OL | Organic Letters

Cite This: Org. Lett. XXXX, XXX, XXX−XXX

Letter

pubs.acs.org/OrgLett

Click and Release: A High-Content Bioorthogonal Prodrug with Multiple Outputs

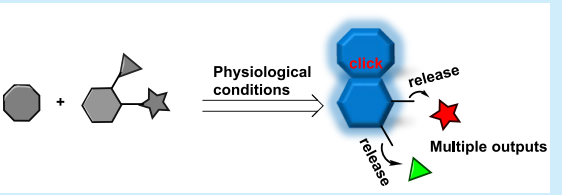
Xingyue Ji,[†,‡] Robert E. Aghoghovbia,[†] Ladie Kimberly C. De La Cruz,[†] Zhixiang Pan,[†] Xiaoxiao Yang,[†] Bingchen Yu,[†] and Binghe Wang*[,†]

[†]Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, Georgia 30303, United States

[‡]Department of Medicinal Chemistry, College of Pharmaceutical Science, Soochow University, Suzhou, Jiangsu 215021, China

Supporting Information

ABSTRACT: A high-content bioorthogonal prodrug with multiple outputs using the "click, cyclize, and release" concept is described. The proof of concept is established by the co-delivery of a gasotransmitter carbon monoxide, an anticancer drug floxuridine, and an in situ generated fluorescent reporter molecule for real-time monitoring of the prodrug activation. Bioorthogonal prodrugs as such are invaluable tools for the co-delivery of other drug payloads for multimodal therapy.



Prodrug strategies have been successfully used in addressing drug developability issues by masking toxicity, improving pharmacokinetic and physiochemical profiles, and affording controlled delivery, among others.[1] Traditional prodrug strategies largely rely on chemical reactions mediated by endogenous enzymes or small molecules to release the parent drug.[2] Recently, click chemistry has been creatively applied to the design of bioorthogonal prodrugs, wherein the parent drug is activated by a bioorthogonal reaction between the prodrug and the another exogenously applied trigger/component.[3] Such bioorthogonal prodrugs have been applied in the delivery of anticancer agents[4] and antibody drug conjugates (ADCs),[5] yielding a controlled release of the payload at the tumor site. Very recently, bioorthogonal prodrugs for gasotransmitters including carbon monoxide (CO),[6] carbonyl sulfide (COS),[7] and sulfur dioxide (SO_2)[8] have also been devised to address the unique challenging issues associated with the delivery of a gaseous molecule. The need for a secondary trigger in using bioorthogonal chemistry in drug delivery has also presented unique challenges associated with the bimolecular nature of the drug activation approach. Recently published pretargeting[5] and enrichment-triggered activation[9] strategies have proven their utility in animal models.

Previously, we have successfully developed a "click, cyclize, and release" system for the delivery of doxorubicin to mitochondria. However, such bioorthogonal prodrugs as well as many others just yield a single output upon activation. Herein, we are interested in going beyond the delivery of a single payload (Figure 1A) by developing a general strategy of prodrugs with multiple outputs, whether in payload, signal, or a combination of both. We herein describe our efforts toward a high-content bioorthogonal prodrug system using a similar "click, cyclize, and release" concept with multiple outputs upon activation, as exemplified by the co-delivery of gasotransmitter

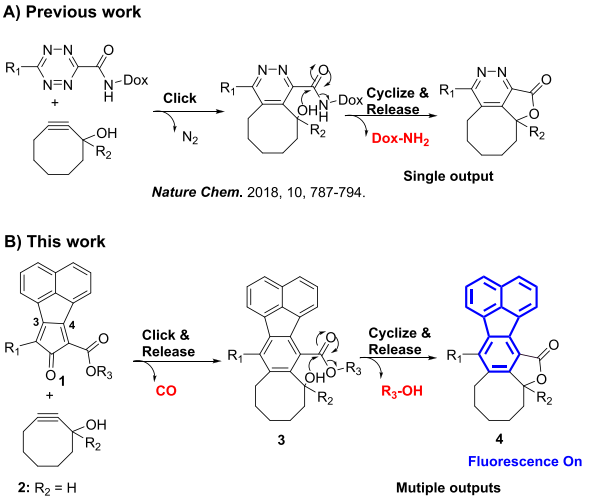


Figure 1. A schematic illustration of the "click, cyclize, and release" concept. (A) Previous work with a single output, and (B) this work with multiple outputs.

