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PAPER

Long conjugated 2-nitrobenzyl derivative caged anticancer prodrugs with visible light regulated release: preparation and functionalizations†

Chunyan Bao,[a] Ming Jin,*[b] Bo Li,[c] Yaodong Xu,[a] Jingyan Jin[a] and Linyong Zhu*[a]

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A series of anticancer prodrugs with different chemical functional groups were prepared, in which the styryl conjugated 2-nitrobenzyl derivatives were introduced as the phototrigger to regulate the drug (chlorambucil) release. Compared to the common 4,5-dimethoxy-2-nitrobenzyl caged compounds, most of the prodrugs exhibited large and redshifted one-photon absorption within the visible range. One-photon excitation for the drug release was studied by measuring UV-vis absorption, FT-IR, and HPLC spectra, which suggested that chlorambucil was released effectively and precisely by manipulating external light conditions. And the introduction of different functional groups made this type of prodrug a good platform to further react with some typical drug carriers and to further form excellent visible light responsive drug delivery systems. Moreover, the drug also could be effectively released under the excitation of two-photon at 800 nm with comparable photorelease efficiencies.

Introduction

Recently, the design and synthesis of stimuli-responsive prodrugs that exhibit reduced toxicity in health tissues and can be transformed into pharmacologically active agents specifically within the tumor regions have aroused considerable interest for the development of modern chemotherapy of malignant tumors.[1–3] Among the stimuli-responsive strategies for prodrugs, light exhibits a predominant advantage. It is an external stimulus that could allow a very mild activation of sensitive molecules and provides a greater selectivity in terms of control over the moment and the location of drug-release without physically disturbing organized biological systems. To date, considerable effort have been put into preparing photoactived prodrugs.[4] For example, we recently reported two types of photoactived prodrugs based on coumarin as phototrigger[5] or *N*-methyl-4-picolinium as photolabile group by photo-induced electron transfer (PET) with semiconductor quantum dots (QDs).[6]

Among the reported photoactivated groups, 2-nitrobenzyl derivatives (ONB) have gained wide acceptance and a lot of caged biologically active compounds that have been prepared belong to this series[4b,c,7,8] due to their versatile modification and well-known photolysis mechanism.[9] However, most of the known ONB-caged chromophores showed UV absorption, which is the major drawback and limitation for the practical application, since the presence of UV light is damaging to cells as it also provides poor penetration due to light scattering and absorbance by intrinsic biological chromophores.[10] Recently, the Jullien and Bolze groups reported that the elongation of the conjugation backbone for the nitro group and the introduction of D–π–A backbone would redshift the maximum wavelength of absorption, and enlarge the corresponding molar absorption coefficient as well as the two-photon absorption (TPA) cross section.[11] Therefore, the present paper would utilize the styryl-2-nitrobenzyl (SNB) platform as cage group to prepare chlorambucil prodrugs photoactivated either by one-photon visible light or two-photon NIR light.

Moreover, to reduce systemic toxicity and enhance therapeutic efficacy, the photoactivated prodrugs always need to incorporate with carriers prior to the actual applications, *e.g.* nanoscale carriers promise both prolonged circulation time due to the nanoscale size and selective tumor accumulation *via* the enhanced permeability and retention (EPR) effects. The widely used carriers mainly contained biocompatible macromolecules,[12] inorganic nanoparticles,[4c,5,7c] liposomes,[13] and protein nanocomposites.[14] To achieve this aim, linker chemistry is developed between the photoactivated prodrugs and carriers because it provides more precise control in terms of the density and orientation of the caged biomolecules and forms a stable linkage under

[a]*Shanghai Key Laboratory of Functional Materials Chemistry, Institute of Fine Chemicals, East China University of Science and Technology, Shanghai 200237, P. R. China. E-mail: linyongzhu@ecust.edu.cn; Fax: +86-21-64253742*
[b]*Institute of Functional Polymer Materials, School of Materials Science and Engineering, Tongji University, Shanghai 200237, P. R. China. E-mail: mingjin@tongji.edu.cn; Fax: +86-21-69580143*
[c]*Key Laboratory of Polar Materials and Devices, Ministry of Education, Department of Electronic Engineering, East China Normal University, Shanghai, 200241, P. R. China*

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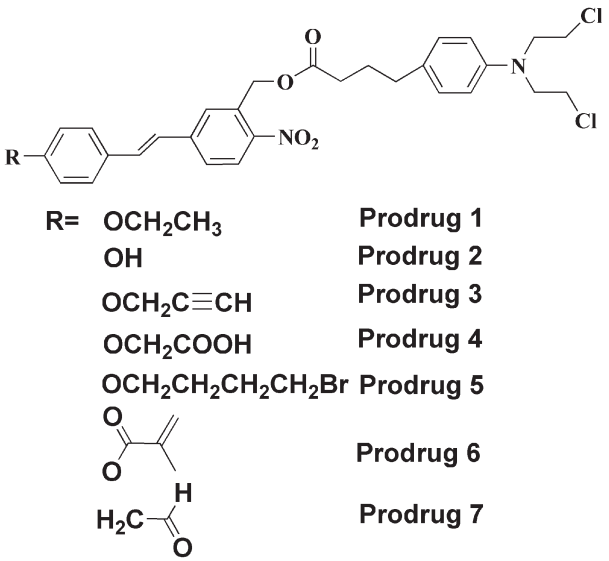


