Original Paper

Gamma-Secretase Inhibitors Attenuate Neurotrauma and Neurogenic Acute Lung Injury in Rats by Rescuing the Accumulation of Hypertrophic Microglia

Hung-Jung Lin^{a,b} Chien-Chin Hsu^{a,b} Chung-Ching Chio^c Yu-Feng Tian^{c,d}
Mao-Tsun Lin^e Ting-Wei Lin^a Chih-Hsien Chang^f Ching-Ping Chang^{e,g}

^aDepartment of Biotechnology, Southern Taiwan University of Science and Technology, Tainan,

^bDepartment of Emergency Medicine, Chi Mei Medical Center, Tainan, ^cDepartment of Surgery, Chi Mei Medical Center, Tainan, ^dDepartment of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, ^eDepartment of Medical Research, Chi Mei Medical Center, Tainan, ^fDepartment of Medicine, Cheng-Kung University School of Medicine, Tainan, ^gThe Ph.D. Program for Neural Regenerative Medicine, Taipei Medical University, Taipei, Taiwan

Key Words

Traumatic brain injury • γ -secretase • Neurogenic acute lung injury • Microglia

Abstract

Background/Aims: In response to traumatic brain injury (TBI), activated microglia exhibit changes in their morphology from the resting ramified phenotype toward the activated hypertrophic or amoeboid phenotype. Here, we provide the first description of the mechanism underlying the neuroprotective effects of γ -secretase inhibitors on TBI outcomes in rats. **Methods:** The neuroprotective effects of γ -secretase inhibitors such as LY411575 or CHF5074 on TBI-induced neurotoxicity were analysed using a neurological motor function evaluation, cerebral contusion assay, immunohistochemical staining for microglia phenotypes, lung injury score and Evans Blue dye extravasation assay of brain and lung oedema. **Results:** Hypertrophic or amoeboid microglia accumulated in the injured cortex, the blood-brain-barrier was disrupted and neurological deficits and acute lung injury were observed 4 days after TBI in adult rats. However, a subcutaneous injection of LY411575 (5 mg/kg) or CHF5074 (30 mg/kg) immediately after TBI and once daily for 3 consecutive days post-TBI significantly attenuated the accumulation of hypertrophic microglia in the injured brain, neurological injury, and neurogenic acute lung injury. **Conclusion:** Gamma-secretase inhibitors attenuated neurotrauma and neurogenic acute lung injury in rats by reducing the accumulation of hypertrophic microglia in the vicinity of the lesion.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Traumatic brain injury (TBI) or ischemic stroke causes prominent neuroinflammation [1, 2]. After a TBI, the ischemic environment drives the infiltration of blood born macrophages and resident activated microglia into the lesion sites in the brain [3]. Extracranial complications, including acute lung injury (ALI), occur in 20-25% of patients with severe TBI [4]. Acute lung injury is an independent predictor of poor outcomes in patients with TBI [4] and strongly affects the mortality rate and neurological outcomes [5].

Microglia, the main immune cells in the brain, are activated by TBI [6, 7]. The morphology of ramified microglia in the resting or relatively inactive status is strikingly similar in humans and rodents [8]. Upon activation, activated microglia retract their ramifications and transform into an amoeboid or hypertrophic morphology. Activated hypertrophic or amoeboid microglia are associated with the overproduction of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), and IL-6 [9]. In contrast, macrophages are less prone to morphological alterations in response to ischemia or injury [10]. Additionally, γ -secretase inhibitors such as CHF5074 [11-13] and LY411575 [14] inhibit microglial activation [1] and attenuate learning deficits in a mouse model of Alzheimer's disease (AD) [13]. A systemic inflammatory response plays an important role in the development of TBI-associated ALI [15-17]. Microglia act as the primary mediators of the response to injury in the brain [9], raising the possibility that inhibition of γ -secretase with CHF5074 or LY411575 may ameliorate both neurological injury and neurogenic ALI.

In the present study, we evaluated contusion brain injuries and microglia activation in a rat TBI model [7, 18], which is closely related to human brain injury, to address this question. We also investigated whether brain injury disrupted blood-brain-barrier (BBB) permeability, induced the accumulation of both polymorphonuclear cells (PMNs) and pro-inflammatory cytokines in the bronchoalveolar lavage fluid (BALF), and induced acute lung pathology. Furthermore, we attempted to ascertain whether inhibition of microglial activation with γ -secretase inhibitors such as CHF5074 and LY411575 attenuated neurological injury and neurogenic ALI following TBI.

Materials and Methods

Animals

We purchased adult male Sprague-Dawley rats (body weight 280-320 g) from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Four animals were housed in each cage at an ambient temperature of $26 \pm 0.5^\circ\text{C}$ with a 12-h light/dark cycle. Pelleted rat chow and tap water were available *ad libitum*. Rats were allowed to become acclimated for at least one week. Experiments were performed in accordance with protocols approved by the Animal Ethics Committee of Chi Mei Medical Center (Tainan, Taiwan) (Institutional Animal Care and Use Committee (IACUC) approval no. 105122608) under Guidelines of the Ministry of Science and Technology of the Republic of China (Taipei, Taiwan) and conformed to the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines.

Induction of Moderate TBI and Experimental Groups

A moderate cortical contusion was performed using the lateral fluid percussion injury (FPI) method [19]. Our fluid percussion injury method was a slight modification from previously described protocols. Briefly, male SD rats were anesthetized with sodium pentobarbital (25 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and intramuscularly injected with a mixture containing ketamine (4.4 mg/kg; Nankuang Pharmaceutical, Tainan City, Taiwan), atropine (0.02633 mg/kg; Sintong Chemical, Taoyuan City, Taiwan) and xylazine (6.77 mg/kg; Bayer AG, Germany). We placed each rat in a stereotaxic apparatus frame and incised its scalp. A 4.8-mm circular craniotomy was performed midway between the lambda and bregma at a location 3.0 mm to the right of the central suture. A cannula (2.6-mm inner diameter) was secured into the craniotomy with cyanoacrylate adhesive and dental acrylic. Subsequently, the FPI was induced (pressure intensity: 2.2 atmospheres) using a fluid percussion device (Virginia Commonwealth University

