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Efficacy of GM6001 as an adjuvant to ceftriaxone in a neonatal rat model of *Streptococcus pneumoniae* meningitis

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Evidence has demonstrated that matrix metalloproteinases (MMPs) contribute to the pathophysiology of bacterial meningitis; therefore, MMP inhibitors may be a neuroprotective treatment for brain injury caused by meningitis because of their anti-inflammatory effects. The objective of this study was to evaluate the effect of the MMP inhibitor GM6001 in a rat model of *S. pneumoniae* meningitis. For these experiments, 7-day-old Sprague–Dawley rats were randomly divided into an uninfected group, meningitis group, antibiotic group and GM6001+antibiotic group. Uninfected animals were sham infected with sterile saline. Rats in the other three groups were inoculated with *S. pneumoniae* and left untreated, treated with ceftriaxone, or treated with ceftriaxone combined with GM6001. Rats in the meningitis group were severely ill, and MMP-9 was significantly up-regulated. The change in brain water content was consistent with the MMP-9 level. A significant loss of neurons and impaired learning function were observed in the meningitis group. Treatment with the antibiotic and GM6001 significantly down-regulated the level of MMP-9, decreased the brain water content, attenuated neuronal injury and improved learning. Conclusions: GM6001 protected the brain from damage caused by *S. pneumoniae*, and this effect may occur *via* down-regulating MMP-9 and decreasing brain water content.

Key words: bacterial meningitis, cytokines, MMP inhibitors, brain injury

INTRODUCTION

Streptococcus pneumoniae is one of the main causative agents of bacterial meningitis in humans (Meli et al. 2002, Scheld et al. 2002), and *S. pneumoniae* meningitis remains a significant clinical problem despite recent advances in therapy. This is because of the occurrence of antibiotic-resistant *S. pneumoniae* strains and its ubiquitous nature. *S. pneumoniae* meningitis is associated with a high degree of mortality (Bashir et al. 2003), and patients who survive *S. pneumoniae*-associated meningitis often exhibit serious neurological sequelae (Østergaard 2007).

After bacteria have entered the central nervous system, the presence of multiplying bacteria within the subarachnoid and ventricular space compartments triggers an intense inflammatory host response to kill the invading microorganisms. We and others have previously confirmed that proinflammatory mediators released during *S. pneumoniae* meningitis include tumor necrosis factor alpha (TNF-α) and matrix metalloproteinases (MMPs) (Leib et al. 2001, Meli et al. 2004, Liu et al. 2008), a family of zinc-dependent endopeptidases with substrate affinity for different components of the extracellular matrix that function as sustainers of the inflammatory host response by activating cytokines and cleaving cytokine receptors. Therefore, manipulation of MMPs is essential to improve disease outcome. Reports indicate that the MMP synthetic inhibitor GM6001 suppresses autoimmune encephalomyelitis and prevents brain edema after intracerebral hemorrhage (Gijbels et al. 1994, Wang et al. 1996). In the present study, we evaluated the effect of combination therapy of ceftriaxone and the MMP inhibitor GM6001 in a rat model of *S. pneumoniae* bacterial meningitis.

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METHODS

Animals and experimental design

The study was formally approved by the Institutional Guidelines of the Shandong University for the Care and Use of Laboratory Animal and was conducted in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals by the APA Board of Scientific Affairs Committee on Animal Research and Ethics. A total of 96 7-day-old Sprague–Dawley rats were obtained from the Medical Experimental Animal Center of Shandong University.

For the short-term study, the rats were randomly divided into an uninfected group meningitis group antibiotic group and GM6001+antibiotic group (12 rats in each group). GM6001 (Calbiochem, Germany) was dissolved in dimethyl sulfoxide (DMSO). Uninfected animals were sham infected with 1 µl sterile saline. Rats in the meningitis group were inoculated with *S. pneumoniae* (1 µl saline containing 1×10^6 bacteria). The antibiotic group was inoculated with *S. pneumoniae* (1 µl saline containing 1×10^6 bacteria) and treated with the vehicle DMSO and ceftriaxone (100 mg kg^{-1} subcutaneously; Roche Pharma) once daily for 2 days beginning 24 h after inoculation. Rats in the GM6001 group were inoculated with *S. pneumoniae* (1 µl saline containing 1×10^6 bacteria) and treated with the vehicle DMSO, ceftriaxone (100 mg kg^{-1} subcutaneously) and GM6001 (GM6001 65 mg kg^{-1} intraperitoneally, twice daily) as previously described (Leib et al. 2000) beginning 24 h after inoculation for 2 days. On the third day post-infection, all rats were assessed clinically using the following scores (Leib et al. 2001): (1) coma; (2) not turning upright; (3) turning upright within 30 s; (4) minimal ambulatory activity, turning upright in <5 s; and (5) normal. Cerebrospinal fluid (CSF) was obtained by puncturing the cisterna magna for analysis of MMP-9 by SDS-PAGE. Subsequently, the animals were killed and all brain tissues were removed. The brain tissues were prepared for microscopy or assessment of brain water content.

