Published in final edited form as:

Angew Chem Int Ed Engl. 2016 December 23; 55(52): 16080–16083. doi:10.1002/anie.201609731.

Photo-Triggered Secretion of Membrane Compartmentalized Bioactive Agents

Prof. Robert M. Hughes[a],[b], **Christina M. Marvin**[a], **Dr. Zachary L. Rodgers**[a],[b], **Dr. Song Ding**[a], **Dr. Nathan P. Oien**[a],[b], **Dr. Weston J. Smith**[a],[b], and **Prof. David S. Lawrence**[a]

[a]Department of Chemistry, Division of Chemical Biology and Medicinal Chemistry, and Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599 (USA)

Abstract

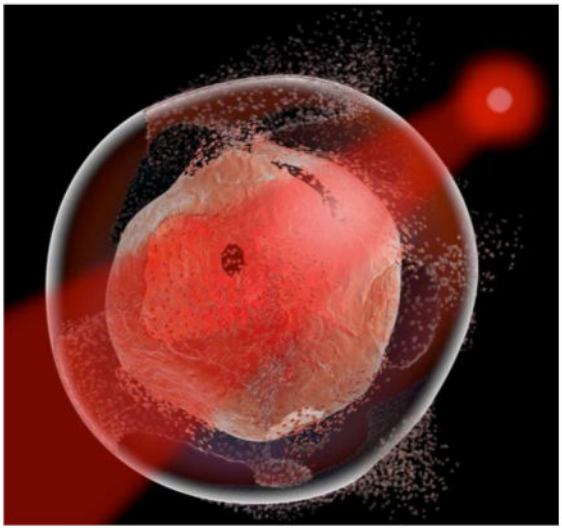
A strategy for the light-activated release of bioactive compounds (BODIPY, colchicine, paclitaxel, and methotrexate) from membrane-enclosed depots is described. We have found that membrane permeable bioagents can be rendered membrane impermeable via covalent attachment to cobalamin (Cbl) through a photo-cleavable linker. These Cbl-bioagent conjugates are imprisoned within lipid-enclosed compartments in the dark, as exemplified by their retention in the interior of erythrocytes. Subsequent illumination drives the secretion of the bioactive species from red blood cells. Photo-release is triggered by wavelengths in the red, far red and near IR, which can be pre-assigned by affixing a fluorophore with the desired excitation wavelength to the Cbl-bioagent conjugate. Pre-assigned wavelengths allow different biologically active compounds to be specifically and unambiguously photo-released from common carriers.

Graphical abstract

Correspondence to: David S. Lawrence.

[b]Present Addresses: RMH: Department of Chemistry, 300 Science & Technology Building, East Carolina University, Greenville, NC 27858. ZLR: Center for Nanotechnology in Drug Delivery, Department of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599. NPO: KBI Biopharma, 1101 Hamlin Rd, Durham, NC 27704. WJS: Skaggs School of Pharmacy, 12850 E. Montview Blvd, University of Colorado, Aurora CO 80045.

Supporting information for this article is given via a link at the end of the document.



Membrane-impermeable, photo-responsive, conjugates have been developed that can be inserted into and retained by membrane-enclosed compartments. Illumination generates a membrane permeable biologically active agent, which is rapidly secreted. Furthermore, the wavelength of photo-release can be unambigously pre-assigned, enabling the specific discharge of different bioagents from a common carrier.

Keywords

Photolysis; Cobalamines; Membranes; Drug Delivery; Prodrugs

Light serves as a stimulus for nearly all forms of life, as exemplified by phototropism,[1] displayed by plants and insects, and the circadian clock,[2] which is found in organisms that range from cyanobacteria to humans. Light, which can be applied with exquisite spatial and temporal precision, possesses photophysical properties (intensity, wavelength) that are readily modulated. These attributes, when applied in conjunction with chemically and biologically engineered photo-responsive molecules, have been used to probe and perturb biological phenomena in cells, tissues, and organisms. A wide variety of bioactive compounds have been converted into light-responsive derivatives by either (1) modifying essential functional groups with photocleavable moieties or (2) introducing light-triggered configurational switches that reversibly convert the inactive and active forms of the bioagent.[3] By contrast, genetically encoded “optogenetic” proteins are typically constrained or transferred to specific intracellular sites required for their mechanism of action.[4] Is the opposite possible? Can bioagents be restricted to specific inert compartments and then use light to trigger their release? The advantage of this approach is that it is not necessary to impair, alter, or modify the innate biochemical activity of the agent under study.

