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A Novel Carboline Derivative Inhibits Nitric Oxide Formation in Macrophages Independent of Effects on Tumor Necrosis Factor α and Interleukin-1β Expression

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ABSTRACT

Neuropathic pain is a maladaptive immune response to peripheral nerve injury that causes a chronic painful condition refractory to most analgesics. Nitric oxide (NO), which is produced by nitric oxide synthases (NOSs), has been implicated as a key factor in the pathogenesis of neuropathic pain. β-Carbolines are a large group of natural and synthetic indole alkaloids, some of which block activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), a predominant transcriptional regulator of NOS expression. Here, we characterize the inhibitory effects of a novel 6-chloro-8-(glycinyl)-amino-β-carboline (8-Gly carb) on NO formation and NF-κB activation in macrophages. 8-Gly carb was significantly more potent than the NOS inhibitor NG-nitro-L-arginine methyl ester in inhibiting constitutive and inducible NO formation in primary rat macrophages. 8-Gly carb interfered with NF-κB–mediated gene expression in differentiated THP1-XBlue cells, a human NF-κB reporter macrophage cell line, but only at concentrations severalfold higher than needed to significantly inhibit NO production. 8-Gly carb also had no effect on tumor necrosis factor α (TNFα)–induced phosphorylation of the p38 mitogen-activated protein kinase in differentiated THP1 cells, and did not inhibit lipopolysaccharide- or TNFα-stimulated expression of TNFα and interleukin-1β. These data demonstrate that relative to other carbolines and pharmacologic inhibitors of NOS, 8-Gly carb exhibits a unique pharmacological profile by inhibiting constitutive and inducible NO formation independent of NF-κB activation and cytokine expression. Thus, this novel carboline derivative holds promise as a parent compound, leading to therapeutic agents that prevent the development of neuropathic pain mediated by macrophage-derived NO without interfering with cytokine expression required for neural recovery following peripheral nerve injury.

Introduction

Neuropathic pain is a clinical condition characterized by spontaneous pain, hyperalgesia, and allodynia that is often resistant to nonsteroidal anti-inflammatory drugs or even opioids (Zimmermann, 2001). Neuropathic pain can develop secondary to acute peripheral nerve injury, and the mechanisms contributing to this syndrome involve the activation of resident and peripheral immune cells that mediate inflammatory responses associated with neurochemical and electrophysiological changes in the neurons in the dorsal root ganglia (DRG) and dorsal horn of the spinal cord (Zimmermann, 2001; Austin and Moalem-Taylor, 2010; Pope et al., 2013). Several studies implicate nitric oxide (NO) generated by peripheral macrophages in the DRG and microglia in the spinal cord as a key factor in initiating and sustaining the inflammation associated with the establishment of neuropathic pain (Hu and McLachlan, 2002; Milligan and Watkins, 2009; Patro et al., 2010; Kuboyama et al., 2011). NO also modulates the release of neurotransmitters and excitatory neuropeptides and thus contributes to the altered neuronal properties associated with neuropathic pain (Garthwaite, 1991; Haley et al., 1992; Meller et al., 1992; Yamamoto and Shimoyama, 1995). There is, therefore, significant interest in NO as a therapeutic target in neuropathic pain (Mukherjee et al., 2014).

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ABBREVIATIONS: 8-Gly carb, 6-chloro-8-(glycinyl)-amino-β-carboline; CAPE, caffeic acid phenethyl ester; DMSO, dimethylsulfoxide; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; Erk1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; IL-1β, interleukin-1β; iNOS, inducible NOS; LDH, lactate dehydrogenase; L-NAME, NG-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAP, mitogen-activated protein; ML-102B, *N*-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methylnicotinamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative polymerase chain reaction; RT, room temperature; SEAP, secreted embryonic alkaline phosphatase; TFA, trifluoroacetic acid; TNFα, tumor necrosis factor α.

NO is synthesized from L-arginine by three isoenzymes of nitric oxide synthase (NOS; EC 1.14.13.39): type I or neuronal NOS, type II or inducible NOS (iNOS), and type III or endothelial NOS. The type I and III enzymes are constitutively expressed in numerous cell types and require Ca^{2+}/calmodulin for activity, while expression of iNOS is upregulated in macrophages and other immunomodulatory cell types in response to proinflammatory cytokines or endotoxin and its activity is Ca^{2+} independent (Cho et al., 1992) (Fig. 1). Induction of iNOS is transcriptionally regulated by nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (Xie et al., 1993, 1994). Tumor necrosis factor α (TNFα) and lipopolysaccharides (LPS) trigger nuclear translocation of the NF-κB RelA(p65) subunit promoting transcription of NF-κB responsive genes, which include iNOS and cytokines that trigger NO production (Verma et al., 1995; Pope et al., 2013) (Fig. 1). Thus, in addition to direct inhibition of NOS activity, blocking NF-κB activation is another strategy that has been examined for decreasing NO production but has been hampered by the pleiotropic downstream effects following NF-κB activation (Aktan, 2004).

β-Carbolines encompass a large group of natural and synthetic indole alkaloids that possess a common tricyclic pyrido [3,4-β]indole ring structure (Cao et al., 2007). A number of naturally occurring and modified carbolines have been reported to alter NF-κB activation (Lee et al., 2000; Yoon et al., 2005; Oh et al., 2013; Tran et al., 2014). The 6-chloro-β-carboline derivative ML-102B [*N*-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methylnicotinamide] inhibited the production of TNFα and interleukin-2 cytokines in peripheral blood monocytes by blocking NF-κB nuclear translocation (Wen et al., 2006). Biologic screening of 50 functionalized tetrahydro-β-carboline derivatives identified a significant number of these compounds, which decreased NO production coincident with inhibition of NF-κB (Shen et al., 2011). In this study, we investigated the effects of a novel β-carboline derivative, 6-chloro-8-(glycinyl)-amino-β-carboline (8-Gly carb), on NO production, NF-κB activation, and cytokine expression in macrophages. Our findings demonstrate that 8-Gly carb has a unique pharmacologic profile relative to other β-carbolines in that it blocks NO production independent of effects on NF-κB activation and cytokine production. Thus, this novel carboline derivative may be a useful tool for delineating the relative contributions of NO versus inflammatory cytokines in the pathogenesis of neuropathic pain, and it may provide a novel therapeutic strategy for treating this debilitating condition.

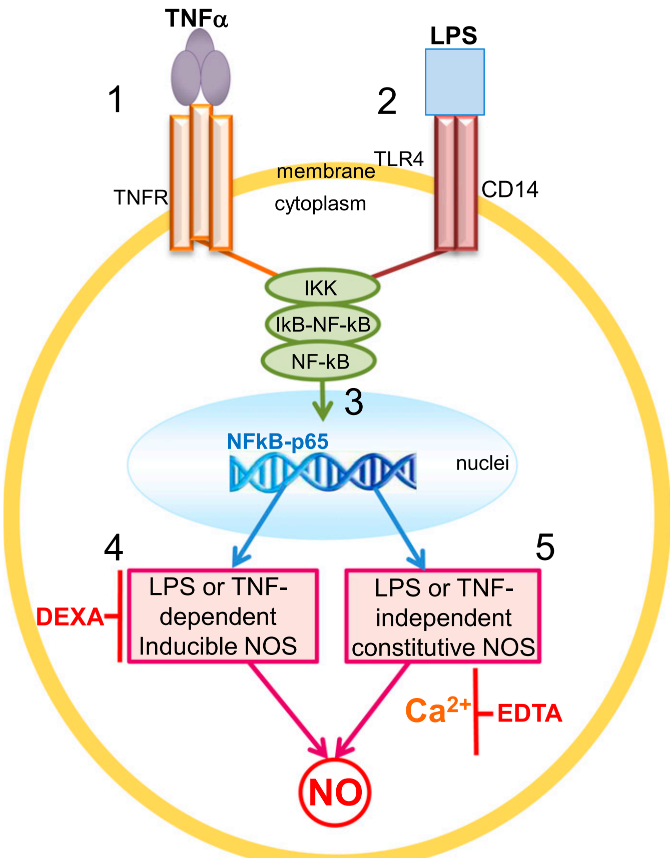
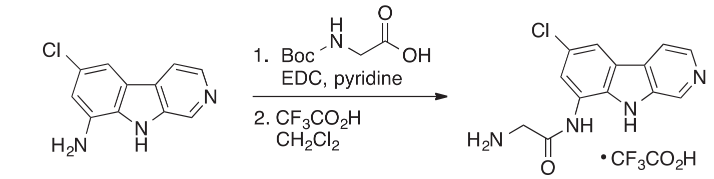


