SUPPLEMENTAL MATERIAL

1) Supplemental Methods:

Stability and Solubility of CXL-1020

The stability and equilibrium solubility of CXL-1020 in aqueous solution was evaluated in water for injection (WFI), 5 % dextrose (D5W), 0.9 % saline, and in 100 mM citrate buffer (pH 4.0, 4.5. and 5.0). Quantities of CXL-1020 were dispensed into microcentrifuge tubes and the vehicle of interest was added to target a nominal CXL-1020 concentration of approximately 25 mg/mL. The samples were rotated at ambient laboratory conditions over 48 hours. Sample pH was recorded at each time point (2, 24, and 48 hours) and the samples were centrifuged to pellet undissolved solids prior to analysis. CXL-1020 is soluble up to concentrations of at least 30 mg/mL when prepared in an approximately 1:1 molar ratio with the β-cyclodextran, Captisol®. A solution of Captisol (150 mg/mL) was prepared in WFI and the pH was adjusted to 4.0. CXL-1020 (15 mg/mL) was added and the solution was stirred until homogeneous and the pH was readjusted to 4.0. The stability of this stock solution was evaluated. Aliquots of this solution were diluted into D5W and 0.9% saline to a concentration of 1.5 mg/mL CXL-1020 and the stability of these solutions was also evaluated. The concentration and purity of all samples were assessed by RP-HPLC using a UV absorbance detector at 272 nm using a step gradient of aqueous acetonitrile containing 0.1 % (v/v) formic acid. The standard curve of CXL-1020 concentration vs peak area (272 nm) was linear from 5 μg/mL to 225 μg/mL. Purity of CXL-1020 samples was approximated by percent peak area, defined as percent of CXL-1020 peak area versus the total area of peaks detected in the chromatogram.

The solid state stability of CXL-1020 was assessed by storing samples of CXL-1020 under anhydrous conditions at -20° C. At 2 month intervals samples were thawed, weighed, dissolved

in aqueous acetonitrile containing 0.1% (v/v) formic acid and analyzed by RP-HPLC as described above. CXL-1020 was stable (>99.5%) for 2 years at -20° C (longest time point tested).

Decomposition of CXL-1020 in Phosphate Buffered Saline (PBS)

The half-life for decomposition of CXL-1020 and concurrent formation of HNO and the byproduct CXL-1051 was measured in PBS. HNO rapidly dimerizes in aqueous solution. HNO production is measured indirectly via gas chromatography headspace analysis by quantifying nitrous oxide, the end product of dimerization reaction. A 20 mg/mL stock solution of CXL-1020 was prepared in dimethylformamide (DMF). Aliquots (50 μL) were transferred into 20 mL headspace vials containing 5.0 mL of PBS. At time points (0, 1, 2, 4, 8, 15, 30, 90 min) the reactions were quenched by addition of 100 mL of formic acid (an amount that drops the pH of a 5 mL sample solution to between pH 2 and 3). The amount of nitrous oxide formed was measured by gas chromatography against bracketing external standards and 1.5 mL samples of the solution were transferred to HPLC auto-sampler vials and the amount of CXL-1020 and CXL-1051 present was analyzed by RP-HPLC against bracketing external standards which gave a %RSD (n=4) for the peak area of <1.0%.

HPLC Assay

The HPLC conditions qualified for analysis of CXL-1020 are summarized in Supplemental Table 1 and a representative chromatogram at the target assay concentration of 0.100 mg/mL is displayed in Supplemental Figure 1. The HPLC method was evaluated over the concentration

range 0.005 – 0.225 mg/mL of CXL-1020 and demonstrated to be highly linear (Supplemental Figure 2).

Decomposition of CXL-1020 in Human Whole Blood

The half-life for decomposition of CXL-1020 and concurrent formation of the byproduct CXL-1051 was measured in human whole blood. Because HNO react with thiols, it's formation could not be quantitatively measured. A 15 mg/mL (w/v) stock solution of CXL-1020 was prepared in 15% (w/v) Captisol in WFI, pH 4.0. Aliquots (67 μL) were added to 1 mL samples of K_2EDTA-treated whole human blood pre-incubated at 37° C. At time points (0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 7, 10 min) the reactions were quenched by addition of 100 μL of 0.5M citric acid. The blood samples were stored on an ice block and centrifuged at approximately 4°C to obtain plasma. Samples were centrifuged within 15 minutes of collection. From each sample, exactly 150 μL of plasma was transferred to each of two tubes containing 15 μL of 10% formic acid. Each blood sample yielded duplicate plasma samples. All samples were stored frozen at approximately -70°C in tightly capped, pre-labeled polypropylene tubes/vials until analysis. Samples for analysis were thawed, and aliquots of plasma were transferred to auto-sampler vials. Internal standard [^{13}C]CXL-1020 or [^{13}C]CXL-1051 was added and the samples were analyzed by LC-MS-MS.