We recently reported that vitamin B12 (cobalamin; “Cbl”) serves as a molecular platform for the photo-release of drugs due to the presence of an extraordinary photo-cleavable Co-C bond[5].[6] B12 is not membrane permeable, but rather is transported into cells via a sophisticated protein carrier, receptor-mediated, endosome-dependent mechanism.[7] We

wondered whether Cbl-bioagent conjugates might retain the membrane impermeability properties of the parent B12 and thus could be ensconced within lipid-enclosed compartments until needed. Previously, we've shown that the light responsiveness of Cbl conjugates can be tuned to specific wavelengths deep into the red, far red, and near IR.[6b] These wavelengths penetrate tissue more effectively than UV and short visible wavelengths, a decided advantage under *in vivo* conditions.[8] With these features in mind, we chose the interior of erythrocytes as a membrane enclosed environment to explore the conjecture that illumination can be used to control the secretion of biologically active compounds (Figure 1). We anticipated that loading Cbl-bioagent conjugates into the interior of erythrocytes would be straightforward since red blood cells swell upon exposure to a hypotonic buffer, creating pores in the plasma membrane.[9] Subsequent resealing with an isotonic buffer should entomb the anticipated membrane impermeable Cbl-bioagent in the erythrocyte.

BODIPY®650 (BODIPY) is an extensively employed membrane permeable fluorescent dye.[10] We prepared the Cbl-BODIPY conjugate (**2**, Scheme 1) from **1a** (where R = NH_2) and the corresponding BODIPY carboxylic acid derivative. Photolysis of **2** furnished the anticipated product (scission at the Co-C bond) as confirmed by LC-MS (Figure S1). Erythrocytes were loaded with **2** using a modified hypotonic/isotonic literature protocol (SI).[9] Subsequent washing of the erythrocytes with serum-containing cell culture medium removed free Cbl-BODIPY that had not been installed within the erythrocyte interior (Figures S2 - S4). Light-triggered (660 nm; LED power = 3.30 ± 0.02 mW/cm^2; 0 – 5 min) BODIPY release was monitored from loaded erythrocytes in 10% hematocrit solutions. The time dependent release of BODIPY from **2**-loaded erythrocytes was quantified by spinning down the blood cells after illumination and measuring the fluorescence in the supernatant (released BODIPY) and the pellet (retained BODIPY; Figures 2, S5). Control experiments were performed under identical conditions in the dark. As anticipated, Cbl-BODIPY **2** is retained by erythrocytes in the dark, with only a negligible amount of BODIPY present in the supernatant (<1%) as a percentage of total Cbl-BODIPY loaded (Figure 2). By contrast, 660 nm illuminated (3 min) samples display robust BODIPY release (80.7 ± 6.4%). Various illumination times were explored and we found that maximal Cbl-BODIPY photolysis occurs at 3 min of illumination (Figure S5).