Fig. 1. Schematic of NF-κB signaling pathways in macrophages. NF-κB activation by (1) TNFα binding to the TNF receptor or (2) LPS-mediated activation of Toll-like receptor 4 (TLR4). Both pathways trigger NF-κB p65 (RelA) nuclear translocation (3), which is required for inducible NO formation, which can be inhibited by dexamethasone (4), and for Ca^{2+}-dependent constitutive NO formation, which is inhibited by EDTA (5). DEXA, dexamethasone; TNFR, TNF receptor.

Materials and Methods

The Griess reaction kit was purchased from Promega (Madison, WI). The antibiotics normocin and zeocin, QUANTI-Blue medium, and LPS-EK Ultrapure (from the *Escherichia coli* K12 strain–Toll-like receptor 4 ligand) were purchased from InvivoGen (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO), and human recombinant and rat recombinant TNFα was purchased from PeproTech (Rocky Hill, NJ). Primers and probes were purchased as part of the PrimeTime predesigned quantitative polymerase chain reaction (qPCR) assays from Integrated DNA Technologies, Inc. (San Diego, CA). Interleukin-1β (IL-1β) and TNFα enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN). Antibodies for NF-κB p65, p44/42 mitogen-activated protein (MAP) kinase, phosphor-p44/42 MAP kinase, p38 MAP kinase, and phosphor-p38 MAP kinase were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

The synthesis and structure of 6-chloro-8-(glycinyl)-amino-β-carboline is illustrated in Fig. 2. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide•HCl (96 mg, 0.50 mmol) was added in one portion to a solution of Boc-Gly-OH (89 mg, 0.50 mmol) and 6-chloro-8-amino-β-carboline (Castro et al., 2003) (gift from Millennium Pharmaceuticals) (100 mg, 0.46 mmol) in dry pyridine (7 ml) at 0°C. The reaction mixture was stirred at 0°C for 2 hours and then at room temperature (RT) for 16 hours. The pyridine was removed under a vacuum. Methanol (1 ml) and saturated aqueous $NaHCO_3$ (7 ml) were added to the residue, and the resultant mixture was stirred for 15–20 minutes before extraction with dichloromethane (3 × 100 ml). The combined organic layer was dried (Na_2SO_4) and then concentrated by rotary evaporation. The crude product was purified by column chromatography (SiO_2, eluent 2–4% methanol in dichloromethane) to obtain the corresponding 6-chloro-8-(Boc-Gly)amino-β-carboline (134 mg, 78%). High-performance liquid chromatography analysis of the product: 5% CH_3CN/H_2O–95% CH_3CN/H_2O [static 0.1% trifluoroacetic acid (TFA)] over a 30-minute flow rate = 1 ml/min; retention time = 17.8 minutes (Atlantis C_{18} column); [^{1}H]NMR [dimethylsulfoxide (DMSO)-d_6] δ 11.39 (s, 1H), 10.06 (s, 1H), 9.02 (br s, 1H), 8.38 (br s, 1H), 8.12–8.18 (m, 2H), 7.90 (s, 1H), 7.15 (t, J = 5.5 Hz, 1H), 3.90 (d, J = 5.5 Hz, 2H), and 1.40 (s, 9H). TFA (1.5 ml) was added dropwise to a solution of the 8-(Boc-Gly) amino-β-carboline analog (100 mg) in dry CH_2Cl_2 (7 ml) at 0°C. The reaction mixture was stirred at 0°C for 1 hour and then warmed to RT. After stirring at RT for 1 hour, the solvent was removed by rotary evaporation. Ethanol (50 ml) was added and then removed by rotary evaporation, and this process was repeated two more times to remove any remaining TFA. Finally, 50 ml of



6-chloro-8-amino-β-carboline

6-chloro-8-[(2-aminoacetyl)amino]-β-carboline·TFA (8-Gly carb)

Fig. 2. Synthetic strategy and structure of 8-Gly carb.

ether was added and the product, TFA salt (88 mg, 92%), was collected as a precipitate. High-performance liquid chromatography analysis: 5% CH_3CN/H_2O–95% CH_3CN/H_2O (static 0.1% TFA) over a 30-minute flow rate = 1 ml/min; retention time = 12.4 minutes (Atlantis C_{18} column); [^{1}H]NMR (DMSO-d_6) δ 12.9 (s, 1H), 10.96 (s, 1H), 9.30 (s, 1H), 8.70 (d, J = 6 Hz, 1H), 8.61 (d, J = 5.5 Hz, 1H), 8.28–8.50 (m, 4H), 8.02 (d, J = 1.5 Hz, 1H), and 4.0 (s, 2H).

Animals. All experiments involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institutes of Health and as approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Adult Sprague-Dawley female rats, 1–2 weeks postpartum, were purchased from Charles River Laboratories (Hollister, CA) and housed individually in standard plastic cages in a temperature (22 ± 2°C) controlled room on a 12-hour light/dark cycle. Food and water were provided ad libitum.

Cell Culture. To set up primary cultures enriched for macrophages, female Sprague-Dawley rats were euthanized by CO_2 asphyxiation and the peritoneal cavity was rinsed with 30 ml of phosphate-buffered saline (PBS) at pH 7.4 (Gibco/Invitrogen Corporation, Carlsbad, CA) to collect resident peritoneal macrophages. Cells were washed once and resuspended in RPMI 1640 medium without phenol red (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 U/ml streptomycin (Gibco). Cells were plated (1×10^5 per well) into a 96-well flat-bottomed tissue culture plate (Corning Costar, Tewksbury, MA) and allowed to adhere for 1 hour at 37°C in a moist atmosphere of 5% CO_2 in air. The nonadherent cells were removed by washing the plates twice with warm RPMI without serum. The remaining adherent cells were highly enriched for macrophages (> 95%) (Donnelly et al., 2005).

THP1-XBlue cells derived from the human monocytic THP1 cell line were obtained from InvivoGen. The THP-1 cell line was originally isolated from the peripheral blood of a 1-year-old male patient suffering from acute monocytic leukemia (Tsuchiya et al., 1980). THP1-XBlue cells are an NF-κB reporter cell line in which activation of the transcription factor NF-κB results in the release of secreted embryonic alkaline phosphatase (SEAP), which is detected using the Quanti-Blue reagent (InvivoGen). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin, 1% streptomycin, 100 μg/ml normocin, and 200 μg/ml of zeocin at 37°C in a humidified incubator with 5% CO_2 in air. Prior to experimentation, cells were treated for 48 hours with PMA at 20 ng/ml to trigger their differentiation into macrophages (Rovera et al., 1979; Schwende et al., 1996). A stock solution of PMA at 40 μg/ml in DMSO (Sigma-Aldrich) was diluted in a tissue culture medium to a final DMSO concentration of 0.05%. The addition of DMSO at 0.05% did not cause THP1-XBlue cells to undergo macrophage differentiation, nor did it affect their viability, as assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay (data not shown). After differentiation, cells were washed twice with RPMI 1640 with 10% FBS and used immediately for experiments.

