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Visible Light-activated Quinolone Carbon Monoxide-releasing Molecule: Prodrug and Albumin-assisted Delivery Enables Anti-cancer and Potent Anti-inflammatory Effects

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Abstract

The delivery of controlled amounts of carbon monoxide (CO) to biological targets is of significant current interest. Very few CO-releasing compounds are currently known that can be rigorously controlled in terms of the location and amount of CO released. To address this deficiency, we report herein a new metal-free, visible light-induced CO-releasing molecule (photoCORM) and its prodrug oxidized form, which offer new approaches to controlled, localized CO-delivery. The new photoCORM, based on a 3-hydroxybenzo[*g*]quinolone framework, releases one equivalent of CO upon visible light illumination under a variety of biologically-relevant conditions. This non-toxic compound can be tracked prior to CO release using fluorescence microscopy and produces a non-toxic byproduct following CO release. An oxidized prodrug form of the photoCORM is reduced by cellular thiols, providing an approach toward activation in the reducing environment of cancer cells. Strong non-covalent affinity of the non-metal photoCORM to albumin enables use of an albumin:photoCORM complex for targeted CO delivery to cancer cells. This approach produced cytotoxicity IC₅₀ values among the lowest reported to date for CO delivery to cancer cells by a photoCORM. This albumin:photoCORM complex is also the first CO delivery system to produce significant anti-inflammatory effects when introduced at nanomolar photoCORM concentration.

TOC Image:

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Supporting Information. NMR, IR, UV-vis, fluorescence, and mass spectral data for **1-3**; BSA binding data; docking study views; MTT and fluorescence imaging data for **1-3** and Flav-1 in HUVECs and RAW 264.7 cells. The Supporting Information (PDF) is available free of charge on the ACS Publications website.

Introduction

The delivery of carbon monoxide (CO) in controlled amounts to targeted locations remains a challenge for using this gaseous molecule for biomedical research and applications.¹ Key to delivering a localized, controlled amount of CO is the development of carbon monoxide releasing molecules (CORMs) that exhibit CO release *only* when triggered.² While many approaches to triggered CO release have been investigated, including light-,^{2–7} enzyme-,⁸ and magnetic heating,⁹ most studies reported to date have been performed using metal carbonyl-based CORMs.¹⁰ A weakness in this approach is that metal carbonyls often exhibit background CO release due to ligand exchange processes, even when encapsulated in macromolecular structures such as proteins, polymers, metal organic frameworks (MOFs), and nano-sized materials.¹¹ Metal-free, triggered organic CORMs offer an alternative approach for highly controlled CO release.^{2,12} In this regard, visible light-triggered CO-releasing molecules (photoCORMs) are especially attractive.^{13–17} As we have recently shown, a flavonol-based organic photoCORM can easily be modified for targeted, localized, and controlled CO release in applications involving environmental sensing or mitochondrial targeting.^{18,19}

The targeted delivery of toxic doses of CO offers a promising approach that could limit toxicity to healthy cells while treating tumors.³ To date, while non-metal spontaneous CO-releasing molecules have been reported that can deliver CO to cancer cells,²⁰ non-metal photoCORMs have not been investigated for anti-cancer applications. Several metal carbonyl photoCORMs have been evaluated for their ability to eradicate cancer cells.^{21–29} However, for the reasons noted above regarding the leakiness of metal carbonyls as CO-release units, localized and targeted CO-delivery remains challenging. Loss of any CO prior to controlled release would necessitate that a higher concentration of CO-delivery compound must be used to deliver an effective amount to the target. Uncontrolled CO release also enhances the likelihood of toxic effects to healthy cells.

To date, metal-free organic photoCORMs have not been explored for triggered delivery of CO to cancer cells or for CO release to produce anti-inflammatory effects. Four types of visible light-induced organic photoCORMs have been reported in the literature (Figure 1).

We envisioned that a quinolone analog of Flav-1 (Figure 1(d)) containing a secondary amine within the heterocyclic ring (**1**, Figure 2) could offer a new multi-faceted approach for controlled, targeted CO delivery in cancer cells by taking advantage of their highly reducing environment. Specifically, this type of compound has the potential to be introduced either as the reduced photoCORM **1**, or as an oxidized prodrug form **2** (Figure 2) that would be acted upon by the reducing environment within cancer cells to reveal the photoCORM. We note that reductive-based approaches have been previously employed to target metal-based anti-cancer compounds to tumors, wherein free thiol concentrations are higher than in normal tissues.³⁰ However, reduction-based generation of a non-metal photoCORM for anti-cancer applications has not previously been reported.

