Organic Carbon Monoxide Prodrugs

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ORGANIC CARBON MONOXIDE PRODRUGS

by

ROBERT AGHOGHOVBIA

Under the Direction of BINGHE WANG, PhD

ABSTRACT

Ongoing efforts towards the development of CO-based therapeutics have resulted in the generation of novel CO prodrugs that release CO via different triggers or stimuli. Here in, we designed and synthesized organic CO prodrugs with a tethered cytotoxic agent to achieve a co-delivery of CO and the cytotoxic drug under physiological conditions by leveraging on the inverse electron-demand Diels-Alder reaction as the trigger. We also attempted to characterize, kinetically and spectroscopically, some of the organic CO prodrugs synthesized by our lab. The results showed that these prodrugs released CO under near physiological condition with controllable and tunable release rate.

INDEX WORDS: Co-delivery, Triggered-release, Bioorthogonal, Carbon monoxide, Cytotoxic drug, Quantum yield.
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ROBERT AGHOGHOVbia

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
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ORGANIC CARBON MONOXIDE PRODRUGS

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Georgia State University
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DEDICATION

First, I would like to dedicate my thesis work to Almighty God who has been faithful and has seen me through this journey. I would also like to dedicate this to my wonderful parents for their support, prayers, and unconditional love for me. And to my elder sister, who is not just a sister but a mom, for your guidance, support, and belief in me. And to the rest of my siblings. You all have been wonderful!

This thesis is also dedicated to my roomies Edwin and Tunde for their support, advice, and brotherly love. I am grateful to you guys.

I also dedicate this work to my friends, Brandon, Emem and to my best friend who I consider a brother, Issa Frampton and his mom, Ama Tavernier for being there for me. I appreciate all you have done and I am forever grateful.
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1 STRATEGIES EMPLOYED IN PRODRUG ACTIVATION

1.1 Introduction

Despite the achievements made thus far by chemotherapy in the fight against cancer, its reliability has been limited by issues such as poor selectivity, toxicity, and inefficiency in the treatment of slow growing solid tumors. The activity of chemotherapy drugs is mainly based on anti-proliferating effect, thus making their use unsuitable for prolonged treatment of cancer, as this results to lethal damage to proliferating non-malignant cells.¹

Targeted prodrug strategies have been employed as a panacea to circumvent the problems associated with cancer chemotherapy. In this approach, the cytotoxic drug is conjugated to a carrier ligand which has high affinity for tumor-associated markers such as antigens or receptors. The drug-ligand conjugate is transported by the carrier to the target cell where it is released either extracellularly or intracellularly to initiate its cytotoxic effect.

Activation of prodrugs can be achieved via cleavage of chemical linkers between the drug and the carrier ligand (promoiety). This usually relies on certain triggerable conditions in the tumor such as the presence of an overexpressed enzyme or changes in pH.² However, activation triggered by enzymes has its own drawback as the tumor cell may be deficient in the target overexpressed enzyme or the target enzyme may be expressed inside the cell which necessitates the entering of the prodrug. Moreover, the subtlety of the changes in the tumor environment hampers selective activation of the prodrug, leading to low tumor-to-background ratio,¹ and many of the linkers have been shown to be susceptible to off-target hydrolysis.²

Another approach that has been used to address the lack of selectivity of chemotherapy drugs is the use of antibody-drug conjugates (ADCs). This approach relies on the high affinity of the antibody to effectively transport the prodrug to the tumor cell.¹ Although ADCs have been
successfully utilized over the years to overcome the challenges of chemotherapy, factors such as immunogenic potential, cell selectivity, and cell permeability are hurdles to overcome in the design of ADCs.\textsuperscript{2} Also, the biodistribution of antibody-drug conjugate prodrugs is limited by heterogeneity of the tumor with respect to vascularization and interstitial pressure, and dictates the concentration of the drug at the tumor site.\textsuperscript{3} These issues can be bypassed with the use of bioorthogonal-triggered release in pre-targeted strategies.\textsuperscript{4-9}

Bioorthogonal chemical reaction has received an avalanche of interest and applications in \textit{in vivo} studies, including detection of DNA and RNA synthesis, visualization of site-specific tagging of proteins, detection of post-translational modifications in proteins, and monitoring of cellular processes.\textsuperscript{10, 11} Some examples of prodrug activation strategies that have employed bioorthogonal-triggered release in \textit{in vivo} application include: activation of a doxorubicin prodrug in which the Staudinger reaction was used as the prodrug activator.\textsuperscript{12} Here the active doxorubicin was released in a cell culture environment after the reaction between an azido-functionalized prodrug (1) and a triphenylphosphine (2) (\textbf{Scheme 1}). Although this method was efficient in terms of its selective cleavage of the prodrug, it is, however, hindered by its slow reactivity, and the phosphine reagents are prone to oxidation.\textsuperscript{4} Another example is the trans-cyclooctene (TCO)/tetrazine ligation for prodrug activation,\textsuperscript{4} in which the active drug was released via an intermediate rearrangement. In spite of its fast kinetics, a number of issues related to off-target hydrolysis of the ADC due to low ratio of drug to tumor, and reduced reactivity of TCO-conjugate in the presence of bulky substituents, have been identified to impede its \textit{in vivo} application.\textsuperscript{9} A more recent example of bioorthogonal prodrug activation strategy is the one driven by strain-promoted 1, 3-dipolar cycloaddition where a trans-cyclooctene-antibody conjugate (5) was “click” with a prodrug (6) that encompasses the cytotoxic drug conjugated via the carbamate self-
immolative linker to the p-azidobenzyl group. Controlled release of the active drug was achieved via unstable triazole intermediate (7) which undergo a rearrangement to an acid-labile imine (9). The in situ generated imine, in the tumor environment, subsequently, undergo acid-catalyzed hydrolysis, resulting to 1, 6-elimination of the active drug (12) (Scheme 2). Again, dependence of the drug release on the tumor environment constitute a limiting factor.

Scheme 1: Activation of a doxorubicin prodrug using the Staudinger reaction between a triphenylphosphine (1) and an azido-functionalized prodrug (2).

Scheme 2: Prodrug activation using 1, 3-dipolar cycloaddition as the trigger.

2 CO AS A GASOTRANSMITTER: PHYSIOLOGICAL EFFECTS AND THERAPEUTIC POTENTIAL

Carbon monoxide (CO) has had a long-standing reputation as a toxic, poisonous gas. This is not unconnected to its strong affinity for hemoglobin, reducing the oxygen-carrying capacity of
the protein, and as an air pollutant. Hence, for decades, scientists have paid little attention to carbon monoxide research. However, recent studies and research have demonstrated a lot of biological and therapeutic relevance of the gas.\textsuperscript{13-19}

Carbon monoxide is one of a family of gasotransmitters with importance on par with that of NO and H\textsubscript{2}S in mammalian systems.\textsuperscript{20, 21} Endogenous CO is generated by heme metabolism, which is catalyzed by the heme oxygenase HO. The products of this catabolic pathway are ferrous iron, carbon monoxide, and biliverdin. There are two isoforms of heme oxygenase (HO-1 and HO-2) that mediate the degradation of heme, while the HO-1 isoform is implicated in oxidative stress or pathophysiological conditions, and thus, referred to as the inducible form, the HO-2 isoform is expressed constitutively in various tissues, including neurons, liver, kidney, and the vascular endothelium.\textsuperscript{13, 22} Experimental investigation into the physiological relevance of CO reveals the roles of CO in mediating physiological processes such as vasodilation, neurotransmission, inhibition of platelet aggregation and anti-proliferating effects on vascular smooth muscle cells. For instance, CO regulates the activity of soluble guanylate cyclase (sGC), an important regulator of neurotransmission, by inducing a conformational change of the protein.\textsuperscript{19} Increased generation of reactive oxygen species (ROS) has been associated with CO regulation of the cellular levels of superoxide.\textsuperscript{23} CO regulates cellular behavior by the enhancement of mitochondria biogenesis, and increase in the cellular level of ATP.\textsuperscript{19}

The anti-inflammatory effects of CO can be seen in its ability to efficiently block the expression of the proinflammatory proteins interleukin-1β, lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α and macrophage inflammatory protein-1β. CO prevents cell and tissue injury, and plays important role in cell proliferation.\textsuperscript{14}
In terms of its therapeutic potential, CO has important roles, and has imparted the treatment of cancer in so many ways. For instance, enzymatically dormant HO-1, localized in the nucleus of cancer cells, has been associated with tumor aggressiveness, thus, revealing its potential as a modulator of cancer progression. Indeed, histopathological studies revealed that long GT repeat in the promoter region of HO-1 and lower HO-1 expression are associated with chronic state of cancer. Also, renal cancer, gastric cancer, and melanoma have all been associated with overexpression of HO-1. However, CO can stimulate the expression of HO-1, in this way, acting as a regulator of tumorigenesis.\textsuperscript{24} In addition, HO-1, through its antioxidant mechanism, has been shown to exhibit anti-proliferative and proapoptotic effects. Furthermore, CO has been shown to induce anti-Warburg effect by switching the metabolic state of cancer cells to fuel their bioenergetics and suppress biogenesis.\textsuperscript{24} This ultimately results to metabolic exhaustion. Cancer cells make use of the Warburg effect, a phenomenon that favors the lactic acid dehydrogenase (LDH) pathway owing to high glucose uptake and elevated levels of glycolysis under hypoxia condition, to promote their growth and metastasize to other tissues and organs, and to develop resistance against chemotherapy drugs such as taxols, doxorubicin, and cisplatin. Their growth is also supported by suppressed mitochondrial activities and biogenesis. Defects in reactive oxygen species (ROS) metabolism has also been associated with cancer metastasis.\textsuperscript{24} CO reverses these processes via enhancement of mitochondria activities of cancer cells, leading to metabolic exhaustion, cell arrest, and apoptosis. CO exhibited chemosparing property by providing protection for heart tissues against doxorubicin-induced cardiotoxicity.\textsuperscript{25} It is also shown to sensitize cancer cells to chemotherapeutics, thus improving their therapeutic efficacy, while shielding the surrounding normal proliferating cells.\textsuperscript{24-26} Given the therapeutic profile of CO in
cancer therapy, in addition to its chemosparing property, we intend to take advantage of the synergistic effect of combining CO with chemotherapy in cancer therapy.