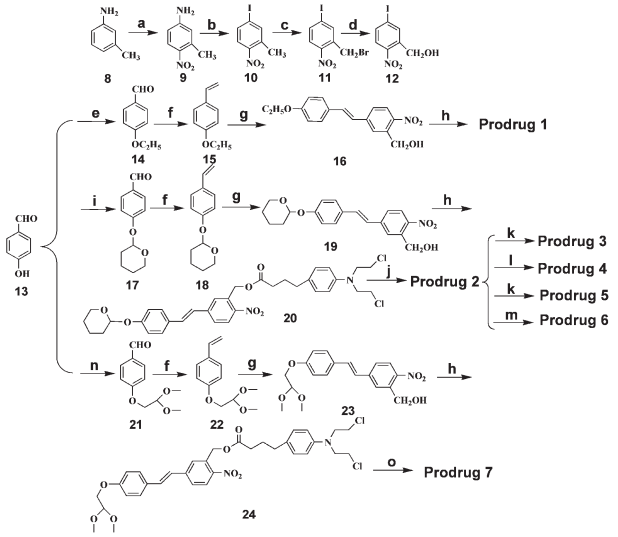
Scheme 1 Structures of designed SNB based prodrugs **1–7**.

in vivo conditions. Herein, we designed and synthesized a series of styryl conjugated 2-nitrobenzyl-caged (SNB) chlorambucil prodrugs (**1–7**) with different functional linkage (as shown in Scheme 1) that expected to conjugate with carriers. The successful controllable drug release regulated by either one-photon visible light or two-photon NIR laser light suggested that the prepared model prodrug served well as a photo-cage for the drug whose release can be regulated precisely by controlling the irradiation conditions. And the different chemical functionalization was hopeful to provide a new promising strategy for conjugating the prodrugs with carriers to further form excellent photo-responsive drug delivery system.

Results and discussion

Design and synthesis of SNB prodrugs

Thinking about molecular engineering to extend the maximum absorption wavelength and increase the molar absorptivity, elongation of the conjugated π-system or an increase in the power of the donor and acceptor side groups for ONB photo-trigger was required. Although the introduction of the stilbene decreased the photolysis quantum yield Φ,[11] the advantages – visible light absorption and suitable two-photon sensitivity – made them still regarded as an excellent photo-cage, especially for bioactive systems. Here, a styrene group was introduced to increase the length of the 2-nitrobenzyl conjugated system and unfunctionalized ethoxyl was used as the electron-pushing group for prodrug **1** which would be treated as model prodrug in our paper to investigate photochemical properties, such as the UV-Vis, FT-IR, and HPLC spectra for the photorelease of the combined chlorambucil drug. Furthermore, considering the incorporation with carriers, several functional groups including hydroxyl, alkynyl, carboxylic acid, alkyl bromide, methacrylate, aldehyde (see prodrug **2–7**) were introduced to form pre-functional prodrugs. The introduction of these functional groups were proposed to conjugated with the corresponding carboxylic



Scheme 2 The schematic synthesis procedures for the prodrugs **1–7**. *Regeants and conditions*: a, H_2SO_4 (85%), guanidinium nitrate, at 0–5 °C; b, H_2SO_4/H_2O, $NaNO_2$, 0 °C for 10 min, then KI at 0 °C for 30 min, rt for 3 h; c, BPO/NBS, CCl_4, reflux for 36 h; d, Na_2CO_3, acetone/water, reflux for 48 h; e, K_2CO_3/KI, bromoethane, acetone, reflux; f, $Ph_3P^+CH_3Br^-$, *t*-BuOK, THF, rt; g, **12**, $Pd(OAc)_2/N(OEt)_3$, 110 °C for 12 h; h, chlorambucil, DCM/DCC/DMAP, rt for 12 h; i, *p*-hydroxybenzaldehyde **13**, PPTS/3,4-2*H*-dihydropyran/DCM, rt; j, TFA, DCM, rt; k, 3-bromopropyne or 1,4-dibromobutane, K_2CO_3/acetone or acetonitrile; l, *tert*-butyl 2-bromoacetate/K_2CO_3/acetonitrile, rt for 24 h, then TFA/DCM; m, methacryloyl chloride, $N(Et)_3$/DCM, 0 °C, 12 h; n, *p*-hydroxybenzaldehyde, 2-bromo-1,1-dimethoxyethane K_2CO_3/KI/DMF, 100 °C overnight; o, TFA/DCM, rt for 2 h.

acid, azide, amino, hydroxyl, and thiol contained carriers by simple esterification, amidation, efficient "click chemistry", and thiol-ene reactions, respectively.[15] Especially, the introduction of methacrylate was also expected to copolymerization in some biocompatible macromolecules besides the conjugation with thiol-containing carriers by thiol-ene reaction.

The synthetic methods for the prodrugs were described in Scheme 2. Prodrug **1** was obtained by the esterification of chlorambucil with benzyl alcohol **16** in the presence of dicyclohexylcarbodiimide (DCC) and 4-*N*,*N*-dimethylaminopyridine (DMAP) in 78.7% yield. The latter was obtained by Heck reaction between 4-ethoxystyrene (compound **15**) and 5-iodo-2-nitrobenzyl alcohol (compound **12**) in the presence of a palladium catalyst.[16] Compound **15** was synthesized in two steps from 4-hydroxylbenzaldehyde by (1) alkylation with bromoethane to provide compound **14**; (2) reduction by Witting reaction. Compound **12** was obtained in four steps from m-toluidine by (1) nitration utilising guanidinium nitrate in 85% sulfuric acid as a nitrating agent;[17] (2) diazotation with sodium nitrite followed by nucleophilic substitution with iodide to provide 4-iodo-2-methyl-1-nitrobenzene **10**; (3) bromination by *N*-bromosuccinimide (NBS) to afford 4-iodo-2-bromomethyl-1-nitrobenzene **11**; (4) hydrolysis to 4-iodo-2-hydroxymethyl-1-nitrobenzene **12**. Prodrug **2** was also synthesized similar as for the prodrug **1** except 3,4-dihydro-2H-pyran was used to protect and finally release of the hydroxyl group.[18] Prodrugs **3–6** were prepared from prodrug **2** by different alkylation and esterification,

