Bioconjugate Chemistry

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Synthesis and Characterization of an Anti-Apoptotic Immunosuppressive Compound for Improving the Outcome of Islet Transplantation

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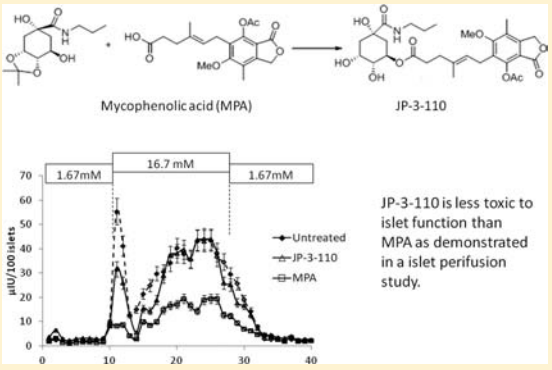
Supporting Information

ABSTRACT: Mycophenolic acid (MPA) is a commonly used immunosuppressive drug for human islet transplantation. However, it is toxic to transplanted islets, causing primary nonfunction. We recently synthesized a quinic acid derivative, 1,3,4,5-tetrahydroxy-N-propylcyclohexanecarboxamide (KZ41), which has anti-inflammatory and anti-apoptotic effects. We hypothesized that the conjugate (*E*)-2,3,5-trihydroxy-5-(propylcarbamoyl) cyclohexyl 6-(4-ethoxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate (JP-3-110), which is composed of KZ41 and MPA through esterification, can suppress the immune rejection while inducing less toxicity. Early characterization showed that the solubility of JP-3-110 was significantly higher than that of MPA, though JP-3-110 was still poorly water-soluble. The ester bond connecting KZ41 and MPA is stable for a limited duration (<4 weeks). Pharmacological studies demonstrated that JP-3-110 induced significantly less activated caspase 3 and apoptotic cell death of human islets than MPA, while maintaining an equally potent immunosuppressive effect. A similar immunosuppressive effect of JP-3-110 and MPA in humanized NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD *scid* gamma, NSG) mice with adoptively transferred human immunity was observed. Taken together, our results demonstrated that JP-3-110 can be a safer immunosuppressive agent for human islet transplantation.



INTRODUCTION

Clinical islet transplantation has met great success from benchtop to bedside in the last two decades for treating type I diabetes. More than 500 patients with type I diabetes have received human islet transplantation worldwide and demonstrated improved quality-of-life afterward. However, the wide application of human islet transplantation is still hindered by two major barriers, the limited supply of donor islets and the inadequate means to prevent immune rejection.[1] Immune rejection is the most important reason for graft failure after islet transplantation. Although the immunosuppressive drugs such as tacrolimus, sirolimus, and mycophenolic acid (MPA) can effectively prevent immune rejection, these drugs also impair insulin release from human islets, and long-term injections of these drugs may cause loss-of-function of human islets, a status characterized as the primary nonfunction (PNF) of human islets.

MPA is among the most commonly used immunosuppressive drugs in human islet transplantation. It inhibits inosine 5′-monophosphate dehydrogenase (IMPDH), an essential enzyme mediating the purine synthesis in T cells and B cells. It also induces a downregulation of anti-apoptotic factors such as B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-xL) and an accumulation of pro-apoptotic mediators such as caspase 3 and small mitochondria-derived activator of caspases (SMACs), suggesting that MPA impairs islet function through the activation of the apoptotic pathway in human islets.[2,3] We recently synthesized a quinic acid derivative, 3,4,5-tetrahydroxy-*N*-propylcyclohexanecarboxamide (KZ41), and demonstrated its anti-inflammatory effect in A549 cells.[4] KZ41 inhibited the nuclear translocation of NF-κB, while MPA is known to suppress the phosphorylation of NF-κB,[5] suggesting potential synergistic effect in preventing the immune rejection of islet grafts. On the other hand, KZ41 suppresses iNOS and caspase 3 activity, suggesting that KZ41 may counteract the pro-apoptotic effect of MPA and mitigate the pro-apoptotic effect of MPA to transplanted human islets. Hereby, we synthesized (*E*)-2,3,5-trihydroxy-5-(propylcarbamoyl)cyclohexyl 6-(4-ethoxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate (JP-3-110) by conjugating KZ41 and MPA via ester bond and evaluated the safety and effectiveness of this new compound to inhibit the alloreactivity of human peripheral blood mononuclear cells (PBMCs) and to protect insulin release ability of human islets. We hypothesized that the conjugate would be friendlier to transplanted islet without compromising the

Received: August 6, 2013
Revised: November 9, 2013
Published: November 20, 2013

   dx.doi.org/10.1021/bc400369t | *Bioconjugate Chem.* 2013, 24, 2036−2044

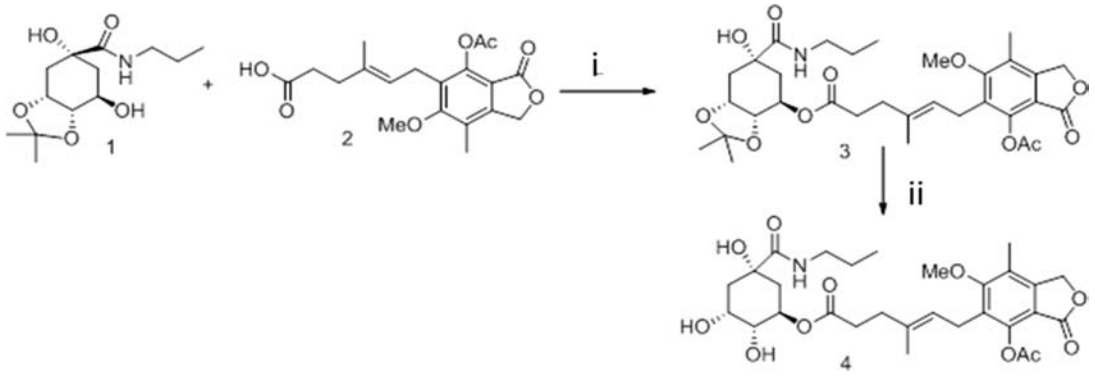


Figure 1. Synthetic scheme of JP-3-110. (i) DIC, DMAP, DCM, 73%; (ii) IN HCl/THF, 12 h, 21%.

immunosuppressive effect. We also expected the conjugate to serve as an initial trial to deliver two active compounds simultaneously to two distinct therapeutic targets.

