Differential modulation of microglia superoxide anion and thromboxane B₂ generation by the marine manzamines

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Abstract

Background: Thromboxane B₂ (TXB₂) and superoxide anion (O₂⁻) are neuroinflammatory mediators that appear to be involved in the pathogenesis of several neurodegenerative diseases. Because activated-microglia are the main source of TXB₂ and O₂⁻ in these disorders, modulation of their synthesis has been hypothesized as a potential therapeutic approach for neuroinflammatory disorders. Marine natural products have become a source of novel agents that modulate eicosanoids and O₂⁻ generation from activated murine and human leukocytes. With the exception of manzamine C, all other manzamines tested are characterized by a complex pentacyclic diamine linked to C-1 of the β-carboline moiety. These marine-derived alkaloids have been reported to possess a diverse range of bioactivities including anticancer, immunostimulatory, insecticidal, antibacterial, antimalarial and antituberculosis activities. The purpose of this investigation was to conduct a structure-activity relationship study with manzamines (MZ) A, B, C, D, E and F on agonist-stimulated release of TXB₂ and O₂⁻ from E. coli LPS-activated rat neonatal microglia in vitro.

Results: The manzamines differentially attenuated PMA (phorbol 12-myristate 13-acetate)-stimulated TXB₂ generation in the following order of decreasing potency: MZA (IC₅₀ <0.016 µM) > MZD (IC₅₀ = 0.23 µM) > MZB (IC₅₀ = 1.6 µM) > MZC (IC₅₀ = 2.98 µM) > MZE and F (IC₅₀ >10 µM). In contrast, there was less effect on OPZ (opsonized zymosan)-stimulated TXB₂ generation: MZB (IC₅₀ = 1.44 µM) > MZA (IC₅₀ = 3.16 µM) > MZC (IC₅₀ = 3.34 µM) > MZD, MZE and MZF (IC₅₀ >10 µM). Similarly, PMA-stimulated O₂⁻ generation was affected differentially as follows: MZB (IC₅₀ = 1.44 µM) > MZA (IC₅₀ = 3.16 µM) > MZC (IC₅₀ = 3.43 µM) > MZD, MZE and MZF (IC₅₀ >10 µM). In contrast, OPZ-stimulated O₂⁻ generation was minimally affected: MZB (IC₅₀ = 4.17 µM) > MZC (IC₅₀ = 9.3 µM) > MZA, MZD, MZE and MZF (IC₅₀ >10 µM). From the structure-activity relationship perspective, contributing factors to the observed differential bioactivity on TXB₂ and O₂⁻ generation are the solubility or ionic forms of MZA and D as well as changes such as saturation or oxidation of the β-carboline or 8-membered amine ring. In contrast,
Background

The hallmark of brain inflammation is the activation of microglia, particularly microglia, the resident immune cells of the brain [1]. Microglia activation in brain pathologies, as caused by infectious diseases, inflammation, trauma, brain tumors, ischemia and AIDS, may result in neuronal injury and ultimately neurodegeneration [1]. Similar to other tissue macrophages, when microglia become activated they release potentially neurotoxic mediators [2], followed by sublethal and lethal injury to the central nervous system. The two different phenotypic forms of microglia, namely the activated but nonphagocytic microglia found in inflammatory pathologies and the reactive or phagocytic microglia present in trauma, infection and neuronal degeneration, appear to have the capacity to express cell-surface receptors and release mediators of inflammation, such as cytokines, coagulation factors, complement factors, proteases, nitric oxide, eicosanoids and reactive oxygen species [2].

Over the last three decades, the marine environment has been demonstrated to be a source of novel therapeutic agents, many of which have anti-inflammatory properties [3]. We have previously shown that selected marine natural products modulate eicosanoids [4,5] and O2- generation from activated rat [6] and human neutrophils [7], as well as liver [8] and alveolar macrophages [9]. Based on these observations we hypothesized that selected marine natural products might potentially attenuate activated brain microglia [2]. Since the discovery by Sakai and Higa that the marine sponge-derived manzamine A (MZA) had potent antitumor activity [10], there has been a sustained interest in the chemistry [11] as well as the pharmacology of the manzamines, a class of β-carboline marine-derived alkaloids. More than 40 manzamine-type alkaloids have been isolated from 9 different genera of marine sponges and as such are of interest in the chemistry [11] as well as the pharmacology of the manzamines, a class of β-carboline marine-derived alkaloids. More than 40 manzamine-type alkaloids have been isolated from 9 different genera of marine sponges. In preliminary communications we have reported that MZA, isolated from the Okinawan marine sponge Haliclona sp., potently inhibited TXB2 and O2- generation by activated rat neonatal microglia while showing very low concomitant toxicity [18-20]. We now extend these previous communications by reporting the results of a structure-activity relationship study with manzamines A, B, C, D, E and F on agonist-stimulated release of O2- and TXB2 from LPS-activated rat neonatal microglia.

