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A light-activatable antibiotic with high activation efficiency and uncompromised bactericidal potency in the activated state

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Abstract

Achieving activatable antibiotics represents one promising solution to tackle the occurrence of side effects, one major issue now plaguing antibiotic usage in collagen-based biomaterials. Despite considerable effort, however, rationale design of activatable antibiotics that display high activation efficiency and uncompromised bactericidal potency in the activated state remains difficult. Here, we demonstrate a design principle that helps to address this challenge. This strategy differs from previous attempts by underscoring photolytic removal of a functionality directly conjugated to the pharmacophore of an antibiotic, enabling not only an activation efficiency significantly improved beyond previous light-activatable antibiotics, but also bactericidal activity in the activated state as potent as the parent drug.

Keywords: Antibiotic, Light, Activatable, Ciprofloxacin, Collagen, Biomaterials

1 Introduction

By virtue of haemostatic properties, low antigenicity, and the ability to stimulate tissue regeneration, collagen has gained increasing popularity in preparing pharmaceutical products that can be used in wound management, orthopaedic, and oral surgeries [1, 2]. However, naturally-occurring collagen is vulnerable to microorganisms, and thus loading collagen-based biomaterials with antibiotics that prevent microbial contamination is currently one standard medical procedure. In spite of their potency in killing microorganisms, always-active antibiotics in the collagen matrix are also toxic to eukaryotes, thus potentially causing injuries to healthy issues [3]. Therefore, to tackle the occurrence of side effects, one major issue now plaguing antibiotic usage in collagen-based biomaterials, novel molecular approaches that enable activatable antibiotics for on-demand drug administration from the collagen matrix are highly desirable.

To render an antibiotic activatable, previous endeavors [4–6] underscored the necessity of a photoisomerizable scaffold permanently attached to the nonpharmacophore site of the parent drug. As the scaffold isomerized upon light irradiation, the amphiphilicity of the drug varied accordingly, which was then translated into an abrupt increase in its antibacterial potency. Despite important advances this strategy represents, it still suffers from two major limitations. First, permanently attaching an exogenous scaffold to a drug even at a nonpharmacophore site hampers optimal interaction with its biological target, thus leading to a significant decrease in antibacterial potency when activated relative to the parent drug. A typical example highlighting this limitation is light-activatable ciprofloxacin. Conjugating a photoisomerizable spiropyrane to the nonpharmacophore piperazine of ciprofloxacin conferred activatability, but the minimum inhibitory concentration (MIC) of the conjugate in the activated state was 50-fold higher than that of the parent drug [5], making this strategy problematic in practical application. Second, the amphiphilicity change induced by photoisomerization of the attached scaffold was so

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subtle that the engineered drug invariably exhibited a low activation efficiency; the ratio of its MIC in the inert state to that in the activated state was typically in the range of 2 to 4 [4–9].

Here, we report a design principle that helps to overcome these limitations. This strategy differs from previous initiatives in two aspects. Instead of harnessing the nonpharmacophore group for modification, we decide to attach a photolabile scaffold directly to the pharmacophore of the drug, which interferes drug interaction with its biological target. This interference effect is so profound that it inactivates the drug to a great extent. Another essential feature of this strategy is the attachment of a photoremovable rather than a photoisomerizable scaffold. Once the scaffold is removed in response to light, the engineered drug restores its original structure, and hence, potency. In this manner, fabrication of an activatable antibiotic with unprecedentedly high activation efficiency and uncompromised bactericidal potency in the activated state is enabled.

