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Organic CO-prodrugs: Structure CO-release rate relationship Studies

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Abstract

Carbon monoxide (CO) is an endogenously produced gasotransmitter in mammals, and may have signaling roles in bacteria as well. It has many recognized therapeutic effects. A significant challenge in this field is the development of pharmaceutically acceptable forms of CO delivery with controllable and tunable release rates. Herein, we describe the structure-release rate studies of the first class of organic CO-prodrugs that release CO in aqueous solution at neutral pH.

Graphical Abstract

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Structure CO-release rate relationship studies of organic CO prodrugs reveals that CO releasing rate can be tuned by modifying the nature of the linker, substituents on dienone ring or linker, and the length of the linker.

Keywords

Carbon monoxide; release rate; metal free; prodrug; click and release

Introduction

Carbon monoxide (CO) has long been recognized as a toxic gas due to the fact that many die from CO poisoning every year. Beyond this perception is the fact that CO is also generated endogenously in mammals at a rate of 500 $\mu\text{mol/day}$ through the degradation of heme by heme oxygenase.^[1] Now, CO is recognized as an important gasotransmitter with importance on par with that of hydrogen sulfide (H_2S) and nitric oxide (NO).^[2] Moreover, past decades have witnessed the demonstration of a myriad of therapeutic indications for CO, including cytoprotective,^[3] anti-inflammatory,^[4] anti-bacterial^[5] and anti-cancer effects,^[6] among many others. However, work in taking CO into clinical applications is still in its infancy. There have been clinical trials using inhaled form of CO, clearly demonstrating the feasibility of CO's safe usage in human. However, inhaled CO is not an ideal delivery form for wide-spread applications due to difficulties in safe administration and in controlling doses, lack of portability, and the dependence on individual patient's respiratory function to deliver precise amounts. Safety issues in case of device malfunction are non-trivial either. Therefore, a number of labs have been working on developing pharmaceutically acceptable delivery forms of CO,^[7] including the usage of CO releasing molecules (CO-RMs) so that CO can be administered orally or through a parenteral route.^[8] Since CO is chemically inert it mainly coordinates with transition metals.^[9] Most of the CO-RMs reported so far are transition metal based. Undoubtedly, metal-based CORMs have made substantial contributions to the understanding of CO's biological effects. However, their clinical applications as therapeutic agents are somewhat hampered due to possible or perceived metal toxicity issues. Consequently, encapsulated CO-RMs and photosensitive organic CO-RMs have been developed, aimed at addressing metal toxicity and targeted delivery issues.^[8g, 8h, 8k, 8o, 10] For example, P. Klán and co-workers developed one elegant photosensitive CO prodrug, which could release CO both *in vitro* and *in vivo* upon irradiation with near infrared light (730 nm), and therefore shows potential for the precise spatiotemporal delivery. All these endeavors pushed CO-RMs closer to human clinical applications. As is true in any drug discovery efforts, structural diversity is critical to the eventual success of a therapeutic area because one would want to avoid the scenario that a major stumbling block in one approach would halt the whole field.

For this purpose, we are interested in the development of organic CO-prodrugs, which do not require light activation, can release CO under physiological condition with tunable release rates, and are amenable to structural optimization for the purpose of permeability, solubility, stability, and metabolism. Among the various features that we are interested in working on, we view the tunability of release rates being a critical one.^[7] Because of the volatile nature of CO, the release half-life plays an important role in determining the effective CO concentration and the duration of CO's action. We have demonstrated this point using another gasotransmitter, hydrogen sulfide.^[11] Moreover, it is widely acknowledged that CO-RMs/CO prodrugs with different releasing profiles are required in different biological milieu.

