Cell Transplantation
Volume 29: 1–9

DOI: 10.1177/0963689720906417
journals.sagepub.com/home/cll
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# In Vitro Evaluation of a Novel Synthetic Bilirubin Analog as an Antioxidant and Cytoprotective Agent for Pancreatic Islet Transplantation

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**Abstract**

Bilirubin is a natural cytoprotective agent and physiologic doses have proven to be beneficial in various models of organ and cellular transplantation. Recently, we showed that bilirubin has protective effects in models of pancreatic islet transplantation, preventing cell death associated with islet stress and suppressing the release of damage-associated molecular patterns. Despite these promising therapeutic attributes, the natural bilirubin used in these research studies is animal-derived (porcine), making it unsuitable for clinical application. In the current study, we synthesized two bilirubin analogs that can be produced without the use of animal-derived products. Antioxidant activity for the analogs was measured using the ferric-reducing-ability-of-plasma (FRAP) and 2,2V-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assays. Dose-dependent cytotoxicity and cytoprotective effects were then demonstrated in isolated rat islets. Compound **I** showed similar antioxidant activity to natural bilirubin. Dose-dependent cytotoxicity was seen following treatment with Compound **I** and natural bilirubin at doses >40 μM, resulting in significantly increased cell death when compared to control islets ($P < 0.05$) or islets treated with doses ≤20 μM ($P < 0.05$). Following hypoxic challenge, islet cell death was reduced in islets treated with Compound **I** at 10 μM (17.27% ± 0.26%) compared to natural bilirubin at 10 μM (51.36% ± 0.71%; $P < 0.0001$) or 20 μM (59.02% ± 0.83%; $P < 0.0001$) and control islets (36.51% ± 0.44%; $P < 0.0001$). Compound **I** was found to have promising antioxidant and cytoprotective effects, limiting islet cell death in a model of islet transplantation hypoxic stress. Compound **I** may serve as a synthetic drug lead for clinical islet transplantation and further evaluation of this molecule and its analogs is warranted.

**Keywords**

bilirubin, synthetic, antioxidant, cytoprotection, islet transplantation

## Introduction

Diabetes mellitus (DM) is a debilitating and progressive disease that has profound effects not only on the affected individual, but on the entire healthcare system[1]. Treatment with insulin injections is tedious and fails to replicate normal physiology and affected patients are at risk of developing progressive hypertension, vascular complications, kidney disease, and blindness[1–3]. Pancreatic islet cell transplantation offers a potential noninvasive cure for patients with type 1 diabetes mellitus (T1DM), although application of this therapy has been limited because of a significant loss of islet cell mass and function occurring after implantation[4,5]. A prolonged period of ischemia occurs from the time of donor harvest of islet cells until the point of

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Submitted: September 5, 2019. Revised: January 23, 2020. Accepted: January 24, 2020.

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revascularization within the recipient. Hypoxemia suffered by the islet cells during the transplantation process results in the formation of numerous reactive oxygen species, ultimately causing cellular necrosis and apoptosis via intracellular signaling pathways. Beta cells are particularly vulnerable to free radical injury due to relatively weak activity of catalase, superoxide dismutase, and glutathione peroxidase in these cells[5,6], making cell death following islet isolation a critical hurdle to successful islet transplantation in patients with T1DM.

Induction of the heme oxygenase-1 (HO-1) system during various forms of cellular insult and injury is an important endogenous cytoprotective mechanism against oxidative stress, and upregulation of this system in transplanted islet cells has shown improved antiapoptotic effects and in vivo function[7–9]. Biliverdin, one of the three byproducts of the breakdown of heme by heme oxygenase, is rapidly reduced to bilirubin via biliverdin reductase in what is thought to be an evolutionary adaptation to produce this cytoprotectant during cell stress[10]. Bilirubin has been shown to be an important mediator of cytoprotection via powerful endogenous antioxidant activity and anti-inflammatory effects[7,11–13]. Bilirubin has not only been shown to provide potent cytoprotection of transplanted islets via induction of HO-1 and significant scavenging of free oxygen and nitrogen radicals, but it has also been shown to suppress the innate immune system resulting in improved donor tolerance to islet grafts[14–16].

Although bilirubin has been established as a promising adjuvant therapy to survival of islets during the transplantation process, continued research into the value of bilirubin as a pharmaceutical agent has been hindered by the fact that bilirubin is currently only available as a naturally derived compound from porcine sources[17]. Strict federal regulations placed on medical devices that are composed of, or are exposed to, animal-derived materials has deterred scientists from pursuit of compounds such as bilirubin for medicinal use[18]. Synthesis of novel compounds, structurally similar to bilirubin, could serve as candidates for safely sourced drugs with similar cytoprotective effects to natural bilirubin, without the concerns and limitations associated with procurement from a live animal source.

