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# Visible Light Controlled Release of Anticancer Drug through Double Activation of Prodrug

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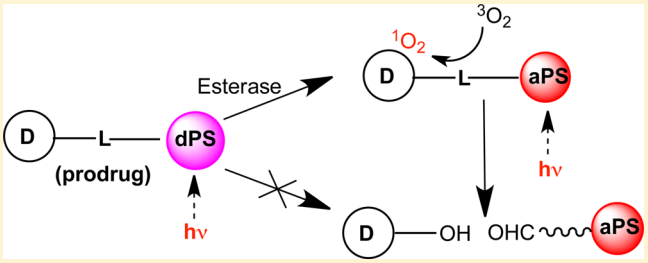
Supporting Information

**ABSTRACT:** We designed and synthesized a novel double activatable prodrug system (drug−linker−deactivated photosensitizer), containing a photocleavable aminoacrylate-linker and a deactivated photosensitizer, to achieve the spatiotemporally controlled release of parent drugs using visible light. Three prodrugs of CA-4, SN-38, and coumarin were prepared to demonstrate the activation of deactivated photosensitizer by cellular esterase and the release of parent drugs by visible light (540 nm) via photounclick chemistry. Among these prodrugs, nontoxic coumarin prodrug was used to quantify the release of parent drug in live cells. About 99% coumarin was released from the coumarin prodrug after 24 h of incubation with MCF-7 cells followed by irradiation with low intensity visible light (8 mW/cm$^2$) for 30 min. Less toxic prodrugs of CA-4 and SN-38 killed cancer cells as effectively as free drugs after the double activation.




**KEYWORDS:** *Prodrug, anticancer drug, aminoacrylate, photounclick, visible/near IR, photosensitizer*

To improve therapeutic effects of drugs and to minimize side effects, prodrugs (PDs) can be designed to be activated by a specific stimulus.[1−5] Light is a very attractive tool for activation of deactivated forms of drugs such as PDs and drugs entrapped in delivery vehicles,[6] and it acts as an external tool for more active control for spatiotemporal activation.[7,8] UV light has been used for spatiotemporal activation of PDs and caged compounds at the cellular level.[9−12] However, it could not be applied to tissues due to the limited tissue penetration, up to 1 mm, and toxicity.[6] While visible and near IR light can reach deeper tissue, about 1 cm,[7] its energy is too low to directly cleave protecting groups. To circumvent this problem, an innovative idea was proposed, taking advantage of a unique chemistry of singlet oxygen that can be generated by irradiation of visible and near IR light to photosensitizers (PSs) of PDs. Drugs are conjugated with singlet oxygen-labile olefinic linkers and then singlet oxygen cleaves the linkers via a spontaneous cleavage of a dioxetane formed by [2 + 2] cycloaddition reaction of singlet oxygen with the olefins.[13−15] However, the idea has not been successfully demonstrated using biological systems due to the limitation of available olefinic linkers and difficulties in their synthesis.

We recently reported a photocleavable aminoacrylate linker that can be synthesized via click chemistry and then cleaved by singlet oxygen.[16] Because singlet oxygen can be generated by the combination of visible/near IR light (400−800 nm) and a corresponding PS, an aminoacrylate linker can be cleaved by such low energy light via singlet oxygen. In fact, the cleavage of an aminoacrylate linker by the combination of 690 nm and dithiaporphyrin PS was verified in our previous experiments.[16] On the basis of the photounclick chemistry, here we first successfully demonstrate the visible light-triggered PDs of anticancer compounds. In particular, we prepared a double activatable PD system to prove the concept of dPS, which could be further engineered to improve specificity of activation.[17−19] As a model activation stimulus in vitro, we used cellular esterase, by which only intracellular dPS will be activated. Thus, the dPS of the PD will be activated first by intracellular esterase and then the drug can be released upon irradiation (Figure 1). In addition, we expected dPS makes the conjugates less vulnerable to unwanted degradation under normal room light condition, which was a tedious problem of the conjugates with non-dPSs.