Measurement of NO Formation. Rat peritoneal macrophages cultured in serum- and phenol red–free RPMI 1640 in 5% CO_2 at 37°C were incubated for 2 hours in the absence or presence of 8-Gly carb, NG-nitro-L-arginine methyl ester (L-NAME), EDTA, or dexamethasone. After the 2-hour incubation, the cells were stimulated overnight with LPS at 1 μg/ml or TNFα at 10 ng/ml. NO was measured in culture supernatants using the Griess reaction system (Tsikas, 2007). The Griess reaction quantifies NO indirectly by measuring the concentration of nitrite (NO^{2-}), which is one of the primary stable and nonvolatile breakdown products of NO (Grisham et al., 1996). Briefly, supernatants (50 μl) were incubated for 5 minutes with the same volume (50 μl) of sulfanilamide solution at RT prior to adding 50 μl of an N-1-napthylethylenediamine dihydrochloride solution. The absorbance at 540 nm was measured within 30 minutes after the addition of the N-1-napthylethylenediamine dihydrochloride solution using a Synergy H1 hybrid multi-mode microplate reader from BioTek U.S. (Winooski, VT). The concentration of nitrite in the supernatants was determined using a nitrite standard reference curve.

Cytotoxicity Assays. The cytotoxicity of 8-Gly carb was assessed using two mechanistically distinct assays: the MTT assay and lactate dehydrogenase (LDH) release assay. The MTT assay measures the reduction of MTT (Sigma-Aldrich) to formazan by mitochondrial reductases. After incubation for 5 hours with 8-Gly carb followed by overnight stimulation with LPS or TNFα, MTT (500 μg/ml) was added to cultures, which were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 hours. The medium was then aspirated from each well and DMSO was added. After 1 hour at 37°C, the absorbance at 562 nm was determined using the Synergy H1 hybrid multi-mode microplate reader.

LDH is a stable cytosolic enzyme that is released upon cell lysis. To quantify the effects of 8-Gly carb on the release of LDH, cultures were incubated for 5 hours with 8-Gly carb and then stimulated overnight with LPS or TNFα. The culture medium was collected from each well, and LDH in these samples was measured using the CytoTox-ONE homogenous membrane integrity assay (Promega) according to the manufacturer's protocol.

NF-κB p65 Nuclear Translocation. Rat peritoneal macrophages and THP1-XBlue cells cultured at 37°C in RPMI 1640 supplemented with 10% FBS and 5% CO_2 were incubated with 8-Gly carb for 2 hours. After the 2-hour incubation, the cells were stimulated by LPS at 1 μg/ml or TNFα at 10 ng/ml for 30 minutes, fixed in 4% paraformaldehyde for 20 minutes, and rinsed twice with PBS. Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 5 minutes, washed 3 times with PBS, and blocked for 1 hour with 5% bovine serum albumin in PBS. After blocking, the cells were incubated with the primary antibody NF-κB p65 (RelA) (rabbit monoclonal antibody D14E12–XP; Cell Signaling Technology) overnight at 4°C, rinsed 3 times with PBS, and then incubated for 1 hour at RT with goat anti-rabbit IgG Ab conjugated to Alexa 647 (Invitrogen) diluted 1:1000. After immunostaining, the cells were washed twice and counterstained with Hoechst (Invitrogen) at 3 μg/ml and phalloidin conjugated to Alexa 488 (Invitrogen) at 8 U/ml. The cells were rinsed twice with PBS and kept at 4°C until imaged using an ImageXpress Micro XL high content imaging system (Molecular Devices, Sunnyvale, CA). The nuclear localization of the NF-κB p65 subunit was analyzed in 16 fields per well in 12 wells per treatment using a custom module designed specifically for colocalizing the NF-κB p65 subunit in nuclei using MetaXpress 5.0 software (Molecular Devices).

NF-κB Activation. PMA-differentiated THP1-XBlue cells were rinsed twice with PBS, incubated for 5 hours with 8-Gly carb and caffeic acid phenethyl ester (CAPE), and then stimulated overnight with 1 μg/ml of LPS or 10 ng/ml of human recombinant TNFα. The activation of the NF-κB pathway was monitored by quantifying the NF-κB–dependent expression of SEAP in culture supernatants using the Quanti-Blue mix colorimetric assay. Absorbance at 630 nm was measured using a SpectraMax spectrophotometer (Molecular Devices).

RNA Isolation and Real-Time qPCR. THP1-XBlue cells were plated at 2.5×10^5 cells/well in 24-well plates and differentiated with PMA for 48 hours. The cells were washed twice with PBS, incubated for 5 hours with 8-Gly carb, and then stimulated for 90 minutes with LPS at 1 μg/ml or TNFα at 10 ng/ml. After the 90-minute stimulation, total RNA was extracted using the RNAeasy Mini Kit (Qiagen Inc., Valencia, CA), and the quality and concentration of extracted RNA were evaluated using Nanodrop 1000 (Thermo Scientific, Rockford, IL). All samples were of high purity (260/280 ratio >2). RNA samples (0.5 μg) were reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen) and random primers at an annealing temperature of 65°C. The resultant cDNA was amplified by quantitative real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems). The concentration of cDNA was selected according to a dilution curve established using the specific primers and probes to determine the highest efficiency for the reference and target genes. The specific sequences of the 18S rRNA primers were 5′-ATC GCT CCA CCA ACT AAG AAC-3′ and 5′-ACG GAC AGG ATT GAC AGA TTG-3′, and for the probe, the sequence was 5′-/56-FAM/ACC ACC CAC/ZEN/GGA ATC GAG AAA GAG/3IABkFQ/-3′. The specific sequences for the TNFα primers were 5′-TTC GAG AAG ATG ATC TGA CTGC-3′ and 5′-AGC CTC TTC TCC TTC CTGAT-3′, and for the probe, the sequence was 5′-/56-FAM/CGC CAC CAC/ZEN/GCT CTT CTGC/3IABkFQ/-3′. The specific sequences for the IL-1β primers were 5′-GTC ATC CTC ATT GCC ACT GTA-3′ and 5′-CAG CCA ATC TTC ATT GCT CAAG-3′, and for the probe, the sequence was 5′-/56-FAM/AGA AGT ACC/ZEN/TGA GCT CGC CAG TGA/ 3IABkFQ/-3′. All qPCR experiments were performed in duplicate. The expression ratio was calculated according to the efficiencies for each gene and normalized to the 18S efficiency. The 18S gene did not show any ΔCt variation with stimulation. To confirm the results, the data were also analyzed using REST 2009 gene quantification (http://www.gene-quantification.de/rest-2009.html), a software tool developed by M. Pfaffl (Technical University Munich) for the analysis of gene expression data from quantitative real-time PCR experiments, in which gene induction is determined using automated statistical randomization and bootstrapping tests (Pfaffl, 2001; Pfaffl et al., 2002).

ELISA. THP1-XBlue cells plated at 10×10^5 cells/well in six-well plates were PMA differentiated for 48 hours. PMA-differentiated THP1-XBlue cells were washed twice with PBS, incubated for 5 hours with 8-Gly carb, and then stimulated for 24 hours with LPS at 1 μg/ml or TNFα at 10 ng/ml in a total volume of 2 ml/well. The supernatants were collected and centrifuged to remove cellular debris. Supernatants were then concentrated to a final volume of 200 μl using an Amicon ultra-centrifugal filter with a molecular mass cutoff of 10 kDa (Millipore, Billerica, MA) to analyze for IL-1β secretion or diluted 20-fold with RPMI 1640 media to measure TNFα. The amount of secreted IL-1β and TNFα in the supernatants was determined using Quantikine ELISA kits specific for human IL-1β and TNFα, as described by the manufacturer (R&D Systems). Cytokine concentrations were determined from standard curves generated using the corresponding standards at 450-nm absorbance corrected to 540 nm using the Synergy H1 hybrid multimode microplate reader software.