The objective of this study was to design and synthesize simplified structural fragments of bilirubin and to screen the candidate compounds for relative antioxidant activity using standard laboratory methods. Selected compounds were then evaluated for dose-dependent cytotoxic effects in isolated murine islets. Finally, in vitro antioxidant and cytoprotective effects were assessed in a relevant model of hypoxic stress. We hypothesized that successful development and synthesis of such a compound could be achieved and that cytotoxicity in islet cells would be dose-dependent and similar to cytotoxicity seen with natural bilirubin. We further hypothesized that the antioxidant and cytoprotective effects of these analogs would be similar to natural bilirubin.

## Materials and Methods

### Development and Synthesis

As an initial evaluation as to whether the effects of bilirubin could be mimicked by simplified bis-pyrroles, we targeted Compounds **1** and **2**. These two molecules were identified as targets for synthesis due to their simplicity and the hypothesized requirement for the bis-pyrrole moiety.

Briefly, synthesis of Compound **1** was as follows: To a resealable glass pressure tube were added 3,4-diethyl-2,5-diformyl pyrrole (0.15 g, 1.0 mmol), 3,4-diethyl 3-pyrrolin-2-one (0.26 g, 2.0 mmol), and EtOH (6.6 ml) followed by piperidine (0.20 ml, 2.0 mmol). The tube was sealed and brought to 100°C and stirred for 48 h. The reaction was concentrated, dissolved in $CH_2Cl_2$, and washed with 5% HCl, saturated. $NaHCO_3$ (×3), and brine. The combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated in vacuo. The crude residue was passed through a short plug of silica with $CH_2Cl_2$ as eluent to afford 126 mg (49%) of Compound **1** as a yellow solid.

Compound **2** was synthesized, in brief, as follows: To a resealable glass pressure tube were added 3-methyl-1H-indole-2-carboxaldehyde (0.15 g, 0.90 mmol), 3,4-diethyl 3-pyrrolin-2-one (0.23 g, 1.8 mmol), and EtOH (6.0 ml) followed by piperidine (0.18 ml, 1.8 mmol). The tube was sealed and brought to 100°C and stirred for 72 h. The reaction was brought to room temperature and filtered. The solid was triturated in MeOH, EtOAc, and $CH_2Cl_2$ to afford 183 mg (76%) of Compound **2** as a yellow solid.

Structure and purity of both bis-pyrrole compounds were confirmed via $^1H$ nuclear magnetic resonance spectroscopy using established methods[19]. Complete compound characterization data for each compound is provided in the supplemental information for this manuscript.

### Relative Antioxidant Screening

Following synthesis, candidate molecules were screened for relative antioxidant activity and compared to natural bilirubin as well as to standard antioxidant assay controls (quercetin and Trolox) using the ferric reducing ability of plasma (FRAP) assay and the 2,2V-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation assay. Stock solutions of each compound were prepared for both assays and concentrations of 800, 600, 400, 300, 200, and 100 μmol were produced by dilution of the stock solution with 100% ethanol. Briefly, stock solutions were prepared for each compound as follows: Bilirubin (Sigma-Aldrich, St. Louis, MO, USA)—5.8 mg was suspended in 1 ml 50% ethanol in water + 10 μl 2 M NaOH, the mixture was sonicated for complete dissolution, then 100% ethanol was added to a final volume of 10 ml; Compound **1** [molecular weight (MW) 258.36]—2.58 mg dissolved in 1 ml dimethyl sulfoxide (DMSO) with sonication until complete dissolution. Ethanol (100%) was added to a final volume of 10 ml; Compound **2** (MW 267.33)—2.58 mg dissolved in 1 ml DMSO with sonication

until complete dissolution. One hundred percent ethanol was added to a final volume of 10 ml; Quercetin (Sigma-Aldrich)—3.02 mg dissolved in 10 ml 100% ethanol; Trolox (Sigma-Aldrich)—2.5 mg was dissolved in 10 ml 100% ethanol.

### FRAP Assay

The FRAP assay was completed evaluating all bis-pyrrole compounds following guidelines as previously reported[20]. The FRAP assay is based on electron-donating antioxidants that reduce ferric-tripyridyltriazine ($Fe^{3+}$ TPTZ) complex, a colorless compound, to ferrous complex ($Fe^{2+}$-TPTZ), resulting in the development of an intense blue color with an absorption maximum at 593 nm. FRAP reagent was prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3 \cdot 6H_2O$ and then prewarmed to 37°C prior to use in the assay[20]. Sample stock solutions were prepared as above. Reagent–sample reaction mixtures were created in a 96-well microplate and were incubated at 37°C. Absorbance was measured at 593 nm and subtracted from the absorbance of the buffer-only blank well after 2 h.

