VIP Photoactivatable Agents Very Important Paper

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Tunable Visible and Near-IR Photoactivation of Light-Responsive Compounds by Using Fluorophores as Light-Capturing Antennas**

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Abstract: *Although the corrin ring of vitamin B_{12} is unable to efficiently absorb light beyond 550 nm, it is shown that commercially available fluorophores can be used as antennas to capture long-wavelength light to promote scission of the Co–C bond at wavelengths up to 800 nm. The ability to control the molecular properties of bioactive species with long visible and near-IR light has implications for drug delivery, nanotechnology, and the spatiotemporal control of cellular behavior.*

Photoresponsive species are used to manipulate intracellular biochemical pathways, light-sensitive nanoparticles are employed to site-selectively deliver cytotoxic agents, and photoinitiated hydrogels are applied as biomaterial replacements.[1] However, the application of these molecular entities is dependent upon short wavelengths of light (< 450 nm), which inflict biological damage and are unable to take advantage of the optical window of tissue (600–1000 nm).[2] Furthermore, the narrow wavelength–response range limits the ability to design a family of photoactivatable species that can be orthogonally triggered at distinct wavelengths.[3] We recently reported expansion of the range of photoresponsive compounds to green light (approximately 560 nm),[4] however these compounds do not absorb light that falls within the optical window of tissue. With these challenges in mind, we report herein wavelength-tunable photoresponsive species based on the vitamin B_{12} cobalamin ring framework. These species are easily encoded, using commercially available fluorophores,[5] to respond to specific wavelengths throughout the visible spectrum and into the near IR.

A fluorescence increase is observed upon photolysis of a 5-carboxytetramethylrhodamine (TAMRA)-substituted cobalamin (Cbl-1) as a result of the ability of cobalamin to quench the fluorescence of attached fluorophores (Figure 1).[4,6] This effect is likely a consequence of contact quenching between the fluorophore and the corrin ring system.[6b] Contact quenching, unlike fluorescence energy resonance transfer, does not require an overlap between the emission and absorption wavelengths of the fluorophore and quencher, respectively, for energy transfer to transpire. Given the fact that the cobalt–alkyl bond is weak (< 30 kcal mol^{-1}),[7] it occurred to us that fluorophores excited at wavelengths beyond that absorbed by cobalamin (> 560 nm) could transfer their excited state energy to the corrin ring and thus promote Co–C bond scission (Figure 1). Indeed, the rate of photolytic conversion of Cbl-1 ($(8.0 \pm 0.4) \times 10^{-3}\ s^{-1}$) is twice that of its non-fluorophore-containing counterparts MeCbl and Cbl-2 ($(3.8 \pm 0.2) \times 10^{-3}\ s^{-1}$ and $(4.2 \pm 0.2) \times 10^{-3}\ s^{-1}$, respectively; Figure 1, and Figures S1–S4 in the Supporting Information), thus suggesting that excitation of an appended fluorophore could play a role in promoting photocleavage of the Co–C bond.