Western Blot Analyses. Western blot analyses were performed using lysates of PMA-differentiated THP1-XBlue cells to assess the effects of 8-Gly carb on the activation of the MAP kinases extracellular-signal-regulated kinase (Erk) 1/2 and p38. THP1-XBlue cells plated at 10×10^5 cells/well in six-well plates were PMA differentiated for 48 hours. PMA-differentiated THP1-XBlue cells were washed twice with PBS, incubated for 5 hours with 8-Gly carb, and then stimulated for 30 minutes with TNFα at 10 ng/ml. Cultures were rinsed with ice-cold PBS and then triturated in an ice-cold lysis buffer [PBS supplemented with 1% Igepal (Sigma-Aldrich), 0.5% sodium deoxycholate (Fisher Scientific, Fair Lawn, NJ), 0.1% SDS (Fisher Scientific), 100 μg/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich), 300 μg/ml aprotinin (Sigma-Aldrich), and a phosphatase inhibitor cocktail (Thermo Scientific)]. Cell lysates were centrifuged in an Eppendorf 5417R microfuge (Eppendorf, Hauppauge, NY) at maximum speed for 5 minutes, and the resultant supernatant was collected. Protein concentration was determined using the microBCA protein assay kit (Thermo Scientific). Lysate samples (20 μg of protein) were resolved by 12% SDS-PAGE and electroblotted onto Immobilon-FL membranes (Millipore). Blots were blocked at RT for 1 hour in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) diluted 1:1 with PBS, and then incubated overnight at 4°C in Odyssey blocking buffer diluted 1:1 with PBS containing 0.1% Tween 20 and primary antibodies (Erk1/2, phosphor-Erk1/2, p38, and phosphor-p38 at 1:1000 dilution; Cell Signaling Technology). Blots were washed 4 times with PBS containing 0.1% Tween 20 and incubated at room temperature for 1 hour in Odyssey blocking buffer diluted 1:1 with PBS containing 0.1% Tween 20 and goat anti-rabbit infrared 700 or goat anti-mouse infrared 700 diluted 1:1000. Subsequently, blots were washed 4 times as described above, visualized, and quantified using the Studio Imaging system (LI-COR Biosciences). Densitometric values of bands immunoreactive for the phosphorylated forms of each MAP kinase were normalized to densitometric values of bands that reacted with antibodies that recognize both the phosphorylated and nonphosphorylated forms of the corresponding MAP kinase.

Statistical Analyses. All data are presented as mean ± S.E.M. The differences between the two treatment groups were analyzed using nonparametric Student's *t* test, whereas the differences between more than two groups were determined using one-way analysis of variance with post hoc Tukey's test or post hoc Kruskal Wallis' test and Dunn's multiple comparison test (Prism 4; GraphPad Software, San Diego, CA). Statistical analyses for qPCR data were performed using REST2009 (Qiagen). Data from NF-κB nuclear translocation, NF-κB reporter secretion, and qPCR analyses were also analyzed by SPSS to confirm results. *P* values < 0.05 were considered statistically significant.

Results

8-Gly Carb Inhibits Nitric Oxide Formation in Rat Peritoneal Macrophages. Recent gene inactivation studies have shown that both constitutive and inducible NO formation contributes to the early development of neuropathic pain (Kuboyama et al., 2011). The efficacy of 8-Gly carb in inhibiting NO formation by either mechanism was determined by measuring nitrite release in primary rat peritoneal macrophages. The activation of the constitutively expressed isoform of NOS is Ca^{2+} dependent (Radomski et al., 1990), and treatment of primary rat peritoneal macrophages with EDTA decreased NO production by 50% (Fig. 3A). Treatment with 8-Gly carb also

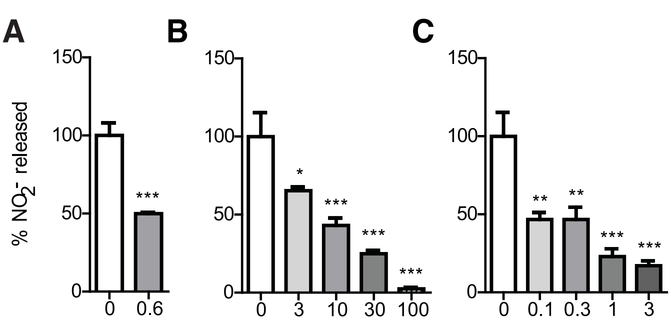


Fig. 3. 8-Gly carb inhibited constitutive NO formation in a concentration-dependent manner. NO production was measured in rat peritoneal macrophages (1×10^5 cells/well in 96-well plates) following 24-hour incubation with vehicle (0.05% v/v DMSO), EDTA (0.6 mM) (A), 8-Gly carb (B), or L-NAME (C). Data presented as mean ± S.E.M. (*n* = 4 wells per treatment). Significantly different from vehicle control (white bar) at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way analysis of variance with post hoc Tukey's test.

significantly reduced constitutive NO formation (Fig. 3B). This effect was concentration dependent, with an IC_{50} of $\cong$ 7 μM. L-NAME is a widely used enzymatic inhibitor of NOS (Rees et al., 1989), which has been previously demonstrated to be deesterified by peritoneal macrophages to NG-nitro-L-arginine, the competitive pseudosubstrate inhibitor of NOS (Pfeiffer et al., 1996). We observed that L-NAME also inhibited constitutive NO formation in primary rat peritoneal macrophages in a concentration-dependent manner; however, L-NAME was approximately 43 times less potent than 8-Gly carb (Fig. 3C).

We next investigated whether 8-Gly carb inhibited inducible NO formation. Stimulation of primary rat peritoneal macrophages with either TNFα or LPS for 24 hours significantly increased NO formation (Fig. 4A). Preincubation with 8-Gly carb for 2 hours inhibited both TNFα- (Fig. 4B) and LPS-induced (Fig. 4C) NO formation in a concentration-dependent manner, with an IC_{50} of $\cong$ 7 and 20 μM, respectively. NO formation induced by LPS was significantly inhibited by dexamethasone in a concentration-dependent manner, and this inhibition was augmented by coincubation with EDTA (Fig. 4D) or 8-Gly carb (Fig. 4E). 8-Gly carb is approximately 15 times more potent in inhibiting LPS-induced NO formation than L-NAME (IC_{50} = ~300 μM) (Fig. 4F). Cotreatment with 8-Gly carb and L-NAME caused a significantly greater inhibition of LPS-induced NO formation than was observed with 8-Gly carb alone (Fig. 4G).

To exclude the possibility that 8-Gly carb interferes with the assay used to measure NO production, supernatants from primary rat peritoneal macrophages not previously exposed to 8-Gly carb were spiked with a vehicle or varying concentrations of 8-Gly carb immediately prior to processing samples in the Griess reaction. Results obtained with the Griess reaction were not altered by the addition of 8-Gly carb (Fig. 5A). The inhibition of NO production observed in macrophages treated with 8-Gly carb was also not secondary to cytotoxic effects of this carboline derivative. A 24-hour exposure to 8-Gly carb at micromolar concentrations that significantly reduced NO production had negligible cytotoxic effects, as assessed by LDH release (Fig. 5B) or MTT reduction (Fig. 5C).

