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Sophorolipid Butyl Ester Diacetate Does Not Affect Macrophage Polarization but Enhances Astrocytic Glial Fibrillary Acidic Protein Expression at Micromolar Concentrations in Vitro

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Abstract

Peritoneal macrophages (PMACs) and spinal cord astrocytes were exposed to varying concentrations of soluble sophorolipid butyl ester diacetate (SLBEDA) in vitro. Macrophages and astrocytes demonstrated no decrease in viability in response to SLBEDA. Studying pro- and anti-inflammatory genes, PMACs did not show a shift toward a pro-inflammatory phenotype. However, at higher concentrations (3 and 30 μ M), astrocytes showed an increase in their expression of glial acidic fibrillary protein. This novel category of compounds poses low risk to PMAC and astrocyte viability; however, the effect on PMAC polarization and astrocyte reactivity requires more elucidation.

Graphical abstract

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[†]Author Contributions: A.M.Z. and M.K.G. contributed equally to this work. All authors designed the study. A.M.Z., M.K.G., and F.T. completed experimental work. A.M.Z., M.K.G., F.T., and C.M.H. analyzed and interpreted the data. A.M.Z. and M.K.G. drafted the manuscript. All authors provided a critical revision of the manuscript.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.6b00451. Additional methods and results for SLBEDA synthesis and characterization and plasticity of PMAC polarization test (PDF)

Notes

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

The authors declare no competing financial interest.

Keywords

Macrophage; astrocyte; sophorolipid; spinal cord injury

Every year in the United States, 17 000 new people are afflicted with spinal cord injury (SCI).¹ The mechanical insult accompanying injury produces hemorrhaging, ischemia, and edema that result in an expanding lesion.² After approximately 2 h, the injury progresses to a secondary injury phase characterized by inflammation. Neutrophils first infiltrate the injury site followed by the activation of microglia and the recruitment of blood monocytes that differentiate and respond to the environment by polarizing to a pro-inflammatory or an anti-inflammatory phenotype. M1, pro-inflammatory, macrophages are prevalent during the first several days postinjury³ but persist weeks postinjury.^{2,4} These macrophages produce high levels of reactive oxygen species (ROS) and pro-inflammatory cytokines.³ Ultimately, this M1 macrophage pro-inflammation is neurotoxic and results in axonal dieback^{3,4} and causes neuronal and glial necrosis.⁵

In addition to macrophages, astrocytes also play an important role post-SCI. Astrocytes are a heterogeneous population of glial cells present in the central nervous system (CNS) and are primarily responsible for the overall maintenance of homeostasis in the CNS.⁶ Following SCI, astrocytes at and around the site of the injury are activated, leading to the formation of a glial scar which acts as a chemical and physical barrier to regenerating axons.⁷ The activation/reactivity of astrocytes is characterized by an increase in migration/proliferation^{8,9} along with an increase in the production of the intermediate filament glial fibrillary acidic protein (GFAP)¹⁰ and extracellular chondroitin sulfate proteoglycans (CSPGs).¹¹ GFAP synthesis is a meaningful aspect of the developmental plan of astrocyte differentiation, and it is a part of the reactive response to several CNS injuries.¹² After the injury, astrocytes show a decrease in GFAP until 1 day postinjury (dpi) followed by a steady increase and peaking of GFAP at 7 dpi.¹³ This increased GFAP, in addition to the increase in CSPGs and astrocytic proliferation, leads to the formation of a mature glial scar by 14 dpi.^{9,11} Although the process of reactive astrogliosis is known to play an important role in stabilizing the CNS tissue postinjury,^{14,15} the formation of a mature glial scar limits the regeneration of the axons. Hence, potential therapies targeting pro-inflammatory M1 macrophages and reactive astrocytes could promote axonal regeneration and functional recovery post-SCI.