Results

Effect of manzamine A on LPS-activated neonatal brain microglia TXB2, O2- and LDH release

As shown in Fig. 1, MZA has a pentacyclic diamine group attached to the β-carboline moiety and it was tested as its hydrochloride salt. As is shown in Fig. 2A, MZA potently inhibited PMA-stimulated TXB2 generation (IC50 = 0.016 µM), with a maximum 95.5% inhibition observed at 10 µM (MZA vs. vehicle, respectively 78.3 ± 45 vs. 1,413 ± 439 pg of TXB2 per 200,000 microglia per 70 min, P < 0.01, n = 4). Furthermore, as depicted in Fig. 3A, MZA inhibited PMA-stimulated O2- generation with an apparent IC50 = 0.1 µM (MZA vs. vehicle, respectively 7.5 ± 1.8 vs. 13.8 ± 1.9 nmol of O2- per 200,000 microglia per 70 min, P < 0.01, n = 4). Significantly, increasing MZA concentrations to 10 µM resulted in O2- inhibition of 55.9 ± 6.1%, P < 0.01, n = 4.

In contrast, as shown in Fig. 2A, the effect of MZA on OPZ-stimulated TXB2 generation was weaker (apparent IC50 = 3.16 µM), with a maximum 58% inhibition at 10 µM (MZA vs. vehicle, respectively 1,927 ± 474 vs. 4,504 ± 308 pg of TXB2 per 200,000 microglia per 70 min, P < 0.05, n = 2). Similarly, as depicted in Fig. 3A, MZA did not appear to affect OPZ-stimulated O2- generation even at 10 µM (MZA vs. vehicle, respectively 9.5 ± 1 vs. 9.4 ± 0.7 O2- nmol per 200,000 microglia per 70 min, P > 0.05, n = 2).

As shown in Fig. 2A and 3A, the cytotoxicity of MZA to neonatal brain microglia measured as LDH release was not significantly different from controls even at 10 µM (MZA vs. vehicle, respectively 21.3 ± 7% vs. 13.9 ± 3.7% of total LDH released by 0.1% Triton X-100 treated-
The chemical structures of manzamines A, B, C, D, E and F. Manzamines are indole-derived alkaloids isolated from the marine sponges *Haliclona* sp. [10], *Amphimedon* sp. [66] and *Xestospongia* sp. [10,67]. Molecular weights are respectively = 585.2, 550.8, 347.5, 591.3, 564.7, 580.8.

Figure 1

The chemical structures of manzamines A, B, C, D, E and F.
**Figure 2**

Differential effects of manzamines A, B, C, D, E and F on PMA and OPZ-stimulated TXB2 generation by LPS-activated rat neonatal microglia. Rat neonatal microglia (200,000 cells/well) were activated with LPS (0.3 ng/mL) for 17 hours. Manzamines were added 15 min before stimulation with either PMA (1 µM) or OPZ (0.5 mg/mL). After 70 min, agonist-triggered TXB2 was measured as described in Materials and Methods. LDH release, indicator of cytotoxicity, was determined as described in Materials and Methods. Data are expressed as percentage of control TXB2 release triggered by either PMA (MZA: 1,423 ± 439 pg TXB2/70 min; MZB, C, D, E, F: 3,279 ± 281 pg TXB2/70 min), or OPZ (4,504 ± 308 pg TXB2/70 min). Data show mean ± SEM of indicated number (n) of experiments. * P < 0.05, ** P < 0.01.

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**Manzamine A**

- PMA (n=4) ▲ OPZ (n=2) □ LDH (n=6)

**Manzamine B**

- PMA (n=3) ▲ OPZ (n=2) □ LDH (n=5)

**Manzamine C**

- PMA (n=3) ▲ OPZ (n=2) □ LDH (n=5)

**Manzamine D**

- PMA (n=3) ▲ OPZ (n=2) □ LDH (n=5)

**Manzamine E**

- PMA (n=3) ▲ OPZ (n=2) □ LDH (n=5)

**Manzamine F**

- PMA (n=3) ▲ OPZ (n=2) □ LDH (n=5)
Figure 3
Differential effects of manzamines A, B, C, D, E and F on PMA and OPZ-stimulated $O_2^-$ generation by LPS-activated rat neonatal microglia. Rat neonatal microglia (200,000 cells/well) were activated with LPS (0.3 ng/mL) for 17 hours. Manzamines were added 15 min before stimulation with either PMA (1 µM) or OPZ (0.5 mg/mL). After 70 min, agonist-triggered $O_2^-$ release was determined as described in Materials and Methods. LDH release, indicator of cytotoxicity, was measured as described in Materials and Methods. Data are expressed as percentage of control $O_2^-$ release triggered by PMA (MZA: 13.8 ± 1.9 nmoles $O_2^-$ /70 min; MZB, C, D, E, F: 10.8 ± 0.6 nmoles $O_2^-$ /70 min), or OPZ (9.4 ± 0.7 nmoles $O_2^-$ /70 min). Data show mean ± SEM of indicated number (n) of experiments. * P < 0.05, ** P < 0.01.
Effect of manzamine B on LPS-activated neonatal brain microglia TXB₂, O₂⁻ and LDH release