Biochemical Engineering, Richmond, VA, USA) [20]. A moderate percussion injury was produced by rapidly injecting a small volume of saline into the closed cranial cavity. This reproducible and consistent model is associated with a $>100\text{-mm}^3$ cerebral contusion volume and neurological motor deficit within the first 3 days after injury [21]. Sham-operated animals received all the aforementioned surgical procedures, except the injury. Body temperature was maintained at 37°C during surgery using a heating pad and was monitored with a rectal thermometer. After surgery, animals were allowed to completely recover from anesthesia in a warmed chamber before being transferred to their home cages. The second group included TBI rats that were treated with a dose of vehicle solution immediately (within 5 minutes) after injury and an additional dose of vehicle solution daily for 3 consecutive days after the injury. The third and the fourth groups were the TBI rats that were treated with CHF5074 (30 mg/mL/kg, subcutaneous (S.C.) injection) and LY411575 (5 mg/mL/kg, S.C. injection) immediately (within 5 minutes) after injury and for 3 consecutive days after injury.

Drugs and Dosing

We dissolved CHF5074 ($\text{C}_{16}\text{H}_{11}\text{Cl}_2\text{FO}_2$; Sigma-Aldrich, St. Louis, MO, USA) or LY411575 ($\text{C}_{26}\text{H}_{23}\text{F}_2\text{N}_3\text{O}_4$; Sigma-Aldrich, St. Louis, MO, USA) in DMSO. CHF5074 (30 mg/kg) or LY411575 (5 mg/kg) was administered S.C.. A S.C. injection 30 mg/kg of CHF5074 [11] or 5 mg/kg of LY411575 [22] inhibited β -amyloid peptide production and restored visual memory in rodents.

Experimental Procedures

In Experiment 1, we S.C. injected LY411575, CHF5074, or vehicle solution into the rats immediately after TBI and once daily for 3 consecutive days post-TBI, and the effects of these compounds on the rats' neurological motor functions were assessed on 1 day before surgery and on the 1st, 2nd, 3rd, and 4th days after TBI. In Experiment 2, we performed the same treatments and observed their effects on the cerebral contusion volume, lung injury score, lung oedema, or microglial activation in the injured brains of rats 4 days after TBI.

Neurological Motor Function Evaluation

In all rats, both a modified neurological severity score (mNSS) test and an inclined plane test were performed 1 day before neurotrauma and 4 days after neurotrauma by an investigator who was unaware of the experimental groups. The mNSS is a composite of balance, sensory, reflex, and motor tests. It was graded on a scale of 0 to 18 ("0" denotes normal score, whereas "18" denotes maximal deficit score) [23]. We also tested the ability of each animal to maintain its position at a given angle on an inclined plane [24]. Rats were placed head down on a platform (40 cm x 60 cm) with a rubber surface followed by a progressive increase in the platform angle. The last angle at which the rat was able to maintain its position for 5 s was documented. Thus, the higher the angle, the more muscle strength in a rat's limbs.

Cerebral Contusion Assay

On the 4th day after TBI, the anaesthetized rats were intracardially perfused with normal saline. Their brain tissues were removed, immersed in cold saline for 5 min, and sliced into 2.0-mm thick sections with a tissue slicer. Triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA) staining procedures were used to determine the extent of the cerebral contusion caused by a TBI, as detailed in a previous study [25]. The contusion volume, as revealed by negative TTC stains that indicated dehydrogenase-deficient tissue, was measured in each slice and summed using Image-Pro Plus analysis software (Media Cybernetics, Silver Spring, MD, USA).

The corrected contusion volume (CCV) was calculated using the following equation to avoid overestimating the contusion volume:

$$\text{CCV} = (\text{CH} - (\text{IH} - \text{ICA})) \times d$$

where CH represents the area of the contralateral hemisphere in square millimetres, IH is the area of the ipsilateral hemisphere in square millimetres, ICA is the area of ipsilateral contusion in square millimetres, and d is the thickness of the slices (1 mm).

Immunohistochemical Staining for Microglia

Rats were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) and transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by perfusion with and immersion in 4% paraformaldehyde to assess microglial activation using immunohistochemical staining. Immunohistochemical staining was performed using a previously described method [25]. Each brain tissue was cut into 3 equally spaced (5 mm) coronal blocks, and each block was embedded in paraffin. A series of adjacent 10- μ m sections corresponding to coronal coordinates located 1.7 mm anterior to the bregma to 4.3 mm posterior to the bregma were cut from each block. Sections were incubated in 2 mol/L HCl for 30 min, rinsed with 0.1 mol/L boric acid (pH 8.5) for 3 min at room temperature and then incubated with an anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody (1:200; GeneTex Inc., San Antonio, Texas, USA) in PBS containing 0.5% normal bovine serum overnight at 4°C. After the slides were washed with PBS, a 1:200 dilution of the biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) in blocking serum was applied for 1 hour and then reacted with the avidin-biotin-peroxidase complex (ABC) (Vectastatin ABC-Kit, Vector Laboratories, Burlingame, CA, USA). Finally, the sections were incubated with the 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich) substrate, rinsed with running tap water for 10 minutes, stained with haematoxylin, and mounted in glycerol-gelatin medium (Sigma-Aldrich). The numbers of Iba-1-positive cells throughout the frontal and parietal cortices were counted. Images of the red brown-coloured immunohistochemical staining of immune cells were captured using an upright microscope system (Carl Zeiss GmbH, Göttingen, Germany) with a 20X objective (N.A. 0.5) and counted using ZEN2.3 software (Carl Zeiss). We modified the methodology of Resende et al [26]. to assess the size of the microglia cell body (soma). In Fig. 3B, each microglia cell body was identified using the counter tool and expressed in μ m². As shown in Fig. 3C, any Iba-1-positive cell with a cell body area greater than the average of sham+vehicle group (110 μ m²) was defined as a hypertrophic or amoeboid microglia cell. The counts obtained from three different images per section were averaged. Results are expressed as a percentage of total ramified or amoeboid microglia in the analysed regions of interests.

