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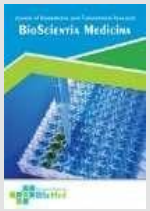

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The Efficacy of NeuroAid™ (MLC601) in Modulating NF-κB Expression and Improving Outcomes in Traumatic Brain Injury: A Preclinical Study

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A B S T R A C T

Background: Traumatic brain injury (TBI) represents a significant global health concern, leading to substantial mortality and long-term disability. The intricate pathophysiology of TBI involves primary mechanical damage followed by a cascade of secondary injury events, including neuroinflammation, apoptosis, and oxidative stress. The nuclear factor kappa B (NF-κB) signaling pathway plays a pivotal role in orchestrating the inflammatory response post-TBI and has emerged as a potential therapeutic target. This preclinical study aimed to investigate the efficacy of NeuroAid™ (MLC601), a traditional herbal medicine, in modulating NF-κB expression and improving outcomes in a rat model of TBI. **Methods:** This study employed a true experimental in vivo design with a post-test only control group. Male Wistar rats (n=18) were randomly divided into two groups: a control group (n=9) subjected to TBI via a weight-drop method, and an experimental group (n=9) subjected to the same TBI procedure followed by intraperitoneal administration of NeuroAid™ (MLC601) at a dose of 2.5 mg/kg body weight at 5 minutes, 8 hours, and 16 hours post-injury. NF-κB expression in brain tissue samples collected 1 hour after the final dose was assessed using immunohistochemistry and quantified by an immunoreactivity score considering both the intensity and percentage of NF-κB expression. **Results:** Immunohistochemical analysis revealed the presence of NF-κB expression in both the nucleus and cytoplasm of neurons in both the control and experimental groups. While the experimental group treated with NeuroAid™ (MLC601) exhibited a lower average immunoreactivity score (0.93) compared to the control group (1.29), the difference in NF-κB expression between the two groups was not statistically significant ($p = 0.122$). **Conclusion:** In this preclinical study using a Wistar rat model of TBI, the administration of NeuroAid™ (MLC601) did not result in a statistically significant reduction in NF-κB expression compared to the untreated control group. Although a trend towards lower NF-κB expression was observed in the NeuroAid™-treated group, further research with larger sample sizes, different dosages, and extended treatment durations is warranted to fully elucidate the potential therapeutic effects of NeuroAid™ (MLC601) in the management of traumatic brain injury.

1. Introduction

Traumatic brain injury (TBI) is a significant and complex neurological condition that results from an external mechanical force impacting the brain. This insult to the brain can lead to a wide spectrum of temporary or permanent impairments, affecting the cognitive, physical, and psychosocial functions of an individual. The global impact of TBI is substantial, posing a major public health challenge due to its significant contribution to morbidity, mortality, and

economic burden on societies worldwide. The prevalence of TBI is a critical concern. It is estimated that approximately 10 million new cases of TBI occur globally each year, highlighting the widespread nature of this condition. Projections have indicated that, by the year 2020, TBI would rank as the third leading cause of the global disease burden, underscoring its growing importance in global health considerations. In the United States, the impact of TBI is particularly evident, where millions of individuals live with disabilities resulting from TBI. It remains a primary cause of death and disability among individuals under the age of 45, a demographic that represents a significant portion of the workforce and family structures. The consequences of TBI are not limited to the immediate aftermath of the injury. They frequently extend into long-term neurological and psychological deficits, affecting both children and adults and presenting ongoing challenges for individuals, their families, and healthcare systems. To fully understand the complexities of TBI, it is essential to consider its intricate pathophysiology. The pathophysiology of TBI is a dynamic process that unfolds in two main phases, primary and secondary injury. The primary injury phase is the direct result of the mechanical forces applied to the brain tissue at the time of the initial impact. These forces can cause immediate and irreversible structural damage, including contusions (bruising of the brain tissue), lacerations (tearing of brain tissue), diffuse axonal injury (damage to nerve fibers), and intracranial hemorrhage (bleeding within the skull). The extent and nature of this primary damage depend on various factors, such as the force of the impact, the mechanism of injury, and the specific areas of the brain affected.[1-3]