MZB differs from MZA in having a tetracyclic diamine complex and an epoxide ring system (Fig. 1). As shown in Fig. 2B, MZB which was tested as a free base, was less potent than MZA in affecting PMA-stimulated TXB₂ generation (IC₅₀ = 1.44 µM). MZB 10 µM reduced TXB₂ release to 25.6% of control (MZB vs. vehicle, respectively 1,160 ± 207 vs. 4,504 ± 308 pg of TXB₂ per 200,000 microglia per 70 min, P < 0.01, n = 2). Furthermore, as shown in Fig. 3B, MZB was less potent than MZA in affecting PMA-stimulated O₂⁻ generation (IC₅₀ = 3.16 µM). MZB 10 µM reduced O₂⁻ release to 20% of control (MZB vs. vehicle, respectively 2.14 ± 2.14 vs. 10.8 ± 0.6 nmol of O₂⁻ per 200,000 microglia per 70 min, P < 0.01, n = 3). Similarly, as depicted in Fig. 3B, MZB was less potent than MZA in affecting PMA-stimulated O₂⁻ generation (IC₅₀ = 3.34 µM). MZB 10 µM reduced O₂⁻ generation to 16.5% of control (MZB vs. vehicle, respectively 546.3 ± 281 vs. 3,279 ± 281 pg of TXB₂ per 200,000 microglia per 70 min, P < 0.01, n = 3). As shown in Fig. 3B, MZB reduced O₂⁻ generation to 41.6% of control (MZC vs. vehicle, respectively 3.9 ± 0.3 vs. 9.4 ± 0.7 O₂⁻ nmol per 200,000 microglia per 70 min, P < 0.05, n = 2).

As shown in Fig. 2B and 3B, in contrast to MZA, MZB was cytotoxic to neonatal brain microglia though not as much as MZB. Substantial LDH release was observed at 10 µM (59.8 ± 11% of total LDH released by 0.1 % Triton X-100 treated-microglia, n = 5, P < 0.05). In summary, similar to MZB, the data suggests that the reduction of both O₂⁻ and TXB₂ generation resulted from both pharmacological and toxic effects of MZC on LPS-activated microglia cells.

Effect of manzamine D on LPS-activated neonatal brain microglia TXB₂, O₂⁻ and LDH release

MZD differs from MZA in having a tetrahydrocarboline group in the molecule (Fig. 1). As shown in Fig. 2D, MZD that was tested as its hydrochloride salt, strongly affected PMA-stimulated TXB₂ generation (IC₅₀ = 0.23 µM). MZD 10 µM reduced TXB₂ release to 18.8% of control (MZD vs. vehicle, respectively 611 ± 342 vs. 3,279 ± 281 pg of TXB₂ per 200,000 microglia per 70 min, P < 0.01, n = 3). Furthermore, as shown in Fig. 3D, MZD also strongly affected PMA-stimulated O₂⁻ generation. MZD 0.1 µM reduced O₂⁻ release to 14% of control (MZD vs. vehicle, respectively 0.7 ± 0.7 vs. 10.8 ± 0.6 nmol of O₂⁻ per 200,000 microglia per 70 min, P < 0.01, n = 3).

In contrast, as shown in Fig. 2D the effect of MZD on OPZ-stimulated TXB₂ generation was limited. MZD 10 µM reduced TXB₂ generation to 79.9% of control (MZD vs. vehicle, respectively 3,613 ± 469 vs. 4,504 ± 308 pg of TXB₂ per 200,000 microglia per 70 min, P > 0.05, n = 2). Furthermore, as shown in Fig. 3D, MZD did not affect OPZ-stimulated O₂⁻ generation even at 10 µM.

As shown in Fig. 2D and 3D, and in contrast to MZB and C, MZD was very cytotoxic to microglia when PMA was used as an agonist to trigger O₂⁻ and TXB₂ release: MZD at 0.1 µM caused 61.5 ± 13% of total LDH released by 0.1 % Triton X-100 treated-microglia (n = 5, P < 0.05). In contrast to the limited effect of MZD on OPZ-stimulated microglia, the data suggests that the reduction of PMA-
stimulated \( \text{O}_2^- \) and TXB\(_2\) generation resulted from both pharmacological and toxic effects of MZD on LPS-activated microglia cells.

