Manuscript Number:

Title: The sp2-iminosugar glycolipid 1-dodecylsulfonyl-5N,6O-oxomethylidenennojirimycin (DSO2-ONJ) as selective anti-inflammatory agent by modulation of hemoxygenase-1 in Bv.2 microglial cells and retinal explants.

Article Type: Full Length Article

Keywords: Diabetic retinopathy, inflammation, p38 MAPK, sp2-iminosugar, glycolipid, heme oxygenase-1

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Abstract: Neuroinflammation is an early event during diabetic retinopathy (DR) that impacts the dynamics of microglia polarization. Gliosis is a hallmark of DR and we have reported the beneficial effects of 1R-DSO-ONJ, a member of the sp2-iminosugar glycolipid (sp2-IGL) family, in targeting microglia and reducing gliosis in diabetic db/db mice. Herein, we have analyzed the effect of DSO2-ONJ, another family compound incorporating a sulfone group that better mimics the phosphate group of phosphatidylinositol ether lipid analogs (PIAs), in Bv.2 microglial cells treated with bacterial lipopolysaccharide (LPS) and in retinal explants from db/db mice. In addition to decreasing iNOS and inflammasome activation, the anti-inflammatory effect of DSO2-ONJ was mediated by direct p38 α MAPK activation. Computational docking experiments demonstrated that DSO2-ONJ binds to p38 α MAPK at the same site where the PIAs and the alkyl phospholipid perfosine activators do, suggesting a similar mechanism of action. Moreover, treatment of microglial cells with DSO2-ONJ increased both heme-oxygenase (HO)-1 and IL10 expression regardless the presence of LPS. In retinal explants from db/db mice, DSO2-ONJ also induced HO-1 and reduced gliosis. Since IL-10-mediated induction of HO-1 expression is mediated by p38 α MAPK activation, our results suggest that this molecular mechanism is involved in the anti-inflammatory effects of DSO2-ONJ in microglia.

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Dear Editor:

Find enclosed the electronic submission of the manuscript entitled ***“The sp^2 -iminosugar glycolipid 1-dodecylsulfonyl-5N,6O-oxomethylidenennojirimycin (DSO₂-ONJ) as selective anti-inflammatory agent by modulation of hemeoxygenase-1 in Bv2.microglial cells and retinal explants”*** to be considered for publication in Food and Chemical Toxicology.

Neuroinflammation has been described as an early event in the progression of diabetic retinopathy (DR). Associated with inflammation, gliosis is a hallamark of DR and recently we have reported the beneficial effects of the bicyclic compound (1R)-1-dodecylsulfinyl-5N,6O-oxomethylidenennojirimycin (1R-DSO-ONJ), a member of the sp^2 -iminosugar glycolipid (sp^2 -IGL) family, in targeting microglia, the immune resident cells of the retina, and reducing reactive gliosis in diabetic *db/db* mice. In the present study we have analyzed the effect of DSO₂-ONJ, another compound of the family incorporating a sulfone group that better mimics the phosphate group of phosphatidylinositol ether lipid analogs (PIAs), in Bv.2 microglial cells treated with bacterial lipopolysaccharide (LPS) and in retinal explants from *db/db* mice. In addition to decreasing iNOS and targeting nuclear factor kappa B (NFκB)-mediated signaling and the inflammasome, the anti-inflammatory effect of DSO₂-ONJ was likely mediated by direct p38α MAPK activation. We performed computational docking experiments revealing that DSO₂-ONJ binds to p38α MAPK at the same site where the PIAs and the alkyl phospholipid perifosine activators do, suggesting a similar mechanism of action. Importantly, treatment of Bv.2 microglial cells with DSO₂-ONJ increased both heme oxygenase (HO)-1 protein and *Il10* mRNA levels regardless the presence of LPS. In retinal explants from *db/db* mice, this compound also induced HO-1 expression, decreased iNOS levels and reduced reactive gliosis. Since IL-10-mediated induction of HO-1 expression is dependent on p38α MAPK activation, our results strongly suggest that this molecular mechanism is likely involved in the anti-inflammatory effects of DSO₂-ONJ in microglia. Altogether, our results provide new molecular insights on the therapeutic effects of the sp^2 -iminosugar glycolipid DSO₂-ONJ against retinal neuroinflammation during DR.

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Looking forward to the Editorial decision of the manuscript and with our thanks for reviewing this work.

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The sp²-iminosugar glycolipid 1-dodecylsulfonyl-5*N*,6*O*-oxomethylidenennojirimycin (DSO₂-ONJ) as selective anti-inflammatory agent by modulation of hemeoxygenase-1 in Bv.2 microglial cells and retinal explants

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ABSTRACT

Neuroinflammation is an early event during diabetic retinopathy (DR) that impacts the dynamics of microglia polarization. Gliosis is a hallmark of DR and we have reported the beneficial effects of 1R-DSO-ONJ, a member of the sp^2 -iminosugar glycolipid (sp^2 -IGL) family, in targeting microglia and reducing gliosis in diabetic *db/db* mice. Herein, we have analyzed the effect of DSO₂-ONJ, another family compound incorporating a sulfone group that better mimics the phosphate group of phosphatidylinositol ether lipid analogs (PIAs), in Bv.2 microglial cells treated with bacterial lipopolysaccharide (LPS) and in retinal explants from *db/db* mice. In addition to decreasing iNOS and inflammasome activation, the anti-inflammatory effect of DSO₂-ONJ was mediated by direct p38 α MAPK activation. Computational docking experiments demonstrated that DSO₂-ONJ binds to p38 α MAPK at the same site where the PIAs and the alkyl phospholipid perifosine activators do, suggesting a similar mechanism of action. Moreover, treatment of microglial cells with DSO₂-ONJ increased both heme-oxygenase (HO)-1 and *Il10* expression regardless the presence of LPS. In retinal explants from *db/db* mice, DSO₂-ONJ also induced HO-1 and reduced gliosis. Since IL-10-mediated induction of HO-1 expression is mediated by p38 α MAPK activation, our results suggest that this molecular mechanism is involved in the anti-inflammatory effects of DSO₂-ONJ in microglia.

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Neuroinflammation is an early event during diabetic retinopathy (DR) that impacts the dynamics of microglia polarization. Gliosis is a hallmark of DR and we have reported the beneficial effects of 1R-DSO-ONJ, a member of the sp^2 -iminosugar glycolipid (sp^2 -IGL) family, in targeting microglia and reducing gliosis in diabetic *db/db* mice. Herein, we have analyzed the effect of DSO₂-ONJ, another family compound incorporating a sulfone group that better mimics the phosphate group of phosphatidylinositol ether lipid analogs (PIAs), in Bv.2 microglial cells treated with bacterial lipopolysaccharide (LPS) and in retinal explants from *db/db* mice. In addition to decreasing iNOS and inflammasome activation, the anti-inflammatory effect of DSO₂-ONJ was mediated by direct p38 α MAPK activation. Computational docking experiments demonstrated that DSO₂-ONJ binds to p38 α MAPK at the same site where the PIAs and the alkyl phospholipid perfosine activators do, suggesting a similar mechanism of action. Moreover, treatment of microglial cells with DSO₂-ONJ increased both heme-oxygenase (HO)-1 and *Il10* expression regardless the presence of LPS. In retinal explants from *db/db* mice, DSO₂-ONJ also induced HO-1 and reduced gliosis. Since IL-10-mediated induction of HO-1 expression is mediated by p38 α MAPK activation, our results suggest that this molecular mechanism is involved in the anti-inflammatory effects of DSO₂-ONJ in microglia.

