Photoregulated Drug Release

International Edition: DOI: 10.1002/anie.201508901
German Edition: DOI: 10.1002/ange.201508901

A *p*-Hydroxyphenacyl–Benzothiazole–Chlorambucil Conjugate as a Real-Time-Monitoring Drug-Delivery System Assisted by Excited-State Intramolecular Proton Transfer

Shrabani Barman, Sourav K. Mukhopadhyay, Sandipan Biswas, Surajit Nandi, Moumita Gangopadhyay, Satyahari Dey, Anakuthil Anoop,* and N. D. Pradeep Singh**

Abstract: *Among the well-known phototriggers, the p-hydroxyphenacyl (pHP) group has consistently enabled the very fast, efficient, and high-conversion release of active molecules. Despite this unique behavior, the pHP group has been ignored as a delivery agent, particularly in the area of theranostics, because of two major limitations: Its excitation wavelength is below 400 nm, and it is nonfluorescent. We have overcome these limitations by incorporating a 2-(2′-hydroxyphenyl)benzothiazole (HBT) appendage capable of rapid excited-state intramolecular proton transfer (ESIPT). The ESIPT effect also provided two unique advantages: It assisted the deprotonation of the pHP group for faster release, and it was accompanied by a distinct fluorescence color change upon photorelease. In vitro studies showed that the p-hydroxyphenacyl–benzothiazole–chlorambucil conjugate presents excellent properties, such as real-time monitoring, photoregulated drug delivery, and biocompatibility.*

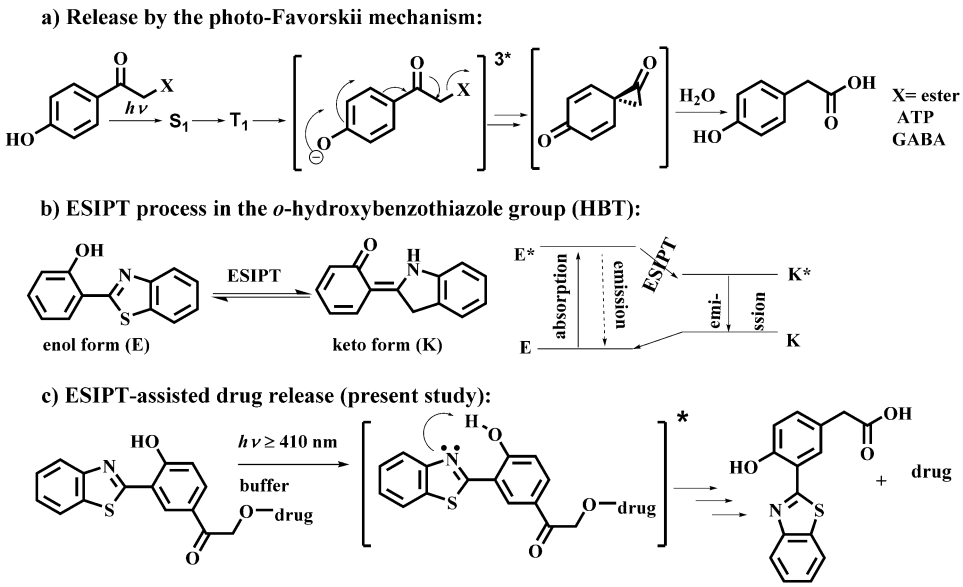
The *p*-hydroxyphenacyl (pHP) group is an efficient phototrigger for the study of very fast biological processes, such as the release of neurotransmitters and secondary messengers,[2] enzyme switches, and the release of nucleotides.[3,4] On excitation, cleavage of the pHP group proceeds efficiently through four steps:[5] I) formation of the triplet intermediate, II) deprotonation of the phenolic group, III) bond reorganization to a putative spirodienedione (Favorskii intermediate), and IV) hydrolytic ring opening of the spirodiketone to yield *p*-hydroxyphenylacetic acid [Scheme 1, Eq. (a)]. The pHP group has been used extensively to cage biomolecules owing to its salient features:[6] I) rapid and clean release, II) high photochemical efficiency, III) synthetic accessibility, IV) ready installation on most substrates, and V) the formation of a transparent (no inner-filter effect) biocompatible photoproduct. Despite these unique properties, its use as a delivery agent in theranostics has remained unexplored, mainly because of two major limitations of the pHP group: Its excitation wavelength is below 400 nm, and it is nonfluorescent. If we could modify the pHP group to obtain a fluorescent phototrigger that can be excited at wavelengths greater than 400 nm, while maintaining its salient features, the resulting phototrigger might exhibit rapid and clean release of active molecules in the visible wavelength region with strong fluorescence. This modified pHP group might be a promising alternative to the existing *o*-nitrobenzyl,[7,8] coumarylmethyl,[9] and quinoline[10] derivatives for the design of photoresponsive drug-delivery systems (DDSs).