**Effect of manzamine E on LPS-activated neonatal brain microglia TXB\(_2\), \text{O}_2^- and LDH release**

As shown in Fig. 1, MZE differs from MZA in having a saturated ketone functionality in the eight-membered amine ring. As depicted in Fig. 2E, MZE inhibited PMA-stimulated TXB\(_2\) generation with a maximum 49.4 \% inhibition observed at 10 \( \mu \)M (MZE vs. vehicle, respectively 1.614 \pm 628 vs. 3.279 \pm 281 pg of TXB\(_2\) per 200,000 microglia per 70 min, \( P > 0.05 \), \( n = 3 \)). Furthermore, as shown in Fig. 3E, MZE inhibited PMA-stimulated \text{O}_2^- generation with a maximum 26.3 \% inhibition observed at 10 \( \mu \)M (MZE vs. vehicle, respectively 8.5 \pm 2.1 vs. 10.8 \pm 0.6 nmol of \text{O}_2^- per 200,000 microglia per 70 min, \( P > 0.05 \), \( n = 3 \)).

As shown in Fig. 2E, MZE had limited effect on OPZ-stimulated TXB\(_2\) generation, with a maximum 43.1 \% inhibition observed at 10 \( \mu \)M (MZE vs. vehicle, respectively 20.1 \pm 3.6 vs. 12.0 \pm 1.1 nmole/30 min, \( n = 2 \), \( P < 0.05 \)). Similarly, as depicted in Fig. 3E, MZE did not affect OPZ-stimulated \text{O}_2^- generation even at 10 \( \mu \)M.

As shown in Fig 2E and 3E, cytotoxicity of MZE to microglia measured as LDH release was low even at 10 \( \mu \)M (MZE vs. vehicle, respectively 20 \pm 6.7 \% vs. 19.8 \pm 7.7 \% of total LDH released by 0.1 \% Triton X-100 treated-microglia, \( P > 0.05 \), \( n = 5 \)).

**Effect of manzamine F on LPS-activated neonatal brain microglia TXB\(_2\), \text{O}_2^- and LDH release**

MZF differs from MZA in having a saturated ketone functionality in the eight-membered amine ring and hydroxylation at the C-8 position of the \( \beta \)-carboline ring system (Fig. 1). As shown in Fig. 2F, MZF did not inhibit PMA-stimulated TXB\(_2\) generation. In the presence of 10 \( \mu \)M MZ, TXB\(_2\) release was 104.1 \pm 24.7 \% of control TXB\(_2\) generation (MZF vs. vehicle, respectively 3.202 \pm 1.139 vs. 3.279 \pm 281 pg of TXB\(_2\) per 200,000 microglia per 70 min, \( P > 0.05 \), \( n = 3 \)). Similarly, as shown in Fig. 3F, MZF did not inhibit PMA-stimulated \text{O}_2^- release. In the presence of 10 \( \mu \)M MZF, \text{O}_2^- release was 113 \pm 14.5 \% of control \text{O}_2^- generation (MZF vs. vehicle, respectively 12.8 \pm 1.6 vs. 10.8 \pm 0.6 nmol of \text{O}_2^- per 200,000 microglia per 70 min, \( P > 0.05 \), \( n = 3 \)).

As shown in Fig. 2E, MZF effect on OPZ-stimulated TXB\(_2\) generation was weak, with a non-statistically significant 26.2 \% inhibition at 10 \( \mu \)M (MZF vs. vehicle, respectively 3.317 \pm 121 vs. 4.504 \pm 308 pg of TXB\(_2\) per 200,000 microglia per 70 min, \( P > 0.05 \), \( n = 2 \)). Similarly, as depicted in Fig. 3F, MZF was minimally effective in inhibiting OPZ-stimulated \text{O}_2^- generation, only a non-statistically significant 15.7 \% inhibition observed at 10 \( \mu \)M.

As shown in Fig. 2F and 3F, cytotoxicity of MZF to neonatal brain microglia measured as LDH release was low even at 10 \( \mu \)M (MZF vs. vehicle, respectively 19 \pm 7.2 \% vs. 19.8 \pm 7.7 \% of total LDH released by 0.1 \% Triton X-100 treated-microglia, \( n = 5 \)).