We subsequently prepared several Cbl conjugates containing methotrexate (MTX; **3**), colchicine (COL; **4**) and paclitaxel (PTX; **5**). These green light-sensitive Cbl-bioactives were prepared by coupling Cbl-propylamine (**1a**) to MTX and succinyl-PTX (Scheme 1). By contrast, Cbl-butyrate (**1b**) was appended to the free amine of the deacetylated form of colchicine (Schemes S1 – S3; Figures S6 – S8). We confirmed (LC-MS) that these species suffer photolysis via scission of the Co-C bond (Figures S9 – S11). These species were introduced into erythrocytes using the hypotonic-loading protocol described in the previous paragraph. The bioactive agents were subsequently released from erythrocytes via illumination at 525 nm, and their biological impact on co-plated HeLa cells monitored. The effect of photo-released MTX on HeLa cells was assessed by the "CETSA assay" (Figures 3, S12).[11] Following exposure to MTX-containing erythrocytes, HeLa cells were exposed to elevated temperatures, lysed, and the lysate centrifuged to separate soluble proteins from their insoluble counterparts. In the absence of MTX, elevated temperatures denature

dihydrofolate reductase (DHFR) rendering it insoluble and poorly observable by western blot. By contrast, MTX-bound DHFR is more resistant to heat-induced denaturation. Only a small amount of DHFR is detected when HeLa cells are incubated in the dark with **3** embedded within erythrocytes. On the other hand, illumination results in MTX release as evidenced by the dramatic increase in stabilized DHFR (Figure 3A–C). Likewise, 525 nm-illuminated erythrocytes containing Cbl-COL induced COL take-up by HeLa cells. In the absence of COL, microtubules extend from the nucleus to the cell surface (Figure 3D). By contrast, upon COL photo-release, microtubule polymerization is blocked, leading to shortened and disrupted microtubule morphology (Figure 3E, S13). Finally, erythrocytes conveying Cbl-PTX released an active paclitaxel derivative upon 525 nm exposure, which stabilizes microtubules against depolymerization as revealed by coalescence (Figures 3F–G, S14).

Having established the methodology for the encapsulation and on-command light-triggered release of bioactive compounds from erythrocytes, we sought to extend the wavelength window of release into the 650 – 800 nm realm. We've previously shown that Co-C photolysis can be induced by red, far red, or near IR light using Cbl derivatives possessing appended long wavelength, light-harvesting fluorophores.[6b] Given the unique excitation wavelengths of individual fluorophores, we investigated whether specific agents could be released from erythrocytes in a wavelength-designated fashion.

Red light-sensitive analogs of Cbl-MTX and Cbl-PTX were prepared using Cy5 as an appended long wavelength antenna (**6 – 7**; λ_{ex} = 660 nm, Scheme 2). We also synthesized a corresponding near IR responsive analog of Cbl-COL by affixing "FL800" to the ribose ring (**8**; λ_{ex} = 780 nm; Schemes 2 and S4, Figure S15). The synthetic routes to Cy5-Cbl-MTX **6,** Cy5-Cbl-PTX **7** and FL800-Cbl-COL **8** employ compounds **1a** and **1b** as the starting materials. In addition, the ribose 5′-OH was activated (1,1′-carbonyl-di-triazole) and subsequently reacted with ethylenediamine, to provide a handle upon which the long wavelength fluorophores are secured (Schemes S5 – S11; Figures S16 – S18). All three fluorophore-Cbl-bioactive conjugates furnish the photolyzed Co-C cleaved products at their assigned wavelengths (Figures S19 – S21). The phototherapeutic agents were encapsulated in erythrocytes via exposure to a hypotonic solution. In contrast to the Cbl derivatives **3** – **5**, analogs **6** – **8** respond to wavelengths beyond which the Cbl ring absorbs light. Both erythrocyte-embedded Cy5-Cbl-MTX **6** and Cy5-Cbl-PTX **7** release their bioactive cargo at 660 nm. When co-cultured with HeLa cells, light-triggered MTX secretion from erythrocytes was confirmed by the CETSA assay and PTX release was demonstrated by microtubule immunostaining (Figures S22 – S23). In addition, FL800-Cbl-COL **8** is designed to respond to near IR wavelengths. 780 nm exposure of erythrocytes containing FL800-Cbl-COL triggered COL secretion and depolymerization of HeLa cell microtubules (Figure S24).

