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A self-immolative and DT-diaphorase-activatable prodrug for drug-release tracking and therapy

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Bowen Li† [a], Peilian Liu† [a], Donghang Yan [a], Fang Zeng* [a] and Shuizhu Wu* [a]

DT-diaphorase, which catalyzes reduction of various biological substances like quinones, is overexpressed in some malignant tumors. However, exploiting this attractive property for the controlled release of an active drug from prodrug has yet to be fully taken advantage of. Herein we report a DT-diaphorase-based prodrug for concomitant drug-release imaging and cancer chemotherapy. This prodrug system is composed of two camptothecin (CPT) moieties as the active anticancer drug, a DT-diaphorase-responsive quinone propionic acid moiety and a set of self-immonlative linkers. The presence of DT-diaphorase leads to the release of two CPT molecules and restore the fluorescence of the latter, thereby realizing the fluorescent monitoring of DT-diaphorase level as well as the tracking of CPT release. Upon internalization by DT-diaphorase overexpressing cells, the prodrug can release fluorescent CPT and exhibit high cytotoxicity (half-maximal inhibitory concentration 0.71 μM) towards the cancer cells. This prodrug features on-demand enzyme-biomarker-triggered drug release as well as self-monitoring of drug release, therapeutic effect and biomarker level. This new strategy may provide an effective approach for constructing prodrugs with enhanced drug loading as well as controllability for drug release and tracking.

1. Introduction

Over the past several decades, chemotherapy remains a front-line therapy for cancer. However, a major problem of chemotherapy is its severe side-effects (high toxicity, non-specificity and etc.) to normal cells.[1] Development of prodrug strategies for overcoming side-effects have been deemed to be a promising approach to indirectly enhance therapeutic efficiency;[2] and the even more preferable strategy is to develop delivery systems which can achieve real-time tracking of the drug's release and localization.[3]

A prodrug, which can be triggered by the unique tumor microenvironment and monitored by an imaging reagent, is an elegant strategy in biomedicine and thus can significantly improve the therapeutic efficacy of anticancer drugs.[1,2] The special environments of tumor cells include lower pH,[4] intracellular reduction,[5] over-expressed enzymes,[6-8] active oxygen species,[9] or hypoxia.[10] Among the over-expressed enzymes, DT-diaphorase is a cytosolic flavoprotein which catalyzes an obligatory two-electron reduction of some biological substances like quinones, by utilizing nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) as the co-factors.[11] DT-diaphorase has recently attracted considerable attention due to its remarkable high expression in some malignant tumors, such as non-small cell lung carcinoma, liver cancers and breast carcinomas; and in some cancer cells highly elevated DT-diaphorase levels (up to 50-folds compared to normal cells) have been observed.[12] Despite the fact that the DT-diaphorase level assay has been employed for cancer diagnosis,[13] there are few reports on the exploitation of this level difference for developing prodrug.[13d-f]

On the other hand, self-immolative prodrugs, which have been regarded as new platforms for drug delivery, show a valuable ability to achieve controllable release of multiple drug molecules with a single bond cleavage.[14] Some of these prodrugs comprise self-immolative linkers, drug molecules as the tail units, as well as an enzyme substrate as the trigger; and following the enzymatic activation, a cascade of self-eliminations take place, thereby excluding the active drug as a result of a number of subsequent steps.[15,16] The prodrugs characterized by biomarker-triggered on-demand drug release through self-immolative reactions, which can simultaneously monitor drug release/therapeutic effect and the biomarker level, are highly desirable.

Camptothecin, a natural product originally extracted from camptotheca acuminata, which stabilizes the covalent attachment of topoisomerase I to single-strand DNA breaks, can disrupt the DNA replication process in cancer cells, thus demonstrating strong anticancer activity.[17a] This anticancer drug has demonstrated to be effective against many types of tumors, but its poor water solubility and its rapid conversion to a less active carboxylate form under physiological conditions severely limit its clinical application.[17b] Hence, development of a new prodrug based camptothecin derivative, which

[a] State Key Lab of Luminescent Materials & Devices, College of Materials Science & Engineering, South China University of Technology, Guangzhou 510640, P. R. China. Tel: +86 20 22236262; E-mail: mcfzeng@scut.edu.cn; shzhwu@scut.edu.cn; Fax: +86 20 22236363.
† These authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: Synthesis scheme, ^{1}H NMR spectra, Mass spectra, zeta potential, absorption spectra, cell viabilities and determination of solubility. See DOI: 10.1039/x0xx00000x

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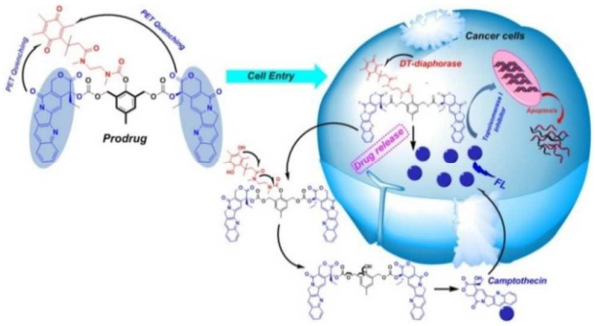
integrates biomarker-triggered self-immolative drug release and concomitant in-situ monitoring of drug release/biomarker level, may circumvent these limitations and greatly improve therapeutic efficacy.[17c]



Scheme 1. Schematic overview of the prodrug for detecting DT-diaphorase, real-time drug tracing and anticancer action.

Previously, we successfully designed a DT-diaphorase-based molecular prodrug for concomitant drug-release imaging and cancer chemotherapy in the prodrug system, one molecule of active drug was coupled with quinone propionic acid moiety.[13f] In an effort to further improve the loading capacity of the molecular prodrug, we postulate that, by employing a self-immolative unit with multiple reaction sites like 1,3-dihydromethyl-2-phenol, we could simultaneously incorporate more than one active drug molecules and the triggering group, thus establishing a DT-diaphorase-based prodrug system with enhanced drug loading and eventually better therapeutic efficacy. To substantiate this idea, herein, we demonstrate a molecular prodrug with high drug load and enhanced efficacy by adopting a self-immolative architecture, as illustrated in Scheme 1. This prodrug system, which is an enzyme-biomarker triggered release system, is composed of two anticancer drug camptothecin (CPT) moieties as the active anticancer drug, a DT-diaphorase-responsive quinone propionic acid and a set of self-immonlative linkers (Scheme 1 and Scheme 2). For this prodrug, due to the photoinduced electron transfer (PET) process the CPT's fluorescence is efficiently quenched by the quinone propionic acid. The presence of DT-diaphorase and the cofactor leads to the release of the two CPT molecules and restore the fluorescence of the latter (as demonstrated in Scheme 1 and Scheme S1, ESI†), thereby affording the two CPT moieties as the reporter of DT-diaphorase level and their own release. Moreover, the quinone propionic acid moiety acts as the trigger for the drug release and ensures eventual fluorescent restoration, it can be selectively reduced by DT-diaphorase into hydroquinone, [14] which will subsequently be cleaved and turn into lactone, thereby causing the release of the active and fluorescent CPT via a 1,4-elimination reaction (Scheme S1, ESI†). Therefore, this prodrug can realize on-demand enzyme-biomarker-triggered drug release as well as self-monitoring of drug release, therapeutic effect and biomarker level.

