increases apoptosis in cancer cells as a result of its anti-cancer effects.

To understand the mechanism of **7h**-induced apoptosis, we further examined the changes of the intracellular proteins related to apoptosis such as caspase-3 and poly(ADP-ribose)polymerase (PARP) in HCT-8 cells treated with **7h** (0,

Fig. 4 Effects of **7h** on the induction of apoptosis. HCT-8 cells were treated with the indicated concentrations of **7h** or vehicle (control) for 24 h. The cells were stained with annexin V-FITC and propidium iodide (PI), followed by flow cytometry analysis.

Fig. 5 Effect of **7h** on the expression of apoptotic markers. The relative protein expression levels of PARP and caspase 3 were measured following the **7h** treatment in HCT-8 cells. Cells were treated with increasing concentrations of **7h** or the control (0.1% DMSO) for 24 h and immunoblotting of the whole cell lysate was carried out to determine the protein levels.

2.5, 5, and 10 μM). As shown in Fig. 5, **7h** significantly induced the cleavage of both caspase 3 and PARP in a dose-dependent manner in the cells. Overall, our results confirm that compound **7h** inhibits the growth of CRC cells by inducing apoptosis in addition to its effects on the cell cycle.

Compound **7h** increased ROS levels in CRC cells

Previous studies have demonstrated that the anticancer effect of chalcones is associated with the increase in ROS levels.^{35,79,80} To investigate whether the induced cytotoxic effect of **7h** is related to ROS production, we measured ROS levels in **7h**-treated CRC cells by flow cytometry analysis. Again, we treated HCT-8 cells with different concentrations of **7h** and measured the ROS levels. As shown in Fig. 6, a dramatic increase (from 3.67 to 53.64) in the levels of ROS generation in HCT-8 cells was observed after treatment with **7h**. These results indicate that the induction of cell death by **7h** may be due to its effect on ROS production.

In vivo anticancer activity of **7h**

To evaluate the *in vivo* anticancer activity of **7h**, BALB/c nude mice were inoculated subcutaneously with HCT-8 cells. After the establishment of solid tumors, the mice were randomly treated intraperitoneally with **7h** (60 mg kg⁻¹) or the vehicle consisting of Tween 80/PBS (5:95) daily for 32 consecutive days, respectively. Treatment with 60 mg kg⁻¹ of **7h** significantly reduced the volume of implanted colon tumors ($P < 0.001$ vs. the vehicle-treated control, Fig. 7A and B). Moreover, the tumor weights in the mice treated with **7h** at 60 mg kg⁻¹ were 42% (w/w) less than that in the vehicle-treated controls (1.95 ± 0.42 g vs. 1.13 ± 0.07 g, $P < 0.001$, Fig. 7C) while the body weights of the mice were not significantly changed by the treatment with **7h** (Fig. 7D). Together, our data clearly demonstrated that **7h** inhibited the growth of implanted tumors *in vivo* with little off-target cell toxicity.

Fig. 6 Effect of compound **7h** on ROS levels in CRC cells. Flow cytometry analysis of HCT-8 cells treated with the indicated concentrations of **7h** for 24 h and stained with the fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFH-DA).

Fig. 7 Anticancer effects of 7h in mice inoculated with HCT-8 cells. (A) Images of the tumors. The effect of intraperitoneal 7h (60 mg kg⁻¹) on tumor volumes (B), tumor weight (C) and body weight (D) in a HCT-8 xenograft model. Data are expressed as the mean $\pm$ SD from each group of mice ($n = 6$ per group). (***) $P < 0.001$ vs. the vehicle-treated.

Conclusion

In summary, we have designed and synthesized a novel group of hybrids derived from aspirin and chalcones 7a–p. We found that compound 7h had potent and selective antiproliferative activity against CRC cells *in vitro*. In addition, treatment with 7h induced CRC cell cycle arrest in the G1 phase and apoptosis *in vitro* and increased the relative levels of cleaved caspase 3 and PARP as well as the ROS in HCT-8 cells. Finally, treatment with 7h significantly inhibited the growth of implanted CRC cells in mice. These findings may provide a proof of principle that 7h may be a potential chemotherapeutic agent for the intervention of CRC.