Sphorolipids (SLs) are a novel category of compounds primarily studied for antibacterial purposes.^{16–18} SLs are also antiviral¹⁹ and can modulate the severity of sepsis.²⁰ However, their potential for CNS applications has not yet been explored. SLs are glycolipids produced via yeast fermentation. The natural form of SL can be chemically modified in a ring-opening reaction to form esters. One such example of this modification produces a specific SL, SL butyl ester diacetate (SLBEDA). As lipids are commonly studied for CNS drug delivery applications, as delivery vehicles,^{21,22} we explored the potential of SLBEDA for use as an anti-inflammatory compound for SCI. We aimed to determine whether SLBEDA would be an appropriate therapeutic to both dampen the pro-inflammatory macrophage response and the reactive astrocyte response following SCI. As SL decreased macrophage nitric oxide production in a sepsis model,²⁰ we hypothesized that SLBEDA would shift M1 macrophages to an anti-inflammatory phenotype. Additionally, due to the crosstalk between macrophages and astrocytes,^{23–27} we hypothesized that astrocytes would shift to a less reactive phenotype. We studied peritoneal macrophage (PMAC) and spinal cord astrocyte viability as well as PMAC polarization and astrocytic GFAP expression in response to varying concentrations of SLBEDA in vitro.

RESULTS AND DISCUSSION

Effect of SLBEDA on the Viability of M1 PMACs

We first assessed the effect of SLBEDA (Supporting Information and Figure S1) on the survival of PMACs in culture. We polarized PMACs to an M1 phenotype and used the resultant M1-polarized PMACs (M1 PMACs) in this study as they represent the pro-inflammatory macrophages observed following SCI. M1 PMACs were incubated with increasing concentrations of SLBEDA (30 nM to 30 μ M) to determine the potential toxicity of SLBEDA. Media only and media + DMSO (the SLBEDA solvent, 0.1% final dilution) groups were included as controls. Cells were loaded with calcein and their nuclei labeled with Hoescht 33342 to determine the number of viable and total cells, respectively (Figure 1A). The media only control group showed an average cell viability of $97.60 \pm 0.56\%$, demonstrating that the culture conditions did not adversely affect the M1 PMACs (Figure 1B). Exposure to DMSO did not reduce the viability of M1 PMACs ($97.68 \pm 1.08\%$). The viability of all SL-treated groups were not significantly different compared to the media only control group ($F[5, 12] = 1.18, p = 0.375$), suggesting that SLBEDA has no detrimental effect on the survival of M1 PMACs at any of the concentrations tested.

Effect of SLBEDA on the Polarization of M1 PMACs

We next studied the ability of SLBEDA to shift the polarization of M1 PMACs to a pro-resolving phenotype. A pro-resolving, or an anti-inflammatory, macrophage phenotype characteristically promotes cell proliferation, extracellular matrix synthesis, angiogenesis, remyelination, and injury site stabilization.³ M1 PMACs were treated with a range of SLBEDA (3 nM – 30 μ M) for 24 h. Cell lysates were prepared, and mRNA levels were quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Message levels were quantified for the M1 markers: inducible nitric oxide synthase (iNOS) and interleukin-12 p40 (IL-12 p40; p40 subunit present in both IL-12 and IL-23) as well as the M2 markers: arginase-1 (Arg-1) and interleukin-10 (IL-10).³ For all the genes assessed, the

relative expression of SLBEDA-treated groups was not significantly different from the controls (Figure 2), implying that SLBEDA does not alter the polarization of M1 PMACs (iNOS: $F[6, 27] = 1.19$, $p = 0.342$; IL-12 p40: $F[6, 27] = 0.51$, $p = 0.793$; Arg-1: $F[6, 27] = 0.26$, $p = 0.952$; IL-10: $F[6, 27] = 1.86$, $p = 0.125$). To ensure that this lack of a change in M1 PMAC polarization was not due to macrophage inactivity, we confirmed the plasticity of PMAC polarization. M1 PMACs were reprogrammed using lipopolysaccharide (LPS) and interleukin-4 (IL-4); the relative expression displayed a significant increase in IL-12 p40 with LPS exposure and Arg-1 with IL-4 exposure, suggesting that PMAC polarization could be shifted (Figure S2).