Histology and Lung Injury Scoring

At the end of the experiments, we euthanized the animals, excised their lungs *en bloc*, and then fixed the specimens with glutaraldehyde and embedded them in paraffin. A portion of each lobe of the lung was sectioned, stained with haematoxylin-eosin (HE), and scored by a pathologist who was blinded to the identity of the groups using the Lung Injury Scoring System of Matute-Bello and colleagues [27]. Lung injury was assessed in a blinded manner using a modified scoring system based on the number of neutrophils detected in the alveolar space or interstitial space, the deposition of hyaline membranes and other proteinaceous debris filling the airspaces, and alveolar septal thickening. According to the criteria established by Matute-Bello et al [27], we scored the extent of lung injury as shown in Table 1. Two investigators examined a minimum of 20 random high-power fields (400x magnification) in a blinded manner. The resulting scores from these investigators were added and averaged to obtain the lung injury score.

*Evans Blue Dye (EBD)
Extravasation*

Rats were injected with 2% EBD (Sigma-Aldrich) via the left jugular vein (5 mL/kg) 30 min before the end of the 4-day recovery period after a TBI, as detailed in a previous study [28]. After 30 min, the animals were sacrificed, and the lungs and brain were perfused with 1 mL of PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA). The lungs and brain were excised *en bloc* and frozen in liquid nitrogen and

Table 1. Lung Injury Scoring System. Score=[(20xA)+(14xB)+(7xC)+(7xD)+(2xE)]/(number of fieldsx100). (adapted from Matute-Bello et al., 2011[27])

Parameters	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	none	1-5	>5
B. Neutrophils in the interstitial space	none	1-5	>5
C. Hyaline membranes	none	1	>1
D. Proteinaceous debris filling the airspaces	none	1	>1
E. Alveolar septal thickening	<2x	2x-4x	>4x

homogenized in 2 mL of PBS. The homogenate was diluted with formamide and incubated at 60°C for 2 h, followed by centrifugation at 5,000×g for 30 min. The supernatant was collected, and the absorbance was measured at 620 and 740 nm using a dual-wave spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Contaminating heme pigments were corrected using the formula $E_{620}(\text{EBD}) = E_{620} - (1.426 \times E_{740} + 0.030)$. Data are reported as micrograms EBD per gram tissue [29].

Determination of PMN Numbers in the BALF

Animals were subjected to bronchoalveolar lavage to collect the BALF using previously described methods [30]. BALF was collected three times through a tracheal cannula using 0.5 mL of autoclaved PBS, which was instilled to a total volume of 1.3 mL. Samples were centrifuged at 3000 rpm for 10 min at 4°C. Aliquots of supernatants were stored at -20°C until the analysis of cytokine levels. Cell pellets were resuspended in PBS to obtain total cell counts. Cells were counted with the aid of a haemocytometer. Slides were visualized using Wright-Giemsa staining (Fisher Scientific Co., Middletown, VA), and PMNs were identified by a certified laboratory technologist in a blinded fashion.

Determination of Cytokine Levels in BALF

Protein levels in the BALF were quantified (Bio-Rad Laboratories, Hemel Hempstead, UK) and TNF- α , IL-1 β , and IL-10 levels in the supernatants of the BALF were determined using ELISA assay kits (R&D Systems, Abingdon, UK), according to the manufacturers' instructions.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) or Sigma Plot (Systat Software Inc., San Jose, CA, USA) software. All data are presented as means $\pm$ standard deviations (SD). Behavioral data (mNSS and inclined plane) were analysed using either Student's *t* test or two-way ANOVA followed by Tukey's multiple comparisons test. Biochemical data (brain contusion, lung permeability, lung injury score, and cytokine expression) were analysed using one-way ANOVA followed by Dunnett's multiple comparisons test. Differences between values were considered significant if $p < 0.05$.

Results

The average intensity of the fluid pulse delivered to rats in the injured group was 2.2 ± 0.2 atm (mean $\pm$ SD). Immediately after a TBI, all rats had a short period of apnoea. No differences were observed among the three TBI groups. However, sham-TBI group rats did not experience apnoea.

γ -Secretase Inhibitors Reduce TBI-induced Brain Contusions

We generated a unilateral FPI of the parietal cortex in rats to examine the neuroprotective effects of LY411575 or CHF5074 on TBI. Immediately (or within 5 minutes) after trauma, two groups of rats ($n=10$ of each group) received LY411575 (5 mg/kg) or CHF5074 (30 mg/kg). These two groups of rats received an additional dose of LY411575 (5 mg/kg) or CHF5074 (30 mg/kg) daily for consecutive 3 days after trauma. A third group of rats received the solvent alone ($n=10$) and was termed the vehicle group. After injury, the extent of the brain contusion was determined by TTC staining and volumetry (Fig. 1A). Compared to the contusion volume of the primary lesion, which was determined 15 minutes after TBI, the contusion volume in the vehicle group increased six-fold over 4 days (TBI+vehicle group), revealing substantial progression of secondary brain damage in the FPI model (Fig. 1B). A reduced volume of the secondary brain damage was noted in rats treated with LY411575 or CHF5074 (Fig. 1C).

γ -Secretase Inhibitors Attenuate TBI-induced Neurological and Motor Deficits

Four days after the animals had been subjected to brain injury, behavioural tests revealed that the TBI+vehicle group had higher mNSS values and a lower maximal angle at which a rat could maintain its position on the inclined plane than the Sham+vehicle group (Fig. 2;

Fig. 1. Inhibition of γ -secretase with LY411575 or CHF5074 attenuates brain contusions in the rat model of moderate TBI. (A) Each panel represents an image of TTC staining in a rat that received the sham operation or TBI and was euthanized 4 days after the sham operation (Sham+vehicle [4 days]), a TBI+vehicle rat euthanized 15 minutes post-TBI (TBI+vehicle [15 min]), a TBI+vehicle rat euthanized 4 days post-TBI (TBI+vehicle [4 days]), a TBI+LY411575 rat euthanized 4 days post-TBI (TBI+LY411575 [4 days]), and a TBI+CHF5074 rat euthanized 4 days post-TBI (TBI+CHF5074 [4 days]). Cerebral contusion volumes (B) and areas (C) were evaluated using TTC staining and volumetry. Each column and bar represent the mean $\pm$ SD of 10 rats per group. * $p < 0.05$, TBI+vehicle (15 min) group compared with the Sham+vehicle (4 days) group; * $p < 0.01$, TBI+vehicle (4 days) group compared with the TBI+vehicle (15 min) group; and # $p < 0.05$, TBI+LY411575 (4 days) group compared with the TBI+vehicle (4 days) group or TBI+CHF5074 (4 days) group compared with the TBI+vehicle (4 days) group.