Herein we outline chemical and cell-based studies of the use of **1** and **2** for controlled and localized CO release. To further facilitate accumulation of **1** and **2** in cancer cells, we have investigated the interactions of these compounds with serum albumin protein, which is known to facilitate delivery of small molecules to cancer cells via the enhanced permeability and retention effect.³¹ Our results demonstrate that **2** is readily reduced to **1** by biological thiols (cysteine, glutathione) that are abundant in the reducing environment of cancer cells. Bovine serum albumin binds **1** with a high affinity similar to naturally occurring flavonols. Notably, bovine serum albumin (BSA) also reduces **2** to **1**, likely via reactivity with a surface exposed cysteine residue (Cys-34). Visible light-induced CO release from the BSA:**1** complex enables eradication of cancer cells at a concentration of the photoCORM that is among the lowest reported to date for any CORM. Compounds **1** and **2** are both non-toxic to both normal and cancer cells. Notably, illumination of cells to which BSA and subsequently **1** or **2** has been added also produces significant anti-inflammatory effects at unprecedented nanomolar concentrations. Control experiments demonstrate that this effect is clearly due to CO and not the organic CO release product **3** (Figure 2). Overall, we demonstrate herein that the new quinolone-type photoCORM **1** can be used in combination with serum albumin protein to deliver specific amounts of carbon monoxide to produce desired biological effects. This approach leverages a biologically-relevant CORM that has a high affinity to a ubiquitous protein carrier. Coupled with its high level of control for CO delivery, this natural system is a unique tool to advance studies and applications of the role CO in biology.

Results

CO-release reactivity of **1**.

Compound **1** has been previously reported as a dye.³² While we prepared this compound following the literature procedure, we report additional characterization data including ¹³C{¹H} NMR, IR, and mass spectral features (Figure S1-S6). The lowest energy absorption band for **1** in CH₃CN is centered at 449 nm (Figure 4(a) and Figure S4). Excitation into this band produces dual-emission features, with maxima at ~500 and 600 nm (Figure 4(b) and Figure S5).³² The fluorescence quantum yield of **1** at 600 nm is lower than that of Flav-1 (Figure 1(d), Φ_{PL} = 34.5 and 7.9, respectively), which correlates with the difference in fluorescence lifetimes of Flav-1 (7.4 ns) and **1** (6.4 ns) in O₂-free CH₃CN.

Illumination of an aerobic acetonitrile solution of **1** at 419 nm (2450 lx) or 465 nm (5972 lx) results in the release of 0.90(2) equivalents of CO (as determined by GC headspace analysis) and in the formation of the non-emissive depside (**3**, Figure 2), which was identified by independent synthesis and characterization (Figure S7-S11). The quantum yield for the CO release step using 419 nm light under air is 0.0045(1) ($\phi_e = 33$), which is only slightly lower than that found for Flav-1 (0.007(1); $\phi_e = 43$) under identical conditions.¹⁷ Notably, the reaction quantum yield for **1** is higher than that reported for the visible light-triggered xanthene-9-carboxylic acid derivative (Figure 1(a), $6.8 \pm 3.0 \times 10^{-4}$; $\phi_e = 1-10$; $\lambda_{ex} = 500$ nm) under aerobic conditions when illuminated in aqueous PBS buffer at pH = 7.4.¹³ The BODIPY derivatives (Figure 1(b)) also exhibit lower quantum yields under anaerobic ($2.7 \pm 0.4 \times 10^{-4}$ ($\phi_e = 13$) and $1.2 \pm 0.4 \times 10^{-5}$ ($\phi_e = 0.6$), respectively, $\lambda_{ex} = 500$ nm) and aerobic conditions ($1.1 \pm 0.1 \times 10^{-4}$, $\phi_e = 5$; $R_1 = -CH_3$, Figure 1(b)) in aqueous PBS buffer.¹⁴ The quantum yield for CO release from the diketone photoCORM shown in Figure 1(c) is 0.02.^{15,33}

Compound **1** also exhibits quantitative visible light-induced CO release in aqueous buffer:DMSO (1:1 or 1:0.05; pH = 7.4) and in DMSO under hypoxic (1% O₂) conditions (Figure S12) that are typical of tumors.³⁴ Thus, **1** is a reliable visible light-induced CO-releasing molecule under a variety of conditions. This is noteworthy, as the photoCORMs shown in Figure 1(a)-(c) exhibit lower CO release yields in the presence of O₂ and H₂O, respectively.^{14,15}

Synthesis, characterization, and thiol reactivity of **2**.

The diketone **2** (Figure 2) was synthesized in 60% yield via MnO₂ oxidation of **1**.³⁵ Compound **2** was characterized by ¹H and ¹³C{¹H} NMR, IR, mass spectrometry, UV-vis, and fluorescence (Figures S13-S19). The diketone exhibits different molecular ion peaks depending on the solvent used for ESI-MS analyses. When obtained in methanol, the observed high-resolution m/z 318.1099 (Figure S18) is consistent with a [M+CH₃OH+H]⁺ ion. This is the result of addition of the nucleophilic solvent to the 3-carbonyl moiety resulting in the formation of a hemiketal. In CH₃CN, the low-resolution m/z 286.2 is consistent with a [MH]⁺ ion (Figure S19) for the formulation of **2** as shown in Figure 2. Consistent with the mass spectral results, **2** exhibits solvent-dependent absorption and emission spectra. The red color observed for solutions of **2** in CH₃CN changes to pale yellow when the compound is dissolved in CH₃OH or 4% DMSO in H₂O (Figure 3), solvent conditions under which reversible hemiketal formation can take place. The absorption and emission features of **1** and **2** are distinct indicating that the reduction of **2** to **1** is identifiable by either absorption or emission spectroscopy (Figure 4). When dissolved in either CH₃CN or aqueous buffer:DMSO (95:5) compound **2** is stable with respect to visible light over the time period needed for CO release from **1** (data not shown).