Our lab has a long standing interest in gasotransmitters.^[7, 11–12] Recently, we have disclosed the first class of CO-prodrugs that can release CO under physiological condition.^[12c] Such CO prodrugs can spontaneously release CO upon dissolution in a mixed aqueous solution or in a biological medium, and generate a fluorescent reporter after CO release (Figure 1, Scaffold I). Most importantly, they showed very pronounced anti-inflammatory effects in Raw 264.7 cells. By using one representative compound, it was demonstrated that such CO-prodrugs can be used for the treatment of experimental colitis induced by TNBS in mice with the suppression of TNF- α and MPO expressions, and increased survival. Herein, we describe the first comprehensive study of the relationship between structure and CO release rate for this class of CO-prodrugs. Meanwhile, two other new structural scaffolds (Figure 1, Scaffolds II and III) for the same purpose are also introduced here.

Results and Discussion

Design

This class of CO-prodrugs cages a CO molecule in the form of a ketone carbonyl group in a substituted cyclopentadienone and relies on a Diels-Alder reaction with an alkyne for the release of CO under physiological conditions. In designing new analogs, we separate these CO-prodrugs into three types. Type I represent an extension of our earlier work on fusing a cyclopentadienone ring with a naphthalene ring, which forms a fluorescent product after CO release, and thus allows for the real-time monitoring of CO formation. In this scaffold, the alkyne group is tethered to the cyclopentadienone moiety through a linear linker containing either an ester or amide functional group. The structural modifications to scaffold I were primarily made to the substituent on the cyclopentadienone ring (R_4 , Figure 1), the nature of the tethering linker (X, Figure 1), and the substituents on the tethering linker (R_1 – R_3 , Figure 1). In type II, the linker has a ring structure, providing additional conformational constraints. In addition, a carboxyl group in scaffold II can be used for structural optimization or for tethering additional structures for targeting and other applications. Similar structural modifications were also made to Scaffold II (X, Y and R_1) to probe the structure-release rate relationship. CO prodrugs with Scaffolds I and II both possess a naphthalene group fused with the dienone ring. However, the introduction of naphthalene ring is also accompanied with decreased water solubility due to its rigid and flat structural nature. Moreover, the fluorescent reporter is not necessarily needed for eventual clinical applications. Therefore, CO prodrugs with Scaffold III were also designed. In this class, each of the 3, 4 positions of

the cyclopentadienone moiety is substituted with an individual aryl ring. This affords additional structural diversity and flexibility. Further modifications followed a similar approach as for Scaffolds I and II.

In considering the design, one critical factor is the balance of the reactivity needed for cycloaddition in aqueous solution at neutral pH, and stability in organic solvent and during storage. It has been reported that water and protein binding accelerate Diels-Alder reactions by thousands of folds,^[13] largely driven by hydrophobic forces. This allows the possibility for CO-prodrugs to be prepared in organic solvents, remain stable during storage, and yet readily undergo cycloaddition in an aqueous solution to release CO.

Chemistry

CO prodrugs **BW-CO-104-106**, **108-113** with different tethering linkers and substituents were synthesized according to our previous procedures.^[12c] Briefly, compound **1a-b** were condensed with a variety of alcohol or amine to afford compounds **2a-i**, which were found to exist as a mixture of keto-enol tautomers (Scheme 1). Compounds **2a-i** were then reacted with acenaphthylene-1,2-dione, followed by treatment with an acid for dehydration to yield the desired CO prodrugs **BW-CO-104-106**, and **108-113** in 20–77% yield. CO prodrug **BW-CO-114** with a (2,5,8,11,14,17-hexaoxonadecan-19-yl)oxyl group was also synthesized in order to improve water solubility and biocompatibility (Scheme 2). Briefly, compound **4** was synthesized by alkylation of compound **3**, and was subsequently hydrolyzed to afford compound **5** in 98% yield. Compound **5** was then condensed with 2, 2-dimethyl-1,3-dioxane-4,6-dione using EDC as the coupling reagent to afford compound **6**. **BW-CO-114** was then obtained by a similar dehydration method used for the synthesis of **BW-CO-104-106**, **108 – 113** as shown in Scheme 1.