$p < 0.01$). Compared with those in the TBI+vehicle group, the mNSS values and the maximal angle were significantly lower and higher in the TBI+LY411575 and TBI+CHF5074 groups, respectively (Fig. 2; $p < 0.05$).

γ -Secretase Inhibitors Reduce TBI-induced Microglia Activation

Four days after the rats had been subjected to TBI, the TBI+vehicle group displayed significantly greater numbers of both total Iba-1-positive cells (microglia or macrophages) (Fig. 3D) and cell body area (Fig. 3B) in the ipsilateral frontal and parietal cortices than the sham-TBI group. As shown in Fig. 3B, the average cell body areas for the ramified Iba-1 positive cells and the hypertrophic or amoeboid Iba-1 positive cells were $91 \pm 12 \mu\text{m}^2$ and $287 \pm 27 \mu\text{m}^2$, respectively. We postulate that any an Iba-1-positive cell with a cell body area less than $103 \mu\text{m}^2$ or greater than $110 \mu\text{m}^2$ was defined as displaying ramified phenotype or a hypertrophic (or amoeboid) phenotype, respectively. Compared to the Sham+vehicle group, the TBI+vehicle group displayed significantly higher numbers of hypertrophic Iba-1-positive cells in the ipsilateral frontal cortex and parietal cortex (Fig. 3C). However, the over-accumulation of hypertrophic Iba-1-positive cells in the injured brain was significantly reduced by the CHF5074 or LY411575 treatment (Fig. 3).

γ -Secretase Inhibitors Reduce TBI-induced Acute Lung Injury

Four days after the rats had been subjected to TBI, the microscopic analysis showed that TBI caused substantial changes in the lungs, including alveolar haemorrhaging, alveolar wall

Fig. 2. Inhibition of γ -secretase with LY411575 or CHF5074 inhibits neurological motor deficits in the rat model of moderate TBI. Both a modified neurological severity score (mNSS) test and an inclined plane test were performed 1 day before neurotrauma or sham operation and 4 days after neurotrauma or sham operation in the Sham+vehicle group, TBI+vehicle group, TBI+LY411575 group, and TBI+CHF5074 group. The higher the mNSS score, the more severe the neurological injury. The lower the maximal angle at which a rat could maintain its position on the plane, the more severe the loss of muscle strength. Data are expressed as means $\pm$ SD. * p <0.01, compared with the Sham+vehicle group; + p <0.01, compared with the TBI+vehicle group. N =10 in each group.

thickening, and increased amounts of exudates in the alveolar spaces in the vehicle group (Fig. 4). Using the standard assessment reported by Matute-Bello et al [27], lungs from the TBI+vehicle group had higher ALI scores than the Sham+vehicle controls (Fig. 4). Compared to the TBI+vehicle group, the TBI+LY411575 and TBI+CHF5074 groups had significantly lower ALI scores (p <0.05; Fig. 4).

γ -Secretase Inhibitors Reduce the TBI-induced Increase in Lung and Brain Permeability and Vascular Leakage

An increase in pulmonary or brain capillary permeability was detected at 4 days after a TBI. Compared to Sham+vehicle controls, the TBI+vehicle group exhibited significantly higher EBD extravasation (Fig. 5; p <0.05). Based on gross observations, some areas of bleeding were detected on surfaces of the lung or brain tissues exhibiting EBD extravasation

Fig. 3. Inhibition of γ -secretase with LY411575 or CHF5074 increases the percentage of Iba-1-positive cells displaying the ramified phenotype, but decreases the percentage of Iba-1-positive cells displaying the hypertrophic or amoeboid phenotypes in the injured cortex. Histochemical staining procedures were used to assess the morphology of Iba-1-positive cells in serial 50- μ m coronal sections corresponding to coordinates 1.7 mm anterior to 4.3 mm posterior to the bregma. (A) Representative images of staining obtained 4 days after the sham operation or TBI from a Sham+vehicle rat, a TBI+vehicle rat, a TBI+LY411575 rat, and a TBI+CHF5074 rat. (B) The average soma areas of Iba-1-positive cells in the frontal cortex (anterior 1.7 mm to bregma) and parietal cortex (posterior 4.3 mm to bregma) of the TBI-vehicle group

were significantly higher than the Sham+vehicle group. Increased average cell body areas of Iba-1-positive cells in these regions of interest following TBI were significantly reduced by LY41575 and CHF5074. (C) The percentages of Iba-1-positive cells with amoeboid or ramified phenotypes are presented. *Compared to the Sham+vehicle group, the TBI+vehicle group displayed significantly fewer cells with the ramified phenotype (* p <0.001) but greater numbers of cells with the amoeboid phenotype (* p <0.001) in the injured cortex. However, compared to the TBI+vehicle group, the TBI+LY411575 and TBI+CHF5074 groups displayed significantly greater (* p <0.001) numbers of cells with the ramified phenotype and significantly (* p <0.001) fewer cells with the hypertrophic or amoeboid phenotype in the ipsilateral cortex. (D) The total number of Iba-1-positive cells in the regions of interest from each group. Data are expressed as means $\pm$ SD (n =10 each group). * p <0.01, TBI+vehicle group compared with the Sham+vehicle group; * p <0.05, TBI+LY411575 group compared with the TBI+vehicle group or TBI+CHF5074 group compared with the TBI+vehicle group.

in the TBI+vehicle group (Fig. 5). Compared to those of the TBI+vehicle group, the extents of both haemorrhage and EBD extravasation were significantly reduced in the TBI+LY411575 and TBI+CHF5074 groups (Fig. 5).