Compound **2** is reduced to **1** in the presence of typical cellular thiols. As shown in Figure 5, titrations of aqueous buffer:DMSO (95:5) solutions of **2** at pH = 7.4 in the presence of cetrimonium bromide (CTAB, 20 mM) with excess glutathione and cysteine results in an increase in fluorescence consistent with the formation of **1**.

Serum albumin binding of **1**.

The affinity of **1** for bovine serum albumin was investigated using fluorescence quenching studies in 4% DMSO:Tris buffer (pH = 7.4, [NaCl] = 0.1 M). Monitoring of the tryptophan emission of BSA upon titration with **1**, followed by data analysis using Stern-Volmer and modified Stern-Volmer equations (Figure S20), revealed that the compound binds approximately 900-fold more strongly to BSA than does Flav-1 (Table 1).³⁶ The binding constant for **1** ($2.9 \times 10^6 \text{ M}^{-1}$) is in the range of the strongest binding naturally-occurring flavonoids (10^5 - 10^7 M^{-1}), including quercetin,³⁷⁻³⁹ and is comparable to that of ibuprofen ($3.6 \times 10^6 \text{ M}^{-1}$) binding to BSA.⁴⁰ Compound **1** binds in an approximate 1:1 stoichiometry with BSA similar to quercetin.³⁷⁻⁴⁰ Docking studies performed using AutoDock Vina 1.1.2⁴¹ (using PDB: 3V03) suggest a high affinity-binding site for **1** near Site I in subdomain IIA (Fig. S21). Consistent with binding at this site, **1** shows reduced affinity in the presence of warfarin (Table 1), which is known to bind at Site I.⁴² Two additional possible high affinity-binding sites for **1** were also identified using AutoDock Vina 1.1.2. These are located at Site II in subdomain IIIA and in subdomain IB. Some interaction with Site II is evidenced by a lowering of the K_a value for **1** in the presence of ibuprofen (Table 1), which is known to bind at that site.⁴² Notably, the CO-release depside byproduct **3** (Figure 1) binds strongly to BSA with a binding constant of $1.2 \times 10^6 \text{ M}^{-1}$ (Table 1; Figure S22) and one binding site per protein.

In the presence of one equivalent of BSA (4% DMSO:Tris (pH = 7.4, [NaCl] = 0.1 M)), **1** exhibits similar CO release reactivity (0.84 eq) to the free flavonol. In the presence of five equivalents of BSA, the CO reaction is still quantitative, but is less efficient than that exhibited by free **1** as evidenced by a lower reaction quantum yield ($\Phi = 0.0008(1)$). We note that the tight binding of the CO release product **3** to BSA suggests that following CO release from the BSA:**1** complex any extracellularly released **3** would become bound to the protein. Within the cell, **3** may undergo further biodegradation.

Serum albumin reactivity of **2**.

We discovered that **2** is reduced to **1** in the presence of BSA. Monitoring the emission of **2** upon titration with BSA results in a >10-fold increase in emission (Figure 6(a)). Performing the same study with **1** resulted in only a minimal emission increase (Figure 6(b)), which may be due to protein binding. Overall, the similar features of the final emission spectra indicate that **2** is reduced to **1** in the presence of BSA. Additional support for the reduction of **2** to **1** in the presence of BSA comes from CO release studies. Specifically, while **2** is stable with respect to visible light, addition of BSA to a solution of **2**, followed by visible light illumination, results in the release of 0.82 eq of CO, which is similar to that obtained using **1** in the presence of BSA (0.84 eq). This result provides strong evidence of the formation of **1** from **2** in the presence of BSA. Based on literature precedent, a redox active, surface-exposed cysteine residue, Cys-34, is likely involved in the reduction of **2** to **1**.⁴³ This residue is known to form mixed disulfides with free cysteine or intermolecular disulfide linked proteins involving two Cys-34 residues.

Cellular CO delivery using **1** and **2**. Anti-cancer and anti-inflammatory effects.