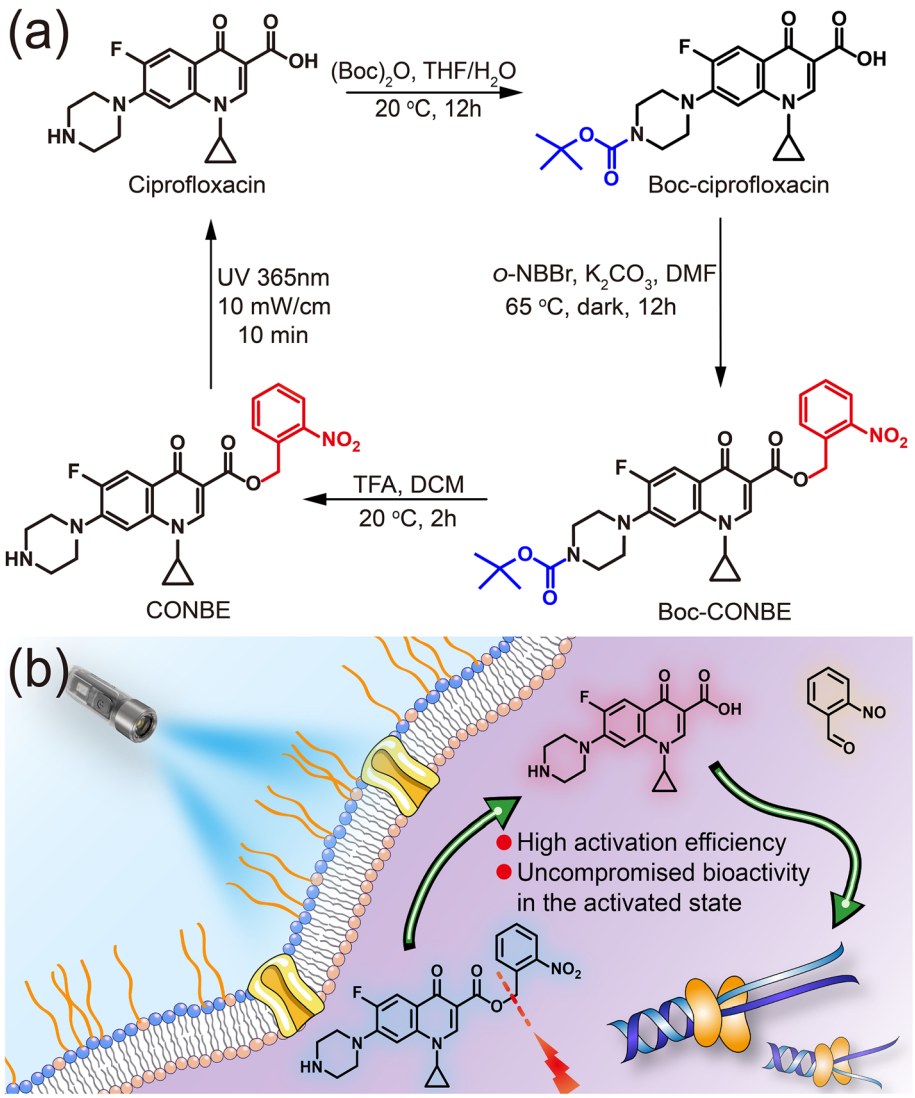
2 Results and discussion

To demonstrate the feasibility and advantage of the proposed strategy, proof-of-concept experiments were conducted herein by still using ciprofloxacin as the model drug. The bactericidal activity of ciprofloxacin stems from their hydrogen binding to a single-stranded DNA pocket created DNA gyrase, with a pharmacophore consisting of a benzene ring fused with a carboxypyridone moiety [10]. To acquire selective and high binding affinity to the target, the drug has to assemble through π-π ring stacking of the pharmacophore in a flip-over manner so that the 4-keto groups point in the same direction toward the DNA while the 3-carboxyl groups are located on the opposite sides to avoid charge repulsion [11]. Molecular stacking of this manner is essential as it allows optimal hydrogen bonding with DNA by using two properly-distanced hydrogen-bond acceptors: the 4-keto in one quinolone ring and the 3-carboxyl in the other [12]. Given this unique mechanism of action, we envision that if a coplanarity-destroying, photolabile substitute is covalently attached to the 3-carboxyl of ciprofloxacin, the drug will be inactivated to a great extent because both close stacking of the pharmacophore and hydrogen bonding with the DNA target are inhibited. Furthermore, once the substitute is removed photochemically, the drug is free to assemble and interact inside the DNA binding pocket again, shifting from being inert to its activated state with bactericidal activity as potent as the parent drug.

To this end, a bulky, photocleavable *ortho*-nitrobenzyl (*o*-NB) group was conjugated to the 3-carboxyl of ciprofloxacin according to the procedures illustrated in Scheme 1. The model drug was first allowed to react with di-tert-butyl dicarbonate ($(Boc)_2O$) at 20 °C in a mixture of tetrahydrofuran (THF) and H_2O that enabled protection of the secondary amine of the 7-piperazinyl ring. In a *N,N*-dimethylformamide (DMF) dispersion of K_2CO_3, coupling between the resulting acid and *ortho*-nitrobenzyl bromide (*o*-NBBr), followed by removal of the butyloxycarbonyl (Boc) group with trifluoroacetic acid (TFA), produced the crude compound. The solvent was then removed by vacuum drying, and the residue purified by preparative high performance liquid chromatography (HPLC, SI, Fig. S1†) to give a pale yellow solid (CONBE) in 70% yield. Detailed procedures for synthesis of CONBE was included in SI, Section S1.2. The chemical structure of the product was verified by ^{1}H nuclear magnetic resonance (^{1}H NMR, SI, Fig. S2†) and mass spectrometry (ESI-MS, SI, Fig. S3†), respectively.