### ABTS Radical Cation Assay

The ABTS assay was performed on all bis-pyrrole compounds using previously reported methods[21]. ABTS solution was combined with 2.45 mmol/l potassium persulfate to produce colored 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$). Dilution of the deep green $ABTS^{\cdot+}$ solution with ethanol to an absorbance of 0.7 at 734 nm was performed. During the assay, the $ABTS^{\cdot+}$ is decolorized by antioxidants according to their concentrations and antioxidant capacities. Stock solutions of antioxidant test bis-pyrrole compounds (as above) were mixed with oxidized $ABTS^{\cdot+}$ and decolorization of the samples (absorptance) was measured at 734 nm, 10 min following reagent–sample mixing.

### Biological Testing

Based on the strong performance of Compound **1** in the antioxidant screening assays, this analog was selected for evaluation in the biological assays. Compound **1** was compared to natural bilirubin in cytotoxicity models and in hypoxia-induced stress using rat pancreatic islets as follows.

### Islet Isolation

Experiments were approved by the Institutional Animal Care and Use Committee at North Carolina State University and were performed according to the NIH Guidelines for Animal Care and Use. Wistar rats were purchased from Charles River Laboratories, Wilmington, NC, USA. Rats were euthanized using $CO_2$ inhalation and harvest of islet cells was performed immediately after confirmation of death. Pancreatic islets were isolated using the standard enzymatic digestion methods described by Zmuda et al.[22] Digestion of pancreatic tissue was performed by injection of 5 ml of 2 mg/ml Collagenase IV (Sigma-Aldrich) into the common bile duct. Pancreata were maintained on ice until digestion was initiated by immersion in a 37°C water bath for 17 min. Islet purification was performed using a Ficoll density gradient separation technique, and islet yield was calculated before plating in standard RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum and 1% Pen-Strep (Invitrogen, Carlsbad, CA, USA). Islets were then incubated at 37°C and 5% $CO_2$ for 24 h to allow stabilization after isolation stress. Experiments were performed in triplicate, and for each experiment, islets from a single rat were hand-picked and divided into separate plates to allow comparison of different compounds and at different concentrations.

### Cytotoxicity

Following post-isolation incubation, islets were washed and replated with 300 islets per 35 $mm^2$ well. Islets were then suspended in 3 ml of media containing either Compound **1** or natural bilirubin at concentrations of 10, 20, 40, and 80 μM and were compared to islets suspended in media containing the same volume of 0.05% DMSO (vehicle control) only. Experiments were repeated three times. Test concentrations were chosen to encompass a range based on previous reports that suggest that bilirubin's antioxidant therapeutic effects occur at 10 to 20 μM when used in organ perfusion solutions, which approximates the physiologic range of bilirubin during upregulation of the HO-1 system as well as concentrations >50 μM, which have been shown to hinder mitochondrial respiration[7,12,15,16,23]. Suspended islets were again incubated under standard conditions (37°C, 21% $O_2$) for 48 h prior to viability assessment.

### Hypoxic Challenge

To assess biologic effects following hypoxic injury, further rat islets were isolated and incubated overnight under standard conditions. Islets were then hand-selected, and aliquots of 300 islets were distributed into individual wells of 12-well cell culture plates. Individual wells ($n = 3$) of islets were assigned to one of five treatment groups; Compound **1** or natural bilirubin at concentrations of 10 or 20 μM, or DMSO vehicle control. Test concentrations were chosen based on the results of cytotoxicity evaluation as well as previously mentioned reports concerning ideal therapeutic doses of bilirubin[7,23]. Islets in test media were incubated in hypoxic conditions (1% $O_2$, 5% $CO_2$) at 37°C for 24 h using a mixed gas incubator (Sanyo MCO-19 M, Sanyo Electric Co., Osaka, Japan). Previous reports have shown that significant islet loss occurs following intermittent incubation for up to 3 h at 1% $O_2$, while more extended periods of hypoxia resulted in almost complete destruction of murine islets[24]. Islets were transferred from the mixed gas incubator to a standard oxygen environment (37°C, 21% $O_2$) and allowed to stabilize for 4 h prior to cell viability assessment.

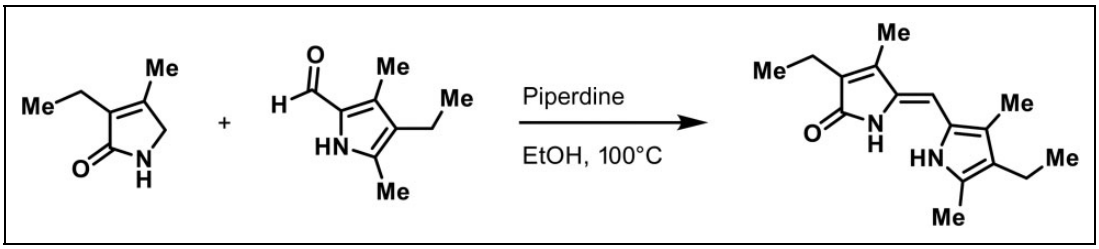


**Fig. 1.** Chemical equation for the synthesis of Compound 1, a simple bis-pyrrole with a system of conjugated double bonds and potentially reactive hydrogen atoms, similar to tetra-pyrrole, bilirubin. Calculated MW for $C_{16}H_{23}ON_2$ $[M + H]^+$ is 259.1805, measured MW 259.1796.
MW: molecular weight.