We began cell-based studies of Flav-1, **1** and **2**, as well as the CO release product **3**, by examining their cytotoxicity toward normal human cells (umbilical vein, HUVECs) using an MTT assay (Figure S23). Cell viability of greater than 50% was observed for each compound up to 100 μ M. We next comparatively examined the intracellular uptake of **1** and **2** (50 μ M) using fluorescence microscopy. Experiments were performed in the absence and presence of an added amount of BSA relevant to physiological conditions (0.6 mM).⁴⁴ As shown in Figure 7, incubation of HUVECs with **1** under standard conditions (in media containing 10% fetal bovine serum) results in the appearance of a precipitate, with green emission being detected from the aggregates (Figure 7, row 2). Compound **1** was not distributed within the cells under these conditions and the aggregates were observed not to lose fluorescence intensity or release CO when illuminated with visible light (data not shown). Use of the diketone **2** under the same conditions resulted in no precipitation and enhanced distributed green emission (Figure 7, row 3), with higher emission intensity localized to cells, suggesting the intracellular uptake of **1**. Importantly, pre-treatment of the HUVECs with BSA (0.6 mM) in addition to the amount typically present in cell culture experiments (estimated to be 0.04 mM for 10% fetal bovine serum in media) eliminated the precipitation for **1** (Figure 8, row 2). Monitoring the green emission produced from **2** in the presence of BSA (0.6 mM) again suggests better intracellular distribution via the reductive generation of **1** (Figure 8, row 4). Evidence that the green intracellular signal is due to the presence of **1** was obtained through visible light-induced CO release (Figure 8, rows 3 and 5). Specifically, the disappearance of the green signal upon illumination with visible light indicates CO release and the formation of **3**, which is non-emissive. The release of CO was further validated through the use of a Nile red-based intracellular CO sensor (1-Ac).⁴⁵ As shown in Figure 9, illumination of cells containing 0.6 mM BSA and **1** or **2** resulted in no remaining signal in the green channel and the appearance of a red emission indicating CO production. This experiment demonstrates the efficacy of CO delivery to cells by **1** or **2** in the presence of added BSA. Moreover, as indicated by the Nile red CO sensor results, both compounds were similarly effective in delivering CO to cells in the presence of physiologically-relevant levels of BSA.

Studies of the anti-cancer properties of CO release from **1** and **2** were initiated using adenocarcinoma human alveolar basal epithelial cells (A549 cells). The toxicity of Flav-1 and **1-3** toward A549s in the presence of added BSA (0.6 mM) was analyzed using an MTT assay (Figure 10(top)). As shown in Table 2, in the absence of visible light illumination, compounds **1**, **2** and the CO-release organic product **3** were non-toxic up to 100 μ M. It is notable that the structurally related Flav-1 was slightly more toxic, with an IC₅₀ value of 82 μ M. Upon visible light ($\lambda_{\text{ill}} = 419$ nm; 2450 lx) illumination to trigger in situ CO release, **1** and **2** exhibited notably enhanced cytotoxicity, with IC₅₀ values of 24 and ~50 μ M, respectively, while the CO-release product **3** remained non-toxic. The IC₅₀ value for **1** is in the range of the lowest reported to date for cancer cells using a molecular photoCORM.³ The CO release from **1** and **2** produced a more pronounced toxic effect than did CO release from Flav-1 under identical conditions. As **1** and Flav-1 each release one equivalent of CO and have similar reaction quantum yields, the difference in CO-induced toxicity toward

cancer cells (A549s) may be due to their differences in serum albumin binding and/or cellular uptake.

To begin to examine the broader applicability of **1** and **2** in the presence of added BSA, we next examined CO-associated anti-inflammatory effects in RAW 264.7 murine macrophage cells.^{21,46} Compounds **1** and **2** were initially screened in the presence of added BSA for their cytotoxicity in the RAW 264.7 cells under non-illumination and illumination conditions using an MTT assay. As shown in Figure S24 and Table 2, without illumination compounds **1** and **2** have IC₅₀ values of ~40 and 33 μM, respectively, whereas **3** is non-toxic up to 100 μM. Flav-1 was nontoxic to RAW 264.7 cells, showing that structural variation within the family of compounds allows for tuning of the toxicological profile features.

Upon visible light-induced CO release, the toxicity was significantly enhanced, with **1**, **2** and Flav-1 exhibiting lower IC₅₀ values of ~12, 4, and 22 μM, respectively. With this data in hand, we examined how effectively the compounds could attenuate LPS-induced inflammation by suppressing production of TNF-α.⁴⁶ Two plates of RAW 264.7 cells were pretreated with **1-3** for 4 h in the presence of 0.6 mM BSA followed by illumination of one of the plates using a blue LED array (66451 lx) for 1 hr. The illuminated and non-illuminated plates were then treated with LPS (1 μg/mL final concentration) for 1 h. The concentration of TNF-α in the supernatant of each well was evaluated using a commercial ELISA kit. CO release from **1** almost completely suppressed the TNF-α level at a concentration of 80 nM (Figure 11). To our knowledge, this is the lowest concentration of a CORM reported to date to produce a demonstrated anti-inflammatory effect. Metal carbonyl CORMs that have been shown to reduce TNF-α expression in RAW 264.7 murine macrophages include CORM-3 and a BSA-Ru(CO)₂ conjugate.²¹ These CORMs start to produce effects at concentrations of 10 and 4.5 μM, respectively.^{21,46} Wang et al. have reported spontaneous metal-free CORMs that exhibit statistically significant TNF-α suppression only at concentrations of 5 μM or higher.⁴⁷⁻⁴⁹ Comparatively, the BSA:**1** complex is more effective at a concentration that is more than 6-times lower. This may be due to differences in intracellular versus extracellular CO release and/or triggered versus non-triggered release, as these factors may influence the local concentration of CO. The lack of any effect from the CO release product **3** also indicates that the observed biological effect is due to the released CO and not the organic product scaffold. However, we do note that the non-illuminated **1** also caused a dose-dependent decrease in TNF-α concentration. This anti-inflammatory activity is associated with the parent structure of **1** prior to CO release and demonstrates the advantages of using the biologically-inspired 3-hydroxybenzo[*g*]quinolone framework.⁵⁰