Isolated Myocyte Studies

Adult cardiac myocytes were freshly isolated from male C57BL/6J mice, 2-6 mo. old. (Jackson Laboratory, ME) with either normal or failing hearts. The latter was generated by 9-week exposure to pressure overload induced by trans-aortic constriction (27G needle to

size the suture constraint). At this time, ventricular hypertrophy was 100-150% over baseline, and the chambers markedly dilated, as reported[1]. Myocytes were isolated following rapid cardiac excision, and aortic root retroperfusion at 100 cmH_2O and 37ºC for ~3 min with a Ca^{2+}-free bicarbonate-based buffer containing (in mM) 120 NaCl, 5.4 KCl, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 1.6 $MgCl_2$; glucose (1 mg/ml), 2,3-butanedione monoxime (BDM, 1 mg/ml), and taurine (0.628 mg/ml), gassed with 95% O_2-5% CO_2. Enzymatic digestion was initiated by addition of 0.9 mg/ml collagenase type 2 (Worthington Biochemical Co., 299 U/mg) and 0.05 mg/ml protease type XIV (Sigma Chemical Co.) to the perfusion solution (6-7 min).

Dispersed myocytes were filtered through a 150 μm mesh and gently centrifuged at 500 rpm for 30 seconds. The pellet was re-suspended in Tyrode's solution with increasing Ca^{2+} (1 mM), and cells then incubated for 10 min with 3 μmol/l Fura 2-AM (Invitrogen, Molecular Probes, Carlsbad CA) in Tyrodes (1 mM Ca^{2+}). After rinsing, cells were placed in a perfusion chamber with a flow-through rate of 2 ml/min, and sarcomere length and whole cell Ca^{2+} transients recorded using an inverted fluorescence microscope (Nikon, TE2000), and IonOptix (Myocam®) software. Twitch amplitude is expressed as a percentage of resting cell length. Twitch kinetics was quantified by measuring the time to peak shortening and the time from peak shortening to 50% relaxation.

Effects of HNO donated by CXL-1020 and its pro-moiety (CXL-1051) as a negative control, on myocyte function was determined in concentrations ranging from 50 to 500 μM, at room temperature. CXL-1020 and CXL-1051 were dissolved in DMSO generating a stock solution of 100 mM, and then diluted to the target concentration immediately prior to each experiment. To test if these effects were cAMP or cGMP dependent, sarcomere shortening was measured in the presence of 100uM Rp-cAMPs (100 mM stock in Tyrode's solution) or 1H-

[1,2,4]Oxadiazolo quinoxalin 1-one (ODQ), 10uM (10 mM stock solution in 100% DMSO) to block protein kinase A (PKA) and soluble guanylyl cyclase (sGC) signaling, respectively. The 0.05-0.5% DMSO vehicle has no independent impact on myocyte function.

For each experimental protocol, at least 10 cells from more than 3 animals were used.

Mouse Heart Failure Model:

C57Bl/6J mice were subjected to trans-aortic constriction using a 27G needle to size the suture constraint. After surgery, animals were followed for 9 weeks during which time chambers both hypertrophied (100-150% over baseline) and chambers dilated, as previously reported[1]. Myocytes were isolated following rapid cardiac excision, and perfusion in a Langendorff-type preparation with collagenase-containing buffer solution as described[2]

Canine Microembolization Heart Failure Model

Heart failure was produced using healthy, conditioned, purpose-bred mongrel dogs (20-26 kg) using a sequential microembolization model as reported[3-5]. Coronary microembolization is performed until LV-ejection fraction (determined angiographically under anesthesia) was ~30% or lower. Two weeks were then provided after the last microembolization to ensure stabilization of the model prior to initiating experiments. An initial dose-finding study (2-100 μg/kg/min for 40 min) was performed in 3 dogs to identify therapeutically relevant doses of CXL-1020. Based on these data, a primary group of six animals were studied, receiving 3 or 10 μg/kg/min over a 4 hour period, followed by one hour washout. Only one dose was studied on a given day, the other at least one-week later, and the order randomized. Hemodynamic, ventriculographic, and echocardiographic measurements were made during left and right heart catheterizations in

anesthetized dogs (induction: hydromorphone (0.22 mg/kg i.v.) and diazepam (0.17 mg/kg iv), maintenance: 1-2% isofluorane). LV end-systolic (ESV) and end-diastolic volumes (EDV) were calculated from ventriculograms using the area-length method. Peak aortic blood velocity was obtained in the ascending aorta using flow Doppler for measurements of peak power index (PPI). LV fractional area of shortening (FAS), was measured from LV short axis view at the level of papillary muscles obtained from 2-dimensional echocardiograms. Measured indexes of LV diastolic function included deceleration time of mitral inflow velocity (DT), ratio of the integral of early mitral inflow velocity (Ei) to velocity during atrial contraction (Ai) (Ei/Ai) and LV end-diastolic circumferential wall stress (EDWS).