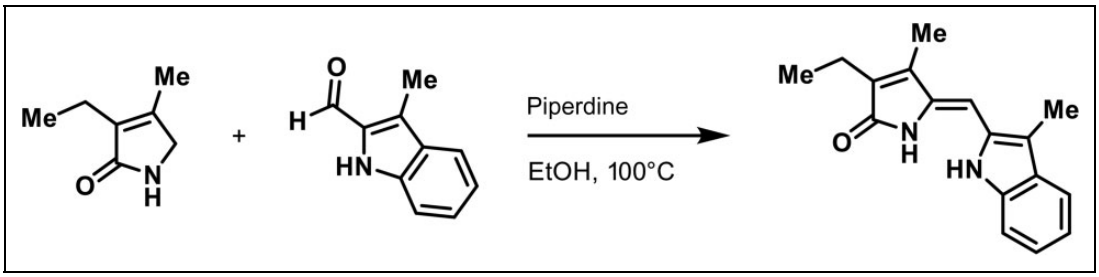


**Fig. 2.** Chemical equation for the synthesis of Compound **2**, a second bi-pyrrole with a slightly more complicated structure, including an indole bicyclic structure. Calculated MW for $C_{17}H_{19}N_2O$ $[M + H]^+$ is 267.14919, found MW 267.14894.
MW: molecular weight.

### *Islet Cell Viability*

Islet cell death following both cytotoxicity and oxidative challenge experiments was determined based on propidium iodide (PI) exclusion. Islets were incubated for 15 min with Hoechst 33258 and PI stains followed by examination via epifluorescent photomicroscopy. Images were analyzed using NIH Image J software, and the percentage of PI-positive cells present in each islet, indicating % cell death in each individual islet, was calculated using a custom islet macro as previously described[24].

### *Statistical Analysis*

Islet viability data were compared between groups using a one-way analysis of variance and post hoc pairwise comparisons were made using Tukey's honestly significant difference test. StatView software was utilized to calculate all statistics (SPSS Inc., Chicago, IL, USA). *P*-values of $\leq 0.05$ were considered statistically significant.

## Results

### *Synthesis*

The synthesis and purification of bis-pyrrole compounds **1** and **2** were successfully completed. Molecular structure for each compound is illustrated in Figs. 1 and 2. Complete characterization data for each compound are found in the supplemental information for this manuscript.

### *FRAP*

Results of antioxidant activities based on the FRAP assay are illustrated in Fig. 3. Order of antioxidant activity of tested compounds from highest to lowest activity was quercetin > bilirubin > Compound **1** > $FeSO_4$ and Compound **2**. As expected, bilirubin exhibited potent antioxidant effects and, as hypothesized, Compound **1** was found to have an antioxidant effect close to natural bilirubin. In contrast, Compound **2** demonstrated minimal antioxidant activity.

### *ABTS*

The antioxidant activity results based on the ABTS assay are illustrated in Fig. 4. Antioxidant effect was similar to that demonstrated in the FRAP assay results and antioxidant activity from highest to lowest was as follows: quercetin > bilirubin > Compound **1** > Compound **2** > Trolox. Compound **1** was found to have antioxidant effects close to natural bilirubin, while Compound **2** was characterized by a lower level of antioxidant activity.

### *Islet Viability Following Cytotoxicity*

Dose-dependent cytotoxicity was evident in murine islets in both treatment groups with significant increases in percent cell death seen with natural bilirubin and Compound **1** at 40 and 80 μM as compared to control islets ($P < 0.0001$ and $P < 0.05$, respectively) (Fig. 5). Islet viability data are expressed as mean cell death (%) ± standard error of the mean. Islet

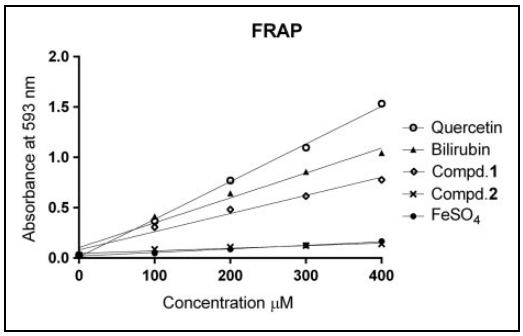


**Fig. 3.** Antioxidant capacity of Compound **1** and Compound **2** vs control compounds based on the FRAP assay; dose–response lines for solutions of Quercetin (open circles), natural bilirubin (triangles), Compound **1** (open diamonds), Compound **2** (crosshairs), and $FeSO_4$ (closed circles). The FRAP assay is based on electron-donating antioxidants that reduce ferric-tripyridyltriazine ($Fe^{3+}$ TPTZ) complex, a colorless compound, to ferrous complex ($Fe^{2+}$-TPTZ), resulting in the development of an intense blue color with an absorption maximum at 593 nm. Reagent–sample reaction mixtures of Compounds **1** and **2**, as well as natural bilirubin and controls were created in a 96-well microplate and were incubated at 37°C. Absorbance was measured at 593 nm and subtracted from the absorbance of the buffer-only blank well. Natural bilirubin and Compound **1** exhibited favorable antioxidant activity. Compound **1** and natural bilirubin may have improved antioxidant activity as compared to Quercetin control in the physiologic dose range (10 to 20 μM).
FRAP: ferric reducing ability of plasma.