KEY WORDS: Diabetic retinopathy, inflammation, p38 MAPK, sp^2 -iminosugar, glycolipid, heme oxygenase-1

ABBREVIATIONS:

ARVO: Association for Research in Vision and Ophthalmology

BSA: Bovine Serum Albumin

CNS: Central Nervous System

DAPI: 4,6-diamidino-2-phenylindole

DR: Diabetic Retinopathy

DSO₂-ONJ: (1*R*)-1-dodecylsulfonyl-5*N*,6*O*-oxomethylidenenojirimycin

EGTA: Ethylene Glycol Tetraacetic Acid

FBS: Fetal Bovine Serum

FELASA: Federation of European Laboratory Animal Science Associations

GFAP: Glial Fibrillary Acidic Protein

HO-1: Heme oxygenase-1

HRP: Horseradish peroxidase

IκBα: Inhibitor of nuclear factor-κB, α

IL1β: Interleukine 1β

IL8: Interleukine 8

IL10: Interleukine 10

iNOS: Inducible Nitric Oxide Synthase

1 JNK: c-Junk N-terminal Kinase
2

3 LPS: Bacterial Lipopolysaccharide
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6 MAPKs: Mitogen-Activated Protein Kinases
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9 MCP-1: Monocyte chemoattractant protein-1
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12 MMFF94: Merck Molecular Force Field 94
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15 MKKs: Mitogen-Activated Protein Kinase Kinases
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18 NADPH: Nicotinamide Adenine Dinucleotide Phosphate
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21 NEDA: N-(1-naphthyl) ethylenediamine
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24 NF- κ B: Nuclear factor- κ B
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27 NLRP3 inflammasome: NACHT, LRR and PYD domains-containing protein 3
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inflammasome complex

Nrf2: Nuclear factor-erythroid 2-related factor 2

PDB: Protein Data Bank

PIAs: Phosphatidylinositol Ether Lipid Analogs

PVDF: Polyvinyl difluoride

qPCR: Quantitative Polymerase Chain Reaction

ROS: Reactive Oxygen Species

RT-PCR: Real Time Polymerase Chain Reaction

SEM: Standard Error of the Measurement

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

sp²-IGL: sp²-Iminosugar Glycolipid

1R-DSO-ONJ: (1R)-1-dodecylsulfinyl-5*N*,6*O*-oxomethylidenennojirimycin

T2DM: Type 2 Diabetes Mellitus

TNF- α : Tumor Necrosis Factor- α

HIGHLIGHTS:

1. The sp²-iminoglycolipid DSO₂-ONJ targets microglial Bv.2 cells and reduced LPS-mediated pro-inflammatory responses.
2. The anti-inflammatory effect of DSO₂-ONJ was mediated by direct p38 α MAPK activation.
3. DSO₂-ONJ increased HO-1 and *Il10* in retinal explants from *db/db* mice, which reduced reactive gliosis.
4. Docking experiments are consistent with DSO₂-ONJ binding at a hydrophobic pocket located at the C'-lobe of p38 α MAPK.
5. Similarly to phosphatidylinositol ether analogs or perifosine, DSO₂-ONJ likely induces autophosphorylation of p38 α MAPK.

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is recognized as a low grade chronic inflammatory disease in which initially the infiltration of immune cells in the adipose tissue triggers a systemic pro-inflammatory milieu that negatively interferes with the insulin action in peripheral tissues, as well as dysregulate whole body energy balance [1]. Inflammation, together with hyperglycemia are responsible for a prolonged metabolic stress that also impacts the central nervous system (CNS), leading to neuroinflammation [2].

Recently, neuroinflammation has been described as an early event in the progression of diabetic retinopathy (DR), a major diabetic complication and one of the leading causes of blindness worldwide [3]. Similar to other neurodegenerative diseases, DR exhibits an inflammatory component associated with changes in retinal expression of inflammatory mediators, which occurs in concert with functional changes in retinal permeability and cellular viability [4]. DR-associated retinal diseases such as proliferative diabetic retinopathy and diabetic macular edema are often accompanied by activation of microglial cells that constitute the immune resident population in the retina [5, 6]. In the *db/db* mouse model that recapitulates the progression of DR [7], we have recently reported that the polarization of microglia is a dynamic process that switches from the anti-inflammatory M2 stage that was increased adaptively in early diabetic retinas attempting to maintain retinal homeostasis to the pro-inflammatory M1 stage in established DR that is characterized by elevations in pro-inflammatory cytokines and the activation of the inflammasome system [8]. In this regard, $\text{TNF}\alpha$, $\text{IL1}\beta$, IL-8 and MCP-1 have been found elevated in the vitreous of diabetic patients [9, 10]. Thus, targeting microglia by switching its polarization towards the M2 stage is a therapeutic strategy to delay and/or prevent the deterioration of visual function in diabetic patients.

Oxidative stress, which is induced by the increased accumulation of reactive oxygen species (ROS) and/or decreased anti-oxidant capacity, has an important role in the pathogenesis of DR [11]. Elevations in ROS in the diabetic retina in humans have been confirmed by several groups [12, 13] as well as increases in the expression and/or activity of the NADPH oxidases Nox2 and Nox4 that constitute one of the major sources of ROS in the retina [14].

Heme oxygenase-1 (HO-1), encoded by HMOX1 gene in humans or *Hmox1* gene in rodents, is an anti-oxidant enzyme regulated by the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2), a master regulator of the cellular anti-oxidant responses [15]. On the other hand, it has been reported that overexpression of HO-1 in the retina was able to restore the visual function in diabetic animal models [16], suggesting that HO-1 represents an important adaptive mechanism of the retinal tissue for moderating the severity of cell damage produced by oxidative stress. Regarding oxidative stress in the retina of *db/db* mice, both HO-1 expression and activation of Nox4 have been found increased in the retinas at the age of 8 weeks, while decreased at 20 weeks compared to their age-matched controls [17], strongly suggesting that during the early stages of DR the retina has anti-oxidant sensors that protect against the oxidative component of the diabetic milieu. Pharmacological induction of HO-1 is therefore a promising approach for therapeutic intervention in DR conditions.