CO, an anticancer drug floxuridine (5-fluoro-2′-deoxyuridine), and a fluorescent reporter (Figure 1B).

Carbon monoxide has been well-established as a gasotransmitter with pleiotropic pharmacologic effects.[10] It has also been shown to have synergistic effects with other drugs such as anticancer,[11] antibacterial,[12] and anti-inflammatory agents.[13] In some cases, CO also alleviates some severe side-effects observed with chemotherapies,[14] making CO a very promising adjuvant in disease treatment. Therefore, it would be highly desirable to develop a drug delivery system for the co-delivery

Received: March 28, 2019

DOI: 10.1021/acs.orglett.9b01086
Org. Lett. XXXX, XXX, XXX−XXX

of CO and another payload for multimodal therapy. Along this line, we describe our efforts toward a high-content bioorthogonal prodrug system for the co-delivery of CO and an anticancer drug payload.

The development of CO prodrugs represents a major challenge due to its gaseous and chemically inert nature. Generally speaking, CO delivery approaches reported so far are classified into five categories: (1) gaseous CO for inhalation administration, (2) CO in a drink (dissolved CO) for oral delivery, (3) metal-immobilized carbonyls (commonly referred to as CO-releasing molecules or CORMS) for oral or parenteral administration,[10a,15] (4) photosensitive organic CO donors for topical application,[16] and (5) organic CO prodrugs for oral and parenteral administration.[3b,17] In developing organic CO prodrugs capable of CO release under physiologic conditions, we have successfully employed the click reaction between cyclopentadienones and (strained) alkynes to trigger CO release without the need for photoirradiation.[6] Herein, we utilize the same click reaction for CO release. Meanwhile, a drug payload is appended to the dienone moiety via an ester bond (**1**, Figure 1B), and a hydroxyl group is installed at the propargyl position of the strained alkyne (**2**). Ideally, the initial click reaction between **1** and **2** leads to CO release and the formation of intermediate **3**, which is expected to readily undergo lactonization to liberate the drug payload and compound **4**. In addition, a naphthalene group is attached at the C3, 4 positions to enable fluorescence turn-on upon the release of CO and the other payload, which can be leveraged for real-time monitoring of payloads release.

In order to examine the feasibility of such a design, a model compound **1a** with benzyl alcohol as the model payload was synthesized (SI). Indeed, the click reaction between **1a** and **2** resulted in a blue fluorescence turn-on. The second-order reaction rate constant was determined to be 0.018 M^{-1} s^{-1} in 30% of DMSO/PBS at 37 °C by monitoring the fluorescence intensity increase, and CO release was validated by a CO-myoglobin assay (Figure S5). In order to further confirm the release of benzyl alcohol, the reaction between **1a** and **2** was studied with ^{1}H NMR in DMSO-d_6 at 37 °C by monitoring the featured protons Ha in **1a**, Hb, c in **3a**, Hb′, c′ in **3a′**, and He, f in **4a**. Noteworthy, protons Hc, Hc′, and He are shifted significantly to high field (6.0−6.3 ppm) because they are in the shielding cone of phenyl ring A. As shown in Figure 2, the click reaction between **1a** and **2** completed within about 4.5 h with the formation of **3a** (Hb, c), **3a′** (Hb′, c′), benzyl alcohol (Hd), and the lactone **4a** (He, f). The peaks of intermediate **3a** (Hb, c) decreased gradually with the increase in intensity for the peaks of benzyl alcohol (Hd) and **4a** (He, f). After 21 h, all the intermediate **3a** was totally consumed with **3a′**, **4a**, benzyl alcohol, and excessive **2** as the only compounds in the solution. Due to a regioselectivity issue, the release yield of the benzyl alcohol was around 58% based on the integration ratio between Hd and Hb′. Altogether, these results unambiguously validated the release of benzyl alcohol via a cascade of click, cyclization, and release process.

