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ORIGINAL RESEARCH

Beneficial Effects of Oral Carbon Monoxide on Doxorubicin-Induced Cardiotoxicity

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BACKGROUND: Doxorubicin and other anthracyclines are crucial cancer treatment drugs. However, they are associated with significant cardiotoxicity, severely affecting patient care and limiting dosage and usage. Previous studies have shown that low carbon monoxide (CO) concentrations protect against doxorubicin toxicity. However, traditional methods of CO delivery pose complex challenges for daily administration, such as dosing and toxicity. To address these challenges, we developed a novel oral liquid drug product containing CO (HBI-002) that can be easily self-administered by patients with cancer undergoing doxorubicin treatment, resulting in CO being delivered through the upper gastrointestinal tract.

METHODS AND RESULTS: HBI-002 was tested in a murine model of doxorubicin cardiotoxicity in the presence and absence of lung or breast cancer. The mice received HBI-002 twice daily before doxorubicin administration and experienced increased carboxyhemoglobin levels from a baseline of ≈1% to 7%. Heart tissue from mice treated with HBI-002 had a 6.3-fold increase in CO concentrations and higher expression of the cytoprotective enzyme heme oxygenase-1 compared with placebo control. In both acute and chronic doxorubicin toxicity scenarios, HBI-002 protected the heart from cardiotoxic effects, including limiting tissue damage and cardiac dysfunction and improving survival. In addition, HBI-002 did not compromise the efficacy of doxorubicin in reducing tumor volume, but rather enhanced the sensitivity of breast 4T1 cancer cells to doxorubicin while simultaneously protecting cardiac function.

CONCLUSIONS: These findings strongly support using HBI-002 as a cardioprotective agent that maintains the therapeutic benefits of doxorubicin cancer treatment while mitigating cardiac damage.

Key Words: anthracyclines ■ cancer ■ carbon monoxide ■ cardiotoxicity ■ heme oxygenase-1

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Despite the wide use and demonstrated efficacy of anthracyclines in cancer, cardiac toxicity from these drugs limits dosing and causes substantial cardiac morbidity. Anthracyclines, such as doxorubicin, have been used for over 45 years and are among the most effective anticancer treatments ever developed. This class of drug continues to be used as the standard of care against more types of cancer than any other class of chemotherapeutic agents, with indications including leukemia and Hodgkin's lymphoma, as well as cancers of the breast, stomach, lung, thyroid, bladder, and soft tissue.[1–4] Perhaps most compelling is that many novel immunotherapies and tyrosine kinase inhibitors are more effective when combined with doxorubicin.[5–7] However, anthracyclines induce life-threatening and irreversible cardiotoxicity, which can appear immediately or years after treatment.[8,9] Unfortunately, 10% to 75% of cancer survivors suffer from chronic cardiovascular issues in later life caused by the cumulative toxicity of their cancer therapy.[1,8,9,10]

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RESEARCH PERSPECTIVE

What Is New?

- HBI-002, an oral liquid product, increases carboxyhemoglobin levels and offers safe delivery of carbon monoxide into the heart tissue.
- HBI-002 not only protected the heart from doxorubicin cardiotoxic effects but also boosted the sensitivity of mammary tumors to doxorubicin without compromising the chemotherapy efficacy.

What Question Should Be Addressed Next?

- Further studies utilizing large animals would enhance our understanding of the safety and efficacy of carbon monoxide across all stages of anthracycline-induced cardiotoxicity, providing excellent relevance and clinical potential for HBI-002 to be used in cancer patients under anthracycline therapy.

Nonstandard Abbreviations and Acronyms

CO	carbon monoxide
HBI-002	oral liquid product containing carbon monoxide
HO-1	heme oxygenase-1
iCO	inhaled carbon monoxide

Moreover, ≈5% of patients treated with anthracyclines have shown evidence of congestive heart failure or a significant dose-dependent decline in left ventricular (LV) function.[11,12] This damage to the heart causes patient morbidity and limits anthracycline dosing, which ultimately affects cancer management. There are no effective treatments to limit doxorubicin-induced cardiotoxicity other than the iron chelator dexrazoxane, the use of which is restricted by the US Food and Drug Administration only for patients with advanced breast cancer on high doses of doxorubicin.[13]

Previous work has shown that low-dose carbon monoxide (CO; ≈12%–15% carboxyhemoglobin) protects the cardiomyocyte from cell death and supports overall cardiovascular health in numerous cardiac-focused studies such as those addressing transplant, right heart hypertrophy, and vascular stenosis.[14–16] Moreover, CO increases the sensitivity of cancer cells to genotoxins, such as doxorubicin or camptothecin, by up to 1000-fold while sparing normal cells in cancer-laden tissue.[17] Endogenous CO is generated in the body by the cytoprotective heme oxygenase (HO) enzymes that are responsible for the degradation of heme.[18] In humans, normal carboxyhemoglobin levels are 0.5% to 1.5% of total Hb (hemoglobin) bound to CO.[19] Despite the demonstrated toxicity associated with higher doses of CO,[20] extensive research conducted by numerous groups over the past 2 decades has resulted in approved clinical testing of low levels of CO[20] (NCT00094406, NCT02425579, NCT03799874, NCT04870125, NCT03926819). These efforts are based on a plethora of preclinical evidence that CO, when used at low concentrations, offers remarkable beneficial effects in a variety of preclinical models of disease.[21] The clinical safety and tolerability of CO have been tested in 23 Phase I and Phase II clinical trials using inhaled and carrier molecule-bound CO,[22,23] and overall conclusions showed no electrocardiogram or neurocognitive changes versus placebo when CO treatment increased the carboxyhemoglobin up to ≈12%.

The cellular mechanisms of the cytoprotective action of CO are not well defined but include induction of protective and antiapoptotic genes, mitochondrial biogenesis, alterations in cellular bioenergetics, and the promotion of tissue repair.[18,21,24] Despite such promising data, the difficulty of administering CO has proven to be challenging. All 23 completed clinical studies with CO and most in vivo studies have used inhaled CO (iCO), CO-releasing molecules, or CO conjugated to a PEGylated Hb.[15,25,26,27,28] Administration of iCO, CO-releasing molecules, or PEGylated CO-Hb has been in development for years and, although offering potential value to the patient, has not yet been shown to be suitable for home use or chronic, long-term administration.[29–32] Thus, oral administration of HBI-002, which is a liquid drug product containing CO with Generally Regarded as Safe excipients, per the Food and Drug Administration, avoids many of the challenges associated with other methods of CO delivery and provides a platform for outpatient administration, appropriate dosing, and compliance. In this light, a Phase I safety study in healthy adults has been completed with the oral liquid drug product containing CO at the dose used for the work presented here and showed no adverse events of clinical significance (www.clinicaltrials.gov, NCT03926819). Here, we tested the ability of HBI-002 to protect against doxorubicin-induced cardiotoxicity without interfering with doxorubicin effects on tumor growth. We find that HBI-002 recapitulates the cardioprotective characteristics and anticancer effects associated with low-dose CO inhalation and, importantly, obviates the concerns associated with other modes of CO administration.

METHODS

The data that support the findings of this study are available from the corresponding or first authors (ralves1@bidmc.harvard.edu) upon reasonable request.

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Animals

Ten- to 12-week-old male C57BL/6 and female Balb/c mice (Jackson Laboratories) were housed under specific pathogen-free conditions with 12-hour day/light cycles. All mouse procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (#083–2021).

