Journal of Materials Chemistry B



View Article Online

PAPER

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Cite this: J. Mater. Chem. B, 2019, 7, 7548

Received 18th September 2019, Accepted 25th October 2019

DOI: 10.1039/c9tb02041a

rsc.li/materials-b

Introduction

Over the past few decades, prodrug design has been developed^{1,2} to overcome many problems of clinical drugs, such as high toxicity, poor selectivity and uncontrolled release. Etoposide is a semisynthetic derivative of podophyllotoxin,³ and one of the first identified topoisomerase II inhibitors, which was widely used in cancer therapy, especially in acute myeloid leukaemia, Hodgkin's or non-Hodgkin's lymphoma, lung cancer, gastric cancer, breast cancer and ovarian cancer. Etoposide can cause cell death by forming a ternary complex with topoisomerase II and DNA.⁴ However, obvious side effects like cardiac, hematological and gastrointestinal toxicity of etoposide have seriously limited its use in clinical therapy.⁵⁻⁷ Over the years, several researchers have tried to overcome this problem. In 2013, Linyong Zhu and coworkers reported a photo-trigger etoposide prodrug.8 And in 2017, Ping Shi and coworkers reported another etoposide prodrug that could be released in the presence of GSH.⁹ However, the in vivo toxicity of their prodrugs was not studied and their activities were mainly verified in vitro. Herein, we

Real-time monitoring of etoposide prodrug activated by hydrogen peroxide with improved safety[†]

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Etoposide is one of the most used first-line chemotherapeutic drugs. However, its application is still limited by its side effects. Herein, we designed a novel H_2O_2 sensitive prodrug **6YT** for selectively releasing the anti-cancer drug etoposide in cancer cells. In this paper, etoposide and a hydrogen peroxide (H_2O_2) sensitive aryl borate ester group were linked by a fluorescent coumarin and finally the prodrug **6YT** was generated. The fluorescence of coumarin was quenched before the connected aryl borate ester group was cleaved by H_2O_2 . However, in the high level H_2O_2 environment of the tumor, the fluorescence could be activated simultaneously with the release of etoposide, and the drug release state of the prodrug was monitored real-time. With the support of **6YT**, we obtained direct and visual evidence of etoposide release in a high H_2O_2 environment both in cells and zebrafish. The prodrug **6YT** was also verified with comparable activity and improved safety with etoposide both in cells and in a mouse model. As a safe and effective prodrug, **6YT** is expected to be one of the promising candidates in chemotherapy against cancer.

designed a novel H2O2-response prodrug to selectively release etoposide in cancer cells. Abundant studies indicated that tumor cells and their microenvironment are different from normal cells in many aspects, including lower pH,^{10,11} overexpressed enzymes,¹²⁻¹⁵ a higher level of glutathione (GSH)^{16,17} and reactive oxygen species (ROS).18,19 Generally, ROS including hydrogen peroxide (H_2O_2) , superoxide anions (O_2^-) and hydroxyl radical (•OH), and H₂O₂ play a major role due to their chemical stability, wide diffusion in and out of cells and tissues and the fact that they can be generated from nearly all sources of oxidative stress.²⁰ Therefore, H₂O₂ plays an important role in ROS dependent prodrugs as the therapeutic target. The aryl boronic acids and their esters can be easily oxidized and cleaved by H₂O₂.²¹ In addition, boronic acids and esters are intrinsically nontoxic, and their metabolite, boric acid, is considered to be nontoxic to humans.²² Depending on the above factors, boronic acids and their esters were widely used in the design of tumor targeted prodrugs²²⁻²⁶ and fluorescent probes.²⁷⁻²⁹ Besides in vitro experiments, we also performed antitumor and safety studies in vivo, which further verified the feasibility of our prodrug design.

There is still a lack of simple and effective means for the prodrugs to detect drug release and distribution directly. Therefore, it is of major significance to create a real-time monitoring prodrug by connecting it with a fluorophore. Herein, we report a novel prodrug to specifically release the antitumor drug etoposide,

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[†] Electronic supplementary information (ESI) available. See DOI: 10.1039/c9tb02041a



 $\mbox{Scheme 1}$ Structure and mechanism of $\mbox{H}_2\mbox{O}_2$ prodrug for etoposide release.

accompanied by *in situ* fluorescence release in the presence of H_2O_2 . In this study, the fluorescence of **6YT** was detected both in living cells and zebrafish, to further verify that the prodrug **6YT** could react with H_2O_2 both *in vitro* and *in vivo*.

In our study, an aryl borate ester was used as the trigger unit in response to H₂O₂, and 7-hydroxy-3-hydroxymethyl-coumarin (7-OH-COU) was used as the fluorophore unit to monitor the release of etoposide as well as imaging (Scheme 1). When substituted with an aryl borate ester group, the fluorescence of A (6YT) is quenched. However, when the aryl borate ester group is cleaved by H_2O_2 , the phenol intermediate **B** is quickly produced, and then a series of electron transfer processes start and B releases a free etoposide and a quinone methide C through a 1,8-elimination mechanism. Subsequently, the quinone methide C is activated with H_2O to form fluorophore D (7-OH-COU), which shows a significant increase in fluorescence. This mechanism has been applied in nitrogen mustard,³⁰⁻³² diazeniumdiolate, 33-35 ciprofloxacin36 and SN-3837 prodrug design. In order to take full advantage of the fluorescence, we further studied the drug release aided by the fluorescence, both in cells and zebrafish. In addition, we evaluated the anticancer efficacy and safety on different cell lines and on a mouse model with human colon cancer HCT-116 cells, to prove the activity and safety of the prodrug 6YT as a chemotherapy drug against cancer.