Having confirmed the feasibility of the "click and release" strategy, we next appended a real drug floxuridine to the dienone moiety. In addition, in order to enhance the reaction rate, an electron-withdrawing group morpholine-4-carbonyl was attached to the C2 position of the dienone ring to further lower its LUMO energy level and thereby to facilitate the cycloaddition reaction.[17] Indeed, the reaction rate constant between **2** and **1b** increased to 0.17 M^{-1} s^{-1}, which is about 9-

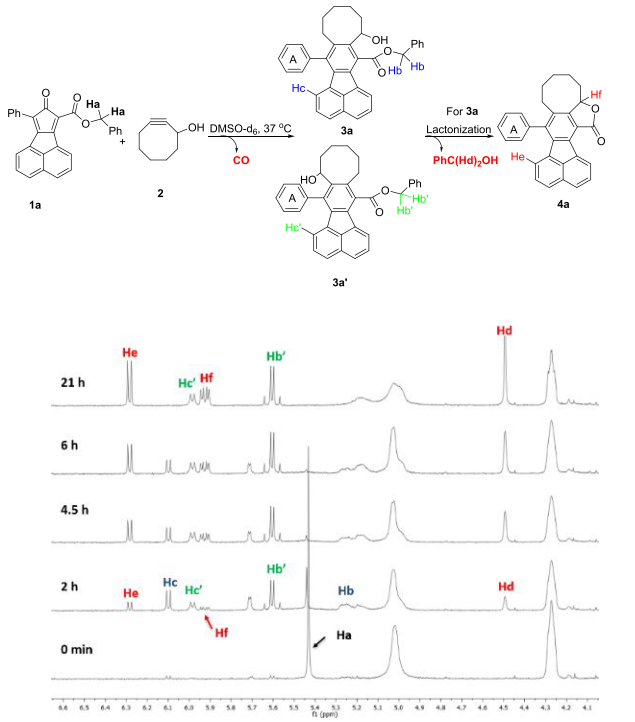


Figure 2. CO release reaction monitored by ^{1}H NMR. A solution of **1a** and **2** in DMSO-d_6 was incubated at 37 °C.

fold higher than that of the reaction between **2** and **1a**. The release of floxuridine from the reaction between **2** and **1b** was monitored using HPLC, and the results showed that the cycloaddition reaction between **2** (5 mM) and **1b** (100 μM) in 30% of DMSO/PBS was finished within about 4 h with the floxuridine recovery yield being about 50% (Figure 3A). The remaining 50% was trapped as compound **4b′**. The chemical structures of **4b** and **4b′** have been thoroughly elucidated by NMR and HRMS (SI), confirming the proposed payload release mechanism (Figure 3A). However, incubation of **1b** only under the same conditions led to <3% of floxuridine

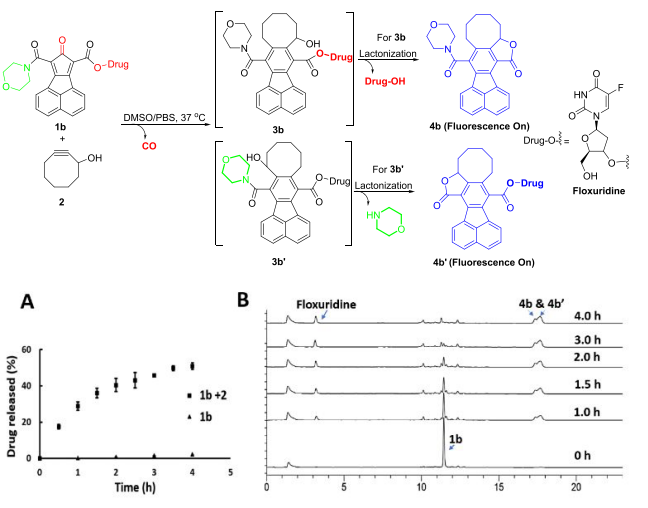


Figure 3. Release profiles of bioorthogonal prodrug **1b** upon clicking with strained alkyne **2**. (A) The drug release yield upon the click reaction between **1b** (100 μM) and **2** (5 mM) in 30% of DMSO/PBS at 37 °C. (B) The HPLC chromatogram of the reaction between **1b** and **2** at different time points.