EXPERIMENTAL PROCEDURES

Materials. Rat insulinoma INS-1E cell is a kind gift from Professor Claes B. Wolheim (University Medical Center, Geneva, Switzerland). Human islets were received from Integrated Islet Distribution Program (Duarte, CA). CMRL-1066 medium for islet culture and DAPI were purchased from Sigma Aldrich (St. Louis, MO). FBS was purchased from MediaTech Cellgro. (Herndon, VA). PBS was purchased from GIBCO-BRL (Gaithersburg, MD). NF-κB SEAPorter Assay Kit was purchased from IMGENEX (San Diego, CA). Human IL-2, IL-2sRa, IL-10 ELISA, TNFα, and IFN-γ ELISA kits were purchased from R&D Systems (Minneapolis, MN). The primary antibodies for CD3, CD4, insulin, and Dylight 488-conjugated secondary antibody were purchased from Abcam (Cambridge, MA). Alexa Fluor 568-conjugated secondary antibody and 0.25% trypsin were purchased from Invitrogen (Carlsbad, CA). Ultrasensitive One Touch glucose test strips and One Touch Ultra glucometer were purchased from LifeScan (Milpitas, CA). Tissue-Tek O.C.T. compounds were purchased from Sakura Finetek (Torrance, CA).

Synthesis and Characterization of Anti-Apoptotic Immunosuppressive Drug. The synthetic scheme of JP-3-110 is shown in Figure 1. All reagents for the synthesis were purchased from commercial sources and were used without further purification. Moisture-sensitive reactions were carried out under an argon atmosphere. Routine TLC was performed on aluminum backed Uniplates (Analtech, Newark, DE). NMR spectra were obtained on a Bruker ARX-400 MHz (Billerica, MA) or a Varian Inova-500 MHz spectrometer (Varian NMR Inc., Palo Alto, CA). Chemical shifts are reported as parts per million (ppm) relative to tetramethylsilane (TMS) (0 ppm) in $CDCl_3$. Temperature was regulated with a general accuracy of ±0.1 °C. Mass spectral data were collected on a Bruker ESQUIRE-LC/MS system equipped with an ESI source. The synthesis of JP-3-110 used in the pancreatic islet protection studies was synthesized as illustrated in Figure 1. The starting compound 5,7-dihydroxy-2,2-dimethyl-*N*-propyl-hexahydrobenzo[*d*][1,3]dioxole-5-carboxamide (amide 1), which has been described before,[6] was coupled with acetylated MPA 2 to obtain quinic acid ester 3 and further deprotection of acetanoid group using 1N HCl in tetrahydrofuran (THF) to give compound 4.

Synthesis of Compound ***2***. Acetic anhydride (0.2 mL, 1.9 mmol) was slowly added to a pyridine (5 mL) solution of MPA (0.2 g, 0.62 mmol) and 4-dimethylaminopyridine (DMAP, 0.03 g, 0.25 mmol) at 0 °C. The reaction mixture was stirred for 2 h and then poured onto crushed ice. The aqueous phase was acidified with 2 M aq. HCl (pH ∼ 2) and extracted with EtOAc (3 × 400 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and the solvent removed under reduced pressure to afford acetylated MPA 2 (0.18 g, 83%) as a white powder mp 155−157 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.18−5.5 (m, 3H), 3.79 (s, 3H), 3.28 (d, J = 6.9 Hz, 2 H), 2.43−2.38 (m, 5H), 2.33−2.28 (m, 2H), 2.21 (s, 3H), 1.79 (s, 3H). Mass: 385.2 (M+Na).

Synthesis of Compound ***3***. A solution of amide 1 (0.32 g, 0.88 mmol), acetylated MPA 2 (0.24 g, 0.88 mmol), *N,N*-dimethyl amino pyridine (0.16 g, 1.32 mmol), and di-isopropyl carbodiimide (0.21 mL, 1.32 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature under Ar atmosphere overnight. The reaction mixture was diluted with $CHCl_3$ (20 mL) and then washed with 1 N HCl (10 mL), water (10 mL), aqueous saturated $NaHCO_3$ solution (10 mL), and brine (10 mL). The organic layer was dried (Na_2SO_4) and evaporated, and the residue was purified by column chromatography (silica gel, 30% acetone in petroleum ether) to afford (*E*)-6-hydroxy-2,2-dimethyl-6-(propylcarbamoyl)-hexahydrobenzo[*d*][1,3]dioxol-4-yl-6-(4-acetoxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate (compound **3**, 0.4 g, 73%) as a white solid mp 71−73 °C.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.08 (t, 1 H), 5.32−5.23 (m, 1H), 5.17 (s, 1H), 5.15−5.08 (m, 1H), 5.32−5.24 (m, 1H), 4.54−4.48 (m, 1H), 4.14−4.08 (m, 1H), 3.84 (s, 1H), 3.78 (s, 3H), 3.5 (d, J = 6.9 Hz, 2 H), 3.24−3.15 (m, 2H), 2.45−2.36 (m, 5H), 2.33−2.28 (m, 2H), 2.23 (s, 3H), 2.03−1.90(m, 2H), 1.76 (s, 3H), 1.58 (s, 3H), 1.55−1.48 (m, 2H), 1.36 (s, 3H). 0.93 (t, 3H). Mass: 640.3 (M+Na).