Results and discussion

Synthetic procedures

Synthesis of compound **6YT** involves three stages (Scheme S1, ESI†). Firstly, fluorophore 3 (7-OH-COU) was synthesized in three steps. 3-Methyl-7-propionate-coumarin was synthesized using 2,4-dihydroxybenzaldehyde and sodium propionate, the methyl in the allyl position was brominated, then the allyl bromide was substituted by sodium acetate. 7-OH-COU was obtained after acidization with 2 N HCl. Second, the H₂O₂-responsive aryl borate ester was connected to the phenolic group of 7-OH-COU. And in the last step, the anti-tumor drug etoposide was linked to it after a chlorination reaction on the hydroxymethyl group. The compound **6YT** was characterized by ¹H, ¹³C NMR and HRMS (Fig. S6–S8, ESI†). All the intermediate compounds were also characterized by ¹H NMR and HRMS (Fig. S1–S5, ESI†).



Fig. 1 (A) UV-absorption and (B) fluorescence emission spectra of compound **6YT** (10 μ M, λ_{ex} = 320 nm, λ_{em} = 458 nm) before and after the addition of H₂O₂ (500 μ M) in PBS buffer after 60 min (pH 7.4, 0.1% DMSO).

Spectral properties of 6YT towards H₂O₂

Firstly, we investigated the spectral properties of compound **6YT**. The UV-vis absorption spectra indicate that compound **6YT** shows a maximum absorption band at about 320 nm both before and after the addition of H_2O_2 (Fig. 1A). Besides, the fluorescence response was detected and is shown in Fig. 1B, from which we can see that compound **6YT** (10 µM) only shows a very weak emission at 458 nm, but after addition of H_2O_2 (500 µM) in PBS buffer, the emission intensity shows a significant increment after 60 min.

In addition, we studied the time-dependent fluorescence spectral change (Fig. 2A and B) of compound **6YT** (10 μ M) after addition of H₂O₂ (500 μ M) in PBS buffer. The result showed that the fluorescence intensity was increased to its maximum at about 100 min with a 32-fold increment, indicating its potential for the rapid detection of H₂O₂ and etoposide release. Moreover, the fluorescence intensities of **6YT** towards H₂O₂ (0–80 eq.) were increased in a dose-dependent manner (Fig. 2C and D). As the H₂O₂ concentration was gradually increased, the fluorescence



Fig. 2 (A) Time-dependent fluorescence spectra of compound **6YT** (10 μ M) upon addition of H₂O₂ (500 μ M) in PBS buffer (pH 7.4, 0.1% DMSO). (B) The fluorescence spectra change of **6YT** (λ_{ex} = 320 nm, λ_{em} = 458 nm) depending on time. (C) Fluorescence spectra changes of compound **6YT** (10 μ M) after treatment with increasing concentrations of H₂O₂ (0–80 eq.) in PBS buffer after 100 min. (D) The linear correlation between the fluorescence intensity of **6YT** and the concentration of H₂O₂ (0–80 eq.) in PBS buffer after 100 min.

intensity increased as well, and the linear correlation between the fluorescence intensity of 6YT (458 nm) and the concentration of H_2O_2 in the range of 0-80 eq. was determined with R^2 of 0.9930. From the results of fluorescence spectra, we find that the aryl borate ester group was easily released by H₂O₂ in a time and dose-dependent manner. Furthermore, we also evaluated the pH dependent response of 6YT toward H2O2 to investigate its practicability for biological imaging (Fig. S9, ESI[†]). There was no obvious change of fluorescence signal at 458 nm above the pH range of 5.0-9.0, indicating the stable structure of the prodrug during acidic to alkaline conditions. And as expected, after addition of H_2O_2 (300 μ M), the emission intensity increase was sensitive to the pH range from 5.0 to 9.0, with a maximum intensity at pH 7.4 and 37 °C. The results indicate that 6YT responded well to H₂O₂ under normal physiological conditions.

Selective response of 6YT towards H₂O₂

To further investigate the fluorescence response specificity of **6YT** towards H_2O_2 , the fluorescence intensity of **6YT** (10 μ M) was detected in the PBS buffer stimulated by other species at regular time intervals (30, 60 and 90 min). As shown by the results (Fig. 3), the addition of other species only resulted in a negligible fluorescence response, and only H2O2 could induce a remarkable fluorescence response, indicating good selectivity of 6YT towards H₂O₂ cleavage.

Drug release ability of 6YT in the presence of H₂O₂

In order to evaluate the ability of the compound 6YT to release free anti-tumor drug etoposide and fluorophore 7-OH-COU upon addition of H₂O₂, reversed-phase HPLC was performed at regular intervals for verification, using methanol/water (50%/ 50%) for the first 6 minutes and methanol/water (90%/10%) for the rest as the mobile phase, with a flow rate of 1 ml min⁻¹. The time-dependent release curve is shown in Fig. S10 (ESI[†]), etoposide can be released from the compound 6YT in the presence of H₂O₂ as determined from HPLC measurements, while no drug release was detected without the addition of H₂O₂.

Fig. 3 Fluorescence response of compound 6YT (10 μ M) after incubated with various reactive analytes (500 $\mu\text{M})$ at 37 $^{\circ}\text{C}$ in PBS (pH 7.4) for 30, 60 and 90 min. PBS: PBS buffer; TBHP: tert-butyl hydroperoxide; OCl-: sodium hypochlorite; TEMPO: 2,2,6,6-tetramethylpiperidinyloxy; H2O2: hydrogen peroxide; •OH: hydroxyl radical; ^tBuO⁻: tert-butoxy radical; ONOO⁻: peroxynitrite; GSH: glutathione; H₂O₂; Cys: cysteine; Hcy: homocysteine; Fe³⁺: FeCl₃; Fe²⁺: FeCl₂.