Excited-state intramolecular proton transfer (ESIPT) processes have generated much attention because of their desirable properties,[1] such as a fluorescence band with a large Stokes shift, a low inner-filter effect, and low self-quenching. Excited-state intramolecular proton transfer is an ultrafast enol–keto phototautomerization process occurring on the excited-state surface of many intramolecularly H-bonded molecules. This process leads from an initial enol form to its phototautomeric keto form or vice versa [Scheme 1, Eq. (b)]. As a result, these chromophoric systems give rise to dual fluorescence emission: fluorescence originating from the enol form and fluorescence with a large redshift from its tautomeric keto form. Because of these interesting fluorescence properties, a variety of applications have been explored in major areas, such as laser dyes, photostabilizers,[11] radiation scintillators, luminescent materials,[12] molecular probes,[13] sensing ions,[14] and photoswitching of polymorphs.[15] Among the various ESIPT molecules, 2-(2′-hydroxyphenyl)benzothiazole (HBT) has been a compound of great interest because of its very fast and efficient ESIPT effect. The HBT moiety also shows dual emission, which is highly sensitive to solvent polarity and the pH value.[16]

For these and other reasons, we have designed a novel photoresponsive DDS, designated as *p*-hydroxyphenacyl–benzothiazole–chlorambucil (pHP-Benz-Cbl), with a built-in ESIPT substituent by incorporating the HBT moiety on the pHP group. This system has a number of advantages: The excitation wavelength of the pHP group is extended to greater than 400 nm, and the combined chromophores can act as an environment-sensitive fluorophore. This fluorophore can assist in the deprotonation of the phenol moiety of the pHP group to effect faster release and is now capable of a distinct fluorescence color change on photorelease, thus leading to excellent real-time monitoring during rapid delivery of the anticancer drug [Scheme 1, Eq. (c)].

[*] S. Barman, S. Biswas, S. Nandi, M. Gangopadhyay, A. Anoop, N. D. P. Singh
Department of Chemistry, Indian Institute of Technology Kharagpur 721302, West Bengal (India)
E-mail: ndpradeep@chem.iitkgp.ernet.in
S. K. Mukhopadhyay, S. Dey
Department of Biotechnology, Indian Institute of Technology Kharagpur 721302, West Bengal (India)

Supporting information for this article can be found under http://dx.doi.org/10.1002/anie.201508901.



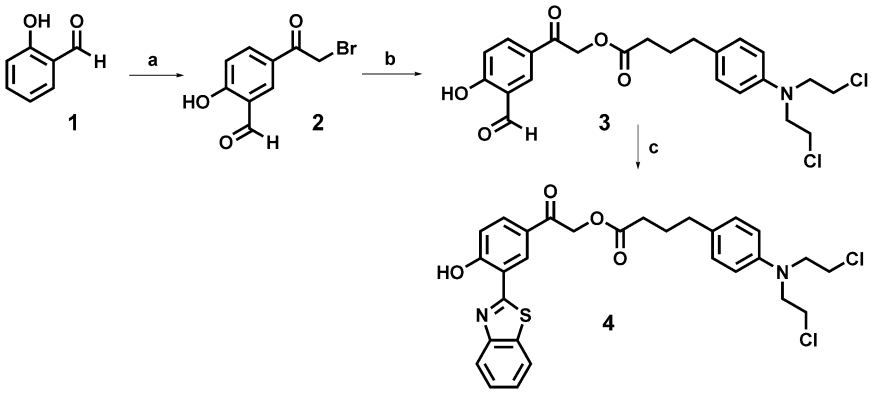
Scheme 1. a) Mechanism of the photorelease of the pHP group. b) ESIPT mechanism of HBT. c) Mechanism of the ESIPT-assisted photorelease of the pHP group. ATP = adenosine 5′-triphosphate, GABA = γ-aminobutyric acid.