Activation of mitogen-activated protein kinases (MAPKs), such as the p38 α MAPK, plays a central role for the induction of HO-1 gene expression [18]. Accordingly, lipid-based activators of p38 α MAPK, which include the phosphatidylinositol ether lipid analogs (PIAs) and the alkyl phospholipid perfosine, have demonstrated a major anti-inflammatory potential [19, 20]. However, side effects associated to hemotoxicity,

cardiotoxicity, gastrointestinal toxicity and alteration of the insulin/glucose metabolism may represent a limitation for clinical translation [21, 22]. Thus, the identification and characterization of compounds featuring a better pharmacological profile that induce HO-1 in a cell-specific and cell context-specific manner remains a current priority.

The World Health Organization (WHO) has estimated that 80% of the world inhabitants utilized traditional medicine for their primary health care needs and the majority of this therapy requires the use of herbal extracts and their active components. Various medicinal plant bioactive extracts and their identified/isolated active constituents have shown a variety of medicinal pharmacological properties against chronic diseases with an inflammatory component such as T2DM [23, 24]. Iminosugar glycosyl hydrolase inhibitors such as 1-deoxynojimycin [25, 26] and castanospermine, bear strong potential in therapies such as cancer, viral infections [27, 28], tuberculosis [29], diabetes and glycosphingolipid storage disorders [30, 31]. We have recently reported the beneficial effects of the bicyclic compound (1*R*)-1-dodecylsulfinyl-5*N*,6*O*-oxomethylidenenojirimycin (1*R*-DSO-ONJ), a member of the sp²-iminosugar glycolipid (sp²-IGL) family, in reducing reactive gliosis in retinal explants from *db/db* mice by increasing the expression of the anti-inflammatory M2 marker arginase-1 [8]. At the molecular level, 1*R*-DSO-ONJ ameliorated the NFκB-mediated pro-inflammatory signaling cascade triggered by LPS in Bv.2 microglial cells, resulting in decreased iNOS expression and pro-inflammatory cytokines. Initially, the biological effects of sp²-iminosugars were assumed to be the result of their interaction with glycosidases, since several representatives have been shown to behave as selective inhibitors or activators of these enzymes [32, 33]. Notwithstanding, neither the anti-inflammatory activity of 1*R*-DSO-ONJ nor the anti-proliferative, anti-metastatic and anti-parasitic activities displayed by other sp²-IGLs could be correlated with glycosidase inhibition [34, 35].

1 Instead, this range of activities is very much reminiscent of that displayed by PIAs,
2 suggesting that sp²-IGLs and PIAs share the same kinase targets. If this hypothesis is
3
4 correct, replacement of the sulfinyl group in 1R-DSO-ONJ by a sulfonyl group (1-
5 dodecylsulfonyl-5*N*,6*O*-oxomethylidenennojirimycin; DSO₂-ONJ, Figure 1A), a closer
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7 neutral isoster of the phosphate group in PIAs, might lead to a better candidate for DR
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9 treatment. As a proof of concept, here we report our results on the evaluation of the
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11 effect of DSO₂-ONJ in Bv.2 microglial cells and retinal explants of *db/db* mice in the
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13 context of inflammation and oxidative stress associated to DR. The data are consistent
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15 with the activation of p38α MAPK leading to the enhancement of HO-1 expression.
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17 Computational docking experiments further support that DSO₂-ONJ binds to
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19 p38α MAPK at the same site where the PIAs and perifosine activators do, strongly
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21 suggesting a similar mechanism of action.
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32 **2. RESEARCH DESIGN AND METHODS**

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35 **2.1. Reagents.** Fetal bovine serum (FBS) and culture media were obtained from
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37 Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA), crystal violet,
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39 bacterial lipopolysaccharide (LPS) and SB203580 were purchased from Sigma-Aldrich
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41 (St Louis, MO, USA). Bradford reagent, acrylamide, immunoblot PVDF membranes
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43 and chemiluminiscent HRP Substrate were purchased from Bio-Rad (Madrid, Spain).
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49 **2.2. Antibodies.** Antibodies against caspase-1 (p10) (sc-514), iNOS (sc- 650), IκBα (sc-
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51 371), p65-NFκB (p65) (sc-732), JNK (sc-571) and phospho-p38α MAPK (Thr
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53 180/Tyr182) (sc-17852-R) were purchased from Santa Cruz Biotechnology (Palo Alto,
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55 CA, USA). Anti-phospho JNK (Thr183/Tyr185) (#4668) and anti-p38α MAPK (#9212)
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antibodies were purchased from Cell Signaling Technology (MA, USA). Anti-arginase-1 (BD610708) antibody was purchased from BD Bioscience (Madrid, Spain). Anti-glial fibrillary acidic protein (GFAP) antibody (Z0334) was obtained from DAKO (Glostrup, Denmark) and anti- α -tubulin (T-5168) antibody was from Sigma-Aldrich (St Louis, MO, USA).

2.3. Synthesis of the sp^2 -iminosugar glycolipid DSO₂-ONJ. (1*R*)-1-Dodecylsulfonyl-5*N*,6*O*-oxomethylidenenojirimycin (referred to as DSO₂-ONJ, Fig. 1A) was synthesized from (1*R*)-1,2,3,4-tetra-*O*-acetyl-5*N*,6*O*-oxomethylidenenojirimycin [36] by *S*-glycosylation with 1-dodecanethiol, oxidation of the resulting sulphide with *m*-chloroperbenzoic acid and final deacetylation, following the procedures previously reported [35]. DSO₂-ONJ was dissolved in DMSO (50%) before storage at -20°C .

2.4. Cell culture. Mouse microglia Bv.2 cell line was supplied by Dr. M. L. Nieto (IBGM, Spain). Bv.2 cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in RPMI supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin (Sigma) and 2 mM L-glutamine (Gibco, Carlsbad, California, USA). Cells were grown up to 70% confluence and then washed twice with PBS and further cultured in serum-free medium and stimulated with LPS (200 ng/ml) with or without DSO₂-ONJ (1, 10 50 μM) for several time-periods.

2.5. Retinal explants. Ex vivo assays were performed with retinas from 8-week-old male *db/+* and *db/db* mice in the C57BL/KsJ genetic background purchased from Charles River Laboratories (Charles River, Barcelona, Spain). Mice were maintained in light/dark (12-hours light/12-hours dark), temperature (22°C) and humidity-controlled rooms, and fed *ad libitum* with free access to water. All animal experimentation followed recommendations of the Federation of European Laboratory Animal Science

Associations (FELASA) on health monitoring in accordance with the regulations of the Association for Research in Vision and Ophthalmology (ARVO). Animals were killed by cervical dislocation and eyes were enucleated. The lens, anterior segment, vitreous body, retinal pigment epithelium and sclera were removed and the retinas were cultured in R16 medium (provided by Dr. P.A. Ekstrom, Lund University, Sweden) with no additional serum. Retinas were stimulated with DSO₂-ONJ as indicated in the figure legends.