Synthesis of Compound ***4***. Compound **3** (0.4 g, 0.64 mmol) was dissolved in THF (3 mL), then aqueous 1 N HCl (5 mL) was added at room temperature, and the reaction mixture was stirred for 12 h and monitored by ESI-MS. After completion of the reaction, solution was saturated with solid NaCl and aqueous phase was extracted with ethyl acetate (3 × 100 mL). The organic layer was dried (Na_2SO_4) and evaporated, and the residue was purified by column chromatography (silica gel, 90% ethyl acetate in petroleum ether) to afford compound **4** (0.08 g, 21%) as a white solid mp 74−76 °C.

dx.doi.org/10.1021/bc400369t | *Bioconjugate Chem.* 2013, 24, 2036−2044

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.08 (t, 1 H), 5.22−5.16 (m, 3H), 5.11−5.05 (m, 1H), 4.84 (s, 1H), 4.29−4.24 (m, 1H), 3.80 (s, 3H), 3.68−3.61 (m, 1H), 3.38 (d, J = 6.9 Hz, 2 H), 3.32−3.30 (M, 1H), 3.25−3.16 (m, 3H), 2.45−2.36 (m, 5H), 2.33−2.28 (m, 2H), 2.23 (s, 3H), 2.15−2.0 (m, 4H), 1.8 (s, 3H), 1.51−1.50 (m, 2H), 0.9 (t, 3H). Mass: 578.3 (M+H).

NF-κB Activity. INS-1E cells which were stably transfected with SEAP gene containing the response-element for NF-κB were used to screen for anti-inflammatory activity. A cytokine cocktail (10 ng/mL TNF-α, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ) was used to mimic the in vivo challenge to the INS-1E cells and human islets by the inflammatory cytokines. Briefly, 10^6 cells/well were seeded overnight followed by treatment with cytokine cocktail and either KZ41 or JP-3-110 (1 μM) at the same time. SEAP activity was measured 18 h later in supernatant samples (50 lL) using NF-κB SEAPorter Assay Kit (IMGENEX, San Diego, CA) and a microplate luminometer. SEAP activity was normalized to the total protein content. Inhibitory potency (IC_{50}) was determined from dose−response curves (n = 3 separate experiments).

Apoptosis Studies. Caspase-Glo 3 assay kits were used to analyze caspase 3 as per the manufacturer's protocol (Promega, Madison, WI). This assay kit provides a proilluminescent caspase substrate, DEVD, that, when cleaved by caspases, will release luciferin to quantitatively determine caspase concentration. Briefly, INS-1E cells were treated with MPA (20 μM), MPA+KZ41 (each of 20 μM) and JP-3-110 (20 μM) for 2 days. 100 μL of Caspase-Glo reagent was added to 100 μL of culture supernatants in 96-well plates and incubated at room temperature for 1 h. The contents were then transferred into culture tubes, and luminescence was determined using a luminometer (Berthold, Germany). The apoptosis of INS-1E cells was analyzed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). After the same treatment mentioned before, INS-1E cells were characterized by DeadEnd Colorimetric TUNEL system (Promega, Madison, WI), in which fragmented DNA from apoptotic cells was labeled with biotinylated nucleotide and detected using hydrogen peroxide and diaminobenzidine. For human islets, following treatment with MPA (20 μM), MPA+KZ41 (each of 20 μM), and JP-3-110 (20 μM) for 5 days, human islets were digested with 0.25% trypsin/EDTA into a single-cell suspension, stained with Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA), and analyzed with flow cytometry. Annexin V binds to phosphatidylserine on the cell surface, which is a feature found in apoptotic cells. Fluorescent intensity was analyzed using CellQuest software (BD Bioscience, Franklin Lakes, NJ). Three sets of independent transduction experiments were carried out for each assay.

Insulin Release. Insulin secretion from human islets was quantified using a dynamic islet perifusion assay as described before.[7] Briefly, 50 islets from each group were handpicked and loaded onto a Swinnex 13 chamber (Millipore, Burlington, MA) and perifused with Krebs-Ringer bicarbonate HEPES buffer of the following composition (in mM): 129 NaCl, 5 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 10 HEPES at pH 7.4. The flow rate was maintained at 1 mL/min with a peristaltic pump (Themo Fisher, Waltham, MA) and the temperature was maintained at 37 °C with a solution heater (Warner Instruments, Hamden, CT). Islets were first perifused with basal glucose for 60 min, stimulatory glucose for 20 min, and basal glucose until insulin release reversed to the basal level. The perifusion speed was set to 2 mL/min and samples were collected once per minute through an automatic fraction collector (Waters, Milford, MA) and analyzed for insulin content by Insulin Ultrasensitive EIA kit (Alpco Diagnostics, Salem, NH).

Mixed Lymphocyte Reaction (MLR). PBMCs were isolated using Ficoll Paque. T cells were isolated from PBMCs using Dynabeads. T cells were stimulated with phytohemagglutinin (PHA) (1 μg/mL) for 24 h alone or in the presence of MPA (5 μM) or JP-3-110 (5 μM) for additional 3 days. The total number of PBMCs was measured using T4 Automatic Cell Counter (Nexcelom, Lawrence, MA). The extent of T cell activation was determined by measuring the level of IL-2, IL-2sRa, TNFα, and IFN-γ in the medium using ELISA at indicated time.

Humanized NSG mice. Human PBMCs were isolated from buffy coats by gradient centrifugation using Ficoll Paque as described before.[8] T cells and B cells of PBMCs were stained with FITC conjugated CD3 antibody and APC conjugated CD19 antibody, respectively, and characterized using flow cytometry. Humanized NSG mouse was generated as described before.[8] Briefly, PBMCs (5×10^6/mouse) were injected peritoneally into NSG mice at the concentration. Potent human IgG in the mouse bloodstream and evident human CD3 staining in the spleen sections were detected at around two weeks after the injection, suggesting the establishment of human immunity. Then, each mouse was subjected to 7 consecutive shots of JP-3-110 (50 mg/kg), MPA (50 mg/kg), and equal amount of saline for 7 days.

To determine the functions of T cells, spleens of humanized NSG mice were isolated, washed with PBS, fixed in 4% paraformaldehyde overnight, and embedded in optimal cutting temperature compound. Frozen sections of 5 μm thickness were cut. The slides were stained with rabbit anti-human CD3 primary antibody (1:200) at 4 °C overnight and Alexa Fluor 568 conjugated goat anti-rabbit secondary antibody (1:500) at room temperature for 1 h. To determine the function of B cells, whole blood of NSG mice was collected. The serum was isolated by immediate centrifugation. The function of B cells was assessed by measuring human IgG concentration in the mouse serum.