Oral CO Therapy

HBI-002 is a novel oral liquid product consisting of CO as well as Food and Drug Administration-defined generally recognized as safe components. Vehicle control was the same formulation without CO. Vials of HBI-002 and vehicle, supplied by Hillhurst Biopharmaceuticals, Inc, were administered to mice by oral gavage, as previously reported.[33] HBI-002 was given at 20 mL/kg, divided into 2 doses of 10 mL/kg, 1 hour apart, due to the maximum dose volume limit to maximize the concentration and duration of CO exposure. Initially, we tested the effects of 2 weeks (5 d/wk) of HBI-002 on healthy animals with hearts harvested 24 hours after the last dose to assess changes in body mass, cardiac function, and heart HO-1 expression (4–8 mice per group). Next, HBI-002 was tested in combination with doxorubicin. In doxorubicin regimens, the second HBI-002 dose preceded doxorubicin administration by 10 minutes. For chronic studies ±tumor burden, this dosing regimen continued throughout the doxorubicin treatment regimen (Figure 1, A to D). HBI-002 was dosed to achieve ≈7% peak carboxyhemoglobin levels. Control groups received vehicle or HBI-002 without doxorubicin administration.

Measurement of CO Levels

To evaluate the pharmacokinetics of CO, 4 male mice per time point were given 2 doses of HBI-002, 1 hour apart (10 mL/kg per dose), and the blood was obtained from a lethal cardiac puncture under anesthesia. Blood collection occurred at baseline and subsequent time points of 10, 15, and 60 minutes following the first administration of HBI-002. After 60 minutes, the animals received a second dose (10 mL/kg), and blood samples were again collected at 70, 80, and 120 minutes after the initial administration. All blood was placed into

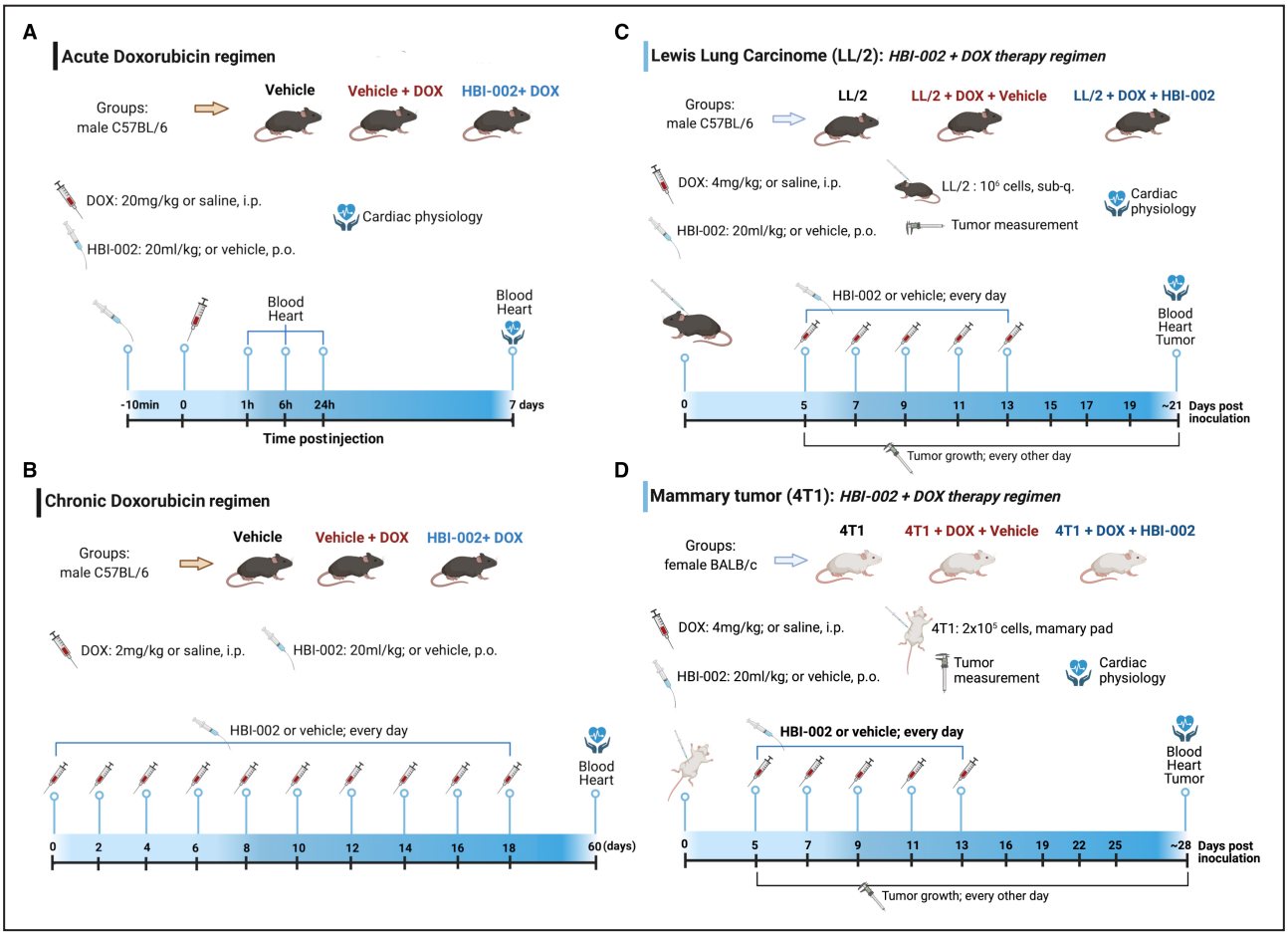

Figure 1. Experimental design of HBI-002 treatment in doxorubicin-treated mice.
HBI-002 effects in acute (**A**) and chronic (**B**) doxorubicin regimen models. Lewis lung carcinoma (LL/2) (**C**) and mammary tumor (**D**) models treated with doxorubicin in combination with HBI-002. Illustration created using BioRender.com.

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1-mL BD syringes filled with 100 U of heparin and run on an ABL80 FLEX CO-OX blood gas analyzer. The concentration of CO in heart tissue was measured in 8 mice per group, as previously described.[34]

Doxorubicin Treatment Regimens

Doxorubicin HCl (D1515, Sigma-Aldrich) regimens were designed to mimic that used in humans, with a recommended therapeutic dose of 60 to 75 mg/m^2 every 21 days in patients with cancer.[35] We tested both an acute toxicity regimen (single dose at 20 mg/kg, IP; ≈60 mg/m^2; 3–7 mice per time point or group) with blood and hearts harvested at 1 hour, 24 hours, and 7 days after doxorubicin (Figure 1A) and a chronic dose regimen where a cumulative dose was administered via injections administered intraperitoneally every 48 hours for 10 doses (2 mg/kg, for a cumulative dose of 20 mg/kg, ≈60 mg/m^2; 4–12 mice per group) for 18 days (Figure 1B). For chronic studies, the animals were then euthanized 6 weeks after their last doxorubicin administration. The chronic dose was also used in the context of a tumor burden, as described later. The presence of doxorubicin-induced cardiomyopathy was confirmed by mRNA quantification of the cardiac dysfunction markers (*BNP* [brain natriuretic peptide]: *Nppb*; ANP [atrial natriuretic peptide]: *Nppa*; cardiac troponin-T: *Tnnt*; and β-MyHC [beta-myosin heavy chain]: *Myh7*) and measurement of cardiac function by echocardiography compared with control vehicle-treated mice (discussed later). Blood serum for biochemical analyses and the ventricles was carefully collected, frozen in liquid nitrogen, and stored at −80 °C until analysis.