DOI: 10.1021/acs.orglett.9b01086
Org. Lett. XXXX, XXX, XXX−XXX

release. It is worth noting that lactonization also occurred for regioisomer **3b′** with the release of morpholine, suggesting the utility of this approach for the delivery of either alcohol- or amine-containing drugs. In both cases (**3b** and **3b′**), the build-up for the initial cycloaddition intermediates **3b** and **3b′** was not observed under the experimental conditions, indicating the rate-limiting nature of the cycloaddition reaction in this cascade reaction sequence. It is worth pointing out that for the click reaction between **2** and **1b**, four different outputs were generated, namely CO, floxuridine, morpholine, and blue fluorescence, albeit release yields for floxuridine and morpholine were moderate (ca. 50% each).

Having confirmed the drug release from the reaction between **2** and **1b**, we moved to test if that is the case in a real biological milieu. The fluorogenic nature of the click reaction greatly facilitated the real-time monitoring of the intracellular payload release. For this purpose, MB-231 cells were co-treated with compounds **2** and **1b** for 24 h. Then cells were fixed for imaging studies under the DAPI channel. As expected, cells co-treated with **2** and **1b** (SI, Figure S6e,g) exhibited much enhanced blue fluorescence as compared to the control cells treated with vehicle (Figure S6a) or **1b** (Figure S6c) only. It is worth pointing out that the turned-on fluorescence can only indicate the release of CO but not floxuridine in that both **4b** and **4b′** are fluorescent.

Having confirmed the intracellular payloads release, we next assayed the antiproliferative activity of the prodrug against MB-231 cells. As shown in Figure 4, co-treatment with **1b** and

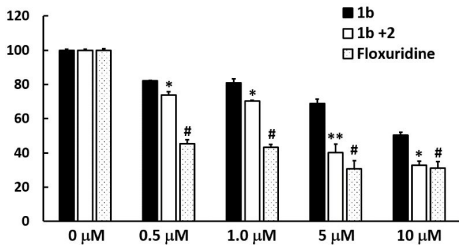


Figure 4. Antiproliferative activities of bioorthogonal prodrug **1b** + **2** (100 μM). The drug exposure time was 72 h. The mean of **1b** + **2** or floxuridine-treated group was compared with the **1b** only group by two-sample *t* test. * $p < 0.05$, **, # $p < 0.01$, vs **1b** group. 100 μM of **2** did not comprise the cell viability (data not shown).

2 (100 μM) presented much enhanced cytotoxicity as compared to **1b** only. Although only 50% of the floxuridine was released upon the click reaction, co-treatment of **1b** and **2** (100 μM) also showed comparable antiproliferative activity to that of the positive control at 5 and 10 μM, presumably due to the release of CO, which might show additive cytotoxicity together with floxuridine. The prodrug **1b** also showed some cytotoxicity, presumably due to the intracellular esterase-mediated activation of the drug floxuridine. However, prodrug **1b** is quite resistant to the esterase-mediated hydrolysis. In the presence of porcine liver esterase (1 unit/mL), no obvious release of floxuridine was detected after 24 h incubation in 5% of DMSO/PBS at 37 °C. Though a small amount of intracellular release of the drug by various enzymes cannot be completely excluded, it is reasonable to assume that the observed cytotoxicity was due to **1b** itself.