Statistical Analysis. Statistical significance of the difference between the two groups was determined by unpaired t test and between several groups by one-way ANOVA.

RESULTS

Synthesis and Characterization of JP-3-110. Quinic acid amide analogue KZ41 was synthesized as described before.[4] JP-3-110 was synthesized by conjugating KZ41 with MPA (Figure 1). The structure of JP-3-110 was confirmed by ^{1}H NMR (Figure S1). The solubility of JP-3-110 was determined to be 0.48 ± 0.16 mg/mL (0.80 ± 0.27 mM) by HPLC (Figure S2), which is practically insoluble according to USP solubility criteria. The drug JP-3-110 and KZ41 were directly dissolved in water. The drug MPA was first dissolved in DMSO as a stock solution then dissolved in water. The concentration of DMSO was controlled below 0.1% percent to make sure that DMSO will not compromise the viability and function of INS-1E cells and human islet.

JP-3-110 Suppressed NF-κB Activity. We first tested whether KZ41 and JP-3-110 suppressed NF-κB activity in insulin producing INS-1E cells which had been stably transfected with SEAP gene containing the response-element for NF-κB. A cocktail of inflammatory cytokines (10 ng/mL

TNFα, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ) was used to mimic the inflammation in vivo and added into the INS-1E cell culture alone or with MPA, KZ41, and JP-3-110 at the same time. We demonstrated that MPA, KZ41, and JP-3-110 at 5 μM significantly reduced NF-κB activity in INS-1E cells after 18 h (Figure 2). Among three groups, JP-3-110 caused the most reduction in NF-κB activity, suggesting the synergistic anti-inflammatory effect of KZ41 and MPA.

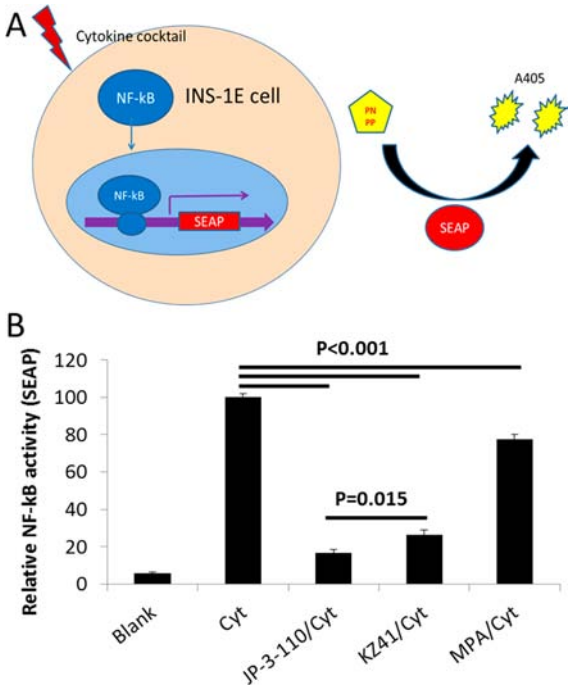


Figure 2. JP-3-110, KZ41, and MPA suppressed NF-kB activity in INS-1E cells. (A) Schematic illustration of the mechanism of SEAP reported gene assay to determine NF-kB activity. Briefly, a plasmid construct containing NF-kB promoter and SEAP gene was transferred into INS-1E cells by lipofectamine. SEAP was then expressed and secreted into culture supernatant and allowed chemiluminescent detection using a substrate PNPP under 405 nm. (B) NF-kB activity was measured 24 h after addition of inflammatory cytokine cocktail (10 ng/mL TNFα, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ) alone or with JP-3-110 (5 μM) or KZ41 (5 μM) or MPA (5 μM). Blank, no cytokine stimulation. Cyt, cytokine cocktail. Results are presented as the mean ± S.D., n = 3.

JP-3-110 Held Similar Immunosuppressive Effect as MPA. Mixed lymphocyte reaction was used to determine the immunosuppressive effects of MPA and JP-3-110. We did not observe any immunosuppressive effect of KZ41 (data not shown). Our results showed that JP-3-110 was equally potent as MPA to suppress the proliferation of T cells in 4 days (Figure 3A). The levels of IL-2, IL-2sRa, TNFα, and IFNγ were detected by ELISA to determine the effects of JP-3-110 and MPA on T cell activation. IL-2 and IL-2R typically peaked at the first 48 h after PHA stimulation as markers for the early stage T cell activation, while TNFα and IFNγ were constitutively expressed by PHA-stimulated T cells as later-stage markers for T cell activation. JP-3-110 and MPA did not have any effect on the levels of IL-2 and IL-2 receptors (Figure 3B), suggesting that JP-3-110 and MPA did not block the IL-2-dependent T cell proliferation. Surprisingly, JP-3-110 and MPA demonstrated moderate inhibition of the levels of TNFα and IFNγ (Figure 3C). However, the results might be due to an