Experimental protocols

Chemical analysis

All chemicals were obtained from commercial suppliers and used without further purification unless noted specifically. Analytical thin layer chromatography was visualized by ultraviolet light at 254 nm. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 400 instrument, and tetramethylsilane (TMS) was used as a reference. Data for ¹H are reported as follows: chemical shift (ppm) and multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Data for ¹³C NMR are reported as ppm. High resolution mass spectrometry (HRMS) spectra were recorded on an Agilent Technologies LC/MSD TOF instrument. Aspirin 2 and α -bromoacetophenones 4a–f were commercially available.

Synthesis of intermediate 3. Intermediate 3 was prepared according to a method described previously⁸¹ with minor modification. To a solution of aspirin (180 mg, 1 mmol) in anhydrous CH₂Cl₂ (2 mL), SOCl₂ (1 mL) was added, and the mixture was refluxed under nitrogen for 2 h. The reaction solution was evaporated *in vacuo* to give the corresponding crude intermediate 3, which was used later without further purification.

Synthesis of intermediates 5a–f. Intermediates 5a–f were prepared according to a method described previously^{82–85} with minor modification. Triphenyl phosphine (PPh₃, 1 mmol) was dissolved in anhydrous THF (2 mL). To this mixture were added α -bromoacetophenones 4a–f (1 mmol). The mixture was stirred at room temperature for 16 h. The resulting white precipitate was collected by filtration and washed with *n*-hexane (3 $\times$ 20 mL). The white solid was dissolved in MeOH (5 mL). To this solution was added KOH (10 mmol) dissolved in H₂O (5 mL). The mixture was allowed to stir at room temperature (rt) for 12 h. The MeOH was removed under reduced pressure and the crude reaction mixture was extracted with DCM (3 $\times$ 20 mL). The combined organic layers were washed with water (3 $\times$ 5 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* to give the corresponding crude intermediates 5a–f, which were used in the next step without further purification.

Synthesis of 6a–p. Chalcones 6a–p were prepared using a known synthetic method procedure.⁸⁵ A mixture of a corresponding aldehyde (1.0 mmol) with intermediates 5a–f (1.0 mmol) in anhydrous toluene (2.0 mL) was stirred at 60 °C under nitrogen for 8 h. The solvent was evaporated *in vacuo* to

give the corresponding crude intermediates **6a–p**, which were directly used in the next step without further purification (data in the ESI[†]).

General procedures for the preparation of 7a–p. A mixture of **6a–p** (1 mmol) and TEA (2 mmol) in anhydrous CH₂Cl₂ (5 mL) was stirred at 0 °C. Intermediate **3** (1 mmol) in anhydrous CH₂Cl₂ (2 mL) was sequentially added dropwise, and the mixture was stirred at room temperature for 12 h. The reaction solution was quenched by adding water and extracted with CH₂Cl₂. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The resulting crude product was purified by flash chromatography on a silica gel column to provide the compounds **7a–p** (53–88%), respectively.

(E)-2-(3-Oxo-3-(p-tolyl)prop-1-en-1-yl)phenyl 2-acetoxybenzoate (7a). The title compound was prepared according to the general procedure, as described above in 66% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.47 (d, *J* = 6.8 Hz, 1H), 8.09 (d, *J* = 15.6 Hz, 1H), 8.01–7.99 (m, 3H), 7.88 (t, *J* = 7.2, 7.6 Hz, 1H), 7.75–7.52 (m, 4H), 7.45–7.36 (m, 4H), 2.57 (s, 3H), 2.48 (m, 3H); ¹³C-NMR (100 MHz, CDCl₃) δ 189.9, 169.7, 162.6, 151.4, 149.5, 143.6, 137.7, 135.3, 134.9, 132.2, 131.3, 129.3, 128.7, 128.5, 127.9, 126.6, 126.4, 124.6, 124.2, 123.4, 122.0, 21.7, 21.0; HRMS (ESI): calcd for C₂₅H₂₁O₅ [M + H]⁺ 401.1389, found 401.1395.