Next, we synthesized CO prodrugs with Scaffold II. As shown in Scheme 3, compound **8** was alkylated under basic condition to afford compounds **9a/b**. CO prodrugs **BW-CO-115-116** were then readily synthesized by a series of reactions similar to those used for the synthesis of **BW-CO-114** (Scheme 2). Meanwhile, in order to probe the effects of substituent on the linker for the CO release rate, CO prodrugs **BW-CO-117/118** were also synthesized as shown in Scheme 4. Initially, we tried to employ methyl 2-(2-aminophenyl)acetate as the starting material to make **BW-CO-117/118**, by following similar approaches used for **BW-CO-115/116**. However, unlike its phenol counterpart (**8**), methyl 2-(2-aminophenyl)acetate is unstable, and is prone to intramolecular lactamization to form a five-membered lactam ring. Consequently, compound **13** was used as the starting material, which was sulfonated and alkylated to afford compounds **15a/b**. The deprotection of the TBS group and subsequent oxidation of the alcohol to acid **16a/b** was accomplished in one pot by using KF and Jones reagent in acetone at room temperature.

With compounds **16a/b** in hand, the synthesis of **BW-CO-117 /118** was straightforward by a series of similar reactions used for **BW-CO-114**. However, in the case of **BW-CO-118**, **BW-CP-118** was afforded as the major product at the dehydration step, presumably due to the fast cycloaddition of **BW-CO-118** under such conditions. The synthesis of CO-prodrugs of Type III is depicted in Scheme 5. Intermediate **2a**, **2e** and **2f** described in Scheme 1 were

further reacted with benzil in the presence of KOH to yield the aldol intermediates, which were used directly for the dehydration step without purifications to afford **BW-CO-119 - 121** as red solid.

CO release kinetics and structure CO release rate relationships

With all the CO prodrugs in hand, we next studied their CO release rates in a mixed aqueous solution (DMSO/PBS = 4/1, 37 °C). For CO prodrugs of Scaffolds I and II, their CO release kinetics were easily determined by monitoring the increase of the fluorescent intensity at different time points. For CO prodrugs of Scaffold III, since the CO prodrugs have a UV absorbance peak at around 360 nm, and yet the cyclized product has no absorbance at this wavelength, the CO release rate was indirectly determined by monitoring the decrease of UV absorbance at 360 nm. The CO release kinetics for the CO prodrugs are summarized in Tables 1, 2 and 3.

As shown in Table 1, all the CO prodrugs of Type I can readily undergo intramolecular cycloaddition to release CO. The CO release was confirmed by the structural elucidation of the fluorescent cycloaddition products **BW-CP-104 - 106, 108 – 114**, a commercial CO detector and a CO myoglobin assay (SI, Figure S1). Three major factors are important in influencing the reaction rates: (1) the nature of the X, which determines whether it is an ester or amide, (2) additional substituents on the linear linker, which may impose additional conformational constraints favoring the cycloaddition reaction and thus CO release rates, and (3) the linker length, which determines whether cycloaddition would lead to a five- or six-membered ring formation. Generally, CO-prodrugs with an amide linker lead to faster CO release as compared to those with an ester linker. For example, the half-lives of **BW-CO-108/109** ($t_{1/2}$ = 55.6 or 0.55 h) with an ester linker were much longer than **BW-CO-110/111** ($t_{1/2}$ = 12 or 0.2 h) with an amide linker. This is understandable because amide bond is not as freely rotatable as an ester bond, and thus provides an entropic advantage in such cycloaddition reactions. It is well known that “gem-dialkyl” effect can greatly accelerate ring-closure reaction.^[14] Indeed, we find that the introduction of a gem-dimethyl group accelerates the cycloaddition rate significantly. For example, the half-lives for **BW-CO-109** and **BW-CO-111** are only around 0.55 and 0.20 h, respectively, which represent a more than 1000-fold difference as compared to **BW-CO-105/106** ($t_{1/2}$ > 10 days), which do not have this gem-dimethyl group. By the same token, the introduction of one methyl group on the tethering linker also enhances the cycloaddition rate. For example, **BW-CO-106** has a half-life of more than 10 days while **BW-CO-110** has a much shorter half-life of 12.0 h after introducing a methyl group.