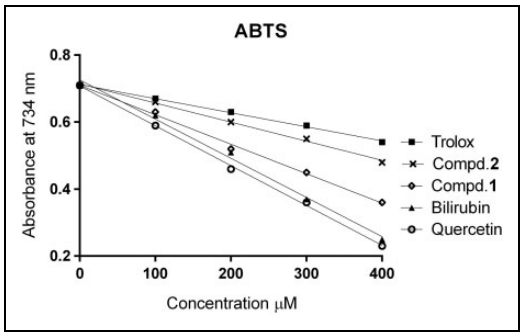


**Fig. 4.** Antioxidant activity of Compound **1** and Compound **2** vs control compounds based on the $ABTS^{*+}$ radical cation assay; dose–response lines for solutions of Trolox (squares), Compound **2** (crosshairs), Compound **1** (open diamonds), natural bilirubin (triangles), and Quercetin (open circles). $ABTS^{*+}$ is decolorized by antioxidants according to their concentrations and antioxidant capacities. Stock solutions of Compounds **1** and **2**, as well as natural bilirubin and $ABTS^{*+}$ controls were mixed with oxidized $ABTS^{*+}$ and absorptance was measured at 734 nm. Natural bilirubin exhibited powerful antioxidant activity, comparable to that of Quercetin control. Compound **1** was also found to have favorable antioxidant activity.
$ABTS^{*+}$: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation.

cells treated with Compound **1** at either 40 μM (59.14% ± 4.80%) or 80 μM (60.8% ± 5.67%) had significantly higher percent cell death compared to islets treated with Compound **1** at 10 μM (27.93% ± 4.63%; $P < 0.001$) or 20 μM (36.18% ± 4.35%; $P < 0.05$). Similarly, islets treated with natural bilirubin at either 40 μM (89.43% ± 4.80%) or 80 μM (84.71% ± 4.80%) had significantly higher cell death rates compared to islets treated with natural bilirubin at 10 μM (46.67% ± 5.18%; $P < 0.0001$) or 20 μM (53.58% ± 5.18%; $P < 0.001$). Interestingly, Compound **1** showed significantly less cell death at 40 μM (59.14% ± 4.80%) and 80 μM (60.8% ± 5.67%) when compared to islets treated with natural bilirubin at 40 μM (89.43% ± 4.80%; $P < 0.006$) and 80 μM (84.71% ± 4.80%; $P < 0.05$), respectively. No significant difference in cell death was found between islets treated with natural bilirubin at 10 μM (46.67% ± 5.18%; $P = 0.6$) or 20 μM (53.58% ± 5.18%; $P = 0.11$) nor between islets treated with Compound **1** at 10 μM (27.93% ± 4.63%; $P = 0.99$) or 20 μM (36.18% ± 4.35%; $P = 0.99$) when compared to vehicle control islets (32.27% ± 5.41%).

### *Islet Viability Following Hypoxic Injury*

Overall, rat pancreatic islets subjected to hypoxic injury showed significantly higher survival ($P < 0.0001$) when treated with Compound **1** at 10 μM as compared to natural bilirubin or vehicle control (Fig. 6). Islet viability data are expressed as mean cell death (%) ± standard error of the mean. In examining dose–response more specifically, islets treated with Compound **1** at either 10 μM (17.27% ± 0.26%) or 20 μM (34.71% ± 0.42%) had significantly decreased cell death when compared to treatment with natural bilirubin at either 10 μM (51.36% ± 0.71%; $P < 0.0001$) or 20 μM (59.02% ± 0.83%; $P < 0.0001$). Although there was a significant difference between islets treated with Compound **1** at 10 μM (17.27% ± 0.26%) and vehicle control islets (36.51% ± 0.44%; $P < 0.0001$), no significant difference was found between islets treated with Compound **1** at 20 μM (34.71% ± 0.42%) and vehicle controls (36.51% ± 0.44%; $P = 0.16$). Interestingly, natural bilirubin at either 10 μM (51.36% ± 0.71%) or 20 μM (59.02% ± 0.83%) did not show a cytoprotective effect against islet loss when compared to vehicle control (36.51% ± 0.44%) in this study. Dose-dependent cytotoxicity was observed in both treatment groups with 20 μM of Compound **1** and natural bilirubin resulting in significantly increased cell death compared to 10 μM dosing ($P < 0.0001$).

