

SUPPLEMENTAL DATA

In vitro and in vivo characterization of a new organic nitrate hybrid drug covalently bound to pioglitazone

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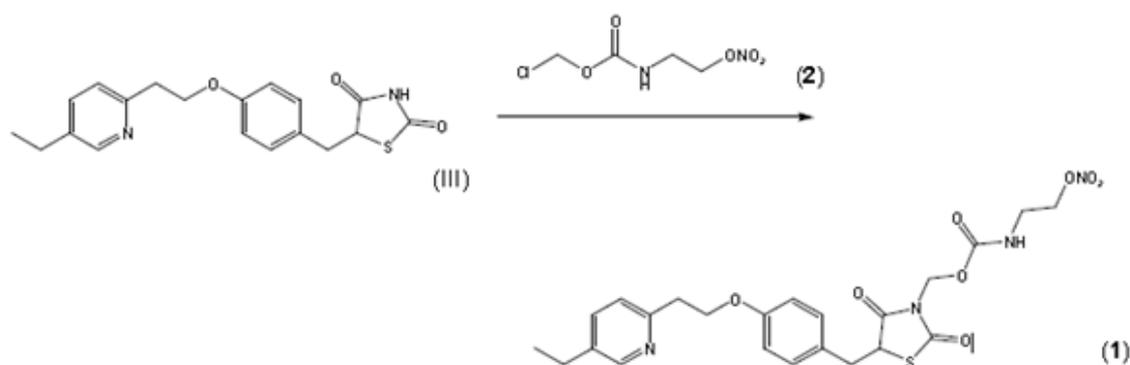
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Extended Methods

Synthesis of organic nitrate linkers and hybrid drugs

(2-Nitroxyethyl)carbamic acid 5-(4-[2-ethylpyridin-2-yl]ethoxy)benzyl)-2,4-dioxothiazolidin-3-ylmethyl ester (pioglitazone mononitrate, CLC-3000). A solution of chloromethyl 2-(nitrooxy)ethylcarbamate (2, 13.37 g, 67.3 mmol) in CH₂Cl₂ (20 ml) is added drop-wise to a suspension of 5-(4-(2-(5-ethylpyridin-2-yl)ethoxy)benzyl)-thiazolidine-2,4-dione (pioglitazone, 12 g, 33.7 mmol) in CH₂Cl₂ (160 ml) and triethylamine (9.38 ml, 67.3 mmol) at 22°C (room temperature) under an argon atmosphere. The reaction is stirred for 8 h. The reaction is diluted with CH₂Cl₂ and H₂O and the layers separated. The organic layer is dried (MgSO₄) and the solvent evaporated to give the crude product. The crude material is purified by column chromatography (silica, 40:60 ethyl acetate:heptane, product R_f 0.3 (80:20 ethyl acetate:heptane) to give the title compound (5-(4-(2-(5-ethylpyridin-2-yl)ethoxy)benzyl)-2,4-dioxothiazolidin-3-yl)methyl 2-(nitrooxy)ethylcarbamate (1), 5.3 g (30%), as a white solid. ¹H NMR: δ 8.41 (s, 1H), 7.50 (d, J = 7, 1H), 7.21 (d, J = 7, 1H), 7.15 (d, J = 7, 2H), 6.82 (d, J = 7, 2H), 5.50 (q, J = 9, 2H), 5.40 (br s, 1H), 4.58 (t, J = 2, 2H), 4.45 (dd, J = 7, 2, 1H), 4.35 (t, J = 7, 2H), 3.55 (q, J = 3, 2H), 3.45 (dd, J = 12, 2, 1H), 3.25 (t, J = 7, 2H), 3.10 (dd, J = 12, 7, 1H), 2.68 (q, J = 7.5, 2H), 1.30 (t, J = 7.5, 3H). ¹³C NMR: δ 172.6, 170.0, 158.4, 155.5, 154.4, 148.8, 137.3, 136.1, 130.4, 127.3, 123.4, 114.9, 71.5, 67.3, 63.6, 51.8, 38.5, 37.6, 37.4, 25.7, 15.3. LCMS: 519 (M⁺H⁺), 443.