As anticipated, the resulting CONBE was photolabile, which could be validated by using ultraviolet-visible (UV-vis) spectroscopy (Fig. 1). The UV-vis absorption spectrum of ciprofloxacin showed two absorption bands centering at λ_{max} = 227 and 277 nm, respectively, in accordance with previous report [13]. Conjugating a photocleavable *o*-NB to the 3-carboxyl of ciprofloxacin imposed no influence on the absorption at 227 nm, but resulted in an intensity increase and a concomitant redshift of the absorption at 277 nm. Exposure of a solution of CONBE to UV light (λ = 365 nm; intensity = 10 mW/cm^2) photochemically cleaved the *o*-NB linker, manifested by a gradual recovery of the absorption at 277 nm. Here, noteworthy was the fact that the intensity of the absorption at 227 nm increased as a function of UV irradiation time. This phenomenon was ascribed to increasing concentration of the photolytic by-product, *ortho*-nitrosobenzaldehyde, in the mixture, which also displayed a λ_{max} at around 227 nm [14, 15]. No further photocleavage could be observed after UV irradiation for 10 min. According to a standard curve generated from the absorbance of ciprofloxacin of different concentrations at 277 nm, the maximal conversion efficiency of CONBE to ciprofloxacin was determined to be ca. 91%. We also monitored the photochemical regeneration of ciprofloxacin from CONBE by HPLC analysis. Upon UV irradiation for 10 min, one could see disappearance of the chromatographic peak (1) corresponding to CONBE, with two new signals (2 and 3) observed. The retention times for these two new signals coincided well with pure ciprofloxacin (**2**) and *ortho*-nitrosobenzaldehyde (3), respectively. This observation was indicative of the photolabile nature of CONBE, which produced free ciprofloxacin by highly efficient photolytic removal of the *o*-NB group.

With an *o*-NB-masked ciprofloxacin in hand, we then turned to investigate light-dependent susceptibility of bacteria to this conjugate. As the bulky, coplanarity-



Scheme 1 a Synthesis procedure and molecular structure of CONBE, a photolabile ciprofloxacin with an *o*-NB covalently conjugated to the 3-carboxyl. **b** Schematic illustration of how light-induced cleavage of the *o*-NB ester influenced bactericidal potency of CONBE

destroying *o*-NB group was attached directly to the pharmacophore of ciprofloxacin, the drug encountered difficulty in molecular stacking and hydrogen bonding with its target, thus becoming biologically inactive. This speculation was verified by a combination of MIC measurement and a LIVE/DEAD Baclight bacterial viability assay. Herein, *Escherichia coli* (*E. coli*) K12 was selected as an indicator in antibacterial experiment in that ciprofloxacin is known to be more effective against Gram-negative bacteria than Gram-positive ones [16]. Using a 2-fold broth microdilution method, MIC values of CONBE before and after UV irradiation against *E. coli* K12 were determined. As tabulated in Table 1, non-irradiated CONBE exhibited a MIC value of 8.0 μM, significantly higher than that of ciprofloxacin (MIC = 0.25 μM) by a factor of 32. Upon UV irradiation for 10 min, the overwhelming majority of CONBE was converted into ciprofloxacin. Thus, the MIC value underwent a remarkable decrease to a level (0.5 μM) quite similar to the parent drug, yielding an activation efficiency of 16, significantly improved beyond previous light-activatable antibiotics with reported activation efficiency being in the range of 2–4 [4–7]. Almost full recovery of antibacterial potency after UV irradiation was not associated with *ortho*-nitrosobenzaldehyde, as this photolytic by-product displayed no measurable inhibitory effect on growth of the strain even at a high concentration (MIC> 100 μM). Also, control experiment indicated that UV irradiation for 10 min had no appreciable influence on viability of the strain. The pronounced light-dependent difference in susceptibility was confirmed by a LIVE/DEAD Baclight bacterial viability