(E)-2-(3-(4-Methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7b). The title compound was prepared according to the general procedure, as described above in 77% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.30 (d, *J* = 8.0 Hz, 1H), 7.94–7.90 (m, 3H), 7.81 (d, *J* = 6.8 Hz, 1H), 7.69 (t, *J* = 8.0, 7.2 Hz, 1H), 7.57 (d, *J* = 16.0 Hz, 1H, COCH=CH), 7.50–7.41 (m, 2H), 7.35 (6.8, 8.0 Hz, 1H), 7.28–7.23 (m, 2H), 6.88 (d, *J* = 8.0 Hz, 2H), 3.83 (s, 3H, Ar-OCH₃), 2.31 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ 188.6, 169.7, 163.4, 162.7, 151.4, 149.4, 137.2, 135.0, 132.2, 131.2, 130.9, 130.7, 128.5, 128.0, 126.7, 126.4, 124.4, 124.3, 123.4, 122.1, 113.8, 55.4, 21.0; HRMS (ESI): calcd for C₂₅H₂₁O₆ [M + H]⁺ 417.1338, found 417.1344.

(E)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7c). The title compound was prepared according to the general procedure, as described above in 80% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.60 (d, *J* = 7.6 Hz, 1H), 8.20 (d, *J* = 15.6 Hz, 1H), 8.15–8.11 (m, 3H), 8.05 (t, *J* = 7.6, 8.0 Hz, 1H), 7.85–7.65 (m, 6H), 7.61–7.56 (m, 2H), 2.63 (s, 3H); ¹³C-NMR (100 MHz, CDCl₃) δ 189.4, 169.6, 162.6, 151.4, 149.5, 139.1, 138.8, 136.1, 135.1, 132.2, 131.6, 130.0, 128.8, 128.7, 127.7, 126.7, 126.4, 124.3, 124.2, 123.4, 121.9, 21.0; HRMS (ESI): calcd for C₂₄H₁₈ClO₅ [M + H]⁺ 421.0843, found 421.0849.

(E)-2-(3-(4-Bromophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7d). The title compound was prepared according to the general procedure, as described above in 60% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.43 (d, *J* = 7.6 Hz, 1H), 8.03 (d, *J* = 16.0 Hz, 1H, COCH=CH), 7.95 (d, *J* = 7.6 Hz, 1H), 7.90–7.88 (m, 3H), 7.68–7.50 (m, 6H), 7.42 (t, *J* = 8.0 Hz, 2H), 2.46 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ

189.7, 169.7, 162.6, 151.4, 149.5, 138.9, 136.5, 135.1, 132.2, 131.8, 131.6, 130.1, 128.7, 127.8, 127.6, 126.8, 126.4, 126.3, 124.3, 124.2, 123.4, 121.9, 21.0; HRMS (ESI): calcd for C₂₄H₁₈BrO₅ [M + H]⁺ 465.0338, found 465.0345.

(E)-2-(3-(4-Cyanophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7e). The title compound was prepared according to the general procedure, as described above in 53% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.23 (d, *J* = 7.6 Hz, 1H), 7.88–7.73 (m, 5H), 7.60 (d, *J* = 7.6 Hz, 2H), 7.52 (t, *J* = 8.0 Hz, 1H), 7.44–7.36 (m, 3H), 7.26 (d, *J* = 7.6 Hz, 2H), 2.29 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ 190.0, 169.6, 162.5, 151.5, 149.6, 141.1, 149.2, 135.3, 132.3, 132.1, 132.0, 129.4, 128.9, 128.8, 127.3, 126.9, 126.4, 124.4, 124.2, 123.5, 121.8, 118.0, 115.6, 21.0; HRMS (ESI): calcd for C₂₅H₁₈NO₅ [M + H]⁺ 412.1185, found 412.1198.