The starting material chloromethyl N-(2-nitrooxyethyl)carbamate (2) is obtained as follows: 20 g Ethanolamine (328 mmol) is added dropwise to 60 g HNO₃ (100%) at -20°C, care being taken that the temperature does not rise above 5°C. The mixture is added to ethyl ether (300 g) and stirred for 1 h at 0°C. The obtained suspension is filtered, the solids washed with ethyl ether and dissolved in ethanol (120 g) at 30°C. On cooling to 0°C, the product precipitates again. The solids are added in portions to 60 g HNO₃ (100%) at -5°C, then cooled to -15°C, and treated with ethanol. Ethyl ether (200 g) is added, and the mixture stirred 1 h at -10°C. The obtained suspension is filtered, the solids washed with ethyl ether, and dried in hot air to give 21.9 g (41%)

white crystals of 2-nitroxyethylammonium nitrate. 4.25 g Chloromethyl chloroformate (33.0 mmol) are added dropwise in 15 min to 5.0 g 2-nitroxyethylammonium nitrate (29.6 mmol) and 7.5 g triethylamine (74.1 mmol) in 130 g CH₂Cl₂ at -15°C. The mixture is stirred at -10°C for 1 h. Water (40 g) is added, the organic phase washed with aqueous citric acid (10%) and saturated aqueous NaHCO₃, then dried over Na₂SO₄, and evaporated to give 4.6 g (78%) of a yellowish oil. ¹H-NMR (200 MHz, CDCl₃): 3.53-3.63 (m, 2 H); 4.57 (t, J = 5.1, 2 H); 5.74 (s, 2 H).

Assessment of Vasodilative Properties – Isometric Tension Studies

After seven days exposure in vivo to the CLC-3000 at different doses or the solvent DMSO alone, aortic rings were prepared as described above and assessed for their responsiveness to various vasodilators. Generally, four ring preparations were made per treated animal, and each preparation was used to generate three concentration-relaxation curves, using vasodilators in the following sequence: acetylcholine (ACh), CLC-3000 itself and nitroglycerine (GTN). The ACh binds to cholinergic receptors on the endothelium and induces production of NO, which passes into the smooth muscle to induce vasodilation. Thus, ACh provides a measure of endothelium-dependent vasodilation. The GTN is biotransformed by mitochondrial ALDH-2 to generate NO (or a related species), which acts directly on smooth muscle, independent of the endothelium. A shift of the concentration-relaxation curve to the right relative to vehicle controls indicates a reduced response of the rings to the vasodilator.

Isometric tension studies were performed in vitro and ex vivo to assess the vasodilative properties of the test compounds. Isolated rat aortic rings were prepared as described in [1]. All preparations were made fresh and experiments were completed the same day. Aortic rings including associated endothelium were dissected from Wistar rats, placed in an aorta tissue bath (25 ml total volume) containing Krebs buffer (118.3 mM NaCl, 4.69 mM KCL, 1.87 mM CaCl₂, 1.2 mM MgSO₄, 1.03 mM KH₂PO₄, 25 mM NaHCO₃, 11.1 mM glucose; pH 7.40) plus 10µM indomethacin as a cyclooxygenase inhibitor. The aorta was attached to a force transducer (TRN001 from Kent Scientific, Isometric Force Transducer from Letica Scientific Instruments or FT03 Force-Displacement Transducer from Grass Instruments). All tension measurements were recorded electronically on a Powerlab 8sp (SP8552) from ADInstruments with ETH-255 Bridge Bio-Amplifier from CB Sciences, or on an Octal Bridge Amplifier and Powerlab 8/30 from ADInstruments. Data were processed using Chart 5 Software for Windows, version 5.4.2. Aortic rings were precontracted with KCl, up to 80 mM, to determine the maximum tone for the preparation. After washing out the KCl, phenylephrine (0.5-1µM) was added to the chamber to pre-constrict the ring to 50%-60% of the maximum tension produced by KCl; this was defined as 0% vasodilation.