Cancer Cell Lines and Tumor Cell Injection

The 4T1 murine breast cancer (ATCC, CRL-2539) and Lewis lung carcinoma (LL/2, ATCC, CRL-1642) cell lines were cultured in high-glucose (4.5 g/L) DMEM supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) at 37 °C and 5% CO_2. For LL/2 cells, we injected 10^6 cells into the flank of 8 to 13 C57BL/6 male mice per group. For the 4T1 mammary tumor model, 4 Balb/c female mice per group were orthotopically inoculated with 2×10^5 cells in the fourth inguinal mammary fat pad (100 μL/mouse) (Figure 1C and 1D). Once the animals from both models had a palpable solitary mass (≈day 5), they were randomized into groups and placed in the study. LL/2 and 4T1 tumor mice models received a treatment consisting of 5 doses of doxorubicin (4 mg/kg, IP), administered every other day. During doxorubicin and non-doxorubicin treatment days, mice were administered HBI-002 or vehicle as described. Mice were randomly enrolled into 1 of the 4 groups, with sham as a control, LL/2 or 4T1 tumor, placebo (vehicle) as negative controls, and HBI-002 as the therapeutic arm: (1) control; (2) LL/2 or 4T1; (3) doxorubicin+ vehicle (10 mL/kg); and (4) doxorubicin+HBI-002. Body weight was determined periodically, and tumor volume was measured with a digital Vernier caliper. Tumor volume was calculated using the following equation: tumor volume (mm^3)=[length×width×height]/2, where the height, length, and width are in millimeters. The doxorubicin protocol lasted for ≈10 days after cancer cell inoculation. LL/2 mice were euthanized at ≈21 days and 4T1 mice at ≈28 days postinoculation for tumor and cardiac harvest.

Echocardiography

Heart structure and function assessment were performed using echocardiography (Vevo 770, Visual Sonics, Toronto, Canada) under isoflurane (2% v/v) anesthesia. Animals assigned to acute doxorubicin regimen, LL/2, and 4T1 tumor models were subjected to echocardiography before and after each experimental protocol (Figure 1A, 1C and 1D). For chronic doxorubicin regimen, echocardiography was performed before doxorubicin initiation, after the last doxorubicin dose, and at the end of the protocol (Figure 1B). Interventricular septum thickness in systole and diastole, LV inner dimensions (LVID) at systole and diastole, LV posterior wall thickness at systole and diastole, LV end-systolic and end-diastolic volume, and ejection fraction (EF) were assessed. LVID at diastole and systole were used to calculate the fractional shortening as follows: fractional shortening (%)=[(LVID diastole – LVID systole)/LVID diastole]×100. All myocardial structures were manually measured in accordance with the leading-edge method of the American Society of Echocardiography.[36] The examiner (R.W.A.d.S.) was blinded to the group's allocation.

Biochemical Analyses

Serum creatine kinase, lactate dehydrogenase), and cTnI (cardiac troponin-I) were used for the evaluation of tissue and myocardial damage. Serum creatine kinase and lactate dehydrogenase were analyzed on an IDEXX Catalyst DX analyzer (IDEXX Laboratories). cTnI was determined by ELISA (MBS766175, MyBioSource) according to the manufacturer's instructions.

Histological Analysis

Heart specimens were fixed overnight in a 4% paraformaldehyde solution at pH 7.4. Following fixation, the hearts were embedded in paraffin and subsequently sectioned serially at a thickness of 5 μm. Routine histological examination was performed by staining the sections with hematoxylin and eosin, enabling visualization of tissue architecture and cellular morphology using a light microscope. Additionally, select sections

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were stained with Sirius red to evaluate collagen deposition. To ensure unbiased analysis, all histological assessments were carried out by a single observer (R.W.A.d.S.) blinded to the identity of the studied mice.

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was extracted from heart ventricles using TRizol reagent (Invitrogen) following manufacturer's instruction. Reverse transcription was performed using a High-Capacity cDNA synthesis kit (Thermo Scientific). After cDNA synthesis, reverse transcription quantitative polymerase chain reaction for target and endogenous genes (Table 1) was run separately, and amplifications were detected using QuantStudio3 (Applied Biosystems, Foster City, CA) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Fold-change was calculated through the ΔΔCt method.

Immunoblotting

Protein extraction, quantification, preparation, and immunoblotting experiments were performed as previously described.[37] Mitochondrial oxidative phosphorylation complexes (OXPHOS, ab110413), Hmox1 (ab13243), and Gapdh (ab9485) primary antibodies were purchased from Abcam. Secondary specific antibodies were goat antimouse IR800 and goat antirabbit IR680 and IR800 (LICOR). Membranes were scanned using the Odyssey infrared imaging system (LICOR).

Statistical Analysis

Statistical analysis was performed using GraphPad (GraphPad Prism version 9, GraphPad Software, La Jolla, CA). The Shapiro–Wilk normality test was used to assess the data distribution. Statistical data were tested for normal distribution and homogeneity. In the HBI-002 studies, for the comparisons between 3 groups, an unpaired 2-tailed Student's *t* test was performed. Two-way ANOVA with repeated measures was used when the effects of experimental factors were analyzed at different time points. In the HBI-002±doxorubicin±cancer studies, 1-way ANOVA with Tukey's post hoc test was performed. No statistical methods were used to predetermine the sample size. All results are depicted as mean±SEM, and *P* values <0.05 were considered significant.

RESULTS

HBI-002 Administration Increases CO Levels in the Blood and Heart

Administration of HBI-002 to mice showed no change in body mass or cardiac function after 2 weeks of daily dosing at 20 mL/kg, PO (Figure 2A and 2B). HBI-002 allows for precise dosing at defined intervals, unlike iCO, which requires consideration of breathing rate and depth of inspiration. Upon a single gavage of HBI-002 (10 mL/kg), carboxyhemoglobin levels surged from ≈1% to 4.3±0.4%. To maximize the concentration and duration of CO exposure, we administered a second

Table 1. Primer Sequences for RT-qPCR mRNA Analysis

Target	PCR primer sequence 5′→3′	Product size (bp)	GenBank accession #
Hmox1	F: CAGAAGAGGCTAAGACCGCC	52	NM_010442
	R: AGCTCCTCAAACAGCTCAATGT		
Nppa	F: GATCTGATGGATTTCAAGAACCTG	65	NM_008725
	R: ACCTCATCTTCTACCGGCATC		
Nppb	F: TTTGGGCTGTAACGCACTGA	113	NM_001287348
	R: CACTTCAAAGGTGGTCCCAGA		
Myh7	F: CCTGCTGTTTCCTTACTTGCT	84	NM_080728
	R: CCAGGCCTGTAGAAGAGCTGTA		
Tnnt	F: AGCCCACATGCCTGCTTAAA	115	NM_011619
	R: TCTCGGCTCTCCCTCTGAAC		
Gpx4	F: CATTGGTCGGCTGCGTGAG	80	NM_008162
	R: TTAAGTAAGCGGCTCAGACGG		
Ptgs2	F: TGAGTACCGCAAACGCTTCT	74	NM_011198
	R: CAGCCATTTCCTTCTCTCCTGT		
Hprt1	F: CAGTCCCAGCGTCGTGATT	138	NM_13556
	R: GCAAGTCTTTCAGTCCTGTCCAT		
Actb	F: CCTTCTTGGGTATGGAATCCTGT	86	NM_007393
	R: GAGGTCTTTACGGATGTCAACG		

bp indicates base pairs; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

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dose of HBI-002 (10 mL/kg; cumulative dose of 20 mL/kg), which resulted in higher carboxyhemoglobin levels (7.4±1.4%) within 10 minutes postgavage (Figure 2C). HBI-002 presented a half-life of ≈30 minutes, similar to studies involving iCO.[38,39] To test if oral HBI-002 dosage also resulted in effective tissue distribution with increased CO concentrations in the heart, perfused heart tissue samples were collected 10 minutes after gavage. Similar to carboxyhemoglobin levels, the CO concentrations, determined by gas chromatography, rose from 7.0±0.2 pmol CO/mg to 31.6±4.6 pmol CO/mg (Figure 2D). Collectively, these results show that HBI-002 is a reliable method for oral delivery of CO that results in rapid diffusion into the blood and cardiac tissue with minimal impact on animal body mass and heart EF.