The illuminated diketone **2** also exhibited a concentration-dependent TNF-α suppression effect that is not seen with **1**. The difference between the compounds likely relates to the reductive step that must first take place between BSA and **2** to produce the photoCORM **1**. The non-illuminated diketone in the presence of BSA shows no effect up to ~50 μM, which is a toxic level for the RAW 264.7 cells.

Discussion

We report herein that a 3-hydroxybenzo[*g*]quinolone photoCORM, or an oxidized prodrug form of the compound, can be used in combination with serum albumin protein to produce anti-cancer and potent anti-inflammatory effects in cellular environments. Compound **1** is the first metal-free photoCORM to produce demonstrated anti-cancer effects upon CO release.³ This compound undergoes controlled CO release only when triggered. This feature addresses a significant drawback of using metal carbonyl CORMs as anti-cancer agents as most metal carbonyls exhibit some CO leakage via ligand exchange thus limiting targeted delivery.¹¹ In terms of localization of CO release, we demonstrate herein that the prodrug **2** undergoes reduction to **1** in the presence of cellular thiols, the concentrations of which are higher in the reducing environment of cancer cells. We also show that a BSA:**1** complex can easily be generated and that this construct offers a straight-forward method for controlled CO delivery to cancer cells. These approaches toward localizing CO delivery to cancer cells are unprecedented in terms of non-metal CORMs and leverage the unique properties of the bioinspired 3-hydroxybenzo[*g*]quinolone framework. Most significant is that we demonstrate that use of the BSA:**1** complex enables the use of low micromolar and nanomolar photoCORM concentrations to achieve anti-cancer and anti-inflammatory effects, respectively.

The biological features of **1** are noteworthy as they distinguish this photoCORM and its oxidized prodrug **2** from the vast majority of CORMs and photoCORMs reported to date. Compound **1** is an extended analog of 1*H*-3-hydroxy-4-oxo-quinolone, the substrate for cofactor-free bacterial 2,4-dioxygenases, which catalyze a CO-releasing reaction.⁵¹ The reductive reactivity of **2** with biological thiols is similar to that of flavins, redox cofactors that are reduced in the presence of thiols (Scheme 1(top)).⁵² Compound **2** also has similarity to dehydroascorbic acid (DHA), the diketone oxidized form of vitamin C (ascorbic acid, AA), which can serve as a prodrug of this essential nutrient.⁵³ Within cells, DHA is reduced to AA by glutathione and other thiols (Scheme 1(middle)). Overall, visible-light induced CO release from **1** has many features in common with natural heme-oxygenase-mediated CO production. These include a dioxygenase-type reaction leading to the release of a single equivalent of CO, and the formation of a single organic byproduct. It is also important to note similarities in the chemistry of the CO release product **3** to bilirubin, the downstream product generated upon heme degradation via heme oxygenase CO-release enzyme activity. Compound **3** and unconjugated bilirubin both bind tightly but non-covalently to albumin, with binding constants of $\sim 10^7$ M⁻¹.⁵⁴ This high affinity binding suggests possible protein-mediated transport of the product from the site of CO-release in both cases.

The BSA:**1** complex is the first example of a non-metal CORM/protein assembly to be used for intracellular CO delivery. It is more straight-forward to generate and use than protein-based CO delivery systems involving metal carbonyl moieties coordinated within protein structures.^{55,56} Serum albumin delivery of drug molecules is an approach currently under investigation, including for the delivery of nitric oxide, for anti-cancer applications.^{31,57–60} Albumin is known to accumulate in cancer cells. Bernardes et al. have previously shown that albumin binding of a metal-carbonyl type photoCORM (CORM-3) to form a BSA-Ru(CO)₂ adduct enabled accumulation of CO at tumor sites in mice.²¹ We have previously reported

that Flav-1 binds to BSA with an affinity that is weaker than naturally-occurring flavonols such as quercetin.³⁶ Studies of the BSA binding properties of **1** showed the replacement of the pyrone ring oxygen atom with a secondary amine produces a ~900-fold enhanced binding affinity to the protein. The binding constant for **1** is in the range of the strongest binding naturally-occurring flavonoids ($\sim 10^5$ - 10^7 M⁻¹), including quercetin.³⁷⁻⁴⁰ This strong affinity of **1** to BSA provides a convenient approach for constructing a CO-delivery complex in situ.