One agitation about the possibility of using CO as a therapeutic has been centered around its safety window. CO has been shown to have a safety margin of more than ten-fold, a safety margin wider than that of nutrients such as glucose, metal ions, and most clinically approved drugs. Studies involving healthy humans showed that carboxyhemoglobin (COHb) levels in blood at 12% due to inhaled CO was well tolerated with no ramifications, and with CO having clear distribution and elimination profiles. All the above information have revealed a lot on the therapeutic potential of CO. However, there is a great amount of work that needs to be done in terms of developing an efficient system that is capable of delivery CO, in a well-controlled manner, at specific target site. This means that the system must encompass a CO-prodrug with tunable release rate.

So far, there are three identifiable forms of CO delivery. They include gaseous delivery at low concentration, use of physiological solutions saturated with CO gas, and molecular entities that are capable of releasing CO in a controlled manner, known as carbon monoxide releasing molecules (CORMs). While the applicability of the second mode of delivery is limited, there are many issues associated with gaseous delivery. Some of which include, but not limited to, lack of targetability, reliability, control, effective concentration and limitation to hospital settings. The carbon monoxide releasing molecules (CORMs) are divided into two groups: the metal-based CORMs which encompass the transition metal carbonyls based around manganese, ruthenium or molybdenum, releasing CO upon exposure to light, water or enzyme; and metal-free CORMs. The metal-based CORMs have been successfully employed in both in vitro and in vivo studies where they provide cardioprotection, safely deliver CO for the treatment of various diseases, and
exhibited anti-inflammatory, vasodilatory and anti-ischemic effects.\textsuperscript{16} The first generation of metal-based CORMs include the dimanganese dicarbonyl (CORM-1), tricarbonyldichlororuthenium dimer (CORM-2), tricarbonylchloro (glycinato) rutheniunm (CORM-3), and the boron-containing carboxylic acid, CORM-A1.\textsuperscript{13} While CORM-1 and CORM-2 are lipid-soluble, releasing CO in DMSO on interaction with deoxymyoglobin or deoxyhemoglobin with half-lives of < 1 min and 1 min respectively, CORM-3 and CORM-A1 which are water-soluble, release CO under physiological conditions with half-lives 1 min and 21 min respectively.\textsuperscript{26} A more recent CORM which is activated by light is the photosensitive CORM, [Mn(pqa)(CO)\textsubscript{3}]\textsubscript{2}(ClO\textsubscript{4}); pqa = 2-pyridylmethyl)(2-quinolymethyl)amine). These metal-based CORMs have helped shaped and broaden our understanding of the biological and physiological functions of carbon monoxide. However, the continuous use of these metal-based CO therapeutics has been relegated by the potential toxicity pose by transition metals and perceived issues that seem to pose life-threatening situations.\textsuperscript{26} The toxicity issue is based on the fact that these metals, which are present in trace amount, are tightly regulated in the body, the source of challenge in the development of these CORMs. Also, the byproduct of some of these metal-based CORMs has unpredictable effect on the pH of the body. With these issues at stake, the need therefore arises for the development of metal-free CORMs with desirable pharmaceutical properties. This class of novel CORMs must have the following features: no toxicity for both the CORM and its inactive products, controlled release of CO with tunable release rate under physiological conditions, triggered release, targeted or localized delivery, and easy to formulate using existing approaches.\textsuperscript{26} Organic CORMs represent a novel class of carbon monoxide releasing molecules with potential to attain these desirable pharmaceutical properties.
Our group has pioneered this direction with the design of an organic CO-prodrug with tunable rate, that releases CO under physiological conditions, via a click-and-release approach based on the inverse electron-demand Diels Alder reaction (DA\textsubscript{inv}).\textsuperscript{27} The reaction is between the strained alkyne, bicyclo[6.1.0]nonyne (BCN, 13a), characterized with high HOMO energy and the diene, tetraphenylcyclopentadienone (TPCPD, 14a) (Fig. 1). TPCPD is known to undergo the DA\textsubscript{inv} reaction, releasing CO under very rigorous conditions such as high temperature; thus, TPCPD is a form of “caged” CO. The ability of this carbonylketone to furnish CO is due to energy strain conferred on the transition state of the DA\textsubscript{inv} reaction.\textsuperscript{28} However, the hash

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The click reaction between BCNs and TPCPDs condition that characterized the reaction makes it unsuitable for therapeutic applications. It is therefore reasoned that if a high-energy HOMO alkyne such as BCN is “click” with TPCPD, it will allow the release of CO under mild conditions, and by extension, physiological conditions. Of course, this is possible as the rate of the DA\textsubscript{inv} reaction depends on the HOMO-LUMO energy gap. The use of a high-energy HOMO alkyne will essentially narrow the energy gap, consequently leading to a higher reaction rate. Indeed, when the BCN (13a) and TPCPD (14a) reaction was examined, the reaction proceeded successfully in methanol at ambient temperature with a second-order rate constant of 0.61 M\textsuperscript{-1} S\textsuperscript{-1}.\textsuperscript{27} However, due to the toxicity of the 13a and 14a, compounds 13b and 14b with the sugar moieties were employed to attenuate the potential toxicity of 13a and}
Cell viability studies showed no toxicity with 13b and 14b. Evaluation of their anti-inflammatory effects in RAW 264.5 cells shows 13b and 14b effectively inhibit LPS-induced TNF-α.27 These data demonstrated the efficiency of the organic CO-prodrug in delivery CO in biological systems, and also its therapeutic potential for in vivo biological applications.

Here, we designed and synthesized organic CO-prodrug with tethering cytotoxic agent, and then tested the release rate and further examined the synergistic effects of our prodrug system.

2.1 Results and Discussion

Motivated by the outcome of our previous work on the organic CO-prodrug and the data we have at our disposal on CO therapeutic effect, we decided to examine the synergistic effects of CO with chemotherapy drug. To do this, we reasoned that by tethering a cytotoxic agent to our organic CO-prodrug system, it would be possible to achieve a co-delivery of CO and the cytotoxic drug at the target site. This would involve the use of the DA_{inv} reaction as the prodrug activator.

The general idea is depicted in Fig. 3a. Here, CO is released spontaneously via a retro-DA/cheletropic reaction after the cycloaddition reaction between a strained alkyne (16) and the prodrug (17). Subsequent lactonization will result in the release of the cytotoxic agent.
Figure 2. a) Proposed strategy for co-delivery of CO and cytotoxic agent using the DAinv as the prodrug activator. b) Examination of the DAinv reaction between the cyclooct-2-ynol, 21 and the model prodrug, 22.

To test our concept, we synthesized compounds 21 and 22 (Fig. 2b). For compound 22, to allow for real-time monitoring of CO release, we installed the naphthalene moiety to the cyclopentadienone as this would generate a fluorophore as a byproduct of the cheletropic reaction.

Scheme 3. i) CHBr₃, tBuOK, anhydrous hexane, 5°C to rt, overnight, 65%, ii) AgClO₄, acetone/H₂O, 10 min, 20°C, 73%, iii) pyridinium-PTSA, DCM, 0°C, 10 min, iv) tBuOK, DMSO, 20°C, 2 min, 71%, v) PTSA, methanol, rt, 1 h, 93%

The synthesis of 21 starts with the cycloheptene, 25. The electrophilic “insertion” of the dibromocarbene, generated by the reaction between bromoform and potassium tert-butoxide, across the double bond of the alkene resulted in the formation of the dibromide 26. The dibromide adduct, upon treatment with silver perchlorate (AgClO₄) in the cosolvent (water/acetonitrile) gave
compound 27. This resulted from the silver (1)-assisted rearrangement of the dibromide, forming an allylic cation. Nucleophilic attack of this position by the solvent (water) generates the allylic alcohol, 27. This type of silver (1)-assisted rearrangement, $S_{N}1$ Ag-type process is a common method used for the synthesis of trans-alkene from exo halobicyclo(n.1.0)alkanes (n ≥ 5) in the presence of strong nucleophilic solvents.$^{29-31}$ Protection of the allylic alcohol was achieved upon treatment of 27 with dihydropyran, 28 in the presence of pyridinium-PTSA, in a catalytic amount, giving both diastereomers of compound 29 in nearly 1:1 ratio. We observed that, in addition to the trans isomers, the cis isomers (both diastereomers) were formed as revealed by the $^1H$ NMR spectrum. This is due to isomerization of the trans to the cis isomer in column during purification. Hence, we proceeded with the crude product of subsequent synthesis of 29 for the next step.