2. Experimental Section

2.1 Materials

2,6-Dihydroxy methyl-4-methyl phenol, t-butyldimethylchlorosilane (TBSCl), imidazole, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloricde (EDC), 4-dimethylaminopyridine (DMAP), p-toluenesulfonic acid monohydrate (PTSA•H_2O) and triphosgene were purchased from Sigma-Aldrich Reagents without further purification. DT-diaphorase (lyophilized powder, recombinant, expressed in E. coli) and β-nicotinamide adenine dinucleotide reduced disodium salt (NADH) were purchased from Sigma-Aldrich. Dichloromethane (DCM), N, N-dimethyl-formamide (DMF) was dried with CaH_2 and distilled under nitrogen atmosphere. The water used throughout the experiments was the triple-distilled water which was further treated by ion exchange columns and then by a Milli-Q water purification system. Other solvents used in this study were analytical grade reagents and used without further purification.

2.2 Prodrug synthesis

Synthesis of compound 1-3: Compound 1-3 were synthesized according to the procedures reported elsewhere.[18]

Synthesis of 2,6-bis(((tert-butyldimethylsilyl)oxy)methyl)-4-methylphenol (4).

2,6-Dihydroxy methyl-4-methyl phenol (1.5 g, 8.9 mmol) and imidazole (1.81 g, 26.3 mmol) was dissolved in 10 mL THF and then added to TBSCl (3.3 g, 22 mmol) in batches. After stirring for 3 h at room temperature, the solvent was removed and diluted with ethyl acetate. The organic phase was washed with water and brine, dried over anhydrous Na_2SO_4 overnight and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (DCM:hexanes 1:10) to give 4 (3.24 g, 92% yield) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 7.90 (s, 1H), 6.78 (s, 2H), 4.70 (s, 4H), 2.13 (s, 3H), 0.82 (s, 18H).

Synthesis of 2,6-bis(((tert-butyldimethylsilyl)oxy)methyl)-4-methylphenyl (4-nitrophenyl) carbonate (5) .

TEA (210 μL, 2.1 mmol) and trace DMAP were added to a solution of compound 4 (200 mg, 0.49 mmol) in dichloromethane and then stirring at 0 ^{o}C for 0.5 h. 4-Nitrophenyl chloroformate was dissolved in dichloromethane and slowly added in the solution. After 0.5 h of reaction, the mixture was diluted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (EtOAc:hexanes 1:100) to give 5 (170 mg, 60% yield) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ 8.32 (d, J = 9.2 Hz, 2H), 7.49 (d, J = 9.2 Hz, 2H), 7.22 (s, 2H), 4.72 (s, 4H), 2.38 (s, 3H), 0.92 (s, 18H), 0.09 (s, 12H). HR-MS (ESI): calcd for $C_{28}H_{43}NO_7Si_2$ ($[M+H]^+$) 562.2651, found: 562.2656.

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Synthesis of N-(2-((1-(2,6-bis(((tert-butyldimethylsilyl)oxy)-methyl)-4-methylphenoxy)ethyl)amino)ethyl)-N,3-dimethyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanamide (6).

Compound 5 (2.6 g, 4.4 mmol) and compound 3 (770 mg, 2.4 mmol) were dissolved in 10 mL DMF and TEA (1 ml, 10 mmol) was then slowly added into the solution. After 3 h, the solution was diluted with dichloromethane, washed with 1 N HCl and saline. The organic layer was collected and dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (EtOAc:hexanes 1:4) to give 6 (820 mg, 52% yield) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ 7.18 (s, 2H), 4.61 (s, 4H), 3.45 (d, *J* = 29.5 Hz, 4H), 3.11-2.90 (m, 8H), 2.34 (dd, *J* = 8.8, 6.3 Hz, 3H), 2.13 (dd, *J* = 14.0, 5.3 Hz, 3H), 1.94 (t, *J* = 12.4 Hz, 6H), 1.44 (s, 6H), 0.92 (s, 18H), 0.08 (s, 12H). HR-MS (ESI): calcd for $C_{40}H_{66}N_2O_7Si_2$ ($[M+H]^+$) 743.4481, found: 743.4479.

Synthesis of N-(2-((1-(2,6-bis(hydroxymethyl)-4-methylphenoxy)ethyl)amino) ethyl)-N,3-dimethyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanamide (7).

Compounds 6 (500 mg, 1 mmol) and PTSA•H_2O (45 mg, 0.25 mmol) were mixed and stirred in methanol (5 mL) at room temperature under nitrogen for 3 h. After completion of reaction, the solvent was removed at room temperature. The crude product was purified by column chromatography on silica gel (EtOAc:hexanes 1:3) to give 7 (560 mg, 90% yield) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$,ppm) δ 7.23-7.18 (m, 2H), 4.52 (dd, *J* = 28.8, 14.7 Hz, 4H), 3.65-3.42 (m, 4H), 3.08 (ddd, *J* = 47.8, 33.7, 19.2 Hz, 8H), 2.35 (t, *J*= 3.6 Hz, 3H), 2.16-2.07 (m, 3H), 1.98-1.81 (m, 6H), 1.48-1.36 (m, 6H). MS(ESI): m/z 513.27 $[M-H]^-$.

Synthesis of the prodrug 8 (the prodrug).

CPT (102 mg, 0.285 mmol) and DMAP (107 mg, 0.85 mmol) were suspended in 10 mL anhydrous dichloromethane at ice water under nitrogen. Triphosgene (34 mg, 0.114 mmol) was rapidly added and stirred for 30 min at room temperature. Then compound 7 (60 mg, 0.12mmol) was dissolved in 5 mL anhydrous dichloromethane and stirred overnight. The mixture was diluted with ethyl acetate and washed with water, saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc) to give 8 (61 mg, 33% yield) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.42 (s, 2H), 8.23 (s, 2H), 7.96 (s, 2H), 7.86 (s, 2H), 7.70 (s, 2H), 7.36 (s, 2H), 7.16 (s, 2H), 5.66 (s, 2H), 5.40 (s, 2H), 5.30 (s, 4H), 5.05 (s, 2H), 4.96 (s, 2H), 3.44 (t, *J* = 55.5 Hz, 4H), 3.08-2.75 (m, 8H), 2.30- 2.12 (m, 7H), 2.02 (s, 4H), 1.81 (s, 6H), 1.32 (s, 6H), 0.98 (s, 6H). HR-MS (ESI): calcd for $C_{70}H_{66}N_6O_{17}$ ($[M+H]^+$) 1263.4557, found: 1263.4565.

2.3 Cell culture

A549 cells (human nonsmall cell lung cancer) and L929 cells (murine aneuploid fibrosarcoma cells) were maintained according to the supplier's (ATCC) protocols. The two cell lines were incubated with 10% FBS (fetal bovine serum, GIBCO) - containing Dulbecco's modified eagle medium (DMEM) with 1% penicillin and streptomycin (GIBCO) at 37 °C in a humidified atmosphere containing 5% of CO_2. When the cell density reached 70-80% of confluence, a subculture was considered complete. The medium was changed approximately every 1 to 2 days.

