The non-antibiotic tetracycline COL-3 prevents microglial inflammatory responses by reducing glucose-mediated oxidative stress.

Maurício dos-Santos-Pereira[1], Elaine Del Bel[1], Rita Raisman-Vozari[1], patrick michel[1], florencia gonzalez-lizarraga[1], Nilson Carlos Ferreira-Junior[1], Nour Francis[1], Paola Ramirez[1], and Rosana Chehín[1]

[1]Affiliation not available

May 19, 2021

Nilson Carlos Ferreira Junior[1,2,3], Maurício dos Santos Pereira[1,2,3], Nour Francis[1], Paola Ramirez[1], Florencia González-Lizarraga[4], Rosana Chehín[4], Elaine Del Bel[2,3], Rita Raisman-Vozari[1]* and Patrick Pierre Michel[1]*

[1]*Paris Brain Institute – CNRS UMR 7225 – Inserm U 1127 – Sorbonne Université UM7 Paris, France.*

[2]*Department of Basic and Oral Biology, FORP, Campus USP, University of São Paulo, Av. Café, s/no, 14040-904, Ribeirão Preto, SP, Brazil.*

[3]*USP, Center for Interdisciplinary Research on Applied Neurosciences (NAPNA), Brazil.*

[4]*Instituto de Investigación en Medicina Molecular y Celular Aplicada (IMMCA) (CONICET-UNT-SIPROSA), Tucumán, Argentina.*

*() Patrick Michel: Pharm.D., Ph.D.; Sorbonne Université, Paris Brain Institute, Paris, France; F-75013. Phone: +33(0)157274550, email: patrick-pierre.michel@upmc.fr

Rita Raisman-Vozari: Research Director; Sorbonne Université, Paris Brain Institute, Paris, France; F-75013. Phone: +33(0)157274555, email:

ritaraisman@gmail.com

Abbreviations: 4-Dedimethylaminosancycline: COL-3 (CMT-3,6-demethyl-6-deoxy-4-de[dimethylamino]-tetracycline); 2-deoxy-D-glucose: 2-DG; Apocynin : APO; dexamethasone: DEX; doxycycline: DOX; Dulbecco's modified Eagle's medium: DMEM; Fetal calf serum: FCS; Lipopolysaccharide (LPS); Parkinson disease: PD; Polyethyleneimine: PEI; Reactive oxygen species: ROS; α-synuclein: αS; αS aggregates: αSa; αS monomers: αSm; Trolox: TROL.

ABSTRACT (274 words)

Tetracyclines have recently emerged as possible therapeutic agents for several diseases of the central nervous system. Chemically modified tetracycline 3 (also known as Incyclinide, CMT-3 or COL-3), a tetracycline derivative without antibiotic activity that crosses the blood–brain barrier, has been recently validated as a potent anti-inflammatory molecule. The present study discloses a possible mechanism through which COL-3 induces an anti-inflammatory response in cultured microglial cells activated by two different inflammogens: the standard bacterial component lipopolysaccharide (LPS) and the amyloid fibrils of the synaptic protein α-Synuclein (αSa). Under LPS and αSa treatment, COL-3 effectively reduced the production of prototypical

proinflammatory markers, as the cytokine TNF-α and the microglial protein Iba-1. COL-3 effects were reproduced by doxycycline (DOX), a tetracycline used as reference molecule and dexamethasone, a classic anti-inflammatory drug. Surprisingly, COL-3 avoided the increase in glucose uptake induced by LPS and αSa in microglial cells, an outcome also observed with the inhibitor of NADPH oxidase apocynin, high concentration of glucose and with its non-metabolized analog 2-deoxy-glucose. COL-3 and DOX, as well as apocynin and 2-deoxy-glucose also reduced glucose-derived NADPH, a cofactor required for NADPH oxidase activation and reactive oxygen species (ROS) generation. Comforting these data, our results showed that LPS- and αSa-induced ROS production was also blunted in the presence of COL-3 and DOX, as well as apocynin and the anti-oxidant vitamin E analog trolox. Accordingly, apocynin and 2-deoxy-glucose mimicked the TNF-α reduction induced by COL-3 in LPS and αSa-treated cells. In this way, we propose that the anti-inflamatory mechanism of action of COL-3 is the consequence of its capacity to block glucose consumption and consequent glucose- and NADPH-dependent ROS production in microglial cells.

Keywords: 2-deoxyglucose uptake; α-synuclein; CMT-3; doxycycline, glia; glucose metabolism; incyclinide; microglia; NADPH; NADPH oxidase; neuroinflammation; pentose-phosphate shunt; ROS.

INTRODUCTION

Tetracyclines are a family of antibiotics that inhibit bacterial protein synthesis by binding to the small ribosomal subunit and blocking the binding of aminoacyltRNAs to the ribosome A site [1]. Although tetracycline effects as antibiotics are rather old, there has been a renewing interest in these molecules, as it has been reported that, in addition to their antibacterial activity, they induce important pharmacological effects which may have clinical applications to various disease states. In recent years, preclinical studies have suggested that tetracyclines have neuroprotective properties that are worth reusing for neurodegenerative diseases [2-4].

In particular, doxycycline (DOX), an antibiotic belonging to the tetracycline family, has not only been reported to exhibit neuroprotective activity in Parkinson's disease (PD) animal models like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine, attenuating the loss of dopaminergic neurons in the*substantia nigra pars compacta* and striatal nerve terminals [5,6], but it also remodels toxic α-synucein oligomers into a non-toxic, non-seeding form [7], interferes with tau amyloid aggregation [8] and decreases the expression of the main markers of inflammation in microglial cultures treated with LPS [9], indicating that its underlying mechanism is the ability of DOX to reduce neuroinflammation [2,10].

A new family of chemically modified tetracyclines (CMTs) has been structurally rearranged to eliminate their antimicrobial activities while retaining their complementary mechanisms, such as inhibition of inflammation, proteolysis, angiogenesis, among others [11-14]. To date, more than eight CMTs are available [15]. Because CMTs might represent a safer treatment compared to antimicrobial treatment with tetracyclines, since their administration in laboratory animals does not produce tetracycline resistant microorganisms in the oral and intestinal flora [16], some of them, as CMT-1, CMT3 and CMT-8 have been tested in pre-clinical and clinical applications, including cancer trials and neurological disease studies [10,17,18].

Chemically modified tetracycline 3 (CMT-3,6-demethyl-6-deoxy-4-de[dimethylamino]-tetracycline) might be a promising drug for oxidative stress-related disorders, as neurodegenerative diseases, due to the advantage of being able to be used in chronic treatments without generating serious side effects. CMT-3, also called Incyclinide or COL-3 is produced by deletion of the dimethylamino group from carbon 4 in the A ring of tetracyclines, which eliminates the antibiotic activity without affecting other important properties of the molecule [19] (Figure 1). COL-3 is the only CMT tested in clinical trials in cancer patients [20,21], and its pleiotropic properties offer impressive therapeutic potential to reduce the excessive degradation of connective tissue during various pathological processes, including inflammatory conditions [22-25]. COL-3 molecule is highly lipophilic, so it can also cross the blood-brain barrier and thus act within the brain [14,26]. COL-3 mechanism of action in the central nervous system includes anti-apoptotic effects, anti-protein aggregation, removal of ROS, inhibition of matrix metalloproteinase, and protection against mitochondrial dysfunctions, among others [2,17]. COL-3 has been also proposed as an anti-inflammatory molecule, as it can inhibit

lipopolysaccharide (LPS)-induced microglial activation and cytokine expression in the brain [26]; however, the mechanism through which this molecule reduces neuroinflammation is yet to be described.

In the present study, we used COL-3 to further explore the mechanisms underlying its anti-inflammatory action and compare its effects with a reference tetracycline that presents antibacterial activity, DOX. To this aim, we used culture of microglial cells isolated from postnatal mouse brains and an activation paradigm using the classical inflammogen LPS. Since the aggregation process that affects α-synuclein (αS) synaptic protein may represent a potential trigger for the activation of microglial cells, which is involved in the pathogenesis of synucleinopathies including PD [27-29], we also used the aggregated version of αS (αSa) as an inflammogen. We observed that COL-3 exerts robust anti-inflammatory effects on microglial cells by inhibiting ROS signaling events and glucose-dependent synthesis of NADPH, a cofactor required for activation of NADPH oxidase and the generation of ROS. Surprisingly, the anti-inflammatory action of COL-3 was significantly higher than that evoked by DOX, as lower concentrations of COL-3 were sufficient to induce the same anti-inflammatory effect that a higher concentration of DOX.

MATERIAL AND METHODS

Pharmacological and cell culture reagents

Lipopolysaccharide (LPS; Escherichia coli strain O26:B6; #L8274), 2-deoxy-D-glucose (2-DG; #D8375), Trolox (TROL; #238813), dexamethasone (DEX; #D4902) and doxycycline hyclate (DOX; #D9891) were obtained from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). 4-Dedimethylaminosancycline (COL-3; #HY-13648) was purchased from MedChemExpress (Monmouth Junction, NJ). Apocynin (APO; #4663) was purchased from R&D Systems Europe (Lille, France). Dulbecco's modified Eagle's medium (DMEM) and the Trypsin (0.05%)-EDTA solution used to produce subcultures were obtained from ThermoFisher Scientific (Saint Aubin, France). Fetal calf serum (FCS) was provided by Biowest LLC (Eurobio, Les Ulis, France). Deoxy-D-glucose 2-[1,2-3H (N)]- (NET549A) was purchased from Perkin Elmer (Courtaboeuf, France).

Microglial cell cultures

Ethics statement

To generate microglial cell cultures, we used newborn C57BL/6J mice from Janvier LABS (Le Genest St Isle, France). Mice were housed, handled, and cared for in strict accordance with the European Union Council Directives (2010/63/EU). Experimental protocols were approved by the Committee on the Ethics of Animal Experiments Charles Darwin no. 5.