An additional observation related to water solubility and CO-release rate is also very interesting. The introduction of a PEG linker to the phenyl ring in **BW-CO-114** slightly decreases the CO release rate ($t_{1/2}$ = 97 h vs $t_{1/2}$ = 56 h of **BW-CO-108**). It is possible that the introduction of a hydrophilic unit close to the reaction site disfavors a process that is partially driven by hydrophobic forces. Unexpectedly, substituting the phenyl group (**BW-CO-109/111**) on the dienone moiety with a methylthio group (**BW-CO-112/113**) does not make much of a difference in terms of CO release rate. Compared to a phenyl group, the methylthiol group is, albeit weak, an electron donating group, and should increase the

LUMO energy level of the dienone compound, resulting in a decrease in the cycloaddition rate. Indeed, calculations of the partial charge distributions show that the electron density of dienone ring with a methylthiol group (**BW-CO-112/113**) is higher than that of **BW-CO-109/112** (Figure S17). However, the results show that there is no significant difference in CO release rate between these two types of analogues, indicating that entropy factors dominated in rate acceleration instead of electron density on the dienone ring in such cases. This also opens a door for introducing substituents of varying electronic properties on the phenyl ring for structural diversity.

As shown in Table 2, all the synthesized CO prodrugs with scaffold II can release CO and generate a fluorescent reporter in DMSO/PBS (4:1) at 37 °C as well. The CO release was verified by structural elucidation of the cyclized products, a commercial CO detector and a CO myoglobin assay (SI, Figure S2). As for the CO release rate, the introduction of a ring structure on the linker did not seem to afford general rate enhancement. The CO release rates are in the same general range as that of Type I. Among the three analogs studied in Table 2, the *N*-methanesulfonyl aniline linker seems to provide faster CO release rates. For example, **BW-CO-117** with an *N*-methanesulfonyl group as “X” has a release half-life of 0.78 h. In comparison, compound **BW-CO-115**, which has an oxygen as the “X” group has a release half-life of 6.74 h. Such results are in agreement with the conformational constraints imposed by the *N*-methanesulfonyl aniline structure as compared to a phenol oxygen in the same place. The introduction of one methyl group into the tethering linker also enhanced the CO release. For example, **BW-CO-115** has a half-life of 6.74 h while **BW-CO-116**, which has a methyl group on the linker, has a half-life of only 2.25 h. This is the same trend as observed in the CO prodrugs of scaffold I.

As shown in Table 3, the CO prodrugs with Scaffold III also readily release CO in DMSO/PBS (4:1) at 37 °C. Intriguingly, the CO release rates for prodrugs of Scaffold III are much faster than that of the corresponding CO prodrugs of Scaffold I. For example, the half-life for **BW-CO-104**, **105** and **108** of Scaffold I is more than 6 h, 10 days and 55 h, respectively, and yet the half-life for their Scaffold III counterparts is only 1 h, 4 min, and 7 min, respectively. Interestingly, computed partial charges indicated that tethering a phenyl ring would lower the electron density of the dienone ring especially at the C₄ position (Figure S17). It is possible that the conjugation afforded by the phenyl ring in Scaffold III prodrugs increases the reactivity of the cyclopentadienone structure in a Diels-Alder reaction by lowering the LUMO energy level of the dienone ring. In addition, **BW-CO-121** ($t_{1/2}$ = 0.97 h) with an amide linker is found to release CO at a slower rate than **BW-CO-119** ($t_{1/2}$ = 0.12 h), despite the fact that it has an amide group, while **BW-CO-119** has an ester linker. Such results further indicate that the size of the ring formed from the tether plays an important role as well. It is clear that lactam/lactones of five-membered rings formed by **BW-CO-119** is favored over six-membered ring formed by **BW-CO-121**, leading to enhanced CO release rates. Besides, introduction of one methyl group on the tethering linker increases the CO release rate ($t_{1/2}$ = 0.073 h of **BW-CO-120** vs $t_{1/2}$ = 0.12 h of **BW-CO-119**), which is similar to our previous findings in Scaffold I.