**Effect of manzamine A, B, C, D, E and F on hypoxanthine-xanthine oxidase generated \text{O}_2^-**

In order to determine a potential scavenging effect of MZA, B, C, D, E and F on \text{O}_2^-\, a standard hypoxanthine-xanthine oxidase system was used as a cell-free source of \text{O}_2^- [21]. As shown in Fig. 4, \text{O}_2^- generation by incubation of purified xanthine oxidase with hypoxanthine was abolished by superoxide dismutase. Furthermore, DMSO, the vehicle used to prepare the manzamines, did not affect \text{O}_2^- formation (control vs. DMSO, respectively 12.3 \pm 2.3 vs. 12.2 \pm 1.1 nmole/30 min, \( n = 2 \), \( P > 0.05 \)). Similarly, MZA, B, and E did not significantly affect \text{O}_2^- generation (MZA, B, E vs. control, respectively, 15.3 \pm 4.4, 15.3 \pm 2.4, 16.3 \pm 2.2 vs. 12 \pm 1.1 nmole/30 min, \( n = 2 \), \( P > 0.05 \)). In contrast, MZC, D and F appeared to enhance \text{O}_2^- formation (MZC, D, F vs. control, respectively 20.7 \pm 3.1, 22.2 \pm 2, 20.1 \pm 3.6 vs. 12 \pm 1.1 nmole/30 min, \( n = 2 \), \( P < 0.05 \) (MZC, F), \( P < 0.01 \) MZD). Thus we conclude that the inhibition of either PMA or OZ stimulated-\text{O}_2^- release from LPS-activated microglia by the manzamines was not the result of a direct \text{O}_2^- scavenging effect.

**Discussion**

The important role of neuroinflammation and glial activation in the pathogenesis of brain disorders has progressively been established [1,2,22]. Because *in vitro* LPS-activated microglia appear to mimic the functions of activated microglia found in neuroinflammatory conditions *in vivo* [23], we used LPS-activated rat microglia as a relevant *in vitro* paradigm to search for marine natural products that may modulate the enhanced release of TXB\(_2\) and \text{O}_2^- from activated microglia [24]. Using this *in vitro* model we have previously communicated that MZA, a secondary metabolite isolated from the Okinawan marine sponge *Haliclona* sp[10], inhibited TXB\(_2\) and \text{O}_2^- generation by microglia [18]. The current study extends our initial observations, and reports a structure-activity relationship study with manzamines A, B, C, D, E and F on both PMA and OPZ-stimulated release of TXB\(_2\) and \text{O}_2^- from LPS-activated rat neonatal microglia.

Members of the eicosanoid family (i.e. prostaglandins, leukotrienes and thromboxanes) are important mediators of inflammation that would appear to be play a causative role in the pathogenesis of several CNS disorders [25-27]. Increased levels of eicosanoids have been observed in...
manzamine analogs inhibited OPZ-stimulated TXB₂ generation with reduced potency: MZB>MZA>MZC>MZD, MZE and MZF. Thus, with the exception of MZB and MZC which modulated both PMA and OPZ-stimulated TXB₂ release with similar potency, MZA, MZD, MZE and MZF inhibited TXB₂ release with lower potency when OPZ was used as an agonist.

It is interesting to compare our differential results with the MZA, B, C, D, E, and F with those reported for other agents that have been shown to modulate microglia eicosanoid release by targeting the cyclooxygenase I and II enzymes which are expressed in activated rat and human microglia [35,44]. PGE₂ and TXB₂ synthesis that occurs concomitantly with LPS-induced activation of rat [38,45,46] and human microglia [35], has been shown to be attenuated by nonsteroidal anti-inflammatory drugs (NSAIDs) with differing activities towards the two isoforms of COX. Thus, LPS-induced microglia PGE₂ synthesis was reduced by COX-1 inhibitors: acetylsalicylic acid (aspirin) (IC₅₀ = 3.12–10 μM) [38,46], flurbiprofen (apparent IC₅₀ = 100 nM) [45] and indomethacin (apparent IC₅₀ = 1 nM) [38,], and the COX-2 inhibitor NS-398 (apparent IC₅₀ = 1–5 nM) [38]. Even though NSAIDs have been reported to attenuate neurotoxicity in vitro [47] and neuroinflammation in animal models [48-50], an important caveat is the fact that determining the best NSAIDs for clinical neurodegenerative disease management appears to remain a matter of considerable debate in view of their well known adverse effects [51-53]. Thus, although the molecular mechanism by which the manzamines inhibit TXB₂ release in LPS-activated cells remains currently undetermined, MZA inhibited PMA-stimulated eicosanoid generation in vitro with potency similar to that of the COX-1 inhibitor indomethacin [38], potency that was higher than that of other NSAIDs that have been reported to modulate enhanced eicosanoid release in both activated rat and human microglia [35,38,45,46].