(E)-2-(3-(4-Nitrophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7f). The title compound was prepared according to the general procedure, as described above in 67% yield. ¹H-NMR (400 MHz, DMSO-d₆) δ 8.27–8.20 (m, 6H), 7.92 (d, *J* = 15.6 Hz, 1H, COCH=CH), 7.83 (t, *J* = 6.8, 8.0 Hz, 1H), 7.73 (d, *J* = 15.6 Hz, 1H, COCH=CH), 7.61 (t, *J* = 6.8, 8.0 Hz, 1H), 7.53 (t, *J* = 8.0, 6.8 Hz, 1H), 7.46 (t, *J* = 7.6, 7.2 Hz, 1H), 7.40–7.35 (m, 2H), 2.22 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, DMSO-d₆) δ 189.0, 169.6, 162.8, 151.2, 150.2, 149.9, 142.2, 138.3, 136.0, 132.8, 132.2, 130.2, 128.9, 127.4, 127.3, 127.1, 124.9, 124.6, 124.2, 123.9, 121.9, 21.1; HRMS (ESI): calcd for C₂₄H₁₈NO₇ [M + H]⁺ 432.1083, found 432.1094.

(E)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)-5-methoxyphenyl 2-acetoxybenzoate (7g). The title compound was prepared according to the general procedure, as described above in 88% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.38 (d, *J* = 8.0 Hz, 1H), 7.96 (m, 5H), 7.56–7.35 (m, 5H), 7.02 (d, *J* = 8.0 Hz, 1H), 6.90 (s, 1H), 3.98 (s, 3H, Ar-OCH₃), 2.42 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ 189.5, 169.7, 162.4, 151.5, 150.9, 138.9, 136.4, 135.2, 132.2, 129.9, 128.7, 126.4, 124.3, 121.8, 121.6, 120.1, 113.3, 108.6, 55.8, 21.0; HRMS (ESI): calcd for C₂₅H₂₀ClO₆ [M + H]⁺ 451.0948, found 451.0955.

(E)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)-4-methoxyphenyl 2-acetoxybenzoate (7h). The title compound was prepared according to the general procedure, as described above in 78% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.51 (d, *J* = 8.0 Hz, 1H), 8.06–8.04 (m, 3H), 7.96 (t, *J* = 8.0 Hz, 1H), 7.70–7.66 (m, 2H), 7.59–7.57 (m, 2H), 7.50–7.48 (m, 2H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 4.13 (s, 3H, Ar-OCH₃), 2.55 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ 189.5, 169.7, 163.0, 157.6, 151.4, 143.1, 139.1, 138.9, 136.0, 135.0, 132.1, 130.0, 128.8, 128.3, 126.4, 124.4, 124.3, 124.2, 122.0, 117.2, 112.9, 55.8, 21.0; HRMS (ESI): calcd for C₂₅H₂₀ClO₆ [M + H]⁺ 451.0948, found 451.0953.

(E)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)-4-fluorophenyl 2-acetoxybenzoate (7i). The title compound was prepared according to the general procedure, as described above in 59% yield. ¹H-NMR (400 MHz, CDCl₃) δ 8.14 (d, *J* = 7.2 Hz, 1H), 7.72–7.67 (m, 3H), 7.60 (t, *J* = 7.2, 8.0 Hz, 1H), 7.37–7.29 (m, 3H), 7.23 (d, *J* = 8.0 Hz, 2H), 7.13–7.07 (m, 3H), 2.19 (s, 3H, CH₃CO); ¹³C-NMR (100 MHz, CDCl₃) δ 188.9, 169.6,

162.6, 160.4 (d, $J = 245$ Hz), 151.4, 145.4, 139.3, 137.4, 135.8, 135.2, 132.1, 130.4, 130.0, 129.3, 128.9, 126.4, 125.0, 124.9, 124.3, 121.7, 118.3 (d, $J = 24.4$ Hz), 114.4 (d, $J = 24.4$ Hz), 21.0; HRMS (ESI): calcd for $C_{24}H_{17}ClFO_5$ $[M + H]^+$ 439.0749, found 439.0757.