Cytotoxicity studies

The cytotoxicity of **BW-CO/CP-104** has been tested in our previous work and these compounds showed no cytotoxicity at up to 100 μM .^[12c] Cytotoxicity of the other CO prodrugs along with their corresponding products after CO release was studied in Raw 264.7 cells with a drug exposure time of 24 h. The results (Figure S18–19) revealed that most of the compounds tested did not present obvious cytotoxicity at 100 μM , and only a few CO prodrugs (**BW-CO-112**, **113**, **115**, **116**, **120** and **121**) and inactive products (**BW-CP-111/115/120**) showed cytotoxicity with IC_{50} values in the range of 50–100 μM . Clearly, there is no general intrinsic toxicity issues related to this class of compounds, but idiosyncratic toxicity may occur with individual compounds, which can also be addressed by structural optimizations.

Intracellular CO release

Having confirmed that all the prodrugs readily undergo intramolecular cycloaddition to release CO in a mixed aqueous solution, we next probed whether they would release CO in a biological milieu. To this end, prodrugs **BW-CO-109** (Scaffold I), **117** (Scaffold II), and **119** (Scaffold III) were chosen as representatives to study CO release in Raw 264.7 cell culture. For **BW-CO-109/117**, CO release was monitored by the blue fluorescence of the cyclized product. For **BW-CO-119**, CO release was verified by using a reported CO fluorescent probe **COP-1**.^[15] As shown in Figure 3, the cells treated with **BW-CO-109** and **117** showed dose-dependent blue fluorescence formation, which indicated CO release intracellularly. For **BW-CO-119**, the cells treated with prodrug and **COP-1** showed strong green fluorescence in a dose dependent fashion, and the cells treated with **COP-1** alone showed only negligible green fluorescence. Moreover, fluorescence enhancement was assessed quantitatively. As shown in Figure 2d, cells treated with **BW-CO-119** and **COP-1** showed obvious increased in fluorescence intensity compared to the vehicle or **COP-1** only group. Similar results were observed when the cells were treated with **BW-CO-109** (Figure 3e) or **BW-CO-117** (Figure 3f). Altogether, these results indicated that all the CO prodrugs would release CO in a biological matrix.

Conclusions

In conclusion, CO prodrugs of different structural scaffolds were designed and synthesized, and their CO release rates were also determined. The CO release rate of these prodrugs is readily tunable with half-lives ranging from minutes to hours and even days. The structure-CO release rate relationships are summarized in Figure 4. Generally, entropic factors that each tethering linker imposes on the system seem to play very critical roles in determining the CO release rate. The more rigid tethering linkers, gem-dimethyl group and 5-membered ring formation, favor the cycloaddition reaction, and hence increase CO release rate. Apart from entropic factors, other factors could also be used to tune the CO release rate. For example, the presence of two phenyl rings at R_2 positions also substantially increased CO release rate. The understanding of the structure-CO release rate relationships achieved should allow us to further tune CO release rate and optimize our CO prodrugs. This study also provides a diverse group of CO-prodrugs for biology studies to examine the relationship among release rates, CO concentration, and efficacy and biological responses.

Experimental Section

General information

Fluorescence spectra were recorded on a Shimadzu RF-5301PC fluorometer and UV absorption spectra were obtained with Shimadzu UV-1700. **COP-1** was synthesized following literature procedures.^[15]

CO detection using a house-hold CO detector—BW-CO-113, 117 or 121 (~10 mg) were prepared in a solution of DMSO/PBS (4:1, 10 mL) and the solution was kept in a sealed 250 mL desiccator together with the CO detector (Drager Pac 7000). After 30 minutes, the detector began to beep because of CO levels exceeding the alarm threshold (35 ppm).

Myoglobin-CO assay—CO release from compounds **BW-CO-113**, **BW-CO-117** and **BW-CO-121** was confirmed by the myoglobin-CO assay. The reported “Two compartment” myoglobin-CO assay system was employed here.^[12c] A myoglobin solution in PBS (0.01 M, pH = 7.4) (1.7 mg/mL, 2.9 mL) was degassed by bubbling with nitrogen for at least 20 min and a freshly prepared solution of sodium dithionite (17mg/mL, 300 µL) was added to the myoglobin solution. Then the CO prodrug (1 mM) was added to the inner vial. After incubation for 1 h (**BW-CO-113**), 2 h (**BW-CO-117**) and 30 min (**BW-CO-119**) at 37 °C, respectively, the solution was cooled in an ice bath for 10 min to increase the solubility of CO in water and the UV absorption spectra was recorded (Figure S1–S3).