2.4 Cell imaging

A549 and L929 cells were incubated in DMEM supplemented with 10% FBS for 24 h incubation. Then cells were seeded in a 6-well plate and each well containing polylysine-coated cell culture glass slides. Cells on glass slides was allowed to 50–70% confluence and washed with DMEM, and the cells were incubated with the prodrug in a humidified incubator (37 °C, 5% CO_2) for 12 h or 24 h. Afterwards, the 6-well plate were washed with PBS for three times and the glass slides were taken out. Fluorescence images were obtained using an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD.

2.5 Cell viability assay

Cells (5000 cells/well) were seeded in 96-well plates and incubated with the prodrug of varied concentrations from 0.1 to 80 μM at 37 °C. The cells without the treatment were used as control. After incubation for 48 h, the wells were washed three times with PBS buffer and treated with DMEM medium containing 0.5 mg/mL MTT for another 4 h. The resulting formazan crystal was dissolved in 150 μL of DMSO after carefully removing the medium and the absorbance was recorded at 570 nm. The cell viability assays were performed using a Thermo MK3 ELISA plate reader. The independent experiments performed in in six replicates were used to obtain the statistical mean and standard deviation. As for the dicoumarol-involved MTT assays, the cells were co-treated with 15 μM dicoumarol and the prodrug of varied concentrations for 24 h before being subject to MTT assay.

2.6 Apoptosis Analysis by Annexin V-FITC and Propidium Iodide (PI) Double Staining

A549 and L929 cells were seeded in 6-well plate at the density of 1.0 ×10^6 cells/well for 12 h at 37 °C. When the cell density reached 80-90% of confluence, the medium was replaced with different concentrations of prodrug or CPT for 24 h. The cells were then washed with PBS and harvested after trypsinization and centrifugation at 2000 rpm for 10 min. Subsequently, the cells were collected and resuspended in 1 ml PBS and then mixed with Annexin V Binding buffer (400 μL) containing 5 μL Annexin V-FITC and 10 μL PI. Finally, the flow cytometry

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analyses were performed using BD Accuri C6 flow cytometer and the data were analyzed using the BD Biosciences software.

2.7 Measurements

^{1}H NMR spectra were recorded on a Bruker Avance600 MHz NMR spectrometer. All chemical shifts are reported in ppm value using the peak of residual proton signals of TMS as an internal reference. Mass spectra were obtained on a Bruker Esquire HCT Plus mass spectrometer. High resolution mass spectra were obtained on AB Sciex Triple TOF 5600+ mass spectrometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescence images were obtained using an Olympus IX 71 with a DP72 color CCD.

3. Results and discussion

3.1 Synthesis of the prodrug

The synthesis route for the prodrug (compound **8**) is shown in Scheme 2. Compound **1-3** were synthesized according to previously reported methods.[18] 2,6-Dihydroxy-4-methyl phenol was allowed to react with tert-butyldimethylsilyl chloride to yield compound 4. Compound **5** was then obtained through a nucleophilic reaction between 4-nitrophenyl chloroformate and compound **4**; afterwards compound **5** was allowed to react with **3** via a transesterification reaction to give a key intermediate **6**. Compound **7** was obtained by deprotection of **6**. Finally, the CPT was incorporated into compound **7** in the presence of triphosgene to yield the prodrug (compound **8**). The structures of all the intermediates and the final product were confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (Fig. S1–S9, ESI†). The zeta potential for the prodrug and CPT were determined and the result is given in Fig. S10, which indicates that in aqueous medium with 1% DMSO, the two compounds were negatively charged. The solubility of the prodrug and CPE was investigated, and it was found that the prodrug was soluble in common organic solvents and slightly soluble in water; while CPT exhibited poor solubility in these solvents (Table S2, ESI†).

3.2 Drug release studies in vitro

To demonstrate the release of active drug CPT from the prodrug in the presence of DT-diaphorase, absorption and fluorescence spectra for the prodrug solutions were determined under physiological condition. The absorption spectra were shown in Fig. S10, which indicate that both the prodrug and CPT show a strong absorption band at 365 nm. Fig. 1A shows the fluorescence spectra of CPT, the prodrug and the prodrug in the presence of DT-diaphorase. This prodrug molecule is designed as an electron donor-acceptor system in which the initial photoexcitation is localized on the acceptor. In the absence of DT-Diaphorase, the HOMO of the donor (CPT moiety) lies higher in energy than that of the acceptor (quinone propionic acid moiety) and can transfer an electron to the acceptor's HOMO. This photoinduced electron transfer (PET) process competes favorably with radiative decay to the ground state, substantially diminishing the fluorescence quantum yield and thus substantially quenching the CPT's fluorescence. In the presence of DT-diaphorase, a 6-fold increase in fluorescence intensity at 446 nm can be observed in the presence of DT-diaphorase and its cofactor NADH (100 μM) at 37 °C in phosphate buffer (pH 7.4, 10 mM), and this spectrum is similar to that of CPT. This is because, the released CPT restores its fluorescence in the absence of PET process due to drug release, and hence the released drug (restored fluorescence) can act as a turn-on fluorescent reporter for drug release. In addition, a red-shift in fluorescence band can be observed upon treatment with the enzyme; and this red-shift is due to the cleavage of the carbonate bond which leads to the change in the "push-pull" electronic configuration of CPT and eventually causes the red-shift in fluorescence band.

Furthermore, the concentration and time dependence of the prodrug's fluorescence spectra were also recorded, as shown in Fig. 1B and 1C. Upon addition of 60 μg/mL DT-diaphorase into the solution with 5 μM of prodrug, the fluorescence intensity at 446 nm gradually increases with time and reaches a plateau in 24 h (Fig.1 B).

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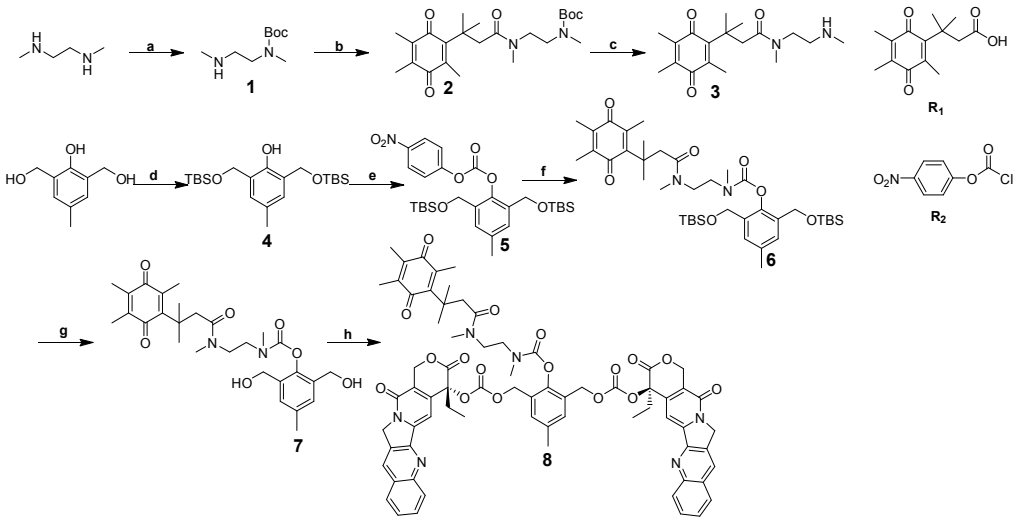