In summary, we have successfully developed a bioorthogonal prodrug system for multipayload delivery. The proof of concept was established by the activation of CO and an anticancer drug floxuridine. The fluorescent nature of the byproduct after prodrug activation can also be used as a reporter molecule for real-time monitoring of payload release. Such a multimodality of this click and release system would be invaluable for drug delivery, especially for combination therapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01086.

Synthetic procedure and copies of spectra, CO-myoglobin assay, and biology experimental details (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wang@gsu.edu.

ORCID

Binghe Wang: 0000-0002-2200-5270

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge GSU Molecular Basis of Disease (Z.P.), Brains and Behaviors (B.Y.), and University 2CI (L.K.C.d.l.C.) fellowships for their support.

■ REFERENCES

(1) Wang, B.; Hu, L.; Siahaan, T. *Drug Delivery: Principles and Applications*, 2nd ed.; John Wiley and Sons: Hoboken, NJ, 2016.

(2) (a) Cheng, R.; Feng, F.; Meng, F.; Deng, C.; Feijen, J.; Zhong, Z. Glutathione-responsive nano-vehicles as a promising platform for targeted intracellular drug and gene delivery. *J. Controlled Release* **2011**, *152* (1), 2−12. (b) Chen, W.; Meng, F.; Cheng, R.; Zhong, Z. pH-Sensitive degradable polymersomes for triggered release of anticancer drugs: a comparative study with micelles. *J. Controlled Release* **2010**, *142* (1), 40−6. (c) Qi, H.; Lu, J.; Li, J.; Wang, M.; Xu, Y.; Wang, Y.; Zhang, H. Enhanced Antitumor Activity of Monophosphate Ester Prodrugs of Gemcitabine: In Vitro and In Vivo Evaluation. *J. Pharm. Sci.* **2016**, *105* (9), 2966−2973.

(3) (a) Li, J.; Chen, P. R. Development and application of bond cleavage reactions in bioorthogonal chemistry. *Nat. Chem. Biol.* **2016**, *12* (3), 129−37. (b) Ji, X.; Pan, Z.; Yu, B.; De La Cruz, L. K.; Zheng, Y.; Ke, B.; Wang, B. Click and release: bioorthogonal approaches to "on-demand" activation of prodrugs. *Chem. Soc. Rev.* **2019**, *48* (4), 1077−1094.

(4) (a) Versteegen, R. M.; Rossin, R.; ten Hoeve, W.; Janssen, H. M.; Robillard, M. S. Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation. *Angew. Chem., Int. Ed.* **2013**, *52* (52), 14112−14116. (b) Tu, J.; Xu, M.; Parvez, S.; Peterson, R. T.; Franzini, R. M. Bioorthogonal Removal of 3-Isocyanopropyl Groups Enables the Controlled Release of Fluorophores and Drugs in Vivo. *J. Am. Chem. Soc.* **2018**, *140* (27), 8410−8414. (c) Bernard, S.; Audisio, D.; Riomet, M.; Bregant, S.; Sallustrau, A.; Plougastel, L.; Decuypere, E.; Gabillet, S.; Kumar, R. A.; Elyian, J.; Trinh, M. N.; Koniev, O.; Wagner, A.; Kolodych, S.; Taran, F. Bioorthogonal Click and Release Reaction of Iminosydnones with Cycloalkynes. *Angew. Chem., Int. Ed.* **2017**, *56* (49), 15612−15616. (d) Jimenez-Moreno, E.; Guo, Z.; Oliveira, B. L.; Albuquerque, I. S.; Kitowski, A.; Guerreiro, A.; Boutureira, O.; Rodrigues, T.; Jimenez-Oses, G.; Bernardes, G. J. Vinyl Ether/Tetrazine Pair for the Traceless Release of Alcohols in