Compound 30 was formed in an elimination step involving dehydrobromination of 29 in the presence of the strong base, potassium tert-butoxide. Compound 21 was formed after the deprotection of 30 in 93% yield (Scheme 3).

Scheme 4. i) pyridine, DCM, 0°C to rt, overnight, 97%, ii) methanol, toluene, reflux, 3 h, 81%, ii) Et$_3$N, THF/Methanol (1:1), rt, 3 h, 84%

The synthesis of 22 starts with 32, meldrum’s acid. Acylation of the meldrum’s acid in the presence of pyridine, gave the acyl Meldrum’s acid, 33 in quantitative yield. This method was reported by Scribner et al.,$^{32}$ as a general method for the synthesis of $\beta$-keto esters. Meldrum’s acid has a remarkable acidity and for this reason, it can react with electrophile under mild conditions
or in the absence of a strong base. Upon interaction with the pyridine, the mono-anion is generated, which then attack the benzoyl chloride (31), ultimately resulting in the formation of the acyl Meldrum’s acid. Subsequent treatment of 33, in toluene, with methanol, under reflux, afforded the β-keto ester, 34 with evolution of carbon dioxide. The formation of the β-keto ester is facilitated by the enolization of the acyl group of the acyl Meldrum’s acid, thus, making it less electrophilic.32 Finally, condensation of the β-keto ester, 34 with acenaphthylene-1, 2-dione, 35 afforded compound 22 in 84% yield (Scheme 4).

With the synthesis of compounds 21 and 22, we then proceeded to test the click reaction. The cycloaddition reaction between compounds 21 and 22 in 30% DMSO/PBS at 37°C generated the two fluorescent compounds, 22a and 22b (Fig. 2b). This shows that there was less than 100% conversion of the intermediate that resulted from the retro-DA reaction. This is due to the relative orientation between compound 21 and compound 22 and thus, dictates the regiochemistry of the cycloaddition reaction (Fig. 3). Furthermore, the second-order rate constant of the cycloaddition reaction between 21 and 22 was determined to be 0.018 M$^{-1}$s$^{-1}$.

Figure 3. Manner of approach of diene 22 to dienophile 21.

After confirming the occurrence of the cycloaddition reaction and subsequent lactonization of the model prodrug 22, we were encouraged to synthesize compound 23 which encompasses the 5-fluoro-2’-deoxyuridine, used as a modality for cancer therapy, as the cytotoxic drug. The introduction of the hydrophilic drug to compound 23 helps to overcome the solubility issues associated with the model prodrug 22 due to the hydrophobic nature of the naphthalene moiety.
To further improve the slow kinetics of the reaction between compounds 21 and 22, we also synthesized compound 24 which has the phenyl group of compound 22 replaced with an amide.

Scheme 5. i) chlorobenzene, reflux, 3.5 h, 85%, ii) THF, conc. HCl, rt, 45 min, 92%, iii) Et$_3$N, THF/MeOH (1:1), rt, 2 h, 65%, iv) imidazole, TBDMS-Cl, DMF, rt, 1.5 h, 71%

The synthesis of compound 23 starts with 33. The primary alcohol of the nucleoside, 36 was selectively protected with tert-butylidemethylsilylchloride (TBS) in the presence of imidazole, resulting in the formation of 37. Treatment of the acyl Meldrum’s acid, 33 with 37 gave the β-keto ester, 38. The TBS group of the β-ketoester 38 was then removed in acid-catalyzed hydrolysis using concentrated hydrochloric acid to give compound 39. Compound 39 was then treated with the diketone, acenaphthalene-1, 2-dione, 35 in a condensation reaction to give the final product, compound 23 in 65% yield (Scheme 5).
The synthesis of compound 24 starts with Meldrum’s acid, 32. The nucleophilic attack by methanol on the carbonyl of Meldrum’s acid gave the ester of malonic acid, 40 with concomitant formation of acetone. Amidation of 40 with morpholine in the presence of the coupling agent, EDC, and DMAP (functioning as a nucleophilic catalyst) gave the amide, 41. Basic hydrolysis of compound 41 led to the formation 42. Compound 43 was formed from the acylation reaction between 42 and Meldrum’s acid in the presence of EDC and DMAP. Nucleophilic attack on the carbonyl of compound 43 by the hydroxyl group of the nucleoside, 37 gave compound 44. After removal of the TBS group of 44 to afford compound 45, the final compound, 24 was obtained in 60% yield from the condensation of compound 45 with acenaphthalene-1, 2-dione, 35 (Scheme 6).
Figure 4. a) Click reaction between compounds 21 and 23. b) Click reaction between compounds 21 and 24. c) Click reaction between compounds 21 and 47

Next, we tested the click reaction, first, between compounds 21 and 23, and then between compounds 21 and 24 in 30% DMSO/PBS at 37°C. The cycloaddition reaction between 21 and 23 released CO with the concomitant formation of the two fluorescent, regioisomers 23a and 23b, and release of the cytotoxic drug (Fig. 4a). The second order rate constant between compounds 21
and 23 in 30% DMSO/PBS at 37°C was determined to be 0.048 M·s⁻¹. The click reaction between compounds 21 and 24, upon incubation in 30% DMSO/PBS at 37°C furnished CO with the formation of two fluorescent compounds 24a and 24b resulting from different regiochemistry, and release of the cytotoxic drug and morpholine respectively (Fig. 4b). The regiochemistry of the click reaction and outcome of the subsequent lactonization, as earlier acknowledged, were due to the relative orientation between the diene and dienophile. The second order rate constant between compound 21 and compound 24 was determined to be 0.168 M·s⁻¹. As we expected, the result showed higher reaction rate, with 9-fold and 4-fold increase compared to that between 21 and 22, and between 21 and 23 respectively. This increased reaction rate between compound 21 and compound 24 was presumably due to fine tuning of the electronics of the cyclopentadienone ring achieved by the introduction of the amide substituent. This has the effect of decreasing the LUMO energy of the diene, thus, narrowing the HOMO-dienophile–LUMO-diene energy gap, ultimately resulting in higher reaction rate.

To determine the ratio of the cyclized product, 22a to the unyclized product, 22b, we synthesized compound 47 and monitored the click reaction between 21 and 47 (Fig. 4c) using ¹H NMR spectroscopy Fig. 13. We used compound 47 instead of 22 for this study because of the solubility issue associated with compound 22. Within 2 hrs, we observed the disappearance of the methylene peak (5.43 ppm) of compound 47, indicative of the cycloaddition. New resonances corresponding to benzylalcohol (BnOH), compound 47a, compound 47b, and the intermediate, the byproduct of the retro-DA reaction, at 4.49 ppm, 5.90–5.95 ppm and 6.30 ppm, 5.98–6.00 ppm and 5.60 ppm, and 6.1 ppm and 5.7 ppm respectively were observed. We noticed that because of the chirality of compound 47b at the carbon atom, a, the two methylene protons at carbon a’ are diastereotopic, hence the quartet at 5.60 ppm, confirming the formation of the regioisomer 47b.
The presence of the BnOH peak confirms the cyclization reaction. After 21 hrs, the ratio of 47a to 47b is 1:1, and 57% of the cyclized product 47a was obtained.

We subsequently employed HPLC to test the release of the cytotoxic drug from compound 24 (100 µM) as induced by 21 (5 mM) in 20% DMSO in PBS at 37°C (Fig. 5). The reaction was completed in 220 ± 10 min and afforded the cytotoxic drug in 45 ± 0.4 % as determined from the calibration curve. To ensure that the release is not due to instability of the prodrug, due to the susceptibility of the ester linker to hydrolysis, we incubated the prodrug, compound 24 (50 µM) for at least 21 h in 20% DMSO in PBS at 37°C. We observed 76 % of the prodrug 24 after 8 h Fig. 14. This implies that the release of the drug from compound 24 was mainly triggered by the DA\textsubscript{inv} reaction as the release of the drug was completed in 3.5 h.

![Drug Release](image)

**Figure 5.** Drug release profile from compound 24 (100 µM) with 5 mM of 21 in 20% DMSO/PBS at 37°C.
The solution (100 µM) of compounds 24 and 21 was diluted to 2, 4, 6, 8, and 10 µM. After confirming the release of the cytotoxic drug, we then proceeded to evaluate the synergistic effect between CO and the cytotoxic drug. This study was done by one of our collaborators at Emory. The cytotoxicity assay was carried out using the human breast tumor cell line, mb231. We first assessed the cytotoxic effect of compounds 21 and 24 separately. Compound 21 showed no decrease in cell viability at 100 µM concentration (figure not shown). However, compound 24 showed a 50% decrease in cell viability at 10 µM concentration after 72h treatment (Fig. 6). Upon co-treatment of the cells with 10 µM of compound 24 and 100 µM of compound 21 for 72 h, there was a decrease in cell viability by 30%, although, we did not observe any synergistic effect between CO and the cytotoxic drug. Barbara Wegiel et al., demonstrated the synergistic effect between CO and the genotoxin, doxorubicin in human prostrate and lung cancer. They showed that with low CO concentration in combination with camptothecin or doxorubicin, there was an increased synergy between CO and camptothecin or doxorubicin in inducing cell death in prostate cancer cell line with an approximate 1000-fold over chemotherapy-alone-treated.
cells. In addition, they also showed that CO offered protection to normal cells against doxorubicin-induced cell death while enhancing the killing of tumor cells. Based on this, we posited that the lack of synergistic effect of our system might be due to two possible reasons: first, the ratio of CO to the cytotoxic agent released might not be the right ratio to elicit any beneficial effect by the combo. Although, to the best of our knowledge, there has not been any publication that clearly defines the right ratio. Secondly, we reasoned that 5-fluorouracil may not have been the “right” drug to use for this evaluation since, with the use of doxorubicin and camptothecin, such synergistic effect was clearly demonstrated and observed, suggesting that CO can sensitize cancer cells only to doxorubicin- or camptothecin-induced DNA damage.