Conclusions

Development of metal-free molecular tools for localized and targeted CO delivery in biological systems is important to the further development of CO as a therapeutic. Using a biologically-inspired photoCORM and protein-based delivery, we have developed a highly controlled system for CO delivery to living cells. This approach can be used to produce potent anti-cancer and anti-inflammatory effects at micromolar and nanomolar photoCORM concentrations, respectively.

Experimental Section

Materials and Methods.

All reagents were purchased and used as received unless otherwise noted. Polyphosphoric acid, anthranilic acid, and bovine serum albumin (BSA, heat shock fraction) were purchased from Sigma Aldrich. Ibuprofen and 2-bromoacetophenone were purchased from Alfa Aesar. 3-amino-2-naphthoic acid was purchased from Combi-Blocks. Warfarin was purchased from Cayman Chemicals. Distilled or double distilled water was used in all experiments. Compound **1** was prepared following the literature procedure.³²

¹H and ¹³C{¹H} NMR spectra were collected using a Bruker Avance III HD Ascend-500 spectrometer. UV-vis spectra were measured at ambient temperature on a Cary 50Bio or a Hewlett-Packard 8453A diode array spectrometer. FTIR spectra were recorded on a Shimadzu FTIR-8400 spectrometer. Fluorescence emission spectra were collected using a Shimadzu RF-530XPC spectrometer in the range of 295–800 nm, with the excitation wavelength corresponding to the absorption maxima of the analyte. The spectra were collected using 1.0 cm quartz cells with excitation and emission slit widths set at 3.0 nm. Mass spectral data were obtained in house using a Shimadzu LC-MS2020 (LR-MS) or at the Mass Spectrometry Facility, University of California, Riverside (ESI-APCI HRMS). A Rayonet photoreactor equipped with RPR-4190A or LED-BL (465 nm) lamps was used for all photochemical reactions. Quantum yields were measured using ferrioxalate as a standard to measure photon flux.^{61,62} Serum albumin binding and molecular docking studies were performed as previously described.³⁶

3-hydroxybenzo[g]quinolone (**1**).

¹H NMR (CD₃CN, 500 MHz) δ ppm 8.90 (s, 1H), 8.12 (s, 1H), 8.10 (d, J = 8.5 Hz, 1H), 7.97 (d, J = 9.0 Hz, 1H), 7.93 (d, J = 7.5 Hz, 2H), 7.61 (t, J = 7.5 Hz, 2H), 7.56 (t, J = 6.5 Hz, 2H), 7.46 (t, J = 7.5 Hz, 1H); ¹³C{¹H} NMR DMSO-*d*₆, 125 MHz) δ ppm 171.2, 135.8, 135.5, 134.0, 133.9, 132.5, 129.8, 129.4, 129.2, 128.3, 127.4, 126.9, 124.7, 124.5, 122.2,

114.1 (17 signals expected, 16 observed due to overlap in the aromatic region); UV-vis (CH₃CN, nm) (ϵ , M⁻¹cm⁻¹) 445 (7800); ESI/APCI-MS (CH₃CN) (relative intensity) calcd. for [MH]⁺: 288.098; found: 288.101 (100%); FTIR (KBr, cm⁻¹) 3237 ($\nu_{\text{O-H}}$), 3396 ($\nu_{\text{N-H}}$), 1640 ($\nu_{\text{C=O}}$), 1199 ($\nu_{\text{C-N}}$).

3,4-benzo[g]quinolonedione (2).

Compound **1** (50 mg, 0.17 mmol) was dissolved in 25 ml of dry CH₂Cl₂. Activated MnO₂ (71 mg, 0.82 mmol) was added to a reaction mixture which was stirred at room temperature. Thin layer chromatography (TLC; 1:2 hexanes:ethyl acetate) was used to monitor the reaction. When no starting material was identified by TLC, the MnO₂ was removed by filtration and was washed using CH₂Cl₂. The solvent was then removed under reduced pressure (60%, 29 mg). ¹H NMR (CD₃CN, 500 MHz) δ ppm 8.66 (s, 1H), 8.17 (s, 1H), 8.12 (d, J = 8.5 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 8.02 (d, J = 7.5 Hz, 2H), 7.73 (t, J = 7.5 Hz, 1H), 7.67 (t, J = 7.5 Hz, 1H), 7.5–7.6 (m, 3H); ¹³C{¹H} NMR (CD₃CN, 125 MHz) δ ppm 178.6, 177.2, 161.1, 142.8, 138.2, 135.6, 133.5, 133.3, 131.8, 131.8, 131.8, 131.6, 130.3, 129.8, 129.2, 126.9 (17 signals expected, 16 observed due to overlap in the aromatic region); ESI-MS (CH₃CN) (relative intensity) calcd. for [MH]⁺: 286.1; found: 286.2 (100%); ESI/APCI (MeOH) (relative intensity) calcd. for [M+CH₃OH+H]⁺: 318.1130; found: 318.1099 (100%); FTIR (KBr, cm⁻¹) 1683 ($\nu_{\text{C=O}}$), UV-vis (CH₃CN, nm) (ϵ , M⁻¹cm⁻¹) 482 (6500); emission (CH₃CN) λ_{max} = 555 nm (λ_{ex} = 482 nm).