Table 1 Photochemical properties of prodrugs **1**–**7**[a]

Prodrugs	λ_{max} (nm)	ε_{max} ($\times 10^4$)[b]	ε_λ = 400 nm ($\times 10^4$)[c]	Φ_{chem} ($\times 10^{-4}$)[d]	$\Phi_{chem} \times \varepsilon_\lambda$ = 400 nm[e]
1	376	2.33	1.73	6.7	11.6
2	374	2.17	1.61	5.2	8.4
3	369	2.22	1.03	11.4	11.7
4	372	2.46	1.56	3.2	5.0
5	375	2.06	1.51	6.0	9.1
6	351	2.27	0.51	15.4	7.9
7	372	2.27	1.20	7.3	8.8

[a] All experiments were done in acetonitrile solution with concentration of 10^{-4} M. [b] Molar absorptivity at maximum absorption wavelength. [c] Molar absorptivity at 400 nm. [d] Photolysis quantum yield for the consumption of prodrugs upon the irradiation of 400 nm (10 mw cm^{-2}). [e] The overall photolysis efficiency: the photolysis quantum yield and molar absorptivity at 400 nm.

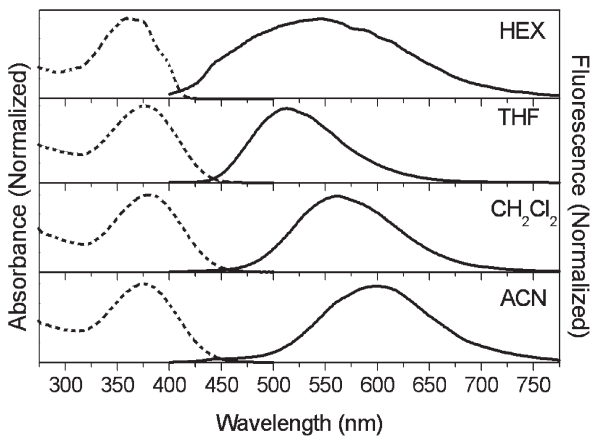


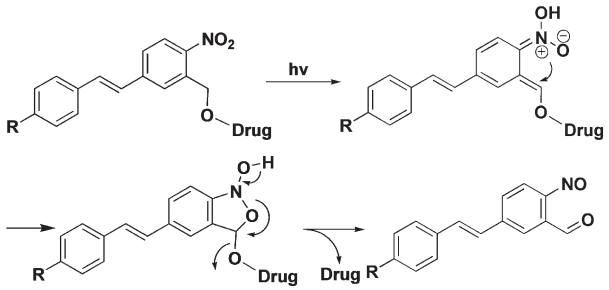
Fig. 1 Normalized absorption (dashed) and fluorescence spectra (λ_{ex} = 380 nm) (solid) of prodrug **1** in solvents of different polarity.

respectively. Prodrug **7** was obtained by the similar synthesis procedure as for prodrug **1** and **2** excepting 2-bromo-1,1-dimethoxyethane was used to react with 4-hydroxylbenzaldehyde, and finally aldehyde substituted prodrug was generated by TFA-mediated deacetalization.[19]

Photophysical and photolysis results

The absorption properties of the prodrugs are summarized in Table 1. Except for prodrug **6** (electron-drawing substitution), the maximum absorption of the prodrugs is located around 370 nm and the absorption band extended up to 450 nm which is red-shifted about 20 nm compared with the popular 4,5-dimethoxy-2-nitrobenzyl phototriggers, as well as the molar absorption coefficient increased four times.[11a,20] It suggested that the introduction of donating power and π-conjugated stilbene prolonged the absorption wavelength and enlarged the molar absorption coefficient, as well as provided the opportunity for the one-photon photorelease by visible light. Due to the similar absorption properties for the different substitutions, prodrug **1** was selected as the model to investigate the concrete photophysical and photochemical properties in the following experiments as mentioned above.

First, the normalized UV-vis and fluorescence spectra of prodrug **1** in various solvents of increasing polarity are shown in



Scheme 3 A schematic presentation for photocleavage of the prodrugs.