The concentration of phenylephrine and the resulting tension were recorded. To determine the concentration-relaxation curve, the test compound at the lowest concentration was added to the chamber and allowed to equilibrate prior to measuring the tension. The concentration of the test compound was then increased in 0.5 log steps and allowed to equilibrate for each of the subsequent readings of the tension measurement. Vasodilation was represented as reduction in the phenylephrine-induced tension (i.e., a reduction in tension to the resting tension = 100%). After the highest concentration was measured in the in vitro experiments, the test compound was removed by exchanging the chamber buffer three times. The same amount of phenylephrine as used prior to the concentration-relaxation curve was added to the chamber and the tension again recorded. For the in vitro experiments, determination of the concentration-relaxation curve was then repeated. For the in vivo experiments, concentration-relaxation curves, as described above, were determined using aortic rings from animals that had been treated for seven days with the test compounds. Each aortic ring was used for three successive concentration-relaxation curve assessments. The first curve was generated using acetylcholine (ACh) to assess endothelium-dependent activity. The second curve used CLC-3000 itself, while the third curve was generated with glyceryl trinitrate (GTN) to measure endothelium-independent activity.

Induction of Oxidative Stress

Generation of Mitochondrial Reactive Oxygen Nitrogen Species via Succinate (Complex II Substrate)

The assay is based on the reaction of the chemiluminescence dye L-012 with RONS generated by the complex II substrate, succinate and subsequent emission of chemiluminescence light (ECL). The signal is counted in photons/time (=counts/30s) for 5 or 20 minutes. The data are based on experiments with cardiac mitochondria from at least six rats on three different days. Briefly, mitochondria were prepared as previously described in Raha et al, 2000 [2]. Wistar rat hearts were homogenized in HEPES buffer (50 mM HEPES, 70 mM sucrose, 220 mM mannitol, 1 mM EGTA, and 0.033mM bovine serum albumin, pH 7.4) and centrifuged at 1500g for 10 minutes, followed by 2000g for 5 minutes at 4°C. The pellet was discarded after each centrifugation. The supernatant was then centrifuged at 20,000g for 20 minutes and the pellet was resuspended in 1 mL HEPES buffer. The latter step was repeated and the pellet was resuspended in Tris buffer (10 mM Tris, 340 mM sucrose, 100 mM KCl, and 1 mM EDTA, pH 7.4) to a concentration of 5-10 mg/mL. The isolated mitochondrial preparation was diluted to a concentration of approximately 0.1 mg/mL in 0.5 mL PBS as described in [3]. The dye L-012 was added to the mitochondrial prep (100 µM), and for the in vitro experiments, the test compound was added at the indicated concentration. The reaction was initiated by adding the complex II substrate, succinate (5 mM). Chemiluminescence was measured over 5 minutes using a Lumat LB9507 from

Berthold Technologies, and expressed as counts/30 seconds at 5 minutes. In the same mitochondria (1 mg/ml final protein concentration), the ALDH-2 activity was assessed by an HPLC-based method by the conversion of 2-hydroxy-3-nitrobenzaldehyde to its benzoic acid product with UV/Vis detection at 360 nm as described [4].

Activity of Cardiac NADPH Oxidase and Generation of Reactive Oxygen and Nitrogen Species

The first assay measures NADPH-generated superoxide via its reaction with the chemiluminescent dye lucigenin, which has very high specificity for superoxide. The second assay uses luminol/HRP to measure hydrogen peroxide resulting from the disproportionation of 2 superoxide molecules. Isolated cardiac membranous preparations were prepared according to [5]. Wistar rat hearts were homogenized in homogenization buffer containing 50 mM Tris HCL, pH7.4, 10 mM dithiothreitol (DTT), and a protease inhibitor cocktail for general use. The homogenate was centrifuged at 2000g for 5 minutes at room temperature, followed by 20,000g for 20 minutes at 4°C. The pellet was discarded after each centrifugation. The resulting supernatant was then centrifuged at 100,000g for 60 minutes at 4°C. The final pellet was resuspended in homogenization buffer without DTT and diluted with PBS to give a final protein concentration of approximately 7 mg/mL. The membranous preparation was diluted to a concentration of approximately 0.2 mg/mL in 0.5 mL PBS. The dye lucigenin (5 µM) or luminol (100 µM)/HRP (0.1 µM) was added, followed by the NADPH oxidase substrate, NADPH (to 200 µM), as previously described [6]. Chemiluminescence was measured after 5 minutes using a Lumat LB9507 from Berthold Technologies, and expressed as counts/30 seconds.