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402 ON Buo. ONOÓ Jesh in Fig. 4, the retention time of compound 6YT was identified at 9.20 min, and after addition of H₂O₂ (10 mM) for 30 min, the peak of 6YT completely disappeared, and three new peaks concomitantly appeared at 4.53 min (peak1, verified to be 7-OH-COU), 9.22 min (peak2, verified to be etoposide) and 9.67 min (peak3). In contrast to the corresponding peaks after 60 min and 120 min, we found that peak3 reduced gradually whereas peak1 and peak2 increased concomitantly, indicating that peak3 was a phenol intermediate, as shown in Fig. 4. In addition, the peak of quinone methide C (in Scheme 1) was not found, while peak1 (7-OH-COU) increased gradually, indicating that the quinone methide C was transient and easily converted to 7-OH-COU. The evolution of these peaks in the HPLC pattern indicated a two-step mechanism. First, the aryl borate ester group was cleaved by H2O2 and the phenol intermediate was formed. Immediately after that, the spontaneous 1,8-elimination reaction started and led to the release of the anti-tumor drug etoposide and coumarinyl quinone methide, which will rearrange itself to the fluorophore 7-OH-COU spontaneously in an aqueous medium as shown in Scheme 1. The peaks of released 7-OH-COU and etoposide were verified by both retention time and HR-ESI-MS. Cytotoxicity assay

In addition, the time-dependent release curve was highly con-

sistent with the fluorescence curve shown in Fig. 2B, indicating

the synchronization of fluorescence and drug release. As shown

In order to evaluate the cytotoxicity activity of 6YT, the antitumor activities of 6YT and etoposide were assessed in cancer cell lines (A549 and HCT-116) and a normal cell line (MCF-10A), using MTT assays. As shown in Fig. 5, compared with etoposide, 6YT exerted similar cytotoxicity activity against both A549 and HCT-116 cancer cells with IC_{50} values of 8.91 and 4.81 μ M, but has much lower cytotoxicity toward MCF-10A cells (normal cells, $IC_{50} = 60.66 \ \mu M$) than etoposide (3.98 μM), whereas for etoposide, no such selectivity was observed, and the cytotoxicities toward A549, HCT-116 and MCF-10A cell lines were similar with IC_{50} values of 4.53, 2.63 and 3.98 μM (Table 1), respectively,





oci

600

400

200

Intensity (a.u.)

(10 mM). Peaks in the chromatograms were detected by monitoring the absorption at 280 nm



Fig. 5 Cytotoxicity study of etoposide and **GYT** in A549 (A), HCT-116 (B) and MCF-10A (C) cell lines. Values are presented as means \pm standard deviations of three independent observations.

Table 1 Cytotoxicity activity in different cell lines

Compounds	IC_{50} (μ M)		
	A549	HCT-116	MCF-10A
6YT Etoposide 7-OH-COU Compound 4	$\begin{array}{c} 8.91 \pm 0.89 \\ 4.53 \pm 0.58 \\ > 80 \\ > 80 \end{array}$	$\begin{array}{c} 4.81 \pm 0.63 \\ 2.63 \pm 0.35 \\ > 80 \\ > 80 \end{array}$	$60.66 \pm 1.70 \\ 3.98 \pm 0.43 \\ > 80 \\ > 80$

Cytotoxicity activity in different cell lines were determined by the MTT assay, $IC_{50}\pm SD\,(\mu M)$, all experiments were independently performed at least three times.

suggesting that **6YT** was selectively activated in cancer cells, which have comparatively higher ROS levels than normal cells.

To further investigate if the etoposide was the main source of cytotoxicity, we also tested the cytotoxicity of 7-OH-COU and compound 4 (structure shown in Scheme S1, which was not connected to etoposide, ESI†), the IC_{50} of which were both beyond 80 μ M in A549, HCT-116 and MCF-10A cell lines (Table 1), indicating that etoposide was the only source of cytotoxicity.

ROS level evaluation of different cell lines

To verify that the differences in the cytotoxicity of compound **6YT** in different cells result from the ROS difference among different cell lines, we measured the ROS of different cells by using the ROS sensitive dye, 2',7'-dichloro-fluorescein diacetate (DCFH-DA) and then detected the levels by flow cytometry using a BD Accuri C6 flow cytometer. The ROS levels of HCT-116 and A549 were obviously higher than that in the normal cell line MCF-10A (see Fig. 6), which was consistent with the cytotoxicity results, indicating that the high-level ROS in tumor cells triggered the release of etoposide to kill the cells.

Fluorescence imaging of 6YT in cells

Further, we applied compound **6YT** (10 μ M) to assess the applicability of a prodrug in increasing the concentration of





exogenous H_2O_2 (0 μ M, 100 μ M and 200 μ M) in A549 cells using ImageXpress Micro Confocal analysis. As shown in Fig. S11 (ESI[†]), the fluorescence intensity increased with increasing concentration of H₂O₂ indicating that the prodrug 6YT shows good response to exogenous H2O2. Next, we investigated the response of the prodrug 6YT to endogenous H₂O₂ at different time intervals (0 h, 2 h, 4 h, 8 h and 12 h) in A549 and HCT-116 cell lines. As shown in Fig. S12 and S13 (ESI⁺), the fluorescence signal increased in a good time-dependent manner. Cancer cells have a higher level of ROS compared with normal cells and H_2O_2 is an important component of ROS. To further investigate the different ROS content between cancer cells and normal cells, MCF-10A (normal cell line) cells were also incubated with the prodrug 6YT at different time intervals (0 h, 2 h, 4 h, 8 h and 12 h) (Fig. S14, ESI⁺). Compared with MCF-10A cells, the fluorescence signal intensity in A549 and HCT-116 cells was much stronger (Fig. 7), indicating that the compound 6YT in cancer cells was more active than that in normal cells, which is consistent with the results of cytotoxicity studies in cancer and normal cells. In addition, we also tested the fluorescence response at different concentrations of 6YT after incubation in A549 cell lines for 12 h. The results are shown in Fig. S15 (ESI[†])



Fig. 7 (A) Fluorescence images of (a) MCF-10A, (b) HCT-116 and (c) A549 cell lines incubated with compound **6YT** (10 μ M) for 12 h. Scale bar = 20 μ m. (B) The relative fluorescence intensities of (a–c) were measured at three regions in each dish. Error bars represent standard deviation (n = 3).



