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Original article

Synthesis and antitumor activity of conjugates of 5-Fluorouracil and emodin

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ABSTRACT

A series of conjugates of 5-Fluorouracil (5-FU) and emodin were synthesized by coupling trimethyl emodin with N^1, N^3 dialkylated 5-FU. The 5-FU moiety contained various substituents at the N^3-position were linked to the 2-position of trimethyl emodin via a methylene linkage. Their cytotoxicity against three cancer cell lines and one noncancerous cell were studied. The results revealed that some of conjugates exhibited better or comparable in vitro antitumor activity to 5-FU and emodin and low toxicity in the normal cell. The structure–activity relationship study showed N^3-aromatic substituent was important for their cytotoxic activity.

1. Introduction

Despite the fact that some tumors are sensitive enough to be cured by single drugs, the single drug therapy has fallen short in treating cancers due to serious toxic side effects and poor selectivity. On the other hand, the development of resistance is a common problem of cancer chemotherapy, especially when using a single drug. Nowadays effective cancer chemotherapy often relies instead on combination therapies [1]. The simultaneous use of two or more active drugs is common today for the treatment of cancer. If a combination is synergistic, lower doses can be used to achieve the same or better efficacy with lower toxicity [2]. In addition to producing better therapies, chemical combinations also may be used to study disease and reveal insight into the cellular pathways at their root. Many combinations, which were named mutual drug, in clinical use consist of an antimetabolite with one other antineoplastic agent. The two agents may be combined directly or by means of a linker [3].

5-Fluorouracil (5-FU) is an antimetabolite of the pyrimidine analog type, which has been widely used in the treatment of solid tumors [4]. Although 5-FU has had clinical success as a single agent, it has been modified by different ways to synthesize its derivatives which may improve its therapeutic index [5–8] because of its well-known side effects such as short half-life, wide distribution, low selectivity, and various toxic side effects. Recently chemists have paid more attention to the conjugates of 5-FU with a wide spectrum of low- or high-molecular-weight carriers. Some conjugates of 5-FU have been reported as an approach to develop effective anticancer nucleoside analogs such as 5-FU-podophyllotoxin [9], 5-FU-cyclotriphosphazene [10], 5-FU-cholic acid [11], 5-FU-amino acid ester [12], and 5-FU-pectin [13], *etc* (Fig. 1).

Emodin is a naturally occurring anthraquinone present in the rhubarb as well as the roots and bark of numerous plants of the genus *Rhamnus* [14]. Structurally it belongs to the anthraquinone group along with daunorubicin and mitoxantrone, which have been in clinical use over 30 years for the treatment of cancers [15]. Attempts to use emodin for the treatment of cancer were mostly unsuccessful due to the severe side effect, which the treatment kills healthy cells as well as cancerous ones, hence emodin has been used as a lead compound for the design and development of anticancer agents. Many emodin derivatives, in which most modifications were focused on the methyl group and hydroxyl group, have been synthesized in order to improve efficacy and decrease the adverse effect potential (Fig. 2) [16–20].

We recently reported a hydroxymethylation of 6,8-*O*-dimethyl emodin, which introduced a hydroxymethyl group into the 2-position of emodin [21]. We therefore considered whether we

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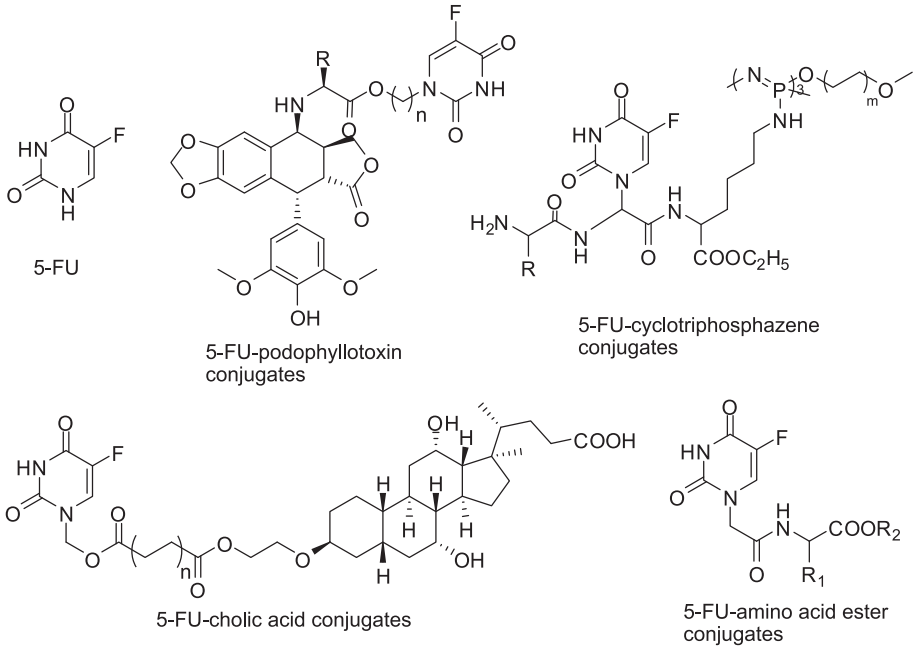