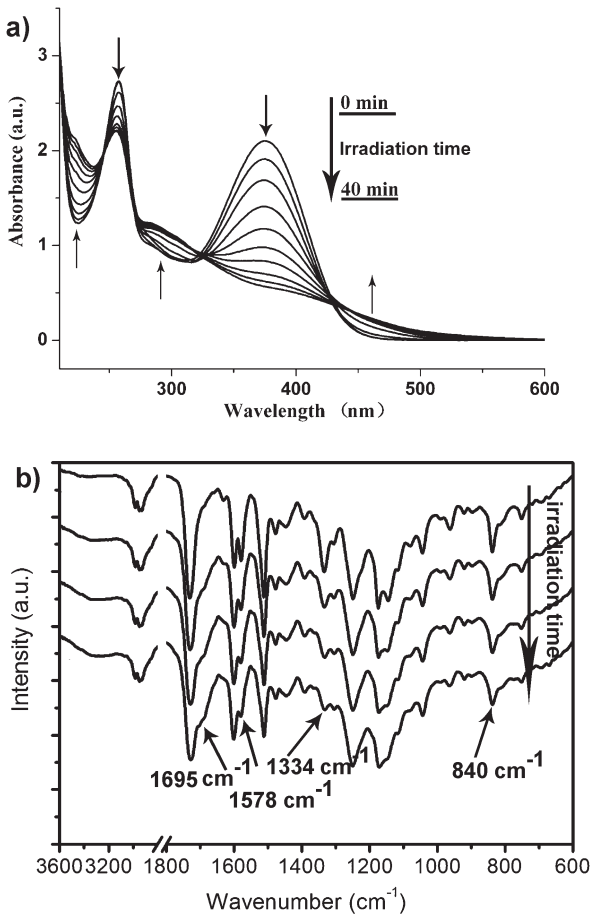


Fig. 2 One-photon irradiation ($\lambda \geq 400$ nm, 120 mW cm^{-2}) of a solution of prodrug **1** in acetonitrile (10^{-4} M). (a) Evolution of the UV-vis absorption spectra of the solution as a function of time (t (min) = 0, 1, 5, 10, 15, 20, 25, 30, 35, 40). (b) Evolution of the IR spectra of the solution as a function of time (t (min) = 0, 5, 15, 30), the sample was prepared by dropping the solution on a KBr pellet and tested after drying under vacuum.

Fig. 1. It is found that the polarity influenced the position of the longest absorption and emission peaks.[21] Then, drug release experiments of prodrug **1** were proceeded by exposing an acetonitrile solution (10^{-4} M) to the visible light ($\lambda \geq 400$ nm). Based on the photolysis mechanism for ONB phototriggers,[11a] prodrug **1** was photolyzed to yield the parent chlorambucil and corresponding 2-nitroso-5-styrylbenzaldehyde derivatives as photo byproducts (as shown in Scheme 3). Fig. 2a and 2b

displayed typical evolution of the UV-vis and FT-IR spectra as a function of time upon illumination of visible light ($\lambda \geq 400$ nm, 120 mW cm^{-2}). In Fig. 2a, a rapid decrease in absorbance around 376 nm was observed, which suggested the photolysis of SNB was proceeded effectively and gradually. And the appearance of isosbestic points at 246, 269, 324, and 431 nm indicated a relatively clean photochemical reaction for prodrug **1** upon visible light irradiation. In the FT-IR spectra shown in Fig. 2b, the signal of the characteristic peaks belonging to NO_2 groups at 1578 cm^{-1} and 1334 cm^{-1} were reduced and a new peak at 1695 cm^{-1} belonging to nitrosoaldehyde appeared and increased as a function of irradiation time, which further confirmed the drug release proceeded well upon illumination of visible light.

To check the kinetics of the photorelease process, HPLC analysis was performed. As illustrated in Fig. 3a, the release processes progressed effectively for prodrug **1** upon the irradiation with visible light ($\lambda \geq 400$ nm, 120 mW cm^{-2}), and the maximum release of chlorambucil reached 68% after 50 min exposure. The incomplete photolysis for prodrug **1** may be attributed to an internal filtering effect from the photo-byproduct. Precise control of the photolytic release was demonstrated by monitoring the progress of chlorambucil release after periods of exposure to light and dark conditions, as shown in Fig. 3b. The distinctive "stepped" profile revealed that the drug release only proceeded under light conditions, thus realizing "light-regulated precise release". The photolysis was further studied in PBS buffer solution containing 50% acetonitrile. As shown in Fig. 3c, the photorelease was also effectively carried out, although the release rate was a little slower than that in acetonitrile. This may be attributed to the relatively bad solubility for prodrug in PBS solution. The photorelease stability was also validated with the high remaining rate of the prodrugs (larger than 95%) by keeping the prodrug solution in the dark for 48 h. All these results indicated that this kind of SNB-based phototriggers were stable enough for the biological application and would act as the photo-responsive linker between drug and carrier, and the drug release could be precisely controlled by manipulating external light conditions.

The sensitivity of prodrug **1** to two-photon excitation was also studied and quantified as the product of TPA cross-section δ_a and the uncaging quantum yield Φ. The δ_a value was determined to 20 GM by using a 80 fs-pulsed, mode-locked Ti:sapphire laser at 800 nm by the method of Z-scan technique. Photorelease experiment with two-photon excitation for prodrug **1** was measured by HPLC and graphed as a function of time as shown in Fig. 4. It showed that the release of chlorambucil was progressed effectively and it reached 20% after 2 h with a ~0.2 mm diameter beam spot. Although the overall photorelease efficiency, $\delta_a\Phi$, was not high (about 14 mGM at 800 nm), there were some successful examples to support the biological applications, even at the single-cell level.[8a,22]