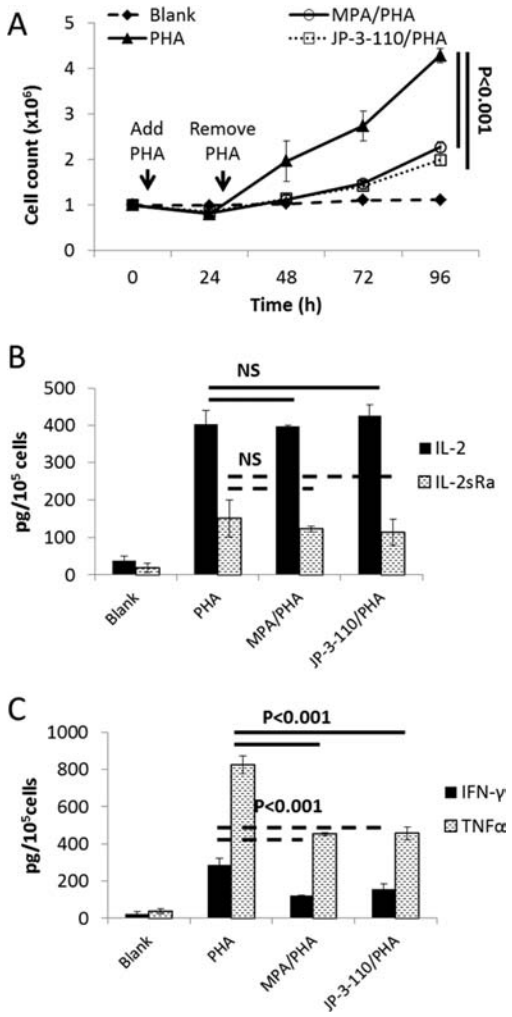


Figure 3. JP-3-110 suppressed the proliferation but not the activation of T cells. (A) The proliferative curve of the PHA-stimulated T cells under the treatment of JP-3-110 (5 μM) and MPA (5 μM). Briefly, human peripheral blood mononuclear cells PBMCs were isolated from human buffy coat using Ficoll-Paque. T cells (5×10^5) were isolated from PBMCs using Dynabeads for human CD3+ T cells and subjected to PHA (1 μg/mL) stimulation for 24 h alone or with JP-3-110 (5 μM) and MPA (5 μM) for additional 3 days. The PHA-stimulated proliferation of T cells is characterized by a quick drop in the early stage (24 h) and a following fast-proliferative stage. (B-C) The levels of IL-2, IL-2 receptor, TNFα, and IFNγ in the media of T cells at 48 h following PHA stimulation. IL-2 and IL-2 receptor were transient expressed markers for early stage T cell activation, while TNFα and IFNγ were stably expressed markers for later-stage T cell activation. Results are presented as the mean ± S.D., n = 6.

overlap between the immunosuppressive pathway and the anti-inflammatory pathway of JP-3-110 and MPA. Taken together, the results indicated that JP-3-110 retained the similar immunosuppressive effect as MPA.

JP-3-110 Suppressed the Function of T Cells and B Cells in Humanized NSG Mice. To test the immunosuppressive effect of JP-3-110 in vivo, a humanized mouse model was used as described before.[8] JP-3-110 and MPA were administrated intraperitoneally at 50 mg/kg for 7 consecutive shots. At the end of the study, the amount of human CD3+ T cells in the mouse spleen (Figures 4A-B) and the serum human IgG level (Figure 4C) were significantly reduced in the mice receiving JP-3-110 and MPA administration compared with the

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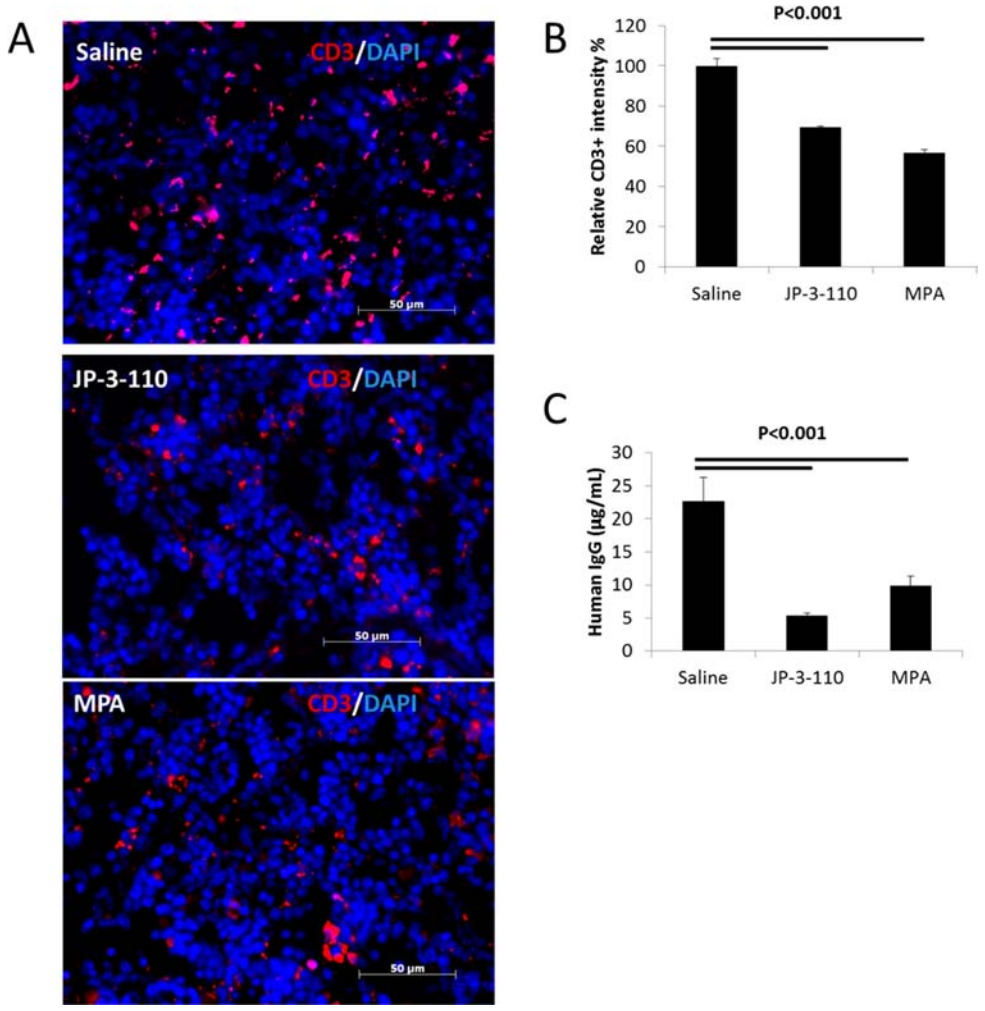

Figure 4. JP-3-110 and MPA inhibited the function of T cells and B cells in humanized NSG mice. Human PBMCs (5×10^6/mouse) were injected intraperitoneally into NSG mice to introduce human immunity. (A) A representative spleen section of humanized NSG mice after receiving 7 consecutive shots of JP-3-110 (50 mg/kg, middle), MPA (50 mg/kg, lower), and equal amount of saline (upper). Sections were stained to indicate human CD3+ T cells (red) and counterstained with DAPI (blue). (B) The relative T cell intensity in the spleen sections of humanized NSG mice was quantified by ImageJ. (C) The serum IgG level of humanized NSG mice. Results are presented as the mean ± S.D., $n = 5$.

mice receiving equal amounts of saline, suggesting the immunosuppressive effect of JP-3-110. MPA showed better immunosuppressive effect than JP-3-110 in humanized NSG mice, a result probably caused by the greater molar amount of MPA injected into each NSG mouse.