Coating procedure with polyethyleneimine

Polyethyleneimine (PEI; Mw: 750,000 and Mn: 60,000; # P3143, Sigma Aldrich) was used to coat culture flasks to spontaneously isolate microglial cells, as previously described (Sepulveda-Diaz et al., 2016). PEI was diluted at 1 mg/ml in a pH 8.3 borate buffer solution prepared with borax decahydrate (#B3545) [30-32]. After at least a 2 h incubation at 37°C, PEI was discarded, and culture flasks washed 4 times with Dulbecco's phosphate-buffered saline (PBS) until further use.

Isolation of microglial cells and production of subcultures

Brains from mouse pups (post-natal day 1) were mechanically dissociated as described before [33] and cells in suspension were plated in PEI-coated T-75 culture flasks (Corning) containing 12 ml of DMEM supplemented with 10% FCS and a penicillin/streptomycin cocktail. Under these conditions, the purification of microglial cells occurs spontaneously after 14-18 days of culture. When required, purified microglial cells were maintained for up to one more week in cell culture flasks by adding 2-3 ml of DMEM supplemented with antibiotics and 1% FCS, only. To produce subcultures, purified microglial cells were trypsinized with 0.05% trypsin during 5 min. After trypsin neutralisation with 10% FCS, cells were plated onto uncoated Nunc 48 plates, at a density of 100,000 cells per well, using DMEM supplemented with antibiotics and 1% FCS as maintenance medium.

Πυριφιςατιον ανδ αγγρεγατιον οφ ρεςομβιναντ αΣ

Expression and purification of recombinant human αS was performed as previously described [7,34]. The purity of the recombinant αS protein was evaluated by SDS-PAGE electrophoresis and protein samples were then applied to a Pierce Spin column (#88275; Thermo Scientific) to remove contaminating endotoxins. The Limulus Amebocyte Lysate assay revealed that monomeric αS stock solutions contained less than 0.1 endotoxin unit (EU)/mg protein at this stage. The protein solutions were then filtered and centrifuged, and the protein content of the supernatant was measured at 280 nm with a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific). The protein solution was finally adjusted to obtain appropriate concentrations.

To produce αSa fibers, recombinant αS samples were incubated at 37°C under orbital agitation for 96 h (Thermomixer comfort; Eppendorf; Montesson, France). Protein aggregates were harvested, sonicated for 2 min and kept at -20°C until further use as previously described [7]. The production of amyloid fibrils through this protocol was previously confirmed by transmission electron microscopy [30,35].

Stimulation protocols with inflammogens and drug treatments

Microglial cells were exposed to either LPS or αSa fibers for 24 hours to model brain inflammatory-type reactions. Treatments with the test compounds and reference molecules were initiated 2 h before addition of the inflammogens and pursued until the end of stimulation protocols.

Transmission electron microscopy (TEM)

Samples of αS solution were adsorbed onto glow-discharged 200 mesh carbon film coated copper grids and stained with uranyl acetate (2%). Excess liquid was removed and grids were allowed to air dry. Images were captured using a Hitachi HT7700 120kV transmission electron microscope.

Immunofluorescence detection of microglial markers

The MAC-1/CD11b antibody (clone M1/70.15) from BioRad (Oxford, UK) and the rabbit anti-ionized antibody of the calcium-1 binding molecule (Iba-1, #019-19741) from Wako Chemicals (Neuss, Germany) were used to monitor microglial cell responses at the cellular level. Briefly, cultures were fixed with 4% formaldehyde in PBS (20 min, at room temperature), washed and incubated sequentially with antibodies against MAC-1/CD11b (1:1000 in PBS for 72 hours) and Iba-1 (1: 500 in PBS-0.2% Triton X-100 overnight). Immunodetection of MAC-1/CD11b and Iba-1 were performed with anti-rat Alexa-Fluor 555 and anti-rabbit Alexa-Fluor 488 secondary antibodies, respectively (Invitrogen). Nuclear counterstaining with Hoechst 33342 (1 µg/ml for 5 min) was performed to enable automated focus during the cell counting procedure with an Arrayscan XTi workstation (Thermo Fisher Scientific, Courtaboeuf, France). Fluorescent images were automatically acquired with a 20x objective, and variations of immunofluorescence signal intensities were quantified at the cellular level using the HCStudio software. For each treatment condition, at least 2,500 cells were processed for image analysis.

ΤΝΦ-α ασσαψ

TNF-α was measured using an ELISA kit from Thermofisher Scientific (#BMS607-3) according to the manufacturer's instructions. Absorbance measurements were performed at 450 nm using a SpectraMax i3X microplate reader (Molecular Devices, Sunnyvale, CA).

Quantification of intracellular NADPH levels

NADPH levels were quantified using the high sensitivity NADPH quantification fluorometric Assay Kit (#MAK216; Sigma Aldrich), as previously described [32]. The fluorescent reaction product was quantified with a SpectraMax i3X microplate reader (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 535 and 587 nm, respectively.

Quantification of intracellular ROS levels

Intracellular ROS levels were measured using the membrane-permeable CellROX Deep Red reagent (Invitrogen Life Technologies), as described before [33]. Signal acquisition was performed an Arrayscan XTI automated workstation fitted with a 20x objective (Thermo Scientific, Courtaboeuf, France). Cellular fluorescence intensity levels were estimated with the HCStudio software [30].

Assessment of [^{3}H]-2-DG uptake

The uptake of [^{3}H]-2-DG was measured based on a protocol previously described [32]. Briefly, after termination of test treatments, microglial cell cultures were incubated at 37°C for 30 min in PBS containing 1 µCi [^{3}H]-2-DG. 2-DG accumulation was terminated by washing the cultures with ice-cold PBS and the radioactivity incorporated by the cells recovered by scraping the cultures in distilled water containing 1% Triton-X. The radioactivity accumulated by microglial cells was quantified with a Tri-Carb 4910TR liquid scintillation counter (Villebon sur Yvette, France). Blank values were obtained by applying 50 mM glucose to control cultures.

Statistical analysis

Experimental values expressed as mean ± SEM were derived from at least two independent experiments performed in triplicate. For statistical analysis, we performed comparisons by One-way ANOVA followed by a Bonferroni post-hoc test using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA).

RESULTS

Τηε τετρασψχλινε ˚ΟΛ-3 ρεδυχες τηε ινφλαμματορψ ρεσπονσε οφ μιχρογλιαλ χελλς χηαλλενγεδ ωιτη ειτηερ ΛΠΣ ορ αΣα

Figure 2A confirms that amyloid fibrils were present in αS shaken samples for 96h (lower image), whereas non-shaken samples did not produce fibrilar αSa species.

To evaluate the anti-inflammatory potential of the non-antibiotic tetracycline COL-3, we used microglial cells activated with the bacterial cell wall component LPS (10 ng/ml), a prototypical agonist for Toll-like receptor-4 (TLR4) [36]. After 24 hours of treatment, LPS strongly stimulated the production/release of the proinflammatory cytokine TNF-α. When the cultures were exposed to either 10 or 20 µM COL-3, the production of TNF-α induced by LPS was significantly reduced by more than 30 and 75%, respectively (Figure 2B). No significant effect was observed, however, at 1 µM, indicating that the suppressive action of the tetracycline derivative was concentration-dependent and only significant after 10 µM. COL-3 at 20 µM appeared as effective as the reference tetracycline DOX, at 50µM. The reference anti-inflammatory drug DEX (2.5 µM) wasmore effective than either 20 µM COL-3 or 50 µM DOX to reduce TNF-α release.

Cellular expression of the phenotypic activation marker Iba-1 was also estimated under the same experimental conditions. We established that COL-3 suppressed the LPS-induction of microglial Iba-1 expression only at 10 or 20 µM (Figure 2B). At 50 µM, DOX also caused a robust decrease of the Iba-1 immunosignal. Note that 2.5µM DEX reduced Iba-1 expression with an efficacy that was similar to that of COL-3 and DOX, at 20 and 50µM, respectively. Photomicrographs illustrated LPS-treated microglial cell cultures, exposed or not to COL-3 (20 µM) or DOX (50 µM) and then processed sequentially for CD11b and Iba-1 fluorescence immunodetection, binding proteins that are constitutively expressed in microglia (Figure 2C).

To model inflammatory reactions as they may occur in PD we used αSa to trigger an inflammogenic response in microglial cell cultures. As expected, a 24-hour exposure to αSa caused a robust increase of TNF-α release whereas the monomeric form of αS (αSm) does not induce a significant effect compared to control levels (Figure 2D). At 10 or 20 µM, COL-3 significant reduced TNF-α release induced by αSa. DOX exerted a comparable repressive effect at 50µM. DEX at 2.5 µM was as effective as COL-3 and DOX at 20 and 50 µM, respectively.

Coherent with previous observations, we also demonstrated that the induction of Iba-1 expression induced by αSa was efficiently curtailed by COL-3 (10 or 20 µM), DOX (50 µM) and DEX (2.5 µM) (Figure 2E).

Micrographs illustrate microglial cultures treated with αSa in the presence or not of COL-3 (20μM) or DOX (50 μM) and then processed sequentially for CD11b and Iba-1 fluorescence immunodetection (Figure 2F). It is interesting to point out that αSm does not induce any effect.