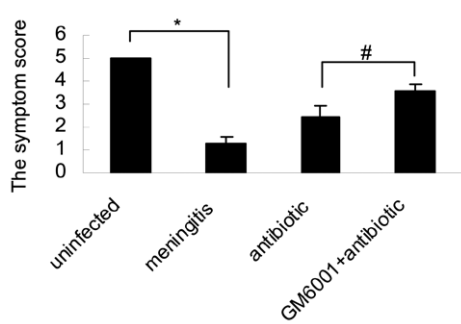

Fig. 1. The symptom score of rats 72 h after infection. The rats in the meningitis group showed lower symptom scores compared to the uninfected group. Treatment with antibiotic only caused a moderate improvement of the symptom score. Treatment with antibiotic and GM6001 significantly increased the symptom score. $^{\#}P<0.05$ *versus* the antibiotic group. $^{*}P<0.05$ *versus* the uninfected group.

To further evaluate adjuvant therapy with GM6001, we investigated the long-term effect of GM6001 on learning capacity using the water maze protocol. Similarly, we randomly divided the animals into the following 4 groups: uninfected meningitis antibiotic and GM6001 (12 rats in each group). The rats in the antibiotic group were treated with the vehicle DMSO and ceftriaxone for 14 days, and the GM6001 group was treated with the vehicle DMSO, ceftriaxone and GM6001 for 14 days. At the end of the third week after infection, the rats were evaluated using the Morris water maze to assess the long-term effects of GM6001 on spatial learning.

Infectious organism

In these studies, we utilized the serotype 3 *S. pneumoniae* strain (provided by the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), one of the most common types that causes neonatal meningitis. This organism was grown on tryptic soy agar with 5% defibrinated sheep blood and cultured overnight in brain heart infusion broth at 37°C with 5% CO_2 as previously described (Liu et al. 2008). The culture broth was centrifuged, pelleted, resuspended in sterile saline to the desired density containing 1×10^6 bacteria/µl and then used for intracisternal injection. The inoculum was routinely checked for purity and density by quantitative cultures.

Model of meningitis

In brief, infection was induced by direct intracisternal injection of 1 µl saline containing 1×10^6 *S. pneumoniae via* a 32-gauge needle as previously described (Liu et al. 2008). Uninfected animals were injected with 1 µl sterile saline. To confirm the development of bacterial meningitis, 24 h after inoculation, CSF was obtained by puncture of the cisterna magna and was

cultured to determine the extent of meningitis. Animals showing signs of coma were killed immediately.

Gelatinase level detection

The gelatinase level in the CSF was measured by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis zymography) as described previously (Liu et al. 2008). Samples of CSF (3 μl) were incubated with sample buffer (0.4 M Tris–HCl, pH 6.8, 5% sodium dodecyl sulfate (SDS), 20% glycerol, 1% bromophenol blue). A total volume of 10 μl was loaded into the well of a precast gel, and the electrophoresis was performed at 20 Ma constant current for 2 h under non-reducing conditions in 10% polyacrylamide-SDS gels containing 1 mg/ml gelatin as the proteinase substrate. The gel was placed in 100 ml 2.5% Triton X-100 for 1 h at room temperature to remove the SDS and then incubated in enzyme buffer containing $CaCl_2$–Tris–NaCl for 18 h at 37°C to allow proteolysis of the gelatin substrate. The gel was then fixed and stained with Coomassie blue. The bands were quantified by densitometric analysis using the BandScan software.