Independent synthesis of 3-(benzoylamino)-2-naphthalenecarboxylic acid (3).

3-amino-2-naphthoic acid (0.50 g, 2.7 mmol) was dissolved in 10 ml dry CH₂Cl₂ and mixed with triethylamine (0.41 ml, 3.0 mmol). A solution of benzoyl chloride (0.31 ml, 2.7 mmol) in 3 ml of dry CH₂Cl₂ was added to the mixture, which was then stirred overnight. A white precipitate was formed. After addition of H₂O (50 ml) to the flask, the organic phase was separated, mixed with ethyl acetate and left open for solvent to evaporate. The precipitate obtained was filtered and washed with water and cold ethyl acetate (57%, 45 mg). ¹H NMR (CD₃CN, 500 MHz) δ ppm 9.32 (s, 1H), 8.80 (s, 1H), 8.05 (d, J = 7.5 Hz, 2H), 7.98 (d, J = 8.5 Hz, 1H), 7.93 (d, J = 8.5 Hz, 1H), 7.57–7.65 (m, 4H), 7.50 (t, J = 7.5 Hz, 1H); ¹³C{¹H} NMR (CD₃OD, 125 MHz) δ ppm 171.9, 167.5, 137.7, 137.7, 136.1, 135.0, 133.2, 130.4, 130.2, 130.2, 130.0, 128.5, 128.3, 126.8, 118.3, 118.0 (16 signals expected and observed); ESI/APCI (CH₃CN) (relative intensity) calcd. for [MH]⁺: 292.093; found: 292.114 (15%); [MNa]⁺: 314.0793; found: 314.0782 (100%); FTIR (KBr, cm⁻¹) 1687 ($\nu_{\text{C=O}}$). Anal Calcd. for C₁₈H₁₃NO₃·0.7H₂O: C, 71.14; H, 4.78; N, 4.61. Found: C, 71.12; H, 4.57; N, 4.63. ¹H NMR confirmed the presence of H₂O in the elemental analysis sample.

CO quantification.

CO gas was detected and quantified using an Agilent 3000A micro gas chromatograph with molecular sieve and Plot U columns and a thermal conductivity detector. CO gas quantification from **1** and **2** was performed in 4% DMSO-Tris solutions. Solutions used for these measurements contained **1** or **2** (2×10^{-3} M) and an equimolar amount of BSA. Each was then illuminated with 419 nm light for 24 hours. Upon illumination, 0.84 and 0.82 equivalents of CO were released from **1** and **2**, respectively, in the presence of BSA.

Photophysical characterization.

Time-resolved emission data for **1** and Flav-1 were collected at room temperature using an Edinburgh Instruments FLS980 spectrometer. The dynamics of emission decay were monitored using the time-correlated single-photon counting capability (1024 channels; 100 ns window) with data collection for 10,000 counts. Excitation was provided by an Edinburgh EPL-360 picosecond pulsed light emitting diode (360 ± 10 nm, pulse width 892 ps) operated at 10 MHz. Emission from the sample was passed through a single grating (1800 l/mm, 500 nm blaze) Czerny-Turner monochromator (0.75 nm bandwidth) and finally detected using a peltier-cooled Hamamatsu R928 photomultiplier tube. Kinetic data was fit with a single exponential function using the Edinburgh software package.

Absolute emission quantum yields were acquired using an integrating sphere incorporated into the FLS980 spectrofluorimeter. The samples were placed in the sphere and a movable mirror was used for direct or indirect excitation, making it possible to measure absolute emission quantum efficiency following the de Mello method.⁶³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Metal-free organic photoCORMs with different CO-releasing motifs. (a) xanthene-9-carboxylic acid; (b) BODIPY carboxylic acid; (c) diketone; (d) 3-hydroxybenzo[*g*]flavone (Flav-1).

Figure 2.

(top) Quinolone-type photoCORM **1** along with its oxidized form (**2**, left) and CO-release product **3** (right). (bottom) Strategy of evaluation of CO delivery by **1** and **2** in the reducing environment of cancer cells in the absence and presence of serum albumin protein. CO release from **1** is induced by visible light in the presence of O₂.

Figure 3.
Absorption spectra of **2** in various solvents.

Figure 4. Absorption (a) and emission (b) spectra of **1** and **2** in CH_3CN ($\lambda_{\text{ex}} = 428 \text{ nm}$). The concentration of both compounds is 0.1 mM.