Measurements of myocardial oxygen consumption were performed at baseline and at 4 hours after 10μg/kg/min infusion using previously described methods[6]. Briefly, arterial and coronary sinus blood samples were simultaneously drawn at baseline and at the end of each study time point. Oxygen content was determined with a hemoximeter. Coronary artery blood velocity was measured with a Doppler flow velocity wire placed in the left circumflex coronary artery proximal to the first marginal branch or in the left anterior descending coronary artery just proximal to the first diagonal branch. Blood flow was estimated by calculating the cross-sectional area of the coronary artery at the site of the velocity measurement from coronary angiography. Total coronary blood flow was assumed to be twice the flow measured in the circumflex or left anterior descending coronary artery. MVO2 was calculated as the product of total coronary blood flow and the oxygen content difference between arterial and coronary sinus blood.

Canine Tachycardia-pacing model

Full details of the model and instrumentation have been reported previously[7]. Heart failure was induced by RV ventricular pacing at 240 bpm for 3 weeks. Data were measured in chronically instrumented dogs in the conscious state. Instrumentation included 3 pairs of sonomicrometers to determine septal/lateral, anterio/posterior, and base/apex dimension, with LV volume calculated by: $V = \pi/6 \bullet d_{sl} \times d_{ap} \times d_{ba}$. An apical micromanometer (Konigsberg, CA) was used to measure LV pressure. Pressure-volume relations were determined before and during transient occlusion of the inferior vena cava by inflation of an implanted perivascular cuff. Indwelling venous catheters provided ports for drug infusion, and an aortic catheter facilitated calibration of the micromanometer. Calculation of end-diastolic volume was from the mean of volumes determined during mid-isovolumic contraction, stroke volume was determined by the mean width of the PV loop measured at mean systolic pressure, and power Index was calculated from the peak value of d(Volume)/dt × LVP/EDV^2, end-systolic elastance from perpendicular regression of the collection of end-systolic pressure-volume points (Pes/[Ves-Vo]), using an iterative method to determine Vo, and preload recruitable stroke work (PRSW) from linear regression of PV loop area (stroke work) versus EDV for multiple cycles measured during preload reduction.

Studies were performed at initial baseline (control state), and after induction of heart failure (i.e. 3 weeks later). Vehicle control was used for the zero-drug dose, and the study performed as a dose escalation study, with each dose allowed to stabilize for 10 minutes before increasing to the next level. Baseline hemodynamics at baseline and after induction of heart failure are provided in Supplemental Table 1.

2) Supplemental Tables

Supplemental Table 1

Conditions used for HPLC Analysis of CXL-1020 and metabolite.

CXL-1020 Analytical Conditions			
Column	Phenomenex Luna C18(2), 3 µm, 150 x 4.6 mm,		
Mobile Phase A	0.1 % (v/v) Formic acid in water		
Mobile Phase B	0.1 % (v/v) Formic acid in ACN		
Diluent	50/50/0.1 (v/v) Water/ACN/Formic acid		
Pump Gradient Program	Time (min)	% A	% B
	0	70	30
	7	60	40
	10	10	90
	12	10	90
	12.1	70	30
	15	70	30
Injection Volume (µL)	20		
Flow Rate (mL/min)	1.00		
Detection Wavelength (nm)	272		
Column Temperature	35		
Sample Temperature	5		
Retention Time (min)	4.2		
Run Time (min)	15		

Supplemental Table 2: Baseline Hemodynamics in Tachycardia-Pacing Canine HF Model

	Baseline	Heart Failure	p-value
Heart Rate (min^{-1})	100.6±4.1	142.7±10.2	0.01
End-systolic Pressure (mmHg)	118.4±8.0	103.0±7.2	NS
End-diastolic Pressure (mmHg)	8.0±2.6	26.6±2.4	<0.01
dP/dt_{max} (mmHg/sec)	1916.3±93.4	1174±139	<0.01
dP/dt_{min} (mmHg/sec)	-1405±96.6	-1344±76	NS
End-diastolic volume (mL)	56.3±8.6	85.5±14	0.07
End-systolic volume (mL)	33.7±5.5	66.8±12.1	<0.05
Maximal LV Power/EDV^2 (PmxI) (mmHg/mL*sec)*100	1160±200	470±150	<0.01
End-systolic Elastance (mmHg/mL)	6.4±1.1	2.5±0.5	<0.05

p-values based on paired T-test, or Wilcoxon if appropriate. N=5

Supplemental Table3. Effect of CXL-1020 in normal canine heart. Control study data in same animals in which heart failure was then induced using tachypacing.