Scheme 8. A different route to the synthesis of trans-alkene
In our earlier effort to synthesize compound 21, we realized that it could be possible to carry out lithium exchange with compound 27, and subsequent protonation would afford compound 48. We proceeded to attempt this reaction and not only did we obtained the desired product, 48, as a single diastereomer, equatorial isomer as confirmed by $^1$H NMR, the yield was also improved- compound 48 was obtained in an overall 74% yield (Scheme 8).

2.2 Conclusion

In conclusion, we have designed and successfully synthesized various organic CO-prodrugs. We demonstrated the proof of concept and co-delivery of CO and the cytotoxic agent by leveraging on the inverse electron-demand Diels Alder reaction as the trigger near physiological conditions. We showed that CO release rate and delivery of the cytotoxic agent can be tuned by manipulating the electronics of our prodrug system. Furthermore, cell viability studies using
human breast cancer cell lines, mb231 showed significant inhibition of tumor growth, however, with no synergy between CO and the cytotoxic drug.

2.3 Experimental

2.3.1 General Information

All reagents and solvents were of reagent grade. Column chromatography was carried out using flash silica gel (Sorbent 230–400 mesh) and P-2 Gel (Bio-Gel, particle size range 45-90 μm). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at 400 MHz for 1H and 100 MHz for 13C on an Avance Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using the respective solvent (1H NMR, 13C NMR) as the internal reference.

**Reagents:** tBuOK- potassium tert-butoxide, THF- tetrahydrofuran, Et₃N- triethylamine, AgClO₄- silver perchlorate, TBDMS-Cl- tertbutyldimethylsilylchloride, AcOH- acetic acid, PTSA-p-Toluenesulfonic acid, EDC-1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, DMAP-4-dimethylaminopyridine, Et₃N-triethylamine.

2.3.2 Synthesis of 26

![Diagram of 26]

Compound 26: 8, 8-dibromodicyclo[5.1.0].3d 5.03g of 25 (52.3 mmol), 11.7g of tBuOK (104.2 mmol), and 12 ml of anhydrous hexane were introduced into a dried round-bottom flask under argon. The flask placed in an ice/salt bath (<-5°C), and the solution was vigorously stirred for 30 min and then 19.8g of bromoform (78.3 mmol) were added dropwise. Once the addition was complete, the mixture was warmed to room temperature overnight under argon and with
vigorous stirring. 40 ml of water was subsequently added followed by the addition of 1M HCl. The reaction mixture was extracted with hexane. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$, and concentrated. The crude product was purified by column chromatography (silica gel, hexane) to afford 26 as a colorless liquid in 65% yield, $^1$H NMR (CDCl$_3$), 2.25-2.31 (m, 2H), 1.81-1.94 (m, 3H), 1.69-1.77 (m, 2H), 1.34-1.45 (m, 2H), 1.13-1.27 (m, 3H).

2.3.3 Synthesis of 27

![Chemical structure of 27]

Compound 27: (E)-2-bromocyclooct-2-en-1-ol.$^{35}$ To a solution of AgClO$_4$ (2.11g, 7.8 mmol) in 5 ml of acetone/H$_2$O (9:1) was added 26 (2.11g, 7.8 mmol) dropwise at 20°C and the reaction mixture was stirred for 10 min. Water was added to the mixture and the silver salt was filtered off under suction. The filtrate was extracted with EA. The combined organic layer was washed with brine and the silver salt was again filtered under suction. The filtrate was dried with anhydrous Na$_2$SO$_4$, and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (20:1)) to afford 27 as a white solid in 73% yield, $^1$H NMR (CDCl$_3$), 6.07 (dd, $J = 12.0$ Hz, 4.0 Hz, 1H), 4.17 (dd, $J = 8.0$ Hz, 4.0 Hz, 1H), 2.88 (s, 1H), 2.58-2.69 (m, 1H), 2.23-2.29 (m, 1H), 1.95-1.99 (m, 2H), 1.62-1.88 (m, 4H), 1.40-1.51 (m, 4H), 0.74-0.82 (m, 1H).

2.3.4 Synthesis of 28

![Chemical structure of 28]
Compounds 29: (Z)-2-((2-bromocycloct-2-en-1-yl)oxy)tetrahydro-2H-pyran. To a solution of 27 (1.49g, 7.2 mmol) in 8 ml of DCM was added pyridinium-PTSA (0.09g, 0.03 mol%). To this solution was added 28 (0.91g, 10.8 mmol) dropwise at 0°C. The reaction mixture was stirred at this temperature for 10 min. Water was added to the mixture and extracted with DCM. The combined organic layer was washed with brine, dried with anhydrous Na2SO4, and concentrated. The crude product was used in the next step without purification.

2.3.5 Synthesis of 30

![Image of compound 30]

Compounds 30: 2-(cycloct-2-en-1-yl)oxy)tetrahydro-2H-pyran. To a stirring solution of t-BuOK (0.47g, 4.2 mmol) in 10 ml of anhydrous DMSO was added a solution of 29 (0.31g, 1.1 mmol) in 1 ml of anhydrous DMSO at 20°C. After adding, the reaction was immediately quenched by pouring the reaction mixture into an ice solution of 1 ml of acetic acid. The mixture was extracted with EA and the combined organic layer was washed with brine, dried with anhydrous Na2SO4, and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (300:1)) to afford 30 as an colorless oil in 71% yield, 1H NMR (CDCl3), 4.69-4.71 (t, 1H), 4.38-4.43 (m, 1H), 3.96-4.01 (m, 1H), 3.51-3.56 (m, 1H), 2.24-2.33 (m, 1H), 2.11-2.20 (m, 2H), 1.91-2.03 (m, 2H), 1.80-1.89 (m, 3H), 1.66-1.79 (m, 2H), 1.49-1.64 (m, 5H), 1.36-1.45 (m, 1H), 2.25-2.32 (m, 1H).

2.3.6 Synthesis of 21

![Image of compound 21]
Compound 21: cyclooct-2-yn-1-ol. To a solution of 30 (0.17g, 0.8 mmol) in 10 ml of methanol was added PTSA (0.008g, 0.04 mmol) at room temperature. The reaction mixture was stirred continuously at room temperature for 1h 45 min. After completion of the reaction as indicated by TLC, the methanol was removed by rotavap, and water was added to the product mixture and extracted with EA. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$, and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (100:1)) to afford 21 as a colorless oil in 93% yield, $^1$H NMR (CDCl$_3$), 4.47-4.50 (t, 1H), 2.25-2.32 (m, 1H), 2.12-2.23 (m, 1H), 1.87-2.01 (m, 2H), 1.51-1.83 (m, 6H).

2.3.7 Synthesis of 33

![Structure of 33](image)

Compound 33: 2, 2-dimethyl-5-(2-phenylacetyl)-1, 3-dioxane-4, 6-dione. To a solution of 32 (2.3g, 15.6 mmol) in 10 ml of DCM was added 2.5 ml of pyridine dropwise at 0°C. To this mixture was added 2.1 ml of 31 dropwise. The mixture was stirred at 0°C for 1 h and then warmed to room temperature and stirred overnight. To the product mixture was added I M HCl and extracted with DCM. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (1:1)) to afford 33 as a white solid in 97% yield, $^1$H NMR (CDCl$_3$), 15.35 (s, 1H), 7.42 (s, 1H), 7.40 (s, 1H), 7.36-7.37 (t, 1H), 7.35 (s, 1H), 7.30-7.33 (m, 1H), 4.45 (s, 1H), 1.74 (s, 6H).
2.3.8 Synthesis of 34

Compound 34: **methyl 3-oxo-4-phenylbutanoate**.\(^{27}\) To a solution of 33 (3.20g, 0.01 mmol) in 10 ml of toluene was added methanol (0.78g, 0.02 mmol). The resulting mixture was heated under reflux for 3h. The reaction mixture was cooled to room temperature and concentrated. The crude product was purified by column chromatography (silica gel, Hexane/EA (2:1)) to afford 34 in 81% yield. \(^1\)H NMR (CDCl\(_3\)), 7.23-7.38 (m, 5H), 3.84 (s, 1H), 3.72 (s, 3H), 3.48 (s, 1H).