HBI-002 Treatment Prevents Doxorubicin-Induced Cardiotoxicity in Mice

Exogenous administration of CO or induction of endogenous CO by increased HO-1 activity is potently cardioprotective.[15,21,24] As an example of the importance of HO-1 in the protection of the heart, transgenic expression of human HO-1 in pigs confers resistance to xenograft rejection,[40] brain injury,[41] doxorubicin cardiotoxicity,[27] and ischemic heart disease.[42] We and others have shown the cytoprotective benefits of CO[22,43,44,45] and the effect of low-dose CO to protect cardiomyocytes from cell death and maintaining overall cardiovascular health.[16,18,24,46] We tested the ability of HBI-002 administration and release of CO to upregulate HO-1 expression in the heart and observed a nearly 4-fold induction in HO-1 mRNA and 2-fold increase in HO-1 protein compared with vehicle control-treated mice (Figure 2E and 2F).

Common biomarkers used for diagnosing myocardial injury are creatine kinase, lactate dehydrogenase, and cTnI.[27,47] Administration of doxorubicin to mice rapidly and reproducibly increases cardiac injury markers in both blood (Figure 3A and 3B) and tissue. Given previous reports that iCO can protect against doxorubicin toxicity, we tested whether HBI-002 given before doxorubicin would attenuate the increase of these markers. We

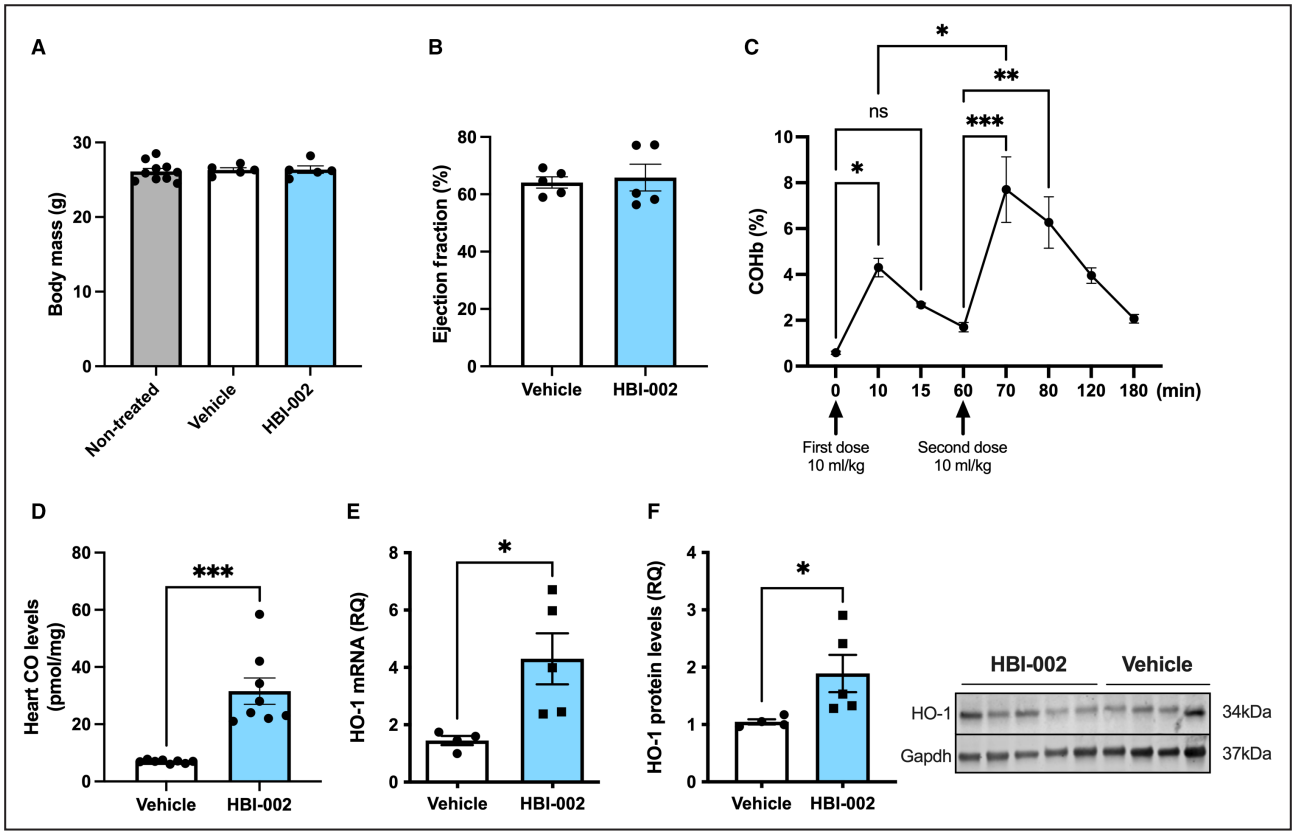

Figure 2. HBI-002 delivers CO and shows no adverse effects on body mass or cardiac function.
Body mass (**A**) and ejection fraction (**B**) measured by echocardiography in healthy mice after 2 weeks of daily HBI-002 (20 mL/kg per day) or vehicle dosing (5–10 mice per group). **C**, Kinetic of carboxyhemoglobin levels after the first and second doses of HBI-002 (20 mL/kg per day). Data represent mean±SEM at each time point; **P*<0.05, ***P*<0.01, ****P*<0.001; 4 mice per time point. **D**, Heart CO concentrations (pmol/mg) were measured by gas chromatography in samples collected 15 minutes after HBI-002 or vehicle administration (8 mice per group). HO-1 mRNA expression (**E**) and protein expression with representative immunoblots (**F**) in the heart after 2 weeks of daily HBI-002 administrations (4–5 mice per group). Mean±SEM; **P*<0.05, ***P*<0.01, ****P*<0.001. CO indicates carbon monoxide; COHb, carboxyhemoglobin; HBI-002, oral liquid product containing carbon monoxide; HO-1, heme oxygenase-1; ns; no significant difference; and RQ, relative quantification.

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observed that HBI-002 effectively prevents doxorubicin-induced elevations in creatine kinase and lactate dehydrogenase (Figure 3C and 3D), within 1 hour after doxorubicin administration and attenuates higher cTnI levels after 7 days (Figure 3E). We next evaluated cardiac function before and 7 days after acute doxorubicin administration, and in agreement with the serum markers, HBI-002 attenuated doxorubicin-elicited deterioration in cardiac function as shown by a significant decrease in fractional shortening and EF when compared with vehicle controls (Figure 3F and 3G). Finally, we evaluated the expression of heart stress markers, including β-MyHC, a known upregulated gene in conditions such as cardiac failure or hypertrophy,[48,49] and *TnnT*, a myocardial injury biomarker,[50] following doxorubicin treatment, both in the presence and absence of HBI-002. We found that HBI-002 prevented doxorubicin-induced upregulation of β-MyHC and *TnnT* mRNAs, compared with vehicle-treated controls (Figure 3H). Collectively, the reduction in serum and cardiac tissue markers associated with HBI-002 administration was coupled with an increased survival rate (Figure 3I), showing that HBI-002 given before a high dose of doxorubicin protects the heart and increases survival rate.