The tetracycline COL-3 reduces glucose accumulation in microglial cells challenged ωιτη ειτηερ ΛΠΣ ορ αΣα

Recent studies have demonstrated that glucose is an essential fuel for microglial inflammatory processes [32,37]. Accordingly, we found that a 24-h treatment with 10 ng/ml LPS leads to an almost 2-fold increase in [^{3}H]-2-DG uptake in microglial cultures (Figure 3A). [^{3}H]-2-DG uptake was significantly reduced when LPS-treated cultures were exposed to either 10 or 20 μM COL-3, whereas a lower COL-3 concentration (1 μM) was not effective (Figure 3A). A similar effect was obtained with DOX used at a concentration of 50 μM. Surprisingly, DEX (2.5 μM) failed to alter [^{3}H]-2-DG uptake, indicating that the anti-inflammatory effects of the glucocorticoid and the non-antibiotic tetracycline derivative may occur through distinct mechanisms. The uptake of [^{3}H]-2-DG was also strongly reduced by inhibiting NADPH oxidase activity with 300μM APO or by exposing the cultures to 500 μM unlabeled 2-DG, a concentration of the glucose analog susceptible to restrain glucose utilization by these cells without affecting their survival (dos-Santos Pereira et al, 2020). As expected, glucose uptake was also efficiently prevented when 50 mM glucose was added acutely to the cultures to compete with [^{3}H]-2-DG during uptake measurement (Figure 3B).

Interestingly, αSa also robustly increased [^{3}H]-2-DG in microglial cell cultures (Figure 3C), suggesting that the greater demand of glucose by microglial cells was not circumscribed to LPS-induced inflammatory events. In contrast, αSm did not induce statistical effects. Noticeably, COL-3 (10 or 20 μM) reduced substantially glucose uptake when applied concomitantly to αSa. This effect was mimicked by DOX at 50 μM. A co-treatment of αSa-treated cultures with 300 μM APO or 500 μM 2-DG also led to a significant reduction in [^{3}H]-2-DG uptake. As expected [^{3}H]-2-DG uptake was also prevented when glucose was added in excess to compete with the radioligand when performing uptake measurements (Figure 3D).

COL-3 prevents the accumulation of NADPH in microglial cells challenged ωιτη ειτηερ ΛΠΣ ορ αΣα

Glucose may have a key impact on microglial inflammatory processes by stimulating the pentose phosphate pathway and consequently the synthesis of NADPH, the requisite substrate for the superoxide producing enzyme NADPH oxidase [32,37]. Coherent with this view, we found that NADPH levels were increased substantially in microglial cells activated by either 10 ng/ml LPS or 70 μg/ml αSa but not with αSm (Figure 4A,B). Of interest, COL-3 (20 μM) caused a significant reduction of NADPH levels in both inflammation paradigms (Figure 4A,B). As expected, the suppressive effect of COL-3 was mimicked by DOX at a concentration of 50 μM.

Both the inactive glucose analog 2DG (500 μM) and the NADPH oxidase inhibitor APO (300 μM) mimicked the inhibitory effect of COL-3 on NADPH production in microglia activated by LPS or αSa, indicating that the non-antibiotic tetracycline may interfere with a glucose-dependent mechanism that promotes NADPH synthesis and as a consequence ROS production via the NADPH oxidase enzyme.

COL-3 prevents intracellular oxidative stress generated in microglial cells in response to inflammatory signals

To test the hypothesis of a ROS-dependent NADPH production, we used a quantitative cell-based fluorescence assay to evaluate ROS in microglial cells exposed to 10 ng/ml LPS or 70 μg/ml αSa (Figure 5). As expected, a 24-h treatment with LPS (10 ng/ml) or αSa (70 μg/ml) induced a significant increase of intracellular ROS levels in microglial cells. In contrast, αSm does not induce significant effects on ROS levels. Of interest, ROS production returned to control values when cultures exposed to either LPS or αSa were previously treated with 20 μM COL-3 (Figure 5A,B), confirming the hypothesis that the non-antibiotic tetracycline may exert anti-inflammatory effects by counteracting oxidative stress-dependent mechanisms. As expected, the inhibitory effect of COL-3 on ROS production was mimicked by DOX at 50 μM. Noticeably,

the NADPH oxidase inhibitor APO (300 μM) and the antioxidant TROL (10μM) reduced ROS production with an efficacy similar to that demonstrated by COL-3.

The anti-inflammatory activity of COL-3 is reproduced by reducing oxidative stress or glucose utilization

Next, we wished to determine whether COL-3 suppressive effects on TNF-α release were reproduced by lowering ROS production or glucose utilization. In line with this possibility, we established that the NADPH inhibitor APO (300 μM) and the non-metabolizable glucose analog 2DG (500 μM) (Figure 6A) were as effective as COL-3 to reduce TNF-α release induced by LPS or αSa, thus further supporting the view that COL-3 exerts its anti-inflammatory effect by interfering with a glucose-dependent mechanism that promotes oxidative stress (Figure 6B).

DISCUSSION

COL-3, a modified tetracycline with non-antibacterial properties [19,38,39], represents a promising molecule to treat neurodegenerative- or inflammation-related diseases, since it is a tetracycline with pleiotropic effects that does not present antibacterial properties and has previous studies demonstrating its safety, toxicity and therapeutic range [40]. In this study, we unveiled COL-3 is a potent inhibitor, in primary microglial cell cultures, of inflammatory reactions prompted by the bacterial cell wall component LPS and by the aggregated form of αS, a potential trigger to PD pathophysiology. COL-3 anti-inflammatory action was observed through TNF-α production/release and Iba-1 expression. Outstandingly, the ability of COL-3 to restrict LPS and αSa-induced microglial inflammation comes, at least partially, from its capacity to inhibit glucose consumption and production of NADPH, the requisite substrate for the superoxide producing enzyme NADPH oxidase. This outcome converts to increased levels of ROS and consequent oxidative stress in the cells, which are also restrained by COL-3 (Figure 7).

ˆOΛ-3 περφορμς ας α ποτεντ αντι-ινφλαμματορψ δρυγ φορ ΛΠΣ- ανδ αΣα-αςτιατεδ μιςρογλιαλ ςελλς

Our group has previously demonstrated, in newborn mice primary microglial cultures, the robust inflammatory action of LPS and αSa as these inflammogens stimulated the production/release of the proinflammatory cytokine TNF-α and glutamate, a non-cytokine inflammatory mediator [30,32,35]. The present study pointedly demonstrates that COL-3, when administered at 10 and 20 μM restrained or almost entirely abolished the release of TNF-α and Iba-1 expression induced by LPS or αSa in microglial cell cultures, an absent effect at 1 μM, indicating that COL-3 presents a threshold concentration.

Previous studies sustain our findings, as COL-3 can restrain the development of paclitaxel-induced thermal hyperalgesia, effect associated to a COL-3 protection against CD11b+ cells increase – a surface protein expressed by many immune cells, such as macrophage and microglial cells – and cytokine production, such as TNF-α and Il-1β [41]. In the study of Cazalis et al. (2009) [42], using an *ex vivo* human whole blood model, it was shown that LPS increased cytokines' secretion, and COL-3 reduced LPS-induced cytokine secretion at several degrees. This outcome was centrally reproduced in an *in vivo* model of inflammation, where COL-3 was able to prevent, at least partially, microglia activation and TNF-α (but not IL-1β) release in the brain [26]. Our results suggest the efficacy of COL-3 is more effective than the reference tetracycline DOX in restraining inflammation produced by LPS or αSa, since the effect of COL-3 lower concentrations (10 and 20 μM) toward TNF-α and Iba-1 expression was sufficient to mimic the effect of a higher DOX concentration (50 μM) or of the classic glucocorticoid anti-inflammatory, DEX.

The anti-inflammatory properties of COL-3 derives from its ability to restrain glucose uptake and consequent glucose-dependent NADPH production

Our data showed that the treatment with inflammogens LPS or αSa substantially elevated glucose consumption by microglial cells, as it seems to be a prerequisite to the shift into an activated profile [43], estimated through the capture of [3H]-2-DG, a synthetic analog of glucose labeled with tritium. This corroborates previous results from our group showing LPS increases glucose uptake as a substrate to NADPH-dependent ROS

production, which leads to an inflammatory state of microglial cells [32]. This is the first time it is shown that the aggregated inflammatory form of αS can increase glucose consumption in microglial cells, which points to a common mechanism of these aggregated form of the protein with LPS to induce the inflammatory state in microglial cells. Of interest, COL-3 strongly inhibited [^{3}H]-2-DG uptake in LPS- and αSa-stimulated cells, indicating the non-antibiotic tetracycline curtailed inflammatory-type responses by reducing glucose uptake. Again, a higher concentration of the reference antibiotic tetracycline DOX reproduced these findings; however, the microglial uptake of [^{3}H]-2-DG was unresponsive to DEX, indicating that the mechanisms of action of the glucocorticoid and the tetracyclines were most probably partly different.

It should be emphasized that the uptake of [^{3}H]-2-DG was also strongly reduced by inhibiting NADPH oxidase activity with APO, which indicates that COL-3 prevented the uptake of glucose through inhibition of ROS dependent-signaling events [44]. Noticeably, COL-3 anti-inflammatory action was also mirrored by unlabeled 2-DG used at concentrations that competitively inhibit hexokinase, the glycolytic enzyme that converts glucose into glucose-6-P [45], and saturating concentrations of GLUT-dependent glucose uptake [46]. Our current data is one more evidence that the control of glucose availability by anti-inflammatory drugs largely contributes to the reduction of microglial cell activation [32,47].

NADPH oxidase (NOX2), a multimeric complex, utilizes NADPH as substrate and reduces molecular oxygen to produce superoxide, that is then further dismutated into stable and diffusible hydrogen peroxide pro-oxidant [48]. The first metabolite product of glucose, glucose-6-phosphate, can be translocated into the pentose phosphate shunt [49], which serves to produce nucleotide precursors and helps regenerate the reducing agent NADPH, which is also a requisite substrate for NOX2 [45,49]. Consistent with previous findings from our group and others, the pentose-phosphate shunt pathway is essential for ROS response in immune cells like macrophages and microglial cells [32,48]. Therefore, we tested the possibility that the increase in glucose uptake observed in primary microglial cells may serve to stimulate an increase in NADPH levels and that COL-3 may possibly resolve inflammatory processes by preventing this effect. Here we observed a robust elevation of NADPH synthesis in LPS- and αSa-challenged microglial cells.

