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A New Trick (Hydroxyl Radical Generation) for an Old Vitamin (B_{12})

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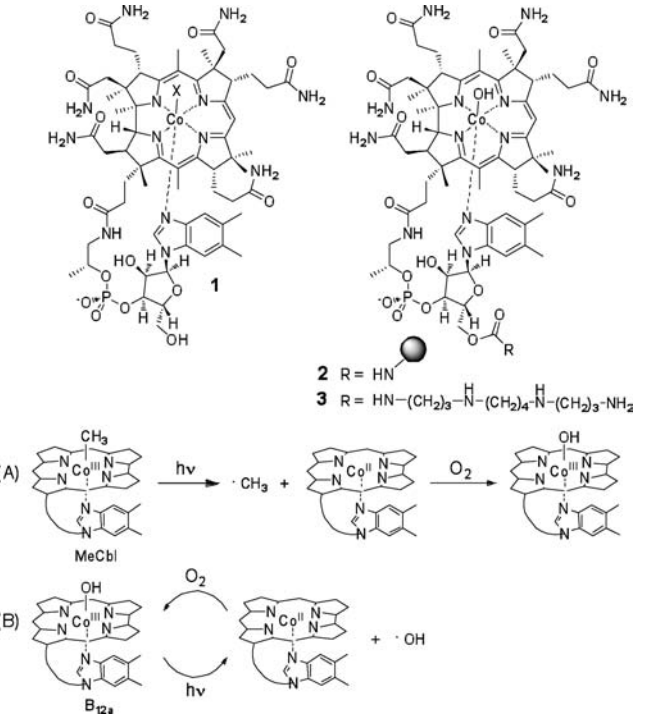
Supporting Information

ABSTRACT: Photolysis of hydroxocobalamin in the presence of plasmid DNA (pBR322) results in DNA cleavage. Temporal control of hydroxyl radical production and DNA strand scission by hydroxocobalamin was demonstrated using a 2-deoxyribose assay and a plasmid relaxation assay, respectively. The light-driven hydroxocobalamin-mediated catalytic formation of hydroxyl radicals was demonstrated using radical scavenging studies of DNA cleavage and via recycling of a hydroxocobalamin−resin conjugate several times without loss of efficacy.

Hydroxyl radicals (·OH) have been used to assess DNA structure[1] and elucidate DNA-binding sites for proteins[2] and small molecules.[3] An example of the Fenton reaction, in which Fe(II)·EDTA reduces H_2O_2, is the most commonly used method to generate ·OH. However, for intracellular purposes, a limitation of this method is the inability to precisely control when the reaction starts and stops. A Fenton-like reaction utilizing a caged Cu(II) ion that can be released by photolysis of the ligand cage has been reported.[4] This strategy furnishes control over the initiation of the Fenton reaction but not the termination step. Several light-mediated "Fenton reagents" have been developed.[5] In contrast to the Fenton reaction, the majority of these compounds have the disadvantage of stoichiometric radical production. TiO_2 does catalytically produce ·OH upon exposure to light,[6] but since TiO_2 induces intracellular oxidative damage in the absence of light,[7] it cannot be used in a light-dependent fashion in cell-based studies. By contrast, synchrotron X-ray[8] and γ[9] irradiation can be used to precisely initiate and terminate ·OH production. Although these methods are advantageous for their ability to provide time-resolved information, they are not widely available and cannot be used to direct oxidative damage to specific intracellular or molecular sites.

Cobalamins, a class of compounds of which vitamin B_{12} is a member, could be useful for generating radical species under cellular conditions. For example, photolysis of the cobalt−carbon bond in methylcobalamin (MeCbl; **1** with X = Me) generates Co(II)-cobalamin (B_{12R}) and methyl radical (·CH_3) (Scheme 1A).[10] On the basis of this precedent, we wondered whether hydroxocobalamin (B_{12a}; **1** with X = OH) could undergo an analogous reaction to generate ·OH and B_{12R} (Scheme 1B). In the presence of oxygen, the latter species is rapidly oxidized to regenerate B_{12a}.[10] We therefore investigated the possibility that the combination of light and B_{12a} could be used to oxidatively damage biomolecules via the generation of ·OH.

Scheme 1. (A) Photolysis of MeCbl To Furnish CH_3· and (B) Proposed Light-Dependent Generation of ·OH by B_{12a}



Initial studies employed a plasmid relaxation assay to observe the ·OH-mediated conversion of circular, supercoiled DNA (form I) to relaxed, circular DNA (form II). A Pyrex filter (>300 nm) was used to photolyze B_{12a} in the presence of pBR322 DNA. Analysis by agarose gel electrophoresis revealed a band corresponding to form II DNA only for light-exposed reaction mixtures containing B_{12a} (Figure S1 in the Supporting Information). In contrast, light in the absence of B_{12a} or B_{12a} in the absence of light did not result in significant DNA damage.