Effect of SLBEDA on the Viability of Spinal Cord Astrocytes

We then determined the effect of SLBEDA on spinal cord astrocytes, due to their crosstalk with macrophages. Astrocytes were plated onto glass coverslips and treated with increasing concentrations of SLBEDA (30 nM to 30 μ M); media only and media + DMSO controls were run in parallel. After 1 and 4 days in culture, the cells were loaded with calcein, their nuclei labeled with Hoechst 33342 as above (Figure 3A), and astrocyte viability was quantified (Figure 3B). We found that the astrocyte viability was similar across all the concentrations of SLBEDA assessed at the 1 day postplating time point ($H = 9.30$, $p = 0.098$) as well as the 4 days postplating time point ($H = 1.89$, $p = 0.865$). This implies that the viability of the SLBEDA-treated groups are comparable to that of the corresponding media control and DMSO control groups. These results suggest that SLBEDA does not affect the viability of astrocytes in culture. We also found that the percentage of viable cells was significantly higher at the 4 day time point compared to the corresponding 1 day time point for all groups except for the DMSO control group, implying that SLBEDA does not hamper astrocytic proliferation. Taken together, these results suggest that the concentrations of SLBEDA used in this study do not adversely affect the viability/proliferation of astrocytes in culture. In addition, since the astrocytes in the DMSO control group did not proliferate, SLBEDA may be promoting astrocyte proliferation by counteracting the potential inhibitory effect of DMSO (Figure 3B).

Effect of SLBEDA on the GFAP-Immunoreactivity of Spinal Cord Astrocytes

To determine if SLBEDA induces any changes in the GFAP expression of astrocytes, cells were plated onto glass coverslips and treated with increasing concentrations of SLBEDA (300 nM to 30 μ M); media only and media + DMSO were used as controls. After 4 days in culture, the cells were stained for GFAP, and the average intensity of GFAP-immunoreactivity (ir) per cell in a view field was calculated (Figure 4). Astrocytes treated with higher concentrations of SLBEDA (3 and 30 μ M) had a significantly higher average intensity of GFAP-ir compared to the control group, suggesting that SLBEDA increases astrocytic GFAP expression at higher concentrations. There was also a significant increase in the average intensity of GFAP-ir in the cells treated with 3 and 30 μ M SLBEDA compared to the DMSO control group, implying that the changes in GFAP expression are indeed due to the SLBEDA and not because of the DMSO solvent.

While SLBEDA (3 nM to 30 μ M) did not affect the viability of M1 PMACs and astrocytes, it increased astrocyte GFAP expression at higher concentrations while being unable to shift

the polarization of M1 PMACs. The effect of lipids on the macrophage response has been studied most extensively in atherosclerosis research using monocytes; saturated fatty acids present within atherosclerotic plaques enhance the pro-inflammatory response of monocytes.²⁸ Free fatty acids, such as palmitate, increase ROS production, increase toll-like receptor expression, and activate the transcription factor, nuclear factor- κ B,²⁹ in addition to increasing the production of interleukin-1 β .³⁰ However, oleic acid, an unsaturated fatty acid with a similar tail to SLBEDA, increased the expression of M2 markers in both PMACs³¹ and bone marrow-derived macrophages.³² These studies suggest that conjugation of fatty acid tails plays a role in macrophage polarization and that unsaturated SLs have the potential to dampen the pro-inflammatory response.