Given the fact that the long wavelength excitation spectra of the BODIPY and FL800 fluorophores in this study are non-overlapping (Figure S25) we investigated the orthogonal light-triggered secretion of bioactive compounds by simply modulating the wavelength of illumination. We considered two possible loading strategies: (1) Cbl derivatives co-loaded into the same erythrocytes and (2) Cbl derivatives loaded in different erythrocytes and

subsequently mixed to create a single pool of blood cells. Both possibilities were explored using the 650 nm sensitive Cbl-BODIPY **2** and the 780 nm responsive FL800-Cbl-COL **8**. Both approaches furnished erythrocyte-encapsulated Cbl-bioagent conjugates that differentially release their cargo in response to orthogonal wavelength-embedded commands (Figures 4, S26 – S28). For example, erythrocytes loaded with both **2** and **8,** when exposed to 660 nm, selectively release BODIPY, which is taken up by HeLa cells (Figure 4A). By contrast, 780 nm exposure triggers COL discharge and subsequent structural disruption of HeLa microtubules (Figure 4B). *Both compounds resist release from erythrocytes when illuminated at non-absorbing wavelengths* (Figures S126, S28). Analogous studies were performed with erythrocytes that were separately loaded with **2** or **8** and subsequently mixed into a single pool (Figures S27 – S28). These results confirm wavelength-specified photo-secretion of specific bioagents from membrane-delimited compartments.

In conclusion, we've demonstrated that long wavelength responsive bioactive species can be constructed by appending membrane impermeable Cbl via a photo-cleavable Co-C bond. We anticipate that this strategy could prove applicable to other membrane-enclosed compartments, such as endosomes, the cytoplasm, or various organelles in conjunction with robust delivery moieties. These studies are in progress. In addition, we've shown that photo-secretion can be triggered by preassigned wavelengths, an easily modulated photophysical property of light. Finally, it has not escaped our attention that on-command selective release of therapeutic agents from biocompatible delivery vehicles[12] offers a strategy that addresses many of the challenges encountered by drug targeting to diseased sites.

Experimental Section

See the online Supporting Information for Materials and Methods and Supporting Figures and Schemes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Center for Advancing Translational Sciences, National Institutes of Health (UL1TR001111) and the UNC Lineberger Comprehensive Cancer Center for generous financial support. CMM was supported by an NIH T32 Cancer Cell Biology Training Grant (5T32CA071341-19). ZLR acknowledges support from the NIH sponsored Carolina Cancer Nanotechnology Training Program (1T32CA196589).

References

1. Chen M, Chory J, Fankhauser C. Annu Rev Genet. 2004; 38:87–117. [PubMed: 15568973]
2. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. Nat Rev Genet. 2005; 6:544–556. [PubMed: 15951747]
3. a) Brieke C, Rohrbach F, Gottschalk A, Mayer G, Heckel A. Angewandte Chemie. 2012; 51:8446–8476. [PubMed: 22829531] b) Klan P, Solomek T, Bochet CG, Blanc A, Givens R, Rubina M, Popik V, Kostikov A, Wirz J. Chem Rev. 2013; 113:119–191. [PubMed: 23256727] c) Lee HM, Larson DR, Lawrence DS. ACS chemical biology. 2009; 4:409–427. [PubMed: 19298086] d) Lerch MM, Hansen MJ, van Dam GM, Szymanski W, Feringa BL. Angewandte Chemie. 2016; 55:10978–10999. [PubMed: 27376241]