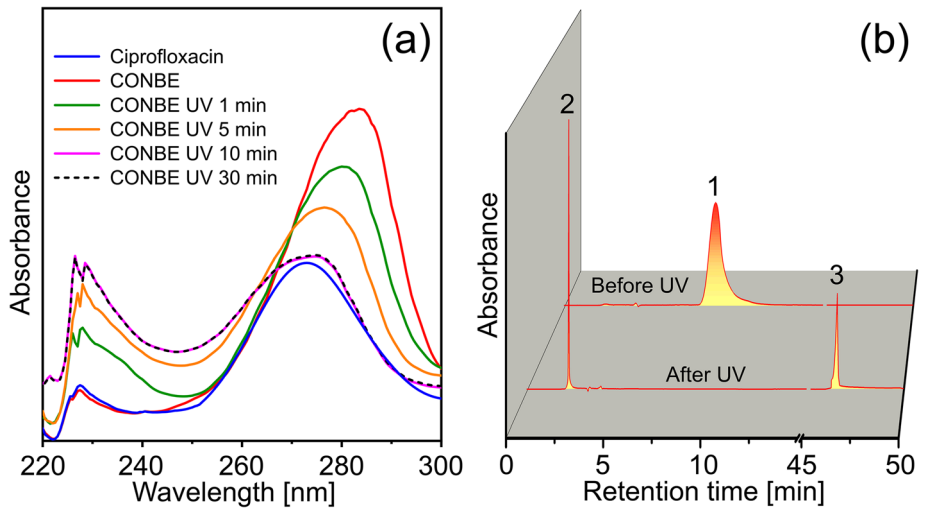


Fig. 1 **a** UV-vis spectra of CONBE (4.0 μM) in 5% (v/v) dimethyl sulfoxide aqueous solution as a function of UV irradiation time (λ = 365 nm; intensity = 10 mW/cm^2). For comparison, the UV-vis spectrum of ciprofloxacin was also included. **b** HPLC chromatograms of CONBE before and after UV irradiation for 10 min

assay (Fig. 2), which differentiated live and dead cells using a two-color fluorescence assay. After incubated with non-irradiated CONBE at a concentration of 4.0 μM, the bacteria remained viable and hence fluoresced green. This observation indicated that masking the 3-carboxyl of ciprofloxacin using an *o*-NB group abolished its antibacterial activity. Upon UV irradiation for 10 min, light-induced unmasking re-generated ciprofloxacin, and hence switched the drug to the activated state. In this case, only dead bacteria featuring red fluorescence were observed.

An activatable antibiotic exhibiting a large difference in activity upon light irradiation enables facile external control of bacterial growth with high spatiotemporal resolution. In general, altering the concentration of active drug in a biological system at a desired time is difficult to accomplish without disrupting the experiment. To demonstrate precise temporal control of bacterial growth using photolabile CONBE, experiments were performed that remotely activated the *o*-NB-masked drug during the incubation process using light (Fig. 3). The bacteria were initially incubated with non-irradiated CONBE of concentrations (0.5–4.0 μM) below its MIC. As expected, the strains experienced normal growth as if the engineered drug were not present. After the incubation lasted for 360 min, the wells were illuminated with UV light (λ = 365 nm; intensity = 10 mW/cm^2) for 10 min, switching the CONBE back to its bioactive form. Thereafter, the bacteria within the cells ceased growth immediately; the extent of bacteria number decline was positively correlated with the initial CONBE concentration. This observation suggested that bacteria viability could be controlled with high temporal resolution by using CONBE.

Table 1 MIC values of CONBE against *E. coli* K12 before and after exposure to UV light (λ = 365 nm; intensity = 10 mW/cm^2) for 10 min

Compound	MIC[a] (μM)
Ciprofloxacin	0.25
Non-irradiated CONBE	8.0
CONBE after UV exposure[b]	0.5
Ortho-nitrosobenzaldehyde[c]	>100

[a] According to the 2-fold broth microdilution method employed in this study, a 2-fold shift in the measured MIC was considered insignificant [10]
[b] Control experiment indicated that UV irradiation for 10 min had no appreciable influence on viability of the strain
[c] *Ortho*-nitrosobenzaldehyde was not commercially available; it was obtained from *ortho*-nitrobenzyl alcohol by UV irradiation for 10 min according to a previous report [17]

The light-responsive nature of CONBE also allowed a unique approach to control bacterial growth in a spatial-specific manner. To demonstrate this advantage, an eosin-methylene blue agar plate containing *E. coli* K12 and inactive, non-irradiated CONBE (4.0 μM) was prepared. A Chinese character mask was then placed on top of the agar plate, with the assembly being irradiated using UV light (λ = 365 nm; intensity = 10 mW/cm^2) for 10 min to activate the engineered drug only in the exposed areas. After that, the plate was incubated overnight at 37 °C, and bacterial colonies observed only where the area of the plate was covered to prevent UV irradiation. These experiments underlined that when CONBE was coupled with light of specific wavelength, the active antibiotic could be confined to a particular place at a desired time (Fig. 4).