Scheme 2. Synthetic route for the prodrug (compound 8). Reagents and Conditions: a) $(Boc)_2O$, DCM, r.t., overnight; b) R_1, EDC, pyridine, r.t., overnight; c) 5% TFA, DCM, r.t., 3 h; d) TBSCl, DCM, imidazole, THF, r.t., 3 h; e) R_2, TEA, DMAP, DCM, 0 oC to r.t., 1 h; f) 3, TEA, DMF, r.t., 3h; g) PTSA, r.t., 3 h; h) CPT, Triphosgene, DMAP, DCM, 0 ^{o}C to r.t., overnight.

On the other hand, on gradual addition of DT-diaphorase (0 μg/mL to 80 μg/mL) to the solution of prodrug, the fluorescence intensity at 446 nm increases until it reaches a saturation point at 60 μg/mL. These results suggest the successful release of CPT from the prodrug.

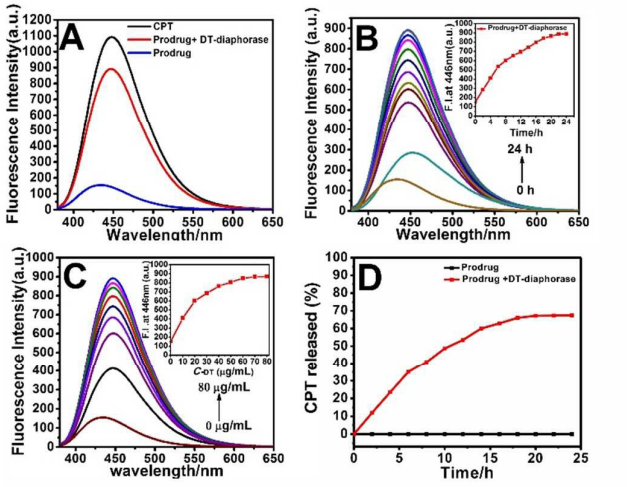

Figure 1. (A) Fluorescence spectra of the prodrug (5 μM), CPT (5 μM) and prodrug (5 μM) with DT-diaphorase (60 μg/mL). (B) Time course of fluorescence of the prodrug (5 μM) in the presence of DT-diaphorase (60 μg/mL). Inset: Change in fluorescence intensity at 446 nm as a function of time after DT-diaphorase (60 μg/mL) treatment. (C) Fluorescent spectra for prodrug (5 μM) after treatment with varied concentrations of DT-diaphorase (0-80 μg/mL). Inset: Change in fluorescence intensity at 446 nm as a function of DT-diaphorase concentration. (D) Percentage of CPT (as determined by HPLC) released from the prodrug (5 μM) as a function of time in the presence or absence of DT-diaphorase (60 μg/mL). The cofactor NADH was used for all measurements. All fluorescence spectra data were obtained at 37 °C in phosphate buffer (pH 7.4, 10 mM) containing 1% (v/v) DMSO. Excitation wavelength: 365 nm.

To further confirm the release of CPT from the prodrug in the presence of DT-diaphorase, high-performance liquid chromatography (HPLC) was employed and high resolution MS spectrum was recorded as well (Fig. 1D, Fig. 2, and Fig. 3). One can find in Fig. 1D that, CPT is gradually released from the prodrug in the presence of DT-diaphorase (60 μg/mL), whereas there is no CPT detected in the absence of DT-diaphorase. As shown in Fig. 2, the retention time of prodrug and CPT are monitored and identified at 4.74 min and 1.63 min in HPLC chromatogram. For the prodrug, upon treatment with DT-diaphorase (60 μg/mL), the peak intensity at 4.74 min (corresponding to the prodrug) decreases and a new strong peak emerges at 1.63 min which well matches that for CPT. Moreover, the anticipated release of CPT as active cancer drug was also proved by HR-MS (ESI) analyses, as shown in Fig. 3. There is an ionic peak corresponding to the prodrug ($[M+H]^+$=1263.4567) in the HR-MS spectrum as well as that for CPT ($[M+H]^+$=349.1192) after the prodrug being treated with DT-diaphorase and NADH, which provides additional evidence for the release of CPT from the prodrug.

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3.3 Selectivity and anti-interference performance of prodrug

To assess the selectivity of the prodrug towards DT-diaphorase over some potential interferents, the prodrug was allowed to respectively incubate with DT-diaphorase, some metal ions, amino acids, biological reductants or other enzymes for 24 h, and the fluorescent intensity at 446 nm for the samples were then recorded. As shown in Fig. 4I, none of the potential interferents can cause remarkable fluorescence enhancement. This result confirms that this prodrug shows quite good selectivity toward DT-diaphorase over other competitive substances and also demonstrates that the fluorescence enhancement as a result of CPT release was induced by DT-diaphorase.

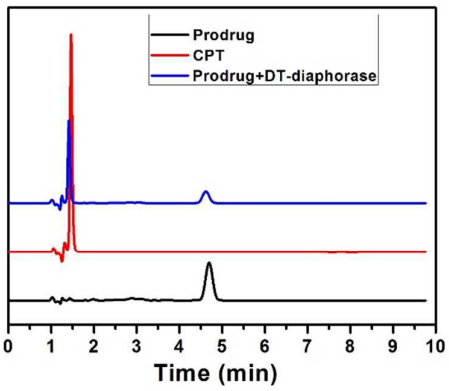

Figure 2. Typical HPLC chromatogram of the prodrug (A), CPT (B) and the prodrug (10 μM) incubated with DT-diaphorase (60 μg/mL) and cofactor NADH for 24 h (C). Peaks in the chromatograms were detected by monitoring the absorption at 365 nm. The mobile phase was 80/20 acetonitrile/water and the flow rate was 1.0 mL/min.

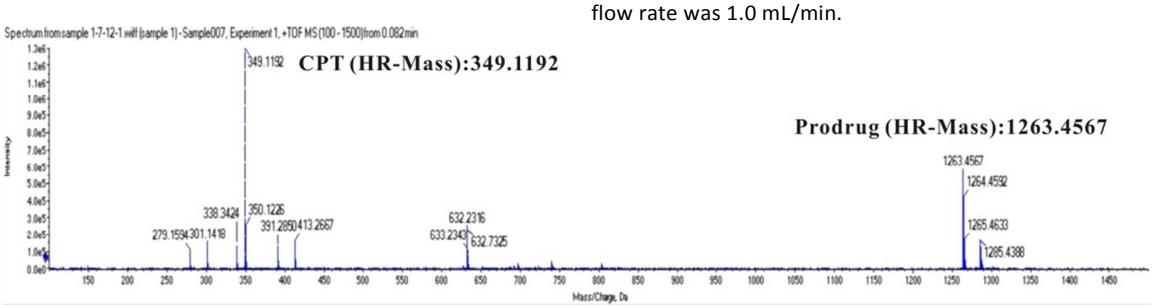

Figure 3. HR-MS (ESI) analyses for the prodrug (10 μM) upon incubation with DT-diaphorase (60 μg/mL) and cofactor NADH for 24 h.