Fig. 1. Structures of 5-FU and its conjugates.

might be able to connect emodin with 5-FU by a methylene linkage at the 2-position to form more effective and less toxic new antitumor compounds. A notable example of this kind was the mutually beneficial modification of the ampicillin and sulbactam to give sultamicillin [22]. Survey of literature revealed that till date enough effort had not been made to connect these moieties as a single molecular scaffold and identify new candidates that may be value in designing new antitumor agents. So in this study, we designed and synthesized a series of new compounds linking emodin with 5-FU through a methylene to screen their antitumor activity against different cancer cell lines and noncancerous cells in vitro in order to get less toxic and selective agents.

2. Results and discussion

2.1. Chemistry

The synthesis of target conjugates is illustrated in Scheme 1. 1-Hydroxy-2-(hydroxymethyl)-6,8-dimethoxy-3-methylanthracene-9,10-dione **3** was prepared using emodin **1** as starting material via methylation and modified Marschalk reaction according to the procedure reported by our group previously [21]. The synthesis of intermediate **4** involved the methylation of **3** with dimethyl sulfate in acetone in the presence of anhydrous K_2CO_3. This reaction yielded **4** in good yield after chromatographic purification. Chlorination of **4** with thionyl chloride in CH_2Cl_2 at room temperature led to the intermediate **5**. The crude product of the reaction was used in the next step without further purification. Subsequently, alkylation of 5-FU with intermediate **5** in the presence of K_2CO_3 and KI gave N^1-substituted 5-FU **6** in good yield. Generally the nucleophilicity of N^3 of 5-FU is higher than that of N^1 under the basic condition due to the higher acidity of the N^1–H proton (N^1–H pKa 7.95 vs N^3–H pKa 12.4) [23]. Because of the two carbonyls at the 2- and 6-position of 5-FU, however, the 3-position of 5-FU becomes difficult to accommodate the bulky group such as the intermediate **5**. Thus the alkylation occurs regioselectively at the 1-position. Baker et al. observed that the orientation of alkylation in the uracil ring also depended on the nature of the alkyl halide, that is, allylic

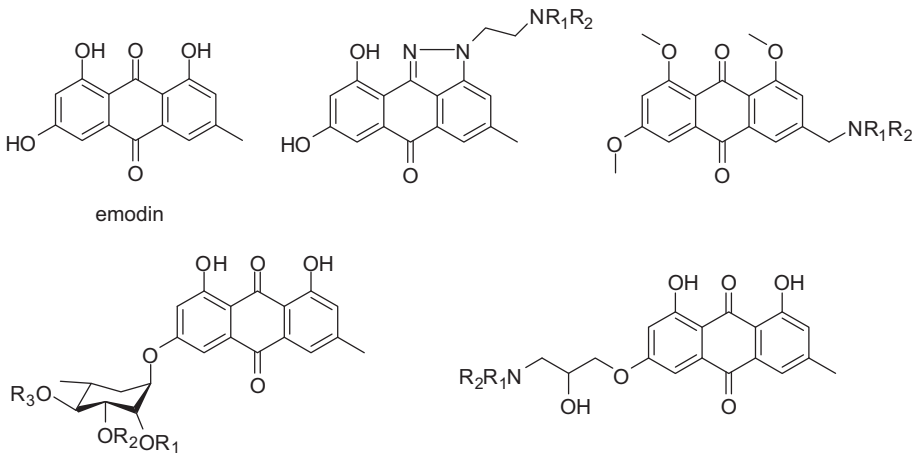
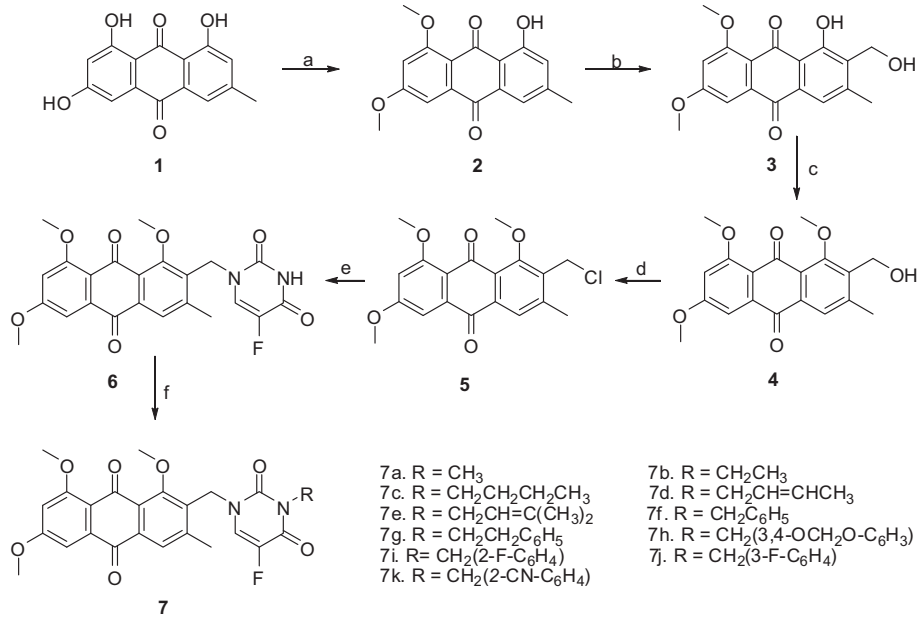