8-Gly Carb Blocks NF-κB Activation Only at the Highest Concentration (100 μM) Tested. LPS- or cytokine-induced NO formation is transcriptionally regulated by NF-κB (Xie et al., 1993, 1994; Xie and Nathan, 1994). 6-Chloro-β-carbolines have been shown to block NF-κB activation by inhibiting the

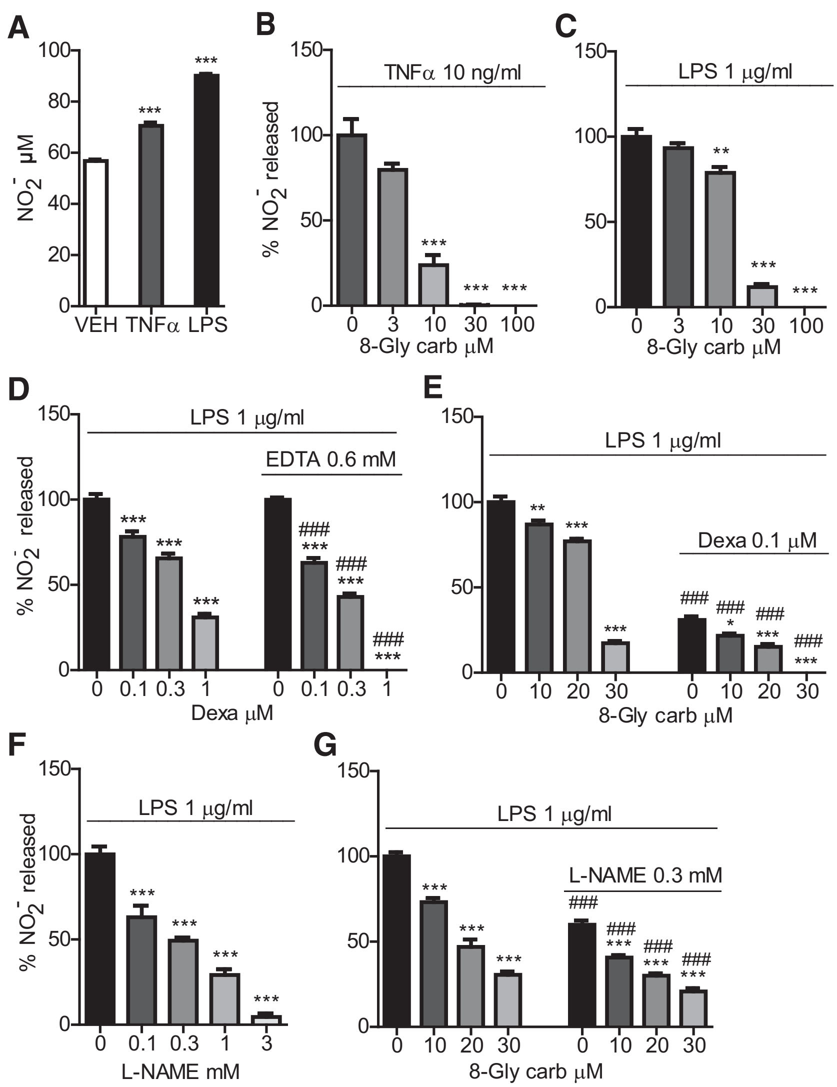


Fig. 4. 8-Gly carb inhibited inducible NO formation in macrophages in a concentration-dependent manner. (A) NO production was measured in rat peritoneal macrophages (1 × 10^5 cells/well in 96-well plates) following 24-hour incubation with vehicle (0.05% v/v DMSO), TNFα (10 ng/ml), or LPS (1 μg/ml). In subsequent experiments, peritoneal macrophages were incubated for 2 hours with one of the following: vehicle, varying concentrations of 8-Gly carb (B, C, E, and G), L-NAME (F and G), EDTA (D), and/or dexamethasone (Dexa) (D and E) prior to 24-hour exposure to TNFα (B) or LPS (C–G). NO formation was measured at 24 hours, and data are presented as mean ± S.E.M. (n = 4). Data presented in (A) were analyzed using Student's t test. Significantly different from vehicle at $***P < 0.001$. Data presented in (B–G) were analyzed using one-way analysis of variance with post hoc Tukey's test. Significantly different from vehicle control at $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; significantly different from LPS + same concentration of dexa (D) or LPS + same concentration of 8-Gly carb (E and G) at $^{###}P < 0.001$. VEH, vehicle.

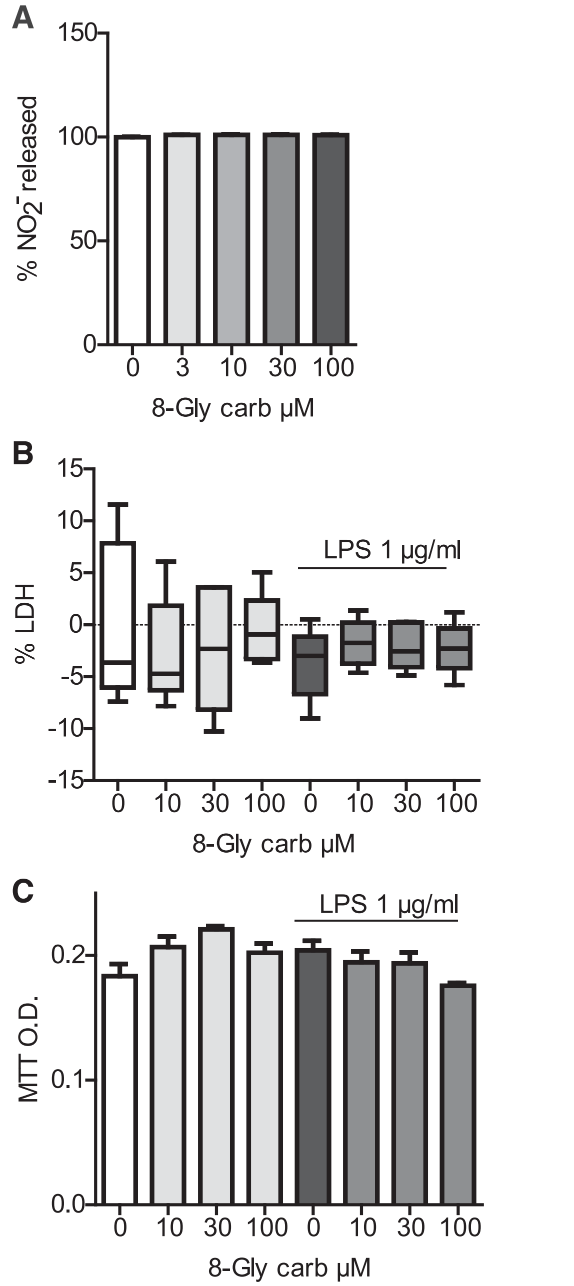


Fig. 5. 8-Gly carb did not interfere with the assay used to quantify NO and did not cause cytotoxicity. (A) To determine whether 8-Gly carb interfered with the assay used to quantify NO production, supernatant collected from rat peritoneal macrophages (1×10^5 cells/well in 96-well plates) following a 24-hour incubation was spiked with vehicle (0.05% v/v DMSO) or varying concentrations of 8-Gly carb. Samples were then immediately analyzed using the Griess reaction. Data are presented as the percentage of vehicle control. To determine whether 8-Gly carb caused cytotoxicity, LDH release (B) and MTT reduction (C) were quantified in rat peritoneal macrophages incubated with vehicle or 8-Gly carb in the absence or presence of LPS at 1 μg/ml for 24 hours. Data are presented as mean ± S.E.M. (n = 6 wells per treatment). No statistically significant differences between treatment groups were identified by one-way analysis of variance with post hoc Tukey's test.

inhibitor of the κB kinase complex (Castro et al., 2003; Wen et al., 2006). Here, we investigated the effects of 8-Gly carb on NF-κB activation by immunocytochemical analyses of NF-κB p65 (RelA) nuclear translocation (Xie and Nathan, 1994) and quantification of NF-κB–mediated gene transcription in the NF-κB reporter cell line THP1-XBlue (Grodzki et al., 2013).