2.6. Analysis of the cellular viability by crystal violet staining. After cell treatments, the medium was discarded, and the remaining viable adherent cells were stained with crystal violet (0.2% w/v in 2% ethanol) for 20 min. After this time, plates were rinsed with tap water and allowed to dry, and 1% SDS was added to solubilize them. The absorbance of each plate was read spectrophotometrically at 560 nm.

2.7. Analysis of Nitrites (NO₂⁻). Levels of NO₂⁻ were measured by using the Griess method [37]. Briefly, nitrites turn into a pink compound in contact with an acid solution containing 1% sulphanilamide and 0.1% N-(1-naphthyl) ethylenediamine (NEDA), and can be quantified by a colorimetric method at 540 nm in a microplate reader (Versamax Tunable Microplate reader, Molecular Devices, Sunnyvale, CA, USA).

2.8. Immunofluorescence. Bv.2 microglial cells were seeded on coverslips 24 h before adding LPS and/or DSO₂-ONJ in serum-free medium. After treatments, cells were washed in PBS, fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature, washed in PBS and permeated with 0.4% (w/v) Triton X-100 (Sigma) in PBS for 20 min. Non-specific binding was blocked in PBS containing 3% (w/v) BSA and 0.1% (w/v) Triton X-100 for 2 h and the cells were then left overnight in a humid chamber at 4 °C with rabbit anti-p65NFκB antibody in blocking buffer. Subsequently,

1 the cells were washed and incubated in darkness for 2 h with anti-rabbit conjugated
2 Alexa 488 antibody (Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA)
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4 The cells were then washed, stained with 4,6-diamidino-2-phenylindole (DAPI) and
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6 mounted with medium (Fluoromount G). Immunofluorescence was examined in a
7
8 Nikon Eclipse 90i microscope with imaging software Nis elements (Nikon, Nikon
9
10 Instruments Europe B.V.).
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15 For immunofluorescence analysis in retinal explants, eyes were fixed in 4% (w/v)
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17 paraformaldehyde for 24 h at 4 °C and infiltrated with sucrose 25% (w/v). Retinal
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19 explants were washed in TBS containing 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100
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21 (this buffer was used for all subsequent washes), and then blocked and permeated for 2
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23 h in TBS containing 3% (w/v) BSA and 1% (v/v) Triton X-100. The retinal explants
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25 were then incubated overnight in a humid chamber at 4 °C with rabbit anti-GFAP
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27 antibody in blocking solution. Retinal explants were washed and incubated for 90 min
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29 with anti-rabbit immunoglobulin antibody conjugated to Alexa 488 (1:2000; Molecular
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31 Probes, Eugene, OR). After washing, retinal explants were mounted with medium
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33 (Fluoromount G) containing DAPI. Staining was observed with an inverted laser
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35 confocal microscope LSM710 (Carl Zeiss Microscopy GmbH, Göttingen, Germany).
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43 **2.9. Quantitative real-time PCR (qPCR) analysis.** Total RNA was extracted with
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45 Trizol® reagent (Invitrogen, Madrid, Spain) and reverse transcribed using a
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47 SuperScript™ III First-Strand Synthesis System for qPCR following the manufacturer's
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49 indications (Invitrogen). qPCR was performed with an ABI 7900 sequence detector.
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51 Primer-probe sets for mouse *Tnfa*, *Il6*, *Il1b*, *Il10*, *Nlrp3*, *Nos2*, *arginase-1* and *18s* were
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53 purchased as predesigned TaqMan gene expression assays (Applied Biosystems).
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2.10. Western blot. Whole retinas or Bv.2 microglial cells were homogenized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 1mM NaF, 1 mM EGTA, 15% (w/v) NP40 and 0.25% (w/v) sodium deoxycholate, supplemented with protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, and 100 µg/ml phenylmethylsulphonyl fluoride). All debris was removed by centrifugation at 14,000 x g for 10 min at 4 °C and protein concentration was quantified using the Bio-Rad protein assay with BSA as a standard. Equivalent amounts of protein were resolved using denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to PVDF membranes (Bio-Rad). Membranes were blocked using 5% non-fat dried milk or 3% BSA in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS), and incubated overnight with several antibodies (1:2000 unless otherwise stated) in chemiluminescence reagent (Bio-Rad).

2.11. Statistical analysis. Densitometry of the Western blots was performed using the ImageJ program. Values in all graphs represented the mean ± SEM. Statistical tests were performed using SPSS 21.0 for Windows (SPSS Inc. IBM, Armonk, NY, USA). Data were analyzed by one-way ANOVA followed by Bonferroni t-test or by paired t-test when comparisons were among two groups. Differences were considered significant at *†#p ≤ 0.05.

2.12. Docking experiments. Protein-ligand docking calculations were performed with the AutoDock4 program [38]. For the DSO₂-ONJ docking experiments, two slightly different sets of coordinates corresponding to the p38α MAPK receptor were used: those from p38α MAPK in complex with PIA23 (PDB ID 4E6A) and those from the p38α MAPK:perifosine complex (PDB ID 4E6C) [39]. The calculations predict a better binding of DSO₂-ONJ to the 4E6A structure (Supplementary Figure 2) and, thus, these

will be the results presented in the Results section; the ones corresponding to the 4E6C protein coordinates are included in the Supplementary Information (Supplementary Figure 3). The initial coordinates of the DSO₂-ONJ ligand were built with the Chem3D program, assuming a ⁴C₁ conformation for the six-membered ring. A MMFF94 energy minimization in a continuum solvent environment ($\epsilon = 78$) was used to optimize the DSO₂-ONJ structure. For the protein, hydrogen atoms were added and the protonation states of the ionizable residues were assigned with PROPKA through the PDB2PQR server [40]. For the docking calculations, the volume for exploration was defined by a grid box centred at a point in the middle of the hydrophobic cavity ($x = 6.5$, $y = 2.0$, $z = -2.2$, according to PDB ID 4E6A coordinates), with 86 x 102 x 112 points and a grid spacing of 0.253 Å. Gasteiger charges were used. The docking calculations were carried out using the Lamarckian genetic algorithm, with rigid side chains for the receptor and randomly changing the torsion angles (15 rotatable bonds for DSO₂-ONJ) and overall orientation of the ligand. 20 solutions were generated for each ligand. For the genetic algorithm, the recommended default values were used for all the parameters except for the number of energy evaluations, which was set to a larger value (2.5×10^6) to better account for the large dimensionality of the systems under study. For comparison, docking calculations of perifosine (which presents a similar number of rotatable bonds, 19) to the 4E6C protein structure were also conducted. The same protocol was followed, except for the fact that the coordinates of perifosine were taken from the PDB ID 4E6C, when available, and the ones for the missing atoms were built with Chem3D. The corresponding results are given in the Supplementary Information (Supplementary Figure 4). Molecular representations in figures were generated with the USCF Chimera package [41].

3. RESULTS

3.1. Effect of the sp²-iminosugar glycolipid DSO₂-ONJ on the cellular viability and the expression of iNOS in LPS-stimulated Bv.2 microglial cells.