HBI-002 Attenuates the Chronic Cardiotoxic Effects of Doxorubicin

Motivated by the acute doxorubicin findings, we next tested the effects of HBI-002 in a doxorubicin regimen

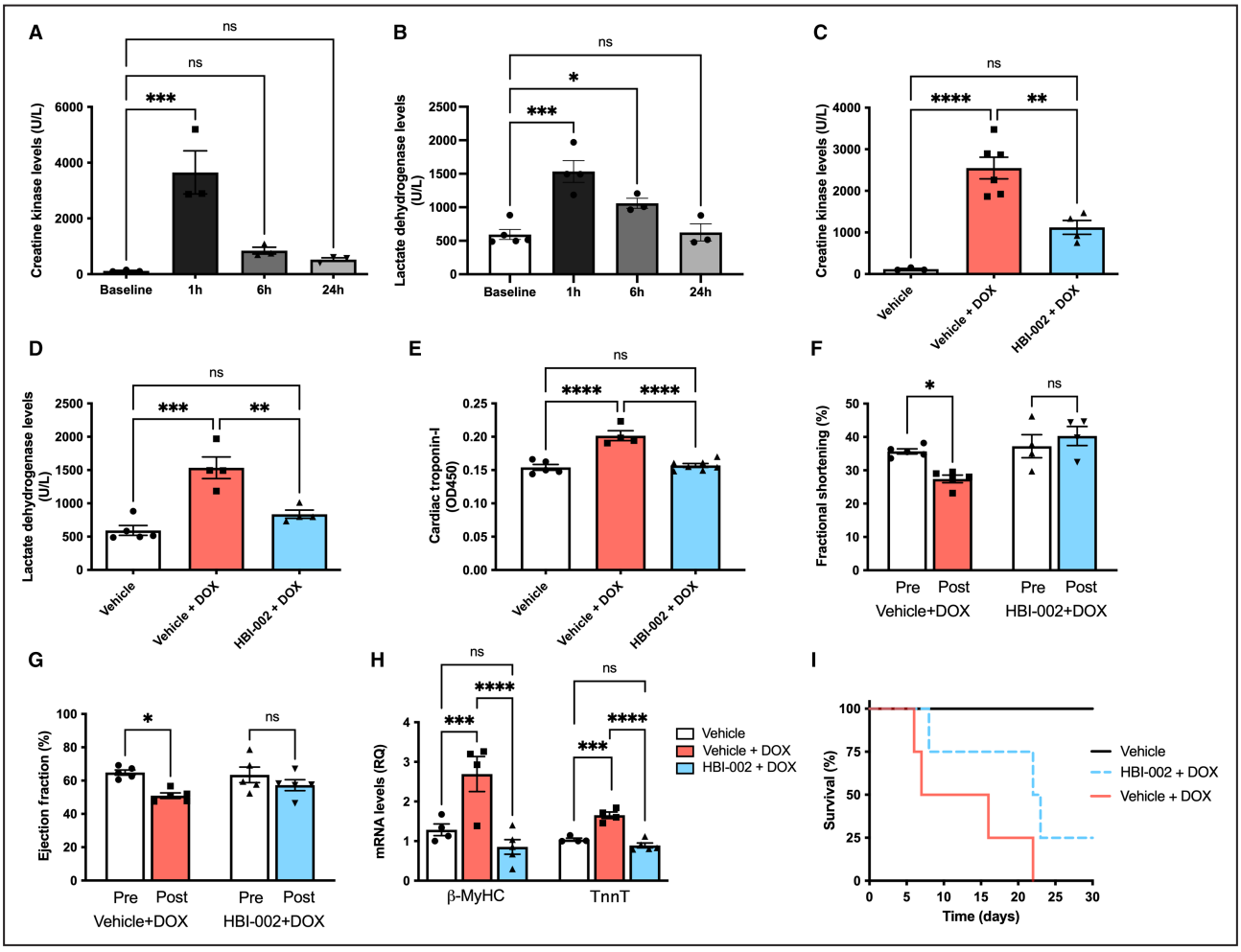

Figure 3. HBI-002 prevents acute doxorubicin toxicity.
Serum creatine kinase (**A**), and lactate dehydrogenase (**B**) levels at different time points after acute doxorubicin injection (20 mg/kg per IP). Data represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs baseline; 3 to 5 mice per time point. HBI-002 attenuated doxorubicin-induced increases in serum creatine kinase (**C**), lactate dehydrogenase (**D**), and cTnI (cardiac troponin-I) levels (**E**) 1 hour after a single dose of doxorubicin (20 mg/kg, IP) for creatine kinase and lactate dehydrogenase, and 7 days for cTnI. HBI-002 prevented doxorubicin-induced cardiac dysfunction after 7 days, evidenced by less fractional shortening (**F**) and ejection fraction (**G**). **H**, HBI-002 decreased doxorubicin-induced upregulation of the cardiac dysfunction markers β-MyHC and TnnT. **I**, HBI-002 improved overall survival. Data represent mean±SEM; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; 4–7 mice per group. DOX indicates doxorubicin; β-MyHC, beta-myosin heavy chain; HBI-002, oral liquid product containing carbon monoxide; ns, no significant difference; RQ, relative quantification; and TnnT, cardiac troponin T.

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in mice that recapitulates chronic cardiomyopathy observed in patients.[35] Treatment with this doxorubicin regimen (10 doses at 2 mg/kg, IP) resulted in a decrease in EF (29.6%) and fractional shortening (31.9%), as assessed by echocardiography 3 and 9 weeks following the initial doxorubicin dosing (Figure 4A and 4B). At the conclusion of doxorubicin dosing, doxorubicin-treated mice displayed the prototypical cardiac remodeling, evidenced by decreases in both interventricular septum thickness in systole and LV posterior wall thickness at systole, and increases in both LVID systole and LV end-systolic volume. In contrast, animals treated with daily dosing of HBI-002 (see Figure 1) showed significant attenuation in the elevation of the LVID systole and LV end-systolic volume (Table 2). In addition, molecular markers of cardiac dysfunction showed that HBI-002 prevented doxorubicin-induced upregulation in the expression of the cardiac stress-response markers, including ANP, BNP, and β-MyHC (Figure 4C).[48,49] Qualitative analysis of histopathologic changes in the heart in response to chronic doxorubicin administration showed that HBI-002 given before doxorubicin attenuated doxorubicin-induced cardiomyopathy, as reflected in less inflammatory cell infiltration and deposition of fibrotic tissue (Figure 4D). Moreover, considering the well-characterized effects of doxorubicin on mitochondrial dysfunction,[3,27,47,51] we found that doxorubicin downregulates protein expression of all mitochondrial complexes, and HBI-002 prevented this decrease (Figure S1).

Effects of HBI-002 on Doxorubicin-Induced Cardiotoxicity in the Presence of a Tumor Burden

Breast and lung tumor incidence represents a significant proportion of total cancers seen clinically, and treatment of these patients continues to depend on traditional chemotherapies. Thus, to further assess the benefits of HBI-002 in protecting the heart, we next evaluated the role of HBI-002 in combination with doxorubicin in the context of murine Lewis lung carcinoma (LL/2) and 4T1 mammary tumors. Animals harboring a tumor burden were exposed to a regimen of HBI-002 (20 mL/kg, p.o.) ±doxorubicin (injected 10 minutes after HBI-002 treatment). Doxorubicin administration was highly effective at reducing LL/2 lung tumor growth as expected, and importantly, this effect on tumor growth was not influenced by the administration of HBI-002 (Figure 5A and 5B). HBI-002 decreased doxorubicin-induced increases in ANP, BNP, and β-MyHC compared with vehicle controls (Figure 5C). In contrast, HBI-002 enhanced the sensitivity of the 4T1 tumor to doxorubicin (Figure 5D and 5E). This enhanced benefit of CO is similar to our previous report that showed that iCO sensitized tumors to doxorubicin nearly 1000-fold.[17,52] Importantly, and in agreement with the LL/2+doxorubicin model, HBI-002 also protected the heart against doxorubicin toxicity in the presence of a 4T1 tumor burden, preventing β-MyHC upregulation and improving cardiac function (Figure 5F and 5G). These results strongly support the use of HBI-002 to deliver CO at low doses of CO that elicit cardioprotective effects and, in certain cancers, simultaneously enhance the chemotherapeutic benefit of doxorubicin.