		CXL-1020 mg/kg/min			
		Δ 10	Δ 30	Δ 100	p
Heart Rate	(min^{-1})	-6.3 ± 3.2	2.1 ± 8.9	11.0 ± 4.3	0.1
End-systolic Pressure	(mmHg)	-5.7 ± 2.6	-13.0 ± 3.4*	-15.3 ± 3.8*	<0.005
End-systolic Volume	(mL)	-0.5 ± 0.8	-2.7 ± 1.0†	-4.5 ± 1.1*	<0.0002
End-diastolic Volume	(mL)	-1.6 ± 1.1	-4.7 ± 1.4*	-8.0 ± 2.2*	<0.0001
End-diastolic Pressure	(mmHg)	-0.5 ± .44	-1.6 ± 0.3*	-2.8 ± 0.4‡	<0.0001
Systemic Resistance	(mmHg/ml/s)	0.08 ± 0.3	-0.1 ± 0.24	-0.25 ± 0.15	0.21
End-systolic Elastance	(mmHg/mL)	0.76 ± 0.15‡	1.3 ± 0.41*	3.1 ± 0.9*	<0.0001
dP/dtmx/EDV	(mmHg/s*mL)	5.0 ± 1.3‡	11.9 ± 1.9*	24.1 ± 5.8*	<0.0001
Tau	(msec)	2.3 ± 3.1	-6.2 ± 1.7*	-7.4 ± 2.2*	0.02

P value in table is for drug effect in repeated measures analysis of covariance.
* $p \leq 0.01$; † $p<0.05$; ‡ $p<0.001$ versus vehicle baseline by Kruskal Wallis test.

Supplemental Table 4. Arrhythmia Threshold Analysis in Ischemic CM dogs administered CXL-1020.

	Vehicle		CXL-1020 (5 μg/kg/min)	
	Baseline	40min Infusion	Baseline	40min Infusion
Heart Rate (bpm)	82±3	82±2	78±3	80±2
Systolic Aortic Pressure (mmHg)	91±3	90±5	88±3	87±3
Diastolic Aortic Pressure (mmHg)	66±2	66±4	62±2	60±2
Mean Aortic Pressure (mmHg)	78±3	79±4	73±2	73±2
Fractional Area Shortening (%)	29.5±3.2	29.8±3.9	29.4±3.6	36.1±4.6*
Threshold Score[†]	—	4.7±0.9	—	8.4±2.2
Conversion by DC Shock (%)[#]	—	5/7 (71)	—	4/7 (57)
Spontaneous Conversion (%)[#]	—	2/7 (29)	—	3/7 (43)

Threshold Score calculation: see below . *, P<0.001 versus Baseline. Values mean±sem, N=7. #, Note that all animals successfully converted to normal rhythm with both treatments.

Programmed ventricular stimulation (PVS) was performed using an active fixation pacing lead advanced from the external jugular vein and positioned in the right ventricular apex (guided by fluoroscopy) and connected to a Contak Renewal 3 CRT-D Model 174 generator (Guidant) and a Zoom Latitude Programming System (Guidant 3120). The stimulation protocol included delivery of up to 4 extrastimuli at progressively shorter coupling intervals (starting at 350 msec, in step of 10 msec). The extrastimuli was delivered following 8 ventricular paced beats with drive cycle length between 600 and 200 msec. The protocol was terminated with the induction of a sustained ventricular tachycardia (VT) lasting more than 30 seconds or ventricular fibrillation. In either case, an external direct current (DC) shock with 350-400 J was applied to terminate the arrhythmia and restore sinus rhythm.
Threshold data were quantified based on a linear scaling factor, designating a low value of "1" to S1 of 600 msec and S2 of 350 msec, and progressively increasing the score based on severity of stimulation to the highest score of "20" designated for S1 of 250 msec and S5 of 200 msec. The scoring schema is shown in the table below. Based on this scoring schema, the threshold scores were compared in each dog in pair-wise fashion (CXL-1020 versus Vehicle).

Threshold Scoring Schema				
S1 600	**S1 500**	**S1 400**	**S1 300**	**S1 250**
S2 350-x =1	S2 300-x=5	S2 250-x=9	S2 200-x=13	S2 200-x=17
S3 350-x =2	S3 300-x=6	S3 250-x=10	S3 200-x=14	S3 200-x=18
S4 350-x =3	S4 300-x=7	S4 250-x=11	S4 200-x=15	S4 200-x=19
S5 350-x =4	S5 300-x=8	S5 250-x=12	S5 200-x=16	S5 200-x=20

Supplemental Table 5. Baseline Demographics for Dosing Cohorts

	Placebo	CXL-1020 Cohort 1	CXL-1020 Cohort 2
Infusion rate (µg/kg/min)	none	3,10,20	1,3,10
Number	7	12	12
Age (year)			
Mean	55	55	55
SD	12	14	11
Gender			
Male	5 (71%)	9 (75%)	10 (83%)
Female	2 (29%)	3 (25%)	2 (17%)
Race			
White	3 (43%)	8 (67%)	7 (58%)
Black or African American	2 (29%)	4 (33%)	5 (42%)
Asian	1 (14%)	0	0
Hispanic or Latino	1 (14%)	0	0
Etiology of Heart Failure			
Coronary artery disease	2 (29%)	6 (50%)	5 (42%)
Hypertension	4 (57%)	8 (67%)	8 (67%)
Other	2 (29%)	2 (17%)	2 (17%)
NYHA functional class			
Class III	4 (57%)	5 (42%)	4 (33%)
Class IV	3 (43%)	7 (58%)	8 (66%)

Supplemental Table 6 Baseline Hemodynamics in Three Patient Groups.