To test the double activatable PD concept, two PDs **10** and **11** were prepared from two cytotoxic compounds (SN-38 and CA-4) as shown in Scheme 2. SN-38 is an active metabolite of irinotecan (CPT-11, topoisomerase I inhibitor), and it is at least 1000 times more active than irinotecan.[20] CA-4 is the active component of combretastatin A-4 phosphate, an antiangiogenic and antimitotic agent.[21] However, due to the highly toxic nature of these drugs (CA-4 and SN-38), detailed studies for the first activation (hydrolysis of dPS by esterase) and the second activation (release of free drug by irradiation) were hampered by cell death at the experimental concentration

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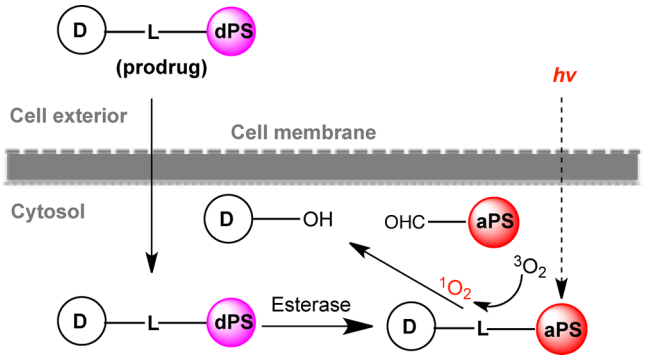
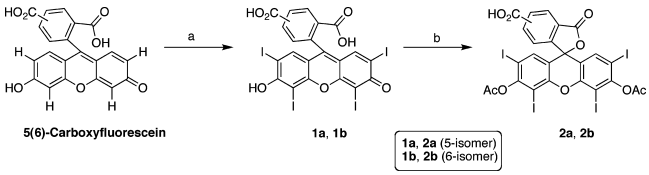



**Figure 1.** Schematic representation of the mechanism involved in the prodrug for the double activation: hydrolysis and "photo-unclick chemistry". **D**, drug; **L**, linker; dPS, deactivated photosensitizer; aPS, activatable photosensitizer; *hv*, light.

(10 $\mu$M). Thus, the prodrug **9** from nontoxic coumarin was prepared for the quantitative studies of the two activation steps, avoiding the interference by the released drug (coumarin). The esterase-activatable PS **2** was prepared in two steps from 5(6)-carboxyfluorescein: tetraiodination followed by diacetylation (Scheme 1). Respectively, the hydroxyl group of drugs was

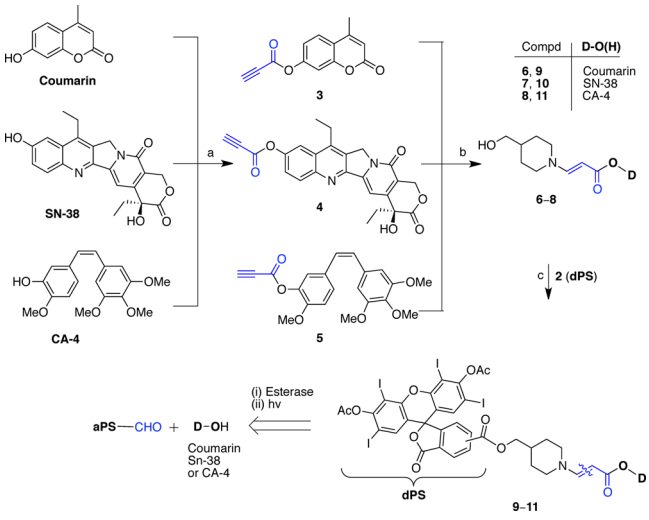
**Scheme 1. Preparation of dPS (2)**[a]




[a]Reagents: (a) iodine, NaI, $NaHCO_3$; (b) dry pyridine, anhydrous acetic anhydride.

esterified by Steglich esterification[22] with propiolic acid in the presence of DCC and DMAP to yield **3**–**5** (Scheme 2). The intermediates **6**–**8** were then synthesized via a click (yne-amine) reaction by stirring compounds **3**–**5** and 4-piperidinemethanol. Finally, the esterification between the hydroxyl group of intermediates **6**–**8** and the carboxyl group of **2** gave PDs **9**–**11** (Scheme 2). The PDs were prepared in three steps under mild conditions with high yields (Scheme 2 and Supporting Information). The purity of PDs **9**–**11** was verified to be above 95% by HPLC.