While primary injury establishes the initial damage, the secondary injury phase plays a critical role in the subsequent progression of TBI. Secondary injury is a complex cascade of cellular and molecular events that evolve over hours and days following the primary insult. These secondary processes contribute significantly to the overall brain damage and the resulting functional deficits observed in TBI patients. The key mechanisms involved in secondary injury include neuroinflammation, oxidative stress, excitotoxicity, apoptosis (programmed cell death), and disruption of the blood-brain barrier (BBB). These processes are interconnected and can amplify the initial damage, leading to further neuronal dysfunction and loss. Neuroinflammation, a prominent component of the secondary injury response, is a complex process with a dual role in TBI pathophysiology. An appropriate and controlled inflammatory response is essential for the initiation of tissue repair and remodeling processes in the injured brain. However, in the context of TBI, the inflammatory response can often become excessive and prolonged, leading to detrimental effects. This dysregulated inflammation can exacerbate brain edema (swelling), disrupt the integrity of the blood-brain barrier, and contribute to further neuronal injury, thereby worsening the overall outcome. The inflammatory cascade involves the release of various pro-inflammatory mediators, including a range of cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6. These signaling molecules contribute to the complex interplay of inflammatory processes that can either aid in recovery or exacerbate damage following TBI.[4-6]

The nuclear factor kappa B (NF-κB) signaling pathway has been identified as a critical regulator of the inflammatory response following TBI. Previous research has consistently demonstrated that the expression of acute-phase cytokines, which play a central role in the inflammatory cascade, is largely regulated by the NF-κB signaling pathway. This pathway is rapidly activated following TBI, initiating a series of downstream events that contribute to the inflammatory response. The NF-κB family of transcription factors plays a central role in regulating a wide array of cellular processes beyond inflammation, including immune responses, cell proliferation, and apoptosis. In the context of TBI, the activation of NF-κB leads to the transcription of pro-inflammatory genes, further contributing to the neuroinflammatory cascade that amplifies secondary

brain damage. Given its pivotal role in mediating the detrimental effects of inflammation, the NF-κB signaling pathway has emerged as a promising therapeutic target for mitigating secondary injury following TBI. In the pursuit of effective therapeutic interventions for TBI, there is growing interest in exploring the potential of traditional medicines. NeuroAid™ (MLC601) is one such traditional herbal medicine formulation that has gained attention for its potential neuroprotective and neurorestorative properties. This formulation comprises a combination of both herbal and non-herbal extracts and has been traditionally used in China and other parts of Asia, primarily for post-stroke recovery. Preclinical studies have provided evidence suggesting that MLC601 possesses neuroprotective and neurorestorative properties in experimental models of ischemic stroke and TBI. Notably, these studies have shown that MLC601 can reduce phosphorylated NF-κB levels, which is a key indicator of NF-κB activation. Furthermore, MLC601 has been shown to inhibit neutrophil recruitment, a process that can contribute to inflammation and oxidative stress, both of which are significant factors in secondary brain injury. Given the significant role of NF-κB in the complex pathophysiology of TBI and the potential of MLC601 to modulate its activity, there is a strong rationale for further investigation in this area.[7-10] This preclinical study was designed to address this need by investigating the efficacy of NeuroAid™ (MLC601) in modulating NF-κB expression and potentially improving outcomes in a rat model of traumatic brain injury.

2. Methods

This study was designed as a true experimental in vivo investigation. The study utilized a post-test only control group design. In this experimental setup, a controlled traumatic brain injury was induced in a group of Wistar rats. Following the injury, the rats were randomly assigned to either the control group, which received a placebo treatment, or the experimental group, which was administered NeuroAid™ (MLC601). The primary outcome measure for this study was the level of NF-κB expression within the brain tissue. This expression was assessed at a predetermined time point following the induction of injury and the subsequent treatment.

The study was conducted at the LPPT UGM research facility in February 2022. The study utilized male Wistar rats (*Rattus norvegicus*) as the experimental subjects. The rats were selected to be 2–3 months of age, with a weight range of 150–200 grams. A total of eighteen rats were included in the study. These rats were randomly divided into two groups: a control group and a treatment group, with nine rats in each group. The animals were housed in standard laboratory conditions. These conditions included a 12-hour light/dark cycle and ad libitum access to food and water. All animal care and experimental procedures adhered to the ethical guidelines established by the Universitas Gadjah Mada (UGM).

To ensure the consistency and validity of the study, specific inclusion and exclusion criteria were defined. Inclusion criteria; The rats were required to be 2–3 months of age; The rats needed to have a body weight between 150–200 grams; The rats were required to exhibit active movement and show no apparent neurological deficits before the experiment began; The rats could not have any anatomical defects. Exclusion criteria; Any test animal that died during the study period for reasons unrelated to the experimental procedure was excluded.