## Discussion

Bilirubin has been established as a powerful endogenous cytoprotectant, mediating improved outcomes in several models of health and disease including ischemia reperfusion injury, endothelial damage, and myocardial infarction, as well as solid organ transplantation via significant antioxidant activity and immunomodulatory effects[7,12–16,25–27]. However, our study is the first to evaluate the feasibility of using a synthetic, significantly simplified, bilirubin analog, which could provide a safe, sterile, easily mass-produced drug for

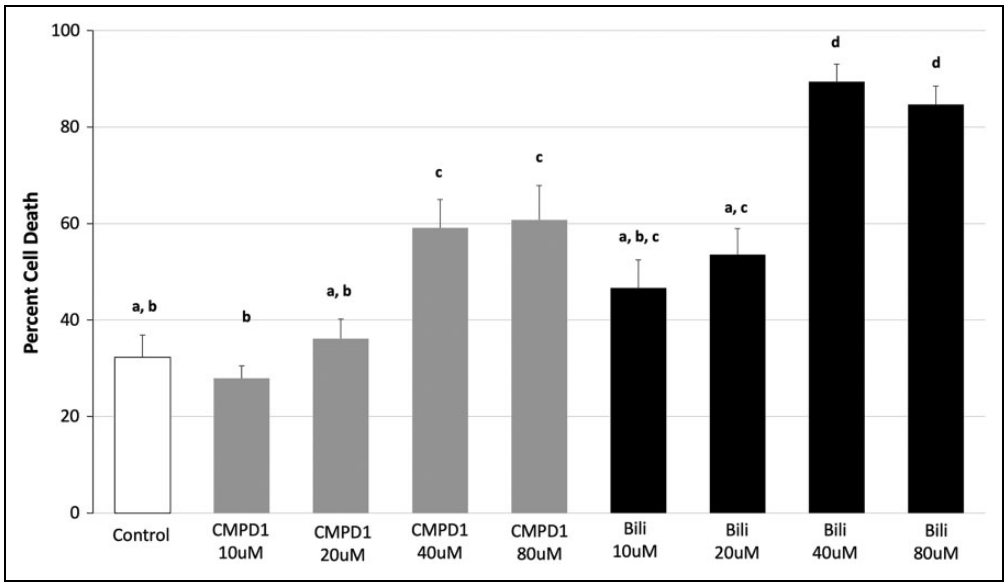


**Fig. 5.** Islet cell death (%) following treatment with increasing concentrations (μM) of Compound **1** and natural bilirubin compared to untreated (vehicle control only) islets. Islets were placed in media containing vehicle control or varying concentrations of Compound 1 or natural bilirubin following by incubation under standard conditions for 48 h. Cell death was evaluated using propidium iodide and is presented as mean ± standard error of the mean % islet cell death for each group. Significant differences are indicated by groups with differing letters (a, b, c, d). Significant dose-dependent cytotoxicity is seen with increasing concentrations (≥40 μM) of both Compound **1** and natural bilirubin with the lowest levels of cytotoxicity seen with islets treated with Compound **1** at 10 μM.

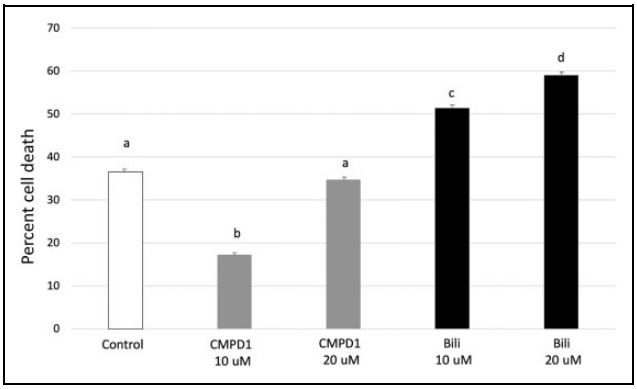


**Fig. 6.** Islet cell death (%) following hypoxic injury and treatment with Compound **1** and natural bilirubin compared to untreated (vehicle control only) islets. Islet cells were placed in media containing vehicle control or varying concentrations of Compound **1** or natural bilirubin and were subjected to 24 h of hypoxia. Cell death was evaluated using propidium iodide staining and is presented as mean ± standard error of the mean % islet cell death for each group. Significant differences are indicated by groups with differing letters (a, b, c, d). Significant cytoprotection was noted for islets treated with Compound **1** at 10 μM compared to natural bilirubin and control. Natural bilirubin was not found to exhibit islet cytoprotection in this study.

use in islet cell transplantation while avoiding the shortcomings of bilirubin procured from an animal source. We were able to successfully synthesize a novel bis-pyrrole compound and demonstrate that Compound **1** had a similar relative antioxidant activity to natural bilirubin as well as improved cytoprotective effects at doses of 10 to 20 μM in a model of hypoxic injury suffered by islets during transplantation.