Fig. 2. Structures of emodin and its derivatives.



Scheme 1. Reagents and conditions: (a) Me_2SO_4, K_2CO_3, acetone, reflux, 5 h; (b) $Na_2S_2O_4$, CH_2O (37%), MeOH, NaOH (1 N), 0 °C, 30 min, subsequent H_2O_2 oxidation; (c) Me_2SO_4, K_2CO_3, acetone, reflux, 16 h; (d) $SOCl_2$, CH_2Cl_2, r.t., 30 min; (e) 5-FU/K_2CO_3, KI, DMSO, r.t., 5 h; (f) alkyl halide or benzyl halide, K_2CO_3, KI, DMSO, r.t., overnight.

halides orienting to the 1-position and alkyl halides to the 3-position [24]. The regiochemical outcome observed here is consistent with that reported by Baker and coworkers. A final alkylation of intermediate **6** and the appropriately alkyl halides or benzyl halides yielded the target conjugates **7a**–**7k**. This alkylation was successfully carried out by using the same method for the synthesis of intermediate **6**. It should be noted that the alkylation of N^3 of 5-FU by using chlorocyclohexane under the aforementioned conditions was unsuccessful, possibly due to the greater steric bulk of chlorocyclohexane.

2.2. Biological activities

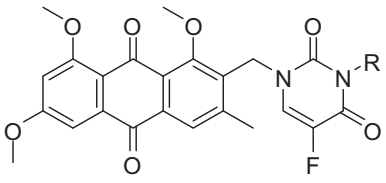
The in vitro cytotoxicty of the target conjugates **6**, **7a**–**7k** were evaluated over tumor cell lines HO-8910 (human ovarian cancer cells), SGC-7901 (human gastric cancer cells), and HepG2 (human liver cancer cells) by the Alamar Blue assay [25], in comparison with emodin and 5-FU. To assess the selectivity of the tested compounds, cytotoxicity of some conjugates was further evaluated against one normal cell line: 293 (human embryonic kidney cells) (Table 1).

As shown in Table 1, some of the conjugates exhibited better antitumor activity than the positive controls. For instance, conjugate **7g** was much potent than lead compound emodin and 5-FU against HO-8910 cells. Conjugates **7f** and **7k** were much active than emodin and 5-FU against SGC-7901 cells. Conjugate **7k** also enhanced the inhibitory effect on HepG2 cells in comparison with 5-FU. The combination result suggest that it should be possible that emodin derivatives can be combined with known anticancer drug 5-FU of low concentration to treat cancer more effectively by lowering the toxic side effects of 5-FU that comes from the usage of high concentration.

When these compounds were screened against SGC-7901 cells, conjugates **7k** and **7f** were found to be approximately 3 and 1.5-fold more active with the IC_{50} values of 21.4 and 39.5 μM, respectively, than emodin (IC_{50} = 61.3 μM). Conjugate **7i** displayed essentially the same value (IC_{50} = 61.0 μM) with the lead compound emodin. All the three compounds were more active than 5-FU (IC_{50} = 76.0 μM). The conjugates were then screened against HO-8910 cells. While compound **7g** exhibited significant cytotoxicity (IC_{50} = 11.6 μM) in comparison with emodin (IC_{50} = 16.0 μM) and 5-FU (IC_{50} = 85.5 μM), compounds **7i**, **7f**, and **7k** showed moderate to good inhibitory effect (IC_{50} = 91.1 μM, 45.1 μM, 22.6 μM respectively). Similarly, compounds **7h**, **7g**, and **7k** displayed moderate to good cytotoxicity against HepG2 cell line, which was better or comparative to 5-FU.