SLBEDA was only studied from 3 nM to 30 μ M here due to solubility constraints. Previous studies have assessed the micelle activity of various types of SLs.^{33–36} Zhang et al. found that SLBEDA had a critical micelle concentration of approximately 3 μ M.³⁶ Thus, it is possible that there was a significant change in astrocyte GFAP expression at 3 and 30 μ M SLBEDA due to the presence of micelles, which may have interacted with astrocytes via a different mechanism than soluble SLBEDA. Although higher concentrations of SLBEDA cause an increase in the expression of GFAP, they do not enhance the proliferation of astrocytes, implying that SLBEDA does not necessarily lead to reactive astrogliosis under the conditions tested. On the other hand, it has been shown previously that GFAP plays an important role in vesicular trafficking; GFAP loss led to a reduction in cell surface trafficking of glutamate transporters to the plasma membrane.³⁷ This increase in GFAP caused by SLBEDA could cause an increase in the trafficking of glutamate transporters to the surface, potentially reducing neuronal cell death due to glutamate excitotoxicity. This effect has been shown previously using carbon nanotubes and cortical astrocytes in culture.^{38–40}

Nanoparticles coated with lipids and lipid nanocapsules have been used as delivery agents to enhance the permeability of the blood-brain barrier (BBB),^{41,42} which is primarily maintained by astrocytes, or to target gliomas. Lipid-coated ionically charged nanoparticles caused a 3–4-fold increase in BBB crossing compared to the uncoated particles while maintaining the integrity of the BBB.⁴³ Furthermore, several groups have reported that lipid nanocapsules, at lower concentrations, promoted the survival of astrocytes in culture.^{44,45} However, the GFAP expression of astrocytes was not assessed in these studies. We show here that SLBEDA does not hinder astrocytic survival, even at the highest concentration tested, suggesting a potential advantage of SLBEDA over the lipids traditionally used for CNS drug delivery applications.

We studied the effect of SLBEDA on cell types recruited and activated post-SCI: macrophages and astrocytes. Neither cell type showed a decrease in cell viability in the presence of SLBEDA, nor was macrophage polarization significantly affected. Astrocytes increased GFAP expression in response to the higher concentrations of SLBEDA. Other compounds in the SL library should be characterized for anti-inflammatory potential in CNS injury. Examples of variations in modified SLs that are available have (i) other ester groups at the carboxyl group lipid terminus, (ii) different sophorose headgroup substituents, and (iii)

varied degree of unsaturation of the lipid moiety. Future studies can also explore the efficacy of macrophage- and/or astrocyte-targeting therapeutics delivered via SL carriers.

METHODS

Macrophage Isolation and Cell Culture

Peritoneal macrophages (PMACs) were isolated from C57BL/6 mice, originally purchased from Jackson Laboratory (Bar Harbor, ME) then bred in house at Albany Medical Center, Albany, NY. All animal care and procedures were approved by the Albany Medical Center Institutional Animal Care and Use Committee (IACUC). Mice were injected with thioglycolate (3% in water, autoclaved then oxidized), and PMACs were extracted in sterile phosphate buffered saline (PBS, 10 mL) 72 h later using a 23G needle. Red blood cells were removed by using ACK lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA ; pH 7.2–7.4). PMACs were centrifuged (1000 rcf, 5 min) and then resuspended in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher, Waltham, MA) supplemented with fetal bovine serum (10% v/v; Thermo Fisher) and gentamicin (50 $\mu\text{g}/\text{mL}$). For the viability assay, freshly isolated PMACs were plated onto 15 mm square glass coverslips at a density of 5×10^4 cells/coverslip. At 15 min after seeding, PMACs were polarized to an M1 phenotype with interferon- γ (IFN- γ ; 100 ng/mL; Peprotech, Rocky Hill, NJ; cat # 315-05; lot # 061398) for 24 h at 37 °C in a 95% air/5% CO_2 incubator. The media was replaced with media containing varying concentrations of SL butyl ester diacetate (SLBEDA) in dimethyl sulfoxide (DMSO, 1000-fold dilution) and returned to the incubator for 24 h. The maximum concentration of the SLBEDA used was 30 μM as the concentration approached the solubility limit in water. For qRT-PCR experiments, PMACs were plated at a density of 1×10^6 cells/well in tissue culture treated 12-well plates (Celltreat) and were polarized and treated with SLBEDA as described above. In an additional control experiment, the plasticity of PMAC polarization was confirmed. PMACs were first polarized to an M1 phenotype using IFN- γ as described above. Next, lipopolysaccharide (LPS; 100 ng/mL; Sigma, St. Louis, MO; cat # L4391; lot #115M4090 V) and interleukin-4 (IL-4; 25 ng/mL; Peprotech; cat # 214-14; lot # 111249) were used to increase and decrease the M1 polarization of PMACs, respectively. For all PMAC experiments, M0 (unpolarized) PMACs were used as a control to confirm M1 polarization.