In a recent study from our group, the phytocannabinoid anti-inflammatory cannabidiol prevented the rise in NADPH synthesis elicited by LPS treatment, indicating that the anti-inflammatory action of cannabidiol may be also dependent of this suppressive effect [32]. Our current results sustain our previous data, as the tetracyclines COL-3 and DOX reduced NADPH levels produced after LPS and αSa stimulation in microglial cells. Like cannabidiol, COL-3 operated by interfering with an alternative glucose-dependent mechanism. Therefore, it is possible to imply that the inhibitory effects that COL-3 exerts on glucose consumption and NADPH synthesis may strengthen the intrinsic antioxidant potential of this compound in a self-reinforcing process.

Reinforcing our hypothesis, we found that compounds capable of restricting LPS and αSa-induced TNF-α release and Iba-1 expression were also effective in preventing not only [^{3}H]-2-DG uptake, but also the consequent production of intracellular NADPH derived from the preferential activation of the pentose-phosphate shunt pathway. In this way, the NOX2 inhibitor APO and the saturating concentration of unlabeled 2DG prevented the production of NADPH, confirming the resulting inflammatory process caused by these inflammogens results from a signaling event that requires an increase in glucose uptake and consequent NADPH production, and the anti-inflammatory effect of COL-3 comes from blocking the signaling cascade of the pentose-phosphate shunt pathway.

Our results also show that DEX, despite reducing inflammatory factors, was ineffective in avoiding LPS- and αSa-dependent [^{3}H]-2AG uptake increase. Although there are reports showing DEX inhibits glucose uptake in contracting myotubes [50], glucocorticoids classically suppress the transcription of several genes that encode pro-inflammatory cytokines and chemokines, therefore suggesting it may not have a direct action over glucose uptake and consumption and oxidative stress response.

˜OΛ-3 πρεεντς POΣ προδυςτιον ανδ ςονσεχυεντ ΛΠΣ ορ αΣα-εοκεδ οξιδατιε στρεσς

To finally assume that the anti-inflammatory effect of COL-3 was acquired from an underlying and indirect

anti-oxidant action, we observed LPS and αSa-increased ROS levels after treatment with COL-3. Coherent with the previous results, COL-3 (20 μM) strongly inhibited ROS production in both scenarios. An intrinsic antioxidant effect from COL-3 would implicate in a passive diffusion from this compound into the intracellular content. Despite being shown that from the eight chemically modified tetracyclines existent, COL-3 is the most lipophilic and consistently exhibited the greatest in vivo efficacy in animal models of tissue breakdown and tissue uptake [51], if COL-3 has an intrinsic antioxidant ability or if it is only an indirect mechanism through the reduction of glucose uptake and NADPH production remains to be determined.

It has been well described by our group and others that the inflammatory profile of microglial cells evoked by LPS and αSa is associated with a robust elevation of intracellular oxidative stress [30,32,35,52,53]. Interestingly this response was efficiently inhibited by COL-3, that reduced microglial inflammatory-type responses, and by APO and TROL, an inhibitor of the superoxide producing enzyme NADPH oxidase, and an antioxidant analog of vitamin E, respectively [54,55]. As previously hypothesized, these data point that ROS production through a NOX2-dependent manner – i.e., the NADPH oxidase isoform that is most abundant in microglial cells [56] – is a critical participant of the inflammatory response to the inflammogens LPS and αSa, and that COL-3 potentially hindered with this signaling event. The expression of the phosphorylated form of the p65 subunit of the NF-ϰB transcription factor is an important sequential event in the ROS signaling cascade, as ROS operate as a trigger for NF-ϰB activation [57]. In addition to ROS direct toxic impact on biological macromolecules, they can trigger the inflammatory response – i.e., microglial activation and cytokine release – by stimulating a number of genes which are regulating the inflammatory-signaling cascades, including NF-ϰB [58].

Again, we noticed COL-3 was proportionally more efficacious to inhibit oxidative stress in LPS- and αSa-activated microglial cells than DOX, as a higher concentration of the last (50 μM was necessary to mimic COL-3 (20 μM) action in avoiding ROS production. Several studies have compared the pleiotropic actions od COL-3 and DOX, with converging results. Protasoni et al. [1] performed a comparative study of COL-3 and DOX to investigate the role of the mitochondrial energy generating capacity in an anticancer mechanism and found that both drugs caused a severe decrease in the levels of mitochondrially encoded cytochrome-c oxidase subunits and cytochrome-c oxidase activity. In addition to that, COL-3 was the only drug to produce a marked drop in the level of nuclear-encoded succinate dehydrogenase subunit A and citrate synthase activity, indicating that COL-3, differently from DOX, had multiple inhibitory effects. Opposingly, Onoda et al. [59], investigating whether tetracyclines could induce apoptosis in human HT29 colon cancer cells, described that both COL-3 and DEX inhibited the proliferation of six different colorectal cancer cell lines in a dose-dependent manner; however, COL-3 had a stronger effect on cancer cells than DOX. This study is the first to suggest a more effective action of COL-3 when compared to a tetracycline with antibiotic activity such as DOX in an inflammatory context for microglial cells.

Our results point to a higher efficacy of COL-3 than DOX in their anti-inflammatory properties. That might be explained by the structural differences of these two molecules: the presence of methyl groups that play a role in the antibiotic activity of DOX might not influence or even reduce the anti-inflammatory/antioxidant response shaped by this tetracycline. Gonzalez-Lizarraga et al. [17] described that COL-3 is also more effective than DOX as an antiaggregant molecule due to these structural distinctions. In this way, COL-3 not only efficiently prevented the formation of toxic α-synuclein amyloid aggregates *in vitro* , but also disaggregated α-synuclein amyloid fibrils, while DOX was only efficient in the former analysis. This suggests that COL-3 and DOX structural differences can impact the efficacy of each compound concerning their anti-inflammatory responses.

Conclusion

This study supports the prominent anti-inflammatory profile of the promising tetracycline with no antibacterial activity, COL-3, on microglial cells activated by ether LPS or αSa. These anti-inflammatory effects derive from the inhibition of glucose uptake and NADPH synthesis-dependent mechanism, which culminates in the production of ROS by NADPH oxidase, necessary for the change of microglial cells into their pro-inflammatory state. Thus, the complex and multifaceted action of COL-3 can be useful in clinical trials in

addition to those already carried out for cancer studies, including different neuroinflammatory conditions of the central nervous system.

REFERENCES

1. Protasoni, M.; Kroon, A.M.; Taanman, J.W. Mitochondria as oncotarget: a comparison between the tetracycline analogs doxycycline and COL-3.*Oncotarget* **2018** , *9* , 33818-33831, doi:10.18632/oncotarget.26107.

2. Bortolanza, M.; Nascimento, G.C.; Socias, S.B.; Ploper, D.; Chehin, R.N.; Raisman-Vozari, R.; Del-Bel, E. Tetracycline repurposing in neurodegeneration: focus on Parkinson's disease. *Journal of neural transmission* **2018** , *125* , 1403-1415, doi:10.1007/s00702-018-1913-1.

3. Orsucci, D.; Calsolaro, V.; Mancuso, M.; Siciliano, G. Neuroprotective effects of tetracyclines: molecular targets, animal models and human disease. *CNS & neurological disorders drug targets* **2009** , *8* , 222-231, doi:10.2174/187152709788680689.

4. Orsucci, D.; Mancuso, M.; Filosto, M.; Siciliano, G. Tetracyclines and neuromuscular disorders. *Current neuropharmacology***2012** , *10* , 134-138, doi:10.2174/157015912800604498.

5. Cho, Y.; Son, H.J.; Kim, E.M.; Choi, J.H.; Kim, S.T.; Ji, I.J.; Choi, D.H.; Joh, T.H.; Kim, Y.S.; Hwang, O. Doxycycline is neuroprotective against nigral dopaminergic degeneration by a dual mechanism involving MMP-3. *Neurotoxicity research* **2009** , *16* , 361-371, doi:10.1007/s12640-009-9078-1.

6. Lazzarini, M.; Martin, S.; Mitkovski, M.; Vozari, R.R.; Stuhmer, W.; Bel, E.D. Doxycycline restrains glia and confers neuroprotection in a 6-OHDA Parkinson model. *Glia* **2013** , *61* , 1084-1100, doi:10.1002/glia.22496.

7. Gonzalez-Lizarraga, F.; Socias, S.B.; Avila, C.L.; Torres-Bugeau, C.M.; Barbosa, L.R.; Binolfi, A.; Sepulveda-Diaz, J.E.; Del-Bel, E.; Fernandez, C.O.; Papy-Garcia, D.; et al. Repurposing doxycycline for synucleinopathies: remodelling of alpha-synuclein oligomers towards non-toxic parallel beta-sheet structured species. *Scientific reports* **2017** , *7* , 41755, doi:10.1038/srep41755.

8. Medina, L.; Gonzalez-Lizarraga, F.; Dominguez-Meijide, A.; Ploper, D.; Parrales, V.; Sequeira, S.; Cima-Omori, M.S.; Zweckstetter, M.; Del Bel, E.; Michel, P.P.; et al. Doxycycline Interferes With Tau Aggregation and Reduces Its Neuronal Toxicity. *Front Aging Neurosci* **2021** , *13* , 635760, doi:10.3389/fnagi.2021.635760.