The induction of traumatic brain injury in the experimental animals was achieved using a weight-drop impact method. Each rat was initially anesthetized through an intraperitoneal injection of ketamine at a dosage of 80 mg/kg, combined with xylazine at a dosage of 10 mg/kg. The effectiveness of the anesthesia was confirmed before proceeding with the injury induction. Following the confirmation of adequate anesthesia, the rat's head was secured in a stereotaxic frame to ensure precise and consistent administration of the injury. A single strike was administered to the exposed skull. The strike was

centered between the bregma and lambda points on the sagittal suture. The impact was generated by a 245-gram weight dropped from a height of 35 cm, with the weight dropped at a 90° angle. This particular method is widely employed to induce a moderate closed-head injury in rodents, effectively mimicking certain aspects of the pathophysiology observed in human TBI.

The treatment group, designated as K1, received NeuroAid™ (MLC601) through intraperitoneal injection. The dosage of NeuroAid™ (MLC601) was 2.5 mg/kg of body weight. The administration of the treatment followed a specific schedule. The first dose was administered 5 minutes post-impact. Subsequent doses were given at 8 hours and 16 hours after the initial injury. In contrast, the control group, designated as N, received an equivalent volume of sterile saline solution. The saline solution was administered at the same time points as the NeuroAid™ (MLC601) in the treatment group. The total duration of the treatment period for both the control and the treatment groups was one day.

One hour following the final administration of either NeuroAid™ (MLC601) or saline, the rats were subjected to deep anesthesia. After the induction of deep anesthesia, the rats underwent transcardial perfusion. The perfusion process involved the use of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. After the perfusion, the brains were carefully extracted from the rats. The extracted brains were then post-fixed in 4% paraformaldehyde overnight at a temperature of 4°C. Following the post-fixation process, the brains were cryoprotected. This cryoprotection was achieved by immersing the brains in a 30% sucrose solution until they sank. The brains were then sectioned into coronal sections using a cryostat. These sections had a thickness of 30 µm. The sections were stored at a temperature of -20°C until they were used for immunohistochemical analysis.

Immunohistochemical staining was performed on the prepared brain tissue sections. This staining procedure was conducted to assess the expression of NF-κB within the brain tissue sections. The sections were initially washed with PBS. Following the wash, the sections were incubated in a blocking solution for 1 hour at room temperature. The blocking solution consisted of 5% normal goat serum in PBS with 0.3% Triton X-100. After the blocking step, the sections were incubated overnight at 4°C with a primary antibody. The primary antibody used was against the NF-κB p65 subunit, with a dilution of 1:200 (Abcam, Cambridge, UK). The next day, the sections were washed again with PBS. Following this wash, they were incubated with a biotinylated secondary antibody for 1 hour at room temperature. The secondary antibody was used at a dilution of 1:200 (Vector Laboratories, Burlingame, CA, USA). After another wash with PBS, the sections were incubated with an avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) for 1 hour at room temperature. The peroxidase activity was then visualized. This visualization was achieved using 3,3'-diaminobenzidine (DAB) as the chromogen. The sections were then dehydrated using graded ethanol solutions. Following dehydration, the sections were cleared in xylene. Finally, the sections were coverslipped with DPX mounting medium to preserve the staining and prepare them for microscopic examination.

The stained brain sections were examined using a light microscope (Olympus BX51). The sections were examined at a magnification of 400x. For each brain tissue sample, a total of six randomly selected observation areas within the peri-contusional cortex were examined. This examination was conducted to ensure that the sampling of the injured tissue was representative. The expression of NF-κB was identified by brown staining observed in the cell nuclei or cytoplasm of neurons. To quantify the expression of NF-κB, an immunoreactivity score was determined. This score was calculated by multiplying the intensity of NF-κB expression by the percentage of NF-κB expression in each observation area. The intensity score was graded on a scale from 0 to 3. In this scale, 0 represented no staining, 1 represented weak staining, 2 represented moderate staining, and 3

represented strong staining. The interpretation of the intensity score was aided by comparison with a positive control image. This positive control image was obtained following the standard product protocol for the staining product, helping to minimize subjectivity in the assessment. The percentage score was determined by comparing the number of brown-stained cells to the total number of cells within a single observation area. The average immunoreactivity score for each animal was calculated. This calculation involved averaging the scores obtained from the six observation areas. This approach to quantifying immunohistochemical staining in brain tissue is consistent with methodologies used in previous studies.