The CO release kinetics of BW-CO-108-121 in DMSO/PBS (pH 7.4, 4:1) at 37 °C—All the CO prodrugs were dissolved in DMSO/PBS (pH 7.4, 4:1) to a final concentration of 100 µM. The CO release was monitored by fluorescent intensity at 450 nm for **BW-CO-108-117** (Figure S4–S13). For CO prodrugs with scaffold **III**, the CO release was determined by the decrease of UV absorbance at 360 nm (Figure S14–S16). Each experiment was repeated three times independently.

Cytotoxicity studies of CO prodrugs and corresponding products—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 vehicle (1% DMSO) and compounds (0 – 100 µM) for 24 hours. After removal of the medium, 150 µL of DMEM containing 10 µL CCK-8 was added to each well and then the cells were incubated for another three hours at 37 °C. The absorbance at 450 nm was then measured by using a Perkin Elmer 1420 multi-label counter. The cell viability was measured and the results were normalized to the vehicle group. The experiment was triplicated and the results are expressed as mean ± SEM (n = 3).

Intracellular CO release study of BW-CO-109, 117 and 119—All the experiments were triplicated to confirm the CO release in the cell environment. Raw 264.7 cells were seeded in 6-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. CO prodrug was dissolved in DMSO and added into the cell culture media to give different final concentrations (25, 50 μ M) with 1% DMSO. For **BW-CO-119**, the cells were co-treated with **BW-CO-119** and COP-1 (CO fluorescent probe). The cells were incubated with the compound for 4 h at 37 °C. After that, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were then washed with PBS again twice and the coverslips with cells were immersed in DI water. The coverslips were mounted onto glass slides using the mounting media without DAPI (ProLong® Live Antifade Reagent; P36974). The fluorescent imaging was performed on a Zeiss fluorescent microscope, using DAPI (**BW-CO-109/117**)/FITC (**BW-CO-119**) imaging channel. The concentration-dependent images were taken using the oil objective.

Quantification of fluorescence emission in Raw 264.7 cells—Raw 264.7 cells were seeded on coverslips in 96-well plates one day before the experiment. CO prodrug was dissolved in DMSO and added into the cell culture media to give different final concentrations (25, 50 μ M) with 1% DMSO. For **BW-CO-119**, the cells were co-treated with **BW-CO-119** and **COP-1**. The cells were incubated with the compound for 4 h at 37 °C. The fluorescence intensity was then measured by using a Perkin Elmer 1420 multi-label counter. The results were normalized to the vehicle group (1% DMSO). The experiment was triplicated and the results are expressed as mean $\pm$ SEM (n = 3).

Calculation of partial charges of select molecules—All calculations were performed using the Gaussian 09 program.^[16] Initial geometry optimizations were carried out by DFT calculations with the use of B3LYP^[16b, 17] and the standard 6-31G* basis set. HF/6-31G* calculation was used to generate the electrostatic potential. Partial charges were produced fitting to the electrostatic potential at points selected according to the Merz-Singh-Kollman scheme.^[18] All calculations were conducted on Georgia State University cluster Orion with 4 CPU cores.^[19]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
General structures of CO prodrugs and their CO release mechanism

Figure 2.

Fluorescence imaging of fixed Raw 264.7 cells treated with **COP-1** (a: 1 μM) only, or **COP-1** (1 μM) + **BW-CO-119** (b: 25 μM, c: 50 μM) under FITC channel; d) quantitative fluorescence changes after treatment with COP-1 (1 μM) and **BW-CO-119**.