Histopathology

The animals were anesthetized with 0.15 to 0.3 ml/100 g 10% chloral hydrate and perfused through the heart with 50 ml phosphate buffered saline followed by 200 ml ice-cold 4% paraformaldehyde in the same fixative for 4 h at 4°C. The brains were then placed in 30% sucrose in phosphate buffered saline 3 days post-infection. The brains were embedded in paraffin and sectioned serially (5–7 μm). The cell number of the hippocampus and cortex was assessed for the same cortical/hippocampus locations in animals from each experimental group using an Olympus BH-2 microscopy system and the Image-pro Plus software.

Quantification of brain water content

Infected and non-infected rats were killed. After removal of the brains, the cerebellum was separated and the hemispheres were cut along the interhemispheric plane. Both hemispheres were weighed to obtain their wet weight. The hemispheres were dried for 24 h at 110°C to determine the dry weight. Based on the gravimetrical differences, the brain water content was obtained using the following equation (Thal et al. 2009): hemispheric water content (%) = (WW−DW)/WW×100, where WW is the wet weight (g) and DW is the dry weight (g) of the brain hemispheres.

Morris water maze

At the end of the third week after infection, all rats used to determine the long-term effect of GM6001 were assessed for spatial memory performance with the Morris water maze (MWM) test (Vorhees et al. 2006). The swimming pool used for the test was 150 cm in diameter and 70 cm deep. The pool was filled with water (24±2°C) to a height of 60 cm. The water was darkened with white floating resin beads covering the surface. The pool was divided into four quadrants, and the escape platform was fixed in a permanent position 1.5 cm under the water surface. The test was performed in two phases, the training trial and probe trial, as described below. The training trial was performed using a 4-trial-per-day regime for 5 consecutive days. The rats were placed in the water facing the inner wall of the tank at one of four different starting points and

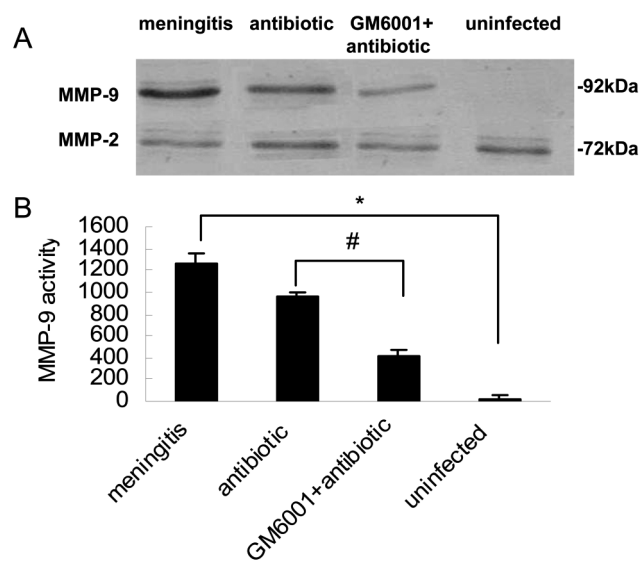

Fig. 2. (A) Detection of MMP-9 level by gelatin zymography. (B) The bands were quantified by densitometry, and the solid columns represent the relative MMP-9 level. The MMP-9 level was significantly increased in the rats infected with *S. pneumoniae* compared to the uninfected rats. The up-regulation of MMP-9 of rats in the antibiotic group was moderately attenuated. Treatment with antibiotic and GM6001 caused a significant reduction of the MMP-9 level. $^{\#}P<0.05$ *versus* the antibiotic group. $^{*}P<0.05$ *versus* the uninfected group.

the escape latency was recorded in each trial. To determine the memory retention, a probe trial was performed 1 day after the training trial was completed. The rats were allowed to swim freely for 120 s without the platform present. The ratio of the amount of time spent in the previous target quadrant to that spent in the other three quadrants was measured.

Statistical analysis

SPSS 13.0 was used for all statistical analyses. All results are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with least significant differences post hoc tests was performed to compare multiple groups for gelatinase level, brain water content and probe trial data. The water maze escape latency data were analyzed using ANOVA for repeated measures, with the test day as the within-subject factor. The cell counts and clinical scores were evaluated with the nonparametric Kruskal-Wallis test followed by the Bonferroni-corrected Mann-Whitney *U* test. A *P*-value <0.05 was considered statistically significant.