PMAC Viability Assay

After a 24 h exposure to SLBEDA, M1 PMACs were incubated with the vital fluorescent dye, calcein acetoxymethyl (AM) ester (1 $\mu\text{g}/\text{mL}$; Thermo Fisher) with the addition of pluronic F-127 (0.25% w/v; Thermo Fisher) for 15 min followed by the cell permeant nuclear stain Hoechst 33342 trihydrochloride trihydrate (10 $\mu\text{g}/\text{mL}$; Thermo Fisher) for another 15 min. Cells were imaged at room temperature (RT) in external imaging solution (10 mM HEPES, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 5 mM D-glucose) using an Olympus IX-81 confocal microscope equipped with epifluorescence illumination (Metal Halide lamp, 120 W) driven by Metamorph Premier 7.7.3.0 imaging software (Molecular Devices, Sunnyvale, CA). The fluorescence of calcein and Hoechst 33342 were visualized and imaged using fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) filter sets, respectively, and a 20 $\times$ LUC Plan FLN

objective. For each condition, six coverslips were imaged with cells isolated from three different animals. Five fields of view ($\sim 0.168 \text{ mm}^2$) per coverslip were imaged to ensure a representative population of cells. The number of cells per view field were counted using ImageJ 1.49v software (National Institutes of Health, Bethesda, MD) (background subtracted using sliding paraboloid with rolling ball radius = 50.0 pixels, threshold established, image made binary, watershed, and cells analyzed with size = 0–500 pixel²). The cells positive for calcein and Hoechst 33342 were considered live, while the cells positive for Hoechst 33342 and negative for calcein were considered dead. Average viability of the 10 fields of view per condition, per animal was calculated, and the mean viability per animal was reported.

PMAC qRT-PCR Polarization Assay

M1 PMACs were lysed with Trizol (Ambion, Waltham, MA), and RNA was isolated per manufacturer's instructions. cDNA was prepared using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA), and the PCR reaction run with the primer sets listed in Table 1. Primer sets were obtained from Integrated DNA Technologies (Coralville, IA) after conducting BLAST analyses. The cDNA obtained was amplified using PerfeCTa SYBR Green FastMix ROX (Quanta Biosciences) and Applied Biosystems 7300 Real Time PCR System. The relative gene expression was calculated using $\Delta\Delta\text{Ct}$ method with conditions normalized to β -actin and the M1 controls.

Spinal Cord Astrocyte Isolation and Cell Culture

Astrocytic cell cultures were prepared using a modified version of a previously described procedure.⁴⁶ All animal care and procedures were approved by the Rensselaer Polytechnic Institute IACUC. Spinal cords were isolated from 2-day-old Sprague–Dawley rat pups (Taconic, Rensselaer, NY) and treated with TrypLE express (Thermo Fisher) for 30 min at 37 °C and neutralized with cell culture media. The resulting cell suspension was plated into 75 cm² tissue culture flasks. One day postplating, the cell suspension was replaced with fresh culture media containing DMEM supplemented with heat-inactivated horse serum (10% v/v; Thermo Fisher), penicillin (100 IU/mL), and streptomycin (100 $\mu\text{g/mL}$). Cells were maintained at 37 °C in a 95% air/5% CO₂ incubator until they reached $\sim 70\%$ confluency after which the cell cultures were purified for astrocytes using a previously described procedure.⁴⁷ Briefly, the flasks were shaken twice on an orbital shaker, first for 1.5 h followed by an exchange with culture media and again for 18 h, at 37 °C and 220 rpm. Purified astrocytes were detached from the flasks by adding TrypLE express for 3 min and pelleted by centrifugation at 500 rcf for 5 min. The pellet was resuspended in culture media, and the cells were plated onto glass coverslips precoated with fibronectin (10 $\mu\text{g/mL}$; Sigma) at a density of 20 000 cells per coverslip and maintained in the incubator for 1 or 4 days until used for experiments. Two hours postplating, the culture media was carefully pipetted and replaced with fresh culture media containing varying concentrations of the SLBEDA.