There was a significant difference in term of their cytotoxicity affected by the type of N^3-substituent of 5-FU (aliphatic or

Table 1
Cytotoxicity of conjugates of emodin derivatives and 5-FU against three tumor cell lines and one non-tumor cell line.



Compd.	R	IC_{50} (μM)[a]			
		HO-8910	SGC-7901	HepG2	293
6	H	>100	>100	>100	>100
7a	methyl	>100	>100	>100	N. D.[b]
7b	ethyl	>100	>100	>100	N. D.
7c	butyl	>100	>100	>100	N. D.
7d	crotyl	>100	>100	>100	N. D.
7e	prenyl	>100	>100	>100	N. D.
7f	benzyl	45.1	39.5	>100	>100
7g	phenylethyl	11.6	>100	68.6	4.6
7h	piperonyl	>100	>100	91.0	30.0
7i	2-fluorobenzyl	91.1	61.0	>100	66.6
7j	3-fluorobenzyl	>100	>100	>100	>100
7k	2-cyanobenzyl	22.6	21.4	39.9	29.6
5-FU		85.5	76.0	76.7	42.9
emodin		16.0	61.3	13.1	1.3

[a] IC_{50} values were calculated from three independent experiments.
[b] N. D., not determined.

aromatic). In general, compounds having an aromatic substituent were more potent than the corresponding compounds bearing an aliphatic substituent. Compounds **6**, **7a**–**7e**, which contain only the aliphatic groups at N^3, showed no cytotoxicity against all three cancer lines. When the substituent was replaced with aromatic groups, the resulting compounds, **7f**–**7i**, **7k**, demonstrated improvement in cytotoxicity. Furthermore, it seemed that the increase in the distance between the phenyl ring and uracil fragment led to an increase in antitumor activity, especially for HO-8910 cell line. For instance, compound **7g** with the insertion of an ethyl group displayed better inhibitory activity against HO-8910 when compared to compounds **7f**, **7h**–**7k**.

Antitumor drugs may be considered potentially more useful when they are more active to tumor cell and lower cytotoxicity to non-tumor cell. Therefore, six compounds: **7f**–**7k** showing good activity in Alamar Blue assay together with compound **6** were selected for further evaluation to test their cytotoxicty on normal cell line. It could be seen from the Table 1 that some of tested compounds exhibited less toxic than positive controls. The most notable compound of the conjugates resulted compound **7f**, which showed a good activity against HO-8910, even more potency against SGC-7901 (IC_{50} = 39.5 μM) than emodin (IC_{50} = 61.3 μM) and 5-FU (IC_{50} = 76.0 μM), but no cytotoxicity against the non-tumor cell 293. Similarly, compound **7i** exhibited comparable antitumor activity against HO-8910 and SGC-7901 to 5-FU, but about 1.5 times less potent than 5-FU on 293. Compound **7k** is about 3 times potent than emodin against SGC-7901 (IC_{50} = 21.4 μM vs IC_{50} = 61.3 μM), but about 23 times less potent than emodin on 293 (IC_{50} = 29.6 μM vs IC_{50} = 1.3 μM). Although emodin showed better antitumor activity against HepG2 and HO-8910 than compound **7k**, no selectivity was reflected between cancer cells tested and the normal cell (IC_{50} = 1.3 μM). These results indicated that emodin derivatives with a methylene-linked nucleosides at 2-position enhanced the selectivity in comparison with the positive control, in spite of the decreased cytotoxic activity of some compounds against cancerous cells with varying degrees. It is noteworthy that although the antitumor activity was improved by increasing the distance between the phenyl ring and the uracil fragment, the toxicity was increased accordingly as seen from the IC_{50} value on 293 of compound **7g** (IC_{50} = 4.6 μM).

3. Conclusions

In summary, we have designed and synthesized twelve conjugates of 5-FU and emodin, and evaluated their antitumor activities against three cancer cell lines and one normal cell. Biological evaluation revealed that some of conjugates exhibited better or comparable in vitro antitumor activity to positive controls with low toxicity in the normal cell. For instance, the conjugate **7f** showed selective cytostatic activity in HO-8910 and SGC-7901 cell lines and no cytotoxicity to human normal embryonic kidney cell 293. Compounds **7k** were shown to have a broad spectrum of antitumor activity against the tested three tumor cell lines and much lower toxic activity toward normal cell compared to emodin. The structure–activity relationship study showed N^3-aromatic substituent was important for their cytotoxic activity.