Fig. 8 Subcellular localization of **6YT** in A549 cells. Cells were treated with 10 μ M **6YT** for 12 h. Propidium iodide (PI) was used to identify the nucleus. Scale bar: 20 μ m.

and indicate that the fluorescence signal was increased in a dosedependent manner.

Intracellular localization of compound 6YT in cells

In order to study the intracellular localization of compound **6YT**, cell imaging experiments were tested in A549 cells utilizing the ImageXpress Micro Confocal analysis system. Cells were incubated with 10 μ M **6YT** for 12 h, and subsequently, 1 μ M propidium iodide (PI) for 30 min. A significant fluorescence signal could be observed, which overlapped with the fluorescence signal of PI (fluorescence marker for the nucleus) (Fig. 8). The results suggest that **6YT** was preferentially activated in the nucleus. This result could be explained by the characteristics of etoposide since the target of etoposide, topoisomerase II, is in the nucleus; the **6YT** or its phenol intermediate was brought into and aggregated in the nucleus, then the fluorophore 7-OH-COU and anti-tumor drug etoposide were continuously released into the nucleus.

In vivo imaging of 6YT in zebrafish

Next, we explored the potential of **6YT** for *in vivo* imaging (Fig. 9) in zebrafish. Zebrafish have many advantages as a fluorescence model animal, such as the 87% homology with the human genome and transparent embryos, which enables observation of the fluorescence in real time. The zebrafish were pretreated with H_2O_2 and incubated for 30 min, then treated with **6YT** for another 30 min, and a significant fluorescence was observed. By contrast, the blank zebrafish exhibited almost no fluorescence, and the zebrafish treated with only probe **6YT** exhibited only a weak fluorescence. These results are consistent with the observations in living cells, which further confirmed that the probe **6YT** could be exploited to be a promising tool for monitoring drug release in living systems. Images were taken using the DAPI channel.



Fig. 9 Confocal fluorescence images of zebrafish (a–c) incubated with PBS for 30 min; (d–f) incubated with **6YT** (10 μ M) for 30 min; (g–i) pretreated with H₂O₂ (300 μ M) for 30 min, subsequently treated with **6YT** (10 μ M) for 30 min. Scale bar: 500 μ m.



Fig. 10 Confocal fluorescence images of zebrafish incubated with H_2O_2 (300 μ M) for 0 (a–c), 10 (d–f), 20 (g–i) and 30 min (j–l), and subsequently treated with **6YT** (10 μ M) for 30 min. Scale bar: 500 μ m.

In addition, *in vivo* real-time monitoring of fluorescence was also performed in zebrafish (Fig. 10). The zebrafish were pretreated with H_2O_2 and incubated for 0, 10, 20 and 30 min, then treated with **6YT** for another 30 min. A gradually increased fluorescence response was observed in zebrafish in a time dependent manner. The results demonstrate that our prodrug **6YT** could respond to H_2O_2 in zebrafish, and could be utilized for real-time detection of the release of the drug in zebrafish.

In vivo tumor studies

We further evaluated the *in vivo* effect against HCT-116 xenografts in the BALB/c nude mice model. Etoposide was used as a comparison control. Human colon cancer HCT-116 was implanted into the hind flank of female nude mice and allowed establishing a sizeable tumor. After the solid tumor developed, the mice were randomized and divided into four groups consisting of six mice/group. As shown in Fig. 11, **6YT** dramatically inhibited tumors (46.19% at 10 mg kg⁻¹ and 59.12% at 20 mg kg⁻¹), which was comparable to etoposide (63.23%). Meanwhile, the body weight of the mice was decreased gradually when treated with etoposide, however, for the **6YT** group, the body weights increased normally, indicating the better safety of **6YT** than etoposide. Then we further compared the toxicity of etoposide and **6YT**, the prodrug of etoposide, *in vivo*.

In vivo acute and short-term toxicity studies in mice

Finally, in order to evaluate the toxicity of **6YT** *in vivo*, acute toxicity and short-term toxicity tests were performed in normal ICR mice. In the acute toxicity test, mice were divided into eight treatment (intraperitoneal) groups (six mice/group, three male and three female) from 52.43 mg kg⁻¹ to 250 mg kg⁻¹ at a multiple ratio of 0.8. These animals were observed for abnormal behavior and mortality for 2 weeks after treatment. The results show that the anti-tumor drug etoposide has a maximum tolerated dose (MTD) of 81.92 mg kg⁻¹, but no death was found after treatment with **6YT** even at a concentration of 250 mg kg⁻¹, which indicates at least a 3-fold increase in MTD of **6YT** than etoposide. Moreover, in the short-term toxicity test, thirty mice were divided into three groups (ten mice/group, five male and five female) randomly. Group I was treated with saline as the control, group II was treated with etoposide at a concentration of



Fig. 11 *In vivo* effects of **6YT** on tumor growth in the xenografted nude mice. (A) Anatomical nude mice's tumor tissue untreated or treated with **6YT** (10 and 20 mg kg⁻¹) or etoposide (20 mg kg⁻¹). (B) Inhibition percentage of mice's tumor tissues upon treatment of **6YT** (10 and 20 mg kg⁻¹) or etoposide (20 mg kg⁻¹), ***p < 0.0001. (C) The growing curves of mice's tumor volume. (D) The growing curves of body weight.