The FRAP and ABTS*$^{+}$ assays are established methods of evaluating antioxidant activity of complex biologic substances, such as those found in plasma, and the ABTS*$^{+}$ assay has been used previously to confirm the powerful antioxidant capacity of bilirubin[20,21,28]. Consistent with previous reports, natural bilirubin was found to have robust antioxidant activity based on these methods, and, encouragingly, the antioxidant activity of Compound **1** was only shown to be marginally less than that of natural bilirubin, making it a potentially promising therapeutic with similar antioxidant-driven cytoprotective properties. Compound **2**, however, was not found to have noteworthy antioxidant capacity. The antioxidant properties of bilirubin are thought to be the product of free radical scavenging, inhibition of NADPH oxidase, which prevents superoxide production, as well as through the activity of the bilirubin–biliverdin redox cycle[29]. Bilirubin, a tetrapyrrole, operates as a free radical scavenger by donating a hydrogen atom attached to the C-10 bridge, forming a carbon-centered radical[11]. We sought to synthesize simpler, bis-pyrrole molecules that would retain this reactive hydrogen atom and antioxidant properties. Both Compounds **1** and **2** were bis-pyrroles with a system of conjugated double bonds and potentially reactive hydrogen atoms; however, Compound **2** did not perform as favorably in the relative antioxidant screening. Antioxidant activity, as

it relates to chemical structure, can occur via several mechanisms: by reacting with peroxyl radicals that have weak O-H or N-H bonds, by reacting with alkyl radicals, decomposing hydrogen peroxide, deactivating metals, cyclic chain termination, and a combination of the above actions[30,31]. When considering antioxidant activity of our bis-pyrroles, the carbon-centered radical formed by oxidation is stabilized by the conjugated pi system and the ability of the pyrrole to donate electron density into this system. Due to the indole contained in Compound **2**, the stabilization of the carbon-centered radical may be diminished, making the C-10 bridged carbon less prone to oxidation, which may explain why Compound **2** underperformed in the relative antioxidant evaluation. However, both of our bis-pyrroles are aromatic in chemical structure and the definitive reason for improved antioxidant activity in Compound **1** as compared to Compound **2** remains unknown. Regardless, this result demonstrates that there are key attributes to Compound **1** that allow for it to mimic the function of bilirubin, and further studies are warranted to probe this structure–activity relationship.

Quercetin, a flavonoid with known vigorous antioxidant activity, was used as a positive control in our study when evaluating the relative antioxidant capacity of our novel analogs[32]. It is worth noting that, although both natural bilirubin and Compound **1** show less antioxidant capacity than quercetin throughout most of the concentration range used in these assays, when scrutinizing the activity within the physiologic range of bilirubin (10 to 20 μM), results would suggest that both natural bilirubin and Compound **1** may have improved antioxidant effect as compared to the quercetin control. However, this finding is an extrapolation based on best-fit equations for the data collected during these assays, as specific data points within the physiologic range of bilirubin were not evaluated. In vivo doses of natural bilirubin are unlikely to exceed 20 μM, as higher doses lead to significant cytotoxicity, making the 10 to 20 μM therapeutic range the most important when considering antioxidant capabilities of bilirubin and analogs for use in transplantation. Unfortunately, the FRAP and ABTS*$^{+}$ screening assays are performed at much higher concentrations and, although natural bilirubin and Compound **1** may have more potent antioxidant effects within this clinically relevant range than are obvious here, more specific conclusions within that narrow range cannot be made. Regardless, it is clear based on these results that, throughout a massive range of concentrations, natural bilirubin and Compound **1** show compelling antioxidant activity.

Dose-dependent cytotoxicity was seen in islet cells treated with increasing concentrations of both natural bilirubin as well as Compound **1**, with doses above 20 μM showing significant cell death when compared to control cells. Supraphysiologic doses of natural bilirubin exceeding 25 to 50 μM have been shown to hinder mitochondrial cellular respiration and induce cellular apoptosis and necrosis via intracellular signaling mechanisms[7,33]. It is likely that similar biologic mechanisms, consistent with those seen in clinically icteric patients, resulted in the toxicity seen with both natural bilirubin and Compound **1** treatment. A trend toward improved cytotoxicity was noted when islets were treated with Compound **1** vs natural bilirubin, with significant differences in cell death rates at 40 and 80 μM. A narrow safe therapeutic dose range for natural bilirubin between 8.5 and 10 μM is currently accepted to avoid lack of cytoprotective effects as well as cytotoxic effects that can be seen when administered outside this range[15]. Our results suggest that Compound **1** may be less toxic than natural bilirubin when used ex vivo in transplant solutions and may provide a wider safety margin and therapeutic range for medicinal use.