Figure 3. Fluorescence imaging of fixed Raw264.7 cells treated with **BW-CO-109** (a: 25 μ M; b: 50 μ M) or **BW-CO-117**(c: 25 μ M; d: 50 μ M) under DAPI channel; e) Fluorescence quantification of CO with treatment of **BW-CO-109**; f) Fluorescence quantification of CO release after treatment with **BW-CO-117**.

Figure 4.

A summary of the relationship between structure and CO release rate

- BW-CO-104:** X = -N-iso-propyl, R₁ = H, R₂ = H, R₃ = Me, n = 2;
BW-CO-105: X = O, R₁ = H, R₂ = H, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-106: X = NH, R₁ = H, R₂ = H, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-108: X = O, R₁ = Me, R₂ = H, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-109: X = O, R₁ = Me, R₂ = Me, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-110: X = NH, R₁ = Me, R₂ = H, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-111: X = NH, R₁ = Me, R₂ = Me, R₃ = H, R₄ = phenyl, n = 1;
BW-CO-112: X = NH, R₁ = Me, R₂ = Me, R₃ = H, R₄ = methylthio, n = 1;
BW-CO-113: X = O, R₁ = Me, R₂ = Me, R₃ = H, R₄ = methylthio, n = 1;

Scheme 1.

Synthesis of CO prodrugs **BW-CO-104-106, 108 – 113** with scaffold **I**. Reagents and conditions: i) toluene, substituted alcohol or amine, reflux, 1–2 h; ii) 1) acenaphthylene-1,2-dione, Et₃N, MeOH/THF (1:2), r.t., 1–3 h, 2). H₂SO₄, Ac₂O, 0 °C to r.t., 1–2 h

Scheme 2.

Synthesis of CO prodrugs **BW-CO-114** with scaffold **I**. Reagents and conditions: i) 2,5,8,11,14,17-hexaoxonadecan-19-yl 4-methylbenzenesulfonate, NaH, THF, 0 °C - r.t., 24 h; ii) LiOH, MeOH/H₂O, r.t., 24h; iii) 2,2-dimethyl-1,3-dioxane-4,6-dione, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), DCM, 0 °C - r.t., 7 h; iv) toluene, but-3-yn-2-ol, reflux, 45 min; v) acenaphthylene-1,2-dione, Et₃N, MeOH/THF (1:2), r.t., 3 h, then H₂SO₄, Ac₂O, 0 °C to r.t., 1 h.

Scheme 3.

Synthesis route of **BW-CO-115 - 116** with scaffold **II**. Reagents and conditions: (i) propargyl bromide or 3-butyn-2-yl 4-methylbenzenesulfonate, K_2CO_3 , ACN, reflux, 4 h; (ii) KOH , H_2O /methanol (1:1), r.t., 1 h; (iii) 2,2-dimethyl-1,3-dioxane-4,6-dione, DMAP, EDC, DCM, r.t., 12 h; (iv) morpholine, trimethylsilyl chloride (TMSCl), chlorobenzene, reflux, 3 h; (v) acenaphthylene-1,2-dione, Et_3N , MeOH/THF (1:2), rt, 3 h, then H_2SO_4 , Ac_2O , 0 °C to r.t., 1 h.

Scheme 4.

Synthesis route of **BW-CO-117 - 118** with scaffold **II**. Reagents and conditions: (i) MsCl, pyridine, DCM, 0°C to r.t, 20 h; (ii) propargyl bromide or 3-butyn-2-yl 4-methylbenzenesulfonate, K₂CO₃, ACN, reflux, 4 h; (iii) KF, Jones reagent, acetone, rt, 20 h; (iv) 2,2-dimethyl-1,3-dioxane-4,6-dione, DMAP, EDC, DCM, rt, 12 h; (v) morpholine, TMSCl, chlorobenzene, reflux, 3 h; (vi) 1). acenaphthylene-1,2-dione, Et₃N, MeOH/THF (1:2), r.t, 3 h; 2). H₂SO₄, Ac₂O, 0 °C to rt, 1 h.

BW-CO-119: X = O, R₁ = H, R₂ = H, R₃ = H, n = 1;

BW-CO-120: X = O, R₁ = Me, R₂ = H, R₃ = H, n = 1;

BW-CO-121: X = -N-iso-propyl, R₁ = H, R₂ = H, R₃ = Me, n = 2;

Scheme 5.