Fig. 4. Inhibition of γ -secretase with LY411575 or CHF5074 attenuates ALI in the rat model of TBI. At 4 days after injury, lung sections were stained with haematoxylin and eosin; lung specimens from TBI+vehicle group developed significant histological changes including neutrophil infiltration (orange arrow) and deposition of fibrin strands (blue arrow) in the alveolar space, hyaline membrane (green arrow), and alveolar capillary membrane thickening (red arrow). In contrast, the addition of LY411575 or CHF5074 after injury attenuated the severity of this injury to a level observed in the Sham+vehicle animals. The ALI severity scores in the TBI+vehicle group were significantly higher than of the scores for the Sham+vehicle group (* $p < 0.01$). The scores for the TBI+LY411575 and TBI+CHF5074 groups were significantly lower than the scores for the TBI+vehicle group (* $p < 0.05$). Each column and bar represent the meanSD of 10 rats per group.

γ -Secretase Inhibitors Normalized TBI-induced Alterations in the Number of PMNs and Cytokine Levels in the BALF

Four days after the rats had been subjected to TBI, the PMN count and TNF- α , IL- β , and IL-10 levels in the BALF from vehicle-treated rats were significantly higher than those in the Sham+vehicle controls (Fig. 6). Compared to the TBI+vehicle group, the TBI+LY411575 and

Fig. 5. Inhibition of γ -secretase with LY411575 or CHF5074 reduces EBD permeability in the lungs and brain of the rat model of TBI. Lung or brain permeability to EBD was measured in the rat model of TBI. Representative EBD permeability in the lungs (A) or brain (B) 4 days after the sham operation or TBI in a sham+vehicle rat, a TBI+vehicle rat, a TBI+LY411575 rat, and a TBI+CHF5074 rat. (C & D) The TBI+vehicle group rats showed a significant increase in the EBD extravasation (* $p < 0.05$) compared with the sham+vehicle group. However, compared with the rats in the TBI+vehicle group, rats in the TBI+LY411575 and CHF5074 groups displayed significantly lower EBD extravasation (* $p < 0.05$). Each column and bar represent the mean $\pm$ SD of 10 rats per group.

TBI+CHF5074 groups displayed significantly fewer PMNs and lower levels of TNF- α and IL-1 β in the BALF (Fig. 6). However, the LY411575 and CHF5074 treatments further increased the IL-10 levels in the BALF after TBI (Fig. 6).

Discussion

FPI is one of most commonly used TBI models and has been modulated to generate injuries with the characteristics of mild or severe TBI [31]. The adult rat brain showed a 52% decrease in cortical cerebral blood flow (CBF) within 15 minutes of FPI, which persisted for 4 hours and then returned to the original CBF level at 24 hours post-injury [32]. Compared to the contusion volume of the primary lesion, which was determined 15 minutes after TBI, the contusion volume in the mouse controlled cortical impact (CCI) model increased approximately two-fold over 24 hours [33]. In a rat model of FPI, the contusion volume increased approximately six-fold over 4 days compared to that in the primary lesion (as shown in the present study). A reduced volume of secondary brain damage was observed in rats treated with the γ -secretase inhibitors LY411575 or CHF5074 in the present study. In addition, Evans Blue staining revealed significantly reduced formation of brain oedema in the lesioned cortical hemisphere of LY411575-treated or CHF5074-treated rats 4 days after TBI (present results).

Fig. 6. Inhibition of γ -secretase with LY411575 or CHF5074 attenuates TBI-induced inflammation in the rat model. The induction of inflammation in the lung tissue was determined by assessing the number of PMNs and TNF- α , IL-1 β , and IL-10 levels in the BALF. Rats in the TBI+vehicle group showed a significant increase in the number of PMNs and TNF- α , IL-1 β , and IL-10 levels in the BALF compared with the rats in the sham+vehicle group (* $p < 0.05$). Compared with the rats in the TBI+vehicle group, rats in the TBI+LY411575 and TBI+CHF5074 groups exhibited significantly fewer PMNs and lower TNF- α and IL-1 β levels, but significantly higher IL-10 levels in the BALF (* $p < 0.05$). Each column and bar represent the mean $\pm$ SD of 10 rats per group.

Clinical data have revealed a link between brain injury and lung inflammation [4, 34]. Kalsotra and colleagues [15] provided the first experimental evidence that brain injury directs pulmonary neutrophil mobilisation. Additionally, brain injury causes increased BBB permeability up to 3 days post-injury. In the present study, brain injury also increased BBB permeability and increased the numbers of PMNs and interleukin-1 β and TNF- α levels in the BALF. These results led us to hypothesize that systemic release of pro-inflammatory cytokines and certain mediators after brain injury might cause ALI following TBI. Furthermore, systemic administration of γ -secretase inhibitors exerted an anti-inflammatory effect by inhibiting both central nervous system and peripheral inflammation induced by TBI. Our results are consistent with the findings reported by Li et al [35], who showed that TBI caused an inflammatory reaction, including increased levels of IL-1 β , IL-6, and TNF- α , as well as microglial cell activation. Simvastatin attenuates microglial cell activation and decreases IL-1 β levels after TBI. Gamma-secretase inhibitors may share similar mechanisms with simvastatin in treating TBI.

The most striking finding of the present study is probably that brain injury induces neurogenic ALI by promoting microglial activation. Resting, inactive microglia are characterized by an arborized or ramified morphology, which allows them to communicate with their surroundings [36]. On the other hand, activated microglia assume their characteristic hypertrophic or amoeboid morphology during inflammation [37]. Amoeboid or hypertrophic microglia, a macrophage phenotype, are able to engage in phagocytosis or the pro-inflammatory process. Low blood flow [38], thrombin and other components

of the blood, as well as damage-associated molecular patterns (DAMPs) [39], are able to activate microglia. Indeed, as shown in the present study, more than 95% of total microglia in the contused brain displayed the amoeboid or hypertrophic morphology following brain injury. Importantly, activated microglia engulf endothelial cells and cause the disruption of the BBB, enabling the release of pro-inflammatory mediators into the bloodstream [40, 41]. Activated amoeboid or hypertrophic microglia are associated with overproduction of pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β in response to brain injury [9]. Thus, following brain injury, microglial activation cause overproduction of pro-inflammatory cytokines in injured brain tissues and BBB disruption, resulting in pulmonary inflammation and ALI. Accordingly, CHF5074- and LY411575-mediated reductions in the accumulation of hypertrophic microglia reverses TBI-induced overproduction of pro-inflammatory cytokines in the brain, BBB disruption, pulmonary inflammation, and ALI in a rat model.