RESULTS

Clinical parameters

The efficacy of GM6001 was assessed using the symptom score of the animals. The rats in the meningitis group (n=10) were severely ill and showed lower symptom scores, whereas in the uninfected group (n=12) the rats were all healthy (1.3±0.23 for meningitis *versus* 5 for uninfected, P<0.05). The symptom score was moderately increased with the antibiotic (2.4±0.52). However, treatment with the antibiotic together with GM6001 (n=12) showed a significant improvement of the symptom score (3.6±0.52, P<0.05) (Fig. 1).

MMP-9 level in CSF

The gelatinase level in the CSF was investigated by gelatin zymography. One-way ANOVA revealed differences among the four groups for the MMP-9 level ($F_{3,\ 42}$=12.76, P<0.01). *Post-hoc* analysis indicated a significant increase in MMP-9 in the CSF at 72 h post-infection with *S. pneumoniae* compared to the uninfected group (P<0.01). The up-regulation of MMP-9 in the CSF of rats in the antibiotic group was moderately attenuated compared to the meningitis group. However, treatment with antibiotic and GM6001 showed a significant reduction of MMP-9 in the CSF compared to the antibiotic only group (P<0.05) (Fig. 2).

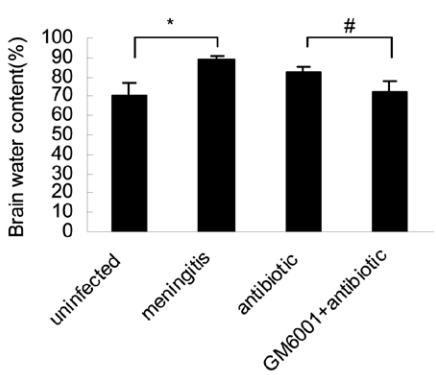

Fig. 3. Brain water content in rats on the third day post-infection as a percentage of water in the brain. The brain water content was significantly increased in the meningitis group compared to the uninfected group. Treatment with antibiotic only slightly decreased the brain water content. Combination treatment with antibiotic and GM6001 significantly decreased the brain water content. #P<0.05 *versus* the antibiotic group. *P<0.05 *versus* the uninfected group.

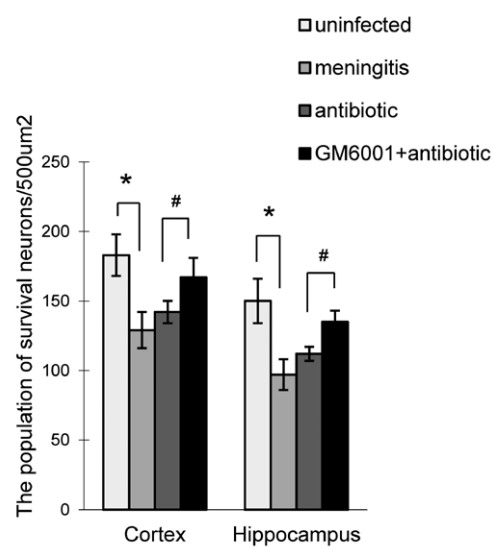

Fig. 4. The population of surviving neurons in rat cerebral cortex and hippocampus. The cortical and hippocampal neurons in the meningitis group were significantly decreased compared to the uninfected group. Treatment with antibiotic moderately attenuated the loss of cortical and hippocampal neurons. Treatment with antibiotic and GM6001 significantly decreased the reduction in cortical and hippocampal neurons. #P<0.05 *versus* the antibiotic group. *P<0.05 *versus* the uninfected group.

Brain water content

The change in brain water content was consistent with the MMP-9 level. One-way ANOVA revealed differences among the four groups for the brain water content ($F_{3, 42}$=18.12, P<0.01). *Post-hoc* analysis indicated that inoculation with *S. pneumoniae* elicited significantly increases of brain water content compared to the uninfected group (P<0.05). This *S. pneumoniae*-induced brain water content was slightly reduced in the antibiotic group. However, there was a marked reduction of brain water content in the antibiotic and GM6001 group. The brain water content was significantly decreased in rats treated with antibiotic plus GM6001 *versus* antibiotics only (Fig. 3) (P<0.05).

Histology and morphometry

The meninges of the infected rats showed that the subarachnoid space was enlarged and filled with granulocytes, and the subarachnoid vessels exhibited marked dilation. The arrangement of cortical and hippocampal neurons in the meningitis group was disordered, and there was a significant loss of hippocampal and cortical neurons in the meningitis group. The population of surviving neurons in the hippocampus (97±11) and cortex (129±13) in the meningitis group decreased compared to the uninfected group (150±16 for hippocampus, 183±15 for cortex). For animals treated with antibiotics only, the cortical (142±8) and hippocampal (112±5) injury was slightly reduced. However, there was a significant reduction in the loss of cortical (167±14) and hippocampal (135±8) neurons for the rats receiving adjuvant therapy with GM6001 (P<0.05) (Fig. 4).