9. Santa-Cecilia, F.V.; Socias, B.; Ouidja, M.O.; Sepulveda-Diaz, J.E.; Acuna, L.; Silva, R.L.; Michel, P.P.; Del-Bel, E.; Cunha, T.M.; Raisman-Vozari, R. Doxycycline Suppresses Microglial Activation by Inhibiting the p38 MAPK and NF-kB Signaling Pathways.*Neurotoxicity research* **2016** , *29* , 447-459, doi:10.1007/s12640-015-9592-2.

10. Bortolanza, M.; Nascimento, G.C.D.; Raisman-Vozari, R.; Del-Bel, E. Doxycycline and its derivative, COL-3, decrease dyskinesia induced by L-DOPA in hemiparkinsonian rats. *British journal of pharmacology***2021** , doi:10.1111/bph.15439.

11. Golub, L.M.; Lee, H.M.; Ryan, M.E.; Giannobile, W.V.; Payne, J.; Sorsa, T. Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. *Adv Dent Res* **1998** ,*12* , 12-26, doi:10.1177/08959374980120010501.

12. Golub, L.M.; Ramamurthy, N.S.; Llavaneras, A.; Ryan, M.E.; Lee, H.M.; Liu, Y.; Bain, S.; Sorsa, T. A chemically modified nonantimicrobial tetracycline (CMT-8) inhibits gingival matrix metalloproteinases, periodontal breakdown, and extra-oral bone loss in ovariectomized rats. *Annals of the New York Academy of Sciences***1999** , *878* , 290-310, doi:10.1111/j.1749-6632.1999.tb07691.x.

13. Golub, L.M.; Suomalainen, K.; Sorsa, T. Host modulation with tetracyclines and their chemically modified analogues. *Curr Opin Dent* **1992** , *2* , 80-90.

14. Sapadin, A.N.; Fleischmajer, R. Tetracyclines: nonantibiotic properties and their clinical implications. *Journal of the American Academy of Dermatology* **2006** , *54* , 258-265, doi:10.1016/j.jaad.2005.10.004.

15. Gu, Y.; Lee, H.M.; Sorsa, T.; Salminen, A.; Ryan, M.E.; Slepian, M.J.; Golub, L.M. Non-antibacterial tetracyclines modulate mediators of periodontitis and atherosclerotic cardiovascular disease: a mechanistic link between local and systemic inflammation. *Pharmacological research* **2011** , *64* , 573-579, doi:10.1016/j.phrs.2011.06.023.

16. Golub, L.M.; Ramamurthy, N.S.; McNamara, T.F.; Greenwald, R.A.; Rifkin, B.R. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Crit Rev Oral Biol Med* **1991** , *2* , 297-321, doi:10.1177/10454411910020030201.

17. Gonzalez-Lizarraga, F.; Ploper, D.; Avila, C.L.; Socias, S.B.; Dos-Santos-Pereira, M.; Machin, B.; Del-Bel, E.; Michel, P.P.; Pietrasanta, L.I.; Raisman-Vozari, R.; et al. CMT-3 targets different alpha-synuclein aggregates mitigating their toxic and inflammogenic effects. *Scientific reports* **2020** , *10* , 20258, doi:10.1038/s41598-020-76927-0.

18. Zhang, H.; Chang, M.; Hansen, C.N.; Basso, D.M.; Noble-Haeusslein, L.J. Role of matrix metalloproteinases and therapeutic benefits of their inhibition in spinal cord injury. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics***2011** , *8* , 206-220, doi:10.1007/s13311-011-0038-0.

19. Islam, M.M.; Franco, C.D.; Courtman, D.W.; Bendeck, M.P. A nonantibiotic chemically modified tetracycline (CMT-3) inhibits intimal thickening. *The American journal of pathology* **2003** ,*163* , 1557-1566, doi:10.1016/S0002-9440(10)63512-2.

20. Syed, S.; Takimoto, C.; Hidalgo, M.; Rizzo, J.; Kuhn, J.G.; Hammond, L.A.; Schwartz, G.; Tolcher, A.; Patnaik, A.; Eckhardt, S.G.; et al. A phase I and pharmacokinetic study of Col-3 (Metastat), an oral tetracycline derivative with potent matrix metalloproteinase and antitumor properties. *Clin Cancer Res* **2004** , *10* , 6512-6521, doi:10.1158/1078-0432.CCR-04-0804.

21. Rudek, M.A.; Figg, W.D.; Dyer, V.; Dahut, W.; Turner, M.L.; Steinberg, S.M.; Liewehr, D.J.; Kohler, D.R.; Pluda, J.M.; Reed, E. Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. *J Clin Oncol* **2001** , *19* , 584-592, doi:10.1200/JCO.2001.19.2.584.

22. Chu, Q.S.; Forouzesh, B.; Syed, S.; Mita, M.; Schwartz, G.; Cooper, J.; Curtright, J.; Rowinsky, E.K. A phase II and pharmacological study of the matrix metalloproteinase inhibitor (MMPI) COL-3 in patients with advanced soft tissue sarcomas. *Invest New Drugs* **2007** ,*25* , 359-367, doi:10.1007/s10637-006-9031-6.

23. Dezube, B.J.; Krown, S.E.; Lee, J.Y.; Bauer, K.S.; Aboulafia, D.M. Randomized phase II trial of matrix metalloproteinase inhibitor COL-3 in AIDS-related Kaposi's sarcoma: an AIDS Malignancy Consortium Study.*J Clin Oncol* **2006** , *24* , 1389-1394, doi:10.1200/JCO.2005.04.2614.

24. Fingleton, B. CMT-3. CollaGenex. *Current opinion in investigational drugs* **2003** , *4* , 1460-1467.

25. Greenwald, R.A.; Golub, L.M.; Ramamurthy, N.S.; Chowdhury, M.; Moak, S.A.; Sorsa, T. In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. *Bone* **1998** , *22* , 33-38, doi:10.1016/s8756-3282(97)00221-4.

26. Edan, R.A.; Luqmani, Y.A.; Masocha, W. COL-3, a chemically modified tetracycline, inhibits lipopolysaccharide-induced microglia activation and cytokine expression in the brain. *PloS one* **2013** ,*8* , e57827, doi:10.1371/journal.pone.0057827.

27. Couch, Y.; Alvarez-Erviti, L.; Sibson, N.R.; Wood, M.J.; Anthony, D.C. The acute inflammatory response to intranigral alpha-synuclein differs significantly from intranigral lipopolysaccharide and is exacerbated by peripheral inflammation. *Journal of neuroinflammation* **2011** , *8* , 166, doi:10.1186/1742-2094-8-166.

28. Lema Tome, C.M.; Tyson, T.; Rey, N.L.; Grathwohl, S.; Britschgi, M.; Brundin, P. Inflammation and alpha-synuclein's prion-like behavior in Parkinson's disease–is there a link? *Molecular neurobiology***2013** , *47* , 561-574, doi:10.1007/s12035-012-8267-8.

29. Zhang, W.; Wang, T.; Pei, Z.; Miller, D.S.; Wu, X.; Block, M.L.; Wilson, B.; Zhang, W.; Zhou, Y.; Hong, J.S.; et al. Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2005** , *19* , 533-542, doi:10.1096/fj.04-2751com.

30. Dos-Santos-Pereira, M.; Acuna, L.; Hamadat, S.; Rocca, J.; Gonzalez-Lizarraga, F.; Chehin, R.; Sepulveda-Diaz, J.; Del-Bel, E.; Raisman-Vozari, R.; Michel, P.P. Microglial glutamate release evoked by alpha-synuclein aggregates is prevented by dopamine. *Glia***2018** , *66* , 2353-2365, doi:10.1002/glia.23472.

31. Rousseau, E.; Michel, P.P.; Hirsch, E.C. The iron-binding protein lactoferrin protects vulnerable dopamine neurons from degeneration by preserving mitochondrial calcium homeostasis. *Molecular pharmacology* **2013** , *84* , 888-898, doi:10.1124/mol.113.087965.

32. Dos-Santos-Pereira, M.; Guimaraes, F.S.; Del-Bel, E.; Raisman-Vozari, R.; Michel, P.P. Cannabidiol prevents LPS-induced microglial inflammation by inhibiting ROS/NF-kappaB-dependent signaling and glucose consumption. *Glia* **2020** , *68* , 561-573, doi:10.1002/glia.23738.

33. Sepulveda-Diaz, J.E.; Ouidja, M.O.; Socias, S.B.; Hamadat, S.; Guerreiro, S.; Raisman-Vozari, R.; Michel, P.P. A simplified approach for efficient isolation of functional microglial cells: Application for modeling neuroinflammatory responses in vitro. *Glia***2016** , *64* , 1912-1924, doi:10.1002/glia.23032.

34. Hoyer, W.; Antony, T.; Cherny, D.; Heim, G.; Jovin, T.M.; Subramaniam, V. Dependence of alpha-synuclein aggregate morphology on solution conditions. *Journal of molecular biology* **2002** ,*322* , 383-393.

35. Acuna, L.; Hamadat, S.; Corbalan, N.S.; Gonzalez-Lizarraga, F.; Dos-Santos-Pereira, M.; Rocca, J.; Diaz, J.S.; Del-Bel, E.; Papy-Garcia, D.; Chehin, R.N.; et al. Rifampicin and Its Derivative Rifampicin Quinone Reduce Microglial Inflammatory Responses and Neurodegeneration Induced In Vitro by alpha-Synuclein Fibrillary Aggregates. *Cells***2019** , *8* , doi:10.3390/cells8080776.

36. Doring, C.; Regen, T.; Gertig, U.; van Rossum, D.; Winkler, A.; Saiepour, N.; Bruck, W.; Hanisch, U.K.; Janova, H. A presumed antagonistic LPS identifies distinct functional organization of TLR4 in mouse microglia. *Glia* **2017** , *65* , 1176-1185, doi:10.1002/glia.23151.

37. Ghosh, S.; Castillo, E.; Frias, E.S.; Swanson, R.A. Bioenergetic regulation of microglia. *Glia* **2018** , *66* , 1200-1212, doi:10.1002/glia.23271.