40 mg kg⁻¹, which is about half-value of the MTD to avoid acute death, and group III were treated with 40 mg kg⁻¹ of **6YT**, accordingly. Mice were treated every other day by intraperitoneal injection for 14 days (Fig. 12A). Body weights were recorded every other day (Fig. 12B), and there were no obvious differences between the control group and the **6YT** group, but the weights of the mice in the etoposide group decreased significantly. After the fifth day, mice in the etoposide group died gradually until all the mice died by the tenth day. However, no deaths occurred in the **6YT** group, and no obvious differences were observed between



Fig. 12 Short-term toxicity test. Thirty mice were distributed into three groups (5 female and 5 male in each group): group I, saline control group; group II, 40 mg kg⁻¹ etoposide; group III, 40 mg kg⁻¹ **GYT**. (A) Survival rate curves of mice treated with **GYT** or etoposide. Mice were treated (intraperitoneal) with 40 mg kg⁻¹ **GYT** or etoposide every other day. (B) Body weight curves of the various groups.

the **6YT** group and the control group. Both the acute toxicity and short-term toxicity tests indicated that compound **6YT** is more safe and less toxic than etoposide. Lastly, this prodrug design strategy might be applicable to more chemotherapy drugs, such as Taxol.

Conclusions

In summary, we designed a novel prodrug **6YT** for selective release of etoposide in cancer cells by targeting H_2O_2 . The emission intensity shows a 32-fold increment after stimulation by H_2O_2 *in vitro*. **6YT** shows highly selective toxicity to cancer cells that overexpress H_2O_2 , whereas neither obvious damage nor fluorescence signal to normal cells has been observed, which was verified by both cytotoxicity and microscopic confocal studies. We also performed *in vivo* imaging in zebrafish, indicating the potential of **6YT** to be used for monitoring drug release in living systems. Finally, further *in vivo* tumor studies and toxicity tests were performed to verify that **6YT** has comparable *in vivo* activity, but much better safety and less toxicity, than etoposide. As a consequence, as a safe and effective prodrug, **6YT** is expected to act as a chemotherapy drug against cancer.

Experimental

Materials

All the solvents and reagents were purchased from commercial suppliers and used without further purification. The reaction was monitored by analytical thin layer chromatography using precoated silica gel GF254 plates (Qingdao Haiyang Chemical Plant, Qingdao, China) and detected under a UV lamp (254 or 365 nm). Column chromatography was performed with silica gel (90–150 mesh; Qingdao Marine Chemical Inc.). ¹H NMR and ¹³C NMR spectra were measured using a Bruker spectrometer (500 and 600 MHz) at 25 °C and referenced to TMS. Chemical shifts are reported in ppm (δ) using the residual solvent line as the internal standard. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded using a Mariner ESI-TOF spectrometer. ROS was detected by flow cytometry (488 nm excitation and 525 nm emission filters) using a BD Accuri C6 flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA). All pH measurements were conducted with a sartorius basic pH-meter PB-10. Data were processed using cell quest software (Becton & Dickinson Company, Franklin Lakes, NJ). Fluorescence spectra were recorded using a Shimadzu FR-6000 luminescence spectrometer. HPLC analysis was performed using Agilent 1100 high-performance liquid chromatography. Zebrafish images were obtained using an OLYMPUS SZX2-ILLB microscope. Cells imaging was performed using ImageXpress Micro Confocal analysis and quantification of fluorescence intensity was done using ImageJ software.

General procedure for the preparation of target compounds

Synthesis of compound 1³³. To a solution of 2,4-dihydroxybenzaldehyde (2.07 g, 15 mmol) in propionic anhydride (50 ml) was added sodium propionate (4.32 g, 45 mmol), piperidine (0.5 ml) and the reaction mixture was further heated under reflux for 12 hours. After the reaction was complete as evidenced by thin layer chromatography analysis, ice water was poured into the solution and the precipitated viscous solid was diluted with EtOAc, the organic phase was washed with brine, dried over Na₂SO₄, and then removed by evaporation under reduced pressure. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate, 4:1) to obtain compound **1** as a white powder (2.12 g, 61%). ¹H NMR (600 MHz, DMSO) δ 7.89 (s, 1H), 7.66 (d, *J* = 8.4 Hz, 1H), 7.25 (d, *J* = 2.1 Hz, 1H), 7.12 (dd, *J* = 8.4, 2.2 Hz, 1H), 2.63 (q, *J* = 7.5 Hz, 2H), 2.09 (d, *J* = 1.1 Hz, 3H), 1.14 (t, *J* = 7.5 Hz, 3H); HRMS (ESI) *m*/z 255.0626 [M + Na]⁺ (calcd for 255.0628, C₁₃H₁₂NaO₄).