DOI: 10.1021/acs.orglett.9b01086
Org. Lett. XXXX, XXX, XXX−XXX

Cells. *Angew. Chem., Int. Ed.* **2017**, *56* (1), 243−247. (e) Matikonda, S. S.; Orsi, D. L.; Staudacher, V.; Jenkins, I. A.; Fiedler, F.; Chen, J.; Gamble, A. B. Bioorthogonal prodrug activation driven by a strain-promoted 1,3-dipolar cycloaddition. *Chem. Sci.* **2015**, *6* (2), 1212−1218. (f) Carlson, J. C. T.; Mikula, H.; Weissleder, R. Unraveling Tetrazine-Triggered Bioorthogonal Elimination Enables Chemical Tools for Ultrafast Release and Universal Cleavage. *J. Am. Chem. Soc.* **2018**, *140* (10), 3603−3612. (g) Neumann, K.; Gambardella, A.; Lilienkampf, A.; Bradley, M. Tetrazine-mediated bioorthogonal prodrug−prodrug activation. *Chem. Sci.* **2018**, *9* (36), 7198−7203.

(5) Rossin, R.; Versteegen, R. M.; Wu, J.; Khasanov, A.; Wessels, H. J.; Steenbergen, E. J.; Ten Hoeve, W.; Janssen, H. M.; van Onzen, A.; Hudson, P. J.; Robillard, M. S. Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice. *Nat. Commun.* **2018**, *9* (1), 1484.

(6) (a) Ji, X.; Zhou, C.; Ji, K.; Aghoghovbia, R. E.; Pan, Z.; Chittavong, V.; Ke, B.; Wang, B. Click and Release: A Chemical Strategy toward Developing Gasotransmitter Prodrugs by Using an Intramolecular Diels−Alder Reaction. *Angew. Chem., Int. Ed.* **2016**, *55*, 15846−15851. (b) Ji, X.; Ji, K.; Chittavong, V.; Yu, B.; Pan, Z.; Wang, B. An esterase-activated click and release approach to metal-free CO-prodrugs. *Chem. Commun.* **2017**, *53* (59), 8296−8299. (c) Pan, Z.; Chittavong, V.; Li, W.; Zhang, J.; Ji, K.; Zhu, M.; Ji, X.; Wang, B. Organic CO Prodrugs: Structure−CO-Release Rate Relationship Studies. *Chem. - Eur. J.* **2017**, *23* (41), 9838−9845.

(7) Steiger, A. K.; Yang, Y.; Royzen, M.; Pluth, M. D. Bio-orthogonal ″Click and Release″ Donation of Caged Carbonyl Sulfide (COS) and Hydrogen Sulfide (H2S). *Chem. Commun.* **2017**, *53* (8), 1378−1380.

(8) (a) Wang, W.; Ji, X.; Du, Z.; Wang, B. Sulfur Dioxide Prodrugs: Triggered Release of SO2 via a Click Reaction. *Chem. Commun.* **2017**, *53* (8), 1370−1373. (b) Ji, X.; El-labbad, E. M.; Ji, K.; Lasheen, D. S.; Serya, R. A. T.; Abouzid, K. A.; Wang, B. Click and Release: SO2 Prodrugs with Tunable Release Rates. *Org. Lett.* **2017**, *19* (4), 818−821.

(9) Zheng, Y.; Ji, X.; Yu, B.; Ji, K.; Gallo, D.; Csizmadia, E.; Zhu, M.; Choudhury, M. R.; De La Cruz, L. K. C.; Chittavong, V.; Pan, Z.; Yuan, Z.; Otterbein, L. E.; Wang, B. Enrichment-triggered prodrug activation demonstrated through mitochondria-targeted delivery of doxorubicin and carbon monoxide. *Nat. Chem.* **2018**, *10* (7), 787−794.

(10) (a) Motterlini, R.; Otterbein, L. E. The therapeutic potential of carbon monoxide. *Nat. Rev. Drug Discovery* **2010**, *9* (9), 728−743. (b) Heinemann, S. H.; Hoshi, T.; Westerhausen, M.; Schiller, A. Carbon monoxide-physiology, detection and controlled release. *Chem. Commun.* **2014**, *50* (28), 3644−3660.