(*E*)-4-Chloro-2-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7j). The title compound was prepared according to the general procedure, as described above in 70% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.39 (d, $J = 8.0$ Hz, 1H), 7.99–7.84 (m, 5H), 7.63–7.55 (m, 3H), 7.50 (d, $J = 7.6$ Hz, 2H), 7.37 (t, $J = 8.0, 7.6$ Hz, 2H), 2.44 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 188.8, 169.6, 162.4, 151.5, 147.9, 139.4, 137.1, 135.8, 135.3, 132.2, 132.1, 131.2, 130.0, 129.3, 128.9, 128.0, 126.5, 125.0, 124.8, 124.3, 121.6, 21.0; HRMS (ESI): calcd for $C_{24}H_{17}Cl_2O_5$ $[M + H]^+$ 455.0453, found 455.0459.

(*E*)-4-Bromo-2-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7k). The title compound was prepared according to the general procedure, as described above in 83% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.13 (d, $J = 8.0$ Hz, 1H), 7.79 (s, 1H), 7.73–7.66 (m, 3H), 7.60 (t, $J = 8.0$ Hz, 1H), 7.46 (d, $J = 8.0$ Hz, 1H), 7.37–7.29 (m, 2H), 7.24 (d, $J = 8.0$ Hz, 2H), 7.12 (d, $J = 7.6$ Hz, 1H), 7.04 (d, $J = 8.0$ Hz, 1H), 2.18 (s, 3H); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 188.7, 169.6, 162.3, 151.5, 148.4, 139.4, 137.0, 135.8, 135.3, 134.2, 132.1, 131.0, 130.0, 129.7, 128.9, 126.5, 125.1, 125.0, 124.3, 121.6, 119.9, 21.0; HRMS (ESI): calcd for $C_{24}H_{17}BrClO_5$ $[M + H]^+$ 498.9948, found 498.9959.

(*E*)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)-6-methoxyphenyl 2-acetoxybenzoate (7l). The title compound was prepared according to the general procedure, as described above in 65% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.26 (d, $J = 7.2$ Hz, 1H), 7.85–7.78 (m, 3H), 7.66 (t, $J = 8.0$ Hz, 1H), 7.44 (d, $J = 15.6$ Hz, 1H, $COCH=CH$), 7.38 (t, $J = 8.0, 7.2$ Hz, 1H), 7.31–7.19 (m, 5H), 7.01 (d, $J = 8.0$ Hz, 1H), 3.78 (s, 3H, $Ar-OCH_3$), 2.25 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 189.3, 169.5, 161.9, 151.8, 151.4, 139.1, 139.0, 138.8, 136.1, 134.8, 132.4, 130.0, 128.8, 127.0, 126.3, 124.4, 124.3, 122.0, 119.8, 114.1, 56.1, 21.0; HRMS (ESI): calcd for $C_{25}H_{20}ClO_6$ $[M + H]^+$ 451.0948, found 451.0953.

(*E*)-2-(3-(4-Chlorophenyl)-3-oxoprop-1-en-1-yl)-4-methylphenyl 2-acetoxybenzoate (7m). The title compound was prepared according to the general procedure, as described above in 75% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.36 (d, $J = 7.6$ Hz, 1H), 7.95–7.90 (m, 3H), 7.81 (t, $J = 6.8, 8.0$ Hz, 1H), 7.68 (s, 1H), 7.57–7.50 (m, 2H), 7.44–7.37 (m, 3H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.24 (d, $J = 8.0$ Hz, 1H), 2.53 (s, 3H, $Ar-CH_3$), 2.39 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 189.5, 169.7, 162.8, 151.4, 147.4, 139.0, 136.5, 136.1, 135.0, 132.4, 132.2, 130.0, 129.0, 128.8, 127.2, 126.4, 124.3, 123.9, 123.1, 122.0, 21.0; HRMS (ESI): calcd for $C_{25}H_{20}ClO_5$ $[M + H]^+$ 435.0999, found 435.1006.