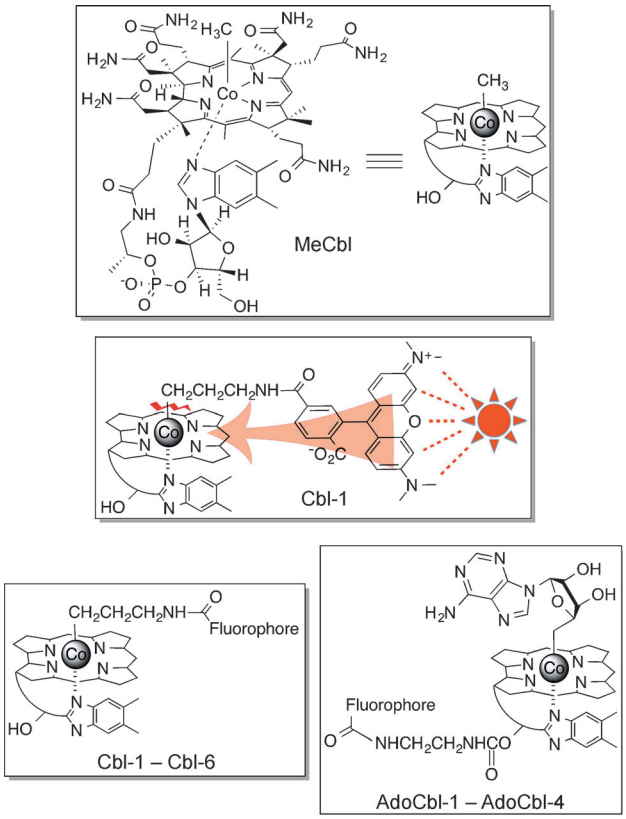

Figure 1. Methylcobalamin (MeCbl) and cobalamin–fluorophore conjugates: Cbl-1 (TAMRA), Cbl-2 (acetyl), Cbl-3 (SulfoCy5), Cbl-4 (Atto725), Cbl-5 (DyLight800), Cbl-6 (Alexa700), AdoCbl-1 (TAMRA), AdoCbl-2 (SulfoCy5), AdoCbl-3 (Atto725), and AdoCbl-4 (DyLight800).

Several fluorophores were appended to the alkylcobalamin framework, all with excitation wavelengths longer than that absorbed by the corrin ring (Cbl-2–Cbl-6). A number of features are apparent from the results presented in Table 1.

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Table 1: Percentage photolysis of cobalamin–fluorophore conjugate upon illumination at 546 nm (5 min), 646 nm (5 min), 700/727 nm (20 min), and 777 nm (10 min). Percentage photolysis was calculated using the maximum observed fluorescence increase as 100% photolysis (see Figures S8, S10, S12, S14, S17, and S24).

Cobalamin conjugate	Fluorophore	$\lambda_{ab/ex}$ [nm][a]	Percent photolysis at applied wavelength [nm]			
			546	646	700/727[b]	777
Cbl-1	TAMRA	546	100 ± 17	[c]	[c]	[c]
Cbl-3	SulfoCy5	546, 646*	13 ± 21	100 ± 21	[c]	[c]
Cbl-4	Atto725	546, 646, 727*, 777	62 ± 8	35 ± 5	100 ± 5	9 ± 5
Cbl-5	DyLight800	546, 727, 777*	22 ± 12	[c]	20 ± 12	100 ± 12
Cbl-6	Alexa700	546, 646, 700*	56 ± 3	48 ± 5	100 ± 3	[c]
Cbl-Bod	Bodipy650	546, 646*	48 ± 12	100 ± 12	[c]	[c]

[a] Absorbance/excitation of the cobalamin–fluorophore conjugate where * is the major fluorophore excitation band. [b] All compounds were photolyzed at 727 nm, except Cbl-6, which was photolyzed at 700 nm (3 min). [c] < 5% photolyzed under the experimental conditions.

First, illumination at the corrin absorption wavelength (546 nm) completely converts all the derivatives to their photolyzed products if sufficient light exposure is applied (25 min under the conditions described in the Supporting Information). However, at a much shorter illumination time (5 min), Cbl-1 is photolyzed to a greater extent at 546 nm than its counterparts, presumably as a result of the significant TAMRA absorbance in this region ($\varepsilon = 90\,000\ cm^{-1}M^{-1}$). Second, all the compounds undergo photolysis at their excitation wavelengths, even when those wavelengths are far beyond the absorbance of the corrin ring. The cobalamin–SulfoCy5 conjugate Cbl-3 (λ_{ex} 650 nm, λ_{em} 660 nm), when illuminated at (646 ± 10) nm, is photocleaved to hydroxocobalamin (B_{12a}) and SulfoCy5 products as assessed by UV/Vis spectroscopy (Figure S5) and LC–MS (Table S2, Scheme S7). The Atto725 (Cbl-4, λ_{ex} 730 nm, λ_{em} 750 nm) and DyLight800 (Cbl-5, λ_{ex} 775 nm, λ_{em} 794 nm) conjugates likewise undergo photolysis at wavelengths absorbed by the appended fluorophores, namely (730 ± 10) and (780 ± 10) nm, respectively (Figures S6, S7, Tables S3 and S4). Third, all the cobalamin–fluorophore conjugates are stable in the dark. Fourth, exposure of these conjugates to wavelengths that they do not absorb has no effect on their structural integrity. The TAMRA derivative, Cbl-1, is stable at 646 nm and longer (Table 1). Cbl-3, with an appended fluorophore that absorbs 646 nm light, is unaffected by 727 and 777 nm illumination.