2.3.9 Synthesis of 22

Compound 22: **methyl 8-oxo-9-phenyl-8H-cyclopenta[a]acenaphthylene-7-carboxylate**.\(^{27}\) To a solution of 34 (0.14g, 0.80 mmol) in 2 ml of THF/MeOH (1:1) was added trimethylamine (0.12g, 1.21 mmol) and 35 (0.14g, 0.80 mmol) at room temperature. After 30 min, the color of the reaction turned green. The reaction was stirred for 3h. The precipitate was filtered, washed several times with methanol and then dried to obtain 22 as a dark solid in 84% yield. \(^1\)H NMR (CDCl\(_3\)), 8.77 (d, \(J = 7.2\) Hz, 1H), 8.09-8.05 (m, 2H), 7.94 (d, \(J = 8.0\) Hz, 1H), 7.84 (d, \(J = 7.2\) Hz, 2H), 7.80 (t, \(J = 7.6\) Hz, 1H), 7.63 (t, \(J = 8.0\) Hz, 1H), 7.54 (t, \(J = 8.0\) Hz, 2H), 7.47 (t, \(J = 7.6\) Hz, 1H), 4.03 (s, 3H).
2.3.10 Synthesis of 37

Compound 37: 1-((tert-butyldimethysilyl)oxy)methyl)-4-hydroxytetrahydrofuran-2-yl)-5-fluoropyrimidine-2, 4(1H, 3H)-dione. To a solution of 36 (1.20g, 4.87 mmol) in 5 ml of DMF was added imidazole (0.99g, 0.01 mmol) and TBDMS-Cl (0.77g, 5.12 mmol) at room temperature the reaction mixture was stirred for 1.5 h. To the product mixture was added water and extracted with DCM. The combined organic layer was washed with brine, dried with anhydrous Na₂SO₄ and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (4:1)) to afford 37 as a white solid in 71% yield. ¹H NMR (CDCl₃), 8.09 (d, J = 8.0 Hz, 1H), 6.37-6.41 (t, 1H), 4.46-4.48 (t, 1H), 4.12 (s, 1H), 3.94 (d, J = 12.0 Hz, 1H), 3.85 (d, J = 12.0 Hz, 1H), 2.44-2.50 (m, 1H), 2.09-2.16 (m, 1H), 0.96 (s, 9H), 0.11 (s, 6H).

2.3.11 Synthesis of 38

Compound 38: 2-((tert-butyldimethysilyl)oxy)methyl-5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl 3-oxo-4-phenylbutanoate. To a solution of 33 (0.37g, 1.41 mmol) in 5 ml of chlorobenzene was added 37 (0.34g, 0.9 mmol) at room temperature. The reaction mixture was heated under reflux for 3.5 hrs. The crude product was concentrated and purified by column chromatography (silica gel, DCM/MeOH (40:1)) to afford 38 as a white solid in 91% yield. ¹H NMR (CDCl₃), 11.86 (s, 1H), 10.25 (d, J = 4.0 Hz, 1H), 8.0 (d, J = 8.0 Hz, 1H), 7.25-7.36 (m, 4H), 7.20 (s, 1H), 6.31-6.35 (t, 1H), 5.26 (d, J = 4.0 Hz, 1H), 3.03 (s, 1H), 0.90 (s, 9H), 0.11 (s, 6H).
3.90 (s, 2H), 3.81 (s, 2H), 3.53 (s, 2H), 2.42-2.48 (m, 1H), 2.07-2.14 (m, 1H), 0.92 (s, 9H), 0.13 (s, 6H), $^{13}$C NMR (CDCl$_3$), 18.3, 25.9, 47.9, 50.3, 76.3, 76.9, 77.5, 85.4, 85.7, 127.5, 128.9, 129.6, 132.9, 149.4, 166.8, 200.3, HRMS (ESI): calculated for; C$_{25}$H$_{33}$FN$_2$O$_7$Si 520.2075, found; 543.1939 [M+Na]$^+$. 

2.3.12 Synthesis of 39

![Compound 39](image)

Compound 39: 5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl 3-oxo-4-phenylbutanoate. To a solution of 38 (0.53g) in about 5 ml of THF was added conc. HCl in drops until the solution became cloudy. The reaction mixture was stirred at room temperature for 45 min. the reaction was quenched with water and extracted with EA. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography (silica gel, DCM/MeOH (20:1)) to afford 38 as white a solid in 98% yield. $^1$H NMR (CDCl$_3$), 2.28-2.41 (m, 2H), 3.54 (s, 2H), 3.81 (s, 2H), 3.86 (s, 2H), 4.09 (s, 1H), 5.34 (d, $J = 8.0$ Hz, 1H), 6.22-6.26 (t, 1H), 7.20 (d, $J = 4.0$ Hz, 2H), 7.24-7.30 (t, 1H), 7.32-7.36 (t, 2H), 8.08 (d, $J = 8.0$ Hz, 1H), HRMS (ESI): calculated for; C$_{19}$H$_{19}$FN$_2$O$_7$ 406.1176, found; 429.1050 [M+Na]$^+$. 
2.3.13 Synthesis of 23

Compound 23: 5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl 8-oxo-9-phenyl-8H-cyclopenta[a]acenaphthylene-7-carboxylate. To a solution of 39 (0.22g, 0.54 mmol) in 2 ml of THF/MeOH (1:1) was added trimethylamine (0.08g, 0.81 mmol) and 35 (0.09g, 0.54 mmol) at room temperature. After 30 min, the color of the reaction turned green. The reaction was stirred for 2h. The precipitate was filtered, washed several times with methanol and then dried to obtain 23 as a dark solid in 65% yield. \( ^1 \)H NMR (DMSO-d6), 1.91 (s, 1H), 1.99 (s, 1H), 3.77 (s, 2H), 4.25 (s, 1H), 4.39 (s, 1H), 5.50-5.56 (m, 1H), 6.31-6.37 (m, 1H), 7.50-7.53 (t, 1H), 7.57-7.61 (m, 2H), 7.75-7.77 (m, 3H), 7.87-7.92 (m, 1H), 7.99-8.13 (m, 2H), 8.27-8.31 (t, 1H), 8.61 (d, \( J = 8.0 \) Hz, 1H), 11.96 (s, 1H), HRMS (ESI): calculated for; C\(_{31}\)H\(_{21}\)FN\(_2\)O\(_7\) 552.1333, found; 575.0784 [M+Na]\(^+\).

2.3.14 Synthesis of 40

Compound 40: 3-methoxy-3-oxopropanoic acid. To a solution of 32 (5.02g, 34.8 mmol) in 10 ml of toluene was added methanol (2.22g, 69.3 mmol) at room temperature. The resulting mixture was heated under reflux for 4h. After completion of the reaction as monitored by TLC, the reaction was cooled to room temperature and then concentrated. The crude product was used directly in the next step.
2.3.15 Synthesis of 41

![Structure of Compound 41](image)

Compound 41: methyl 3-morpholino-3-oxopropanoate. To a solution of 40 (4.10g, 34.7 mmol) in 10 ml of DCM was added morpholine (4.54g, 52.1 mmol) and DMAP (5.09g, 41.6 mmol) at room temperature. The reaction mixture was cooled to 0°C and EDC (7.99g, 41.6 mmol) was added portion-wise for about 5 min. The reaction mixture was then warmed to room temperature and stirred continuously overnight. The reaction was quenched with 1M HCl and then extracted with DCM. The combined organic layer was washed with brine, dried with anhydrous Na₂SO₄, and then concentrated. The crude product was purified by column chromatography (silica gel, Hexane/EA (2:1)) to afford the desired product as a white solid in 75% yield. ¹H NMR (CDCl₃), 3.71 (s, 3H), 3.63-3.66 (m, 6H), 3.40-3.43 (m, 4H), ¹³C NMR (CDCl₃), 167.9, 164.4, 77.4, 76.8, 66.6, 66.5, 52.5, 40.8, 42.2, 40.8

2.3.16 Synthesis of 42

![Structure of Compound 42](image)

Compound 42: 3-morpholino-3-oxopropanoic acid. To a solution of 40 (2.11g, 11.2 mmol) in 10 ml of methanol/H₂O (2:1) was added LiOH (0.40g, 16.7 mmol). The resulting mixture was stirred at room temperature for 1.5h. After completion of the reaction as determined by TLC, the reaction mixture was extracted with EA. To the aqueous layer was added amberlite ion-exchange resin and the mixture was stirred for 30 min. The mixture was filtered under suction and the filtrate was concentrated to afford 42 as a white solid in 92% yield. ¹H NMR (CDCl₃), 3.70-
3.77 (m, 6H), 3.49-3.52 (t, 2H), 3.41 (s, 2H), $^{13}$C NMR (CDCl$_3$), 35.2, 42.5, 45.7, 66.2, 66.5, 76.7, 77.4, 167.6, 168.1, HRMS (ESI): calculated for; C$_7$H$_{11}$NO$_4$ 173.0688, found; 174.0753 [M+H]$^+$. 

2.3.17 Synthesis of 43

![Image of compound 43]

Compound 43: **2, 2-dimethyl-5-(3-morpholino-3-oxopropanoyl)-1, 3-dioxane-4, 6-dione**. To a solution of 42 (1.35g, 77.9 mmol) in 5 ml of DMF was added DMAP (1.17g, 9.5 mmol) and EDC (1.99g, 10.3 mmol) under argon at room temperature. The reaction mixture was cooled to 0°C and a solution of 32 (1.17g, 8.1 mmol) in 2 ml of DMF was added drop-wise to the reaction. The reaction was completed after 4 hrs as determined by TLC. The reaction was quenched with 1M HCl and then extracted with DCM. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$, and concentrated. The crude product was purified by column chromatography (silica gel, acetone) to afford 43 as a white solid in 56% yield. $^1$H NMR (Acetone-d$_2$), 4.13 (s, 2H), 3.82 (s, 1H), 3.60-3.70 (m, 8H), 1.63 (s, 6H), HRMS (ESI): calculated for C$_{13}$H$_{17}$NO$_7$ 300.1039, found; 300.1063.