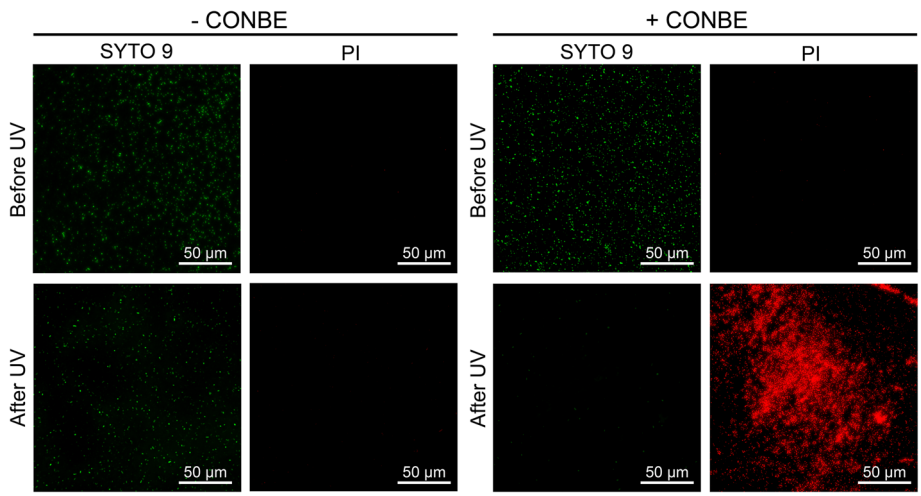


Fig. 2 Fluorescent images illustrating bacteria (*E. coli* K12, 5×10^7 colony-forming units per milliliter) after incubation with non-irradiated and UV-irradiated (λ = 365 nm; intensity = 10 mW/cm^2; 10 min) CONBE (4.0 μM) for 6 h. Control results illustrating UV irradiation for 10 min had no appreciable influence on viability of the strain were also included. The green and red fluorescence indicate live and dead bacteria, respectively

3 Conclusion

In summary, we have demonstrated a strategy for fabrication of a light-activatable antibiotic with high activation efficiency and uncompromised bactericidal potency in the activated state, potentially useful to tackle the occurrence of side effects, one major issue now plaguing antibiotic usage in collagen-based biomaterials. The rationale underlying this strategy centers around temporary conjugation of a photolabile scaffold directly to the pharmacophore of the parent drug, differing from previous initiatives that invariably depended on photoisomerization of a functionality permanently attached to one deliberately selected nonpharmacophore group. The concept is, of course, generalizable to other classes of