Our present hypothesis is consistent with findings from several previous studies. For example, levels of beta-amyloid (A β), a key mediator of microglia activation [42], are substantially increased in response to TBI in both humans and rodents [1]. Brain injury promotes A β accumulation and results in increased γ -secretase expression in microglia [43]. CHF5074, a γ -secretase inhibitor, has also been shown to inhibit A β accumulation and to attenuate memory deficits in mouse models of AD [43].

Bone marrow-derived cell (BMDC)-associated inflammatory responses play a pivotal role in the development of ALI [44-46]. Activation of the adenosine A_{2A} receptor (A_{2A}R) protects against ALI by stimulating BMDC accumulation [47-50]. In a mouse model of neurogenic ALI induced by a TBI, activation of BMDC-associated A_{2A}R exacerbated ALI. In both mouse models of TBI and isolated microglia, a high glutamate concentration in the environment could switch the anti-inflammatory effect of A_{2A}R activation to a pro-inflammatory effect. Plasma glutamate modulates the interaction of A_{2A}R and m-glutamate receptors on BMDC to aggravate TBI-induced ALI [51]. In addition, the release of tissue factor, a potent initiator of the extrinsic coagulation pathway, from the injured brain is also thought to play a key role in coagulopathy after TBI [17]. However, researchers have not determined whether the inhibition of microglial activation with CHF5074 and LY411575 attenuate neurogenic ALI by reducing the accumulation of BMDC and/or tissue factor.

The enzyme γ -secretase processes the Notch protein, allowing Notch to subsequently regulate cell proliferation, development, differentiation, and cell survival [13, 52]. CHF5074, a novel γ -secretase inhibitor, enhances amyloid precursor protein (APP) proteolysis in the brain and attenuates spatial memory deficits in a transgenic mice model of AD [53], without adverse side effects. In addition, CHF5074 has been reported to be a safe and a promising therapeutic agent for 96 patients with mild-to-moderate AD [54].

Conclusion

Based on our data, activated microglia change their morphology from the ramified phenotypes toward the hypertrophic or amoeboid phenotype in response to a TBI. Indeed, the accumulation of activated hypertrophic or amoeboid microglia in the injured brain regions positively correlates with the ensuing BBB disruption (as evidenced by brain Evans Blue dye extravasation), brain contusion volume, neurological motor deficits, and neurogenic acute lung injury in rats with neurotrauma. We concluded that two γ -secretase inhibitors (CHF5074 and LY411575) reduced microglial activation in the vicinity of the lesion, BBB disruption and acute lung injury and promoted acute motor function recovery in TBI rats.

Abbreviations

A β (amyloid-beta); TBI (traumatic brain injury); ALI (acute lung injury); AD (Alzheimer's disease); APP (amyloid precursor protein); PMNs (polymorphonuclear cells); IL-1 β (interleukin-1 β); TNF- α (tumor necrosis factor- α); IL-10 (interleukin-10); BALF (bronchoalveolar lavage fluid); EBD (Evans Blue dye).

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology (Taiwan), MOST 104-2314-B-384-003-MY3 (to CCC) and MOST 106-2314-B-384-001-MY3 (to CCC), and a grant from the Chi Mei Medical Center (Taiwan), CMFHT 10504 (to CPC).

Conception and design of the study: C.C.C. and H.J.L.; drafting the important intellectual content of the manuscript: C.P.C., and H.J.L.; performed the experiments: C.C.H., T.W.L., and C.H.C.; data analysis and interpretation: M.T.L., Y.F.T., and C.P.C.; manuscript revisions: H.J.L.

The use of animals in this study (IACUC No. 105122608) was approved by the IAUCU of Chi Mei Medical Center.

Disclosure Statement

The authors have no conflicting financial interests to disclose.

References

- 1 Mannix RC, Whalen MJ: Traumatic brain injury, microglia, and Beta amyloid. *Int J Alzheimers Dis* 2012;2012:608732.
- 2 Lourbopoulos A, Erturk A, Hellal F: Microglia in action: how aging and injury can change the brain's guardians. *Front Cell Neurosci* 2015;9:54.
- 3 Hanisch UK, Kettenmann H: Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007;10:1387-1394.
- 4 Holland MC, Mackersie RC, Morabito D, Campbell AR, Kivett VA, Patel R, Erickson VR, Pittet JF: The development of acute lung injury is associated with worse neurologic outcome in patients with severe traumatic brain injury. *J Trauma* 2003;55:106-111.
- 5 Mascia L, Zavala E, Bosma K, Pasero D, Decaroli D, Andrews P, Isnardi D, Davi A, Arguis MJ, Berardino M, Ducati A: High tidal volume is associated with the development of acute lung injury after severe brain injury: an international observational study. *Crit Care Med* 2007;35:1815-1820.
- 6 d'Avila JC, Lam TI, Bingham D, Shi J, Won SJ, Kauppinen TM, Massa S, Liu J, Swanson RA: Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. *J Neuroinflammation* 2012;9:31.
- 7 Yang ST, Lin JW, Chiu BY, Hsu YC, Chang CP, Chang CK: Astragaloside improves outcomes of traumatic brain injury in rats by reducing microglia activation. *Am J Chin Med* 2014;42:1357-1370.
- 8 Torres-Platas SG, Comeau S, Rachalski A, Bo GD, Cruceanu C, Turecki G, Giros B, Mechawar N: Morphometric characterization of microglial phenotypes in human cerebral cortex. *J Neuroinflammation* 2014;11:12.
- 9 Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB: ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat Neurosci* 2005;8:752-758.
- 10 Fumagalli S, Perego C, Pischiutta F, Zanier ER, De Simoni MG: The ischemic environment drives microglia and macrophage function. *Front Neurol* 2015;6:81.
- 11 Giuliani A, Beggiato S, Baldassarro VA, Mangano C, Giardino L, Imbimbo BP, Antonelli T, Calza L, Ferraro L: CHF5074 restores visual memory ability and pre-synaptic cortical acetylcholine release in pre-plaque Tg2576 mice. *J Neurochem* 2013;124:613-620.
- 12 Imbimbo BP, Giardino L, Sivilia S, Giuliani A, Gusciglio M, Pietrini V, Del Giudice E, D'Arrigo A, Leon A, Villetti G, Calza L: CHF5074, a novel gamma-secretase modulator, restores hippocampal neurogenesis potential and reverses contextual memory deficit in a transgenic mouse model of Alzheimer's disease. *J Alzheimers Dis* 2010;20:159-173.
- 13 Imbimbo BP, Hutter-Paier B, Villetti G, Facchinetti F, Cenacchi V, Volta R, Lanzillotta A, Pizzi M, Windisch M: CHF5074, a novel gamma-secretase modulator, attenuates brain beta-amyloid pathology and learning deficit in a mouse model of Alzheimer's disease. *Br J Pharmacol* 2009;156:982-993.