4. Experimental

4.1. Chemistry

Regents and solvents were purchased commercially and used without further purified, unless otherwise stated. Reactions were monitored using thin-layer chromatography (TLC) on silica gel plates. Flash column chromatography was performed on silica gel (200–300 mesh) for purification of the compounds. Melting points were measured on an X-4 melting-point apparatus and were uncorrected. The ^1H NMR and ^{13}C NMR spectra were recorded on Bruker Avance spectrometer at 400 MHz and 100 MHz, respectively. Chemical shift values were reported as δ ppm relative to TMS as internal standard. HRMS were recorded on a Bruker TOF-QII spectrometer with electrospray ionization (ESI).

4.1.1. 1-Hydroxy-2-(hydroxymethyl)-6,8-dimethoxy-3-methylanthracene-9,10-dione (*3*)

1-Hydroxy-2-(hydroxymethyl)-6,8-dimethoxy-3-methylanthracene-9,10-dione **3** was synthesized as recently described procedure [21].

4.1.2. 2-(Hydroxymethyl)-1,6,8-trimethoxy-3-methylanthracene-9,10-dione (*4*)

To a solution of **3** (656 mg, 2.0 mmol) in acetone (50 mL) was added anhydrous K_2CO_3 (1.38 g, 10 mmol) and dimethyl sulfate (0.38 mL, 4.0 mmol). The mixture was refluxed for 16 h, then evaporated the solvent, and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried, and concentrated. The residue was purified by flash column chromatography to afford **4** (554 mg, 81%) as yellow solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H), 7.34 (d, J = 2.0 Hz, 1H), 6.78 (d, J = 2.0 Hz, 1H), 4.82 (s, 2 H), 4.02 (s, 3 H), 3.99 (s, 3 H), 3.97 (s, 3 H), 2.53 (s, 3 H).

4.1.3. 2-(Chloromethyl)-1,6,8-trimethoxy-3-methylanthracene-9,10-dione (*5*)

A solution of **4** (547 mg, 1.6 mmol) in dry CH_2Cl_2 (10 mL) was treated with thionyl chloride (0.4 mL, 5.6 mmol) and stirred for 30 min at room temperature. The solution was evaporated in vacuo and the residue was used in the next step without further purification.

4.1.4. 5-Fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)-methyl)pyrimidine-2,4(1H,3H)-dione (*6*)

A stirred mixture of 5-FU (156 mg, 1.2 mmol) and anhydrous K_2CO_3 (166 mg, 1.2 mmol) in DMSO (14 mL) was heated to 80 °C. After stirring for 30 min, the mixture was cooled to room temperature. To the solution was added KI (50 mg, 0.3 mmol) and **5** (108 mg, 0.3 mmol). The mixture was stirred for 5 h at room temperature, then poured into water, and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried, and concentrated. The residue was purified by flash column chromatography to afford **6** (110 mg, 80.8%) as yellow solid. mp: 165–166 °C; ^1H NMR (400 MHz, DMSO-d_6) δ 11.82 (d, J = 5.2 Hz, 1H), 7.94 (d, J = 6.8 Hz, 1H), 7.73 (s, 1H), 7.17 (d, J = 2.4 Hz, 1H), 6.99 (d, J = 2.4 Hz, 1H), 4.90 (s, 2 H), 3.94 (s, 3 H), 3.91 (s, 3 H), 3.79 (s, 3 H), 2.54 (s, 3 H).

4.1.5. General procedure for the preparation of conjugates *7a*–*7k*

A stirred mixture of **6** (136 mg, 0.3 mmol) and anhydrous K_2CO_3 (124 mg, 0.9 mmol) in DMSO (10 mL) was heated to 80 °C. After stirring for 30 min, the mixture was cooled to room temperature. To the solution was added KI (50 mg, 0.3 mmol) and alkyl halides or benzyl halides. The mixture was stirred overnight at room temperature, then poured into water, and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried, and concentrated. The residue was purified by flash column chromatography to afford **7a**–**7k** as yellow solid.