HBI-002 Attenuates the Expression of Markers of Doxorubicin-Induced Ferroptosis

Although the cytoprotective mechanism of action of HBI-002 and CO remains unclear, CO has been shown to prevent normal cell death.[17,18,24] It has been suggested that ferroptosis, a recently discovered form of programmed cell death, plays a crucial role in the development of anthracycline-induced cardiomyopathy.[53] Ferroptosis is driven in large part by the excessive accumulation of prooxidant iron in the cell, which leads to lipid peroxidation and protein damage. In response to doxorubicin, there is a significant increase in HO-1 expression in cardiomyocytes and the heart, suggesting that one mechanism by which iron accumulates in the cell is through HO-1-mediated catalysis of heme.[53] We observed that HBI-002 reduced HO-1 upregulation in cardiac tissue when compared with doxorubicin treatment with vehicle (Figure 6A). We next analyzed the expression in the heart of prostaglandin-endoperoxide synthase 2 (Ptgs2/COX2), a biomarker of ferroptosis,[54] and glutathione peroxidase 4 (Gpx4), an antioxidant enzyme and key regulator of ferroptosis,[55] after a single doxorubicin injection. We found that doxorubicin promoted an increase in the expression of Ptgs2/COX2 mRNA levels and decreased the expression of Gpx4 mRNA ($P<0.01$). HBI-002 treatment abrogated the doxorubicin-induced increase of Ptgs2/COX2 and prevented the Gpx4 downregulation (Figure 6B). These results could suggest that HBI-002 protects against doxorubicin-induced cardiac injury, in part by reducing ferroptosis.

DISCUSSION

Although the pharmacologic effects of CO are investigated with extensive data sets from numerous laboratories and animal models,[56] a key issue remaining is the most effective, safe, and simple modality by which the gas can be delivered for therapeutic purposes.[21,57] Before 2019, there were extensive efforts in developing metal-based CO-releasing molecules[14,15] and organic donors that release CO upon exposure to light.[21,57] Although these CO donors hold promise for patients,

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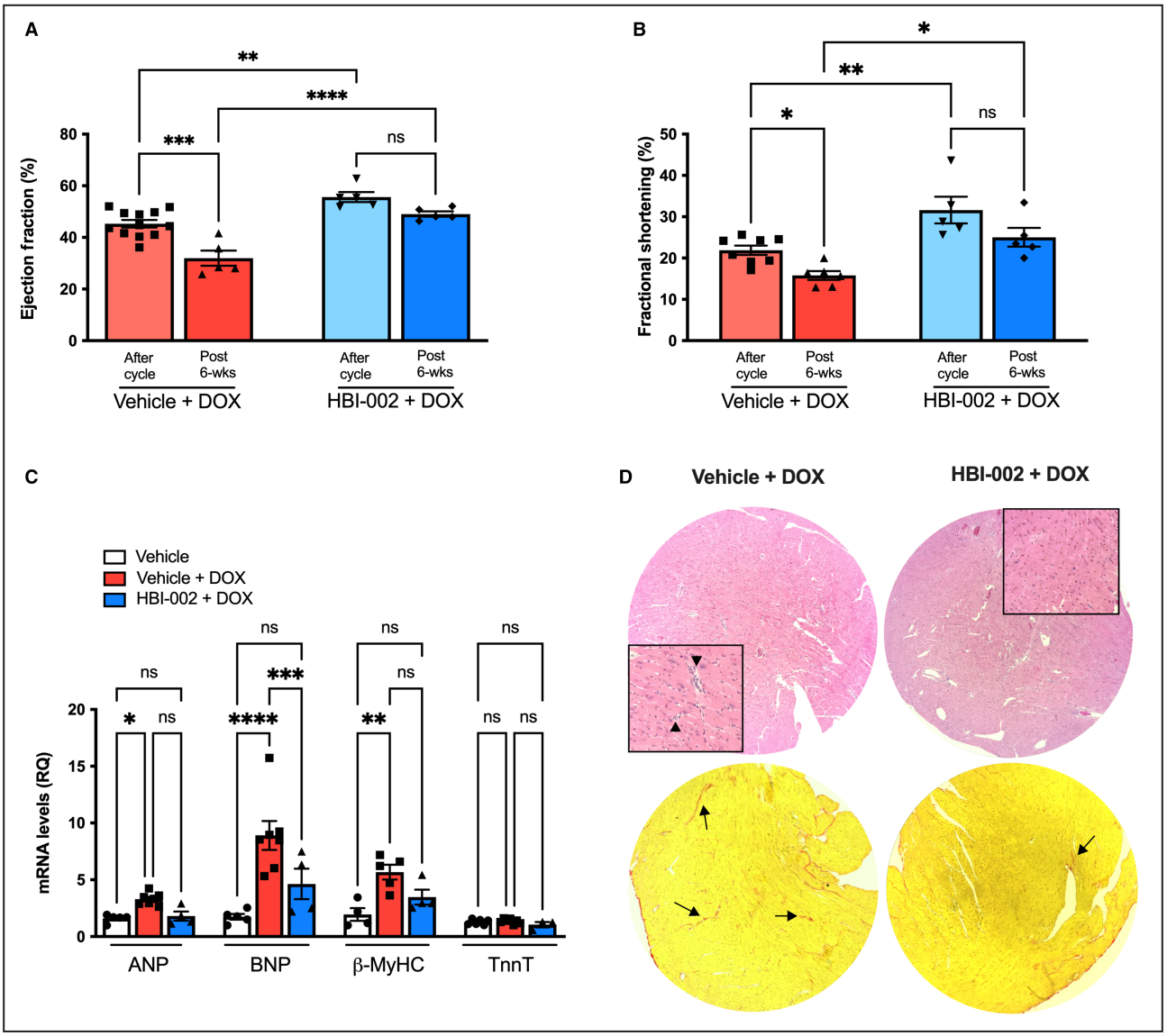

Figure 4. HBI-002 attenuates chronic cardiotoxicity caused by doxorubicin.
HBI-002 (20 mL/kg, PO daily) before doxorubicin (2 mg/kg, IP) administration prevented a reduction in ejection fraction (**A**) and fractional shortening (**B**) compared with vehicle controls measured at 3 weeks (after cycle) and 9 weeks (post 6-wks) post initiation of doxorubicin treatment (5–12 mice per group). **C**, HBI-002 prevented the upregulation of cardiac dysfunction markers after 8 weeks of doxorubicin initiation (4–7 mice per group). **D**, Representative images and qualitative analyses of hematoxylin and eosin and Sirius red staining of heart sections from vehicle+doxorubicin and HBI-002+doxorubicin-treated mice 8 weeks after doxorubicin initiation (3 mice per group, magnification images are ×400, and insets are 400% larger). Hematoxylin and eosin insets show inflammatory cell infiltration (arrowhead). Accumulation of Sirius red positive staining indicative of fibrotic tissue (arrow). Cardiac dysfunction markers: ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), β-MyHC (cardiac beta-myosin heavy chain). Data represent mean±SEM; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; 4 to 12 mice per group. DOX indicates doxorubicin; HBI-002, oral liquid product containing carbon monoxide; ns, no significant difference; RQ, relative quantification; and TnnT, cardiac troponin T.

they have not yet demonstrated suitability for long-term, chronic administration, and their development status has not yet advanced to human trials.[30,31,58,59] Similarly, iCO has met significant challenges related to the consistent ability to dose accurately, reliance on compressed gas cylinders, hospital-only administration, and genuine concern of inadvertent exposure by hospital personnel responsible for its administration. Here, we present HBI-002, a novel oral liquid product containing CO and generally regarded as safe excipients designed to overcome the challenges of dosing and safety barriers associated with iCO and small metal gas carriers. This study shows that HBI-002 is well tolerated in murine cancer models, has sound pharmacokinetic/dynamic attributes, and is bioavailable (Figures 2 and 3). HBI-002 delivers low amounts of CO primarily

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Table 2. Cardiac Structure Parameters 8 Weeks After Initiation of Doxorubicin and HBI-002 Therapy

	Vehicle	Vehicle+doxorubicin	HBI-002+doxorubicin
IVSs, mm	1.37±0.04	1.06±0.06†	1.00±0.15†
IVSd, mm	0.88±0.04	0.78±0.05	0.70±0.10
LVIDs, mm	2.19±0.15	2.73±0.11*	1.95±0.15§
LVIDd, mm	3.54±0.25	3.50±0.11	3.10±0.15
LVPWs, mm	1.35±0.05	0.93±0.07‡	0.88±0.11†
LVPWd, mm	0.95±0.07	0.73±0.05	0.69±0.09
LV Vol s, μL	17.01±2.6	28.36±2.80*	12.33±2.4§
LV Vol d, μL	48.23±7.0	51.34±4.1	42.18±2.0

Data represent mean±SEM. HBI-002 indicates oral liquid product containing carbon monoxide; IVSs and IVSd, interventricular septum thickness at systole and diastole, respectively; LV, left ventricle; LVIDs and LVIDd, LV inner dimensions at systole and diastole; LVPWs and LVPWd, posterior wall thickness at systole and diastole; LV Vol s and LV Vol d, left ventricular end-systolic and end-diastolic volume.