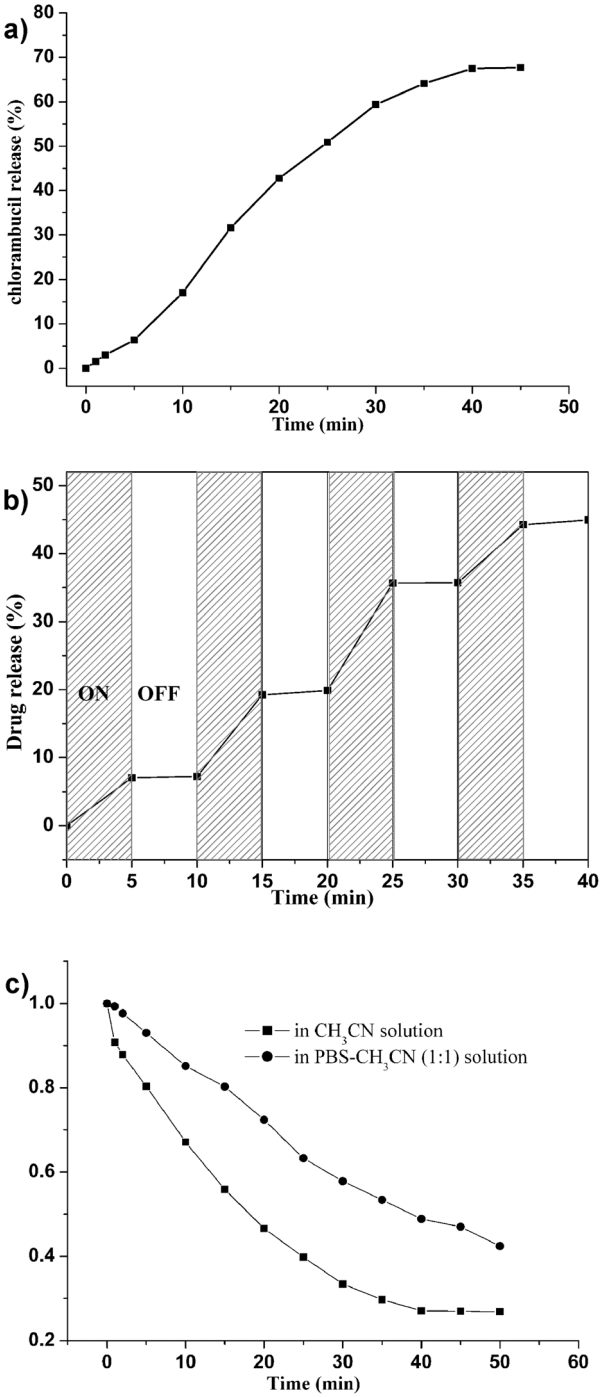


Fig. 3 (a) The time course of drug release for prodrug **1** under visible light irradiation ($\lambda \geq 400$ nm, 120 mW cm^{-2}) determined by HPLC analysis. (b) The partial progress for the release of chlorambucil from prodrug **1** in light and dark conditions. "ON" indicates the beginning of light irradiation; "OFF" indicates the ending of light irradiation. (c) The comparison for time course of drug release of prodrug **1** in different solvent under the same irradiation conditions.

Conclusions

We synthesized a new series of SNB caged prodrugs coupled with various functional groups. Upon either one or two-photon excitation, chlorambucil could be released in a controlled way using visible or NIR light at 800 nm, which avoided the use of UV light and is more suitable for biological applications. In particular, the introduction of different chemical linkage provided opportunity for the prodrugs to further conjugate with different biocompatible carriers, which provided a new promising

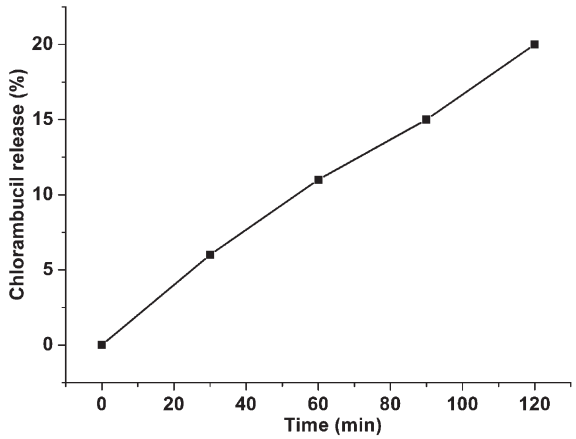


Fig. 4 Time course of two-photon excited drug release from prodrug **1** at 800 nm (0.60 mJ cm^{-2} per pulse). Sample (10^{-4} M) was irradiated in acetonitrile solution.

platform for designing and preparing intelligent drug delivery system with precise modulation by manipulating external light conditions.

Experimental

General

Materials. All reagents were purchased from commercial available sources such as Aldrich, TCI or Fisher and used without further purification. Tetrahydrofuran (THF) was distilled over sodium/benzophenone. Acetonitrile and dichloromethane (CH_2Cl_2) were distilled from calcium hydride before use, and Et_3N was redistilled from and dried over KOH pellets.

Characterization. Proton and carbon nuclear magnetic resonance spectra (^1H, ^{13}C NMR) were recorded on a Bruker Avance 400 MHz spectrometer. Chemical shifts were reported in parts per million (ppm) downfield from the Me_4Si resonance which was used as the internal standard when recording ^1H NMR spectra. Mass spectra were recorded on a Micromass GCTTM and a Micromass LCTTM. Absorption spectra were recorded on a Shimadzu UV-2550 UV-vis spectrometer. FT-IR spectra were obtained on a Nicolet 380 FT-IR spectrometer with KBr pellets. The steady-state fluorescence experiments were performed on a Varian Cary Eclipses fluorescence spectrometer. Samples for absorption and emission measurements were measured using 1 cm × 1 cm quartz cuvettes. Reversed-phase HPLC using a BetaBasic-18 column was analyzed by Agilent technologies 1200 series.