JP-3-110 Showed Less Pro-Apoptotic Effect than MPA. We then determined whether conjugation with KZ41 can reduce the pro-apoptotic effect of MPA. A relatively higher concentration (20 μM) of MPA was used to induce cytotoxicity. We first measured the caspase 3 activities of insulin-producing INS-1E cells under the stimulation of MPA alone, or MPA with free KZ41, or JP-3-110 (MPA conjugated with KZ41). Results showed that the presence of KZ41 in the media, in both a free form and a conjugated form, effectively reversed the elevation of caspase 3 activity caused by MPA (Figure 5A). TUNEL assay also suggested that the apoptotic cell death caused by MPA was effectively reversed by KZ41, in both a free form and a conjugated form (Figure 5B). We then determined the protective effect of KZ41 on human islets against MPA. MPA induced substantial apoptotic cell death in human islets after long-term coculture. However, the conjugated drug, JP-3-110, significantly reduced the percentage of apoptotic cells, suggesting less cytotoxicity to human islets than MPA (Figure 5C).

JP-3-110 Does Not Affect Insulin Release of INS-1E Cells and Human Islets. A static insulin release assay was used to evaluate the function of INS-1E cells. Although MPA (20 μM) did not affect the basal insulin release from INS-1E cells, MPA greatly impaired the stimulatory insulin release from INS-1E cells (Figure 6A) and consequently reduced the stimulation index (Figure 6B). However, JP-3-110 at the same concentration showed significantly less toxicity compared with MPA. A dynamic insulin release assay was used to evaluate the function of human islets. Briefly, 100 handpicked human islets were sequentially perfused with basal and stimulatory glucose. The samples were collected using an autosampler and subjected to ELISA. Fresh islets showed a typical biphasic pattern of insulin release under stimulatory glucose with a sharp and rapid release in the first two minutes and a long-lasting release afterward (Figure 6). The coincubation with MPA impaired the insulin release from both phases and led to a significant reduction in the total insulin release to stimulatory glucose (Figure 6). However, JP-3-110 showed less toxicity to

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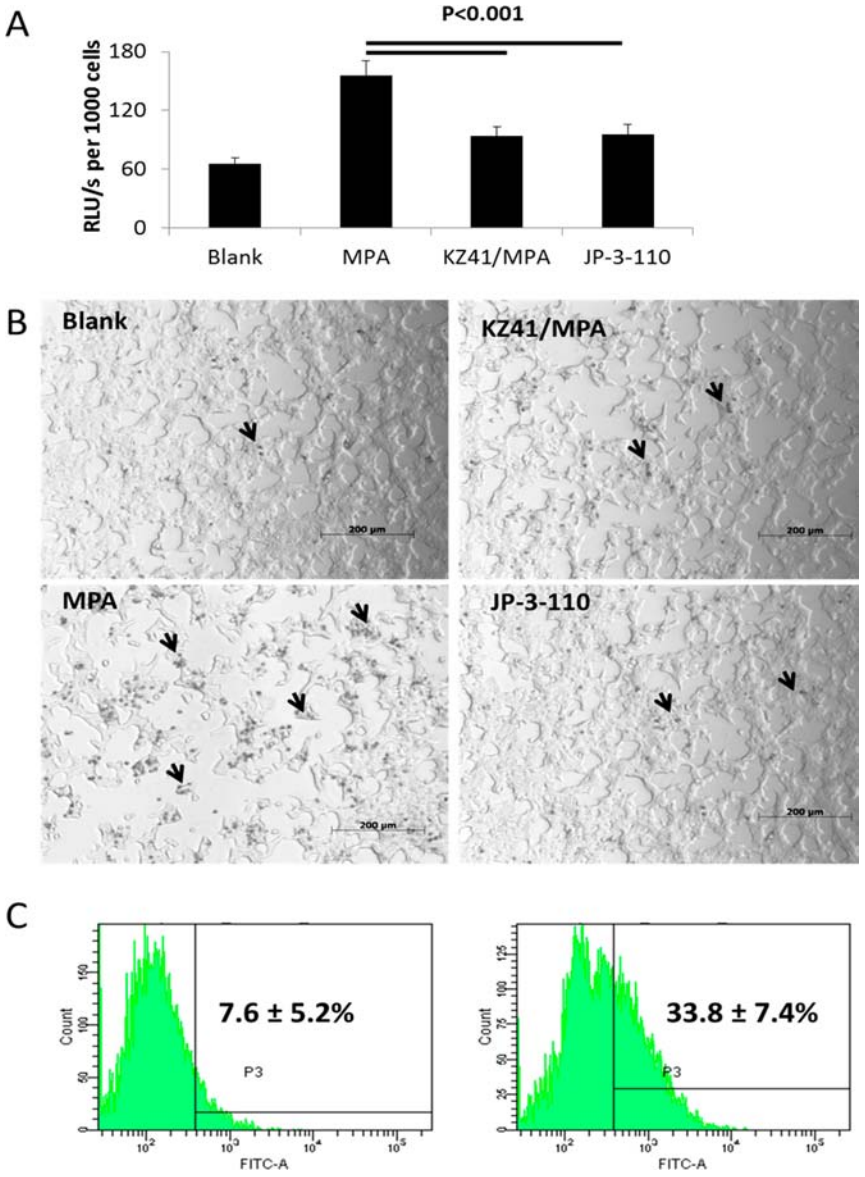

Figure 5. JP-3-110 showed less cytotoxicity to INS-1E cells and human islets than MPA (A) Caspase 3 activities in INS-1E cells after treated with MPA (20 μM), MPA+KZ41 (each of 20 μM), and JP-3-110 (20 μM) for 2 days. Results are presented as the mean ± S.D., n = 3. (B) TUNEL assay of INS-1E cells as determined by DeadEnd Colorimetric TUNEL system. Apoptotic cells were stained in dark (arrows). (c) JP-3-110 was less toxic to human islets than MPA. Briefly, human islets were cultured with JP-3-110 (20 μM) or MPA (20 μM) for 5 days. Islets were collected and dispersed with 0.25% Trypsin/EDTA into single cell suspension. Apoptotic cells were stained with FITC labeled annexin V and counted by flow cytometry. P3 indicated the percentage of apoptotic cells. All experiments were performed in triplicates. Results are presented as the mean ± S.D., *p < 0.05, as determined by an unpaired Student's t test, n = 6.

the insulin release as demonstrated by the clear biphasic insulin release.