Astrocyte Viability Assay

To assess the viability of astrocytes in the presence of SLBEDA, the cells were incubated with calcein and Hoechst 33342 as described above and imaged using a 10 $\times$ LUC Plan FLN objective at 1 day and 4 days postplating. For each condition and time point, six coverslips

were imaged with cells originating from three independent cultures. Five view fields ($\sim 0.67 \text{ mm}^2$) per coverslip were imaged.

Astrocyte GFAP Quantification

To assess the GFAP expression of SLBEDA-treated astrocytes, the cells were labeled for GFAP using indirect immunocytochemistry as described elsewhere.³⁸ Briefly, astrocytes were fixed with 4% paraformaldehyde at RT for 30 min and then permeabilized with 0.25% (v/v) Triton X-100 for 10 min. The cells were incubated with 10% (v/v) goat serum and 0.25% (v/v) Triton X-100 in PBS for 30 min to prevent nonspecific binding followed by an overnight ($>12 \text{ h}$) incubation of the cells at 4°C with primary antibody against GFAP (1:700; Dako, Santa Clara, CA; cat # Z0334; lot # 20015408). Cells were then washed three times with PBS and incubated for 2 h with Alexa Fluor 488 secondary antibody (1:1000; Thermo Fisher; cat # A11008; lot # 1622775) at RT. After a triple wash with PBS and counterstaining the nuclei with DAPI, the coverslips were mounted onto glass microscopic slides in ProLong Diamond antifade reagent (Thermo Fisher) to prevent photo bleaching. Immunoreactivity (ir) of GFAP was visualized using a standard FITC filter set and imaged using a $10\times$ LUC Plan FLN objective. For each condition, six coverslips were imaged with cells originating from three independent cultures and three fields of view were imaged per coverslip. Primary antibody was omitted in a subset of the coverslips to test for the nonspecific binding of the secondary antibody which were used to estimate the threshold value for GFAP positive pixels. The background subtracted mean intensity of the view fields with no primary cells (autofluorescence) + 50 standard deviations was used as the threshold value; the background fluorescence was obtained from regions of coverslips containing no cells. The average intensity of GFAP-ir per cell in each view field was calculated based on the threshold value and normalized to the mean average intensity of the control cells; DAPI labeling of the nuclei was used to estimate the number of cells per view field.

Statistical Analysis

All data are reported as mean $\pm$ standard error of mean or medians with interquartile range. Statistical analysis was performed using JMP Statistical Software (SAS Institute, Cary, NC) and GB-Stat v6.5 software (Dynamic Microsystems Inc., Silver Spring, MD). The number of subjects required for each of the assays was estimated using power analysis (set at 80% and $\alpha = 0.05$). Nonparametric statistics were used for all the subgroups that deviated from normality based on Shapiro-Wilk test. To test the differences between the multiple independent groups, one-way ANOVA followed by post hoc Tukey-Kramer test (Figures 1, 2, and S2) or Kruskal-Wallis one-way ANOVA followed by Newman-Keuls test (Figures 3 and 4) were used. To test the difference between the 1 day and 4 day time points, the two correlated groups were compared using Wilcoxon Signed-Rank test (Figure 3). Significance was established at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

arg-1	arginase-1
BBB	blood-brain barrier
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
GFAP	glial fibrillary acidic protein
IL	interleukin
iNOS	inducible nitric oxide synthase
ir	immunoreactivity
iu	intensity units
LPS	lipopolysaccharide
PBS	phosphate buffered saline
PMAC	peritoneal macrophage
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
SCI	spinal cord injury
SL	sophorolipid
SLBEDA	sophorolipid butyl ester diacetate

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Figure 1.