38. Richards, C.; Pantanowitz, L.; Dezube, B.J. Antimicrobial and non-antimicrobial tetracyclines in human cancer trials.*Pharmacological research* **2011** , *63* , 151-156, doi:10.1016/j.phrs.2010.10.008.

39. Lee, H.M.; Golub, L.M.; Cao, J.; Teronen, O.; Laitinen, M.; Salo, T.; Zucker, S.; Sorsa, T. CMT-3, a non-antimicrobial tetracycline (TC), inhibits MT1-MMP activity: relevance to cancer. *Curr Med Chem***2001** , *8* , 257-260, doi:10.2174/0929867013373660.

40. Cianfrocca, M.; Cooley, T.P.; Lee, J.Y.; Rudek, M.A.; Scadden, D.T.; Ratner, L.; Pluda, J.M.; Figg, W.D.; Krown, S.E.; Dezube, B.J. Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS malignancy consortium study. *J Clin Oncol* **2002** , *20* , 153-159, doi:10.1200/JCO.2002.20.1.153.

41. Parvathy, S.S.; Masocha, W. Matrix metalloproteinase inhibitor COL-3 prevents the development of paclitaxel-induced hyperalgesia in mice.*Med Princ Pract* **2013** , *22* , 35-41, doi:10.1159/000341710.

42. Cazalis, J.; Tanabe, S.; Gagnon, G.; Sorsa, T.; Grenier, D. Tetracyclines and chemically modified tetracycline-3 (CMT-3) modulate cytokine secretion by lipopolysaccharide-stimulated whole blood.*Inflammation* **2009** , *32* , 130-137, doi:10.1007/s10753-009-9111-9.

43. Hsieh, C.F.; Liu, C.K.; Lee, C.T.; Yu, L.E.; Wang, J.Y. Acute glucose fluctuation impacts microglial activity, leading to inflammatory activation or self-degradation. *Scientific reports* **2019** ,*9* , 840, doi:10.1038/s41598-018-37215-0.

44. Dai, C.; Ciccotosto, G.D.; Cappai, R.; Wang, Y.; Tang, S.; Xiao, X.; Velkov, T. Minocycline attenuates colistin-induced neurotoxicity via suppression of apoptosis, mitochondrial dysfunction and oxidative stress. *J Antimicrob Chemother* **2017** , *72* , 1635-1645, doi:10.1093/jac/dkx037.

45. Vander Heiden, M.G.; Cantley, L.C.; Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation.*Science* **2009** , *324* , 1029-1033, doi:10.1126/science.1160809.

46. Jurcovicova, J. Glucose transport in brain - effect of inflammation.*Endocr Regul* **2014** , *48* , 35-48, doi:10.4149/endo_2014_01_35.

47. Fodelianaki, G.; Lansing, F.; Bhattarai, P.; Troullinaki, M.; Zeballos, M.A.; Charalampopoulos, I.; Gravanis, A.; Mirtschink, P.; Chavakis, T.; Alexaki, V.I. Nerve Growth Factor modulates LPS - induced microglial glycolysis and inflammatory responses. *Exp Cell Res***2019** , *377* , 10-16, doi:10.1016/j.yexcr.2019.02.023.

48. Koo, S.J.; Szczesny, B.; Wan, X.; Putluri, N.; Garg, N.J. Pentose Phosphate Shunt Modulates Reactive Oxygen Species and Nitric Oxide Production Controlling Trypanosoma cruzi in Macrophages. *Front Immunol* **2018** , *9* , 202, doi:10.3389/fimmu.2018.00202.

49. TeSlaa, T.; Teitell, M.A. Techniques to monitor glycolysis.*Methods Enzymol* **2014** , *542* , 91-114, doi:10.1016/B978-0-12-416618-9.00005-4.

50. Gong, H.; Liu, L.; Ni, C.X.; Zhang, Y.; Su, W.J.; Lian, Y.J.; Peng, W.; Zhang, J.P.; Jiang, C.L. Dexamethasone rapidly inhibits glucose uptake via non-genomic mechanisms in contracting myotubes. *Arch Biochem Biophys* **2016** , *603* , 102-109, doi:10.1016/j.abb.2016.05.020.

51. Liu, Y.; Ramamurthy, N.; Marecek, J.; Lee, H.M.; Chen, J.L.; Ryan, M.E.; Rifkin, B.R.; Golub, L.M. The lipophilicity, pharmacokinetics, and cellular uptake of different chemically-modified tetracyclines (CMTs).*Curr Med Chem* **2001** , *8* , 243-252, doi:10.2174/0929867013373525.

52. di Penta, A.; Moreno, B.; Reix, S.; Fernandez-Diez, B.; Villanueva, M.; Errea, O.; Escala, N.; Vandenbroeck, K.; Comella, J.X.; Villoslada, P. Oxidative stress and proinflammatory cytokines contribute to demyelination and axonal damage in a cerebellar culture model of neuroinflammation. *PloS one* **2013** , *8* , e54722, doi:10.1371/journal.pone.0054722.

53. Bird, L. Microglial memory. *Nat Rev Immunol* **2018** ,*18* , 358-359, doi:10.1038/s41577-018-0015-5.

54. Stefanska, J.; Pawliczak, R. Apocynin: molecular aptitudes.*Mediators of inflammation* **2008** , *2008* , 106507, doi:10.1155/2008/106507.

55. Lúcio, M.; Nunes, C.; Gaspar, D.; Ferreira, H.; Lima, J.L.F.C.; Reis, S. Antioxidant Activity of Vitamin E and Trolox: Understanding of the Factors that Govern Lipid Peroxidation Studies In Vitro. *Food Biophysics* **2009** , *4* , 312-320, doi:10.1007/s11483-009-9129-4.

56. Bedard, K.; Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews***2007** , *87* , 245-313, doi:10.1152/physrev.00044.2005.

57. Gloire, G.; Legrand-Poels, S.; Piette, J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochemical pharmacology* **2006** , *72* , 1493-1505, doi:10.1016/j.bcp.2006.04.011.

58. Shabab, T.; Khanabdali, R.; Moghadamtousi, S.Z.; Kadir, H.A.; Mohan, G. Neuroinflammation pathways: a general review. *The International journal of neuroscience* **2017** , *127* , 624-633, doi:10.1080/00207454.2016.1212854.

59. Onoda, T.; Ono, T.; Dhar, D.K.; Yamanoi, A.; Nagasue, N. Tetracycline analogues (doxycycline and COL-3) induce caspase-dependent and -independent apoptosis in human colon cancer cells.*International journal of cancer* **2006** , *118* , 1309-1315, doi:10.1002/ijc.21447.

FIGURE LEGENDS

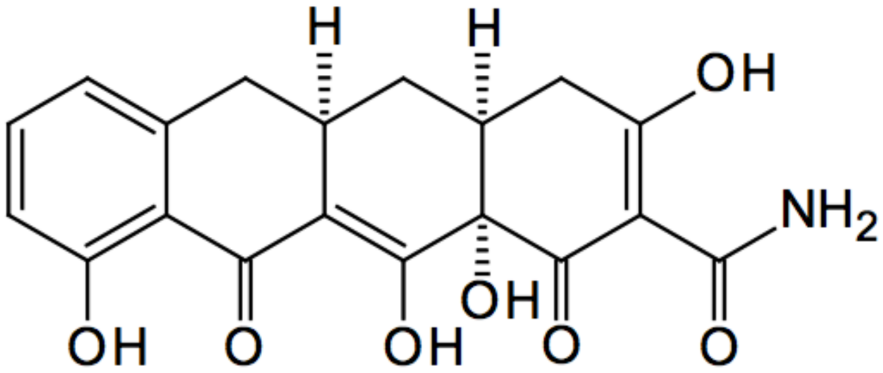

Figure 1: This is a caption

Figure 1: Chemical structure of Chemically Modified Tetracycline (COL-3). While all tetracyclines present an octahydrotetracene-2-carboxamide skeleton, they differ from each other by the presence of chloride, methyl, and hydroxyl groups. COL-3 differs from other tetracyclines in the absence of the dimethylamino group from carbon 4 in the A ring, which confers it the characteristic of a non-antibacterial tetracycline.

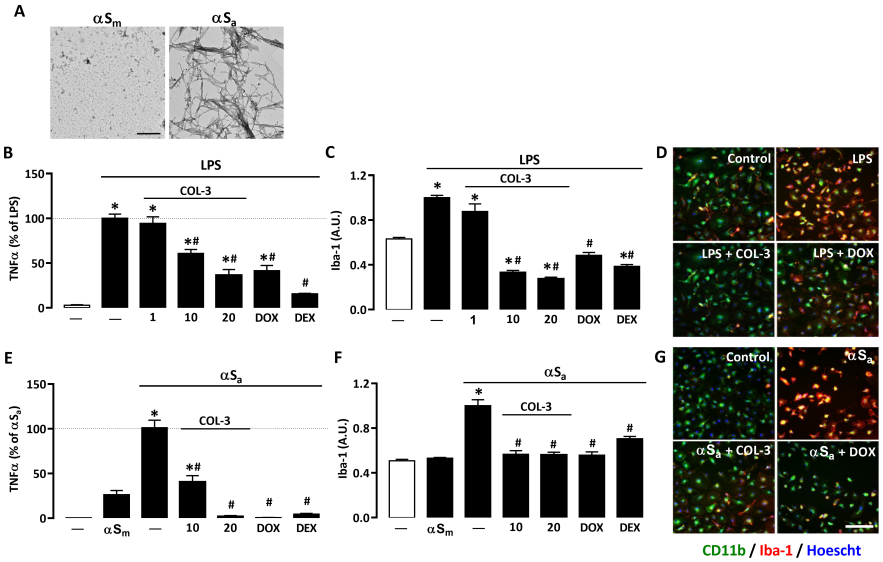

Figure 2: This is a caption

Figure 2: Τηε τετρασψχλινε ˚ΟΛ-3 ρεδυχες τηε ινφλαμματορψ ρεσπονσε οφ μιχρογλιαλ χελλς χηαλλενγεδ ωιτη ειτηερ ΛΠΣ ορ αΣα (A) (b) TEM images showing the presence of αS amyloid fibrils under shaken conditions, only (lower image). Scale bar: 1 μm. **(B)**Quantification of TNF-α levels in microglial cells exposed or not to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (1, 10 or 20 μM), DOX (50μM) or the reference antiinflammatory drug DEX (2.5 μM). Data are means ± SEM (n = 4), *p $<$ 0.05 *vs* control.$^{\#}$p $<$ 0.05 *vs* LPS. One-way ANOVA followed by Bonferroni post-hoc test. **(C)** Quantification of Iba-1 immunofluorescence density in microglial cultures exposed or not to to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (10 or 20 μM), DOX (50μM) or the reference glucocorticoid DEX (2.5 μM). Data are means ± SEM (n = 4), *p $<$ 0.05 *vs* control.$^{\#}$p $<$ 0.05 *vs* LPS. One-way ANOVA followed by Bonferroni post-hoc test. **(D)** Visualization of CD11b (green) and Iba-1 (red) immunofluorescence signals