4. a) Hughes RM, Freeman DJ, Lamb KN, Pollet RM, Smith WJ, Lawrence DS. Angewandte Chemie. 2015; 54:12064–12068. [PubMed: 26418181] b) Hughes RM, Lawrence DS. Angewandte Chemie. 2014; 53:10904–10907. [PubMed: 25156888] c Weitzman M, Hahn KM. Curr Opin Cell Biol. 2014; 30:112–120. [PubMed: 25216352]
5. Barkar HA, Weissbach H, Smyth RD. Proc Natl Acad Sci U S A. 1958; 44:1093–1097. [PubMed: 16590317]
6. a) Shell TA, Lawrence DS. Acc Chem Res. 2015; 48:2866–2874. [PubMed: 26479305] b) Shell TA, Shell JR, Rodgers ZL, Lawrence DS. Angewandte Chemie. 2014; 53:875–878. [PubMed: 24285381] c) Smith WJ, Oien NP, Hughes RM, Marvin CM, Rodgers ZL, Lee J, Lawrence DS. Angewandte Chemie. 2014; 53:10945–10948. [PubMed: 25154925]
7. Fedosov SN. Subcell Biochem. 2012; 56:347–367. [PubMed: 22116708]
8. Tromberg BJ, Shah N, Lanning R, Cerussi A, Espinoza J, Pham T, Svaasand L, Butler J. Neoplasia. 2000; 2:26–40. [PubMed: 10933066]
9. a) Ihler GM, Glew RH, Schnure FW. Proc Natl Acad Sci U S A. 1973; 70:2663–2666. [PubMed: 4354859] b) Rossi L, Serafini S, Pierige F, Antonelli A, Cerasi A, Fraternale A, Chiarantini L, Magnani M. Expert Opin Drug Deliv. 2005; 2:311–322. [PubMed: 16296756] c) Rossi L, Serafini S, Pierige F, Castro M, Ambrosini MI, Knafelz D, Damonte G, Annese V, Latiano A, Bossa F, Magnani M. J Control Release. 2006; 116:e43–45. [PubMed: 17718962]
10. Kowada T, Maeda H, Kikuchi K. Chem Soc Rev. 2015; 44:4953–4972. [PubMed: 25801415]
11. a) Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundback T, Nordlund P, Martinez Molina D. Nat Protoc. 2014; 9:2100–2122. [PubMed: 25101824] b) Martinez Molina D, Jafari R, Ignatushchenko M, Seki T, Larsson EA, Dan C, Sreekumar L, Cao Y, Nordlund P. Science. 2013; 341:84–87. [PubMed: 23828940]
12. Muzykantov VR. Expert Opin Drug Deliv. 2010; 7:403–427. [PubMed: 20192900]

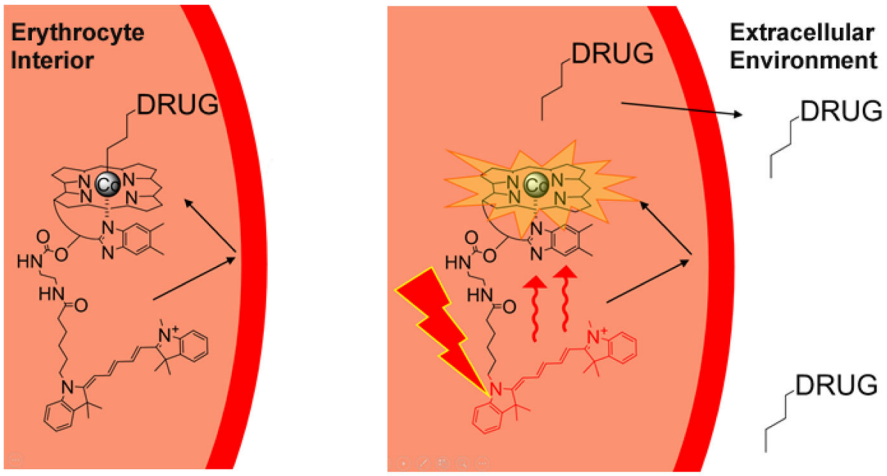


Figure 1.

Light-mediated release of compartmentalized light-responsive compounds. Membrane impermeable Cbl-bioagents are retained inside erythrocytes. Photolysis produces membrane permeable bioagents that can escape the cell.