**Scheme 2. Preparation of PDs (9–11)**[a] **and Schematic Representation of Aminoacrylate Linker Cleavage**




[a]Reagents: (a) propiolic acid, DCC, DMAP, dry $CH_2Cl_2$/dry DMF; (b) 4-piperidinemethanol, THF; (c) **2**, DCC, dry $CH_2Cl_2$.

Both fluorescence spectroscopy and HPLC indicated the effective activation of dPS by cellular esterase. Since dPS **2** has no fluorescence and the activatable PS (aPS) **1** has a fluorescence quantum yield of 0.25 (Table S1 in the Supporting Information), activation of dPS should result in large fluorescence emission. MCF-7 cells were incubated in the presence of 10 $\mu$M of compound (**2**, **9**, or **10**) in 200 $\mu$L of complete medium (DMEM + 10% FBS + 1% antibiotics) per well in a 96-well microplate. After 24 h of incubation, the medium was removed and the cells were lysed with 100 $\mu$L of DMSO. Then, fluorescence emission of the cell lysate was recorded (Figure 2A). Larger emissions were detected from the

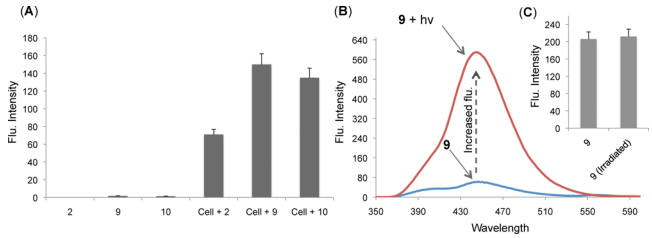



**Figure 2.** (A) Fluorescence emission (570 nm) of **2**, **9**, and **10** (ext. 520 nm): Samples in DMSO (left three bars) and in cell lysate in DMSO (right three bars). (B) Increased coumarin fluorescence intensity of **9** from blue (before irradiated) to red spectrum (after irradiated at 320 nm). (C) Fluorescence emission (440 nm, ext. 320 nm) of **9** with or without irradiation in cell-free medium.

cell lysates compared to the sample solutions in DMSO (10 $\mu$M), presumably, due to the activation of dPS in cells. However, dPS was not activated solely by the complete medium. When these compounds were added to the cell-free complete medium and incubated for 24 h, no significant increase in fluorescence intensity was observed. To quantify the activation of dPS of **9**, **9** and the acetyl-hydrolyzed product of **9** were determined by HPLC (Figures S25 and S27 in the Supporting Information), detected at 11 and 7 min, respectively (mobile phase: 100% acetonitrile and a flow-rate of 0.3 mL/min). On the basis of the standard curve of **9**, it was estimated that 36% of **9** was recovered from incubated MCF-7 cells with 10 $\mu$M of **9**. Accordingly, about 64% of **9** seemed to be activated.

The cleavage of aminoacrylate linker after irradiation was supported by the increased coumarin fluorescence of **9** by irradiation. (The relative stability of drugs (coumarin, CA-4, and SN-38) against oxidation by singlet oxygen was suggested in section 1.3 of the Supporting Information.) PD **9** had negligible coumarin emission compared to that of free coumarin at the equimolar concentration (Figure S24 in the Supporting Information). In the presence of 10 $\mu$M of **9**, MCF-7 cells were incubated for 24 h to activate dPS. Then, the samples were irradiated by visible light (540 ± 10 nm, 8 mW/cm$^2$, 30 min). The irradiated sample showed 10-time increased coumarin fluorescence emission compared to the sample before irradiation, presumably due to the release of coumarin (Figure 2B). In contrast, when **9** was irradiated in the cell-free medium,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it did not show any significant increase of the fluorescence (Figure 2C). The released free coumarin was also detected and quantified by HPLC based on the standard curve of free coumarin (Figures S26 and S27 in the Supporting Information). To our surprise, almost 99% of **9** released coumarin; but only 64% of dPS of **9** was activated when irradiated. It seems that the singlet oxygen in aPS of **9** cleaved not only the linker of itself but also linkers in other PD molecules. These results indicated the activation of dPS by intracellular esterase and the release of coumarin from **9** upon irradiation with light.