2.3.18 Synthesis of 44

![Image of compound 44]

Compound 44: **2-(((tert-butyldimethylsilyl)-5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1 (2H)-yl)tetrahydrofuran-3-yl 5-morpholino-3, 5-dioxopentanoate**. To a solution of 43 (0.18g, 0.5 mmol) in 3 ml of chlorobenzene was added 37 (0.23g, 0.7 mmol) at room temperature. The reaction mixture was heated under reflux for 4hrs. The crude product was
concentrated and purified by column chromatography (silica gel, DCM/MeOH (40:1)) to afford 44 as yellow solid in 73% yield. $^1$H NMR (CDCl$_3$), 14.83 (s, 1H), 9.68 (s, 1H), 8.0-8.03 (t, 1H), 6.33-6.37 (t, 1H), 5.29-5.33 (t, 1H), 4.18 (d, $J = 12.0$ Hz, 1H), 3.90-3.96 (t, 2H), 3.71 (s, 7H), 3.65 (d, $J = 8.0$ Hz, 2H), 3.45-3.47 (t, 1H), 3.28 (s, 1H), 2.49-2.54 (m, 1H), 2.12-2.19 (m, 1H), 0.93 (s, 9H), 0.15 (s, 6H), $^{13}$C NMR (CDCl$_3$), 18.3, 25.9, 38.2, 42.3, 46.7, 48.7, 48.7, 63.7, 66.5, 66.7, 76.7, 77.4, 85.4, 85.6, 149.0, 164.6, 166.7, 196.5, HRMS (ESI): calculated for C$_{24}$H$_{36}$FNO$_5$Si 558.2238, found; 558.2273.

### 2.3.19 Synthesis of 45

[Chemical structure diagram]

Compound 45: 5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl 5-morpholino-3, 5-dioxopentanoate. To a solution of 44 (0.53g) in about 5 ml of THF was added conc. HCl in drops until the solution became cloudy. The reaction mixture was stirred at room temperature for 45 min. The reaction was quenched with water and extracted with EA. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$ and concentrated. The crude product was used directly in the next step.
2.3.20 Synthesis of 24

Compound 24: 5-(5-fluoro-2, 4-dioxo-3, 4-dihydropyrimidin-1(2H)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yl-9-(morpholine-4-carbonyl)-8-oxo-8H-cyclopenta[a]acenaphylene-7-carboxylate. To a solution of 45 (0.82g, 1.85 mmol) in 2 ml of THF/MeOH (1:1) was added trimethylamine (0.28g, 2.77 mmol) and 35 (0.33g, 1.84 mmol) at room temperature. After 30 min, the color of the reaction turned green. The reaction was stirred for 45 min. The precipitate was filtered, washed several times with methanol and then dried to obtain 24 as a dark solid in 60% yield. $^1$H NMR (DMSO-d$_6$), 11.92 (s, 1H), 8.95 (s, 1H), 8.19-8.30 (m, 3H), 7.83-7.90 (m, 3H), 6.33 (s, 1H), 5.44-5.54 (m, 2H), 4.25 (s, 1H), 1 3.53-3.57 (m, 10H), 13C NMR (DMSO-d$_6$), 37.5, 39.4, 39.8, 39.9, 40.2, 40.4, 40.6, 42.4, 47.3, 61.9, 66.6, 67.2, 76.1, 85.0, 85.5, 124.5, 124.8, 125.1, 128.2, 129.5, 129.9, 132.1, 139.5, 141.8, 145.4, 149.6, 155.9, 157.4, 157.6, 161.4, 167.6, 194.1, HRMS (ESI): calculated for; C$_{30}$H$_{28}$FN$_3$O$_9$ 594.1843, found; 595.3818 [M+H]$^+$. 

2.3.21 Synthesis of 46

Compound 46: benzyl 3-oxo-4-phenylbutanoate. To a solution of 33 (505 mg, 1.92 mmol) in 5 ml of toluene was added benzylic alcohol (420 mg, 3.88 mmol). The reaction mixture was refluxed for about 45 min and allowed to cool to room temperature after completion of the reaction as indicated on TLC. The crude product was concentrated on rotavap and then purified by
column chromatography (silica gel, Hexane/EA (10:1)) to afford 46 as a white solid in 84% yield. 

1H NMR (CDCl3), 3.54 (s, 2H), 3.84 (s, 2H), 5.19 (s, 2H), 7.21 (d, J = 8.0 Hz, 2H), 7.33-7.41 (m, 8H); 13C NMR (CDCl3), 48.3, 50.1, 67.2, 76.8, 77.5, 127.4, 128.4, 128.5, 128.7, 128.9, 129.6, 133.2, 135.3, 166.9, 200.3, HRMS (ESI): calculated for; C9H16O3 268.1099, found; 291.0997 [M+Na]+.

2.3.22 Synthesis of 47

Compound 47: benzy 8-oxo-9-phenyl-8H-cyclopenta[a]acenaphthylene-7-carboxylate. To a solution of 46 (132 mg, 0.49 mmol) in 5 ml of THF/MeOH (1:1) was added triethylamine (75.6 mg, 0.75 mmol). To this mixture was added 35 (90.4 mg, 0.49 mmol). The reaction mixture was stirred at room temperature for about 2h. The crude product was concentrated on rotavap and then dissolved in 5 ml of acetic acid anhydride. The resulting mixture was stirred at room temperature, followed by the addition of few drops of concentrated HCl. This resulted to the formation of black precipitates. The product mixture was filtered under suction and the residue was washed several times with MeOH to afford 47 as a black solid in 68% yield. 1H NMR (DMSO-d6), 5.43 (s, 2H), 7.38-7.60 (m, 8H), 7.72-7.81 (m, 4H), 8.00 (d, J = 4.0 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 8.24 (d, J = 8.0 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H), 13C NMR (DMSO-d6), 39.4, 39.8, 39.9, 40.2, 40.4, 40.6, 66.1, 109.7, 121.9, 128.6, 128.8, 129.0, 129.2, 129.5, 129.7, 130.0, 130.5, 132.1, 136.7, 144.8, 150.7, 161.8, 169.6, 169.7, 196.0, 196.7, HRMS (ESI): calculated for; C17H16O3 414.1256, found; 437.1172 [M+Na]+.
2.3.23 Synthesis of 48

![Structure of 48](image)

Compound 49: (E)-cyclooct-2-en-1-ol.\textsuperscript{36} To a solution of 27 (0.66g, 3.20mmol) in about 5 ml of anhydrous THF under argon at -78°C was added butyllithium (5ml) dropwise for about 15 min. the reaction mixture was warmed to room temperature and stirred for 1.5 h. the reaction was quenched with ammonium chloride solution and the product mixture was extracted with EA. The combined organic layer was washed with brine, dried with anhydrous Na\textsubscript{2}SO\textsubscript{4} and concentrated. The crude product was purified by column chromatography (silica gel, hexane/EA (20:1)) to afford 48 as a colorless liquid in 74% yield. \textsuperscript{1}HNMR (CDCl\textsubscript{3}), 0.71-0.79 (m, 1H), 0.85-0.93 (m, 1H), 1.34-1.54 (m, 2H), 1.73-2.00 (m, 4H), 2.12-2.19 (m, 2H), 2.37-2.42 (m, 1H), 4.23-4.29 (m, 1H), 5.54 (dd, \textit{J} = 16.0 Hz, 4.0 Hz, 1H), 5.62-5.65 (m, 1H).

2.4 Click Reaction between 21 and 22

![Click Reaction Scheme](image)

21 (107 mg, 0.32 mmol) and 22 (61.8 mg, 0.49 mmol) were dissolved in 20 ml of ACN/DMSO (1:1) at room temperature. The reaction mixture was heated to about 50°C for about 45 min to dissolve 22. The reaction mixture was cooled to room temperature, and thereafter 0.7 ml of PBS was added. The reaction mixture was stirred overnight at room temperature. To the product mixture was added water and extracted with EA. The combined organic layer was washed
with brine, dried with anhydrous Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography (silica gel, Hexane/EA (20:1)) to afford the desired products as two regioisomers (22a and 22b) in 37% and 22% yields respectively. $^1$H NMR (CDCl$_3$), 22a, 9.12 (d, $J = 4.0$ Hz, 1H), 7.94 (d, $J = 8.0$ Hz, 1H), 7.75-7.83 (m, 2H), 7.60-7.63 (m, 3H), 7.44-7.46 (m, 1H), 7.30-7.36 (m, 2H), 6.37 (d, $J = 8.0$ Hz, 1H), 5.63-5.67 (m, 1H), 2.71-2.80 (m, 2H), 2.60-2.68 (m, 1H), 2.01-2.05 (m, 1H), 1.84-1.90 (t, 1H), 1.44-1.53 (m, 2H), 1.28 (s, 2H), 1.01-1.12 (m, 1H); 22b, 7.83 (d, $J = 8.0$ Hz, 1H), 7.73 (dd, $J = 12.0$ Hz, 8.0 Hz, 2H), 7.57-7.63 (m, 4H), 7.46 (s, 1H), 7.36-7.39 (m, 1H), 7.22-7.24 (t, 1H), 6.03 (d, $J = 8.0$ Hz, 1H), 5.30 (s, 1H), 4.15 (s, 3H), 2.85-2.91 (m, 1H), 1.97 (s, 2H), 1.66-1.83 (m, 5H), 0.91 (s, 1H).