The involvement of reactive oxygen species (ROS) has been documented in CNS pathologies, such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Down's syndrome, cerebral ischemia and reperfusion, amyotrophic lateral sclerosis, multiple sclerosis and meningitis [54]. Prolonged exposure to ROS may potentially damage neurons, particularly their synapses [55] as well as oligodendrocytes, the myelin producing cell of the CNS [56] by overriding normal CNS antioxidant defense mechanisms, e.g. superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, permanently affecting cellular function [57]. Thus the mechanism of ROS generation by CNS leukocytes, i.e. infiltrating neutrophils and monocytes as well as resident microglia production of O₂⁻, hydrogen peroxide and nitric oxide in CNS, has received considerable attention since the mid-
In the 1980s [2]. In fact, during the past 18 years, numerous research groups have shown that O$_2^-$ may be generated by microglia isolated from rat, mice, hamsters, dogs, swine and humans, when stimulated with a variety of agonists such as phorbol ester, opsonized zymosan, calcium ionophore, antiviral antibodies, antibody-coated red blood cells and myelin (reviewed in [2]). We and others have hypothesized that rather than scavenge ROS with antioxidants, the modulation of the signal transduction mechanism leading to microglia ROS generation might be putatively a better therapeutic strategy to turn off or reduce ROS generation that could lead to neuronal injury [2,58,59]. As depicted in Fig. 3, the manzamine analogs attenuated PMA-stimulated O$_2^-$ generation in the following order of decreasing potency: MZD > MZA > MZB > MZC > MZE and MZF. In contrast, and similarly to their weaker effect on OPZ-stimulated TXB$_2$ generation, all the manzamine analogs modulated OPZ-stimulated O$_2^-$ generation with lower potency: MZB > MZC > MZA, MZD, MZE and MZF. Interestingly, as shown in Fig. 4, the effect of MZD, A, B and C on PMA-stimulated O$_2^-$ formation was not the result of any detectable scavenging O$_2^-$, because none of the manzamines inhibited a standard hypoxanthine-xanthine oxidase system that was used as a cell-free source of O$_2^-$, Thus, although the exact mechanism by which the manzamines modulate O$_2^-$ release by microglia remains currently undetermined, we have demonstrated that these compounds clearly modulate the signal transduction pathway that PMA triggers in microglia and ultimately leads to O$_2^-$ generation.

It is of interest to consider the results of our structure-activity relationship (SAR) study with the manzamines, alkaloids characterized by a complex heterocyclic ring system attached to the C-1 of the β-carboline moiety. From the SAR perspective, the potent effect of MZA and D hydrochloride salts on PMA-stimulated O$_2^-$ and TXB$_2$ suggest that the solubility or ionic forms are contributing factors to their bioactivity. Furthermore, the fused 13-membered macrocyclic and octahydroisoquinoline ring system, and any substitutions in these rings would appear to be less important for their in vitro activity. Finally, changes such as saturation or oxidation of the β-carboline or the 8-membered amine ring tended to decrease bioactivity in both O$_2^-$ and TXB$_2$ assays.

Taken together, our current data demonstrates that the most potent and least toxic manzamine analog, namely MZA, was less effective in attenuating O$_2^-$ and TXB$_2$ from LPS-activated microglia when the triggering agonist was OPZ rather than PMA. Similar differential effects between PMA and OPZ-triggered signaling have been observed with other natural products [62]. Furthermore, the current data suggest the following on the as yet undefined mechanism of action of MZA: Firstly, that the MZA molecular target plays a critical role in O$_2^-$ and TXB$_2$ generation initiated by PMA upon binding to PKC [63,64] and activation of the p44/42 mitogen-activated protein kinase signaling pathway [43]; Secondly, that the MZA molecular target probably plays a less critical role in O$_2^-$ and TXB$_2$ release elicited by OPZ, a ligand of the microglial cell surface complement receptor 3 shown to activate the p38 mitogen-activated protein kinase signaling pathway [43]. Studies to determine which element is targeted by MZA in the p38 and/or p44/42 mitogen-activated protein kinase pathways in LPS-activated rat microglia are currently underway in our laboratory.
Conclusion

Our present results provide the first experimental evidence to support the hypothesis that the marine-derived β-carboline alkaloid manzamines differentially modulate both O2·− and TXB2 generated by E. coli LPS-activated rat neonatal microglia. Additional conclusions are the following: Firstly, SAR studies demonstrated that at in vitro concentrations that were non-toxic to E. coli LPS-activated rat neonatal microglia, MZA was the most potent inhibitor of O2·− and TXB2. Secondly, although the mechanism by which MZA inhibited PMA-stimulated TXB2 generation in vitro is as yet unclear, its potency was similar to the COX-1 inhibitor indomethacin [38], and thus higher than other NSAIDs reported to modulate enhanced eicosanoid release in both activated rat and human microglia [35,38,45,46]. Thirdly, although the mechanism by which MZA inhibited PMA-stimulated O2·− generation in vitro remains undetermined, MZA was more potent than propentofylline, a selective phosphodiesterase inhibitor, cabergoline, a potent and selective agonist of D2-dopamine receptors and nicergoline, an ergoline derivative used for cerebrovascular diseases, compounds which have been proposed to confer protective effects against neurodegenerative diseases by affecting O2·− release by activated rat microglia. Fourthly, SAR studies which demonstrated that the ionic forms are a contributing factor to the bioactivity of the complex manzamine heterocyclic ring system attached to a β-carboline moiety may explain the potent effect of MZA and D hydrochloride salts on PMA-stimulated O2·− and TXB2. Interestingly, the fused 13-membered macrocyclic amine and octahydroisoquinoline ring system, as well as substitutions in these rings appeared to be a non-factor for the in vitro activity of the manzamines. Finally, the reported pharmacokinetic properties and the lack of significant in vivo toxicity [14] of MZA, a β-carboline alkaloid whose complete synthesis has been reported [11], would suggest that MZA is a prime candidate for further investigation of its potential utility as a pharmacophore from which new and novel therapeutic agents for neuroinflammatory diseases might be developed.