of LPS (10 ng/ml)-treated microglial cell cultures treated or not with COL-3 (20μM) or DOX (50 μM). Cell nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 120 μm. **(E)** Quantification of TNF-α levels in microglial cells exposed or not to αSa (70 μg/ml, 24-h) in the presence or not of COL-3 (10 and 20 μM), DOX (50μM) or DEX (2.5 μM). Some cultures were also exposed αSm (70 μg/ml). Data are means ± SEM (n = 4), *p < 0.05 *vs* control. #p < 0.05 *vs* αSa. One-way ANOVA followed by Bonferroni post-hoc test.**(F)** Quantification of Iba-1 immunofluorescence signals in microglial cultures exposed or not to to α Sa (70 μg/ml, 24-h) in the presence or not of COL-3 (10 or 20 μM), DOX (50μM) or DEX (2.5 μM). Data are means ± SEM (n = 4), *p < 0.05 *vs* control.#p < 0.05 *vs* αSa. One-way ANOVA followed by Bonferroni post-hoc test. **(G)** Visualization of CD11b (green) and Iba-1 (red) immunofluorescent signals in αSa (70 μg/ml)-treated microglial cell cultures exposed or not to COL-3 (20μM) or DOX (50 μM). Cell nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 120 μm.

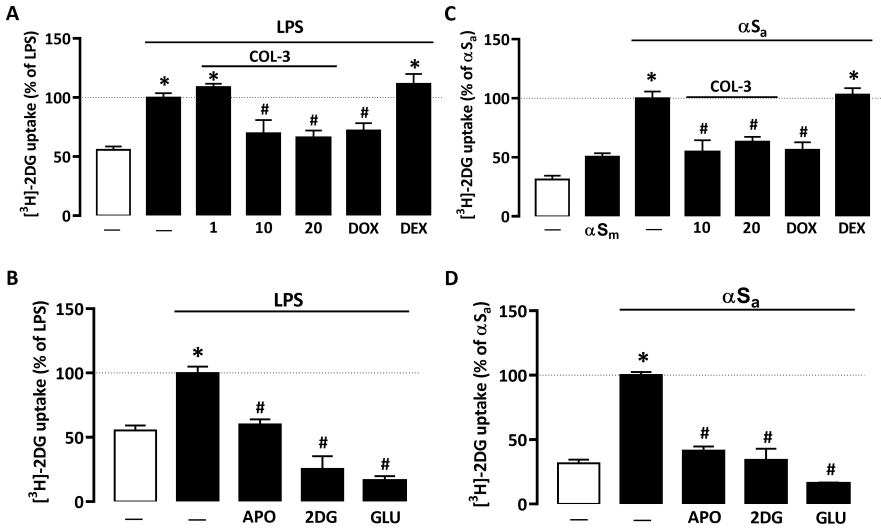

Figure 3: This is a caption

Φιγυρε 3: Τηε τετρασψχλινε ΄ΟΛ-3 ρεδυχες γλυχοσε αχχυμυλατιον ιν μιχρογλιαλ χελλς χηαλλενγεδ ωιτη ειτηερ ΛΠΣ ορ αΣα. (A) Quantification of glucose uptake in microglial cells exposed or not to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (10 or 20 μM), DOX (50μM) or the reference DEX (2.5 μM). **(B)** Quantification of glucose uptake in microglial cells exposed or not to LPS in the presence of APO (300 μM), 2-DG (500 μM). The specificity of the assay was confirmed by acutely exposing LPS-treated cultures to an excess of glucose (50 mM). **(C)** Quantification of glucose uptake in microglial cells exposed or not to aSa (70 μg/ml, 24-h) in the presence or not of COL-3 (10 and 20 μM), DOX (50μM) or the reference glucocorticoid DEX (2.5 μM). **(D)** Quantification of glucose uptake in microglial cells exposed or not to αSa in the presence of APO (300 μM), 2-DG (500 μM). The specificity of the assay was confirmed by acutely exposing αSa-treated cultures to an excess of glucose (50 mM). Data are means ± SEM (n = 6–8), *p < 0.05 *vs* control.#p < 0.05 *vs* inflammogen. One-way ANOVA followed by Bonferroni post-hoc test.

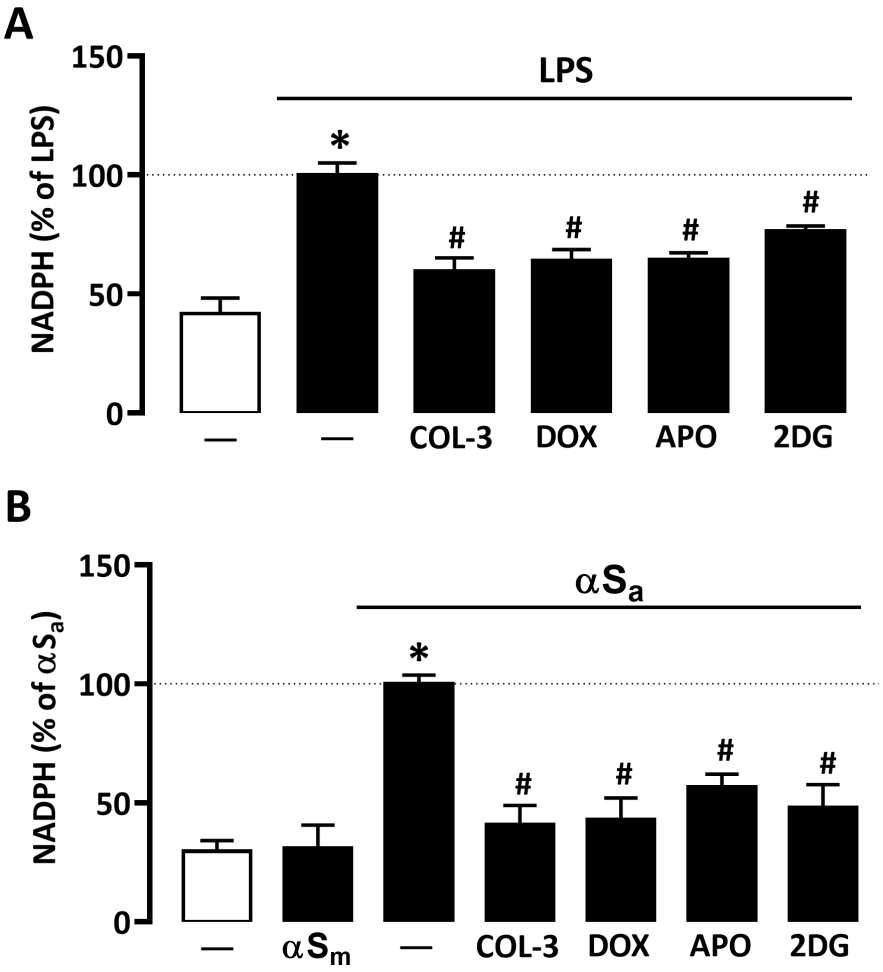

Figure 4: This is a caption

Φιγυρε 4: ΟΛ-3 πρεεντς ΝΑΔΠΗ σψντηεσις ιν μιςρογλιαλ ςελλς ςηαλλενγεδ ωιτη ειτηερ ΛΠΣ ορ αΣα (A) Quantification of NADPH in microglial cells exposed or not to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (20 µM), DOX (50µM), APO (300 µM), 2-DG (500 µM).**(B)** Quantification of NADPH in microglial cells exposed or not to αSa (70 µg/ml, 24-h) in the presence or not of COL-3 (20 µM), DOX (50µM), APO (300 µM), 2-DG (500 µM). Data are means ± SEM (n = 6–8), *p < 0.05 *vs* control. #p < 0.05 *vs* inflammogen. One-way ANOVA followed by Bonferroni post-hoc test.