NF-κB is comprised of five subunits, RelA (p65), RelB, cRel, p50, and p52, but it is the nuclear translocation of RelA (p65) that is widely employed as a functionally relevant biomarker of NF-κB activation (Baeuerle and Henkel, 1994). Therefore, to assess NF-κB activation, macrophages were immunostained for the NF-κB p65 (RelA) subunit and counterstained with Hoechst to identify nuclei and fluorescently labeled phalloidin to identify cytoskeletal actin. In nonstimulated rat peritoneal macrophages (Fig. 6, A and B) and basal THP1-XBlue cells (Fig. 6, D and E), approximately 25% of the cell population exhibited nuclear NF-κB p65 (RelA), as determined by subcellular quantification of colocalized NF-κB p65 (RelA) immunoreactivity (red) with Hoechst nuclear staining (blue). Stimulation with either LPS or TNFα significantly increased the percentage of nuclear localized p65 in rat peritoneal macrophages (Fig. 6, A and C) and THP1-XBlue cells (Fig. 6, D and F). Preincubation with 8-Gly carb did not significantly alter NF-κB p65 nuclear translocation in rat peritoneal macrophages induced by either LPS or TNFα (Fig. 6A). 8-Gly carb did demonstrate a concentration dependent, but not statistically significant, decrease in both LPS- and TNFα-induced NF-κB p65 nuclear translocation in THP1-XBlue cells (Fig. 6D).

We next determined whether 8-Gly carb inhibited NF-κB–driven reporter gene transcription in the THP1-XBlue cell line. THP1-XBlue cells are a human monocyte cell line stably transfected with the NF-κB reporter gene SEAP. LPS or TNFα stimulation triggers nuclear translocation of the NF-κB p65 (RelA) subunit, which then binds to the NF-κB promoter site upstream of the SEAP gene, resulting in the production of SEAP. THP1-XBlue cells stimulated by LPS (Fig. 7A) or TNFα (Fig. 7B) secreted significantly more SEAP than nonstimulated cells, confirming NF-κB activation in these cells via both LPS- and TNFα-triggered pathways (Fig. 1). Exposure to 8-Gly carb or CAPE, previously shown to inhibit NF-κB activation (Jung et al., 2008), for 5 hours preceding stimulation by LPS or TNFα significantly inhibited SEAP secretion only at the highest concentration (100 μM) tested (Fig. 7).

8-Gly Carb Did Not Alter LPS- or TNFα-Mediated Cytokine Production. We next determined whether 8-Gly carb modulated the expression of proinflammatory cytokines at concentrations that did not inhibit NF-κB activation. qPCR was used to quantify TNFα and IL-1β mRNA in THP1-XBlue cells stimulated with either LPS or TNFα. LPS caused an approximately 150-fold increase in TNFα and IL-1β mRNA levels relative to vehicle control cultures (Fig. 8A), whereas TNFα caused about a 15-fold increase in these transcripts as compared with vehicle control cultures (Fig. 8B). Preincubation with 8-Gly carb at 0.1–10 μM did not alter LPS-induced expression of TNFα and IL-1β mRNA (Fig. 8A). 8-Gly carb had no effect on TNFα-induced TNFα mRNA levels but trended toward a concentration-dependent reduction in IL-1β mRNA levels induced by TNFα that was not statistically significant (Fig. 8B). In the presence of 10 μM 8-Gly carb, IL-1β levels remained ~7-fold higher than control THP-1 cell cultures not exposed to TNFα.

We then determined whether 8-Gly carb impaired the secretion of TNFα or IL-1β protein. The amount of TNFα and IL-1β secreted into the media by THP1-XBlue cells stimulated with TNFα in the absence or presence of 8-Gly carb (0.1–10 μM) was measured by ELISA. 8-Gly carb did not alter

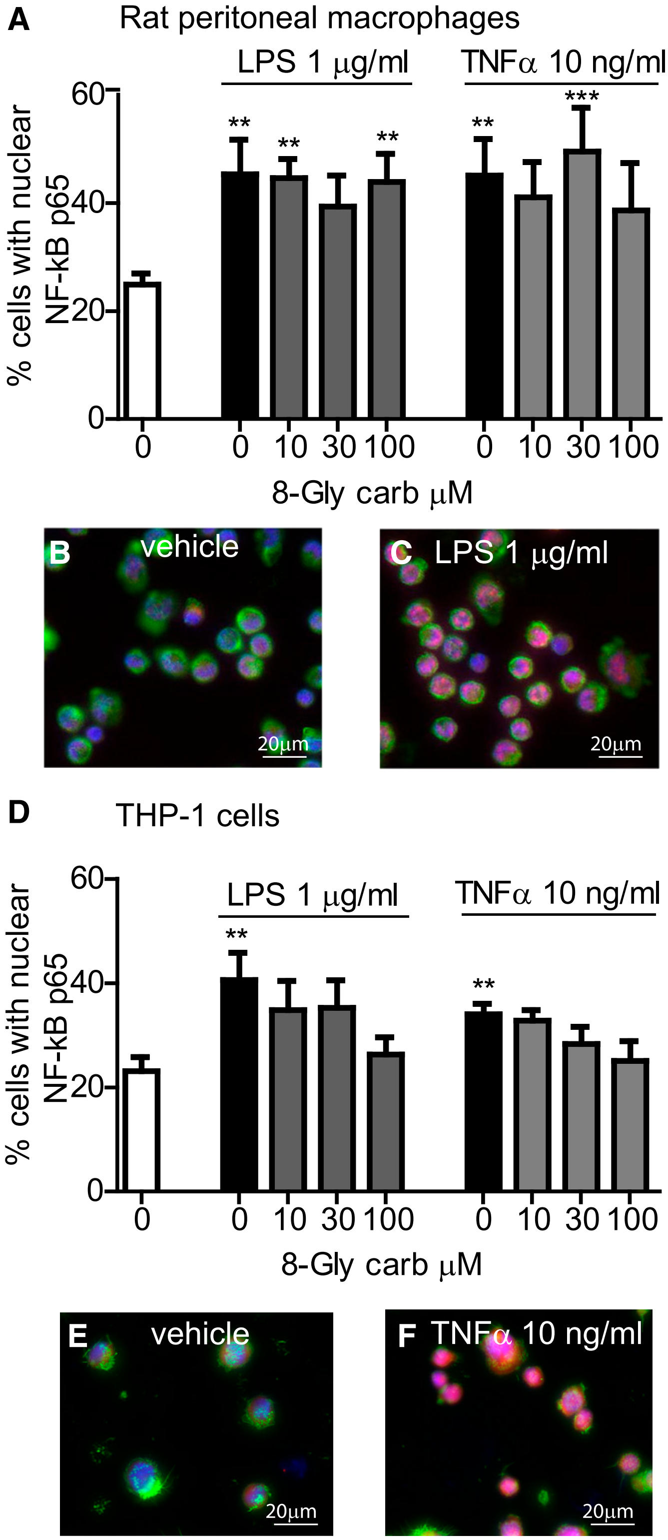


Fig. 6. Nuclear translocation of NF-κB p65 in activated rat peritoneal macrophages and differentiated human THP1 cells following treatment with 8-Gly carb. Rat peritoneal macrophages (A–C) and PMA-differentiated THP1-XBlue cells (D–F) plated in 96-well plates at a density of 1×10^5 cells/well were incubated with vehicle (0.05% v/v DMSO) or 8-Gly carb for 2 hours and then stimulated for 30 minutes with LPS at 1 μg/ml or TNFα at 10 ng/ml. Fixed cells were immunostained for NF-κB p65 (red; B, C, E, and F) and nuclei were counterstained with Hoechst (blue) and fluorescently labeled phalloidin (green). Coregistration of NF-κB p65 and nuclear staining was quantified using high content intracellular imaging and presented as mean ± S.E.M. (n = 12). Significantly different from vehicle as determined using nonparametric one-way analysis of variance with post hoc Kruskal Wallis' test and Dunn's multiple comparison test at $^{**}P < 0.01$; $^{***}P < 0.001$.

the secretion of TNFα (Fig. 8C) or IL-1β (Fig. 8D). 8-Gly carb at 10 μM modestly decreased IL-1β secretion in TNFα-stimulated cultures, but this did not reach statistical significance when compared with the control group.