For the synthesis of pHP-Benz-Cbl, salicylaldehyde (**1**) was first converted into compound **2** by Friedel–Crafts acylation with bromoacetyl bromide according to a previously reported procedure (Scheme 2).[17] Next, **2** was treated with the anticancer drug chlorambucil (Cbl) in the presence of potassium carbonate (K_2CO_3) in dry dimethylformamide (DMF) at room temperature for 4 h to afford **3**. Finally, the treatment of **3** with 2-aminothiophenol at reflux for 1 h in dimethyl sulfoxide (DMSO) yielded our DDS, pHP-Benz-Cbl (**4**), in moderate yield (70%). The product obtained in each step was characterized by ^{1}H NMR, ^{13}C NMR, and FTIR spectroscopy as well as HRMS (see Figures S1–S8 in the Supporting Information).

The photophysical properties of pHP-Benz-Cbl are highly sensitive to the solvent system (Figure 1). An absorption maximum observed at 350 nm in non-hydrogen-bonding solvents was assigned to **6**, the keto form of pHP-Benz-Cbl. However, in polar hydrogen-bonding solvents, we observed a red-shifted absorption maximum at 380 nm, which corresponds to **5**, the enol form of pHP-Benz-Cbl (Figure 1a). The existence of keto–enol tautomers of pHP-Benz-Cbl and their sensitivity to the solvent system are due to the ESIPT effect.[18]

The influence of the ESIPT process is further manifested in the emission behavior of pHP-Benz-Cbl. In non-hydrogen-bonding solvents (cyclohexane, benzene, chloroform), only one emission maximum at 510 nm, corresponding to the keto form of pHP-Benz-Cbl, was observed (Figure 1b). The existence of the keto form of pHP-Benz-Cbl in non-hydrogen-bonding solvents can be attributed to ultrafast proton transfer at intrinsically rapid rates from the hydroxy group of pHP to the nitrogen atom of the benzothiazole moiety (ESIPT). In contrast, in polar aprotic solvents (ACN, THF) and polar protic solvents (EtOH, MeOH), we observed two emission maxima, one near 450 nm (enol form) and another at 515 nm (keto form). The new emission maximum at 450 nm exhibited by pHP-Benz-Cbl in hydrogen-bonding solvents corresponds to the enol form that arises as a result of the restricted ESIPT process. Furthermore, as we increased the hydrogen-bond-forming ability of the solvent, higher fluorescence intensity was observed from the enol form as compared to the keto form (see Figure S11).

We examined photorelease from our DDS in the visible wavelength region by exposing a solution of pHP-Benz-Cbl (1×10^{-4} M) in acetonitrile/HEPES (1:19) buffer (20 mL) to visible light (≥410 nm) from a medium-pressure mercury lamp (125 W, incident intensity (I_0) = 2.886×10^{16} quanta s^{-1}) with a UV cutoff filter (1M $NaNO_2$ solution). The photorelease of Cbl was analyzed by reversed-phase HPLC with acetonitrile as the mobile phase at a constant flow rate (1 mL min^{-1}). The HPLC trace (Figure 2) showed the gradual disappearance of a peak at t_R = 9.15 min corresponding to pHP-Benz-Cbl, thus indicating gradual photodecomposition. On the other hand, the appearance and gradual increase in intensity of two new peaks at t_R = 7.20 and 8.20 min indicated the formation of photoproducts pHP-Benz-COOH (see



Scheme 2. Synthesis of the DDS *p*-hydroxyphenacyl–benzothiazole–chlorambucil (pHP-Benz-Cbl). Reagents and conditions: a) bromoacetyl bromide, $AlCl_3$, CH_2Cl_2, 35 °C, 15 h; b) chlorambucil, K_2CO_3, DMF, room temperature, 4 h; c) 2-aminothiophenol, DMSO, reflux, 110 °C, 1 h.