*4.1.5.1. 5-Fluoro-3-methyl-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7a**).* Yield 64.7%; mp: 256–257 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 7.34 (d, J = 2.4 Hz, 1H), 7.32 (d, J = 5.6 Hz, 1H), 6.80

(d, J = 2.4 Hz, 1H), 5.09 (s, 2H), 3.99 (s, 6H), 3.96 (s, 3H), 3.38 (s, 3H), 2.55 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 159.7, 156.9 (d, J = 29.1 Hz), 150.3, 145.3, 140.0 (d, J = 233.3 Hz), 136.3, 134.6, 133.8, 126.0 (d, J = 33.2 Hz), 125.6, 124.9, 117.8, 105.4, 102.4, 63.1, 56.6, 56.0, 43.7, 28.6, 20.5; HRMS (ESI): 491.1231 for $[M + Na]^+$ (calcd 491.1232 for $C_{24}H_{21}FN_2O_7Na$).

*4.1.5.2. 3-Ethyl-5-fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7b**).* Yield 63.5%; mp: 257–258 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.35 (d, J = 2.4 Hz, 1H,), 7.29 (d, J = 5.6 Hz, 1H), 6.81 (d, J = 2.4 Hz, 1H), 5.09 (s, 2H), 4.04 (q, J = 7.2 Hz, 2H), 4.00 (s, 3H), 3.99 (s, 3H), 3.98 (s, 3H), 2.54 (s, 3H), 1.24 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 159.6, 156.8 (d, J = 25.1 Hz), 149.9, 145.3, 140.2 (d, J = 233.6 Hz), 136.3, 134.6, 134.0, 125.9 (d, J = 33.0 Hz), 125.6, 124.9, 117.8, 105.4, 102.4, 63.1, 56.6, 56.0, 43.4, 37.4, 20.4, 12.7; HRMS (ESI): 505.1392 for $[M + Na]^+$ (calcd 505.1388 for $C_{25}H_{33}FN_2O_7Na$).

*4.1.5.3. 3-Butyl-5-fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7c**).* Yield 84.7%; mp: 214–215 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.35 (d, J = 2.4 Hz, 1H), 7.28 (d, J = 5.6 Hz, 1H), 6.81 (d, J = 2.4 Hz, 1H), 5.10 (s, 2H), 4.00 (s, 6H), 3.98 (s, 3H), 3.97 (t, J = 8.0 Hz, 2H), 2.53 (s, 3H), 1.65–1.57 (m, 2H), 1.42–1.33 (m, 2H), 0.94 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 159.6, 156.9 (d, J = 24.9 Hz), 150.1, 145.3, 140.1 (d, J = 233.5 Hz), 138.9, 136.3, 134.1, 125.9 (d, J = 33.1 Hz), 125.6, 124.9, 117.8, 105.3, 102.4, 63.1, 56.6, 55.9, 43.3, 42.0, 29.5, 20.4, 20.1, 13.7; HRMS (ESI): 533.1710 for $[M + Na]^+$ (calcd 533.1701 for $C_{27}H_{27}FN_2O_7Na$).

*4.1.5.4. (E)-3-(But-2-enyl)-5-fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7d**).* Yield 70.4%; mp: 215–216 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 7.34 (d, J = 2.4 Hz, 1H), 7.26 (d, J = 5.6 Hz, 1H), 6.80 (d, J = 2.4 Hz, 1H), 5.85–5.76 (m, 1H), 5.58–5.50 (m, 1H), 5.10 (s, 2H), 4.50 (d, J = 6.4 Hz, 2H), 3.99 (s, 6H), 3.97 (s, 3H), 2.52 (s, 3H), 1.67 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 159.6, 156.7 (d, J = 24.9 Hz), 149.9, 145.3, 140.2 (d, J = 234.7 Hz), 136.3, 134.6, 134.0, 131.0, 125.9 (d, J = 33.1 Hz), 125.6, 125.0, 123.7, 117.8, 105.4, 102.4, 63.1, 56.6, 56.0, 43.5, 43.2, 20.5, 17.7; HRMS (ESI): 531.1582 for $[M + Na]^+$ (calcd 531.1545 for $C_{27}H_{25}FN_2O_7Na$).

*4.1.5.5. 5-Fluoro-3-(3-methylbut-2-enyl)-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7e**).* Yield 80.5%; mp: 260–261 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 7.34 (d, J = 2.4 Hz, 1H), 7.25 (d, J = 5.6 Hz, 1H), 6.80 (d, J = 2.0 Hz, 1H), 5.23 (t, J = 6.8 Hz, 1H), 5.10 (s, 2H), 4.56 (d, J = 7.2 Hz, 2H), 3.99 (s, 3H), 3.97 (s, 6H), 2.52 (s, 3H), 1.82 (s, 3H), 1.71 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 159.6, 156.8 (d, J = 24.9 Hz), 150.0, 145.4, 140.2 (d, J = 233.6 Hz), 137.9, 136.3, 134.6, 134.1, 125.7 (d, J = 33.1 Hz), 125.6, 124.9, 117.8, 117.7, 105.4, 102.4, 63.1, 56.6, 56.0, 43.2, 40.3, 25.7, 20.5, 18.1; HRMS (ESI): 545.1725 for $[M + Na]^+$ (calcd 545.1701 for $C_{28}H_{27}FN_2O_7Na$).