The results in Table 1 suggest that it should be possible to selectively photolyze specific compounds from a mixture of cobalamin-substituted derivatives by employing the appropriate illumination sequence and/or wavelengths. Owing to the slight photolysis observed for Cbl-4 at 777 nm, the latter was replaced with the Alexa700-appended conjugate Cbl-6, which lacks an absorption band at 777 nm. Only Cbl-5 photolysis occurs when illuminating a mixture of Cbl-5, Cbl-6, Cbl-3, and Cbl-1 at 777 nm (Figure 2a). Subsequent exposure of the mixture to 700 nm elicits the conversion of Cbl-6 to its photoproducts without affecting Cbl-3 or Cbl-1 (Figure 2b). A final sequential illumination at 646 and 546 nm (Figures 2c, d) furnishes the stepwise photolysis of Cbl-3 and Cbl-1, respectively. As expected, 546 nm illumination of a mixture of these Cbl derivatives failed to furnish selective photolysis; a predictable result based on the data in Table 1. By contrast, since Cbl-3 and Cbl-5 are photochemically distinct at 646 nm and 777 nm (Table 1, Figure S19), they can be individually photomanipulated without the need to resort to a specific illumination sequence (Figure S20). The more than 200 nm bandwidth of the cobalamin–fluorophore conjugates in Table 1 furnishes a spectral range in which specific compounds in a mixture can be independently acted upon. Indeed, since the dissociation energy of the Co–C bond is estimated to be less than 30 kcal mol^{-1},[7] it may be feasible to sensitize bond cleavage up to and beyond 1000 nm.

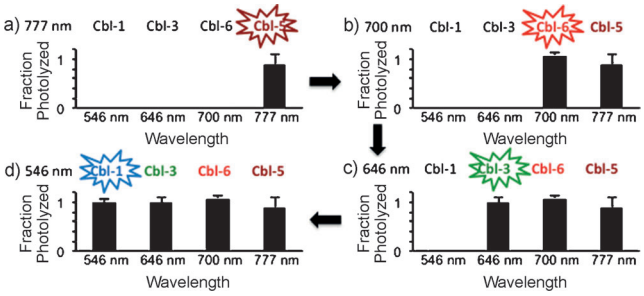

Figure 2. Selective photolysis of individual cobalamin conjugates from a mixture by using serial illumination [(a) 777 nm→(b) 700 nm→(c) 646 nm→(d) 546 nm] sequentially photolyzes Cbl-5, Cbl-6, Cbl-3, and Cbl-1, respectively.

Fluorophores appended to the ribose 5′-OH of alkylcobalamins also promote photocleavage of the cobalt–alkyl linkage (Figure 1, AdoCbl-1–AdoCbl-4). Our initial studies employed coenzyme B_{12} (AdoCbl) derivatives since Schwartz and Frey had previously identified the adenosine products of photolysis.[8] LC–MS confirmed that 546 nm illumination of AdoCbl (no fluorophore) and AdoCbl-1 (TAMRA) generates the expected products (Tables S6, S7). Furthermore, both AdoCbl and AdoCbl-1 are resistant to photolysis at wavelengths beyond 600 nm. In a fashion analogous to the behavior displayed by the Cbl-3–Cbl-6 series, the fluorophore-substituted AdoCbl-2 (SulfoCy5), AdoCbl-3 (Atto725), and AdoCbl-4 (DyLight800) derivatives produce the anticipated adenosine products at the wavelengths absorbed by their appended fluorophores (Tables S8, S10). In short, the photolytic release of compounds attached to the cobalt ion of cobalamin can be tuned in a predictable fashion by using commercially available fluorophores appended to either the ribose ring or the metal ligand.

Light-responsive small molecules are commonly prepared by covalently modifying a functional group essential for biological activity with a photocleavable moiety,[1] whereas genetically expressed light-responsive proteins are typically designed by engineering a link between light-induced con-

formational changes and biochemical activities.[9] Cobalamins offer a potentially attractive alternative since conjugates of cobalamins appear to be retained by endosomes.[10] There has been a huge effort to create delivery strategies that avoid these organelles since endosomally embedded bioactive species are unable to interact with their intracellular targets. This liability represents an opportunity for cobalamin conjugates: cell-permeable bioactive species can potentially be endosomally sequestered, which should preclude their action until released by light. With this in mind, the Bodipy650 derivative Cbl-Bod was prepared (Figure 3, Schemes S2–S4).