■ DISCUSSION

Human islet transplantation has significantly improved the quality-of-life of type I diabetic patients, rendering them insulin-independent for a considerably long period. However, life-long administration of immunosuppressive regimens is still required to prevent the immune rejection.[9]

MPA is a commonly used immunosuppressive drug for organ transplantation, especially human islet transplantation. MPA inhibits the purine synthesis and thereby the proliferation of T cells and B cells.[10] However, MPA also damages the viability and function of human islets in the coculture condition, leading to a status named as PNF of islet transplantation (2). The PNF of human islets is typically caused by the inflammatory cytokines, reactive oxygen species, or poor revascularization and frequently linked with the activation of apoptotic pathway.[11] The administrations of MPA further exaggerate PNF by suppressing Bcl-2 and Bcl-xL and activating caspase 3 and SMACs.[2,3] We previously reported that silencing of caspase 3 or overexpression of X linked inhibitor of apoptosis (XIAP) through gene therapy would lead to a survival advantage of human islets against the PNF caused by inflammatory cytokines.[12,13] Hereby, we reported a new strategy to counteract the pro-apoptotic effect of MPA through chemical conjugation.

KZ41 is a new compound recently synthesized by Zeng et al. who demonstrated its potent anti-inflammatory and anti-apoptotic activities.[4,6] KZ41 suppressed the translocation of

dx.doi.org/10.1021/bc400369t | Bioconjugate Chem. 2013, 24, 2036−2044

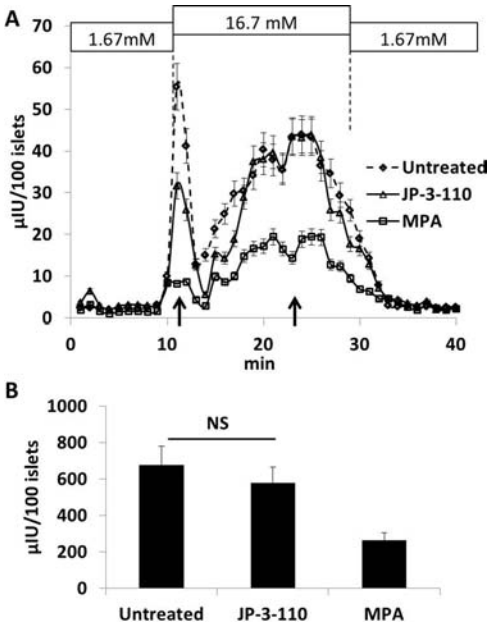

Figure 6. JP-3-110 did not affect the insulin release of INS-1E cells and human islets. (A) A dynamic insulin release assay was used to determine the function of human islets. Briefly, 50 islets from each group were perifused in KRB buffer containing basal glucose (1.67 mM) for 60 min and stimulatory glucose (16.7 mM) for 30 min and finally with basal glucose until insulin release reversed to the basal level. Samples were collected through an automatic fraction collector and analyzed for insulin content by ELISA. The ratio of insulin concentration at stimulatory glucose to that at basal glucose was used to calculate the stimulation index. (B) Cumulative insulin release calculated from (A).

NF-κB and subsequent transcription of inflammatory cytokines (Figure 2B).[4,6] MPA was also reported to be an inhibitor of the phosphorylation of NF-κB,[14] and therefore, we hypothesized that the conjugation of KZ41 with MPA to generate JP-3-110 should not reduce the immunosuppressive effect of MPA. JP-3-110 demonstrated similar immunosuppressive effect as MPA to prevent the proliferation of PBMCs in the mixed lymphocytes reaction (Figure 3A). Although JP-3-110 showed minimal effect in IL-2 dependent T cell activation, JP-3-110 suppressed the levels of TNFα and IFNγ produced by PBMCs (Figures 3B-C), possibly through NF-κB inhibition by KZ41 (Figure 2B). To understand the action of JP-3-110 in vivo, we rebuilt human immunity in NSG mice by adoptively transferring human PBMCs into immunodeficient NSG mice. Results showed that the daily administration of JP-3-110 and MPA suppressed the settlement of human T cells in the spleen and the production of human IgG by B cells in the serum (Figure 4). Taken together, all these results suggested that JP-3-110 suppressed the human immunity in a similar manner as MPA.

Then, we determined and compared the toxicity of JP-3-110 and MPA to insulin producing INS-1E cells and human islets. Briefly, we conducted the experiments through two aspects: (1) the impact of JP-3-110 and MPA on the apoptotic pathway and (2) the impact of JP-3-110 and MPA to insulin release of INS-E cells and human islets. We have previously demonstrated that downregulation of caspase 3 activity can prevent the apoptotic cell death of human islets.[12] Since KZ41 suppressed the caspase 3 activity at 5 μM, while MPA activated caspase 3 at a higher concentration (Figure 5A).[2] We hypothesized that the conjugation of KZ41 to MPA via an ester bond has the potential to reverse caspase 3 upregulation caused by MPA. The results confirmed our hypothesis (Figure 5A), possibly due to caspase inhibition. The percentage of apoptotic cell death in INS-1E cells and human islets caused by MPA was significantly reduced in the presence of KZ41, suggesting less toxicity of JP-3-110 (Figures 5B-C). To understand whether JP-3-110 impairs the function of insulin producing cells, we used a static incubation method for INS-1E cells and a dynamic perifusion method for human islets. The later method provide more information in determining the real-time responses of insulin producing cells but is only suitable for human islets that are cluster of cells. Our studies demonstrated that the coincubation with MPA significantly impaired the responses of insulin producing cells to stimulatory glucose, reducing the stimulation index in the static methods and the stimulatory AUC in the dynamic methods (Figure 6). However, JP-3-110 hardly induced impairment of insulin release, suggesting less toxicity.