A protective effect on murine islets following hypoxic stress was seen when cells were treated with Compound **1** at 10 μM. Compound **1** showed favorable performance in the relative antioxidant screening assays, leading us to suspect that powerful antioxidant activity, similar to that seen with natural bilirubin, is an integral component of Compound **1**'s mechanism of cytoprotection. Interestingly, Compound **1** did not show improved antioxidant activity when compared to natural bilirubin during screening evaluation, and yet Compound **1** had improved cytoprotective effect, showing significantly less cell death when compared to bilirubin in the model of islet transplant hypoxia. One explanation could be that the mechanisms behind Compound **1**'s cytoprotective effect is complex and may include other beneficial properties in addition to antioxidant activity. In addition, improved cytotoxicity with Compound **1** may allow persistence of antioxidant activity without the same degree of hindrance of cellular mechanisms as is seen with natural bilirubin. Further elucidation of all mechanisms contributing to the cytoprotective effect seen with Compound **1** is warranted, and future directions should aim to identify all beneficial cellular protection mechanisms.

In contrast to some previous reports, we were not able to show a significant protective effect on isolated murine islet cells following hypoxic injury treated with natural bilirubin at 10 to 20 μM when compared to vehicle control islets. However, similar reports out of our laboratory have suggested that, although significant improvements in cell survival are seen in murine islets treated with natural bilirubin following subjection to 3 h of hypoxia, prolonged exposure to hypoxic conditions exceeding 24 h may overwhelm bilirubin's protective antioxidant mechanisms[24,34]. Hypoxia and nutrient deprivation due to impaired diffusion, resulting in a gradual transition from apoptosis to necrosis within the central cells of hypoxic pancreatic islets, is thought to be the primary mechanism of death when isolated islets are subjected to prolonged hypoxic conditions[35]. Impaired diffusion of bilirubin, especially to the central cells of the hypoxic islet, likely contributes to islet cell loss under prolonged hypoxic conditions, and perhaps improved diffusion of a smaller and less complex bilirubin analog, such as

Compound **1**, influenced the analog's ultimate improved cytoprotective effect.

These findings also highlight another dilemma with bilirubin as a therapeutic for islet allograft transplantation, namely bilirubin's poor bioavailability, being an insoluble compound in water at physiologic pH and a highly protein-bound substance in plasma[7]. Although Compound **1** was similarly insoluble in water, both bilirubin and Compound **1** were readily dissolved in DMSO, allowing ease of ex vivo therapeutic use in organ transplant solutions, which, some studies have suggested, may be a preferable method to pre-treatment of islet donors[16]. Improved methods of drug delivery of these less bioavailable substances to islet cells are another important area of ongoing research within our laboratory, and even though Compound **1** already shows enhanced cytoprotective effect vs natural bilirubin, which may suggest improved diffusion and bioavailability, continued efforts to optimize drug delivery should be prioritized.

Some limitations should be taken into consideration when interpreting the results of this study. In vitro studies are inherently unable to adequately predict the viability, functionality, and therefore ultimate success of islet cells transplanted in vivo, regardless of therapy employed. Additional practical aspects of Compound **1**, such as solubility, bioavailability, and cellular uptake under physiologic conditions were not assessed in this study and are important to its use in clinical medicine. In addition, although murine islets are commonly used as a model for human and veterinary islet transplantation and diabetes, it is important to keep in mind that the anatomy and physiology of islets vary depending on species, and extrapolation to humans based on murine islet data should be done with caution[36].

Based on the synthetic nature of the bilirubin analog Compound **1** and its demonstrated protective effects in models of transplant-induced stress, it would be appropriate to consider the application of this molecule as an additive to islet preservation media used in cellular transplantation. Future directions should include further identification and understanding of the mechanisms of cellular protection provided by Compound **1**, improved practical methods for drug delivery, and in vivo assessment of the cytoprotective and cytotoxic effects of Compound **1**.

## Conclusions

In conclusion, Compound **1** was found to have promising antioxidant and cytoprotective effects, similar to or improved from that of natural bilirubin, limiting islet cell death in a model of islet transplantation hypoxic stress. Sterile, mass synthesis and production of Compound **1** could provide a safe and effective means of cytoprotection of fragile islets in ex vivo transplant solutions, providing improved outcomes for curative intent transplantation of islets for T1DM. Compound **1** may serve as a synthetic drug lead for clinical islet transplantation and further evaluation of this molecule is warranted.

### Data Availability

The nuclear magnetic resonance data used to support the findings of this article are included within the article and within the supplementary information files. The FRAP, ABTS*$^{+}$, cytotoxicity, and viability following hypoxic injury data are included within the article.

### Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee of North Carolina State University, Raleigh, NC, USA.

### Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the NIH Guidelines for Animal Care and Use, Washington, DC, USA.

### Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the North Carolina State University Research and Innovation Seed Funding Program (grant number 77266).

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### Supplemental Material

Supplemental material for this article is available online.

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