- 14 Henley DB, May PC, Dean RA, Siemers ER: Development of semagacestat (LY450139), a functional gamma-secretase inhibitor, for the treatment of Alzheimer's disease. *Expert Opin Pharmacother* 2009;10:1657-1664.
- 15 Kalsotra A, Zhao J, Anakk S, Dash PK, Strobel HW: Brain trauma leads to enhanced lung inflammation and injury: evidence for role of P4504Fs in resolution. *J Cereb Blood Flow Metab* 2007;27:963-974.
- 16 Weber DJ, Gracon AS, Ripsch MS, Fisher AJ, Cheon BM, Pandya PH, Vittal R, Capitano ML, Kim Y, Allette YM, Riley AA, McCarthy BP, Territo PR, Hutchins GD, Broxmeyer HE, Sandusky GE, White FA, Wilkes DS: The HMGB1-RAGE axis mediates traumatic brain injury-induced pulmonary dysfunction in lung transplantation. *Sci Transl Med* 2014;6:252ra124.
- 17 Yasui H, Donahue DL, Walsh M, Castellino FJ, Ploplis VA: Early coagulation events induce acute lung injury in a rat model of blunt traumatic brain injury. *Am J Physiol Lung Cell Mol Physiol* 2016;311:L74-86.
- 18 Spain A, Daumas S, Lifshitz J, Rhodes J, Andrews PJ, Horsburgh K, Fowler JH: Mild fluid percussion injury in mice produces evolving selective axonal pathology and cognitive deficits relevant to human brain injury. *J Neurotrauma* 2010;27:1429-1438.
- 19 McIntosh TK, Noble L, Andrews B, Faden AI: Traumatic brain injury in the rat: characterization of a midline fluid-percussion model. *Cent Nerv Syst Trauma* 1987;4:119-134.
- 20 Chio CC, Chang CH, Wang CC, Cheong CU, Chao CM, Cheng BC, Yang CZ, Chang CP: Etanercept attenuates traumatic brain injury in rats by reducing early microglial expression of tumor necrosis factor-alpha. *BMC Neurosci* 2013;14:33.
- 21 Cheong CU, Chang CP, Chao CM, Cheng BC, Yang CZ, Chio CC: Etanercept attenuates traumatic brain injury in rats by reducing brain TNF- alpha contents and by stimulating newly formed neurogenesis. *Mediators Inflamm* 2013;2013:620837.
- 22 Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, Bara T, Engstrom L, Pinzon-Ortiz M, Fine JS, Lee HJ, Zhang L, Higgins GA, Parker EM: Chronic treatment with the gamma-secretase inhibitor LY-411575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem* 2004;279:12876-12882.
- 23 Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M: Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001;32:1005-1011.
- 24 Chang MW, Young MS, Lin MT: An inclined plane system with microcontroller to determine limb motor function of laboratory animals. *J Neurosci Methods* 2008;168:186-194.
- 25 Tsai MC, Chang CP, Peng SW, Jhuang KS, Fang YH, Lin MT, Tsao TC: Therapeutic efficacy of Neuro AiD (MLC 601), a traditional Chinese medicine, in experimental traumatic brain injury. *J Neuroimmune Pharmacol* 2015;10:45-54.
- 26 Resende FF, Bai X, Del Bel EA, Kirchhoff F, Scheller A, Titze-de-Almeida R: Evaluation of TgH(CX3CR1-EGFP) mice implanted with mCherry-GL261 cells as an *in vivo* model for morphometrical analysis of glioma-microglia interaction. *BMC Cancer* 2016;16:72.
- 27 Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM: An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011;44:725-738.
- 28 Reino DC, Pisarenko V, Palange D, Doucet D, Bonitz RP, Lu Q, Colorado I, Sheth SU, Chandler B, Kannan KB, Ramanathan M, Xu DZ, Deitch EA, Feinman R: Trauma hemorrhagic shock-induced lung injury involves a gut-lymph-induced TLR4 pathway in mice. *PLoS One* 2011;6:e14829.
- 29 Kaner RJ, Ladetto JV, Singh R, Fukuda N, Matthay MA, Crystal RG: Lung overexpression of the vascular endothelial growth factor gene induces pulmonary edema. *Am J Respir Cell Mol Biol* 2000;22:657-664.
- 30 Patel BV, Wilson MR, Takata M: Resolution of acute lung injury and inflammation: a translational mouse model. *Eur Respir J* 2012;39:1162-1170.
- 31 Namjoshi DR, Good C, Cheng WH, Panenka W, Richards D, Crompton PA, Wellington CL: Towards clinical management of traumatic brain injury: a review of models and mechanisms from a biomechanical perspective. *Dis Model Mech* 2013;6:1325-1338.
- 32 Yamakami I, McIntosh TK: Alterations in regional cerebral blood flow following brain injury in the rat. *J Cereb Blood Flow Metab* 1991;11:655-660.
- 33 Schaible EV, Windschugl J, Bobkiewicz W, Kaburov Y, Dangel L, Kramer T, Huang C, Sebastiani A, Luh C, Werner C, Engelhard K, Thal SC, Schafer MK: 2-Methoxyestradiol confers neuroprotection and inhibits a maladaptive HIF-1alpha response after traumatic brain injury in mice. *J Neurochem* 2014;129:940-954.