*4.1.5.6. 3-Benzyl-5-fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7f**).* Yield 74.2%; mp: 212–213 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.47 (d, J = 6.8 Hz, 2H), 7.33–7.26 (m, 5H), 6.80 (d, J = 2.4 Hz, 1H), 5.15 (s, 2H), 5.09 (s, 2H), 3.99 (s, 3H), 3.97 (s, 6H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.2, 180.9, 164.3, 162.0, 159.5, 157.0 (d, J = 25.1 Hz), 150.1, 145.3, 140.1 (d, J = 233.9 Hz), 136.3, 136.1, 134.6, 134.0, 129.1, 128.5, 127.9, 126.0 (d, J = 33.0 Hz), 125.6, 124.9, 117.7, 105.3, 102.4, 63.1, 56.6, 56.0, 45.2, 43.2, 20.5; HRMS (ESI): 567.1573 for $[M + Na]^+$ (calcd 567.1545 for $C_{30}H_{25}FN_2O_7Na$).

*4.1.5.7. 5-Fluoro-3-phenethyl-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7g**).* Yield 53.2%; mp: 199–200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.33 (d, J = 2.0 Hz, 1H), 7.30 (d, J = 5.6 Hz, 1H), 7.27 (d, J = 2.0 Hz, 4H), 7.23–7.17 (m, 1H), 6.80 (d, J = 2.0 Hz, 1H), 5.07 (s, 2H), 4.20 (t, J = 8.0 Hz, 2H), 3.99 (s, 6H), 3.97 (s, 3H), 2.93 (t, J = 8.0 Hz, 2H), 2.48 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 180.0, 164.3, 162.0, 159.6, 156.8 (d, J = 26.3 Hz), 150.0, 145.4, 140.0 (d, J = 233.8 Hz), 138.0, 136.3, 134.6, 133.9, 129.0, 128.5, 126.6, 126.0 (d, J = 32.9 Hz), 125.6, 124.9, 117.8, 105.4, 102.4, 63.1, 56.6, 56.0, 43.3, 33.5, 29.3, 20.5; HRMS (ESI): 581.1729 for $[M + Na]^+$ (calcd 581.1701 for $C_{31}H_{27}FN_2O_7Na$).

*4.1.5.8. 3-(Benzo[d][1,3]dioxol-5-ylmethyl)-5-fluoro-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7h**).* Yield 83.4%; mp: 189–190 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.35 (d, J = 2.4 Hz, 1H), 7.26 (d, J = 5.6 Hz, 1H), 7.01 (s, 1H), 7.00 (d, J = 8.4 Hz, 1H), 6.81 (d, J = 2.4 Hz, 1H), 6.73 (d, J = 8.0 Hz, 1H), 5.92 (s, 2H), 5.09 (s, 2H), 5.05 (s, 2H), 3.99 (s, 3H), 3.97 (s, 6H), 2.49 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.1, 164.3, 162.0, 159.6, 157.0 (d, J = 25.2 Hz), 150.1, 147.6, 147.2, 145.4, 140.1 (d, J = 234.0 Hz), 136.3, 134.6, 134.0, 129.8, 126.1 (d, J = 33.1 Hz), 125.6, 125.0, 123.1, 117.7, 109.9, 108.1, 105.3, 102.4, 101.0, 63.2, 56.6, 56.0, 45.0, 43.2, 20.5; HRMS (ESI): 611.1476 for $[M + Na]^+$ (calcd 611.1443 for $C_{31}H_{25}FN_2O_9Na$).

*4.1.5.9. 5-Fluoro-3-(2-fluorobenzyl)-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7i**).* Yield 33.2%; mp: 233–234 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.33 (d, J = 2.4 Hz, 1H), 7.33 (d, J = 5.2 Hz, 1H), 7.25–7.21 (m, 2H), 7.08–7.02 (m, 2H), 6.80 (d, J = 2.4 Hz, 1H), 5.24 (s, 2H), 5.11 (s, 2H), 3.99 (s, 3H), 3.98 (s, 3H), 3.97 (s, 3H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.0, 160.8 (d, J = 249.9 Hz), 159.6, 156.9 (d, J = 26.4 Hz), 149.9, 145.4, 140.1 (d, J = 234.4 Hz), 136.3, 134.6, 134.0, 129.4 (d, J = 3.9 Hz), 129.3 (d, J = 9.3 Hz), 126.2 (d, J = 32.9 Hz), 125.6, 125.0, 124.1 (d, J = 3.7 Hz), 122.9 (d, J = 14.3 Hz), 117.8, 115.5 (d, J = 21.4 Hz), 105.4, 102.4, 63.2, 56.6, 56.0, 43.1, 39.2, 20.4; HRMS (ESI): 585.1485 for $[M + Na]^+$ (calcd 585.1451 for $C_{30}H_{24}F_2N_2O_7Na$).