	Placebo	CXL-1020 Cohort 1	CXL-1020 Cohort 2
Number of subjects	7	12	12
Heart Rate (beats/min)	77 ± 7	77 ± 11	75 ± 14
Systolic Blood Pressure (mmHg)	113 ± 17	113 ± 9	116 ± 12
Mean Arterial Pressure (mmHg)	86 ± 8	87 ± 8	87 ± 10
Pulmonary Artery Systolic Pressure (mmHg)	58 ± 17	58 ± 13	65 ± 10
Pulmonary Artery Diastolic Pressure (mmHg)	29 ± 10	39 ± 6	34 ± 9
Pulmonary Artery Mean Pressure (mmHg)	40 ± 6	39 ± 8	45 ± 7
Pulmonary Capillary Wedge Pressure (mmHg)	25 ± 5	28 ± 5	30 ± 6
Mean Right Atrial pressure (mmHg)	16 ± 9	15 ± 7	17 ± 6
Cardiac Index (Fick) (L/min/m^2)	1.6 ± 0.6	2.2 ± 0.6	1.9 ± 0.9
Cardiac Index (Thermodilution) (L/min/m^2)	1.9 ± 0.4	1.9 ± 0.4	1.9 ± 0.3
Systemic Vasculare Resistance (dyne/sec/cm^{-5})	1553 ± 381	1363 ± 249	1232 ± 320

HR, heart rate; SBP, systolic blood pressure; MBP, mean blood pressure; PAS, pulmonary artery systolic pressure; PAD, pulmonary artery diastolic pressure; PA mean, pulmonary artery mean pressure; PCWP, pulmonary artery wedge pressure; RAP, right atrial pressure; CI, cardiac index; SVR, systemic vascular resistance

Supplemental Table 7. Adverse Events in Clinical Studies

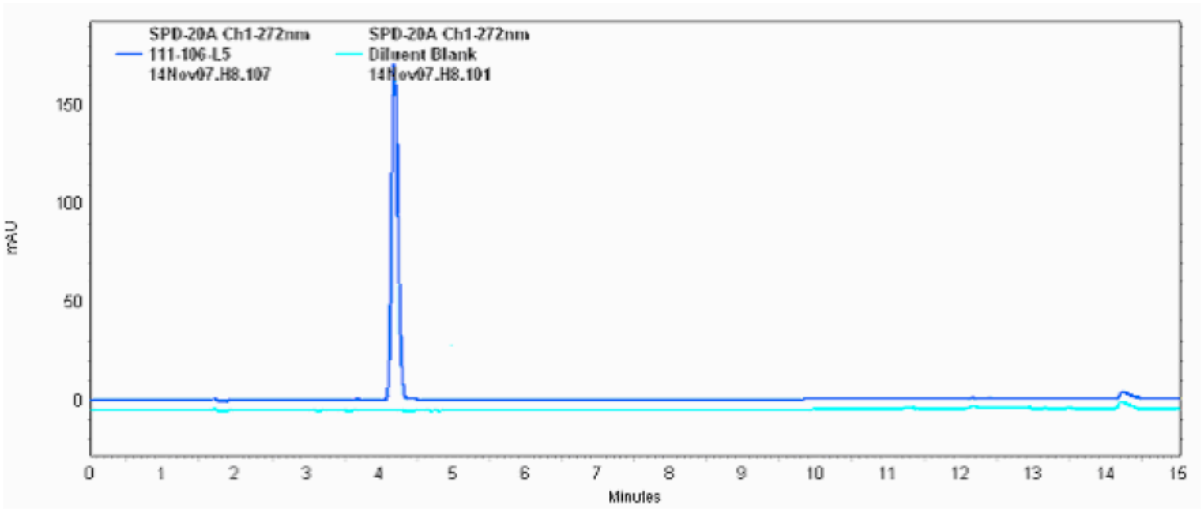
Incidence of Adverse Events (# Patients) by Relationship to Treatment – PA Cath Cohorts

Relationship to Treatment	CXL-1020 (1,3,10ug/kg/min) (N=12)	CXL-1020 (3,10,20ug/kg/min) (N=12)	Placebo (N=7)
Not Related	7 (58.3%)	7 (58.3%)	2 (28.6%)
Possibly related	1 (8.3%) Lymphopenia	1 (8.3%) NSVT	2 (28.6%) Cough, hypoxia
Probably related	0	1 (8.3 %) Headache	0