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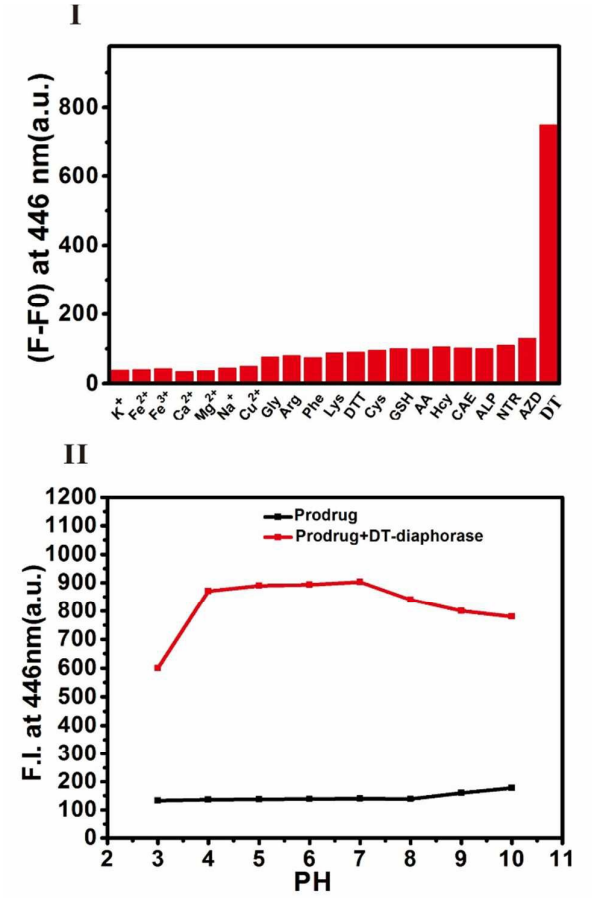

Figure 4. I) Fluorescence intensity for the prodrug (5 μM) at 446 nm in phosphate buffer (pH 7.4, 10 mM, containing 1% (v/v) DMSO) in the presence of various relevant species for 24 h. Excitation wavelength: 365 nm. (60 μg/mL for DT-diaphorase (DT), Nitroreductase (NTR), Azoreductase (AZD), Alkaline phosphatase (ALP), Carboxylesterase (CAE), 5 mM for AA, Hcy, GSH, Gly, Arg, Phe and Cys, 1 mM for others). II) Fluorescence intensity at 446 nm of the prodrug (5μM) as a function of pH in the absence or presence of DT-diaphorase (60 μg/mL) in phosphate buffer (10 mM, containing 1% (v/v) DMSO). The fluorescent intensities were recorded after exposure to DT-diaphorase and NADH for 24 h at 37 °C. Excitation wavelength: 365 nm.

The response of the prodrug to DT-diaphorase under varied pH values was investigated and the result is given in Fig. 4II. In the absence of DT-diaphorase, the fluorescence of prodrug is found stable over the pH range from 3 to 10; whereas in the presence of DT-diaphorase and cofactor, an enhancement of fluorescence intensities are observed, and the values remain fairly stable within the pH range of 4-10. This result shows that the DT-diaphorase-linked camptothecin conjugate (prodrug **8**) may be useful as an indicating agent for delivering camptothecin to cancer cells at biological pH.

3.4 Drug release studies in DT-diaphorase over-expressing cell

We investigated the release of CPT in a DT-diaphorase overexpressing cell line (A549 cell) or a normal cell line without overexpression of DT-diaphorase (L929 cell) by using fluorescent microscope, and the results are presented in Fig. 5. For A549 cells, upon 12 h of incubation with the prodrug (10 μM), blue intracellular fluorescence can be observed; and after 24 h of incubation, the fluorescence become very bright, as shown in Fig. 5A. On the other hand, pretreatment of A549 cells with dicoumarol (a DT-diaphorase inhibitor) causes the disappearance of the intracellular fluorescence (Fig. 5B). While for the L929 cells in which DT-diaphorase is not overexpressed, the incubation with the prodrug for 24 h cannot induce detectable blue fluorescence under a similar experimental condition (Fig. 5B). We can thus conclude that the prodrug can be used to monitor the CPT release in DT-diaphorase over-expressing cells.

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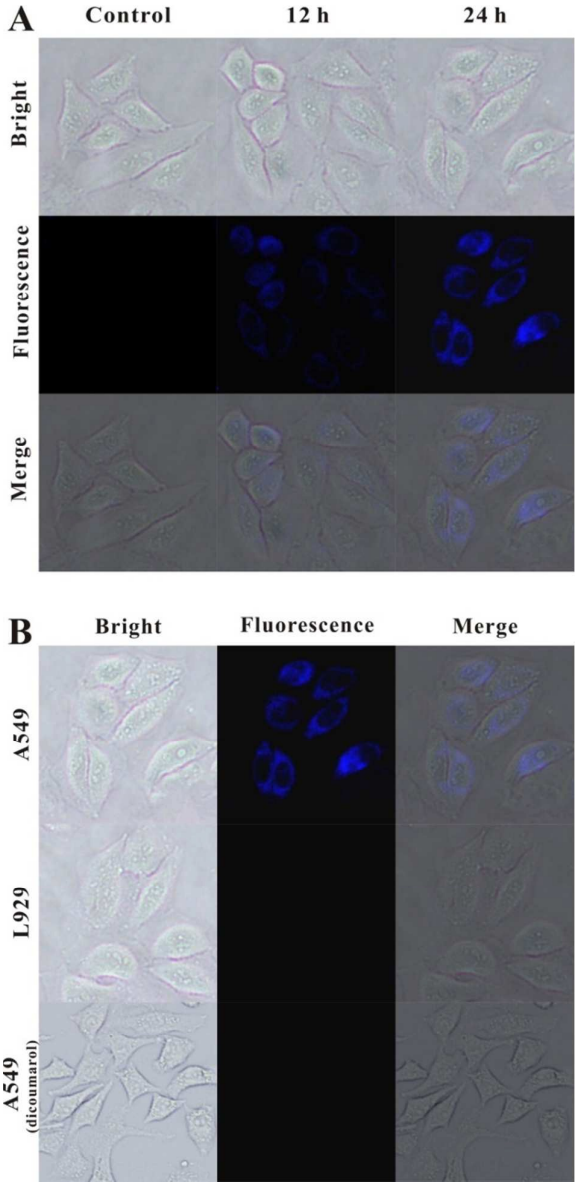
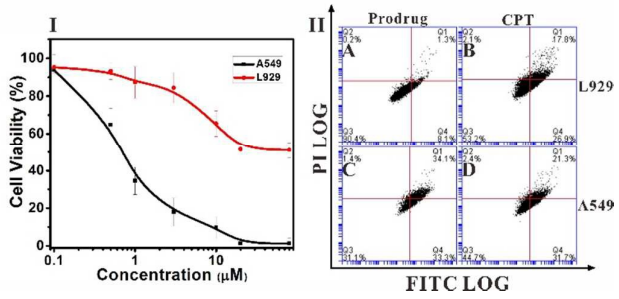


Figure 5. A) Fluorescent microscopic images for A549 cells in the absence (the control) or presence of the prodrug (10 μM, with the incubation time of 12 h or 24 h respectively) B). Fluorescence images of A549 cells and L929 cells upon incubation with the prodrug (10 μM) for 24 h, and of A549 cells pretreated with 20 μM of dicoumarol (the inhibitor of DT-diaphorase) for 30 min followed by the incubation of the prodrug (10 μM) for 24 h.