We first tested the viability of microglia Bv.2 cells when exposed to various concentrations of the sp²-iminosugar glycolipid (sp²-IGL) derivative 1-dodecylsulfonyl-5*N*,6*O*-oxomethylidenennojirimycin (referred to as DSO₂-ONJ, Figure 1A) and found that this effect was dose-dependent, being reduced by 75% at 50 μM concentration of DSO₂-ONJ (Figure 1B). Based on these results, we used the doses of 1 and 10 μM for further experiments.

Figure 1. Effect of DSO₂-ONJ on the cellular viability and the expression of iNOS in LPS-stimulated Bv.2 microglial cells. Bv.2 microglial cells were stimulated with 200 ng/ml LPS and 10 μ M DSO₂-ONJ for 24 h. **A)** Chemical structure of the *sp*²-iminosugar glycolipid 1-dodecylsulfonyl-5N,6O-oxomethylidenenojirimycin, referred as DSO₂-ONJ in the figures. Bv.2 microglial cells were treated for 24 h with DSO₂-ONJ (1-50 μ M). **B)** Viability was determined by crystal violet staining. Colorimetric quantification was performed, and the results are mean $\pm$ S.E.M. The fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni *t*-test. **C)** Bv.2 microglial cells were treated for 24 h with LPS (200 ng/ml) or LPS plus DSO₂-ONJ (1-10 μ M). Nitrites accumulation was analyzed and related to basal levels. Colorimetric quantification was performed, and the results are mean $\pm$ S.E.M. The fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni *t*-test. **D)** mRNA levels of *Nos2* were determined by RT-PCR. The results are means $\pm$ S.E.M. ($n=5$ independent experiments). The fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni *t*-test. **E)** Protein extracts were analyzed by Western blot with the corresponding antibodies against iNOS and α -Tubulin as loading control. Representative autoradiograms are shown ($n= 5$ independent experiments). Blots were quantified by performing scanning densitometry, and the results are mean $\pm$ S.E.M. The

fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni t-test).

In order to get more insights on the effect of DSO₂-ONJ in microglia, Bv.2 cells were stimulated with LPS, a M1 stimulus, in the absence or presence of DSO₂-ONJ. Among other effects, LPS induces a pro-inflammatory environment similar to that found in the obesity-related diabetic context [42]. Bv.2 microglial cells were cultured for 24 h in the presence of LPS (200 ng/ml) or DSO₂-ONJ (1-10 μ M), and the combination of both (LPS + DSO₂-ONJ). As shown in Figure 1C, the elevation of nitrites in the culture medium induced by LPS treatment was significantly reduced by the co-treatment with DSO₂-ONJ in a dose-dependent manner. Likewise, mRNA levels of *Nos2* and protein level of iNOS, which were elevated in LPS-treated Bv.2 microglial cells, significantly decreased in the presence of DSO₂-ONJ (Figure 1D).

3.2. LPS-treated Bv.2 microglial cells induced a pro-inflammatory response and activation of the inflammasome complex that was reduced by DSO₂-ONJ.

The effect of DSO₂-ONJ in the mRNA levels of pro- and anti-inflammatory cytokines in Bv.2 microglial cells was next analyzed. LPS induced a significant increase in *Tnfa*, *Il1b* and *Il6* mRNA that was ameliorated by the co-treatment with DSO₂-ONJ (Figure 2A). As in LPS-stimulated microglia IL1 β is processed via caspase-1 through the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome complex [28], we explored if the anti-inflammatory effect of DSO₂-ONJ was dependent on the inflammasome. The mRNA levels of *Nlrp3* showed a significant increase after LPS stimulation, but were markedly reduced upon treatment with DSO₂-ONJ (Figure 2B). As shown in Figure 2C, the cleavage of pro-caspase-1 in response to LPS was

totally abolished in Bv.2 microglial cells treated with DSO₂-ONJ. Also, the effect of this compound in the inflammasome was assessed by the significant reduction in the cleavage of pro-IL1 β into its active (processed) form which was induced by LPS stimulation (Figure 2D).

A

B

C

D

Figure 2. Protective effects of DSO₂-ONJ against LPS-mediated activation of the inflammasome and elevation of pro-inflammatory cytokines mRNA levels in Bv.2 microglial cells. Bv.2 microglial cells were stimulated with 200 ng/ml LPS and 10 μ M DSO₂-ONJ for 24 h. **A)** *Tnfa*, *Il1b*, and *Il6* mRNA levels were determined by RT-PCR. **B)** *Nlrp3* mRNA levels were determined by RT-PCR. The results are mean $\pm$ S.E.M. (n=5 independent experiments). The fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni t-test). Protein extracts were analyzed by Western blot with antibodies against caspase-1 (**C**) and IL-1 β (**D**). α -Tubulin was used as a loading control. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry, and the results are mean $\pm$ S.E.M. The fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni t-test).

In order to determine the molecular mechanisms involved in the anti-inflammatory effect exerted by DSO₂-ONJ, we examined MAPKs and NF κ B-mediated signaling pathways. Treatment of Bv.2 microglial cells with LPS rapidly activated MAPKs by

inducing the phosphorylation of JNK and p38 α MAPK and induced I κ B degradation; maximal effect being elicited after 30 min (Figure 3A). Co-treatment with DSO₂-ONJ prevented LPS-mediated I κ B α degradation (Figure 3A), as well as the nuclear translocation of p65-NF- κ B (Figure 3B). However, DSO₂-ONJ failed to decrease the phosphorylation of JNK and p38 α MAPK (Figure 3A). On the contrary, co-treatment with LPS and DSO₂-ONJ led to significantly higher levels of phosphorylation of both kinases as compared with the treatment with LPS alone.

A

Figure 3. Effects of DSO₂-ONJ in the activation of MAPKs-mediated signaling in LPS-stimulated Bv.2 microglial cells. Bv.2 microglial cells were stimulated with 200 ng/ml LPS and 10 μ M DSO₂-ONJ for the indicated time periods. **A)** Protein extracts were separated by SDS-PAGE and analyzed by Western blot with antibodies against phosphorylated (p)-JNK, total JNK, phosphorylated (p)-p38 α MAPK, total p38 α MAPK, I κ B α and α -Tubulin. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry and the results are mean $\pm$ S.E.M. The ratio between the indicated proteins and the fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment (two-way ANOVA followed by Bonferroni t-test). **B)** Confocal immunofluorescence assessment of nuclear translocation of p65-NF κ B in Bv.2 microglial cells following stimulation with LPS in the absence or presence of DSO₂-ONJ. Activation of p65-NF κ B nuclear translocation was defined by an increase in immunofluorescence of p65-NF κ B (green channel) in the nuclear regions. Nuclear regions of Bv.2 microglial cells were determined by counterstaining of nuclear DNA with DAPI (blue channel). Nuclear localization of p65-

NFκB appears in LPS treatment at 30 min and 60 min, but not in *Bv.2* microglial cells preincubated with 10 μM DSO₂-ONJ.