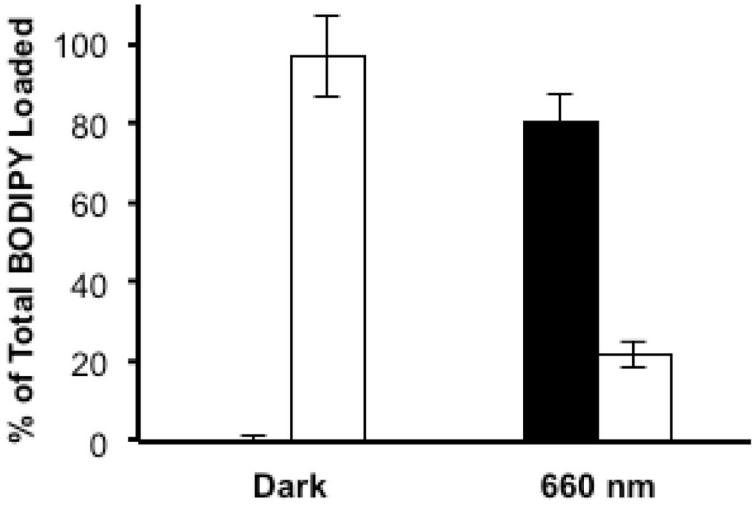


Figure 2.

The percentage of BODIPY retained by (pellet; □) and released from (supernatant; ■) erythrocytes as a function of total loaded Cbl-BODIPY under dark and 660 nm conditions. BODIPY is retained by erythrocytes maintained in the dark. By contrast, 80.7 ± 6.4% of BODIPY is released upon a 3 min exposure to a 660 nm LED array. Compare with Figure S5.

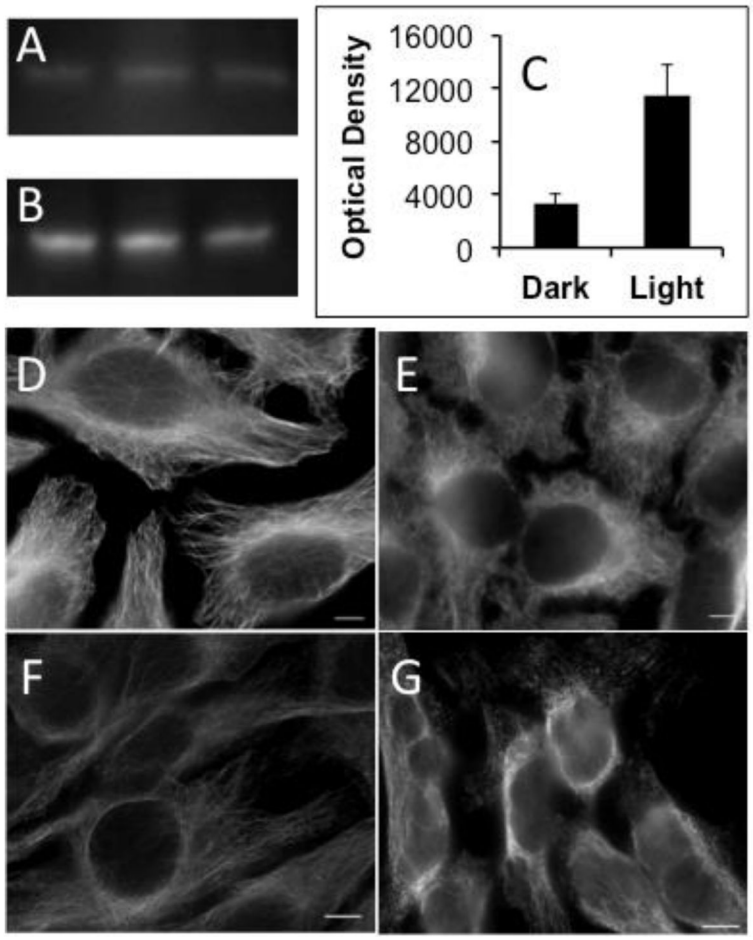


Figure 3.