Methods

Reagents

LPS B (Escherichia coli 026:B6) was obtained from Dičko Laboratories (Detroit, MI); Wright Giemsa stain (modified), ferricytochrome c type III (from horse heart) (FCC), superoxide dismutase (from bovine liver), phosphor 12-myristate 13-acetate (PMA), zymosan and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). PMA was maintained at -80 °C as a 10 mM stock solution in DMSO. Opsonized zymosan (OPZ) was maintained at -20 °C in a stock solution of 15 mg/ml in PBS and prepared as described [65]. Dulbecco’s modified Eagle medium (DMEM) with high glucose (4,500 mg/l), Hank’s balanced salt solution (HBSS), penicillin (P), streptomycin (S), trypsin (0.25%)-EDTA (1 mM) and trypan blue were purchased from Gibco BRL (Grand Island, NY); certified heat-inactivated fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT); a LPS stock of 1 mg/ml was prepared in a 0.9% sodium chloride nonpyrogenic solution from Baxter Healthcare Corp. (Toronto, ONT, Canada) and then diluted with DMEM plus 10% FBS plus P and S to the appropriate concentration used in our experiments. Both the LPS stock solution [10 ng/ml] and dilutions were stored at -80 °C, thawed prior to each experiment and discarded after use.

LPS containment

To inactivate extraneous LPS, all glassware and metal spatulas were baked for 4 hours at 180 °C. Sterile and LPS-free 75- and 162-cm² vented cell culture flasks, 24-well flat-bottom culture clusters, 96-well cell culture clusters and disposable serological pipettes were purchased from Costar Corporation (Cambridge, MA), while polystyrene cell culture dishes (60 × 15 mm) were obtained from Corning Glass Works (Corning, NY). Sterile and LPS-free Eppendorf Biopur pipette tips were purchased from Brinkmann Instruments, Inc. (Westbury, NY).

Manzamines A, B, C, D, E and F

Manzamine A (MZA) was isolated from a marine sponge species of the genus Haliclona collected off Manzamo, Okinawa in waters at a depth of 30 m in April 1985 [10]. Manzamine B (MZB), manzamine C (MZC) and manzamine D (MZD), were isolated from a sponge of the genus Amphimedon collected by SCUBA off Manzamo, Okinawa [66]. Manzamine E (MZE) and manzamine F (MZF) were isolated from a sponge species of the genus Xestospongia collected off the coast of Miyako Island, Okinawa in June 1986 [67]. All manzamines were dissolved in DMSO to prepare a 10 mM stock and stored at 4°C prior to use in the experiments.

Isolation and culture of rat neonatal microglia

All experiments were performed with adherence to the National Institutes of Health guidelines on the use of experimental animals and with protocols approved by Midwestern University’s Research and Animal Care Committee. To isolate rat neonatal microglia, cerebral cortices of 1–2 day-old Sprague-Dawley rats purchased from Harlan (Indianapolis, IN) were surgically removed and placed in cold DMEM + 10% FBS + 120 U/ml P and 12 µg/ml S, the meninges carefully removed, and brain tissue minced and dissociated with trypsin-EDTA at 36 °C for 3–5 min. The mixed astroglial cell suspension was plated in either 75- or 162-cm² vented cell culture flasks with DMEM medium supplemented with 10% FBS + 120 U/ml P + 12 µg/ml S and grown in a humidified 5% CO2 incubator at 36 °C for 12–14 days. On day 14 and every 3–4 days thereafter, microglia were detached using an orbital shaker
(150 rpm, 0.5 hours, 36°C, 5% CO₂), centrifuged (400 × g, 25 min, 4°C), and microglia number and viability assessed by trypan blue exclusion. Microglia were characterized as described earlier [23]. Depending on the particular experimental protocol (see below), microglia averaging greater than 95% viability were plated in 24-well cell culture clusters, with DMEM supplemented with 10% FBS + 120 U/ml P + 12 µg/ml S, and placed in a humidified 5% CO₂ incubator at 36°C 18–24 hours prior to the experiments.