The rapid reaction of aminoacrylate linker with singlet oxygen was further demonstrated by a competitive photooxygenation kinetics study. We determined the second order rate constant ($k$) of the aminoacrylate linker. It was determined to be $2.5 \times 10^7$ $M^{-1}$ $s^{-1}$ (section 1.4 of the Supporting Information). The oxidation by singlet oxygen, not by superoxide radical, was also suggested by kinetic study with specific ROS quenchers. The singlet oxygen quenchers (DABCO and $\beta$-carotene) effectively delayed the oxidation of the aminoacrylate linker, but the superoxide radical quencher (1,4-benzoquinone) did not make any delay of the oxidation of the linker (Figure S23).

We next evaluated the biological activity of PDs with or without irradiation to prove the concept of the double activatable PDs (Table 1). The addition of the bulky group

**Table 1. Toxicities of Drugs SN-38 and CA-4 (in Dark) and the PDs 10 and 11 (Dark Toxicity and Phototoxicity)[a]**

| compd | dark toxicity, $IC_{50}$, nM | phototoxicity, $IC_{50}$, nM |
|---|---|---|
| SN-38 | 170 | nt |
| CA-4 | 8 | nt |
| **10** | 820 | 218 |
| **11** | 116 | 13 |

[a]nt, not tested; $IC_{50}$, 50% inhibitory concentration against MCF-7 cells.

of the dPS-linker effectively reduced the dark toxicity of CA-4 and SN-38. PDs **10** and **11** were 4.8 and 14.5 times less toxic than the parent drugs (SN-38 and CA-4) without irradiation. On the other hand, the irradiation restored the potent activity of SN-38 and CA-4 from the PDs. The $IC_{50}$ values of the phototoxicity of **10** and **11** were very close to those of the dark-toxicity of CA-4 and SN-38. This was consistent with the above cleavage result of **9**, where nearly 100% of **9** released the coumrain after irradiation. We could thus estimate that [**D-L**-dPS]*initial* = [drug]*after irradiation* and, thus, $IC_{50}$ of drug without irradiation ≈ $IC_{50}$ of PD with irradiation. The double activatable PDs worked very well.

It was also indicated that released drugs, not the photodynamic effect (i.e., direct cell damage by singlet oxygen), were primarily responsible for the phototoxicity of the PDs. Since singlet oxygen can also kill cells and the dPS of the PD is activated inside cells, we questioned whether the phototoxicity of PDs came from the released drugs or from singlet oxygen. To examine this idea, the cells were treated with PD **11** in 96-well microplates but only half of each well was irradiated for 30 min. The other half was protected from light using black tape. Fluorescence images of live cells covering both halves were taken after another 96 h of incubation (Figure 3A). Since singlet oxygen has limited diffusion distance (~10−300 nm),[23−25] the singlet oxygen generated in the irradiated half of the wells cannot damage cells in the unirradiated half.

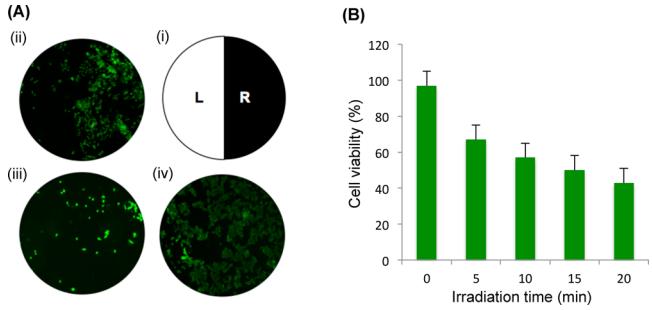


**Figure 3.** (A) Fluorescence-microscopy images (10× magnification) of live cells: (i) L = irradiated area of a well, R = unirradiated area of the same well; (ii) cells treated with **2** (1 $\mu$M) and irradiated; (iii) cells treated with **11** (25 nM) and irradiated; (iv) cells treated with **11** (25 nM) without irradiation. (B) Light-dose dependent cell damage: cells treated with **11** (25 nM) and then irradiated with 540 ± 10 nm at 8 mW/cm$^2$ from 0 to 20 min; the average of at least triplicates.