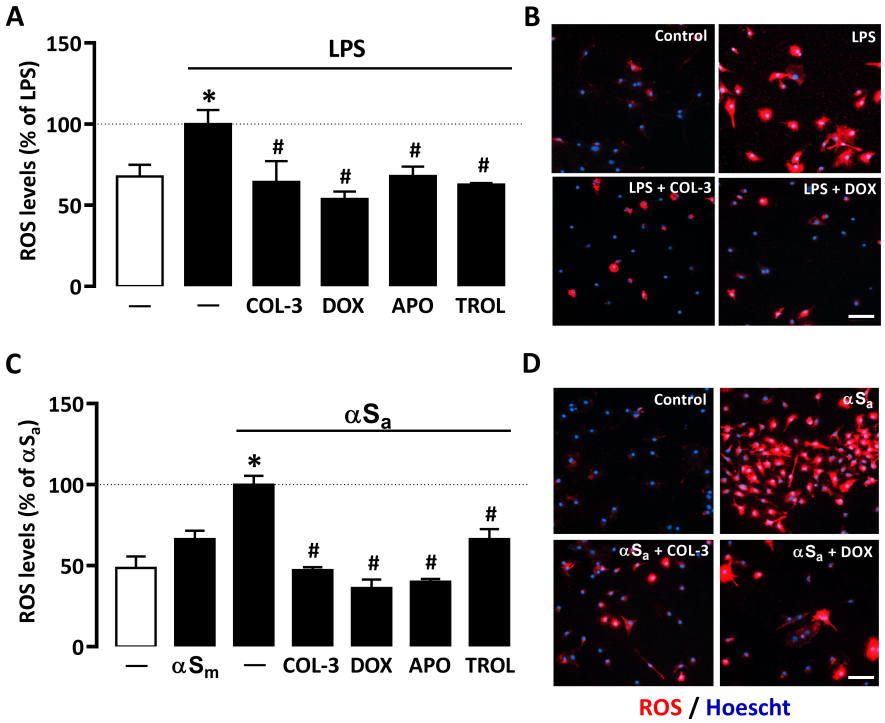

Figure 5: This is a caption

Φιγυρε 5: ΤΟΛ-3 πρεεντς ιντραχελλυλαρ οξιδατιε στρεσς γενερατεδ ιν μιχρογλιαλ χελλς βψ ΛΠΣ ορ αΣα εξποσυρε (A) Quantification of ROS levels in microglial cells exposed or not to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (20 µM), DOX (50µM), APO (300 µM), or TROL (10 µM). Data are means ± SEM (n = 6–8), *p $<$ 0.05*vs* control. #p $<$ 0.05 *vs* LPS. One-way ANOVA followed by Bonferroni post-hoc test. **(B)**Visualization of intracellular ROS levels in LPS (10 ng/ml)-treated microglial cell cultures treated or not with COL-3 (20 µM) or DOX (50 µM). Scale bar: 50 µm**. (C)** Quantification of ROS levels in microglial cells exposed or not to αSa (70 µg/ml, 24-h) in the presence or not of COL-3 (20 µM), DOX (50 µM), APO (300 µM), or TROL (10 µM). Data are means ± SEM (n = 6–8), *p $<$ 0.05 *vs* control. #p $<$ 0.05 *vs* αSa. One-way ANOVA followed by Bonferroni post-hoc test. **(D)** Visualization of intracellular ROS levels in αSa (70 µg/ml)-treated microglial cell cultures treated or not with COL-3 (20µM) or DOX (50 µM). Scale bar: 50 µm**.**

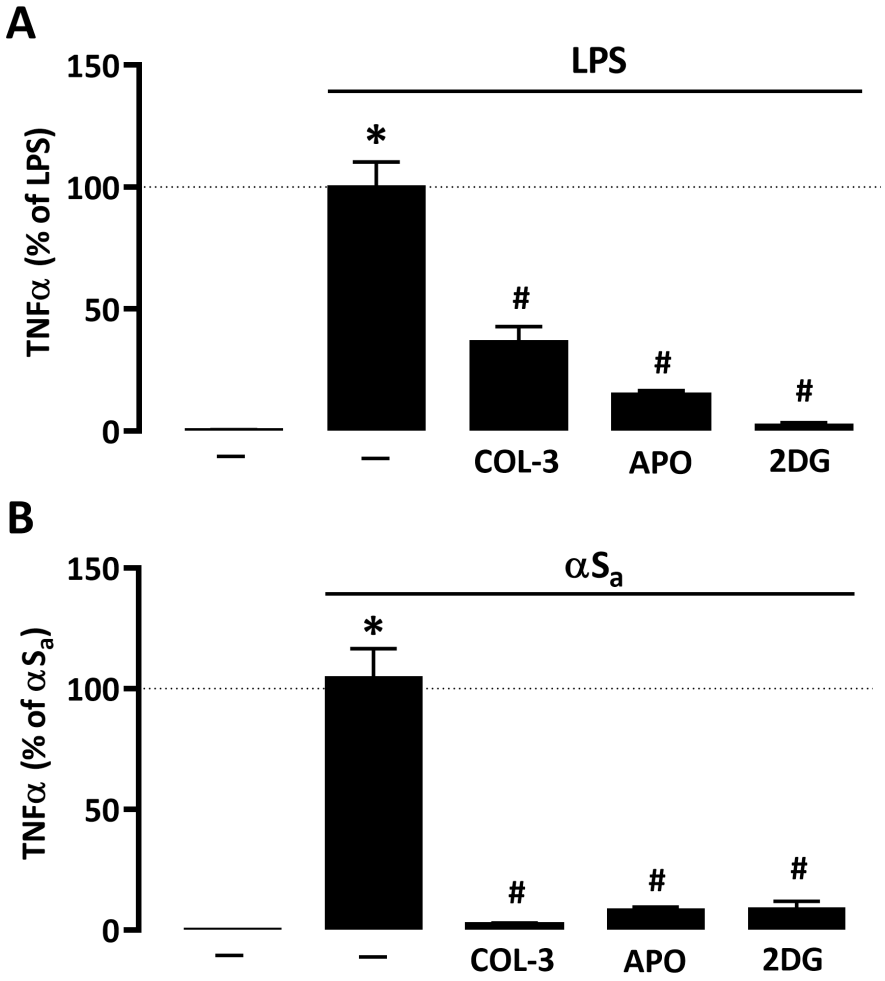

Figure 6: This is a caption

Figure 6: The inhibitory effects of COL-3 ον ΤΝΦ-α ρελεασε αρε ρεπροδυςεδ βψ ειτηερ ρεδυςινγ γλυςοσε μεταβολισμ ορ λοωερινγ ΝΑΔΠΗ οξιδασε αςτιιτψ . **(A)** Quantification of TNF-α levels in microglial cells exposed or not to LPS (10 ng/ml, 24-h) in the presence or not of COL-3 (20 μM), APO (300 μM), or 2-DG (500 μM μM). Data are means ± SEM (n = 6), *p $<$ 0.05 vs control.$^{\#}$p $<$ 0.05 vs LPS. One-way ANOVA followed by Bonferroni post-hoc test. **(B)** Quantification of TNFα levels in microglial cells exposed or not to αSa (70 μg/ml, 24-h) in the presence or not of COL-3 (20 μM), APO (300 μM), or 2DG (500 μM μM). Data are means ± SEM (n = 6), *p $<$ 0.05 vs control.$^{\#}$p $<$ 0.05 vs αSa. A One-way ANOVA followed by Bonferroni post-hoc test.

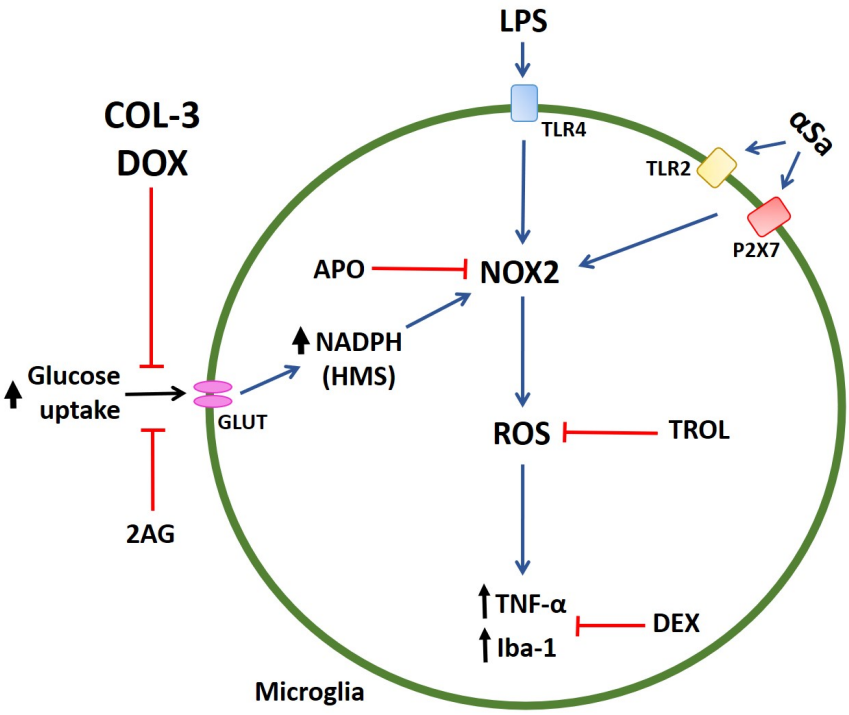

Figure 7: This is a caption

Φιγυρε 7: Προποσεδ σχηεμε ρεπρεσεντινγ τηε μεχηανισμ ΄ΟΛ-3 μαψ ρεδυχε μιχρογλιαλ ινφλαμματιον ιν ρεσπονσε το ΛΠΣ ανδ αΣα. The bacterial inflammogen LPS and the aggregates of αS stimulate ROS production through the activation of TLR4 and TLR2/P2X7, respectively, and indirectly through stimulation of NADPH oxidase (NOX2) activity. Increased levels of NADPH further enhance oxidant stress (seen through ROS production) thus leading to the release of proinflammatory mediators (TNF-α and Iba-1). COL-3 and DOX, by inhibiting glucose uptake, reduces glucose-derived synthesis of NADPH and consequent oxidative stress. Reference drugs can (APO, TROL, DEX, 2AG) affect the inflammation process in different steps (as observed in the figure), resulting in a similar final response to tetracyclines.