### 2.5 Click Reaction between 21 and 24

21 (24.7 mg, 0.23 mmol) and 24 (78.3 mg, 0.13 mmol) were dissolved in 10 ml of DMSO/PBS (30 % DMSO) at room temperature. The reaction mixture was stirred at room temperature for 4h. To the product mixture was added water and extracted with EA. The combined organic layer was washed with brine, dried with anhydrous Na$_2$SO$_4$ and concentrated. The crude product was purified by column chromatography (silica gel, Hexane/EA (5:1)) to afford the desired products as two regioisomers (24a and 24b) in 43% and 32% yields respectively. $^1$H NMR (CDCl$_3$), 24a, 9.114-9.17 (m, 1H), 7.98-8.02 (m, 2H), 7.90-7.93 (t, 1H), 7.78-7.82 (m, 1H), 7.67-
7.71 (m, 1H), 5.61-5.67 (m, 1H), 4.07-4.09 (t, 2H), 3.87-3.97 (m, 2H), 3.37-3.53 (m, 2H), 3.08-3.28 (m, 2H), 2.74-2.86 (m, 3H), 1.81-2.07 (m, 4H), 1.38-1.55 (m, 2H), \(^{13}\text{C}\) NMR (CDCl\(_3\)), 20.9, 24.5, 26.6, 29.4, 39.0, 41.8, 46.7, 66.6, 66.8, 76.7, 82.9, 121.0, 122.7, 127.9, 128.3, 128.4, 129.8, 130.4, 132.6, 133.4, 133.6, 135.0, 136.6, 137.0, 149.5, 167.4, 169.9, HRMS (ESI): calculated for C\(_{28}\)H\(_{25}\)NO\(_4\) 440.1817, found; 440.1845.

22, 23, 24, and 21

The fluorescent property of the cycloaddition products 22a, 22b, 23a, 23b, 24a, and 24b greatly facilitates the determination of the second order reaction rate constants between 22, 23, 24 and 21 (30 % DMSO/PBS, 37 °C). Briefly, the second order reaction was treated as first order reaction by using excessive amount of 21 (> 20-fold), and the obtained \(k'\) was plotted against the concentration of 21 used. The obtained slope is the second order reaction rate constant between 22, 23, 24 and 21 (Figures 8, 10, and 12)
2.6.2 *Determination of the reaction rate constant between 21 and 22*

![Graphs showing fluorescence intensity over time for different concentrations of 21 and 22.](Image)

**Figure 7.** The pseudo first order reaction between 21 and 22: a) 21 (10 mM) + 22 (20 µM); b) 21 (15 mM) + 22 (20 µM); c) 21 (20 mM) + 22 (20 µM); d) 21 (25 mM) + 22 (20 µM).

![Graph showing plots of k' against concentration of 21.](Image)

**Figure 8.** Plot of the obtained k' against the concentration of 21 used. The second order reaction rate constant $k = 0.018 \text{ M}^{-1}\text{s}^{-1}$.
2.6.3 Determination of the reaction rate constant between 21 and 23

Figure 9. The pseudo first order reaction between 21 and 23: a) 21 (500 µM) + 23 (50 µM); b) 21 (750 µM) + 23 (50 µM); c) 21 (1 mM) + 23 (50 µM); d) 21 (1.25 mM) + 23 (50 µM);
Figure 10, Plot of the obtained $k'$ against the concentration of 21 used. The second order reaction rate constant $k = 0.048 \text{ M}^{-1}\text{s}^{-1}$.

### 2.6.4 Determination of the reaction rate constant between 21 and 24

**Figure 11.** The pseudo first order reaction between 21 and 24: a) 21 (500 µM) + 24 (50 µM); b) 21 (750 µM) + 24 (50 µM); c) 21 (1 mM) + 24 (50 µM); d) 21 (1.25 mM) + 24 (50 µM);
Figure 12. Plot of the obtained $k'$ against the concentration of 21 used. The second order reaction rate constant $k = 0.168 \text{ M}^{-1}\text{s}^{-1}$.

2.7 Cytotoxic Studies

Raw 264.7 cells were seeded in 96-well plates and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO₂ for 24 h. Then Raw 264.7 cells were incubated in DMEM containing the vehicle (1% DMSO) and compound 24 (0-10µM) for 24 h. After removal of the media, 150µL of DMEM containing 10µL (Cell Counting Kit-8 CCK-8) was added to each well and the cells were incubated for another 3 h at 37 °C. The absorbance at 450 nm was then measured by using a PerkinElmer 1420 multi-label counter. The cell viability was measured and results were normalized to the vehicle group. The experiment was triplicated and the results are expressed as mean ± standard error of the mean (SEM, n = 3).

2.8 $^1$H NMR Studies for the Click Reaction between 21 and 47

To a solution of 47 (8.20 mg, 0.02mmol) in 3 ml of DMSO-d₆ was added 21 (7.24 mg, 0.06 mmol). The resulting solution was incubated at 37 °C and the $^1$H NMR was taken at different time point. As shown in Fig. 13 after 21 h, the ratio of 47a to 47b is 1:1 and 57% of the cyclized product 47a was obtained.
2.9 Drug Release Experiment

Drug release was induced by the addition of 21 (5 mM) to compound 24 (100 µM) in 20% DMSO in PBS at 37°C. HPLC was used to monitor drug release at different time point. Each experiment was triplicated. Drug stoichiometry was measured using a calibration curve. The concentration of the released drug was obtained by converting the peak area at a given time to concentration using the slope of the calibration curve.

Different concentrations of compound 24, 30, 60, 90, 120 and 150 µM, in 20% DMSO in PBS at room temperature were prepared and used to run the HPLC experiment. Each experiment was triplicated. The calibration curve was plotted using the peak areas obtained from the HPLC experiment against concentrations Fig 14.
2.10 Stability studies for compound 24

50 µM of compound 24 in 20% DMSO in PBS was incubated at 37 °C and the HPLC experiment was run at the different time point. The peak areas were plotted against time Fig. 15.
In furtherance of our effort on the advancement of the medicinal chemistry work on the development of organic CO prodrugs, our lab has developed various prodrugs of CO that release CO based on an intramolecular Diels-Alder reaction. These prodrugs were synthesized by Dr. Ji Xingyue. I only did the kinetic and spectroscopic characterization of these compounds.

3.1 Results and Discussion

To determine the kinetics and fluorescence quantum yield, our lab designed and developed compounds with the general structure shown in Table 1 BW-CO-107, which, under physiological conditions, undergo intramolecular cycloaddition reaction to generate an intermediate that spontaneously releases CO and forms a fluorophore via a cheletropic reaction. From the results shown in Table 1, CO release rates vary with the ring size of the cyclized products, BW-CP-107. For instance, BW-CO-101 and BW-CO-102, each forming a five-membered ring lactam, have half-life of 1.9 min compared to BW-CO-103 and BW-CO-104, each forming a six-membered ring lactam, with half-lives of 72 min and 372 min respectively. The ease of compounds BW-CO-107 to undergo cycloaddition reaction to release CO under mild condition such as physiological conditions in contrast with the vigorous conditions required for that between an alkyne and cyclopentadienone was presumably due to entropic factors. This was displayed in the release rate for BW-CO-102, with TBDPS group in place of the alkyne terminal hydrogen, which happens to be similar with that for BW-CO-101. The CO release rate for BW-CO-105 which leads to formation of a lactone is much slower ($t_{1/2} >$ one week) compared to BW-CO-101 which forms a lactam. The slow kinetics of BW-CO-106 was due to the formation of hydrogen bond between the amide hydrogen and the carbonyl of the cyclopentadienone which locked the molecule in an
unfavourable conformation for the cycloaddition reaction.\textsuperscript{33} The CO release rate for BW-CO-107 with the sugar moiety (t\textsubscript{1/2}, 2.1 h) is slower compared to that for BW-CO-103 (t\textsubscript{1/2}, 1.2 h). However, in an aqueous solution, the CO release rate soared by 11 folds (t\textsubscript{1/2}, 0.18 h). This agrees with the higher reaction rate associated with the cycloaddition reaction in aqueous solution.

After the cyclization reaction, compounds BW-CO-107 led to the formation of blue fluorescent compounds BW-CP-107 which were characterized spectroscopically as shown in Table 1. The fluorescence quantum yield for these compounds are relatively close, and almost half that of the standard used, quinine sulfate with quantum yield of 0.58. These compounds can be used as fluorophores for in vitro and in vivo applications.