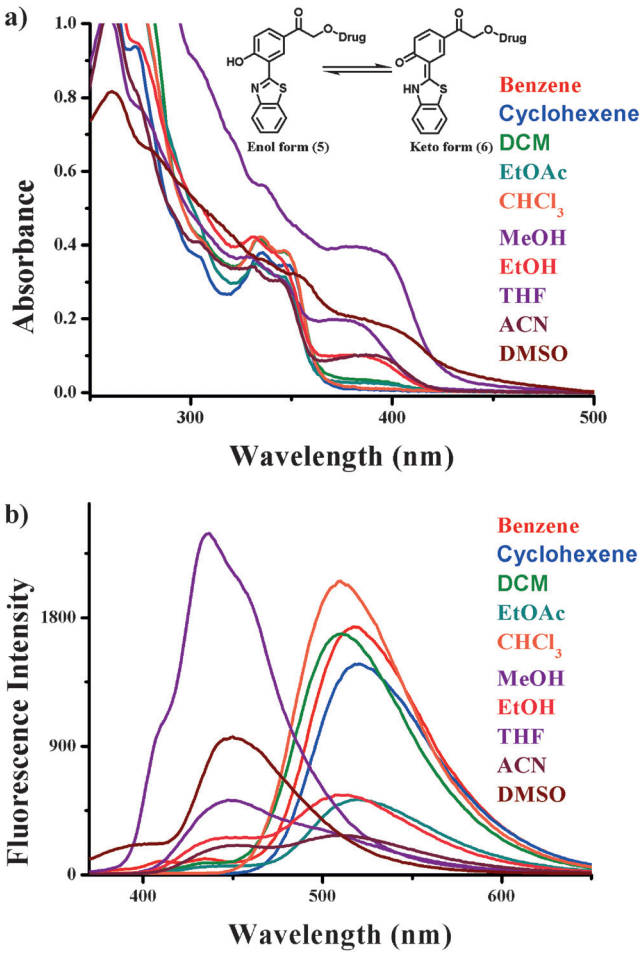


Figure 1. a) Absorption and b) emission spectra of the DDS pHP-Benz-Cbl in different solvents. ACN = acetonitrile, DCM = dichloromethane.

Figures S9 and S10) and Cbl, respectively. The quantum yield for the photorelease of the drug was calculated as 0.46 (see Table S1 in the Supporting Information).

To demonstrate the precise control over the photolytic release of the anticancer drug Cbl, we exposed pHP-Benz-Cbl to light and dark conditions separately, and found that light was entirely responsible for drug release (see Figure S12b).[19] Furthermore, we found that almost 90% of the drug had been released from pHP-Benz-Cbl after irradiation for 15 min with visible light (see Figure S12a).

A possible mechanism for the photochemical release of Cbl from pHP-Benz-Cbl in aqueous solution is summarized in Scheme 3. Except for the initial step involving a rapid ESIPT process and deprotonation, our mechanism is similar to that suggested by Givens and co-workers.[20] Upon irradiation, pHP-Benz-Cbl is excited to the singlet state, where it undergoes rapid ESIPT from the pHP group to the benzothiazole moiety via **7**, thus resulting in deprotonation of the pHP group to give intermediate **8**. The zwitterionic form then passes through efficient intersystem crossing (ISC) to its triplet excited state, which undergoes photo-Favorskii rearrangement to give a putative spirodiketone **10** with the concomitant release of the drug. The spirodiketone is then subject to hydrolytic ring opening to yield pHP-Benz-COOH (**11**).

When the photolysis of pHP-Benz-Cbl was carried out in the presence of the triplet quencher potassium sorbate (50 μM), drug release by pHP-Benz-Cbl was completely arrested, thus clearly indicating that the photorelease occurs from the triplet excited state (see Figure S13). The higher photochemical quantum yield (0.46) is only possible if ESIPT assists the deprotonation process, which further triggers the formation of the zwitterionic form **9** in the triplet excited state on exposure to light.