In clinical human islet transplantation, islets isolated from donor are infused into the portal vein of the liver and settle in the lobules. The liver, as a transplantation site, is highly perfused and thus provides good oxygen and nutrition to the transplanted islets but also makes the islets susceptible to immunosuppressive drugs. Moreover, as a major site of drug metabolism, liver metabolizes a majority of MPA (87−94%) by acryl glucuronidation to generate 7-O-glucuronide of MPA (MPAG) and s small percentage of acyl glucuronide conjugates, both inducing inflammatory cytokine release and toxicity.[10,15,16] In this study, we first replaced 7-hydroxyl with 7-acetoxy to prevent the acryl glucuronidation on that site. We also conjugated KZ41 on the carboxyl group of MPA to prevent the acyl glucuronide conjugates on that site (Figure 1). We hypothesized that such modification can prevent the acryl glucuronidation of MPA and thus reduce the in vivo toxicity. Further work is needed to understand and compare the metabolites of JP-3-110 and MPA to confirm this hypothesis.

Several research groups have synthesized MPA derivatives to increase its bioavailability and specificity.[17] The majority of these derivatives were synthesized based on the carboxyl group of MPA. For example, Lee et al. reported a bioavailability improvement of MPA through amino ester derivatization.[17] Greupink et al. reported the mannose-6-phosphate conjugated MPA to enhance the targeted delivery of MPA to fibrogenic cells.[18] To successfully synthesize a modified MPA, drug−drug interaction must be carefully reviewed and tested to confirm the new compound will not counteract the immunosuppressive effect of MPA. In our studies, we selected KZ41 which was demonstrated to be a NF-κB inhibitor and anti-inflammatory compound (Figure 2A). We believed that the anti-inflammatory effect of KZ41 will not compromise the immunosuppressive effect of MPA (Figures 4−5). Instead, the overlap of inflammatory pathway of KZ41 and the immune rejection pathway of MPA may lead to an intriguing synergistic effect and which requires further elaboration.

This is our first trial to conjugate an immunosuppressive drug to an anti-apoptotic molecule for human islet transplantation. Compared with using two drugs separately, the conjugate held two significant advantages: (1) to mask the pro-apoptotic effect without compromising the immunosuppressive effect of MPA and (2) to deliver two active compounds simultaneously to two distinct therapeutic targets. Generally speaking, in this study, we successfully fulfilled the first purpose by masking the pro-apoptotic effect of MPA, making it more suitable for human islet transplantation. MPA and KZ41

administrated separately may follow different routes of drug distribution and metabolism in vivo. The conjugate would be a safelock making sure that MPA would not compromise the viability and function of human islets in the absence of KZ41. However, for the second purpose, more work is needed to further elaborate the absorption, distribution, and metabolism after the administration of JP-3-110. For example, we still have no idea whether JP-3-110 is as good as its counterpart MPA in islet transplantation. We also do not know the in vivo stability of the ester bond connecting KZ41 and MPA and whether JP-3-110 can reach the desired site and protect human islet effectively. However, our work on the drug conjugate may shed some useful light into the future of immunosuppressants, as the administration of one drug is commonly perceived superior over the administration of multiple drugs, as the former leads to simpler pharmacokinetic study and better patient compliance.

A lot of issues remain unsolved. The first issue is the solubility of JP-3-110. KZ41 is still fairly water-soluble (~0.8 g/mL), but JP-3-110 is not even close to that range with water solubility of only 0.5 mg/mL. The difficulties of working with a compound of such solubility are as follows: (1) the poor solubility of JP-3-110 is unsuitable for systemic administration into animals because a huge amount of water is needed to fully dissolve the compound and (2) a micellar or liposomal delivery system cannot be used to formulate JP-3-110 because the solubility of JP-3-110 is not that poor, and thus JP-3-110 may leak out of the delivery system. To solve these issues, we need to modify the molecular structure of JP-3-110 to drastically change the solubility toward either direction while keeping the pharmacological activities of JP-3-110. The second issue is the stability of the compound. The ester bond of JP-3-110 is susceptible to hydrolytic degradation, releasing KZ41 and MPA as two single drugs. More than 40% of JP-3-110 in the aqueous solution will be degraded when stored in 4 °C for 1 week (Figure S3). Although this degradation has not caused us any problem in efficacy, it may pose a potential problem in the formulation of this drug. The third issue is the difficulty in the synthesis process; therefore, a limited amount of JP-3-110 had been synthesized for each batch. We have synthesized a total amount of 80 mg of JP-3-110, which was just enough for the in vitro characterization but not sufficient enough for human islet transplantation, which lasts for months.

In summary, we demonstrated that the pro-apoptotic effect of MPA can be counteracted by conjugating it to an anti-apoptotic molecule, such as KZ41, to the carboxyl group of MPA. The new compound, JP-3-110, which holds similar immunosuppressive effect as MPA while induces less toxicity, can be a good candidate for human islet transplantation. We demonstrated that a compound drug of an immunosuppressive drug and an anti-apoptotic molecule can be an effective strategy to both prevent immune rejection and PNF of human islets. However, further research is still needed to improve the solubility and stability of this compound and formulate it for in vivo testing.

■ ASSOCIATED CONTENT

Supporting Information

Solubility and stability of JP-3-110 analyzed by HPLC as well as the establishment of human immunity in NSG mice using human peripheral blood mononuclear cells (PBMCs). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the National Institutes of Health (NIH) for financial support (RO1 DK69968, R.M., and NIH5R33AI080534-05, D.M.). We thank Dr. Liguo Song of the University of Tennessee Knoxville for his assistance in mass spectrometry. We thank Meifeng Lu of the Department of Histology at the University of Tennessee Health Science Center for technical support.

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