The data that was analyzed consisted of the immunoreactivity scores for NF-κB expression. These scores were obtained from the brain tissue of both the control and the treatment groups. Before conducting statistical analysis, the normality of the data distribution for each group was assessed. The Shapiro-Wilk test was used for this assessment. Following the normality assessment, the homogeneity of variances between the two groups was assessed. Levene's test was used to assess the homogeneity of variances. If the data distribution was found to be normal and the variances were homogeneous, an independent t-test was used. The independent t-test was used to compare the mean immunoreactivity scores between the control and treatment groups. Statistical significance was set at a p-value of less than 0.05 ($p < 0.05$). All statistical analyses were performed using SPSS software, specifically, version 25.0.

3. Results

Table 1 presents the results of statistical tests conducted to assess the fundamental assumptions required for further data analysis, specifically regarding the distribution of the data and the variability within the groups. These assumptions are critical for ensuring the validity of subsequent statistical tests, such as the independent t-test, which was used to compare the groups. The table is divided into sections, each addressing a specific aspect of these assumptions. The first section details the results of the Shapiro-Wilk Normality Test, a test used to determine if the data within each group follows a normal distribution, which is a common assumption for many parametric statistical tests. The Shapiro-Wilk test was applied separately to the control group and the treatment group. For the control group, the Shapiro-Wilk test yielded a p-value of 0.361. This p-value is greater than the conventional significance level of 0.05. In statistical terms, a p-value above 0.05 indicates that there is insufficient evidence to reject the null hypothesis. The null hypothesis in the Shapiro-Wilk test is that the data is normally distributed. Therefore, the result is interpreted as "Not significantly different from normal," implying that the data in the control group does not significantly deviate from a normal distribution. Similarly, for the treatment group, the Shapiro-Wilk test produced a p-value of 0.114. This p-value is also greater than 0.05. Consequently, the conclusion drawn is the same: "Not significantly different from normal." This indicates that the data in the treatment group also does not show a significant deviation from a normal distribution. The second section of the table presents the result of Levene's Test, which is used to assess the homogeneity of variances between the groups. Homogeneity of variances means that the variability or spread of data is similar across the different groups being compared. This is another crucial assumption for the validity of tests like the independent t-test. Levene's Test was applied to both groups, and the resulting p-value was 0.253. Since this p-value is greater than 0.05, it suggests that the variances between the control group and the treatment group are not significantly different. In other words, the variability of the data in the two groups is considered to be similar. The result is stated as "Variances are homogeneous," confirming that this assumption is met.

Table 1. The normality and homogeneity of variance tests.

Test	Group	p-value	Result
Shapiro-Wilk Normality Test	Control Group	0.361	Not significantly different from normal
	Treatment Group	0.114	Not significantly different from normal
Levene's Test	Both Groups	0.253	Variances are homogeneous

Table 2 presents a comparison of NF-κB expression levels between the control group and the treatment group following the experimental intervention. The table provides key statistical measures that help in understanding the effect of the treatment on NF-κB expression. The table is organized into several columns. The first column, labeled "Group," identifies the two experimental groups being compared: the Control Group and the Treatment Group. The Control Group represents the group that did not receive the experimental treatment (NeuroAid™), while the Treatment Group represents the group that did receive the treatment. The second column, "Mean Immunoreactivity Score," presents the average score for NF-κB expression in each group. The immunoreactivity score is a quantitative measure of the amount of NF-κB protein detected in tissue samples. A higher mean immunoreactivity score indicates a higher level of NF-κB expression. The Control Group has a mean immunoreactivity score of 1.29, while the Treatment Group has a mean immunoreactivity score of 0.93. This shows that, on average, the Treatment Group exhibited a lower level of NF-κB expression compared to the Control Group. The third column, "Standard Deviation," indicates the degree of variability or dispersion of the data within each group. A smaller standard deviation suggests that the data points are clustered closely around the mean, indicating less variability. The Control Group has a standard deviation of 0.35, and the Treatment Group has a standard deviation of 0.28. These values provide a measure of the consistency of the NF-κB expression levels within each group. The fourth column, "p-value," is a critical statistical measure that indicates the probability of observing the obtained results (or more extreme results) if there were actually no real differences between the groups. In other words, it helps to determine if the observed difference in mean immunoreactivity scores is likely due to the treatment or simply due to random chance. The p-value reported is 0.122. The fifth column, "Significance," provides an interpretation of the p-value in terms of statistical significance. A p-value is typically compared to a predetermined significance level, often set at 0.05. If the p-value is less than 0.05, the result is considered statistically significant, meaning that the observed difference is unlikely to be due to chance. In this case, the p-value of 0.122 is greater than 0.05. Therefore, the result is interpreted as "Not Significant ($p > 0.05$)." This indicates that the observed difference in NF-κB expression between the Control Group and the Treatment Group is not statistically significant, meaning that the evidence does not support a strong conclusion that the treatment (NeuroAid™) had a statistically significant effect on reducing NF-κB expression.