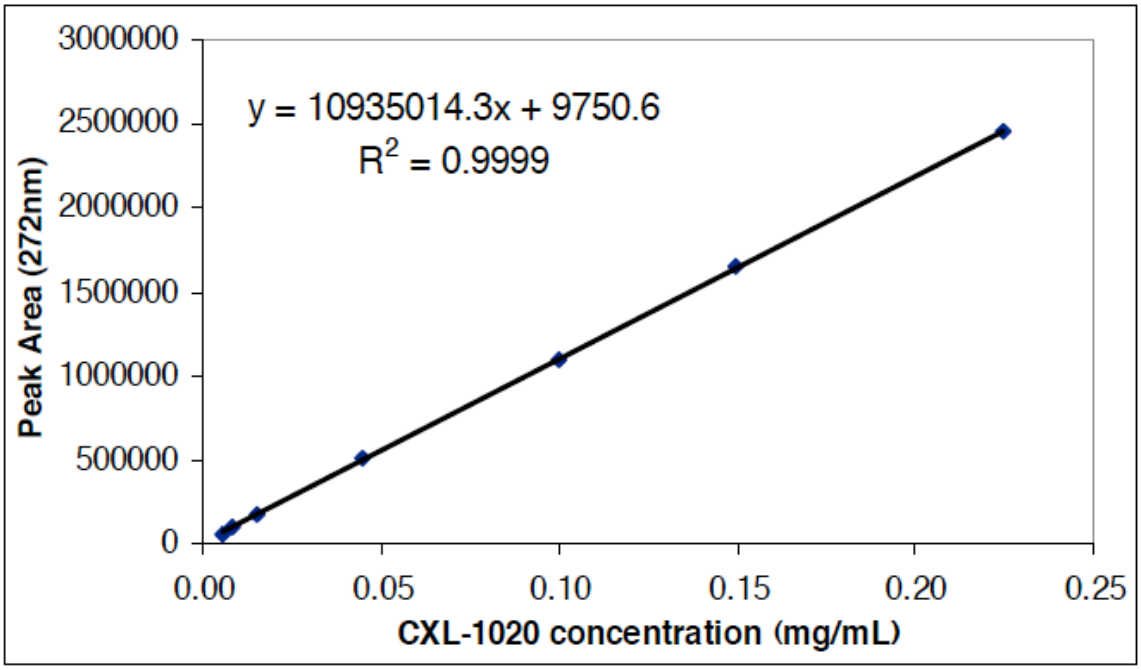
Incidence of Adverse Events (# Patients) by Relationship to Treatment – Echo Cohorts

Relationship to Treatment	CXL-1020 (3ug/kg/min) (N=12)	CXL-1020 (10ug/kg/min (N=12)	CXL-1020 20ug/kg/min (N=9)	Placebo (N=9)
Not Related	5 (45.5%)	4 (44.4%)	4 (44.4%)	3 (33.3%)
Possibly related	0	0	1 (11.1%) Injection Site Sx	0
Probably related	0	1 (11.1%) Injection Site Sx	0	1 (11.1%) Increased Heart Rate

Supplemental Figure 1.