Cell-based assays for internalized phototherapeutic release at 525 nm. (A) DHFR western blot (CETSA assay) of HeLa cells incubated with **3**-loaded erythrocytes in the dark or (B) following exposure to 525 nm light. (C) Quantification of western blot optical density is consistent with photo-release of MTX. (D) Immunostained HeLa cell microtubules incubated with **4**-loaded erythrocytes in the dark or (E) exposed to 525 nm light. Structurally compromised microtubules are consistent with COL photo-release. (F) Immunostained HeLa cell microtubules incubated with **5**-loaded erythrocytes in the dark or (G) exposed to 525 nm light. Condensed microtubules are consistent with PTX photo-release. Scale bars = 10 μm.

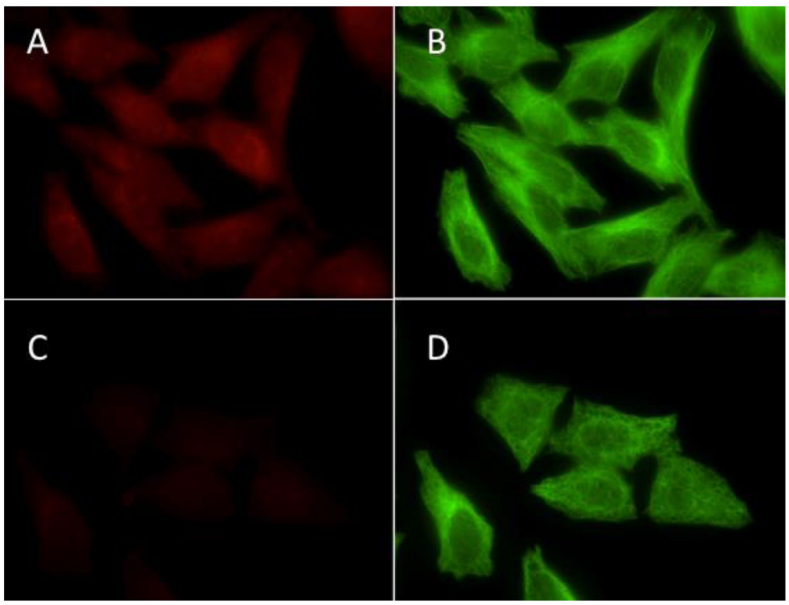
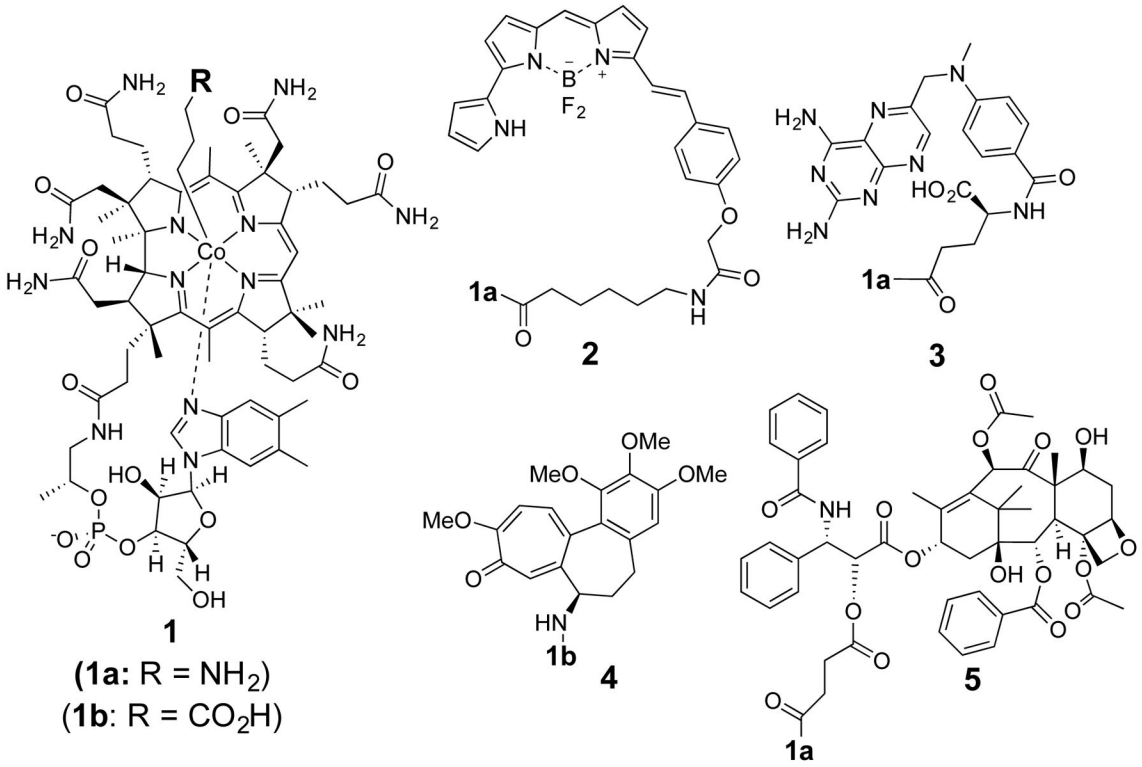