To analyze the direct effect of DSO₂-ONJ in the phosphorylation of JNK and p38α MAPK, we performed a time-course experiment. Figure 4 shows that DSO₂-ONJ induced the phosphorylation of JNK and p38α MAPK, with a maximal effect being elicited at 60 min. Supplementary Figure 1 shows that DSO₂-ONJ-induced p38α MAPK phosphorylation was totally abolished by the inhibitor SB203580.

Figure 4. Direct effect of DSO₂-ONJ in the activation of the MAPKs JNK and p38α MAPK in Bv.2 microglial cells. Bv.2 microglial cells were stimulated with 200 ng/ml LPS and 10 μM DSO₂-ONJ for the indicated time periods. Protein extracts were analyzed by Western blot with antibodies against phosphorylated (p)-JNK, total JNK, phosphorylated (p)-p38α MAPK and total p38α MAPK. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry and the results are mean ± S.E.M. The ratio between the indicated proteins and the fold change relative to Basal condition is shown. *p≤0.05 vs LPS treatment (two-way ANOVA followed by Bonferroni t-test).

3.3. Involvement of p38α MAPK in the effects of DSO₂-ONJ in Bv.2 microglial cells.

As shown in Figure 1E, DSO₂-ONJ reduces the effect of LPS on the induction of iNOS protein levels. As expected, Bv.2 microglial cells treated with the combination of LPS and the p38α MAPK inhibitor SB203580 showed a substantial reduction of iNOS protein expression (Figure 5). Interestingly, protein levels of iNOS were further reduced by the combination of LPS+SB203580+DSO₂-ONJ, reflecting a synergistic effect of SB203580 and DSO₂-ONJ.

Figure 5. Effects of the p38 α MAPK inhibitor SB203580 in the modulation iNOS by LPS and DSO₂-ONJ in Bv.2 microglial cells. Bv.2 microglial cells were pretreated for 30 min with SB203580, followed by stimulation with 200 ng/ml LPS and 10 μ M DSO₂-ONJ for 24 h. The protein extracts were analyzed by Western blot with antibodies against iNOS and α -Tubulin. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry, and the results are mean $\pm$ S.E.M. The ratio between the two indicated proteins and the

fold change relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment. # $p \leq 0.05$ vs LPS + DSO₂-ONJ treatment. † $P \leq 0.05$ vs LPS + SB203580 treatment (two-way ANOVA followed by Bonferroni t-test).

3.4. HO-1 was induced by DSO₂-ONJ in Bv.2 microglial cells and retinal explants from *db/db* mice.

Previous studies have shown that HO-1 induction exerts protective effects in cells stimulated by pro-inflammatory cytokines through the down-regulation of inflammatory mediators [43, 44]. Based on these studies, we analyzed the mRNA levels of the anti-inflammatory cytokine IL10. As shown in Figure 6A, LPS markedly decreased *Il10* mRNA levels, an effect abolished in the presence of DSO₂-ONJ. Next, we analyzed the protein expression of HO-1 in Bv.2 microglial cells stimulated with DSO₂-ONJ in the presence and absence of LPS. As Figure 6B shows, DSO₂-ONJ increased HO-1 levels in microglial cells either alone or in the presence of LPS. However, compound DSO₂-ONJ did not counteract the effect of LPS on *arg-1* mRNA levels (encoding arginase-1), a marker of M2 polarization stage of microglia (Figure 6C).

Figure 6. Induction of an anti-inflammatory response mediated by HO-1 and IL-10 in Bv.2 microglial cells treated with DSO₂-ONJ. Bv.2 microglial cells were stimulated with 200 ng/ml LPS and 10 μ M DSO₂-ONJ for 24 h. **A)** IL10 mRNA levels were determined by RT-PCR. The results are means $\pm$ S.E.M. (n=5 independent experiments). The fold change levels relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment; # $p \leq 0.05$ vs Basal treatment. **B)** Protein extracts were analyzed by Western blot with antibodies against HO-1 and α -Tubulin. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry and the results are mean $\pm$ S.E.M. The fold change of

*HO-1 levels relative to Basal condition is shown. * $p \leq 0.05$ vs LPS treatment; # $p \leq 0.05$ vs Basal treatment. C) Arg-1 mRNA levels were determined by RT-PCR. The results are means $\pm$ S.E.M. ($n=5$ independent experiments).*

We next analyzed the effect of the $\text{sp}^2\text{-IGL DSO}_2\text{-ONJ}$ in whole retinal explants from diabetic *db/db* mice that presented established DR at the age of 8 weeks [7, 8]. Retinal explants from 8 weeks-old *db/+* control and *db/db* mice were treated for 24 h with $\text{DSO}_2\text{-ONJ}$ and the expression of HO-1 was evaluated by Western blot. As shown in Figure 7A, treatment with $\text{DSO}_2\text{-ONJ}$ increased HO-1 expression exclusively in retinal explants from *db/db* mice. Also, the expression of iNOS, which was elevated in retinal explants from *db/db* mice, was significantly reduced by the treatment with $\text{DSO}_2\text{-ONJ}$ compound (Figure 7B).

Figure 7. Treatment of retinal explants from db/db mice with DSO₂-ONJ compound decreased iNOS, increased HO-1 and reduced reactive gliosis. Retinal explants from 8 weeks old db/db and db/+ mice were treated for 24 h with DSO₂-ONJ (20 µM) or vehicle. Western blot was performed with the corresponding antibodies against HO-1 (A) or iNOS (B) using α-Tubulin as loading control. Blots were quantified by performing scanning densitometry and the results are mean ± S.E.M. The fold change levels relative to Basal condition is shown. * $p \leq 0.05$ vs db/db Representative autoradiograms are shown (n=5 retinas per condition). C) Retinal explants from db/db mice at 8 weeks of age were treated for 24 h with DSO₂-ONJ (20 µM) (right panel) or with vehicle (left panel). Immunostaining for GFAP (green) was carried out in whole retinas. Representative images are shown (n=5 retinas per condition).

3.6. Retinal gliosis is reduced by the treatment of retinal explants from db/db mice with DSO₂-ONJ.

To get more insights in the benefit of increasing HO-1 levels in the whole retina by administration of DSO₂-ONJ under a pro-inflammatory environment we evaluated the reactive gliosis, which is a hallmark of neurodegenerative diseases of the retina, including DR [45, 46]. We have previously shown that reactive gliosis is already present in the retinas from obese/diabetic *db/db* mice at 8 weeks of age, and that this effect was maintained in retinal explants [47]. To achieve this, retinal explants from 8 weeks-old *db/db* mice were treated with DSO₂-ONJ for 24 h and subsequently analyzed by immunofluorescence with the anti-GFAP antibody. As shown in Figure 7C, reactive gliosis was totally blunted in retinal explants from *db/db* mice treated with DSO₂-ONJ compound at 20 μ M for 24 h.