To support the singlet-state ESIPT mechanism, we also studied the process on the basis of a model system, a *p*-hydroxyphenacyl–benzothiazole–anisic acid conjugate (pHP-Benz-AA; see Figure S14), by using time-dependent DFT (CAM-B3LYP/6-31 + G (d,p)//CAM-B3LYP/6-31G(d)) with the Gaussian 09 software. We located the transition state on the S1 surface. The computed activation energy for ESIPT is 9.92 kcal mol^{-1}. Proton transfer in the S1 state is exothermic by −2.50 kcal mol^{-1}, and the barrier for the reverse process is 12.42 kcal mol^{-1}. On the other hand, proton transfer in the ground state is unlikely, as it is endothermic by 9.96 kcal mol^{-1}; moreover, the reverse process is nearly barrierless. The subsequent ISC is expected to be more favorable from the S1 state of pHP-Benz-AA K than from pHP-Benz-AA, because the S–T gap (ΔE_{S1-T2}) gap is smaller (2.40 kcal mol^{-1}) after proton transfer than before (ΔE_{S1-T3} = 6.94 kcal mol^{-1}). The mechanism of the next step after the ISC, the photo-Favorskii rearrangement in the triplet state, is well-known[21] and was not calculated. Our computations support singlet-state ESIPT followed by ISC and triplet-state photo-Favorskii rearrangement.

Interestingly, we noted a distinctive fluorescence color change from green to blue during the photolysis of pHP-Benz-Cbl

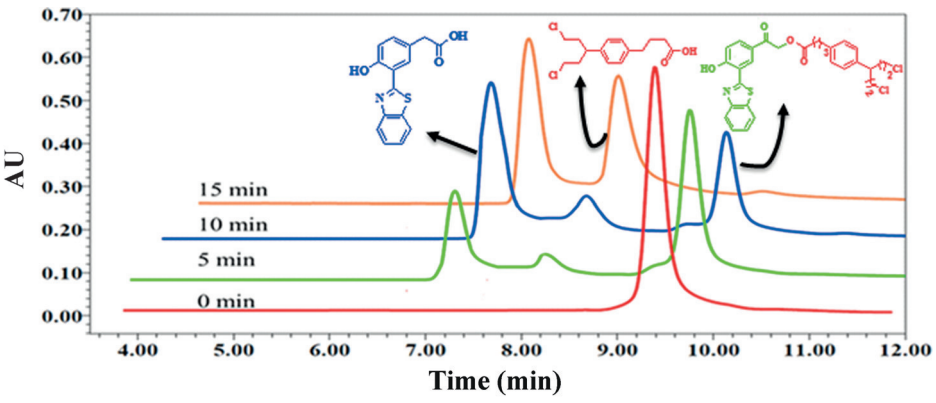
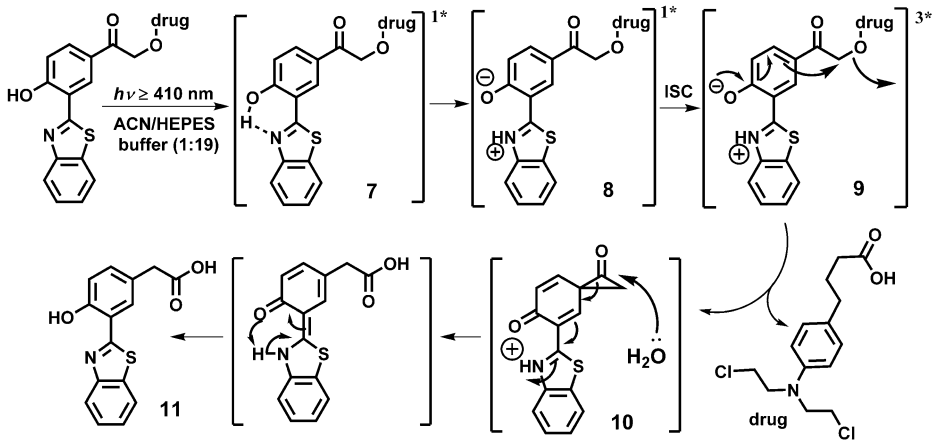


Figure 2. Overlay of HPLC chromatograms of the DDS pHP-Benz-Cbl at regular time intervals of irradiation with visible light (≥410 nm). The *y* axes are offset by 10 mAU and the *x* axes are offset by 15 s to facilitate visualization. AU = arbitrary units.