Synthesis of compound 2³⁵. A solution of compound 1 (1.16 g, 5 mmol) and NBS (0.98 g, 5.5 mmol) in CCl₄ (25 ml) was added to AIBN (0.04 g, 0.25 mmol). The mixture was then refluxed under an argon atmosphere. After 8 hours, the solvent was evaporated, and the residue was washed with water and extracted with ethyl acetate and dried over Na₂SO₄. Then the crude product was added into a solution of NaOAc (0.62 g, 7.5 mmol) in acetic acid (25 ml) and refluxed under an air atmosphere for 5 hours. After the reaction was complete as evidenced using analytical thin layer chromatography, the solution was evaporated and the residue was purified by column chromatography (petroleum ether/ethyl acetate, 4:1) to obtain compound 2 as a yellow powder (0.52 g, 36%). ¹H NMR (600 MHz, CDCl₃) δ 7.74 (s, 1H), 7.50 (d, J = 8.5 Hz, 1H), 7.13 (d, J = 2.1 Hz, 1H), 7.06 (dd, J = 8.4, 2.2 Hz, 1H), 5.05 (d, J = 0.7 Hz, 2H), 2.63 (q, J = 7.5 Hz, 2H), 2.15 (s, 3H), 1.28 (t, J = 7.5 Hz, 3H); HRMS (ESI) m/z 313.0684 $[M + Na]^+$ (calcd for 313.0683, C₁₅H₁₄NaO₆).

Synthesis of compound 3. To a solution of compound 2 (0.44 g, 1.5 mmol) in THF (10 ml) was added 2 N HCl (10 ml), and then the reaction was left under stirring at room temperature for 12 hours. The THF was then evaporated under reduced pressure and the mixture was diluted with EtOAc, the organic phase was washed with brine, dried over Na₂SO₄, and then removed by evaporation under reduced pressure. The residue was purified by column chromatography (petroleum ether/ethyl acetate, 2 : 1) to obtain compound 3 as a yellow powder (0.18 g, 64%). ¹H NMR (600 MHz, DMSO) δ 10.44 (s, 1H), 7.85 (s, 1H), 7.56 (d, *J* = 8.5 Hz, 1H), 6.78 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.72 (d, *J* = 2.2 Hz, 1H), 5.33 (s, 1H), 4.30 (d, *J* = 1.1 Hz, 2H); HRMS (ESI) *m/z* 215.0316 [M + Na]⁺ (calcd for 215.0315, C₁₀H₈NaO₄).

Synthesis of compound 4^{29} . A mixture of compound 3 (0.38 g, 2 mmol), K₂CO₃ (0.41 g, 3 mmol), KI (0.02 g, 0.1 mmol) and 4-(bromomethyl)benzeneboronic acid pinacol ester (0.65 g, 2.2 mmol) in acetonitrile (15 ml) was refluxed for 5 hours. After the reaction was complete as evidenced using TLC chromatography, the solvent was removed by evaporation under reduced pressure. The residue was subjected to silica gel column chromatography (petroleum ether/ethyl acetate, 2:1) to afford compound 4 as a yellow powder (0.62 g, 76%). ¹H NMR (600 MHz, CDCl₃) δ 7.84 (d, *J* = 8.0 Hz, 2H), 7.66 (s, 1H), 7.43 (d, *J* = 8.0 Hz, 2H), 7.39 (d, *J* = 8.6 Hz, 1H), 6.92 (dd, *J* = 8.6, R)

2.4 Hz, 1H), 6.88 (d, J = 2.4 Hz, 1H), 5.15 (s, 2H), 4.58 (d, J = 0.5 Hz, 2H), 1.35 (s, 12H); HRMS (ESI) m/z 431.1640 [M + Na]⁺ (calcd for 431.1636, C₂₃H₂₅¹¹BNaO₆).

Synthesis of compound 5. A solution of compound 4 (0.61 g, 1.5 mmol) and TEA (0.625 ml, 4.5 mmol) in dry dichloromethane was added SOCl₂ (0.164 ml, 2.25 mmol) dropwise and stirred in an ice bath for 2 hours. Then the solvent was removed by evaporation under reduced pressure, and the residue was purified using silica gel column chromatography (petroleum ether/ethyl acetate, 4:1) to afford compound 5 as a yellow powder (0.525 g, 82%). ¹H NMR (500 MHz, DMSO) δ 8.18 (s, 1H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.66 (d, *J* = 8.7 Hz, 1H), 7.47 (d, *J* = 8.0 Hz, 2H), 7.10 (d, *J* = 2.4 Hz, 1H), 7.04 (dd, *J* = 8.6, 2.4 Hz, 1H), 5.27 (s, 2H), 4.59 (s, 2H), 1.28 (s, 12H); HRMS (ESI) *m*/z 427.1478 [M + H]⁺ (calcd for 427.1482, C₂₃H₂₅¹¹BClO₅).