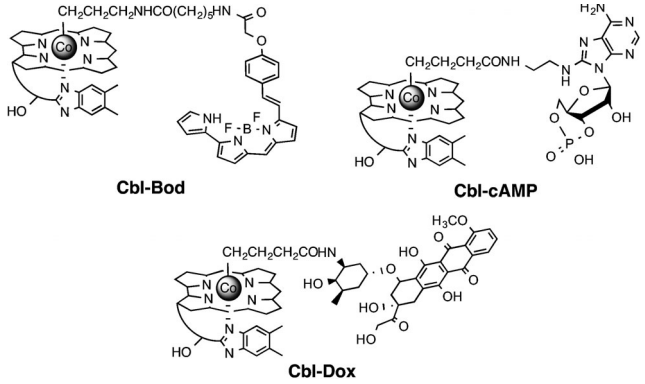

Figure 3. The Bodipy650–, cAMP–, and doxorubicin–cobalamin conjugates Cbl-Bod, Cbl-cAMP, and Cbl-Dox, respectively.

Bodipy650 is cytotoxic through a mechanism based on the targeting of mitochondria.[11] However, the corresponding Cbl-Bod conjugate, unlike Bodipy650, is endosomal as judged by using an endosomal marker (Pearson's coefficient 0.89; Figure 4). Furthermore, it remains endosomally sequestered even after 5 h in the dark. Illumination of cells containing Cbl-Bod at 650 nm furnishes a fluorescence increase ($(230 \pm 6)\%$, Figures S26, S27) similar to that observed in the spectrofluorometer ($(220 \pm 30)\%$), thus signaling liberation of the Bodipy650 from the quenching effect of the cobalamin. Under these conditions, Bodipy650 is simultaneously transferred from endosomes to mitochondria, as assessed by using the mitochondrial marker Mitotracker Green (Pearson's coefficient: 0.81). This supports the notion that the endosomal capture of cobalamin derivatives can be exploited as a means to sequester cell-permeable bioactive agents from their intracellular targets. As an aside, it should be feasible to employ nonendosomal strategies for the sequestration and release of light-sensitive cobalamin derivatives as well.[12]

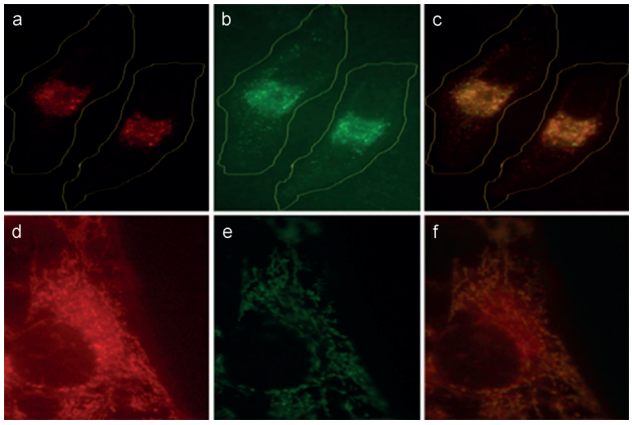

Figure 4. Red light induced translocation of Bodipy 650 in HeLa cells. a) Cbl-Bod before photolysis. b) Rhodamine B-dextran endosomal marker. c) Overlay of (a) and (b). d) Cbl-Bod after photolysis e) Mito-Tracker Green mitochondria marker. (f) Overlay of (d) and (e).

We examined the use of light to control the activity of a cAMP-dependent protein kinase (PKA) signaling pathway. A cobalamin-appended analogue of cAMP, Cbl-cAMP, was prepared (Figure 3, Scheme S10). cAMP-induced activation of PKA is known to promote dramatic morphological changes in cells, including loss of stress fibers and cell rounding.[13] In the absence of illumination, Cbl-cAMP has no effect on the morphology of REF52 cells. Upon illumination in the presence of Cbl-cAMP, REF52 cells undergo the expected loss of their stress fibers as well as cell shrinking and rounding (Figure 5). In addition, an 8-substituted acetylated cAMP analogue (Ac-cAMP, Scheme S9) was prepared and used as a control. Both Ac-cAMP and a commercially available cell-permeable cAMP analogue [8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate; CPT-cAMP] are active in the absence of light, producing morphological effects on REF52 cells analogous to those induced by Cbl-cAMP upon illumination (Figure S28). These results demonstrate that the cobalamin in Cbl-cAMP precludes cytosolic penetration by an appended cAMP derivative (Ac-cAMP is cell permeable) and that illumination releases cAMP from its cobalamin constraint.