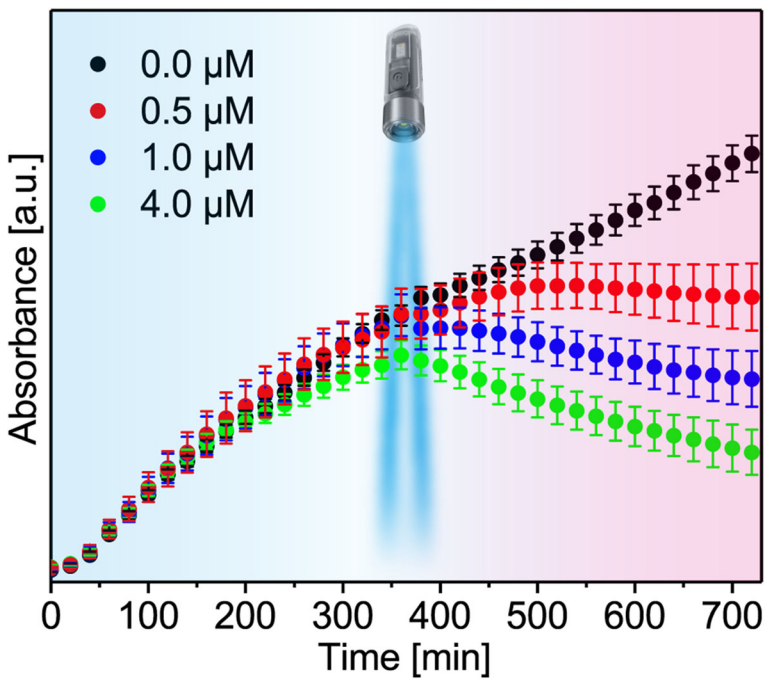


Fig. 3 Bacterial growth curves of *E. coli* K12 (initial concentration = 5×10^5 colony-forming units per milliliter) containing non-irradiated CONBE of varying concentration. After incubated in Luria-Bertani (LB) broth at 37 °C for 360 min, the wells were illuminated with UV light (λ = 365 nm; intensity = 10 mW/cm^2) for 10 min to switch the otherwise inactive CONBE to its activated state. Error bars show standard deviations calculated from measurements in triplicate

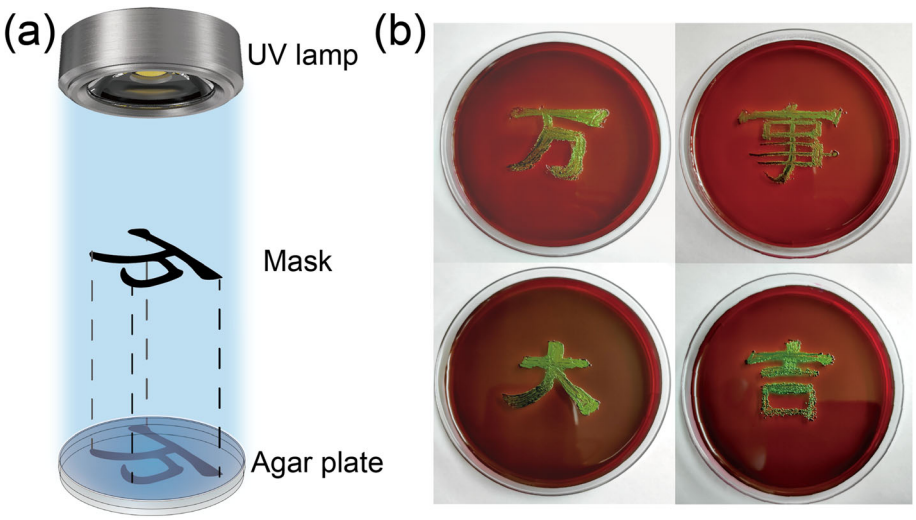


Fig. 4 Spatial control of *E. coli* K12 growth with CONBE and light irradiation. **a** A mask was placed on top of an eosin-methylene blue agar plate containing the bacteria (5×10^5 colony-forming units per milliliter) and CONBE (4.0 μM). The plate was then irradiated (λ = 365 nm; intensity = 10 mW/cm^2) for 10 min, followed by incubation overnight at 37 °C. **b** Results showing bacteria growth (metallic green sheen) only in the unexposed part on the incubated plate. Four Chinese characters meaning "all the best" were used as the masks

antibiotics as long as their pharmacophores are amenable to modification by a photosensitive masking agent.

Nevertheless, UV light has a limited tissue penetration depth. To activate the loaded drug in vivo, the application of CONBE should be confined to collagen-based biomaterials that are intended to cover dermatological lesions or sites endoscopically accessible. Otherwise, a photolabile agent responsive to deep-tissue-penetrating light in the therapeutic window between 650 and 900 nm should be conjugated to the parent drug. The design of such drug that is responsive to near-infrared light (λ = 650–900 nm) will be reported in a follow-up paper.

4 Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42825-021-00051-6.

Additional file 1.

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Not applicable.

Authors' contributions

Delong Hou synthesized CONBE, determined the MIC values, and evaluated spatial control of *E. coli* K12 growth with CONBE and light irradiation. He also interpreted all data, and was a major contributor in writing the manuscript. Rui Wang obtained the NMR and mass spectra. Zhonghui Wang performed the HPLC experiment. Gaofu Yang obtained the UV-vis absorption spectra. Zhou Xu and Qi Zeng carried out the LIVE/DEAD Baclight bacterial viability assay. Yi Chen proposed the technique route to synthesize CONBE and measured the bacterial growth curves of *E. coli* K12 in responsive to UV light. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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