Synthesis of compound 6YT³⁸. A mixture of compound 5 (0.51 g, 1.2 mmol) and sodium iodide (1.8 g, 12 mmol) in 10 ml of acetone was stirred for 1 hour at ambient temperature. The reaction mixture was concentrated under reduced pressure and diluted with water. The suspension was extracted with ethyl acetate, and the organic phase was collected and washed with 10% sodium thiosulfate, and then dried over Na₂SO₄. The residue was redissolved into a mixture of etoposide (0.71 g, 1.2 mmol) and K₂CO₃ (0.5 g, 3.6 mmol) in acetonitrile, the solvent was stirred at room temperature for 6 hours. After the reaction was complete as evidenced by TLC chromatography, the solvent was removed under reduced pressure. The residue was subjected to silica gel column chromatography (DCM/ MeOH, 20:1) to afford 6YT as a yellow powder (0.62 g, 76%). m.p. 169–170 °C; ¹H NMR (500 MHz, DMSO) δ 7.90 (s, 1H), 7.70 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.7 Hz, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.08 (dd, J = 5.2, 2.4 Hz, 1H), 7.01 (dd, J = 6.3, 2.2 Hz, 2H), 6.54 (s, 1H), 6.23 (s, 2H), 6.03 (d, J = 1.4 Hz, 2H), 5.26 (s, 2H), 5.23 (d, J = 5.2 Hz, 2H), 4.93 (d, J = 3.4 Hz, 1H), 4.72 (d, J =5.1 Hz, 1H), 4.70 (d, J = 2.4 Hz, 2H), 4.57 (d, J = 7.7 Hz, 1H), 4.55 (d, J = 5.5 Hz, 1H), 4.27 (dd, J = 9.2, 3.6 Hz, 2H), 4.08 (dd, J = 10.1, 4.9 Hz, 1H), 3.91 (s, 1H), 3.68 (s, 1H), 3.59 (s, 6H), 3.51 (t, J = 10.1 Hz, 1H), 3.26–3.22 (m, 1H), 3.16 (t, J = 9.3 Hz, 1H), 3.09-3.04 (m, 1H), 2.88 (ddd, J = 13.9, 11.7, 3.4 Hz, 1H), 1.29 (s, 12H), 1.24 (d, J = 5.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 175.07, 161.53, 155.04, 153.59, 152.44, 148.26, 146.75, 140.46, 140.14, 136.90, 135.10, 132.78, 129.97, 129.35, 127.50, 127.23, 121.69, 113.59, 113.00, 110.41, 110.26, 108.42, 102.01, 101.93, 101.80, 99.07, 84.19, 80.60, 79.66, 74.89, 73.22, 72.24, 70.14, 69.50, 67.83, 66.25, 56.28, 43.59, 40.62, 40.53, 40.45, 40.36, 40.29, 40.20, 40.12, 40.03, 39.86, 39.69, 39.53, 37.74, 25.44, 25.16, 20.80; HRMS (ESI) m/z 1001.3371 [M + Na]⁺ (calcd for 1001.3374, C₅₂H₅₅¹¹BNaO₁₈).

General procedure for spectra measurements

If there are no special instructions, both absorption and fluorescence spectra were measured in 10 mM PBS buffer (pH 7.4, 0.1% DMSO) at 37 °C. A 10 mM stock solution of **6YT** was prepared in DMSO and the final test solution of **6YT** (10 μ M) was obtained by mixing 1 μ l of stock solution with 999 μ l of PBS buffer.

The stock solutions of various physiologically important species were prepared from H_2O_2 , TBHP, NaOCl, •OH, •OtBu, ONOO⁻, TEMPO, GSH, Cys, Hcy, Fe²⁺ and Fe³⁺.

 H_2O_2 , *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were obtained from 30%, 70%, and 10% aqueous solutions, respectively.

Hydroxyl radical (*OH), and *tert*-butoxy radical (*OtBu) were generated by reaction of 2.5 mM Fe²⁺ with 500 μ M H₂O₂ or TBHP, respectively.³⁹

Peroxynitrite solution (ONOO⁻) was freshly prepared before use as reported. 40

TEMPO stock solutions were prepared by adding 7.8 mg TEMPO to 100 ml $\rm H_2O.$

GSH, Cys and Hcy stock solutions were prepared by adding 30.6 mg GSH, 6.1 mg Cys and 6.8 mg Hcy to 100 ml $\rm H_2O$, respectively.

 Fe^{2+} and Fe^{3+} solutions were prepared by adding 6.3 mg $FeCl_2$ and 8.1 mg $FeCl_3$ to 100 ml $H_2O,$ respectively.

UV-vis absorption spectra were recorded on a Shimadzu UV-2450 spectrometer. Fluorescence spectra were recorded using a Shimadzu FR-6000 luminescence spectrometer. The UV-vis spectra were recorded from 300 to 450 nm, and the fluorescence spectra were recorded at an emission wavelength range from 400 to 600 nm and an excitation wavelength of 320 nm ($\lambda_{\rm em}$ = 458 nm; slit widths, 10 nm/10 nm).

Monitoring of etoposide release by RP-HPLC

The release of the anti-tumor drug etoposide from compound **6YT** was monitored by reversed-phase HPLC measurement at regular time intervals using methanol/water (50%/50%) for the first 6 minutes and methanol/water (90%/10%) for the rest as the mobile phase, with a flow rate of 1 ml min⁻¹. No drug release can be detected in the absence of H₂O₂. HPLC analysis was performed using Agilent 1100 high-performance liquid chromatography.

Cell line and cell culture

All the cell lines were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Both A549 and MCF-10A cell lines were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium and HCT-116 cell lines were incubated in Dulbecco's modified Eagle's medium (DMEM), all the media were supplemented with 10% fetal bovine serum (FBS) at 37 $^{\circ}$ C in a humidified 5% CO₂ incubator.

Cytotoxicity assay

Cell viability was determined using an MTT colorimetric assay. The cells were seeded in 96-well plates at a density of 5000 cells per well. The cells were incubated at 37 $^{\circ}$ C overnight in a humidified 5% CO₂ incubator to attach overnight. After medium removal, different concentrations of test compounds were added in triplicate to the plates in 200 ml fresh medium and incubated at 37 $^{\circ}$ C for 48 h. MTT was added to evaluate cell viability. The absorbance was measured at 570 nm using a microplate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA, USA).

The cytotoxic activity was expressed as the $\rm IC_{50}$ values. All experiments were conducted in triplicate and repeated more than three times.