Preparation of prodrugs

(*E*)-5-(4-Ethoxystyryl)-2-nitrobenzyl 4-(4-(bis(2-chloroethyl)-amino)phenyl)butanoate (Prodrug 1). Under protection of argon, compound **16** (300 mg, 1.0 mmol) and chloroambucil (335 mg, 1.1 mmol) were dissolved in dichloromethane at room temperature in the presence of DCC (248 mg, 1.2 mmol) and DMAP (12 mg, 0.1 mmol), the reaction was kept at rt for 12 hours. Then the mixture was filtered and the solvent was evaporated. The obtained crude was purified by silica gel chromatography (hexane–ethyl acetate, 8 : 1) to afford a yellow solid Prodrug **1** (460 mg, 78.7% yields). ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.10 (d, J = 8.6 Hz, 1H), 7.59 (s, 1H), 7.52 (d, J = 8.6 Hz, 1H), 7.46 (d, J = 8.7 Hz, 2H), 7.18 (d, J = 16.3 Hz, 1H), 7.07 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 16.3 Hz, 1H), 6.91 (d, J = 8.7 Hz, 2H), 6.63 (d, J = 8.5 Hz, 2H), 5.52 (s, 2H), 4.05 (q, J = 7.0 Hz, 2H), 3.66 (t, J = 7.2 Hz, 4H), 3.58 (t, J = 7.2 Hz, 4H), 2.59 (t, J = 7.4 Hz, 2H), 2.46 (t, J = 7.4 Hz, 2H), 2.03–1.93 (m, 2H), 1.43 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 159.6, 145.2, 143.9, 137.7, 133.1, 128.7, 128.4, 127.0, 126.0, 125.4, 123.8, 114.8, 63.6, 62.8, 14.8. ES-HRMS (m/z): calcd for $C_{31}H_{35}Cl_2N_2O_4$, 585.1926 $[M + H]^+$; found, 585.1923.

(*E*)-5-(4-Hydroxystyryl)-2-nitrobenzyl 4-(4-(bis(2-chloroethyl)-amino)phenyl)butanoate (Prodrug 2). In the dark, yellow solid **20** (600 mg, 0.94 mmol) was dissolved in 20 mL dichloromethane and was treated with TFA (1.0 g, 8.8 mmol) at room temperature for 24 hours. Then, the solvent was evaporated and prodrug **2** was obtained as a yellow solid 390 mg (74.6%) after silica gel column chromatography (hexane–ethyl acetate, 6 : 1). ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.14 (d, J = 8.6 Hz, 1H), 7.60 (s, 1H), 7.55 (d, J = 8.6 Hz, 1H), 7.41 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 16.3 Hz, 1H), 7.08 (d, J = 8.2 Hz, 2H), 6.96 (d, J = 16.3 Hz, 1H), 6.86 (d, J = 8.2 Hz, 2H), 6.62 (d, J = 8.2 Hz, 2H), 5.55 (s, 2H), 5.26 (s, 1H), 3.68 (t, J = 7.0 Hz, 4H), 3.60 (t, J = 7.0 Hz, 4H), 2.61 (t, J = 7.4 Hz, 2H), 2.48 (t, J = 7.4 Hz, 2H), 2.05–1.96 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 173.1, 156.6, 145.6, 143.5, 133.1, 132.9, 129.7, 128.7, 126.7, 126.0, 125.6, 123.8, 115.9, 112.5, 63.2, 53.7, 40.4, 34.0, 33.6, 26.7. ESI-HRMS (m/z): calcd for $C_{29}H_{31}Cl_2N_2O_5$, 557.1610 $[M + H]^+$; found, 557.1609.

(*E*)-2-Nitro-5-(4-(prop-2-yn-1-yloxy)styryl)benzyl 4-(4-(bis-(2-chloroethyl)amino)phenyl)butanoate (Prodrug 3). In the dark, yellow prodrug **2** (100 mg, 0.18 mmol) and potassium carbonate (50 mg, 0.36 mmol) were dissolved in acetone (20 mL) at room temperature. After injecting 3-bromopropyne (26 mg, 0.22 mmol), the mixture was kept stirring at room temperature for another 6 hours. Finally, the obtained mixture was filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane–ethyl acetate, 6 : 1) to afford 90 mg prodrug **3** as a yellow solid with 84.1% yield. ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.13 (d, J = 8.4 Hz, 1H), 7.61 (s, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.48 (d, J = 8.1 Hz, 2H), 7.20 (d, J = 16.3 Hz, 1H), 7.07 (d, J = 7.9 Hz, 2H), 7.01 (d, J = 8.1 Hz, 2H), 6.98 (d, J = 16.3 Hz, 1H), 6.61 (d, J = 7.9 Hz, 2H), 5.54 (s, 2H), 4.74 (s, 2H), 3.67 (t, J = 7.0 Hz, 4H), 3.60 (t, J = 7.0 Hz, 4H), 2.60 (t, J = 7.2 Hz, 2H), 2.55 (s, 1H), 2.46 (t, J = 7.1 Hz, 2H), 2.04–1.95 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 172.9, 158.2, 145.7, 144.4, 143.4, 132.9, 130.3, 129.7, 128.4, 126.8, 126.0, 125.7, 124.4, 115.3, 112.2, 78.2, 75.8, 63.1, 55.9, 53.6, 40.5, 34.0, 33.6, 26.7. ES-HRMS (m/z): calcd for $C_{32}H_{33}Cl_2N_2O_5$, 595.1767 $[M + H]^+$; found, 595.1766.