**Experimental protocol to study the effect of manzamines A – F on microglia release of TXB₂ and O₂⁻**

To study the effects of manzamines A, B, C, D, E and F on the generation of TXB₂ and O₂⁻, rat neonatal microglia (2 × 10⁵ cells/24-well cell culture clusters) were treated to the following protocol. Seventeen hours prior to the experiments, microglia cells were treated with LPS (0.3 ng/ml) in a final volume of 1 ml of DMEM supplemented with 10% FBS + 120 U/ml P + 12 µg/ml S. Thereafter, the media was removed and replaced with 1 ml warm HBSS, one of the manzamines (0.1–10 µM final concentration) or vehicle (DMSO) was added, and the microglia incubated for fifteen minutes in a humidified 5% CO₂ incubator at 35.9°C. After the fifteen minute preincubation period with either manzamines or vehicle, PMA (1 µM) or OPZ (0.5 mg/mL) was added and microglia incubated for 70 minutes in a humidified 5% CO₂ incubator at 35.9°C in the presence of the manzamines or vehicle. The final concentration of DMSO did not affect microglia viability or LDH release. O₂⁻, TXB₂ and lactate dehydrogenase (LDH) release were assayed as described below.

**Assay for TXB₂**

Following the incubation of LPS-activated microglia with HBSS, manzamines or vehicle as explained above, PMA (1 µM) or OPZ (0.5 mg/mL) triggered TXB₂ generation in the culture supernatants was measured using immunoassays (Cayman Chemical, Ann Arbor, MI) as indicated by the manufacturer’s protocol. Results were expressed as pg/ml produced after 70 min of PMA or OPZ stimulation.

**Assay for O₂⁻**

O₂⁻ generation was determined by the SOD-inhibitable reduction of FCC [23]. Briefly, PMA (1 µM) or OPZ (0.5 mg/ml)–triggered O₂⁻ release from LPS-activated microglia was measured in the presence of FCC (50 µM) and HBSS, with or without SOD (700 Units) which inhibited >95% of FCC reduction, during the 70 min incubation described above. All experimental treatments were run in triplicate and in a final volume of 1 ml. Changes in FCC absorbance were measured at 550 nm using a Beckman DU-650 spectrophotometer. Differences in the amount of reduced FCC in the presence and absence of SOD were used to determine O₂⁻ generation and expressed in nmol by employing the molecular extinction coefficient of 21.0 × 10⁷ M⁻¹ cm⁻¹.

**Experimental protocol to study the effect of manzamines on superoxide anion by the hypoxanthine-xanthine oxidase system**

A standard hypoxanthine-xanthine oxidase system was used as a cell-free source of O₂⁻. O₂⁻ was generated by incubation of purified xanthine oxidase (0.02 Units/ml) with hypoxanthine (1.5 mM) at 37°C [21]. O₂⁻ formation was assessed spectrophotometrically as the increase in absorbance at 550 nm associated with the SOD (30 U/ml)-inhibitable reduction FCC (50 µM) as described above for rat microglia O₂⁻ generation and expressed in nmol/30 minutes.

**Lactate dehydrogenase assay**

Lactate dehydrogenase (LDH) release from microglia was determined spectrophotometrically as described elsewhere [9]. Microglia LDH release was expressed as a percentage of total LDH. Total LDH resulted from 0.1% Triton X-100-lysed microglia cells (intracellular LDH) plus LDH released to the extracellular medium.

**Statistical analysis of the data**

Data were analyzed with the Prism® software package purchased from GraphPad (San Diego, CA.). One-way analysis of variance followed by Dunnett’s test was performed on all sets of data. Manzamine-treated groups were compared with the vehicle-treated group, shown as 0 or control in the corresponding figure. Differences were considered statistically significant at p < 0.05 and reported in each figure legend.

**Abbreviations**

CNS, central nervous system; DMEM, Dulbecco’s modified Eagle medium; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum certified; FCC, ferricytochrome c type III; HBSS, Hank’s balanced salt solution; LPS, lipopolysaccharide; MZA, manzamine A; MZB, manzamine B; MZC, manzamine C; MZD, manzamine D; MZE, manzamine E; MZF, manzamine F; O₂⁻, superoxide anion; OPZ, opsonized zymosan; P, penicillin; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; S, streptomycin; SOD, superoxide dismutase; TXB₂, thromboxane B₂.

**Authors’ contributions**

A.M.S. M. designed and conducted the experiments described and prepared the manuscript draft.

M.L.H., performed the statistical analysis of the data and prepared Fig. 2, 3 and 4.
S. M. L. helped conduct the xanthine oxidase studies with Manzamines A, B, C, D, E and F.

S.P.G. supplied pure Manzamines A, B, C, D, E and F from the Division of Biomedical Marine Research Depository, prepared Fig. 1, and contributed to various sections of the manuscript.

S.A.P. provided financial support and contributed to various sections of the manuscript.

S.H.S. contributed to various sections of the manuscript.

All authors read and approved the manuscript.

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