\textit{Table 1: The chemical structures and CO release kinetics of CO prodrugs.}

| compounds          | k[h\textsuperscript{-1}]\textsuperscript{[a]} | t\textsubscript{1/2}[h]\textsuperscript{[b]} | \(\lambda_{ex}[\text{nm}]\textsuperscript{[c]} | \(\lambda_{em}[\text{nm}]\textsuperscript{[d]} | \(\phi\textsuperscript{[e]} | \\
|-------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------| \\
| BW-CO-101: X = NMe, R\textsubscript{1} = R\textsubscript{2} = H, n = 1 | 22.4 ± 3.2/2.3 ± 0.2\textsuperscript{[f]} | 0.031 ± 0.004/0.3 ± 0.03\textsuperscript{[f]} | 371 | 450 | 0.21 |
| BW-CO-102: X = NMe, R\textsubscript{1} = TBDPS, R\textsubscript{2} = H, n = 1 | 22.6 ± 0.2 | 0.031 ± 0.0003 | 378 | 456 | 0.21 |
| BW-CO-103: X = N-iso-Pr, R\textsubscript{1} = R\textsubscript{2} = H, n = 2 | 0.57 ± 0.03 | 1.2 ± 0.1 | 373 | 456 | 0.18 |
| BW-CO-104: X = N-iso-Pr, R\textsubscript{1} = Me, R\textsubscript{2} = H, n = 2 | 0.11 ± 0.03 | 6.2 ± 0.2 | 372 | 464 | 0.17 |
| BW-CO-105: X = O, R\textsubscript{1} = H, R\textsubscript{2} = H, n = 1 | - | > one week\textsuperscript{[g]} | 367 | 454 | 0.14 |
| BW-CO-106: X = NH, R\textsubscript{1} = R\textsubscript{2} = H, n = 1 | - | > one week\textsuperscript{[g]} | 370 | 456 | 0.18 |

[a] CO release rate was determined by monitoring the increase of fluorescence intensity in DMSO/PBS (pH 7.4) 5:1 at 37\textdegree{}C. [b] Half-life for CO release. [c] The excitation wavelength of the cycloaddition products BW-CP-101–BW-CP-107. [d] The emission wavelength of the cycloaddition products BW-CP-101–BW-CP-107. [e] The quantum yield of the cycloaddition products BW-CP-101–BW-CP-107 in MeOH was determined using quinine sulfate as the standard. [f] The CO release rate in CH\textsubscript{2}Cl\textsubscript{2} at room temperature. [g] The CO release rate was
determined in [D6 ]DMSO/D2O (10:1) at 37 8C. [h] The CO release rate was determined in 1% of DMSO in PBS (pH 7.4); TBDPS: tert-butyldiphenylsilyl.

3.2 Conclusion

In conclusion, we have taken advantage of the inverse electron-demanded Diels Alder reaction in designing a click and release strategy for a unimolecular system for bioorthogonal CO prodrugs. In this system, both the dienones and non-activated alkyne were conjugated together via various tethering linkers. These series of CO prodrugs were stable during synthesis and storage and yet, readily undergo intramolecular cycloaddition reaction to release CO under physiological condition. The CO release rate varies from minutes to days and can be fine-tuned by varying the size, nature (lactam/lactone), substituents on the tether, and the electronic properties of the alkyne. The cyclized products were characterized spectroscopically, and the result showed that they can be employed as fluorescent probes for cell imaging and diagnostic application.

3.3 Experimental

3.3.1 Spectroscopic properties for cyclized compounds

The fluorescence spectra for the cyclized compounds were taken in different solvents, including CH2Cl2 (DCM), CH3CN (ACN), and DMSO (Figure 16-22).

![Figure 16. The fluorescence spectra for BW-CP-101 (100 µM) in different solvents. Excitation wavelength is 371 nm.](image-url)
Figure 17. The fluorescence spectra for BW-CP-102 (100 µM) in different solvents. Excitation wavelength is 370nm.

Figure 18. The fluorescence spectra for BW-CP-103 (100 µM) in different solvents. Excitation wavelength is 373nm.
Figure 19, The fluorescence spectra of BW-CP-106 (100 μM) in different solvents. Excitation wavelength is 370 nm.

Figure 20, The fluorescence spectra of BW-CP-107 (50 μM) in different solvents. Excitation wavelength is 370 nm.
3.3.2 Quantum Yield Determination

The quantum yields of compounds BW-CP-101 - 109 were determined by a comparative method using a well characterized quantum yield standard quinine sulfate ($\Phi = 0.54$ in 0.05M of H$_2$SO$_4$). Briefly, a series of solutions of compounds and standard with different concentrations (4, 8, 12, 16 and 20 $\mu$M) were prepared, and the UV absorbance at their excitation wavelength was taken. Then the fluorescence of the same solution were recorded. The integrated fluorescence were
plotted against the absorbance, and the quantum yield for the compounds was calculated according to the following equation:

$$\Phi_x = \Phi_{ST}(\text{Grad}_x/\text{Grad}_{ST})(\eta_x^2/\eta_{ST}^2)$$

Where the subscripts ST and x denote standard and compound respectively; $\Phi$ is the fluorescence quantum yield; Grad is the gradient from the plot of integrated fluorescence intensity against absorbance; and $\eta$ is the refractive index of the solvent. Shown in Figures 23-30 are the obtained results.

**Figure 23.** Plot of absorbance against integrated fluorescence intensity for the standard (excitation wavelength = 370 nM)

**Figure 24.** Plot of absorbance against integrated fluorescence intensity for BW-CP-101 (excitation wavelength = 370 nM), $\Phi = 0.21$
Figure 25. Plot of absorbance against integrated fluorescence intensity for BW-CP-102 (excitation wavelength = 378 nM), $\Phi = 0.21$

Figure 26. Plot of absorbance against integrated fluorescence intensity for BW-CP-103 (excitation wavelength = 373 nM), $\Phi = 0.18$
**Figure 27.** Plot of absorbance against integrated fluorescence intensity for BW-CP-104 (excitation wavelength = 372 nM), $\Phi = 0.17$

**Figure 28.** Plot of absorbance against integrated fluorescence intensity for BW-CP-105 (excitation wavelength = 367 nM), $\Phi = 0.14$
Figure 29. Plot of absorbance against integrated fluorescence intensity for BW-CO-106 (excitation wavelength = 370 nM), Φ = 0.18

Figure 30. Plot of absorbance against integrated fluorescence intensity for BW-CO-107 (excitation wavelength = 370 nM), Φ = 0.20

3.3.3 Studies of the CO Release Kinetics

The fluorescence of the cyclized product BW-CO-101 - 104, and 107-109 greatly facilitates the studies of CO release kinetics. Briefly, the fluorescence intensity of the cyclized product were monitored at different time point. The fluorescence intensity was plotted against time, and the obtained curve was fitted using Sigmaplot to get the first order reaction rate constant. The half-life was calculated according to the equation: $t_{1/2} = 0.689/k$. 
3.3.4 The CO release of BW-CO-101 in different solvent

BW-CO-101 was dissolved in different solvent including CH$_2$Cl$_2$ (25 °C), CH$_3$CN (25 °C), DMSO (25 °C), DMSO/PBS (10:1, 25 °C), DMSO/PBS (5:1, 25 °C), and DMSO/PBS (5:1, 37 °C) to give a final concentration of around 100 μM, and CO release was monitored by fluorescence intensity at 450 nm. Each experiment was repeated three times independently.

![Figure 31. The CO release rate in different solvent](image)

As shown in Fig. 31, the CO release rate (first order reaction rate) in DCM, ACN, DMSO, DMSO/PBS (10:1), DMSO/PBS (5:1), and DMSO/PBS (5:1, 37°C) were different and their individual rate constant was calculated to be 2.3±0.2, 3.6±0.4, 6.6±0.6, 7.7±0.6, 11.0±1.4 and 22.4±3.2 h$^{-1}$, respectively. The corresponding half-life for the CO release was 18, 11, 6, 5, 4, and 2 min, respectively. The CO release rate increased along with the polarity of the solvent.

3.3.5 The CO release rate of BW-CO-102-109 under physiological conditions

By a similar method used for studying the kinetics of compound BW-CO-101, the CO release rate constants for BW-CO-102-104 and 107-109 were obtained (Figure 32-38).
Figure 32. The CO release kinetics for BW-CO-102 (100 μM), $t_{1/2} = 1.8$ min.

Figure 33. The CO release kinetics for BW-CO-103 (100 μM), $t_{1/2} = 1.2$ h.

Figure 34. The CO release kinetics for BW-CO-104 (50 μM), $t_{1/2} = 6.3$ h.
**Figure 35.** The CO release kinetics for compound BW-CO-107 (50 μM, DMSO/PBS = 5:1) $t_{1/2} = 0.55$ h.

$\text{Fluorescence Intensity} \quad \text{Time (min)}$

![](bw-co-107.png)

$k = 1.28 \pm 0.18 \text{ h}^{-1}$

**Figure 36.** The CO release kinetics for compound BW-CO-108 (50 μM, DMSO/PBS = 5:1) $t_{1/2} = 12$ min.

$\text{Fluorescence Intensity} \quad \text{Time (min)}$

![](bw-co-108.png)

$k = 3.31 \pm 0.28 \text{ h}^{-1}$
**Figure 37**, The CO release kinetics for compound BW-CO-109 (50 μM, DMSO/PBS = 5:1), $t_{1/2} = 2.1$ h.

**Figure 38**, The CO release kinetics for compound BW-CO-109 (30 μM) in 1% of DMSO in PBS (7.4), $t_{1/2} = 0.18$ h.
REFERENCES


APPENDIX

$^1\text{H}$ and $^{13}\text{C}$ Spectra of compounds in chapter 2
MS of compounds in chapter 2