(*E*)-2-(4-(3-(((4-(4-(Bis(2-chloroethyl)amino)phenyl)butanoyl)-oxy)methyl)-4-nitrostyryl)phenoxy)acetic acid (Prodrug 4). In the dark, a mixture of prodrug **2** (100 mg, 0.18 mmol), *tert*-butyl

2-bromoacetate (60 mg, 0.31 mmol) and potassium carbonate (50 mg, 0.36 mmol) in acetonitrile (20 mL) were stirred at room temperature for 24 hours. The reaction mixture was filtered and the solvent evaporated. The residual was purified by silica gel chromatography (hexane–ethyl acetate, 4 : 1) to afford BOC-protected intermediate as a yellow solid (97 mg, 78.2%).^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.12 (d, J = 8.5 Hz, 1H), 7.60 (s, 1H), 7.56 (d, J = 8.5 Hz, 1H), 7.47 (d, J = 8.7 Hz, 2H), 7.19 (d, J = 16.3 Hz, 1H), 7.07 (d, J = 8.6 Hz, 2H), 6.98 (d, J = 16.3 Hz, 1H), 6.91 (d, J = 8.7 Hz, 2H), 6.61 (d, J = 8.6 Hz, 2H), 5.53 (s, 2H), 4.56 (s, 2H), 3.67 (t, J = 7.0 Hz, 4H), 3.60 (t, J = 7.0 Hz, 4H), 2.60 (t, J = 7.4 Hz, 2H), 2.46 (t, J = 7.4 Hz, 2H), 2.02–1.94 (m, 2H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 172.8, 167.7, 158.6, 145.7, 143.4, 133.0, 132.9, 130.0, 129.6, 128.5, 126.9, 126.0, 124.3, 115.0, 82.6, 65.6, 63.2, 54.4, 39.8, 34.0, 33.5, 33.4, 38.0, 26.7. ES-HRMS (m/z): calcd for $C_{35}H_{41}Cl_2N_2O_7$, 671.2291 $[M + H]^+$; found, 671.2291. Then, the obtained intermediate (80 mg, 0.12 mmol) was dissolved in dichloromethane (10 mL) in dark and was treated with TFA (200 mg, 8.8 mmol) at room temperature for 24 h. Finally, prodrug **4** was obtained as a yellow solid (50 mg, 68.2%) after silica gel column chromatography (hexane–ethyl acetate, 2 : 1). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.12 (d, J = 8.5 Hz, 1H), 7.61 (s, 1H), 7.56 (d, J = 8.5 Hz, 1H), 7.48 (d, J = 8.6 Hz, 2H), 7.19 (d, J = 16.3 Hz, 1H), 7.07 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 16.3 Hz, 1H), 6.95 (d, J = 8.6 Hz, 2H), 6.61 (d, J = 8.6 Hz, 2H), 5.53 (s, 2H), 4.72 (s, 2H), 3.68 (t, J = 6.8 Hz, 4H), 3.59 (t, J = 6.8 Hz, 4H), 2.60 (t, J = 7.4 Hz, 2H), 2.46 (t, J = 7.4 Hz, 2H), 2.02–1.95 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 173.0, 158.0, 145.7, 144.3, 143.2, 132.9, 132.6, 130.5, 130.2, 129.7, 128.6, 126.9, 125.7, 124.8, 115.0, 112.3, 64.7, 63.2, 53.6, 40.5, 34.0, 33.5, 33.4, 26.7. ES-HRMS (m/z): calcd for $C_{31}H_{33}Cl_2N_2O_7$, 615.1665 $[M + H]^+$; found, 615.1667.

(*E*)-5-(4-(4-Bromobutoxy)styryl)-2-nitrobenzyl 4-(4-(bis(2-chloroethyl)amino)phenyl)butanoate (Prodrug 5). In the dark, a mixture of prodrug **2** (100 mg, 0.18 mmol), 1,4-dibromobutane (54 mg, 0.25 mmol) and potassium carbonate (50 mg, 0.36 mmol) in 25 mL acetonitrile was stirred and heated to 55 °C. After keeping at this temperature for 5 hours, the reaction mixture was filtered and the solvent was evaporated. The crude mixture was purified by silica gel chromatography (hexane–acetate, 6 : 1) to afford prodrug **5** as a yellow solid (110 mg, 88.5%). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.12 (d, J = 8.5 Hz, 1H), 7.61 (s, 1H), 7.56 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 8.5 Hz, 2H), 7.20 (d, J = 16.3 Hz, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 16.3 Hz, 1H), 6.91 (d, J = 8.5 Hz, 2H), 6.61 (d, J = 8.4 Hz, 2H), 5.54 (s, 2H), 4.04 (t, J = 6.0 Hz, 2H), 3.68 (t, J = 6.3 Hz, 4H), 3.60 (t, J = 6.3 Hz, 4H), 3.51 (t J = 6.5 Hz, 2H), 2.61 (t, J = 7.5 Hz, 2H), 2.47 (t, J = 7.4 Hz, 2H), 2.13–2.05 (m, 2H), 2.03–1.94 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 172.9, 159.6, 145.6, 144.4, 143.5, 133.1, 132.9, 130.4, 129.7, 128.9, 128.5, 126.7, 126.0, 125.6, 123.9, 114.9, 112.2, 67.0, 63.2, 53.6, 40.5, 34.0, 33.6, 33.4, 29.4, 27.8, 26.7. EI-HRMS (m/z): calcd for $C_{33}H_{37}BrCl_2N_2O_5$, 690.1263 $[M + H]^+$; found, 690.1258.