- 34 Hu PJ, Pittet JF, Kerby JD, Bosarge PL, Wagener BM: Acute brain trauma, lung injury, and pneumonia: more than just altered mental status and decreased airway protection. *Am J Physiol Lung Cell Mol Physiol* 2017;313:L1-L15.
- 35 Li B, Mahmood A, Lu D, Wu H, Xiong Y, Qu C, Chopp M: Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1 β level after traumatic brain injury. *Neurosurgery* 2009;65:179-185; discussion 185-176.
- 36 Boche D, Perry VH, Nicoll JA: Review: activation patterns of microglia and their identification in the human brain. *Neuropathol Appl Neurobiol* 2013;39:3-18.
- 37 Orr AG, Orr AL, Li XJ, Gross RE, Traynelis SF: Adenosine A(2A) receptor mediates microglial process retraction. *Nat Neurosci* 2009;12:872-878.
- 38 Masuda T, Croom D, Hida H, Kirov SA: Capillary blood flow around microglial somata determines dynamics of microglial processes in ischemic conditions. *Glia* 2011;59:1744-1753.
- 39 Gebel JM, Brott TG, Sila CA, Tomsick TA, Jauch E, Salisbury S, Khoury J, Miller R, Pancioli A, Duldner JE, Topol EJ, Broderick JP: Decreased perihematomal edema in thrombolysis-related intracerebral hemorrhage compared with spontaneous intracerebral hemorrhage. *Stroke* 2000;31:596-600.
- 40 Jolivel V, Bicker F, Biname F, Ploen R, Keller S, Gollan R, Jurek B, Birkenstock J, Poisa-Beiro L, Bruttger J, Opitz V, Thal SC, Waisman A, Bauerle T, Schafer MK, Zipp F, Schmidt MH: Perivascular microglia promote blood vessel disintegration in the ischemic penumbra. *Acta Neuropathol* 2015;129:279-295.
- 41 McKeating EG, Andrews PJ, Signorini DF, Mascia L: Transcranial cytokine gradients in patients requiring intensive care after acute brain injury. *Br J Anaesth* 1997;78:520-523.
- 42 Rogers J, Lue LF: Microglial chemotaxis, activation, and phagocytosis of amyloid β -peptide as linked phenomena in Alzheimer's disease. *Neurochem Int* 2001;39:333-340.
- 43 Sivilia S, Lorenzini L, Giuliani A, Gusciglio M, Fernandez M, Baldassarro VA, Mangano C, Ferraro L, Pietrini V, Baroc MF, Viscomi AR, Ottonello S, Villetti G, Imbimbo BP, Calza L, Giardino L: Multi-target action of the novel anti-Alzheimer compound CHF5074: *in vivo* study of long term treatment in Tg2576 mice. *BMC Neurosci* 2013;14:44.
- 44 Abraham E: Neutrophils and acute lung injury. *Crit Care Med* 2003;31:S195-199.
- 45 Grommes J, Soehnlein O: Contribution of neutrophils to acute lung injury. *Mol Med* 2011;17:293-307.
- 46 Nakajima T, Suarez CJ, Lin KW, Jen KY, Schnitzer JE, Makani SS, Parker N, Perkins DL, Finn PW: T cell pathways involving CTLA4 contribute to a model of acute lung injury. *J Immunol* 2010;184:5835-5841.
- 47 Hasko G, Xu DZ, Lu Q, Nemeth ZH, Jabush J, Berezina TL, Zaets SB, Csoka B, Deitch EA: Adenosine A2A receptor activation reduces lung injury in trauma/hemorrhagic shock. *Crit Care Med* 2006;34:1119-1125.
- 48 Reutershan J, Cagnina RE, Chang D, Linden J, Ley K: Therapeutic anti-inflammatory effects of myeloid cell adenosine receptor A2a stimulation in lipopolysaccharide-induced lung injury. *J Immunol* 2007;179:1254-1263.
- 49 Sharma AK, Laubach VE, Ramos SI, Zhao Y, Stukenborg G, Linden J, Kron IL, Yang Z: Adenosine A2A receptor activation on CD4 $^{+}$ T lymphocytes and neutrophils attenuates lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 2010;139:474-482.
- 50 Thiel M, Chouker A, Ohta A, Jackson E, Caldwell C, Smith P, Lukashev D, Bittmann I, Sitkovsky MV: Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury. *PLoS Biol* 2005;3:e174.
- 51 Dai SS, Wang H, Yang N, An JH, Li W, Ning YL, Zhu PF, Chen JF, Zhou YG: Plasma glutamate-modulated interaction of A2AR and mGluR5 on BMDs aggravates traumatic brain injury-induced acute lung injury. *J Exp Med* 2013;210:839-851.
- 52 Wolfe MS: gamma-Secretase as a target for Alzheimer's disease. *Adv Pharmacol* 2012;64:127-153.
- 53 Imbimbo BP, Giardino GA: gamma-secretase inhibitors and modulators for the treatment of Alzheimer's disease: disappointments and hopes. *Curr Top Med Chem* 2011;11:1555-1570.
- 54 Ross J, Sharma S, Winston J, Nunez M, Bottini G, Franceschi M, Scarpini E, Frigerio E, Fiorentini F, Fernandez M, Sivilia S, Giardino L, Calza L, Norris D, Cicirello H, Casula D, Imbimbo BP: CHF5074 reduces biomarkers of neuroinflammation in patients with mild cognitive impairment: a 12-week, double-blind, placebo-controlled study. *Curr Alzheimer Res* 2013;10:742-753.