(*E*)-4-Methoxy-2-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7n). The title compound was prepared according to the general procedure, as described above in 79% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 7.90 (d, $J =$

8.0 Hz, 1H), 7.53 (d, $J = 8.0$ Hz, 2H), 7.45 (d, $J = 16.0$ Hz, 1H, $COCH=CH$), 7.30 (t, $J = 7.6, 8.0$ Hz, 1H), 7.13 (d, $J = 16.0$ Hz, 1H, $COCH=CH$), 7.03 (t, $J = 7.6, 8.0$ Hz, 1H), 6.89 (s, 1H), 6.84 (d, $J = 8.0$ Hz, 1H), 6.78 (d, $J = 8.0$ Hz, 1H), 6.63 (dd, $J = 8.0$ Hz, 1H), 6.49 (d, $J = 8.0$ Hz, 2H), 3.49 (s, 3H, $Ar-CH_3$), 3.45 (s, 3H, $Ar-CH_3$), 1.92 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 188.7, 169.7, 163.4, 163.1, 157.6, 151.3, 143.0, 137.3, 134.9, 132.2, 130.9, 130.6, 128.7, 126.4, 124.6, 124.2, 124.1, 122.1, 116.8, 113.8, 112.7, 55.7, 55.4, 21.0; HRMS (ESI): calcd for $C_{26}H_{23}O_7$ $[M + H]^+$ 447.1444, found 447.1453.

(*E*)-4-Bromo-2-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7o). The title compound was prepared according to the general procedure, as described above in 85% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.23 (d, $J = 8.0$ Hz, 1H), 7.92–7.90 (m, 3H), 7.78 (d, $J = 15.6$ Hz, 1H, $COCH=CH$), 7.65 (t, $J = 7.6, 8.0$ Hz, 1H), 7.53–7.49 (m, 2H), 7.38 (t, $J = 6.8, 8.0$ Hz, 1H), 7.19 (d, $J = 8.0$ Hz, 1H), 7.11 (d, $J = 8.0$ Hz, 1H), 6.86 (d, $J = 8.0$ Hz, 2H), 3.80 (s, 3H, $Ar-CH_3$), 2.27 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 187.9, 169.6, 163.6, 162.4, 151.4, 148.3, 135.5, 135.2, 133.8, 132.2, 130.9, 130.8, 130.4, 130.1, 126.4, 125.3, 125.1, 124.3, 121.7, 119.8, 113.8, 55.5, 21.0; HRMS (ESI): calcd for $C_{25}H_{20}BrO_6$ $[M + H]^+$ 495.0443, found 495.0449.

(*E*)-4-Chloro-2-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl 2-acetoxybenzoate (7p). The title compound was prepared according to the general procedure, as described above in 74% yield. 1H -NMR (400 MHz, $CDCl_3$) δ 8.25 (d, $J = 8.0$ Hz, 1H), 7.92 (d, $J = 8.0$ Hz, 2H), 7.81–7.75 (m, 2H), 7.67 (t, $J = 6.8, 8.0$ Hz, 1H), 7.52 (d, $J = 15.6, 1H$, $COCH=CH$), 7.41–7.40 (m, 2H), 7.19 (t, $J = 6.4, 8.0$ Hz, 2H), 6.87 (d, $J = 8.0$ Hz, 2H), 3.83 (s, 3H, $Ar-OCH_3$), 2.27 (s, 3H, CH_3CO); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 183.3, 164.9, 158.8, 157.7, 146.7, 143.0, 130.9, 130.4, 127.4, 127.4, 126.2, 126.1, 125.7, 124.9, 123.1, 121.7, 120.6, 120.0, 119.5, 116.9, 109.1, 50.7, 16.2; HRMS (ESI): calcd for $C_{25}H_{20}ClO_6$ $[M + H]^+$ 451.0948, found 451.0956.