Figure 5. Emission spectra of **2** (0.1 mM) in the presence of 10 eq. of (a) glutathione or (b) cysteine in 5% DMSO-PBS buffer (10 mM, pH = 7.4) mixture in the presence of 20 mM CTAB ($\lambda_{\text{ex}} = 428$ nm).

Figure 6. Emission spectra of (a) **2** (0.1 mM) and (b) **1** (0.1 mM) in 4% DMSO:Tris (pH = 7.4, [NaCl] = 0.1 M) upon titration with bovine serum albumin ($\lambda_{\text{ex}} = 428$ nm).

Figure 7.

Individual fluorescence microscopy images of HUVECs incubated for 4 h in DMEM/F-12K media with **1** or **2**. Row 1: Media control for all experiments. Row 2: Cells exposed to **1**. Row 3: Cells exposed to **2**. The observed green fluorescence in rows 2 and 3 is from **1**. Cells also co-stained with Hoechst 33342 nuclear dye (blue) to assess cell integrity. Size of bar = 50 μ m.

Figure 8.

Individual fluorescence microscopy images of HUVECs pretreated with BSA (0.6 mM) and incubated with **1** or **2** for 4 h in DMEM/F-12K media under various conditions. Row 1: Media control for all experiments. Row 2: Cells exposed to **1** for 4 hours. Row 3: Cells from second row illuminated (488 nm light, with a light density of 42,620 lx). Row 4: Cells exposed to **2** for 4 hours. Row 5: Cells from fourth row illuminated (488 nm light, with a light density of 42,620 lx). The low observed intensity of the green emission in lines 2 and 4 is in agreement with the low fluorescence quantum yield of **1**. Cells also co-stained with Hoechst 33342 nuclear dye (blue) to assess cell integrity. Size of bar = 50 μ m.

Figure 9.

Fluorescence detection of CO release from **1** and **2** using a Nile red-based sensor in HUVECs in the presence of BSA (0.6 mM). Row 1: Nile red-based CO-sensor control after illumination with 460 nm LED array (66,351 lx) for 1 hour. Row 2: Compound **1** incubated for 4 hours, followed by introduction of the CO sensor and illumination for 1 hour using a 460 nm LED array. Row 3: Compound **2** incubated for 4 hours, followed by introduction of the CO sensor and illumination for 1 hour using a 460 nm LED array (66,351 lx). All cells were co-stained with Hoechst 33342 nuclear dye to assess cell integrity. Size of bar = 50 μ m.

Figure 10.

Plot of A549 cell percent viability versus concentration of **1-3** and Flav-1 in the presence of BSA (0.6 mM) under (top) non-illuminated or (bottom) illuminated conditions. IC_{50} values were determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed. Values displayed represent the average $\pm$ SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol and as such are not seen.

Figure 11.

Anti-inflammatory effects of **1-3** in RAW 264.7 murine macrophage cells in the presence of BSA (0.6 mM) and light (CO release in situ) or under dark conditions. The results are presented as means $\pm$ SEM from three independent experiments. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison post-hoc tests to compare effects of all treatments to the LPS positive control or to compare effects of treatment with compounds **1** and **2** under illuminated and non-illuminated conditions. ★, $p < 0.0001$ compared to LPS positive control; # (blue) or # (red) indicates $p < 0.0001$ compared to corresponding non-illuminated treatment (blue, **1**; red, **2**).

Scheme 1.

(top and middle) Redox reactivity of flavins and DHA with thiols. (bottom) Proposed redox reactivity of **2** with thiols. The heteroatom redox motif in each structure is highlighted for comparison.

Table 1.

Binding constants with bovine serum albumin protein at 22 °C.

Compound	<i>n</i>	K_{SV}, M^{-1}	K_a, M^{-1} (warfarin)	K_a, M^{-1} (ibuprofen)	Ref.
1	1.26	2.9×10^6	6.1×10^5	9.8×10^5	This work
Quercetin	1.29	3.7×10^7			38
3	1.20	1.2×10^6			This work
Flav-1	0.66	3.2×10^3	3.6×10^2	2.6×10^3	36

Table 2.

IC₅₀ values (μM) for **1-3** and Flav-1 in A549 and RAW 264.7 cells under normal and illuminated conditions in the presence of BSA.

	A549	A549 (illuminated) ^b	RAW 264.7	RAW 264.7 (illuminated) ^b
1	ND ^a	24.22 ± 2.03	40.19 ± 1.44	12.17 ± 1.72
2	ND	49.77 ± 1.85	33.34 ± 1.27	4.47 ± 0.21
3	ND	ND	ND	ND
Flav-1	81.91 ± 2.40	95.38 ± 3.91	ND	22.39 ± 2.98

^aND = not determined (> 100 μM).

^bIllumination was performed using a panel of blue LEDs. Values shown are the mean ± SEM of three independent experiments.