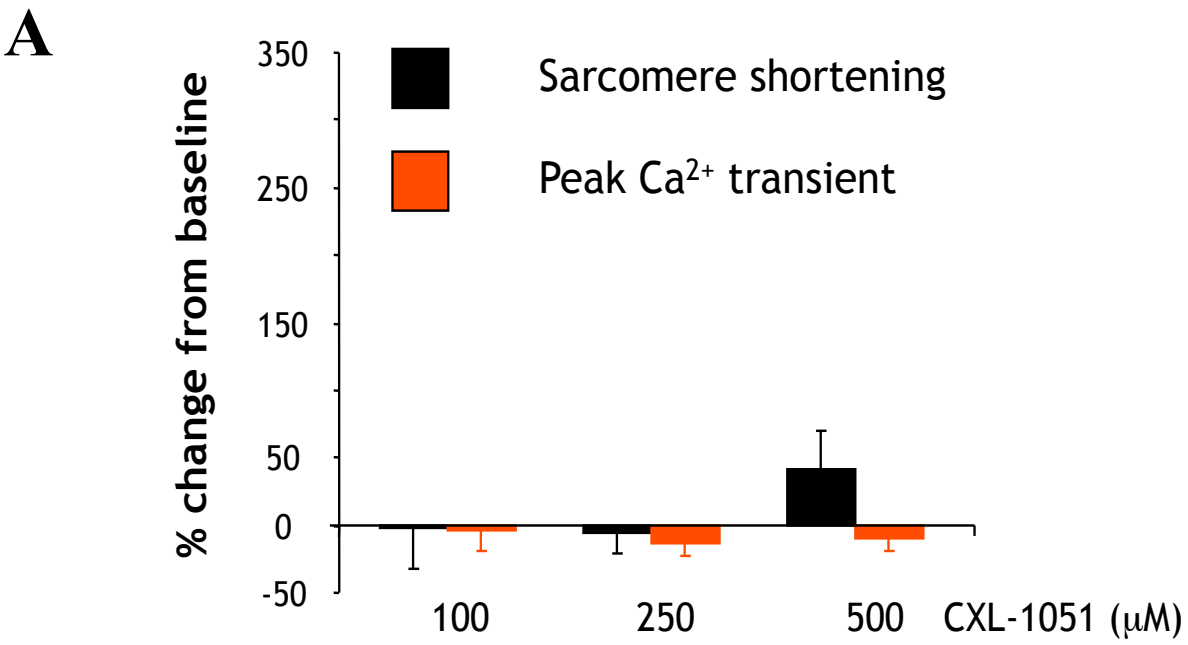


Legend, from bottom to top: Teal = blank injection; Dark Blue = 0.100 mg/mL CXL-1020 standard.

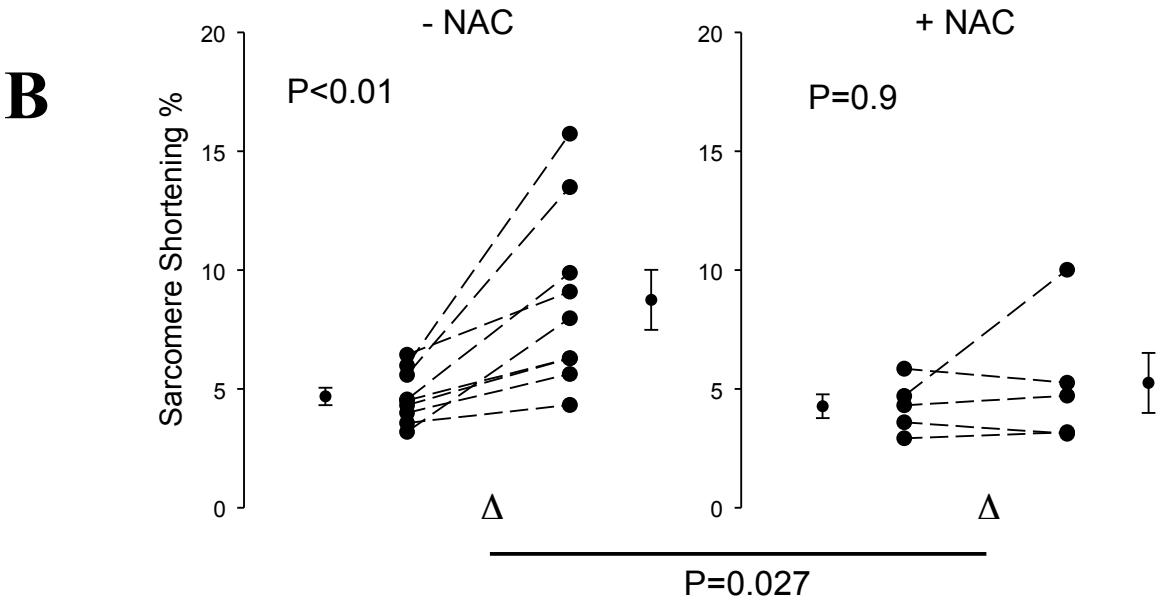


Example of identification of CXL1020 detection by HPLC. The assay was highly linear in the relevant concentration range.