Scheme 3. Possible photorelease mechanism of the DDS pHP-Benz-Cbl.

(Figure 3a). The emission spectrum (Figure 3b) showed only a green-emission band corresponding to pHP-Benz-Cbl ($\lambda_{max} = 517$ nm) at 0 min. As the irradiation time gradually increased (0–15 min), the intense green-emission band gradually decreased, whereas a new blue-emission band at 450 nm corresponding to the photoproduct pHP-Benz-COOH gradually increased in intensity. The blueshift in the fluorescence spectrum of the photoproduct, pHP-Benz-COOH, is due to the disruption of conjugation (from a phenolic hydroxy group to a carbonyl group). A time-resolved fluorescence-decay curve[22] (see Figure S15) showed that the fluorescence lifetime (see Table S2) gradually decreased as the irradiation time increased. We also observed a decrease in steady-state fluorescence intensity[23] (see Figure S16) with an increase in irradiation time.

Real-time monitoring of drug release by our DDS (pHP-Benz-Cbl) inside malignant neoplastic disease cells (MDA-MB 231) was analyzed by confocal microscopy (Figure 4). Initially, the cells showed green fluorescence due to cellular uptake of pHP-Benz-Cbl (see Figure S17). After exposure to visible light (≥410 nm) for 10 min, we observed both green and blue fluorescence, thus indicating the partial release of Cbl by our DDS (pHP-Benz-Cbl). Finally, after irradiation for 15 min, we noted a complete fluorescence color change from green to blue (Figure 4, 2(c)), thus suggesting a greater extent of photorelease of Cbl. We also evaluated in vitro drug release by dialysis. We calculated the percentage of drug release[24] as a function of the change in fluorescence intensity (see Figure S18).

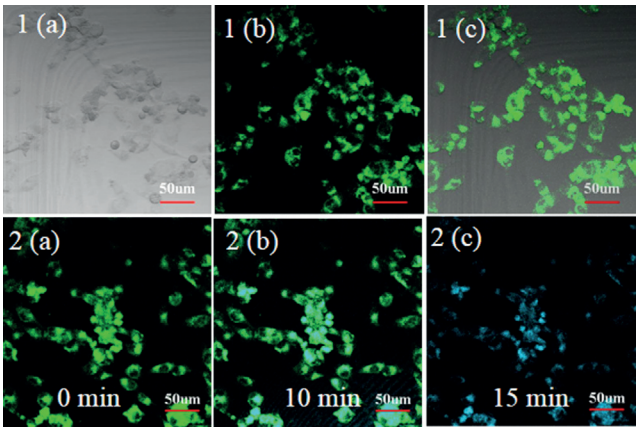


Figure 4. Confocal images of the cellular internalization of pHP-Benz-Cbl: 1 (a) bright-field image, 1 (b) fluorescence image, and 1 (c) overlay of bright-field and fluorescence images; and real-time monitoring (fluorescence images at different times during photoirradiation) of the release of the anticancer drug from pHP-Benz-Cbl on visible-light irradiation (≥410 nm): 2 (a) 0 min, 2 (b) 10 min, and 2 (c) 15 min.