DNA cleavage by B_{12a} did not occur in the absence of O_2, an observation consistent with a radical-mediated mechanism of DNA damage (Figure S3).[11] In addition, the ·OH scavengers sorbitol[12] and sodium benzoate[13] efficiently prevented DNA cleavage (Figure S5), thereby supporting the notion that the damage is mediated by ·OH. The light dependence of DNA cleavage was assessed by photolyzing B_{12a} for various time periods followed by incubation in the dark (Figure S8). Short

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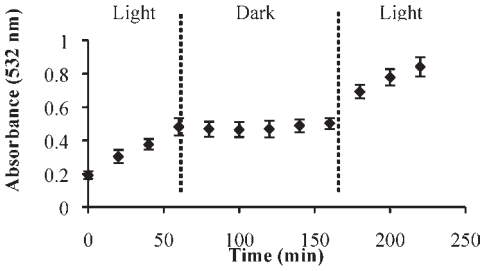

Figure 1. Light-driven ·OH production via photolysis of the B_{12a}–resin conjugate **2**, which was suspended in a deoxyribose solution (50 mM in pH 7.4 PBS). The suspension was sequentially photolyzed (60 min total), stored in the dark (100 min total), and photolyzed (60 min total), and aliquots were removed and analyzed at various time points.

photolysis periods followed by dark incubation resulted in significantly less strand scission than photolysis for the same total time period, demonstrating that the species formed by the photolysis of B_{12a} does not continue to damage DNA without continued exposure to light.

We employed an established ·OH assay to assess the light-mediated production of ·OH.[14] Deoxyribose is attacked by ·OH to generate malondialdehyde, which produces a chromophore (532 nm) upon heating with thiobarbituric acid (TBA).[15] Unfortunately, we found that the presence of B_{12a} during the heating step interfered with the assay. Therefore, B_{12a} was immobilized on an amine-bearing resin using 1,1′-carbonyldi-(1,2,4-triazole) (Figure S10). Deoxyribose was incubated with the B_{12a}–resin conjugate (**2**) under photolytic and nonphotolytic conditions, and the resin was subsequently removed prior to the heating step. The light-driven nature of B_{12a}–resin-mediated ·OH production was evident by the time-dependent production of the TBA–malondialdehyde chromophore in the presence (average absorbance increase of 0.104 per period) but not in the absence (average absorbance increase of 0.003 per period) of light (Figure 1).

Photolysis of the Co–methyl bond in MeCbl generates Co(II)-cobalamin and $\cdot CH_3$ (Scheme 1A). Upon oxidation, the former produces B_{12a},[10] which should generate DNA damaging ·OH under photolytic conditions (Scheme 1B). However, $\cdot CH_3$ is also capable of causing DNA strand scission.[16] We assessed the relative contribution of these two radical species to DNA damage using sorbitol and sodium benzoate (·OH traps) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; a carbon-centered radical trap[17]). TEMPO did not have an appreciable effect on the ability of MeCbl to damage DNA, but sorbitol and sodium benzoate almost completely protected DNA from damage (Figure S11). Therefore, the initial stoichiometric production of $\cdot CH_3$ is small relative to B_{12a}-mediated generation of ·OH.

The notion that B_{12a} catalytically produces ·OH was supported by radical trapping studies of DNA damage involving reuse of conjugate **2** several times without loss in its capacity to damage deoxyribose (Figure S13). Furthermore, a B_{12a} concentration less than the pBR322 concentration was used to demonstrate catalytic production of strand scission events. On the basis of the number of observable strand scission events, B_{12a} induced a minimum of 20 damaging events per molecule during 90 min of light exposure (Figure 2); this represents an underestimate, as plasmid relaxation assays do not visualize all DNA nicks. The amount of DNA damage per molecule increased with longer exposure to light. A band corresponding to linear DNA (form III) resulting from nonrandom double-strand cleavage was observed (Figures S15 and 16),[18] indicative of damage by two radicals that originated near each other in time and space.

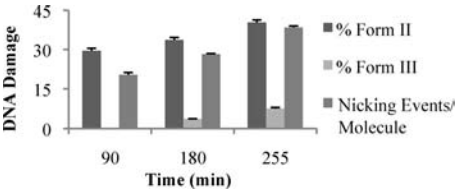

Figure 2. Light-mediated damage of DNA (30 μM/base pair in pH 7.4 PBS) by hydroxocobalamin (300 pM). Samples were irradiated with Pyrex-filtered light from a mercury arc lamp.

A B_{12a}–spermine conjugate (**3**) was synthesized (Figure S17) and proved useful for directing damage to DNA in an environment with a high concentration of a hydroxyl radical trap. Conjugate **3** formed a DNA complex with an apparent binding constant (K_{app} = $1.47 \times 10^8 \pm 3 \times 10^6$ M^{-1}) similar to the reported value for spermine (5.9×10^8 M^{-1}).[19] The B_{12a}–spermine conjugate induced DNA damage more effectively than B_{12a} (Figure S20). More importantly, 10000 equiv of sorbitol failed to significantly inhibit DNA damage induced by **3**, whereas only 100 equiv of sorbitol significantly inhibited strand scission due to B_{12a} (Figure S22), suggesting that **3** generates ·OH near the DNA to which **3** is bound.

Light-dependent ·OH production by B_{12a} enjoys several characteristics that make it potentially useful as a generator of ·OH for biological studies. Light is increasingly employed in modern cell biology to control intracellular processes via cell-embedded photosensitive bioactive reagents.[20] The combination of light and light-sensitive reagents provides the investigator with exquisite temporal control over cellular biochemistry. Spatial control of oxidative damage is afforded by the addition of a binding moiety to B_{12a} because the highly reactive ·OH is generated proximal to the target biomolecule. In addition, light-induced radical generation with B_{12a} is catalytic, whereas radical generation by other systems is stoichiometric. Future research will focus on conjugating recognition moieties to B_{12a} to direct the oxidative damage to specific macromolecules and organelles.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1–S22 and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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