Synthesis of the CO prodrugs **BW-CO-119 - 121** with scaffold **III**. Reagents and conditions:

i) 1). benzil, KOH, N-methyl-2-pyrrolidone (NMP), r.t., 15 h; 2). H₂SO₄, Ac₂O, 0 °C to r.t., 15 min.

Table 1

CO release kinetics for CO prodrugs with scaffold I

Compounds	k (h ⁻¹) [a]	t _{1/2} (h) [b]
BW-CO-104: X = -N-iso-propyl, R ₁ = H, R ₂ = H, R ₃ = Me, n = 2	0.11±0.03	6.2±0.2
BW-CO-105: X = O, R ₁ = H, R ₂ = H, R ₃ = H, R ₄ = phenyl, n = 1	-	> 10 days
BW-CO-106: X = NH, R ₁ = H, R ₂ = H, R ₃ = H, R ₄ = phenyl, n = 1	-	>10 days
BW-CO-108: X = O, R ₁ = Me, R ₂ = H, R ₃ = H, R ₄ = phenyl, n = 1	0.012±0.0003	55.6±1.5
BW-CO-109: X = O, R ₁ = Me, R ₂ = Me, R ₃ = H, R ₄ = phenyl, n = 1	1.28±0.18	0.55±0.08
BW-CO-110: X = NH, R ₁ = Me, R ₂ = H, R ₃ = H, R ₄ = phenyl, n = 1	0.058±0.003	12.0±0.74
BW-CO-111: X = NH, R ₁ = Me, R ₂ = Me, R ₃ = H, R ₄ = phenyl, n = 1	3.31 ±0.28	0.20±0.02
BW-CO-112: X = NH, R ₁ = Me, R ₂ = Me, R ₃ = H, R ₄ = methylthio, n = 1	4.63±0.28	0.15±0.009
BW-CO-113: X = O, R ₁ = Me, R ₂ = Me, R ₃ = H, R ₄ = methylthio, n = 1	1.29±0.07	0.53±0.04
BW-CO-114: X = O, R ₁ = Me, R ₂ = H, R ₃ = H, R ₄ = PEG substituted phenyl group, n = 1	0.0072±0.0003	96.85±4.45

[a] CO release rate was determined by monitoring the increase of fluorescence intensity in DMSO/PBS (pH = 7.4) 4:1 at 37 °C.

[b] Half-life for CO release;

Table 2

CO release kinetics for CO prodrugs with scaffold II

Compounds	$k \text{ (h}^{-1}\text{)}$ ^[a]	$t_{1/2} \text{ (h)}$ ^[b]
BW-CO-115: X = O, R ₁ = H, Y = morpholine	0.10±0.003	6.74±0.21
BW-CO-116: X = O, R ₁ = Me, Y = morpholine	0.308±0.003	2.25±0.03
BW-CO-117: X = NMs, R ₁ = H, Y = morpholine	0.882±0.012	0.78±0.01

^[a] CO release rate was determined by monitoring the increase of fluorescence intensity in DMSO/PBS (pH = 7.4) 4:1 at 37 °C.

^[b] Half-life for CO release;

Table 3CO release kinetics for CO prodrugs with scaffold **III**

Compounds	k (h^{-1}) ^[a]	$t_{1/2}$ (h) ^[b]
BW-CO-119 : X = O, R ₁ = H, R ₂ = H, R ₃ = H, n = 1	5.89±0.91	0.12±0.02
BW-CO-120 : X = O, R ₁ = H, R ₂ = Me, R ₃ = H, n = 1	9.39±1.07	0.073±0.013
BW-CO-121 : X = - <i>N</i> -iso-propyl, R ₁ = Me, R ₂ = H, R ₃ = H, n = 2	0.72±0.08	0.97±0.11

^[a] CO release rate was determined by monitoring the decrease of absorbance at 360 nm in DMSO/PBS (pH = 7.4) 4:1 at 37 °C.

^[b] Half-life for CO release.