It is known that, the low drug loading of prodrug and correspondingly the low drug level locally at the disease site is an obstacle to optimal therapeutic effect. For this prodrug, the drug load is 55.0%, while that for our previous system is 46.0%. This prodrug **8** which can release two equivalent CPT moieties from a single prodrug molecule could enhance the drug concentration locally at disease site through efficient enzyme-biomarker triggered self-immolative release. To investigate the therapeutic effect of this high drug-loading prodrug **8** against the DT-diaphorase over-expressing cancer cells (A549) and normal cells (L929), the cytotoxicity of prodrug is assessed using typical MTT assays and the flow cytometry experiments. The viabilities for A549 and L929 cells upon treatment with the prodrug assessed using MTT assays are shown in Fig. 6I. The prodrug shows high cytotoxicity towards DT-diaphorase over-expressing A549 cells with a molar half-maximal inhibitory concentration (IC_{50}) of 0.71 μM, while that for our prodrug system with only one drug moiety is around 1.2 μM, and the IC_{50} for CPT is around 0.70 μM, suggesting that incorporating two CPT onto one prodrug molecule can enhance anticancer efficacy. On the other hand, in terms of equivalent value of active drug, the current prodrug only has a comparable IC_{50} (1.4 μM) compared to that of our previous one-CPT prodrug. Moreover, as expected, the prodrug exhibits a much higher IC_{50} value (> 80 μM) towards L929 cells, indicating the prodrug is of low cytotoxicity towards normal cells in which DT-diaphorase is not over-expressed. To further verify the role of DT-diaphorase in the prodrug's action, MTT assays were performed for A549 and HeLa cells co-treated with dicoumarol (an inhibitor of DT-diaphorase) and the prodrug for 24 hours, and the result is presented in Fig. S12. Upon pretreatment with dicoumarol, the cells all exhibit high viabilities (over 80% at 10 μM of prodrug). This result suggests that the prodrug is activated by DT-diaphorase. Furthermore, the flow cytometry studies were performed to further verify the pro-apoptotic effect of the prodrug towards DT-diaphorase-overexpressing cells. For L929 cells, after incubation with the prodrug for 24 h, the apoptotic percentage (including the early and late apoptosis, Q1 and Q4 in Fig. 6II and 6III) is 9.4%; whereas incubation with unmodified CPT leads to a much higher apoptotic percentage of 44.7%. While for A549 cells, the apoptotic percentage reaches to 67.4% upon treatment with the prodrug, compared to 53% upon treatment with CPT. The effect of the prodrug's concentration (1 μM to 20 μM) on pro-apoptotic effect towards A549 cells was also assessed by the flow cytometry (Fig. 6III). With the increasing concentration of the prodrug, the apoptotic percentage of A549 cells gradually increases from 26.0 % to 87.1%. The results from flow cytometry are in conformity with the MTT results in that the prodrug exhibits higher cytotoxicity towards DT-diaphorase over-expressing cells.



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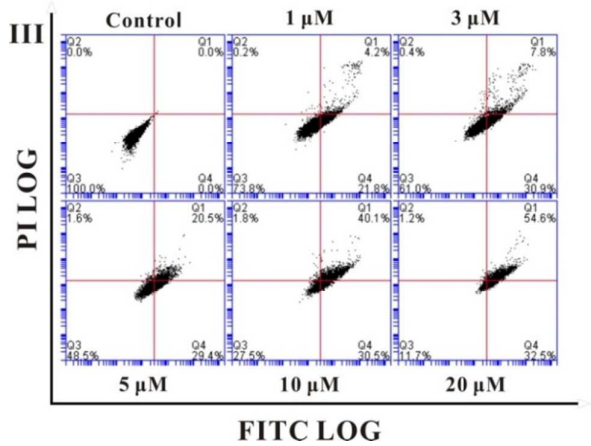

Figure. 6 (I) Cell viabilities for A549 and L929 cell lines upon treatment with the prodrug of varied concentrations(incubation for 48 h). (II) Flow cytometry analysis: representative percent distribution of A549 and L929 cells analyzed by using annexin V-FITC/PI staining. L929 cells were treated with the prodrug (20 μM) (A) and CPT (20 μM) (B) for 24 h and A549 cells were treated by the prodrug (10 μM) (C) and CPT (10 μM) (D) for 24 h. (III) Representative percent distribution of A549 cells respectively treated with the prodrug of varied concentrations for 24 h.

4. Conclusions

In summary, we have successfully developed a self-immolative DT-diaphorase activatable prodrug that can realize on-demand biomarker-triggered drug release and simultaneously monitor drug release/therapeutic effect and the biomarker level. This prodrug has several advantageous features: firstly one prodrug molecule contains two CPT moieties, which greatly improves drug loading and correspondingly the therapeutic effect; secondly CPT moieties in the prodrug not only serve as the active drug but also the turn-on fluorescent reporter for monitoring and imaging of the released drug and enzyme-biomarker level; thirdly the quinone propionic acid moiety of the prodrug not only acts as the trigger for biomarker-responsive on-demand drug release but also as a strong quencher of CPT fluorescence via photoinduced electron transfer (PET), which ensures the eventual fluorescence turn-on imaging and tracking. This new strategy may offer useful insights for developing prodrugs with enhanced drug loading as well as controllability for drug release and tracking.

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Notes and references

1 a) R. Kumar, W. S. Shin, K. Sunwoo, W. Y. Kim, S. Koo, S. Bhuniya J. S. Kim, *Chem. Soc. Rev.,* 2015, **44,** 6670-6683; b) C. Y. Shi, D. D. Guo, K. Xiao, X. Wang, L. L. Wang, J. T. Luo, *Nat. Commun.,* 2015, **6,** 7449; c) C. Li, *Nat. Mater.,* 2014, **13,** 110-115.

2 a) W. A. Denny, *Eur. J. Med. Chem.* 2001, **36,** 577-595; b) L. Bildstein, C. Dubernet, P. Couvreur, *Adv. Drug Deli. Rev.*, 2011, **63,** 3-23; c) X. Xi, S. Hu, Z. Zhou, X. Liu, J. B. Tang, Y. Q. Shen, *J. Mater. Chem. B, 2016,* **4,** 5236-5245; d) J. Xu, F. Zeng, H. Wu, S. Z. Wu, *J. Mater. Chem. B*, 2015, **3**, 4904-4912; e) Q. Chen, H. Ke, Z. Dai, Z. Liu, *Biomaterials,* 2015, **73,** 214-230.