*P<0.05 vs vehicle.

†P<0.01 vs vehicle.

‡P<0.001 vs vehicle.

§P<0.01 vs vehicle+doxorubicin. n=5–8 mice/group.

through the gastrointestinal tract, resulting in highly reproducible amounts of CO in the blood and heart. CO is a highly diffusible molecule, and the carboxyhemoglobin levels depicted in Figure 2C resulted from the absorption in the gastrointestinal tract and binding/release from hemoglobin, with elimination exclusively occurring through the lungs. Notably, the observed half-life is similar to values reported in the literature for inhaled CO.[38,39] Rigorous nonclinical pharmacology and toxicology studies with HBI-002 have resulted in the completion of a Phase I US clinical study (www.clinicaltrials.gov, NCT03926819; unpublished results). The benefits of HBI-002 have previously been reported in models of sickle cell anemia and Parkinson's disease.[33,60] Although CO has been shown to be cardioprotective against acute doxorubicin toxicity (single acute dose),[15] we present a novel CO delivery modality that offers significant advantages. Most importantly, we show the first evidence of the efficacy of CO, through HBI-002, in the setting of a chronic doxorubicin protocol and associated toxicity and the presence of a clinically relevant tumor burden. Many other agents have been shown to mitigate the toxicity of doxorubicin, but this comes with the cost of decreasing effects on tumor burden.[13,61]

Tissue damage results in the release of danger-associated molecular pattern molecules, including mitochondrial fragments (DNA, formyl peptides) as well as heme. It has been recently reported that hemopexin, the heme-scavenging protein found in serum, can reduce doxorubicin cardiotoxicity, thus suggesting elevations in circulating heme are responsible for some of the damaging effects of doxorubicin.[62] It is crucial to remove heme to prevent the accumulation of iron that can lead to inflammation and free radical–driven ferroptosis. Multidose of HBI-002 upregulates HO-1 expression in the heart and, when administered in combination with doxorubicin, attenuates tissue injury, pathologic cardiac remodeling, dysfunction, and mortality (Figures 3 and 4). Further, CO delivered by HBI-002 prevented a decrease in the mitochondrial complex expression in the heart after 8 weeks of chronic doxorubicin protocol (Figure S1), likely related to the maintenance of mitochondrial and cardiac function.[27] Although we observed changes in protein expression, further investigation is required to fully comprehend the impact of HBI-002 on mitochondrial dynamics, activity, and function. One unknown conundrum is whether the activities of HO-1 prevent or promote ferroptosis, a distinctive process marked by an excessive accumulation of free cellular iron, resulting in lipid peroxidation and heightened oxidative stress. Data presented here would argue that induction of HO-1 by doxorubicin would increase iron as heme is metabolized, contributing to the activation of ferroptosis. Simultaneously, HBI-002 protects the heart but increases HO-1. Our interpretation is that the timing of HO-1 induction is critical and that prophylactic induction leads to controlled iron release and appropriate accumulation into ferritin, whereas responsive induction, as observed with doxorubicin toxicity, either reflects overwhelming tissue damage or inappropriate timing of HO-1 upregulation and iron accumulation independent of HO-1 activity. Increasing HO-1 with HBI-002 before doxorubicin allows for the generation of protective molecules such as CO, ferritin, and the antioxidant bile pigments that likely result in the cardioprotection observed in this study.[63-65] Whether induction of HO-1 and endogenous generation of CO and exogenously delivered CO act synergistically to protect the heart is unclear and is the focus of ongoing studies. Regardless, even with our limited data on mRNA levels, a plausible mechanistic hypothesis regarding the CO effects on doxorubicin-induced ferroptosis begins to emerge. The CO delivered by HBI-002 treatment in conjunction with doxorubicin appears to thwart the downregulation of Gpx4 and the upregulation of Ptgs2 levels within the mouse cardiac tissue (Figure 6B). The importance of Gpx4 inactivating, an indispensable antioxidant enzyme responsible for countering lipid peroxides, is well established and is associated with the promotion of ferroptosis.[53,66] Furthermore, Ptgs2 is frequently regarded as an indicative marker downstream of ferroptosis.[67] These collective observations suggest that HBI-002 may have the potential to mitigate doxorubicin-induced ferroptosis.

Very few, if any, existing therapeutics have the plethora of protective effects seen with HBI-002-delivered CO in limiting cardiotoxicity while simultaneously enhancing the anticancer effects of doxorubicin. It is of utmost importance to develop novel strategies that

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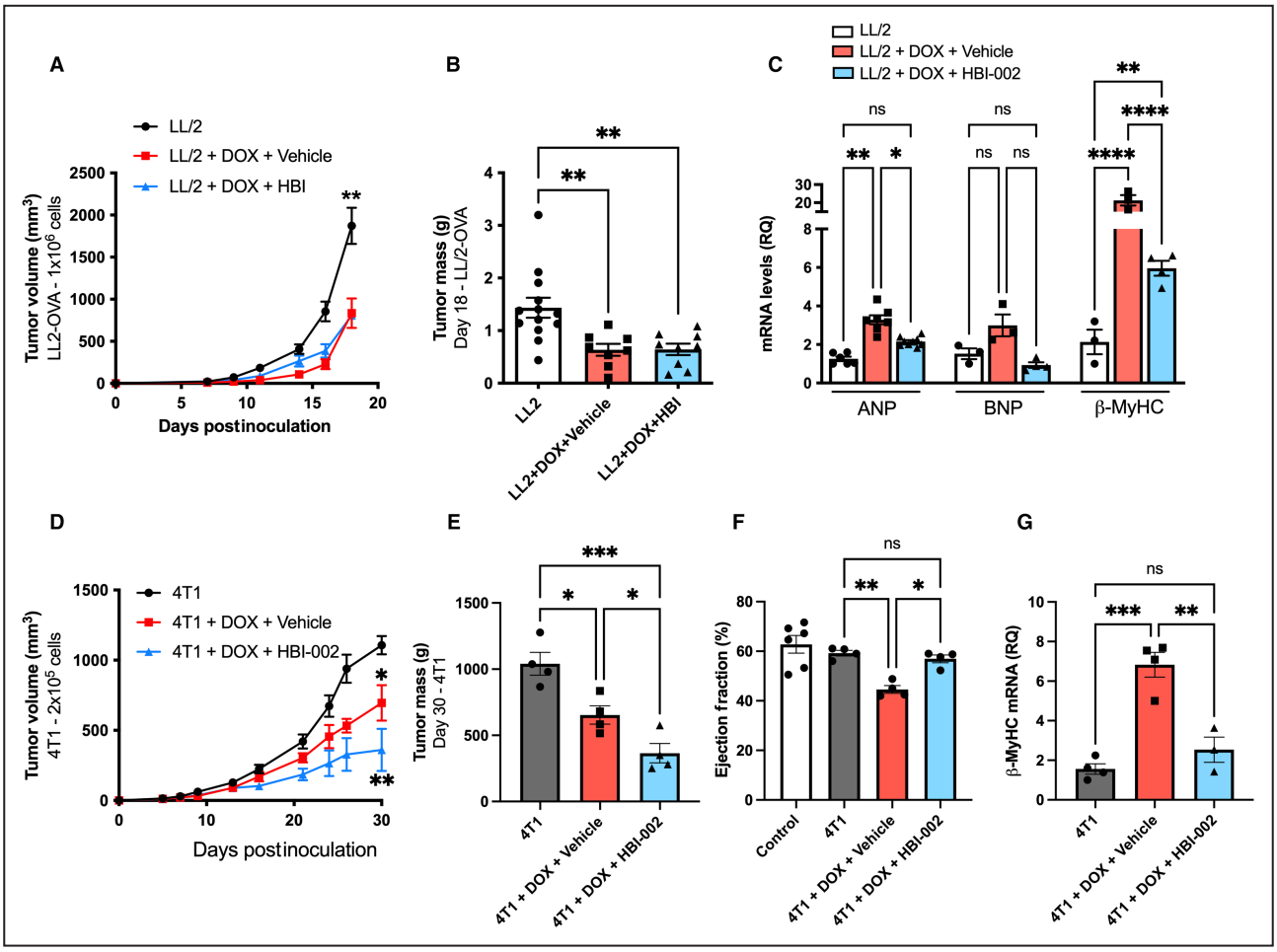