3.7. Docking of DSO₂-ONJ into p38 α MAPK

The overall topology of p38 α MAPK consists of two lobes (N' and C') that form a groove in between hosting the catalytic site (Figure 8A) [48]. The available crystal structures of p38 α MAPK in complex with PIAs and perifosine show that these lipid molecules bind at a hydrophobic pocket located at the C'-lobe distal from the active site [45]. Upon binding, they induce local conformational changes in the α EF/ α F loop region (residues 194-200), leading to p38 α MAPK autophosphorylation and activation. The similar chemical structure of sp²-IGLs and these activating lipid molecules led to the hypothesis that DSO₂-ONJ might bind at the same site in the kinase C'-lobe. To probe this notion, we performed docking experiments between DSO₂-ONJ and p38 α MAPK. Two main binding modes, with similar estimated binding free energies, have been obtained for the binding of DSO₂-ONJ into this hydrophobic pocket (Figure 8). As observed in the 4E6A and 4E6C crystallographic complexes, the polar regions of DSO₂-ONJ are predicted to form only few polar interactions with some main-chain groups. In

the first complex (Figure 8B), these polar regions are more solvent exposed. In the second one (Figure 8C), DSO₂-ONJ binds in a mode reminiscent to that observed for PIA23 in the crystallographic complex (Supplementary Figure 2). These results are consistent with the prediction, supporting that DSO₂-ONJ elicit p38α MAPK autophosphorylation and activation through a mechanism analogous to that evidenced for PIAs or perifosine.

Figure 8. Predicted binding of DSO₂-ONJ into p38α MAPK. Docking calculations of DSO₂-ONJ into the p38α MAPK structure from the PDB ID 4E6A predict two main binding modes, with similar estimated binding free energies ($\Delta G_{\text{binding}}$). **A)** Overall view of p38α MAPK (beige cartoon) with the two predicted binding modes for DSO₂-ONJ shown. The ligand is represented in sticks and coloured by element with purple and cyan carbon atoms for the first and second binding modes, respectively. Hydrogen atoms are not represented for clarity. **B)** and **C)** are closer views to the DSO₂-ONJ first and second binding modes, respectively. Trp197 is shown in beige sticks.

4. DISCUSSION

Diabetic retinopathy has an important pro-inflammatory component associated with alterations in the immune responses, including the dynamics of microglia polarization

[8]. In fact, targeting neuroinflammation by modulating the polarization state of microglia has been suggested as a therapeutic strategy to delay and/or prevent the deterioration of visual function in diabetic patients [49].

Several types of lipid molecules, including PIAs or the alkyl ether lipids (e.g. perifosine) and their glycosylated derivatives, which were initially developed as antitumor agents, have been found to display anti-inflammatory properties [8, 50, 51]. sp^2 -Iminosugar glycolipids (sp^2 -IGLs) represent a recent addition to this list. Although the mechanisms involved in their biological activities are still poorly understood, the structural similarities with the PIAs and their interference with the same biochemical routes suggest that sp^2 -IGLs and PIAs may share the same targets [51]. In the context of DR, we have recently reported the anti-inflammatory effect of 1R-DSO-ONJ, a member of the sp^2 -IGL family, by targeting pro-inflammatory signaling in microglia [8]. In the present study, we have investigated whether DSO₂-ONJ [35], a close analogue incorporating a sulfone group that better mimic the phosphate group in PIAs, exerts anti-inflammatory effects counteracting LPS in Bv.2 microglial cells, and also in an *ex vivo* model of retinal explants from diabetic *db/db* mice. A step further, we have conducted research on the molecular mechanisms involved.

After an initial screening of the dose-dependent effect of DSO₂-ONJ in the cellular viability of Bv.2 microglial cells, we found that at non-toxic concentrations (1-10 μ M) this compound was able to inhibit NO production induced by LPS-stimulation, one of the key features of the inflammatory stages. Regarding the molecular mechanisms by which DSO₂-ONJ elicits the anti-inflammatory effects in microglial cells, it almost completely blocked LPS-induced *Nos2* mRNA, iNOS protein levels, as well as decreased mRNA levels of pro-inflammatory cytokines while, simultaneously, it increased the mRNA levels of the anti-inflammatory cytokine IL10. The analysis of

classical pro-inflammatory signaling cascades revealed that DSO₂-ONJ efficiently inhibited LPS-mediated degradation of IκB and the translocation of p65 NFκB to the nucleus, as well as the NLRP3 inflammasome complex by targeting *Nlrp3* mRNA and IL1β processing by caspase-1. However, DSO₂-ONJ failed to inhibit JNK or p38α MAPK phosphorylation and, more importantly, the latter kinases were directly activated by DSO₂-ONJ.

It is well known that the phosphorylation of JNK and p38α MAPK, key molecules of the MAPKs signaling pathway, is critical for the ability of immune cells to respond to various pro-inflammatory stimuli including LPS [53]. As stated above, DSO₂-ONJ was able to induce the activation of both MAPKs in the same way that LPS. In the light of these results, it has been shown that compounds such as chitosan and its derivatives elicit anti-inflammatory effects in macrophages through the activation of both MAPKs [54], and other studies have reported anti-inflammatory effects mediated by specific activation of p38α MAPK [18]. Although the canonical signaling mediated by upstream MAPK kinases (MKKs) is considered to be the main pathway for MAPKs activation, several alternative mechanisms for the activation of p38α MAPK isoform have been reported [55]. Interestingly, PIAs have been shown to concomitantly induce p38α MAPK autophosphorylation and activation in a MKK3/6 dependent and independent manner [19], and a similar p38α MAPK activation effect has been reported for perifosine [20]. Given the similar chemical nature of DSO₂-ONJ and these activating lipid molecules, we hypothesized that they may all bind at the same site in p38α MAPK. The structures of p38α MAPK in complex with PIAs or perifosine revealed similar mode of binding at a novel hydrophobic binding site in the kinase C'-lobe formed in part by the p38α MAPK insert region and also identified local and significant conformational changes of the αEF/αF loop that could be the basis of the autoactivation

properties [39]. Importantly, this loop, located in the vicinity of the activation loop, has no sequence and conformational similarity within the MAPKs family or to other kinases. In this regard, it is reasonable to speculate that DSO₂-ONJ might also act as an intracellular inductor of p38 α MAPK phosphorylation by binding at the same site in the protein as PIAs or perifosine, thereby activating its downstream signaling pathways. Docking experiments support this hypothesis, suggesting even a more favourable binding energy as compared with PIAs or perifosine (Figure 8 and Supplementary Figures 2-4). Additional experimental evidence for this alternative p38 α MAPK activation by DSO₂-ONJ is supported by the fact that in the presence of SB203580, an inhibitor of the classical activation of p38 α MAPK by dual phosphorylation, DSO₂-ONJ was still able to maintain p38 α MAPK-mediated anti-inflammatory effects in LPS-stimulated Bv.2 cells.