*4.1.5.10. 5-Fluoro-3-(3-fluorobenzyl)-1-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione (**7j**).* Yield 40.1%; mp: 229–230 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.33 (d, J = 2.4 Hz, 1H), 7.31 (d, J = 5.6 Hz, 1H), 7.30–7.23 (m, 2H), 7.19–7.16 (m, 1H), 7.00–6.94 (m, 1H), 6.80 (d, J = 2.4 Hz, 1H), 5.13 (s, 2H), 5.10 (s, 2H), 3.99 (s, 3H), 3.98 (s, 3H), 3.97 (s, 3H), 2.49 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 181.0, 164.3, 162.7 (d, J = 244.8 Hz), 162.0, 159.6, 156.9 (d, J = 25.3 Hz), 150.1, 145.3, 140.1 (d, J = 234.3 Hz), 138.4 (d, J = 7.4 Hz), 136.3, 134.7, 133.8, 130.0 (d, J = 8.1 Hz), 126.2 (d, J = 33.1 Hz), 125.6, 125.0, 124.7 (d, J = 2.8 Hz), 117.8, 116.0 (d, J = 21.7 Hz), 114.9 (d, J = 20.8 Hz), 105.3, 102.4, 63.2, 56.6, 56.0, 44.6, 43.2, 20.5; HRMS (ESI): 585.1473 for $[M + Na]^+$ (calcd 585.1451 for $C_{30}H_{24}F_2N_2O_7Na$).

*4.1.5.11. 2-((5-Fluoro-2,6-dioxo-3-((1,6,8-trimethoxy-3-methyl-9,10-dioxo-9,10-dihydroan thracen-2-yl)methyl)-2,3-dihydropyrimidin-1(6H)-yl)methyl)benzonitrile (**7k**).* Yield 71.7%; mp: 228–229 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.53 (t, J = 7.6 Hz, 1H), 7.37 (d, J = 5.6 Hz, 1H), 7.37 (t, J = 7.6 Hz, 1H), 7.34 (d, J = 2.4 Hz, 1H), 7.26 (d, J = 7.6 Hz, 1H), 6.81 (d, J = 2.4 Hz, 1H), 5.39 (s,

2H), 5.11 (s, 2H), 4.00 (s, 3H), 3.98 (s, 3H), 3.97 (s, 3H), 2.48 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.3, 180.9, 164.4, 162.0, 159.6, 156.9 (d, J = 25.7 Hz), 150.0, 145.3, 139.9 (d, J = 234.8 Hz), 139.5, 136.3, 134.7, 133.7, 133.1, 133.0, 128.0, 127.3, 126.6 (d, J = 33.0 Hz), 125.6, 125.0, 117.8, 117.2, 112.1, 105.4, 102.4, 63.2, 56.6, 56.0, 43.4, 43.4, 20.5; HRMS (ESI): 592.1524 for $[M + Na]^+$ (calcd 592.1497 for $C_{31}H_{24}FN_3O_7Na$).

4.2. Cell culture and cytotoxicity assays

The HO-8910, SGC-7901, HepG2 and 293 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin and incubated under humidified air with 5% CO_2 at 37 °C.

The in vitro cytotoxicity of the target conjugates was determined by the Alamar Blue analysis with minor modifications. Briefly, Cells with same number were inoculated into each well in 96-well plates (Costar, Charlotte, NC) with 100 μL culture medium. After an overnight incubation, the novel synthesized conjugates in 100 μL diluted with the medium were added into the wells for determine the cytotoxicity. Then the cells were incubated for another 2 days. Thereafter, 20 μL Alamar blue solution was added to each well after removal of the sample solution and washing with phosphate-buffered saline (PBS, pH 7.4), and cultured for an additional 4h. Each conjugates was tested in triplicate wells. The absorbance was measured using SpectraMax M2 (Molecular Devices) with a test wavelength of 530 nm and 590 nm. Growth inhibition rate was calculated by the following formula:

$$\text{Growth inhibition rate} = [(A_{\text{Control}} - A_{\text{sample}})/(A_{\text{Control}} - A_{\text{blank}})] \times 100\%$$

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