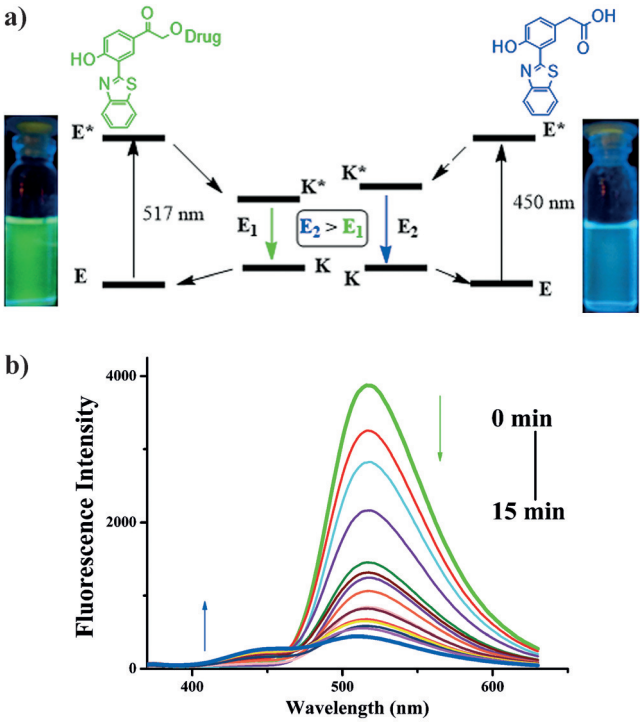


Figure 3. a) Jablonski diagram for the DDS pHP-Benz-Cbl and photoproduct pHP-Benz-COOH. b) Emission spectra of the DDS as the irradiation time is gradually increased (0–15 min).

We examined the cytotoxicity of pHP-Benz-Cbl, Cbl, and Phe-Benz-COOH in vitro by using the MTT[25] assay [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] in the MDA-MB-231 cell line. Cell viability remained above 90% at different concentrations of pHP-Benz-Cbl and pHP-Benz-COOH, whereas increasing cytotoxicity was observed on the addition of increasing amounts of Cbl (see Figure S19a). For the light-exposure experiment, in cells incubated with different concentrations of pHP-Benz-Cbl, Cbl was released on irradiation for 15 min with visible light (≥410 nm), thereby causing toxicity in

cancerous MDA-MB-231 cell lines, as validated by the MTT toxicity data (see Figure S19b) and a live–dead assay (see Figure S20).

On the other hand, no significant cell death was observed when cells were irradiated in the presence of pHP-Benz-COOH, thus indicating that the cytotoxicity was probably caused by the released drug. In comparison, with the same concentration of chlorambucil to that of pHP-Benz-Cbl, pHP-Benz-Cbl showed much lower cytotoxicity than chlorambucil. However, upon irradiation, pHP-Benz-Cbl showed enhanced cytotoxicity towards cancer cells in comparison to Cbl because of the efficient photorelease of Cbl inside the cells. Furthermore, the highest level of toxicity of pHP-Benz-Cbl towards MDA-MB-231 cells was observed for an irradiation time of 15 min (see Figure S19c).

In conclusion, ESIPT-assisted photorelease by the pHP group has been demonstrated. We have utilized the photoremovable pHP protecting group for the first time as a delivery vehicle for a chemotherapeutic agent. The pHP protecting group was designed as an environment-sensitive fluorophore, and the excitation wavelength was extended to the visible wavelength region ($\geq$410 nm). The dramatic fluorescence color change (green to blue) upon the efficient photorelease of the drug demonstrate the excellent photoresponsive DDS ability of the molecule pHP-Benz-Cbl and its outstanding properties for real-time monitoring.

Acknowledgements

We thank DST(SERB) for financial support and DST-FIST for 400 MHz NMR spectroscopy. S. Barman is grateful to IIT KGP for a fellowship.

Keywords: drug delivery · fluorescence · phototriggers · real-time monitoring · theranostics

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 4194–4198
Angew. Chem. **2016**, *128*, 4266–4270