(*E*)-5-(4-(Methacryloyloxy)styryl)-2-nitrobenzyl 4-(4-(bis(2-chloroethyl)amino)phenyl)butanoate (Prodrug 6). In the dark, prodrug **2** (68 mg, 0.12 mmol) with methacryloyl chloride (26 mg, 0.25 mmol) in the presence of triethylamine (37 mg, 0.36 mmol) were dissolved in dichloromethane (20 mL) and kept at 0 °C for 12 hours. The reaction mixture was filtered and the solvent was evaporated in vacuum. The residual was purified by silica gel chromatography (hexane–ethyl acetate, 4 : 1) to afford prodrug **6** as a yellow solid (55 mg, 69.9% yield). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 8.13 (d, J = 8.6 Hz, 1H), 7.64 (s, 1H), 7.58 (d, J = 8.6 Hz, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.23 (d, J = 16.3 Hz, 1H), 7.16 (d, J = 8.4 Hz, 2H), 7.08 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 16.3 Hz, 1H), 6.60 (d, J = 8.4 Hz, 2H), 6.37 (s, 1H), 5.78 (s, 1H), 5.54 (s, 2H), 3.67 (t, J = 7.0 Hz, 4H), 3.59 (t, J = 7.0 Hz, 4H), 2.60 (t, J = 7.5 Hz, 2H), 2.47 (t, J = 7.4 Hz, 2H), 2.08 (s, 3H), 2.03–1.96 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 172.9, 165.7, 151.3, 145.9, 144.4, 142.9, 135.7, 133.7, 133.0, 132.3, 129.7, 128.0, 127.0, 126.2, 126.0, 125.9, 122.2, 112.2, 63.1, 53.5, 40.5, 34.0, 33.6, 26.8,18.4. ES-HRMS (m/z): calcd for $C_{33}H_{35}Cl_2N_2O_6$, 625.1872 $[M + H]^+$; found, 625.1875.

(*E*)-2-Nitro-5-(4-(2-oxoethoxy)styryl)benzyl 4-(4-(bis(2-chloroethyl)amino)phenyl)butanoate (Prodrug 7). In the dark, compound **24** (100 mg, 0.15 mmol) in dichloromethane (15 mL) was treated with TFA (120 mg, 1 mmol) at room temperature for 2 hours, and target prodrug **7** was obtained as a yellow solid (55 mg, 59.2% yield) after column chromatography. ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): δ = 9.88 (s, 1H), 8.13 (d, J = 8.5 Hz, 1H), 7.61 (s, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.49 (d, J = 8.5 Hz, 2H), 7.20 (d, J = 16.3 Hz, 1H), 7.09 (d, J = 8.2 Hz, 2H), 7.00 (d, J = 16.3 Hz, 1H), 6.92 (d, J = 8.5 Hz, 2H), 6.68 (d, J = 8.2 Hz, 2H), 5.54 (s, 2H), 4.63 (s, 2H), 3.68 (t, J = 6.6 Hz, 4H), 3.61 (t, J = 6.6 Hz, 4H), 2.61 (t, J = 7.3 Hz, 2H), 2.46 (t, J = 7.3 Hz, 2H), 2.02–1.94 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): δ = 198.6, 172.9, 158.1, 145.8, 143.2, 132.9, 132.7, 130.1, 129.8, 128.6, 126.9, 126.0, 125.7, 124.7, 114.9, 112.8, 72.7, 63.2, 53.9, 40.2, 34.0, 33.5, 26.7. EI-HRMS (m/z): calcd for $C_{31}H_{32}Cl_2N_2O_6$, 598.1637 $[M]^+$; found, 598.1645.

General methods for the photolysis and drug release

One-photon photolytic reaction of the prodrugs **1–7** was performed by irradiating the solution (10^{-4} M in acetonitrile or acetonitrile-PBS solution (1 : 1)) with a CHFXM-500W lamp ($\lambda \geq 400$ nm with power of 120 mW cm^{-2}). Between certain time intervals, a small aliquot (20 μL) of the suspension was taken out and analyzed by reversed-phase HPLC using a Beta-Basic-18 column eluted with a mixture of 90% acetonitrile and 10% methanol for all prodrugs in acetonitrile solution except prodrug **7** was eluted with 90% methanol and 10% acetonitrile and prodrug **1** in acetonitrile/PBS solution was eluted with 90% methanol and 10% water at a flow rate of 0.5 mL min^{-1}. The chromatogram was plotted by using absorbance detection at 254 nm.

The characterization of the two-photon absorption cross section: two-photon absorption cross-sections were determined by the method of Z-scan technique. The 800 nm pump source was from the fundamental of a fs mode-locked Ti:sapphire laser system (output beam ≈ 80 fs duration and 250 kHz repetition rate). The laser was focused on a quartz cuvette with an optical

path length of 1 mm. The sample was dissolved in CH_3CN at a concentration of 10^{-4} M. Two-photon absorption cross-section (δ) was obtained as follows: by assuming a spatially and temporally Gaussian profile for laser beam, the normalized energy transmittance $T(z)$, for two-photon absorption (2PA) can be given as

$$T(z) = \frac{1}{\sqrt{\pi} q_0} \int_{-\infty}^{\infty} \ln[1 + q_0 \exp(-x^2)] \mathrm{d}x, \qquad (1)$$

where $q_0 = \beta I_0 L_{\mathrm{eff}}$, β is 2PA coefficient, $L_{\mathrm{eff}} = [1 - \exp(-\alpha l)]/\alpha$, α is linear absorption coefficient, and l is the sample path length. The 2PA coefficient can be obtained by fitting the open aperture Z-scan traces by using eqn (1). From the 2PA coefficient β, 2PA cross section (δ_{TPA}) can be deduced using the relation $\delta_{\mathrm{TPA}} = h\nu\beta/N_0$, where N_0 is the number density of prodrug molecules dispersed in the solution.

The two-photon photolysis was performed by irradiating with a 80 fs pulses mode-locked Ti:sapphire laser at 800 nm and at a repetition rate of 250 kHz in a quartz cuvette. The NIR beam was focused onto an ester suspension placed in a stirrer-containing 10 × 10 mm cuvette. With a beam spot diameter of approximately 0.20 mm, the excitation density used for prodrug **1** was 0.60 mJ cm^{-2} per pulse. The data were collected as described as that for one-photon photolysis.

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