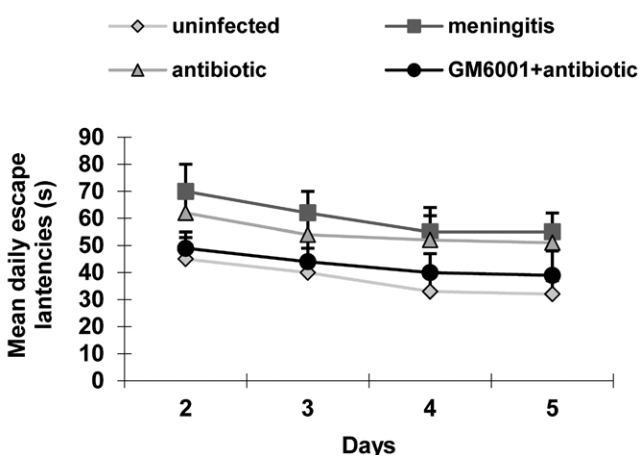

Fig. 5. Mean daily escape latencies from day 2 to day 5 for the Morris water test 3 weeks after pneumococcal meningitis. Learning was significantly impaired in animals of the meningitis group compared to the uninfected group (P<0.05). Treatment with antibiotics plus GM6001 significantly enhanced leaning compared to the animals treated with antibiotics alone (P<0.05).

Spatial learning and memory

The rats were evaluated using the Morris water maze to assess the long-term effects of GM6001 on spatial learning 21 days after infection. Repeated-measures ANOVA revealed differences among the four groups in escape latency ($F_{3, 44}$=10.58, P<0.01). Consistent with previous studies, the escape latency in rats with meningitis was greater than in the uninfected rats (P<0.05). Adjuvant therapy of meningitis with GM6001 significantly improved the performance of the rats compared to the antibiotic-treated group (P<0.05) (Fig. 5). On the day of probe testing (Fig. 6), significant differences were observed for the percentage of time spent in the target quadrant among the four groups ($F_{3, 44}$=12.27, P<0.01). Rats receiving combination treatment with antibiotic and GM6001 spent sig-

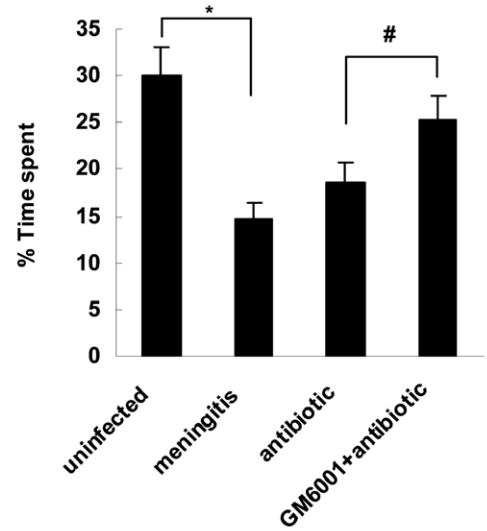

Fig. 6. Evaluation of spatial learning and memory for rats in the water maze test as the percentage of time spent in the target quadrant in the probe trial 3 weeks after pneumococcal meningitis. Memory was significantly impaired in the animals of the meningitis group compared to the uninfected group (P<0.05). Treatment with antibiotics plus GM6001 significantly enhanced memory compared to the animals treated with antibiotics alone (P<0.05). $^{\#}P$<0.05 *versus* the antibiotic group. *P<0.05 *versus* the uninfected group.

nificantly more time (25.2±2.6%) in the target quadrant than those treated with antibiotic only (18.5±2.3%, $P<0.05$).

DISCUSSION

In the present study, we histopathologically confirmed that the cortical and hippocampal neurons of the brain are damaged following bacterial meningitis. These findings are consistent with others who demonstrated that experimental meningitis results in a net loss of neurons, diminished volume, and impaired neurogenesis in the dentate gyrus (Grandgirard et al. 2007). Similarly, histological evaluation of brain sections has revealed apoptosis in the dentate gyrus of the hippocampus in mice with bacterial meningitis caused by *S. pneumoniae* (Hoffmann et al. 2007). This suggests that *S. pneumoniae* meningitis is histopathologically characterized by two forms of neuronal injury that are most prominent in the cortex and hippocampus, although the detailed mechanisms of cellular injury may differ (Leib et al. 2001).