(11) Wegiel, B.; Gallo, D.; Csizmadia, E.; Harris, C.; Belcher, J.; Vercellotti, G. M.; Penacho, N.; Seth, P.; Sukhatme, V.; Ahmed, A.; Pandolfi, P. P.; Helczynski, L.; Bjartell, A.; Persson, J. L.; Otterbein, L. E. Carbon Monoxide Expedites Metabolic Exhaustion to Inhibit Tumor Growth. *Cancer Res.* **2013**, *73* (23), 7009−21.

(12) De La Cruz, L. K. C.; Benoit, S. L.; Pan, Z.; Yu, B.; Maier, R. J.; Ji, X.; Wang, B. Click, Release, and Fluoresce: A Chemical Strategy for a Cascade Prodrug System for Codelivery of Carbon Monoxide, a Drug Payload, and a Fluorescent Reporter. *Org. Lett.* **2018**, *20* (4), 897−900.

(13) Nikam, A.; Ollivier, A.; Rivard, M.; Wilson, J. L.; Mebarki, K.; Martens, T.; Dubois-Randé, J.-L.; Motterlini, R.; Foresti, R. Diverse Nrf2 Activators Coordinated to Cobalt Carbonyls Induce Heme Oxygenase-1 and Release Carbon Monoxide in Vitro and in Vivo. *J. Med. Chem.* **2016**, *59* (2), 756−762.

(14) Soni, H.; Pandya, G.; Patel, P.; Acharya, A.; Jain, M.; Mehta, A. A. Beneficial effects of carbon monoxide-releasing molecule-2 (CORM-2) on acute doxorubicin cardiotoxicity in mice: Role of oxidative stress and apoptosis. *Toxicol. Appl. Pharmacol.* **2011**, *253* (1), 70−80.

(15) (a) Schatzschneider, U. Novel lead structures and activation mechanisms for CO-releasing molecules (CORMs). *Br. J. Pharmacol.* **2015**, *172* (6), 1638−50. (b) Romao, C. C.; Blattler, W. A.; Seixas, J. D.; Bernardes, G. J. Developing drug molecules for therapy with carbon monoxide. *Chem. Soc. Rev.* **2012**, *41* (9), 3571−83.

(16) (a) Palao, E.; Slanina, T.; Muchova, L.; Solomek, T.; Vitek, L.; Klan, P. Transition-metal-free CO-releasing BODIPY derivatives activatable by visible to NIR light as promising bioactive molecules. *J. Am. Chem. Soc.* **2016**, *138* (1), 126−33. (b) Soboleva, T.; Esquer, H. J.; Benninghoff, A. D.; Berreau, L. M. Sense and Release: A Thiol-Responsive Flavonol-Based Photonically Driven Carbon Monoxide-Releasing Molecule That Operates via a Multiple-Input AND Logic Gate. *J. Am. Chem. Soc.* **2017**, *139* (28), 9435−9438. (c) Pan, Z.; Zhang, J.; Ji, K.; Chittavong, V.; Ji, X.; Wang, B. Organic CO Prodrugs Activated by Endogenous ROS. *Org. Lett.* **2018**, *20* (1), 8−11. (d) Ji, X.; De La Cruz, L. K. C.; Pan, Z.; Chittavong, V.; Wang, B. pH-Sensitive metal-free carbon monoxide prodrugs with tunable and predictable release rates. *Chem. Commun.* **2017**, *53* (69), 9628−9631.

(17) (a) Ji, X.; Wang, B. Strategies toward Organic Carbon Monoxide Prodrugs. *Acc. Chem. Res.* **2018**, *51* (6), 1377−1385. (b) Ji, X.; Pan, Z.; Li, C.; Kang, T.; De La Cruz, L. K. C.; Yang, L.; Yuan, Z.; Ke, B.; Wang, B. Esterase-Sensitive and pH-Controlled Carbon Monoxide Prodrugs for Treating Systemic Inflammation. *J. Med. Chem.* **2019**, *62* (6), 3163−3168.

DOI: 10.1021/acs.orglett.9b01086
Org. Lett. XXXX, XXX, XXX−XXX