3 a) D. L. Wang, T. Y. Zhao, X. Y. Zhu, D. Y. Yana, W. X. Wang, *Chem. Soc. Rev.,* 2015, **44,** 4023-4071; b) J. Q. Wang, X. R. Sun, W. W. Mao, W. L. Sun, J. B. Tang, M. H. Sui, Y. Q. Shen, Z. W. Gu, *Adv. Mater.,* 2013, **25,** 3670-3676; c) Y. Liu, Q. Pei, L. Chen, Z. Li, Z. Xie, *J. Mater. Chem. B,* 2016, **4***,* 2332-2337.

4 a) Z. Su, Y. Liang, Y. Yao, T. Wang, N. Zhang, *J. Mater. Chem. B*, 2016, **4,** 1122-1133; b) H. Wang, G. Liu, S. Dong, J. Xiong, Z. Dua, X. Cheng, *J. Mater. Chem. B,* 2015, **3,** 7401-7407; c) J. Z. Du, X. J. Du, C. Q. Mao, J. Wang, *J. Am. Chem. Soc.,* 2011, **133,** 17560-17563; d) J. Q. Fan, F. Zeng, S. Z. Wu, X. D. Wang, *Biomacromolecules,* 2012, **13,** 4126-4137; e)F. Du, J. Xu, F. Zeng, S. Wu, *Acta Chimica Sinica*, 2016, **74**, 241-250; f) Y. Wang, J. W. Du, Y. X. Wang, Q. Jin, J. Ji, *Chem. Commun.,* 2015, **51,** 2999-3002; g) R. J. Dong, S. P. Ravinathan, L. Z. Xue, N. Li, Y. J. Zhang, L. Z. Zhou, C. X. Cao, X. Y. Zhu, *Chem. Commun.,* 2016, **52,** 7950-7953; h) F. Du, Y. Ming, F. Zeng, C. Yu, S. Wu, *Nanotechnology*, 2013, 365101; i) J. Sun, F. Zeng, H. Jian, S. Wu, *Biomacromolecules*, 2013, **14**, 728-736.

5 a) X. M. Wu, X. R. Sun, Z. Q. Guo, J. B. Tang, Y. Q. Shen, T. D. James, H. Tian, W. H. Zhu, *J. Am. Chem. Soc.,* 2014, **136,** 3579-3588; b) L. W. He, Q. Y. Xu, Y. Liu, H. P. Wei, Y. H. Tang, W. Y. Lin, *ACS Appl. Mater. Interfaces,* 2015, **7,** 12809-12813; c) Y. Q. Zheng, B. C. Yu, K. L. Ji, Z. X. Pan, V. Chittavong, B. H. Wang, *Angew. Chem. Int. Ed.,* 2016, **55,** 4514-4518; d) X. L. Hu, J. M. Hu, J. Tian, Z. S. Ge, G. Y. Zhang, K. F. Luo, S. Y. Liu, *J. Am. Chem. Soc.,* 2013, **135,** 17617-17629; e) S. Chen, Q. Lei, S. Y. Li, S. Y. Qin, H. Z. Jia, Y. J. Cheng, X. Z. Zhang, *Biomaterials,* 2016, **92,** 25-35; f) F. Huang, R. Cheng, F. Meng, C. Deng, Z. Y. Zhong, *Biomacrolecules,* 2015, **16,** 2228-2236. g) Z. X. Zhou, J. B. Tang, Q. H. Sun, W. J. Murdochc, Y. Q. Shen, *J. Mater. Chem. B,* 2015, **3,** 7594-7603, h) H. H. Xiao, H. Q. Song, Q. Yang, H. D. Cai, R. G. Qi, L. Yan, S. Liu, Y. H. Zheng, Y. B. Huang, T. J. Liu, X. B. Jing, *Biomaterials,* 2012, **33,** 6507-6519.

6 a) C. E. Callmann, C. V. Barback, M. P. Thompson, D. J. Hall, R. F. Mattrey, N. C. Gianneschi, *Adv. Mater.,* 2015, **27,** 4611-4615; b) S. Y. Li, L. H. Liu, L. Rong, W. X. Qiu, H. Z. Jia, B. Li, F. Li, X. Z. Zhang, *Adv. Funct. Mater.,* 2015**, 25,** 7317-7326; c) W. C. Silvers, B. Prasai, D. H. Burk, M. L. Brown, R. L. McCarley, *J. Am. Chem. Soc.*, 2013, **135,** 309-314.

7 a) M. Roosemboom, J. N. M. Commandeur, N. P. E. Vermeulen, *Pharm. Rev.,* 2004, **56,** 53-102; b) J. Hu, L. Liu, Z. Li, R. X. Zhuo, X. Z. Zhang, *J. Mater. Chem. B*, 2016, **4,** 1932-1940; c) B. T. Adams, S. Niccoli, M. A. Chowdhury, A. N. K. Esarik, S. J. Lees, B. P. Rempel, C. P. Phenix, *Chem. Commun.,* 2015, **51,** 11390-11393; d) Y. Tang, Z. Wu, C. Zhang, X. Zhang, J. H. Jiang, *Chem. Commun.,* 2016, **52,** 3631-3634; e) J. Jang, H. Lee, A. Sharma, S. Lee, T. Lee, C. Kang, J. S. Kim, *Chem. Commun.*, 2016, **52,** 9965-9968; f) X. Hou, J. Peng, F. Zeng, S. Wu, Mater. Chem. Front. 2017. DOI: 10.1039/c6qm00112b.

View Article Online
DOI: 10.1039/C7TB00266A

8 a) E. Secret, K. Crannell, S. Kelly, M. Villancio-Wolter, M. S. Andrew, *J. Mater. Chem. B*, 2015, **3,** 5629-5634; b) S. Thamphiwatana, W. Gao, D. Pornpattananankul, Q. Zhang, V. Fu, J. Y. Li, J. M. Li, M. Obonyo, L. F. Zhang, *J. Mater. Chem. B*, 2014, **2,** 8201-8207; c) P. Huang, D. L. Wang, Y. Su, W. Huang, Y. F. Zhou, D. X. Cui, X. Y. Zhu, D. Y. Yan, *J. Am. Chem. Soc.,* 2014, **136,** 11748-11756; d) J. Wang, Y. Wu, L. Sun, F. Zeng, S. Wu, *Acta Chim Sinica*, 2016, **74**, 910-916; e) P. Díez, A. Sánchez, M. Gamella, P. Martínez-Ruíz, E. Aznar, C. Torre, J. R. Murguía, R. Martínez-Máñez, R.Villalonga, J. M. Pingarrón, *J. Am. Chem. Soc.,* 2014, **136,** 9116-9123.