However, the released drugs are stable and, thus, can damage entire wells by diffusion. Indeed, in wells treated with PS **2**, the unirradiated half looked healthy at a similar density to those of the control wells (Figure 3A-ii). In stark contrast, the wells, treated with **11** (25 nM), showed only a few live cells in the unirradiated half at a similar density to that of the irradiated area (Figure 3A-iii). In addition, the control of **11** (25 nM) was monitored without irradiation (Figure 3A-iv). The data clearly demonstrated that the released drugs, not singlet oxygen, killed the cells in the unirradiated area. On the other hand, the irradiated cells seemed to be more damaged by PDT effects. The live cell density of the irradiated side was less than the unirradiated area treated with **11** (Figure 3A-iii).

We also tested whether the light could control the drug dose, which is a major advantage of an external stimulus. Light-dose dependent cell survival was determined (Figure 3B). MCF-7 cells were treated with **11** (25 nM). After 24 h of incubation, the cells were exposed to the visible light (540 ± 10 nm, 8 mW/cm$^2$) for 0−20 min. The cell damage was dependent on light dose. It seemed that drug dose could be controlled by light dose with this singlet oxygen-mediated release strategy.

Last, we proved that the conjugate (**11**) with dPS was much more stable than a conjugate with non-dPS. Under a low intensity fluorescent lamp (0.8 mW/cm$^2$), 96% of **11** was intact even after 48 h while only 5% of a conjugate with a non-dPS was intact after 3 h.

In conclusion, we successfully proved a novel strategy of double activatable PDs in a tissue culture model with several significant advancements. Almost 99% prodrug released parent drug in 30 min by the irradiation with very low intensity light. Unmodified cancer drugs were released by visible light irradiation via photounclick chemistry while modified drugs (e.g., formylated drugs) were released from previously tested linkers, vinyl diether, or dithioether linkers. The dPS was used to make the double activatable PDs, which could provide more precisely controlled release of drugs in cells. The cell-kill effect of the released drugs was demonstrated, which could be controlled by light dose. In addition, dPS made the conjugate more stable than a conjugate with a non-dPS against the unwanted photodegradation.

We envision that the proposed singlet oxygen-mediated release strategy will be applicable for other drug delivery systems, where a new effective way of drug release control is a key need. The facile synthesis of aminoacrylate linker can be


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easily adopted for polymers, dendrimers, and other nanocarrier platforms. The proposed strategy can provide versatility in terms of drugs, PSs, and activation mechanism of dPS. In particular, since the release was mediated by singlet oxygen, any combination of a PS and corresponding light can be used for release. Given the current availability of PSs, almost any wavelength between 400 and 800 nm can be used for the activation. Preclinical studies with a mouse tumor model are currently in progress.

## ■ ASSOCIATED CONTENT

### Supporting Information

NMR, HPLC, HRMS, experimental details, and biological test methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

A. M. L. H., M. B., and Y. Y. designed the project. A.M.L.H and Y.Y. wrote the manuscript. A.M.L.H. prepared the conjugates and carried out the evaluations of the compounds. M.B. and G.N. prepared starting materials for the conjugate synthesis. S.G.A. carried out the kinetics study with singlet oxygen.


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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

CA-4, Combretastatin A-4; D, drug; DCC, *N*,*N*′-dicyclohexylcarbidiimide; L, linker; PD, prodrug; PDT, photodynamic therapy; PS, photosensitizer (aPS, activatable PS = non-dPS; dPS, deactivated PS); ROS, reactive oxygen species; SN-38, 7-ethyl-10-hydroxy-camptothecin

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