8-Gly Carb Did Not Affect MAP Kinase Signaling in THP1-XBlue Cells Stimulated by TNFα. The MAP kinases are a group of signaling molecules that have been implicated in mediating many cellular responses, including inflammation (Cuschieri and Maier, 2005). There is evidence in macrophages supporting the concept that MAP kinase activation participates in NF-κB–dependent production of NO and proinflammatory cytokines (Kim et al., 2006; Pergola et al., 2006; Suh et al., 2006). Therefore, we investigated if the effects of 8-Gly carb were mediated via MAP kinase signaling pathways. Specifically, we examined the effect of 8-Gly carb on the phosphorylation of p44/42 (ERK1/2) and p38 in TNFα-stimulated THP1-XBlue cells. TNFα stimulation did not increase phosphorylation of ERK1/2 in THP1-XBlue cells, and this was not modulated by treatment with 8-Gly carb (Fig. 9A). Stimulation with TNFα, however, did significantly increase phosphorylation of p38 relative to vehicle controls, and p38 activation remained unaffected by 8-Gly carb, even at 100 μM (Fig. 9B).

Discussion

The most significant findings of this study are 1) the novel 6-chloro carboline derivative 8-Gly carb is approximately 43 times more potent than the competitive NOS inhibitor L-NAME in inhibiting constitutive and inducible NO production in macrophages; and 2) 8-Gly carb inhibits NO production independent of effects on NF-κB activation or cytokine production. Specifically, the IC_{50} value for 8-Gly carb inhibition of constitutive NO production and TNFα-induced NO production and LPS-induced NO production was ~7 and ~20 μM, respectively. Surprisingly, 8-Gly carb did not significantly impair TNFα- or LPS-triggered NF-κB activation in rat peritoneal macrophages, as quantitatively assessed by the extent of nuclear translocation of the NF-κB RelA (p65) subunit in control and treated cells. Therefore, we employed the widely used differentiated THP1-XBlue cell line to more sensitively evaluate NF-κB–regulated gene transcription. The human THP1-XBlue monocytic cell line is stably transformed with the reporter gene SEAP, which is driven by an NF-κB promoter. PMA, a phorbol ester, is used to differentiate these THP1 cells to a macrophage phenotype. Corroborating the observations in rat peritoneal macrophages, 8-Gly carb did not alter nuclear translocation of the NF-κB RelA (p65). Only at the highest concentration tested (100 μM) did 8-Gly carb affect LPS- or TNFα-activated SEAP expression in THP1-XBlue cells.

At concentrations ≤10 μM, 8-Gly carb did not inhibit TNFα- or LPS-induced transcript or protein levels of IL-1β or TNFα. There is the caveat that 8-Gly carb inhibition of NO production was determined in primary rat peritoneal macrophages, whereas the effects on NF-κB activation and cytokine expression were measured primarily in differentiated human THP1 cells. Optimally, 8-Gly carb effects on NO production would be measured in PMA-differentiated THP1 cells, but it has been documented that differentiated human THP1 cells

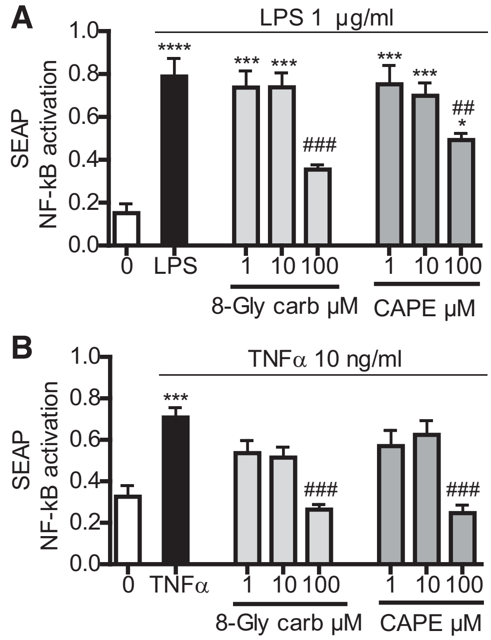


Fig. 7. Inhibition of NF-κB activation in LPS- and TNFα-stimulated THP1 cells. PMA-differentiated THP1-XBlue cells were incubated for 5 hours with either vehicle (0.05% v/v DMSO) or varying concentrations of 8-Gly carb or CAPE followed by 24-hour stimulation with LPS at 1 μg/ml (A) or TNFα at 10 ng/ml (B). The amount of SEAP released into the culture medium was quantified as a measure of NF-κB activation. Data are presented as mean ± S.E.M. (n = 5 independent experiments). LPS- and TNFα-treated cultures were analyzed using Student's t test. Significantly different from vehicle at $^{***}P < 0.001$ (B) and $^{****}P < 0.0001$ (A). Significantly different from vehicle control as determined using non-parametric one-way analysis of variance with post hoc Kruskal-Wallis and Dunn's multiple comparison tests at $^{*}P < 0.05$; $^{***}P < 0.001$; significantly different from LPS- (A) or TNFα-stimulated (B) cultures at $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

do not produce NO when stimulated in vitro (Stuehr and Marletta, 1987), which is an observation we independently corroborated (data not shown). However, collectively, these data indicate that the micromolar inhibition of 8-Gly carb on NO production in basal and TNFα- and LPS-activated cells appears to be independent of NF-κB activation. Importantly, 8-Gly carb does not impair downstream cytokine expression in differentiated THP1 cells, reinforcing the conclusion that 8-Gly carb selectively inhibits NO production in macrophages independent of effects on NF-κB activation and cytokine expression.

Concentrations of 8-Gly carb that inhibited NO production in primary rat peritoneal macrophages did not have cytotoxic or antimetabolic effects, as measured by the LDH and live cell MTT assays, respectively. Previously, it has been reported that naturally occurring carbolines inhibit LPS-induced NO production in macrophages and other cell types by blocking NF-κB activation (Lee et al., 2000; Yoon et al., 2005) or by inhibiting p38 and Erk1/2 MAP kinase activation (Ji, 2004). By contrast, 8-Gly carb does not alter TNFα activation of Erk1/2 or p38 MAP kinases, additionally distinguishing this 6-chloro-8-(glycinyl)-amino-β-carboline from other β-carboline derivatives.

Our observation that 8-Gly carb inhibited both constitutive and inducible NO macrophage production suggested a potential mechanism involving NOS inhibition. However, the inhibitory effects of 8-Gly carb on NO production were additive with those of L-NAME, which generates a competitive enzymatic inhibitor of NOS following 24-hour macrophage incubation. 8-Gly carb is