Vascular Reactive Oxygen Species Formation In situ – Fluorescent Microtopography

For in vivo testing, vascular ROS formation was assessed in situ by dihydroethidine (DHE) dependent fluorescence as previously described [7, 8]. The lamina of the vascular smooth muscle emits a green autofluorescence. Briefly, fresh aortic rings from Wistar rats were embedded in aluminum cups of about 1ml of a polymeric resin (Tissue Tek, USA) and frozen in liquid nitrogen. The rings were cut into 6 µm sections, and DHE (1 µM) was placed on the section prior to it being coverslipped. Fluorescence (at 605 nm) was induced by excitation at 518 nm and recorded with a camera attached to a Leica fluorescence microscope. Quantification was performed using densitometry. Fluorescence of test samples is expressed as percent of intensity of control-treated animals, and representative micrographs are shown for each group of animals.

Extended Discussion

To further explore the potential reason underlying the change in CLC-3000's concentration-relaxation curve, we must consider the underlying theory behind the experimental design. The basis of the concentration-relaxation curve is that increasing concentrations of the vasodilator gradually overcomes partial vasoconstriction induced by phenylephrine. When an aortic ring is placed into the organ chamber, KCl is first added to the bath to a concentration of 80 mM, causing the ring to contract, which is recorded as tension on the attached force transducer. Rat aortic rings typically create a maximum tension of around 3 to 6 grams. The KCl is then washed out of the chamber, allowing the ring to relax. Prior to determining the concentration-relaxation curve, PE is added to the organ bath to constrict the ring until it reaches approximately half the tension induced by KCl (normally achieved with about 0.5-1.0 μ M PE). As increasing concentrations of the vasodilator are added to the organ bath, it overcomes the PE-induced constriction and the recorded tension drops toward the level of the resting tension. After completing a concentration-relaxation curve and washing out the test compound, the same concentration of PE will usually generate a similar level of tension in the ring, although the sensitivity to PE can be altered under various conditions.

Nitrate tolerance, induced by 3-days exposure to GTN *in vivo*, enhances contractile sensitivity to PE, such that a given amount of PE induces greater tension in GTN pre-exposed aortic rings than unexposed rings [9, 10]. Similarly, 10 minutes incubation *in vitro* with endothelin-1 also enhances PE sensitivity [11]. Thus, if the reduced vasodilation seen with CLC-3000 in the second concentration-relaxation curve were due to typical nitrate tolerance, we would expect the aortic rings to have increased PE sensitivity after the first concentration-relaxation curve, i.e., increase in PE-induced tension. Surprisingly, the exact opposite was seen. Compound CLC-3000 showed almost an 80% decrease in PE-induced tension prior to the second concentration-relaxation curve. While it's still unclear why the PE-induced tension should be so dramatically affected by CLC-3000, several possibilities exist. The compound is poorly soluble and may not have been completely removed from the chamber during the washout procedure, leaving it to interfere with the PE-induced tension. Alternatively, it could induce some kind of long-acting vasodilation or anti-vasoconstriction, which PE is unable to overcome. In any case, the reduced PE-induced tension at the start of the second concentration-relaxation curve may be associated with the reduced responsiveness to CLC-3000 during the subsequent procedure, and may explain the reduced vasodilation seen with the compound upon rechallenge.

Endothelial nitric oxide synthase (eNOS) is the enzyme responsible for generating NO from arginine in endothelial cells. Increased expression of eNOS is generally considered a highly protective and beneficial effect, however, in diseased animals (e.g. diabetic or

hypertensive), up-regulation of eNOS has also been postulated as a sign of oxidative stress and viewed as an attempt to counteract dysfunctional signaling pathways [12, 13]. Nox2 is a subunit of NADPH. It's a prominent source of superoxide and its increased expression is considered a marker for oxidative stress. Heme oxygenase-1 (HO-1) breaks down metal porphyrins and thereby produces the antioxidant bilirubin and carbon monoxide, which has anti-inflammatory, antiatherosclerotic and vasodilative effects. Perhaps because of these beneficial effects, HO-1 expression is often elevated in response to stress (e.g., heat-shock, heavy metals, oxidative stress). Consequently, despite its beneficial effects, up-regulation of HO-1 is considered a general marker of stress conditions. Dihydrofolate reductase (DHFR) reduces dihydrobiopterin (BH₂) to tetrahydrobiopterin (BH₄), an essential cofactor of eNOS. Thus, as with eNOS, increased expression of DHFR is both protective and potentially indicative of response to stress [13].

Extended References

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