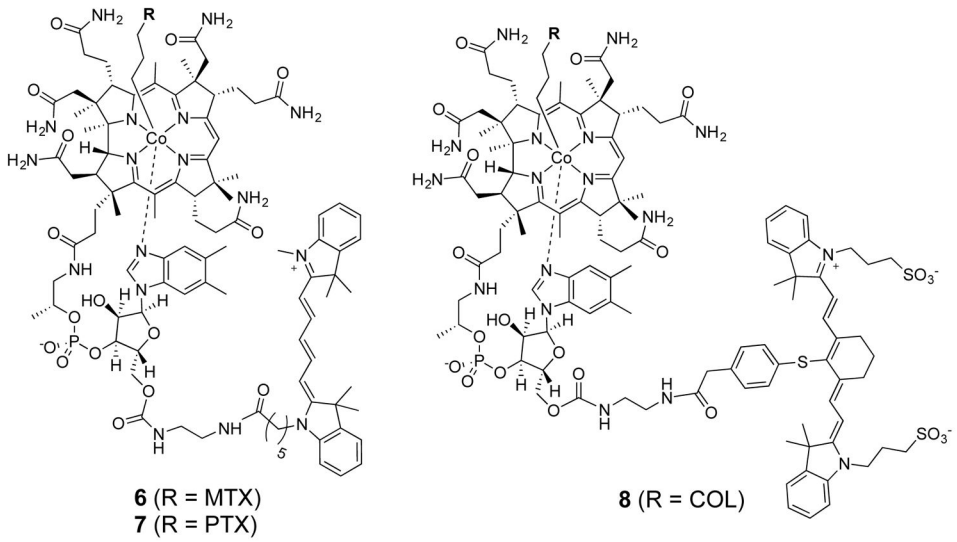
Figure 4.

Orthogonal release of 660 nm-sensitive **2** and 780-sensitive **8** from erythrocytes and uptake by HeLa cells. Left columns show fluorescence of BODIPY650 in HeLa cells and right columns displays HeLa cell microtubules. (A) Erythrocytes exposed to 660 nm light release BODIPY, which is taken up by HeLa cells as demonstrated by red fluorescence. By contrast, (B) no COL is released under these conditions as demonstrated by intact microtubules. (C–D) Erythrocytes exposed to 780 nm light. Under these circumstances, (C) no BODIPY is released. (D) However, COL is delivered to HeLa cells as evidenced by the presence of compromised microtubules. Results from experiments conducted in the dark or simultaneously exposed to both 660 and 780 nm are furnished in Fig. S27. Scale bars = 10 microns.



Scheme 1.

Cbl-Drug conjugates prepared from **1** (where R = NH_2 or CO_2H): Cbl-BODIPY (**2**), Cbl-MTX (**3**), Cbl-COL (**4**), and Cbl-PTX (5).



Scheme 2.

Cy5 derivatives of Cbl-MTX (**6** where R = MTX) and Cbl-PTX (**7** where R = PTX) and FL800 derivative of Cbl-COL (**8** where R = COL).