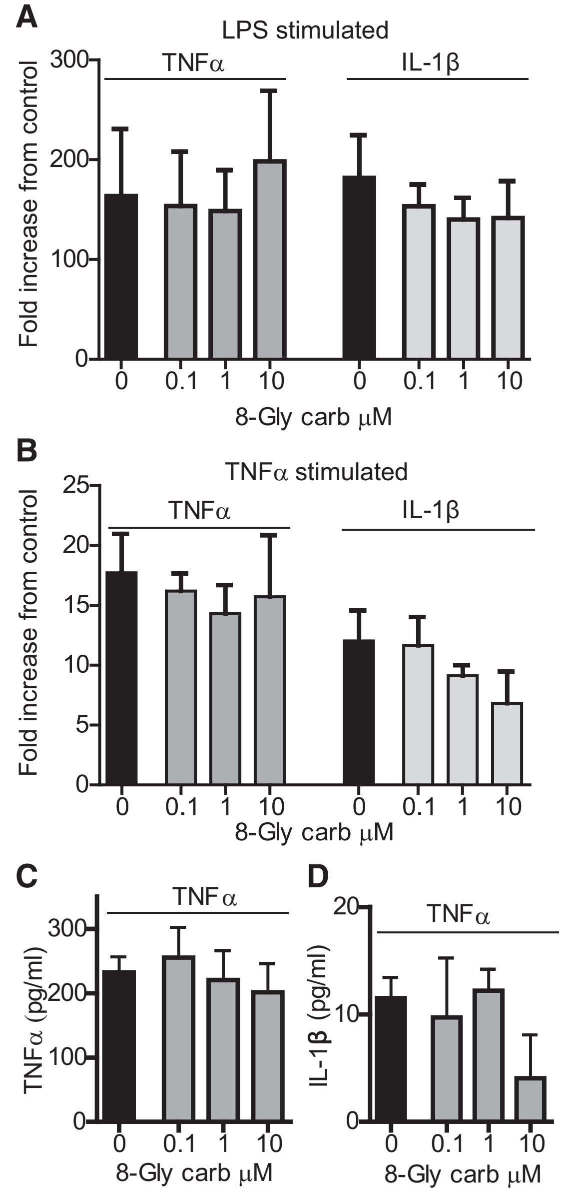


Fig. 8. 8-Gly carb did not block LPS- or TNFα-induced TNFα or IL-1β expression. PMA-differentiated THP1-XBlue cells were incubated with either vehicle (0.05% v/v DMSO) or 8-Gly carb for 5 hours followed by 90-minute stimulation with LPS at 1 μg/ml (A) or TNFα at 10 ng/ml (B). TNFα and IL-1β transcript levels were quantified by qPCR and represented as the fold change from vehicle control. Other cultures were stimulated with TNFα at 10 ng/ml to quantify TNFα (C) and IL-1β (D) expression at the protein level, as determined by ELISA. Data are presented as mean ± S.E.M. (n = 3 independent experiments). There were no statistically significant differences between treatments in either mRNA or protein levels of TNFα or IL-1β levels, as determined using REST2009 analyses (A and B) or one-way analysis of variance (C and D), respectively.

3-fold more potent at inhibiting constitutive NO production than inducible NO production in macrophages stimulated by LPS (Figs. 3 and 4). Further studies are needed to determine if 8-Gly carb acts as a noncompetitive inhibitor of these NOS isozymes, but the likelihood of this possibility is reduced by this latter observation.

It was unexpected that 8-Gly carb inhibited NO production in rat peritoneal macrophages at concentrations that negligibly affected NF-κB activation and cytokine expression. While other

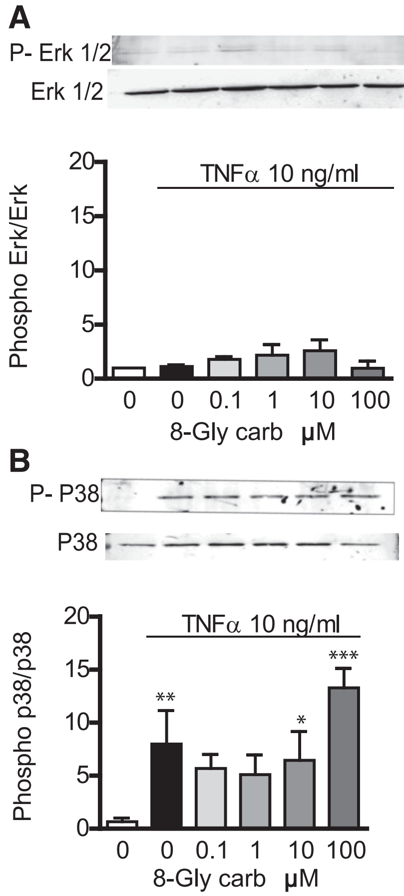


Fig. 9. 8-Gly carb did not block TNFα-stimulated MAP kinase activation. PMA-differentiated THP1-XBlue cells were incubated with 8-Gly carb for 5 hours and then stimulated with TNFα at 10 ng/ml. Representative blots and densitometric analyses of cell lysates separated by gel electrophoresis and immunoblotted for phosphorylated Erk1/2 (P-Erk1/2) and total (phosphorylated and nonphosphorylated) Erk1/2 (A) or phosphorylated P38 (P-P38) and total p38 (B) MAP kinases. Densitometric values of P-Erk1/2 and P-P38 were normalized to total Erk1/2 and P38 protein densitometric values. Data are presented as mean ± S.E.M. (n = 3 independent experiments). Significantly different from vehicle control at $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ as determined using one-way analysis of variance with post hoc Tukey's test.

carbolines have been reported to inhibit NO production in macrophages (Lee et al., 2000; Yoon et al., 2005) and other cell types (Newton et al., 2007; Shen et al., 2011; Wong et al., 2011), this biologic activity has typically been associated with suppressed NF-κB activity (Lee et al., 2000; Yoon et al., 2005; Wen et al., 2006; Newton et al., 2007; Shen et al., 2011; Oh et al., 2013) and reduced expression of cytokines and other inflammatory genes transcriptionally regulated by NF-κB (Newton et al., 2007; Oh et al., 2013). A recent screen of 50 functionalized tetrahydro-β-carboline derivatives (Shen et al., 2011) reported that the most potent carboline derivative had IC_{50} values for inhibition of NF-κB activity and NO production of 4.8 and 2.8 μM, respectively. In comparison, the IC_{50} value of 8-Gly carb for inhibition of NO production (~7 μM) is similar, but the IC_{50} value of 8-Gly carb for inhibition of NF-κB activity would exceed 100 μM. Collectively, these data argue that 8-Gly carb has a unique pharmacologic profile relative to other carbolines.

Whether 8-Gly carb exhibits similar pharmacologic properties in vivo has yet to be demonstrated, but if this proves to be the case, this novel 8-substituted, 6-chloro-β-carboline derivative and subsequent related derivatives could prove to be useful tools for distinguishing the contributions of NO versus inflammatory cytokines in the evolution of neuropathic pain (Austin and Moalem-Taylor, 2010; Ristoiu, 2013). NO formation by macrophage and microglia has been implicated in the activation of nociceptive pathways (Haley et al., 1992; Kitto et al., 1992; Snyder, 1992; Meller and Gebhart, 1993; Dawson and Snyder, 1994; Prast and Philippu, 2001) and as a critical factor in persistent pain hypersensitivity following peripheral injury (Austin and Moalem-Taylor, 2010; Kuboyama et al., 2011; Makuch et al., 2013). Several recent observations suggest that selectively targeting NO production may be an effective therapeutic strategy for treating neuropathic pain. NO directly causes DRG neurons to lower their activation threshold, leading to increased hyperexcitability. Aberrant DRG activation and damage is thought to cause sensory neuronopathy, a diffuse and length-independent neuropathic pain syndrome (Zimmermann, 2001; Podda et al., 2004). Importantly, recent evidence indicates that proinflammatory cytokines, including TNFα and IL-1β, may participate in the resolution of tissue damage and recovery of nerve function (Austin and Moalem-Taylor, 2010; Nadeau et al., 2011). Both neuronal and non-neuronal NO formation antagonizes opioid-mediated reduction of neuropathic pain (Makuch et al., 2013). Genetic deletion of all three NOS isoforms appears to be required to effectively prevent subsequent generation of neuropathic pain and prevents microglial activation in a mouse model of tissue injury–induced pain (Kuboyama et al., 2011). Thus, inhibitors that target selective NOS isoforms may not be as effective in preventing the subsequent evolution of neuropathic pain after peripheral nerve injury. Small molecules like 8-Gly carb that globally impair NO formation in macrophage and microglia formation following LPS or TNFα activation without affecting NF-κB–mediated cytokine expression may provide better therapeutic outcomes in neuropathic pain. Collectively, these observations support the potential importance of 8-Gly carb and subsequent derivatives in preventing the establishment of neuropathic pain or sensory neuronopathy following peripheral nerve injury.

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Authorship Contributions

Participated in research design: Grodzki, Gorin, Lein.
Conducted experiments: Grodzki, Poola.
Contributed new reagents or analytic tools: Nantz.
Performed data analysis: Grodzki, Gorin.
Wrote or contributed to the writing of the manuscript: Grodzki, Pasupuleti, Nantz, Gorin, Lein.

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