[1] L. Xie, Y. Chen, W. Wu, H. Guo, J. Zhao, X. Yu, *Dyes Pigm.* **2012**, *92*, 1361–1369.
[2] R. S. Givens, A. Jung, C.-H. Park, J. Weber, W. Bartlett, *J. Am. Chem. Soc.* **1997**, *119*, 8369–8370.
[3] K. Zou, S. Cheley, R. S. Givens, H. Bayley, *J. Am. Chem. Soc.* **2002**, *124*, 8220–8229.
[4] R. S. Givens, C. H. Park, *Tetrahedron Lett.* **1996**, *37*, 6259–6262.
[5] J. C. Anderson, C. B. Reese, *Tetrahedron Lett.* **1962**, *3*, 1–4.
[6] R. S. Givens, J. F. W. Weber, P. G. Conrad, G. Orosz, S. L. Donahue, S. A. Thayer, *J. Am. Chem. Soc.* **2000**, *122*, 2687–2697.
[7] S. K. Choi, M. Verma, J. Silpe, R. E. Moody, K. Tang, J. J. Hanson, J. R. Baker, Jr., *Bioorg. Med. Chem.* **2012**, *20*, 1281–1290.
[8] G. Liu, C.-M. Dong, *Biomacromolecules* **2012**, *13*, 1573–1583.
[9] S. Atta, A. Jana, R. Ananthakirshnan, P. S. Narayana Dhuleep, *J. Agric. Food Chem.* **2010**, *58*, 11844–11851.
[10] M. J. Devis, C. H. Kragor, K. G. Reddie, H. C. Wilson, Y. Zhu, T. M. Dore, *J. Org. Chem.* **2009**, *74*, 1721–1729.
[11] J. Pospisil, S. Nespurek, *Prog. Polym. Sci.* **2000**, *25*, 1261–1335.
[12] Y. Zhang, J.-H. Wang, W. Zheng, T. Chen, Q.-X. Tong, D. Li, *J. Mater. Chem. B* **2014**, *2*, 4159.
[13] M. B. Cardoso, D. Samios, N. P. da Silveira, F. S. Rodembusch, V. Stefani, *Photochem. Photobiol. Sci.* **2007**, *6*, 99–102.
[14] S. Liu, L. Zhang, W. Zan, X. Yao, Y. Yang, X. Liu, *Sens. Actuators B* **2014**, *192*, 386–392.
[15] T. Mutai, H. Sawatani, T. Shida, H. Shono, K. Araki, *J. Org. Chem.* **2013**, *78*, 2482–2489.
[16] A. J. G. Strandjord, P. F. Barbara, *J. Phys. Chem.* **1985**, *89*, 2355–2361.
[17] A. Liu, L. Huang, Z. Wang, Z. Luo, F. Mao, W. Shan, J. Xie, K. Lai, X. Li, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1548–1552.
[18] S. Barman, S. K. Mukhopadhyay, M. Gangopadhyay, S. Biswas, S. Dey, N. D. P. Singh, *J. Mater. Chem. B* **2015**, *3*, 3490–3497.
[19] A. Jana, K. S. Devi, T. K. Maiti, N. D. Singh, *J. Am. Chem. Soc.* **2012**, *134*, 7656–7659.
[20] P. G. Conrad II, R. S. Givens, J. F. W. Weber, K. Kandler, *Org. Lett.* **2000**, *2*, 1545–1547.
[21] a) P. Klán, T. Šolomek, C. G. Bochet, A. Blanc, R. Givens, M. Rubina, V. Popik, A. Kostikov, J. Wirz, *Chem. Rev.* **2013**, *113*, 119–191; b) C. S. Ma, W. M. Kwok, W. S. Chan, Y. Du, J. T. W. Kan, P. H. Toy, D. L. Phillips, *J. Am. Chem. Soc.* **2006**, *128*, 2558–2570.
[22] C. Banerjee, C. Ghatak, S. Mandal, S. Ghosh, J. Kuchlyan, N. Sarkar, *J. Phys. Chem. B* **2013**, *117*, 6906–6916.
[23] M. L. Viger, W. Sheng, C. L. McFearin, M. Y. Berezin, A. Almutairi, *J. Controlled Release* **2013**, *171*, 308–314.
[24] L. Mora, K. Y. Chumbimuni-Torres, C. Clawson, L. Hernandez, L. Zhang, J. Wang, *J. Controlled Release* **2009**, *140*, 69–73.
[25] S. Karthik, B. Saha, S. K. Ghosh, N. D. P. Singh, *Chem. Commun.* **2013**, *49*, 10471–10473.

Received: September 25, 2015
Revised: January 16, 2016
Published online: February 25, 2016