Table 2. The comparison of NF-κB expression between the control and treatment groups.

Group	Mean immunoreactivity score	Standard deviation	p-value	Significance
Control Group	1.29	0.35	0.122	Not Significant ($p > 0.05$)
Treatment Group	0.93	0.28		

4. Discussion

To fully appreciate the implications of this study's findings, it is essential to consider the well-established and multifaceted role of the nuclear factor kappa B (NF-κB) in the complex pathophysiology of traumatic brain injury (TBI). The intricate cascade of events that unfold following a TBI involves a complex interplay of molecular and cellular mechanisms, and NF-κB stands as a central mediator in many of these processes, particularly in the context of neuroinflammation. Understanding the precise functions of NF-κB in TBI is crucial for interpreting the results of studies investigating potential therapeutic interventions targeting this pathway. Following a traumatic brain injury, the activation of NF-κB is a critical event that occurs relatively rapidly in the secondary injury cascade. This cascade represents the progression of damage to the brain that evolves over time after the initial mechanical insult. Unlike the primary injury, which results directly from the mechanical forces at the time of impact and causes immediate structural damage, the secondary injury cascade involves a series of complex cellular and molecular events that contribute significantly to the overall extent of brain damage and the resulting functional deficits. NF-κB's role in this secondary injury cascade is predominantly pro-inflammatory. The activation of NF-κB leads to a marked increase in the production and release of a variety of pro-inflammatory mediators. These mediators include a wide array of cytokines, which are signaling molecules that facilitate communication between cells, and chemokines, which are a subset of cytokines that attract immune cells to the site of injury. This surge in pro-inflammatory signaling molecules contributes significantly to the inflammatory response within the brain tissue. It is important to recognize that the inflammatory response is not inherently detrimental. In fact, an appropriate and well-regulated inflammatory response is essential for the initiation of tissue repair and remodeling processes in the injured brain. The initial inflammatory processes are part of the brain's attempt to heal and restore itself after the trauma. However, in the context of TBI, this inflammatory response often becomes dysregulated, excessive, and prolonged. Instead of aiding in recovery, it can contribute to further damage and exacerbate the overall injury. The dysregulation of the inflammatory response, largely mediated by NF-κB, plays a significant role in several detrimental processes that occur following TBI. These processes collectively contribute to neuronal dysfunction, cell death, and the long-term neurological deficits observed in TBI patients. A key mechanism through which NF-κB contributes to secondary injury is the disruption of the blood-brain barrier (BBB). The blood-brain barrier is a highly selective semipermeable border of cells that protects the central nervous system (CNS). It separates the circulating blood from the brain and extracellular fluid in the CNS. The BBB is crucial for maintaining the delicate microenvironment of the brain, regulating the entry of substances into the brain tissue, and preventing the entry of harmful substances such as pathogens and toxins. In TBI, the inflammatory response triggered by NF-κB activation can compromise the integrity of the BBB. The increased production of pro-inflammatory mediators disrupts the tight junctions between the cells that make up the BBB, leading to increased permeability. This breakdown of the BBB allows substances that are normally restricted from entering the brain to cross into the brain tissue. The increased permeability of the BBB has several detrimental consequences, one of the most significant being the formation of edema. Edema, or swelling of the brain tissue, is a common and serious complication of TBI. The influx of fluids and proteins into the brain parenchyma due to the disrupted BBB contributes to an increase in intracranial pressure (ICP). Elevated ICP can compress brain tissue, further damaging neurons and glial cells. It can also compromise cerebral blood flow, reducing the delivery of oxygen and essential nutrients to the injured brain. This ischemia, or lack of blood flow, can lead to further neuronal damage and exacerbate the primary injury. Beyond its role in BBB disruption and edema formation, the inflammatory environment