Supplemental Figure 2

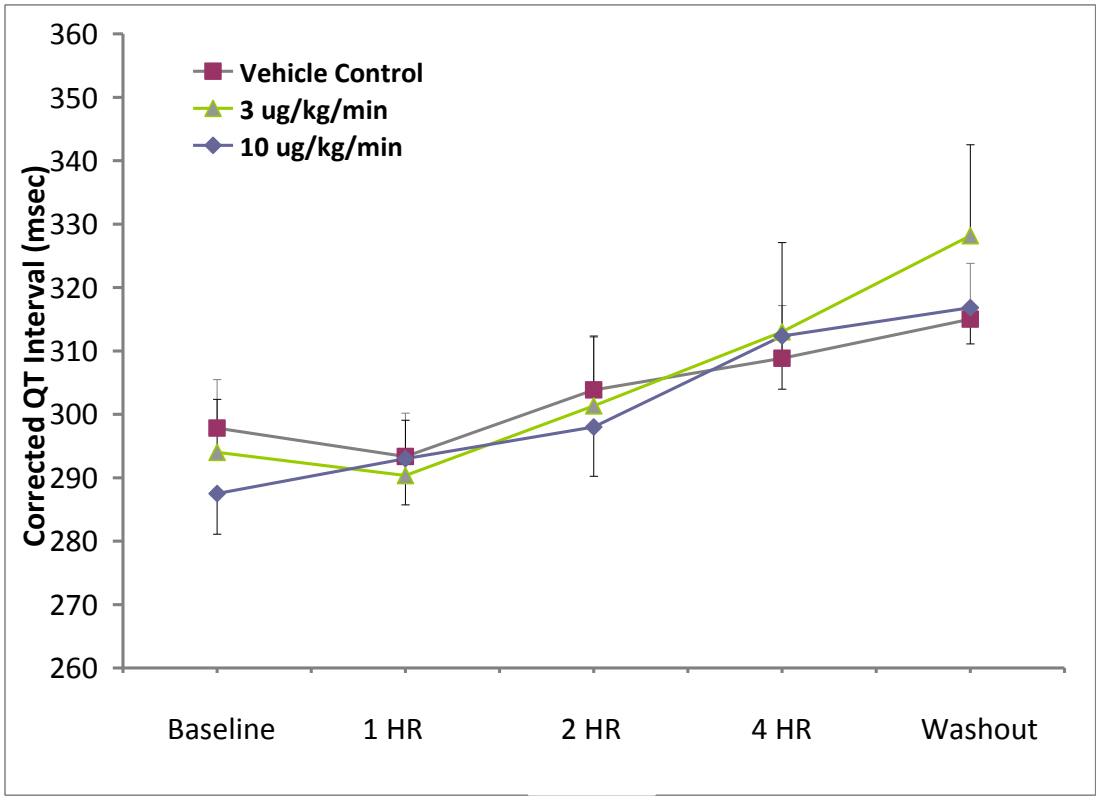


A) Effect of CXL-1051, the breakdown product of CXL-1020 after release of HNO on cardiac myocyte sarcomere shortening and calcium transients.
Unlike CXL-1020, the metabolite had no significant impact on cell behavior.



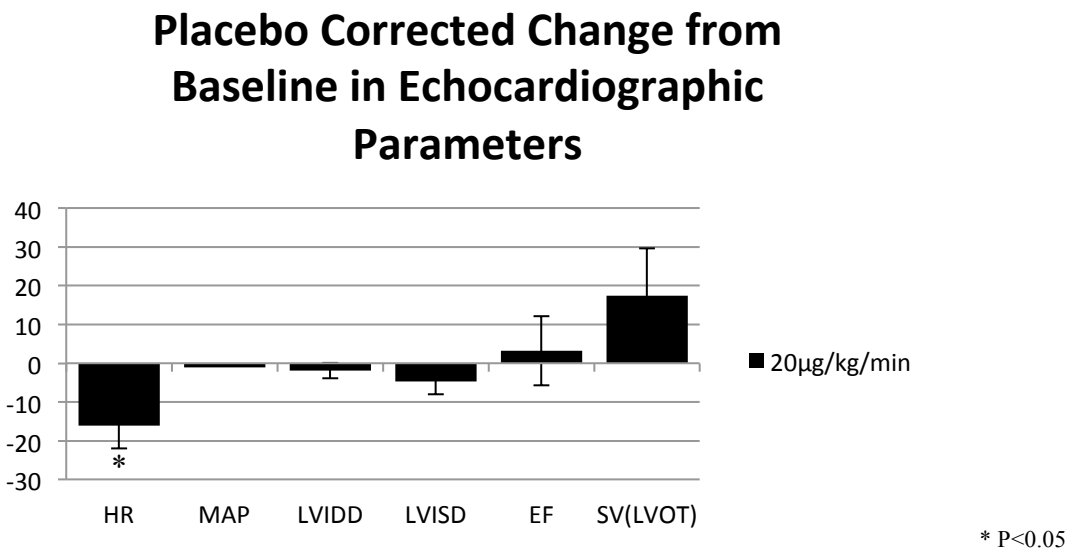
B) Positive inotropic effect from CXL-1020 in isolated C57Bl6/J myocytes is blunted by pre-incubation with N-acetyl cysteine (5 mM). NAC had no impact on resting function. P values in each panel reflect Wilcoxan paired analysis, and for between group change, as Kruskal-Wallis test.

Supplemental Figure 3



Corrected QT interval in dogs with ischemic cardiomyopathy. There was no impact on the QTc in association with CXL-1020 drug infusion.

Supplemental Figure 4



Effect of CXL-1020 on echocardiographic parameters in patients with heart failure (n=9 active vs. n=9 placebo) . Only a decline in heart rate achieved statistical significance, with a trend towards an increase in stroke volume determine from left ventricular outflow tract (Doppler flow) analysis. MAP – mean arterial pressure, LVIDD – left ventricular internal diastolic diameter, LVISD – left ventricular internal systolic diameter, EF – ejection fraction.

Supplemental Material References:

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