9 a) Q. Li, Y. Wen, X. You, F. Zhang, V. Shah, X. Chen, D. Tong, X. Wei, L. Yin, J. Wu, *J. Mater. Chem. B*, 2016, **4,** 4675-4682; b) K. Y. Liu, H. M. Shang, X. Q. Kong, M. G. Ren, J. Y. Wang, Y. Liu, W. Y. Lin, *Biomaterials,* 2016, **100,** 162-171; c) H. Deng, X. Zhao, J. Liu, L. Deng, J. Zhang, J. Liu, A. J. Dong, *J. Mater. Chem. B*, 2015, **3,** 9397-9408; d) X. Liu, J. J. Xiang, D. C. Zhu , L. M. Jiang, Z. X. Zhou, J. B. Tang, X. G. Liu, Y. Z. Huang, Y. Q. Shen, *Adv. Mater.,* 2016, **28,** 1743–1752; e) C. Yu, X. Li, F. Zeng, F. Zheng, S. Wu, *Chem. Commun.*, 2013, 49, 403-405; f) H. Zhang, X. Zhu, Y. Ji, X. Jiao, Q. Chen, L. Hou, H. Zhang, Z. Z. Zhang, *J. Mater. Chem. B*, 2015, **3,** 6310-6326; g) C. Yu, Y. Wu, F. Zeng, S. Wu, *J. Mater. Chem. B*, 2013, **1**, 4152-4159; h) K. Han, J. Zhu, S. Wang, Z. Li, S. Cheng, X. Z. Zhang, *J. Mater. Chem. B*, 2015, **3,** 8065-8069.

10 a) Y. Shao, L. Wang, J. Fu, C. Shi, J. Xu, Y. C. Zhu, J. Mater. Chem. B, 2016, **4,** 5863-5872; b) D. W. Zheng, B. Li, C. X. Li, J. X. Fan, Q. Lei, C. Li, Z. S. Xu, X. Z. Zhang, *ACS Nano*, 2016, **10,** 8715-8722; c) M. Yu, F. Guo, F. P. Tan, N. Li, *J. Control. Release,* 2015, **215,** 91-100**.**

11 a) G. Tedeschi, S. Chen, V. Massey, *J. Biol. Chem.,* 1995, **270,** 1198-1204; b) R. M. Phillips, M. A. Naylor, M. Jaffar, S. W. Doughty, S. A. Everett, A. G. Breen, G. A. Choudry, I. J. Stratford, *J. Med. Chem.,* 1999, **42,** 4071-4080; c) C. Flader, J. W. Liu, R. F. Borch, *J. Med. Chem.,* 2000, **43,** 3157-3167.

12 a) P. Y. Gasdaska, G. Powis, P. Hyman, H. Fisher, *Cancer Res.,* 1993, **53,** 5458-5461; b) V. Fagan, S. Bonham, M. P. Carty, F. Aldabbagh, *Org. Biomol. Chem.,* 2010, **8,** 3149-3156.

13 a) W. C. Silvers, A. S. Payne, R. L. McCarley, *Chem. Commun.,* 2011, **47,** 11264-11266; b) S. U. Hettiarachchi, B. Prasai, R. L. McCarley, *J. Am. Chem. Soc.* 2014, **136,** 7575-7578; c) W. Ong, Y. M. Yang, A. C. Crucian, R. L. McCarley, *J. Am. Chem. Soc.,* 2008, **130,** 14739-14744; d) T. Fryatt, H. I. Pettersson, W. T. Gardipee, K. C. Bray, S. J. Green, A. M. Z. Slawin, H. D. Beallb, Christopher J. Moody, *Bioorg. Med. Chem.,* 2004, **12,** 1667-1687; e) B. H. Huang, A. Desai, S. Z. Tang, T. P. Thomas, J. R. Baker, *Org. Lett.,* 2010, **12,** 1384-1387; f) P. L. Liu, J. S. Xu, D. H. Yan, P. S. Zhang, F. Zeng, B. W. Li, S. Z. Wu, *Chem. Commun.,* 2015, **51,** 9567-9570.

14 a) M. E. Roth, O. Green, S. Gnaim, D. Shabat, *Chem. Rev.,* 2016, **116,** 1309-1352; b) S. Gnaim, D. Shabat, *Acc. Chem. Res.,* 2014, **47,** 2970-2984; c) A. Sagi, R. Weinstain, N. Karton and D. Shabat, *J. Am. Chem. Soc.,* 2008, **130,** 5434-5440; d) M. Shamis, H. N. Lode, and D. Shabat, *J. Am. Chem. Soc.,* 2004, **126,** 1726-1730; e) A. D. Wong, M. A. DeWit, E. R. Gillies, *Adv. Drug Deli. Rev.,* 2012, **64,** 1031-1045; e) M. Grinda, J. Clarhaut, B. Renoux, I. Tranoy-Opalinskia, S. Papot, *Med. Chem. Commun.* 2012, **3,** 68-70.

15 a) I. Tranoy-Opalinski, T. Legigan, R. Barat, J. Clarhaut, M. Thomas, B. Renoux, S. Papot , *Eur. J. Med. Chem.,* 2014, **74,** 302-313; b) T. Legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. L. Pape, S. Lerondel, S. Papot, *Angew. Chem. Int. Ed.,* 2012, **51,** 11606-11610; c) D. N. Duvaz, I. N. Duvaz, F. Friedlos, J. Martin, P. Lehouritis, R. Marais and C. J. Springer, *J. Med. Chem.,* 2003, **46,** 1690-1705; d) A. D. Corso, M. Caruso, L. Belvisi, D. Arosio, U. Piarulli, C. Albanese, F. Gasparri, A. Marsiglio, F. Sola, S. Troiani B. Valsasina, L. Pignataro, D. Donati, C. Gennari, *Chem. Eur. J.* 2015, **21,** 6921-6929.

16 a) A. D. Wong, A. L. Prinzen, E. R. Gillies, *Polym. Chem.,* 2016, **7,** 1871-1881; b) R. A. McBride, E. R. Gillies, *Macromolecules,* 2013, **46,** 5157-5166; c) B. Fan, J. F. Trant, A. D. Wong, E. R. Gillies, *J. Am. Chem. Soc.,* 2014, **136,** 10116-10123.

17 a) M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail, G. A.Sim, *J. Am. Chem. Soc.,* 1966, **88,** 3888−3890; b) Y. H. Hsiang, R. Hertzberg, S. Hecht, L. F. Liu, *J. Biol. Chem.,* 1985, **260,** 4873−4878. c) E. J. Kim, S. Bhuniya, H. Lee, H. M. Kim, C. Cheong, S. Maiti, K. S. Hong, J. S. Kim, *J. Am. Chem. Soc.,* 2014, **136,** 13888-13894.

18 a) L. A. Carpino, S. A. Triolo, R. A. Berglund, *J. Org. Chem.,* 1989, **54,** 3303-3310; b) B. T. Cho, S. K. Kang, M. S. Kim, S. R. Ryu, D. K. An, *Tetrahedron,* 2006, **62,** 8164-8168.

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DOI: 10.1039/C7TB00266A

A DT-diaphorase-activatable theranostic prodrug has been developed for visualizing the release of active drug and enhancing the therapeutic effect.