Figure 5. Effects of HBI-002 on doxorubicin toxicity in the presence of a tumor burden.
Measurements of LL/2 lung tumor volume (**A**) and mass (**B**) ±HBI-002 (20 mL/kg, PO) and ±doxorubicin. Tumor growth was reduced by doxorubicin, which was not affected by the presence of HBI-002. Results are mean±SEM of 8 to 13 mice per group. ***P*<0.01 vs LL/2+doxorubicin±HBI-002 group. **C**, HBI-002 attenuates doxorubicin-induced cardiac dysfunction markers in LL/2 tumor-bearing mice compared with vehicle-treated mice (3–9 mice per group). Measurements of 4T1 breast cancer tumor volume (**D**) and mass (**E**) ±HBI-002 and ±doxorubicin, as previously. Data represent mean±SEM. **P*<0.05, ***P*<0.01 vs 4T1 group. **E**, HBI-002 enhanced the antitumor effects of doxorubicin compared with vehicle control (4 mice per group). HBI-002 preserves ejection fraction (**F**) and prevents β-MyHC (cardiac beta-myosin heavy chain) upregulation (**G**) in response to doxorubicin therapy. Cardiac dysfunction markers: ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), β-MyHC (4 mice per group). All values are presented as mean±SEM; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. DOX indicates doxorubicin; HBI-002, oral liquid product containing carbon monoxide; LL/2, Lewis lung carcinoma; ns, no significant difference; and RQ, relative quantification.

not only safeguard the heart during cancer therapy but also maintain a neutral impact or, ideally, enhance the antitumor efficacy of the treatment. HBI-002 not only preserves the efficacy of doxorubicin in lung and mammary cancer models but also augments the sensitivity of cancer cells to doxorubicin in the 4T1 breast cancer model (Figure 5). Although we did not detect a significant impact of HBI-002 on the lung cancer model, possibly attributed to its rapid tumor growth compared with the mammary orthotopic model, the effects of HBI-002 on the mammary tumor model align with our previous findings, indicating that exposure to CO enhances the sensitivity of prostate cancer cells to doxorubicin, without affecting normal cells.[17] Notably, HBI-002 enhanced doxorubicin antitumor effects while protecting the heart, evident from preserved EF and reduced β-MyHC expression, a factor known to be upregulated in conditions such as cardiac failure or hypertrophy[48,49] (Figure 5). As such, the enhanced effects of the HBI-002 plus doxorubicin regimen on reduction in tumor size would argue for potentially instituting a chemosparing therapy and thus further reducing the adverse side effects of doxorubicin and potentially other chemotherapeutics.

CONCLUSIONS

In summary, we show that CO administered orally via HBI-002 is bioavailable at low levels of carboxyhemoglobin and has similar cardioprotective effects in mice as reported with iCO and CO-releasing

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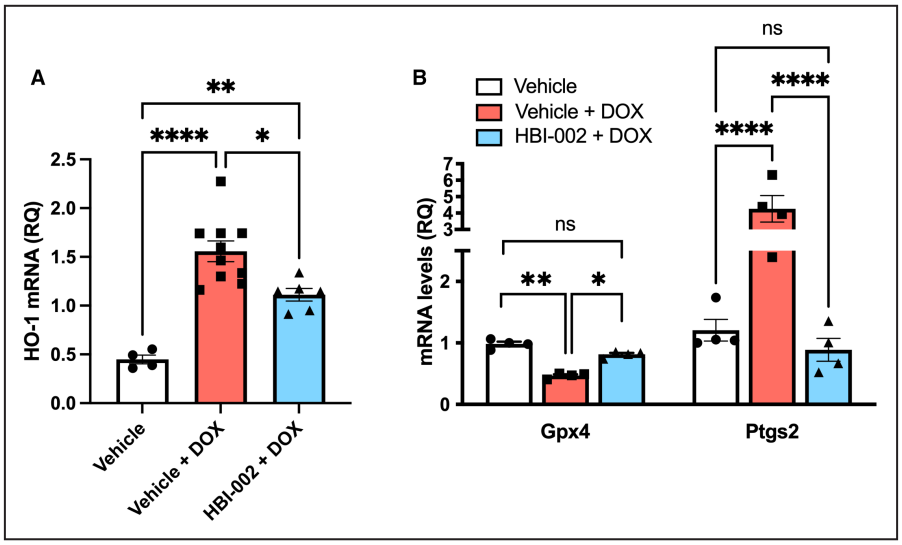

Figure 6. HBI-002 attenuates the expression of markers of doxorubicin-induced ferroptosis.
A, HO-1 mRNA expression in the ventricles of mice after 8 weeks of chronic doxorubicin regimen (4–10 mice per group). **B**, Glutathione peroxidase 4 (Gpx4) and prostaglandin-endoperoxide synthase 2 (Ptgs2) mRNA expression in the ventricle after 24 hours and 7 days, respectively, of a single doxorubicin administration ±HBI-002 (4 mice per group). Expression values were determined by reverse transcription quantitative polymerase chain reaction and normalized to Hprt1. Results represent mean±SEM; **P*<0.05, ***P*<0.01, *****P*<0.0001. DOX indicates doxorubicin; HBI-002, oral liquid product containing carbon monoxide; HO-1, heme oxygenase-1; ns, no significant difference; and RQ, relative quantification.

molecules.[15,24,27,65,68] Importantly, CO is cardioprotective while inhibiting tumor growth in the same animal. We speculate that CO may have pleiotropic effects on cellular mitochondria[69,70] and likely regulates a heme-TLR4 (t oll-like receptor 4) signaling axis in a manner that reduces injury and inflammation.[71,72] These data provide the first evidence showing the potential for safe, low amounts of CO delivered via an oral liquid drug product containing CO that can be used as an adjuvant therapeutic option to limit acute and chronic cardiomyopathy in patients with cancer under anthracycline therapy. Given that HBI-002 has completed a phase I trial, it could be available in the short term for further study in controlled delivery amounts, thus allowing for clinical research in patients with cancer at risk for developing chemo-induced cardiac injury.

ARTICLE INFORMATION

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Drs Alves de Souza, Gomperts, Young, and Otterbein conceived and designed this study. Moreover, Drs Alves de Souza and Voltarelli, with the help of David Gallo and Sidharth Shankar, carried out animal procedures and in vivo experiments. Drs Alves de Souza, Voltarelli, and Tift performed laboratory experiments and analyzed the data. At the end, Drs Alves de Souza and Otterbein wrote the paper.

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Disclosures

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Supplemental Material

Figure S1

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