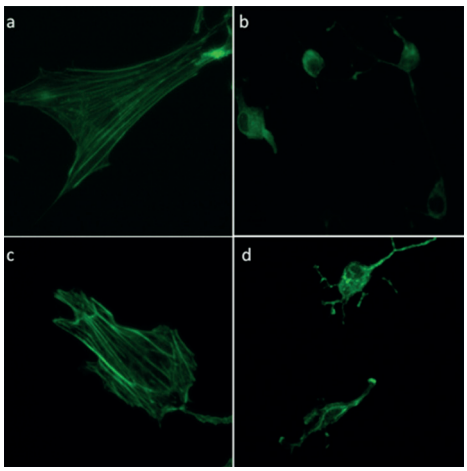

Figure 5. Light-dependent Cbl-cAMP-mediated rearrangement of actin cytoskeleton in rat embryonic fibroblasts (REF52 cells). REF52 cells were exposed to Cbl-cAMP, illuminated at 530 nm, and stained with DyLight488–phalloidin to visualize the actin stress fibers. a) No treatment, media alone. b) CPT-cAMP as a positive control. c) Cbl-cAMP, no illumination. d) Cbl-cAMP with illumination. Images are representative of 10–20 cells.

There is significant interest in the use of light to site-selectively deliver therapeutic agents in vivo. For example, in

the arena of chemotherapy, strategies that limit cytotoxicity to targeted regions offer the promise of reduced side effects and the application of agents that are otherwise too toxic to use. Doxorubicin highlights this need since this valuable anticancer agent displays severe cardiotoxicity.[14] We examined the cytotoxicity of the cobalamin–doxorubicin conjugate Cbl-Dox (Figure 3, Scheme S11) in HeLa cells, after illumination for different periods of time (Figure 6). Light-only treatment

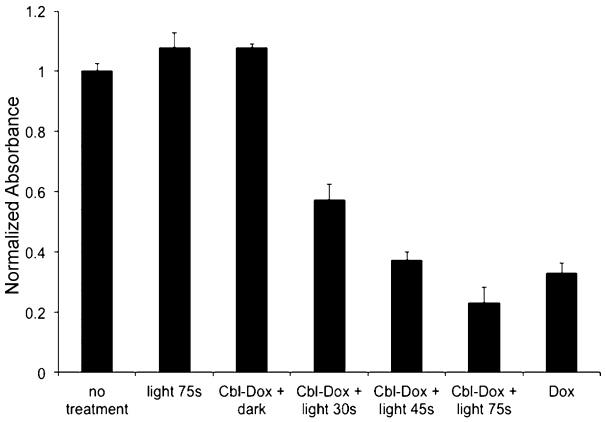

Figure 6. Light-induced release of doxorubicin from Cbl-Dox induces apoptosis of HeLa cells. Cells were loaded with 10 μM Cbl-Dox and illuminated for 30, 45, and 75 s at 530 nm. Cell viability was assessed after 24 h by using an MTS assay (absorbance measured at 520 nm and normalized to the untreated control). The data shown are the averages of three experiments ± SEM.

and Cbl-Dox exposure in the absence of photolysis has no effect on cell viability. By contrast, Cbl-Dox exposure with increasing illumination time furnishes a light-dose-dependent increase in cell death that ultimately recapitulates that produced by doxorubicin alone.

The chemistry of photoresponsive agents has been, for the most part, limited to long UV/short visible wavelengths (300–450 nm).[1,3,4] This narrow region of the electromagnetic spectrum 1) lacks the desired tissue-penetrating depth displayed by 600–1000 nm wavelengths, 2) inflicts biomolecular damage, which can have undesirable short and long-term consequences in vivo, and 3) can support only a limited number of orthogonal wavelength-responsive agents.[15] Although the corrin ring of vitamin B_{12} is unable to efficiently absorb light beyond 550 nm, we found that commercially available fluorophores can be used as antennas to capture long-wavelength light, which promotes scission of the Co–C bond at wavelengths up to 800 nm. Indeed, since the dissociation energy of this bond is estimated to be less than 30 kcal mol^{-1},[7] it may be feasible to sensitize bond cleavage up to and beyond 1000 nm. In summary, the ability to control the molecular properties of bioactive species with long-wavelength visible and near-IR light has implications for drug delivery, material fabrication, nanotechnology, and the spatiotemporal control of cellular behavior.

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