ROS level evaluation of different cell lines

ROS was detected by flow cytometry (488 nm excitation and 525 nm emission filters) using a BD Accuri C6 flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA). Data were processed using cell quest software (Becton & Dickinson Company, Franklin Lakes, NJ). The level of intracellular ROS was measured by using the ROS sensitive dye, 20,70-dichlorofluorescein diacetate (DCFH-DA). Briefly, MCF-10A, HCT-116 and A549 cells were seeded in six-well plates at 3×10^5 cells per well, culturing for 20 h, and then washed three times and incubated with a final concentration of 10 mM DCFH-DA for 30 min at 37 °C in the dark. After incubation, the cells were washed three times and harvested in free-serum medium. The fluorescence of 20,70-dichlorofluorescein (DCF) was detected by flow cytometry (488 nm excitation and 525 nm emission filters) using a BD Accuri C6 flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA). Data were processed using cell quest software (Becton & Dickinson Company, Franklin Lakes, NJ). The data represented is an average of 3 repeats and statistical analysis was done using *t*-test. ***p < 0.0001.

Fluorescence image of compound 6YT activated by different concentrations of exogenous H₂O₂

The cells were seeded in 96-well plates in 100 µl of RPMI/DMEM medium containing 10% FBS at a density of 15 000 cells per well. The cells were incubated at 37 °C overnight in a humidified 5% CO₂ incubator to attach overnight. After medium removal, **6YT** (10 µM) was added in 200 ml fresh medium and incubated at 37 °C for 2 h. After that, different concentrations of H_2O_2 were added (0, 100 and 200 µM) for another 2 h. Cell imaging experiments were tested utilizing the ImageXpress Micro Confocal analysis system at Ex/Em of 360 nm/460 nm and propidium iodide at Ex/Em of 550 nm/620 nm. Quantification of fluorescence intensity was done using ImageJ software.

Fluorescence image of compound 6YT activated by endogenous H_2O_2 in different cell lines

The cells were seeded in 96-well plates at a density of 15 000 cells per well. The cells were incubated at 37 $^{\circ}$ C overnight in a humidified 5% CO₂ incubator to attach overnight. After medium removal, **6YT** (10 μ M) were added in 200 ml fresh medium and incubated at 37 $^{\circ}$ C for different time intervals (0, 2, 4, 8, 12 h). Cell imaging experiments were tested utilizing the ImageXpress Micro Confocal analysis system. Quantification of fluorescence intensity was done using ImageJ software.

Intracellular localization of compound 6YT

Cell imaging experiments were tested in A549 cells utilizing the ImageXpress Micro Confocal analysis system. The cells were incubated with 10 μ M **6YT** for 12 h, and subsequently, 1 μ M propidium iodide (PI) for 30 min. A significant fluorescence

signal could be observed, which was overlapping with the fluorescence signal of PI (fluorescence marker for nucleus).

In vivo imaging of 6YT in zebrafish

Wild type zebrafish were obtained from the Nanjing Xinjia Pharmaceutical Technology Co., Ltd. Zebrafish were fed in E3 embryo media at 28 °C. The 3-day-old zebrafish were incubated with PBS for 30 min, and then imaged as the blank control group. Zebrafish were treated with **6YT** (10 μ M) for 30 min, and then imaged, as the negative control group. Zebrafish were pretreated with H₂O₂ (300 μ M) for 30 min, subsequently treated with **6YT** (10 μ M) for 30 min, and then imaged, as the experimental group. Before imaging, 1% agarose gel was adopted for immobilization of the zebrafish. Fluorescence images were recorded using an OLYMPUS SZX2-ILLB microscope. Images were taken using the DAPI channel. Scale bar: 500 μ m.

In vivo tumor studies of 6YT

All of the animal experiments were approved by the Animal Ethical Committee of China Pharmaceutical University, and the procedures were performed strictly in accordance with the guidelines of the National Institutes of Health on the use of experimental animals (China).

In vivo tumor studies. Six-week-old female nude mice, of Specified Pathogens Free (SPF) grade, were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Nude mice were injected in the right flank area with 3×10^{6} human colon cancer HCT-116 cells in 200 ml of PBS. After 5-10 days, tumors with a diameter of 3 mm were established. The mice were divided randomly into three groups (6 mice/group) and intraperitoneally administered with 6YT (10 or 20 mg kg⁻¹), etoposide (20 mg kg⁻¹) or vehicle and monitored every other day. Tumor growth was determined by tumor volume, which was calculated according to formula $V = 0.52 \times a^2 \times b$ (a is the smallest superficial diameter and b is the largest superficial diameter). After 19 days of treatment, tumors were harvested from the killed mice. Tumor weights were recorded, the data represented is an average of 6 repeats and statistical analysis was done using *t*-test. ***p < 0.0001.

Acute toxicity test

In the acute toxicity test, six-week-old normal ICR mice, of Specified Pathogens Free (SPF) grade, were purchased from Qinglongshan Animal Breeding Ground (Nanjing, China). The mice were divided into eight treatment (intraperitoneal) groups (six mice/group, three male and three female): 52.43, 65.54, 81.92, 102.4, 128, 160, 200 and 250 mg kg⁻¹ at a multiple ratio of 0.8. These animals were observed for abnormal behavior and mortality for 2 weeks after treatment.

Short-term toxicity test

Moreover, in the short-term toxicity test, thirty mice were divided into three groups (ten mice/group, five male and five female) randomly. Group I was treated with saline as the control, group II and III were treated with etoposide and **6YT** at a concentration of 40 mg kg⁻¹, which was about half-value of the MTD of etoposide, to avoid acute death. Mice were treated every other day by intraperitoneal injection for 14 days. The body weights were recorded every other day.

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Experimental data are shown as the mean \pm standard deviation. p < 0.05 was considered to indicate a significant difference.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are sincerely grateful to Nanjing Xinjia Pharmaceutical Technology Co., Ltd, for the help with the zebrafish imaging experiments. This research work was supported by the National Natural Science Foundation of China (Grant No. 81973180 and No. 81673298), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China for Excellent Young Talents (Grant No. BK20180077), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_15R63) and the Drug Innovation Major Project (2018ZX09735002-003).

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