M1 PMAC viability is unaffected by increasing concentrations of SLBEDA. (A) Representative images of M1-polarized PMACs loaded with calcein (left) and their corresponding nuclei labeled with the cell permeable nuclear stain Hoechst 33342 (center). The merged images are shown on the right. The top row shows images of control M1 PMACs, and the bottom row shows M1 PMACs treated with 30 μ M SLBEDA. Scale bar, 100 μ m. (B) Summary graph showing the percentage viability of M1 PMACs treated with increasing concentrations of SLBEDA. Data are presented as mean $\pm$ standard error of the mean ($n = 3$ animals). No statistical difference was observed between the groups based on one-way ANOVA.

Figure 2.

M1 PMAC polarization is not altered by SLBEDA treatment. Summary graphs showing the relative expression of iNOS (A), IL-12 p40 (B), Arg-1 (C), and IL-10 (D) in M1-polarized PMACs in response to increasing concentrations of SLBEDA. All gene expression data were normalized to the housekeeping gene, β -actin, and the corresponding M1 controls using the

Ct method. Data are presented as mean $\pm$ standard error of the mean ($n = 4-5$ animals). For all the polarization markers, no statistical difference was observed between the groups based on one-way ANOVA.

Figure 3.

Viability of rat spinal cord astrocytes in culture is unaffected by increasing concentrations of SLBEDA. (A) Representative images of astrocytes in culture loaded with the vital fluorescent dye calcein (left), and their corresponding nuclei labeled with the cell permeable nuclear stain Hoechst 33342 (middle). The merged images are shown on the right. The top row shows images of control astrocytes, and the bottom row shows images of astrocytes treated with 30 μ M SLBEDA, both at the 4 days postplating time point. Scale bar, 100 μ m. (B) Summary graph showing the percentage viability of astrocytes treated with increasing concentrations of SLBEDA, 1 day and 4 days postplating. Data are represented as medians with interquartile range. Six coverslips were analyzed for each of the conditions and time points. Asterisks indicate a statistical difference between the groups marked by the brackets. Wilcoxon-Signed Rank test (* $p < 0.05$).

Figure 4.

Higher concentrations of SLBEDA augment the GFAP immunoreactivity of rat spinal cord astrocytes in culture. (A) Representative images of control (left) and 30 μ M SLBEDA-treated (right) astrocytes in culture labeled for GFAP using indirect immunocytochemistry, 4 days postplating. Scale bar, 100 μ m. Gray scale on the right is a linear representation of the fluorescence intensities, expressed in fluorescence intensity units (iu), of the pixels in the images. (B) Summary graph showing the average intensity of GFAP-ir per cell in a view field, normalized to the mean intensity of the control group, in the presence of increasing concentrations of SLBEDA. Data are represented as medians with interquartile range. Six coverslips were analyzed for each of the conditions. Asterisks indicate a statistical difference when compared to the control group. The other differences are marked with the brackets. Kruskal–Wallis one-way ANOVA followed by Newman–Keuls test (* $p < 0.05$, ** $p < 0.01$).

Table 1

Primer Sets Used to Study Macrophage Polarization

gene	sense	antisense
<i>β</i> -actin (endogenous control)	TTCCAGCCTTCCTTCTTGG	AGTAATCTCCTTCTGCATCC
iNOS	TCTATCAGGAAGAAATGCAGG	CACCAGCTTCTTCAATGTGG
IL-12 p40	AGCACTCCCCATTCTACTT	CACGCAGACATTCCCGCC
Arg-1	GGAAAGCCAATGAAGAGCTG	GCTTCCAAGTCCAGACTGT
IL-10	TGTGAAAATAAGAGCAAGGCAGTG	GCCTTGTAGACACCTTGGT