A growing body of evidence suggests that MMPs contribute to the pathophysiology of bacterial meningitis (Sulik and Chyczewski 2008, Hsieh et al. 2012, Schubert-Unkmeir et al. 2010, Wang et al. 2010). Accordingly, our laboratory has demonstrated the expression of MMP-9 mRNA and an increase in the level of MMP-9 mRNA in response to *S. pneumoniae* infection of the CNS (Liu et al. 2008). MMPs constitute a family of zinc-binding endopeptidases characterized by their ability to degrade various extracellular matrices (ECM). Neutrophils, neurons, glial cells, vascular smooth muscle cells, and endothelial cells produce MMP upon stimulation (Deb and Gottshall 1996, Gottschall and Debb 1996, Dencoff et al. 1997, Pagenstecher et al. 1998). Infiltrating leucocytes, including eosinophils and macrophages, are important sources of MMP-9 in meningitis (Chen et al. 2005). MMPs have been implicated as mediators of brain injury in a wide variety of disease processes, including multiple sclerosis (Fernandes et al. 2012), Alzheimer's disease (Mizoguchi et al. 2011), stroke (Barber et al. 2012), tumor invasion (Kim et al. 2011), and other inflammatory diseases of the brain. It has become increasingly apparent that MMP-9 plays an important role in the initiation and/or progression of immune responses to pathogen invasion of the CNS *via* subarachnoid space inflammation (Chen et al. 2005, Liu et al. 2008), BBB disruption (Candelario-Jalil et al. 2007, Rosenberg et al. 2007), and brain edema (Candelario-Jalil et al. 2007).

In the present study, we confirmed that MMP-mediated processes are critically involved in the pathogenesis of brain injury in bacterial meningitis. Furthermore, we have provided strong evidence that the MMP inhibitor GM6001 decreases MMP-9 levels, reduces brain water content, improves learning performance and protects neurons in the cortex and hippocampus from inflammatory brain damage during bacterial meningitis. These findings suggest that metalloproteinases are critical for neuronal injury (Leib et al. 2001) and that the administration of the MMP inhibitor GM6001 as an adjuvant therapy may be an effective strategy to reduce neuronal damage and improve sequelae following bacterial meningitis (Leib et al. 2000).

The hydroxamic acid-type MMP inhibitor GM6001 is a broad-spectrum MMP inhibitor with minor tumor necrosis factor α (TNF-α) converting enzyme (TACE) inhibitory activity (Leib et al. 2000). GM6001 acts by chelating the zinc cation in the active site of MMPs and has previously been reported to block leukocyte migration, suppress autoimmune encephalomyelitis, prevent brain edema after intracerebral hemorrhage, abrogate endotoxin-induced death, and reduce neuronal injury following bacterial meningitis (Lai et al. 2005). TNF-α is a key trigger of the inflammatory response and was previously reported to induce the loss of hippocampal neurons in experimental meningitis (Candelario-Jalil et al. 2007). Intracisternal administration of TNF-α resulted in CSF leukocytosis and an increase in cerebral blood flow, whereas intracerebral injection of TNF-α produces a dose-dependent increase in BBB permeability and MMP activity (Leib et al. 2000). TNF-α is activated when the membrane-bound form of TNF-α is converted to its active soluble form by TACE, a metalloproteinase closely related to the MMP family (Leib et al. 2001). Therefore, the significant neuroprotective effect of GM6001 on brain water content, neuropathology and learning suggests that inhibition of both the TNF-α signaling pathway and MMPs occurs.

CONCLUSION

Based on these observations and the current study, we suggest that *S. pneumoniae* meningitis elicits two forms of neuronal injury in the cortex and hippocam-

pus: an increase in brain water content and the development of neurological sequelae and learning defects. Combined MMP and TACE inhibition by GM6001 down-regulated the level of MMP-9, decreased the brain water content, attenuated neuronal injury in the cortex and hippocampus and improved learning. These results suggest that the MMP inhibitor GM6001 is a particularly promising compound for adjuvant therapy in bacterial meningitis.

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