Biological assays

MTT assay

HCT-8, DLD-1, and CCD841 cell lines were purchased from American Tissue Culture Collection (ATCC, Rockville, MD, USA). HCT-8, DLD-1 and CCD841 cells were seeded in 96-well plates and then treated with the vehicle alone or tested compounds for 72 h. Then 20 μ L of MTT (5 mg mL^{-1} , in PBS) was added to each well and further incubated for another 4 h. The MTT formazan formed by viable cells was dissolved in DMSO (150 μ L), and absorbance was measured using a microplate reader (570 nm).

Cell cycle analysis

HCT-8 cells were treated with 7h at concentrations of 0, 2.5, 5 and 10 μ M for 24 h, respectively. The cells were harvested, fixed with 70% ethanol for 2 h, and incubated with PI/RNase staining buffer for 15 min at room temperature. The DNA

content in the different groups of cells was assessed by flow cytometry.

Apoptosis analysis

HCT-8 cells were incubated in six-well plates (1×10^5 cells per well) and treated with DMSO (1%) and compound **7h** at concentrations of 2.5, 5 and 10 μM for 24 h, respectively. The cells were collected, washed with PBS, and stained with FITC-annexin-V and PI. Apoptosis was determined by flow cytometry.

Western blotting

The assay was performed according to a method described previously with minor modification.⁸⁶ HCT-8 cells were incubated in six-well plates (1×10^6 cells per well) overnight and treated with vehicle DMSO (0.1%, v/v) alone or **7h** for 24 h. The cells were harvested and lysed at 4 °C for 30 min in a lysis buffer. The cell lysates were centrifuged at 15 000 rpm for 15 min at 4 °C, and the supernatants were collected. The protein concentration in the cell lysates was determined using a bicinchoninic acid protein assay kit. The protein samples were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel, 20 μg per lane) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with skim milk (5%) in Tris-buffered saline containing 0.05% Tween 20 and sequentially incubated with primary antibodies [anti-caspase 3, anticlaved caspase 3, anti-PARP, anticlaved PARP, anti-GAPDH (Cell Signaling, Boston, MA)], followed by enhanced chemiluminescence detection.

Determination of ROS generation

The assay was performed according to a method described previously with minor modification.⁸⁷ HCT-8 cells were treated with different concentrations of **7h** (0, 2.5, 5, 10 μM) for 24 h. After being loaded with 10 μM DCFH-DA at 37 °C for 20 min, the cells were washed three times with PBS buffer to remove excess dye. The fluorescence intensity, used as a fluorescence indicator of intracellular ROS, was recorded using a plate reader and measured with a flow cytometer with an excitation/emission (Ex/Em) frequency of 488/530 nm.

In vivo tumor growth inhibition experiment

The assay was performed according to a method described previously with minor modification.⁸⁸ Individual male BALB/c nude mice (5–6 weeks, 18–20 g, Experimental Animal Research Center, Chinese Academy of Military Medical Sciences) were inoculated subcutaneously with HCT-8 cells (1×10^7 cells per mouse, American Tissue Culture Collection, ATCC, Rockville, MD, USA). After the solid tumors were established and allowed to reach 180–200 mm^3 , the mice were randomized and treated intraperitoneally with **7h** (60 mg kg^{-1}) or the same volume of the vehicle consisting of Tween 80/PBS (5,95) daily for 32 consecutive days. The tumor

growth was recorded every 4 days from the measurement of length and width using a vernier caliper and calculated as tumor volumes (TV) with the following formula: $\text{TV} (\text{mm}^3) = \text{width}^2 (\text{length}/2)$. The tumor growth inhibition rate (weight per unit weight, w/w, %) was calculated as described previously.⁸⁸ The experimental protocols were evaluated and approved by the Ethics Committee of the Hubei University of Chinese Medicine. All animal experiments complied with the International Guiding Principles for Animals Research (A CIOMS Ethical Code for Animal Experimentation, WHO Chronicle, 39 (2): 51–56, 1985).

Conflicts of interest

There are no conflicts to declare.

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