We next addressed the mechanism by which the direct activation of p38 α MAPK by DSO₂-ONJ could be involved in its anti-inflammatory effects in microglial cells. Activation of p38 α MAPK has been shown to induce HO-1 expression in various cell types including macrophages [46], hepatocytes [47], pulmonary epithelial cells [48], and vascular cells [49]. HO-1 is an important anti-oxidant molecule and its up-regulation is a critical cytoprotective mechanism activated during cellular stress [56, 57]. In this work we have demonstrated that in Bv.2 microglial cells there is a direct relationship between the induction of HO-1 protein expression and elevation of *IL10* mRNA by the treatment with the sp²-iminosugar glycolipid DSO₂-ONJ. It is important to note that HO-1 and IL-10 can perpetuate their anti-inflammatory effects in a reciprocal manner [49]. Moreover, both HO-1-induced IL-10 production and IL-10-induced HO-1 activation are dependent on p38 α MAPK activation [50-53]. In this regard our results strongly suggest that DSO₂-ONJ selectively up-regulates the synthesis

of the anti-inflammatory cytokine IL-10 in a manner likely dependent on HO-1 due to both the canonical and non-canonical p38 α MAPK activation [58].

Our previous data in retinal explants from *db/db* mice at 8 weeks of age have shown that treatment with 1R-DSO-ONJ, another sp²-IGL representative, ameliorated reactive gliosis [8]. A step further, the results presented herein revealed that iNOS expression, which was elevated in the retina of diabetic *db/db* mice, was reduced after treatment of retinal explants with DSO₂-ONJ that in parallel increased HO-1 levels, reinforcing the link between inflammation and HO-1 in the retina. Interestingly, reactive gliosis in retinal explants from *db/db* mice was also reduced by the treatment with DSO₂-ONJ. As reactive gliosis is present in DR [7, 58, 59], our results might have therapeutic implications proposing a novel strategy for its treatment based on the specific induction of HO-1 in the retina.

In conclusion and as summarized in Figure 9, the results of this study have demonstrated a protective effect of DSO₂-ONJ compound in Bv.2 microglial cells and in retinal explants from *db/db* mice that, in addition of targeting NF κ B and the inflammasome, can be mediated through the direct activation of p38 α MAPK. This latter effect leads to the induction of HO-1 and IL-10, boosting the anti-inflammatory efficacy of this compound. Therefore, the induction of HO-1 and its associated anti-inflammatory mechanisms could strengthen the structural and immunological protection of the retina during inflammatory processes such those occurring in DR.

Figure 9. Schematic representation of the signaling pathways involved in the anti-inflammatory effects of DSO₂-ONJ in microglial cells. DSO₂-ONJ compound elicits beneficial effects in microglia during neuroinflammation associated to DR by blocking the pro-inflammatory NFκB pathway. Additionally, DSO₂-ONJ activates p38α MAPK which, in turn, induces HO-1 and IL10 and confers anti-oxidant and anti-inflammatory protection in the retina.

Contributions

AIA researched data and wrote and reviewed the manuscript. AMV provided funding, wrote and reviewed the manuscript. EA-E researched data. EMSF researched data. COM provided funding and researched data. JMGF provided funding and researched data. LM provided funding and conducted the computational experiments. AMV and AIA are responsible for the integrity of the work as a whole.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Acknowledgments

We acknowledge Drs Lucía Sánchez-Ruiloba, and Diego Navarro (IIB Alberto Sols, CSIC, Madrid, Spain) for assistance with microscopy. This work was supported by grants from the Spanish Ministry of Economy and Competitiveness: SAF2015-65267-R (MINECO/FEDER), SAF2016-76083-R (MINECO-FEDER), CTQ2015-64425-C2-1-R (MINECO-FEDER) and CTQ2014-53144-P and grants from the Spanish ISCIII (CIBERdem) and INFLAMES (ISCIII PIE14/00045, co-funded by ERDF, “Investing in your future”).

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SUPPLEMENTARY FIGURES

Figure Supplementary 1. DSO₂-ONJ-induced p38α MAPK phosphorylation is totally abolished by the inhibitor SB203580. Bv.2 microglial cells were pretreated for 30 min with SB203580, followed by stimulation with 200 ng/ml LPS or 10 μM DSO₂-ONJ for the indicated time periods. The protein extracts were analyzed by Western blot with antibodies against pP38α MAPK and P38α MAPK. Representative autoradiograms are shown (n=5 independent experiments). Blots were quantified by performing scanning densitometry, and the results are mean ± S.E.M. The ratio between the two indicated proteins and the fold change relative to Basal condition is shown. *p≤0.05 vs Basal treatment. #p≤0.05 vs DSO₂-ONJ treatment. (two-way ANOVA followed by Bonferroni t-test).

Figure Supplementary 2. Comparison of the predicted binding of DSO₂-ONJ to p38α MAPK (coordinates from PDB ID 4E6A) with that observed for PIA23 in the crystallographic complex PDB ID 4E6A. Docking calculations of DSO₂-ONJ into the p38α structure from the PDB ID 4E6A predicted two main binding modes, with similar estimated binding free energies ($\Delta G_{\text{binding}}$). The ligands are represented in sticks and coloured by element with gray, purple or cyan carbon atoms for PIA23 (A) and the first and second binding modes of DSO₂-ONJ, respectively. Hydrogen atoms are not represented for clarity. Trp197 is shown in beige sticks. In the first complex (B), the polar regions of DSO₂-ONJ are more solvent exposed. In the second one (C), DSO₂-ONJ adopts a binding orientation more similar to that observed for PIA23.

Figure S3. Predicted binding of DSO₂-ONJ to p38α MAPK using the protein coordinates from the PDB ID 4E6C. Two best docking solutions for the binding of DSO₂-ONJ into the p38α structure from the PDB ID 4E6C (based on the estimated binding free energies, $\Delta G_{\text{binding}}$). The binding affinity is predicted to be better in the 4E6A structure. **A)** Overall view of p38α MAPK (blue cartoon) with the two predicted binding modes shown. The ligand is represented in sticks and coloured by element with pink and green carbon atoms for the first and second binding modes, respectively. Hydrogen atoms are not represented for clarity. **B)** and **C)** are closer views to the DSO₂-ONJ first and second binding modes, respectively. Trp197 is shown in blue sticks as a reference.

Figure S4. Docking of perifosine into p38 α MAPK (coordinates from PDB ID 4E6C) and comparison with its crystallographic complex (PDB ID 4E6C). The two best binding modes obtained in the calculations (based on the estimated binding free energies, $\Delta G_{\text{binding}}$), are shown superimposed with the crystallographic structure. Both complexes present similar binding free energies, being the second one more similar to the crystallographic structure. Perifosine (A) is represented in sticks and coloured by element with gray, magenta or green carbon atoms for the crystallographic structure, the first (B) and second (C) calculated binding modes, respectively. Hydrogen atoms are not represented for clarity. The protein is shown in blue cartoon and Trp197 is represented in blue sticks as a reference.

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