promoted by NF-κB activation also contributes to other detrimental processes within the injured brain. These include oxidative stress, excitotoxicity, and apoptosis, all of which play critical roles in the pathophysiology of TBI. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the ability of the body to detoxify these harmful byproducts. ROS are highly reactive molecules that can damage cellular components, including lipids, proteins, and DNA. In TBI, the inflammatory response and other cellular disturbances lead to an overproduction of ROS, overwhelming the brain's natural antioxidant defenses. NF-κB activation contributes to this oxidative stress by influencing the expression of enzymes involved in ROS production and by suppressing the expression of antioxidant enzymes. Excitotoxicity is another process that contributes to neuronal damage following TBI. It involves the excessive release of excitatory neurotransmitters, such as glutamate, which overstimulate neuronal receptors. This overstimulation leads to an excessive influx of calcium ions into neurons, triggering a cascade of intracellular events that ultimately result in neuronal dysfunction and cell death. The inflammatory environment mediated by NF-κB can exacerbate excitotoxicity by impairing glutamate reuptake mechanisms and increasing the vulnerability of neurons to excitotoxic damage. Apoptosis, or programmed cell death, is a tightly regulated process that plays a crucial role in normal development and tissue homeostasis. However, in TBI, apoptosis is dysregulated and contributes to the loss of neurons and other brain cells. The inflammatory signaling mediated by NF-κB can promote apoptosis through various mechanisms, including the activation of death receptors and the upregulation of pro-apoptotic proteins. Given the central and multifaceted role of NF-κB in mediating these detrimental effects following TBI, it has emerged as a promising therapeutic target for interventions aimed at mitigating secondary brain injury and ultimately improving neurological outcomes in TBI patients. Strategies that can effectively modulate NF-κB activation and its downstream signaling pathways hold the potential to reduce neuroinflammation, protect the integrity of the BBB, minimize edema formation, attenuate oxidative stress and excitotoxicity, and ultimately reduce neuronal loss. The rationale for investigating NeuroAid™ (MLC601) in this context stems directly from previous research that has suggested its potential to modulate NF-κB activity and exert neuroprotective effects in other neurological conditions. If MLC601 can effectively modulate NF-κB signaling in TBI, it could represent a valuable therapeutic tool in the treatment of this complex and devastating condition. The potential benefits of such modulation are significant, addressing key aspects of the secondary injury cascade that contribute to long-term disability and mortality following TBI.[11-13]

Previous studies have extensively explored the effects of NeuroAid™ (MLC601) in various neurological conditions, and this existing body of research provides a crucial foundation for the current investigation into its potential therapeutic role in traumatic brain injury. Understanding the effects of MLC601 in other contexts allows for a more informed interpretation of the present study's findings and helps to contextualize the observed trend in NF-κB modulation within a broader perspective. Notably, a significant portion of the previous research has focused on the neurorepair properties of MLC601, particularly in relation to stroke. Research has consistently demonstrated the neurorepair properties of MLC601 in both in vitro studies, which are conducted in a laboratory setting using cells or tissues, and in vivo studies, which are conducted in living organisms, including animal models of stroke. These studies have collectively suggested that MLC601 possesses the capacity to promote neuronal plasticity. Neuronal plasticity refers to the ability of the nervous system to change its structure and function in response to experience or injury. This process is fundamental to recovery following neurological damage, as it allows for the reorganization of neural networks and the restoration of lost functions. The evidence from these stroke

studies indicates that MLC601 can play a beneficial role in enhancing functional recovery following ischemic injury. Ischemic injury occurs when there is a restriction of blood supply to a part of the brain, leading to oxygen deprivation and subsequent tissue damage, as seen in stroke. Furthermore, in addition to investigating the therapeutic effects of MLC601, significant research efforts have been directed towards elucidating the underlying mechanisms of action of MLC601 and a related formulation, MLC901. These mechanistic investigations have provided valuable insights into how these formulations exert their effects at the molecular and cellular level. Notably, studies have revealed their ability to significantly reduce phosphorylated NF-κB levels in experimental models of ischemic stroke. Phosphorylation is a critical biochemical process that involves the addition of a phosphate group to a protein. In the case of NF-κB, phosphorylation is a key step in its activation. When NF-κB is phosphorylated, it undergoes conformational changes that allow it to translocate to the nucleus and initiate the transcription of target genes. Therefore, a reduction in phosphorylated NF-κB levels indicates a decrease in its active state, suggesting a dampening of its pro-inflammatory signaling. This finding, observed in previous research, that MLC601 and MLC901 can reduce phosphorylated NF-κB levels, carries significant implications. It strongly suggests that MLC601 possesses anti-inflammatory effects, at least in the context of stroke, and that these effects are mediated, at least in part, by modulating NF-κB signaling. Given the critical role of NF-κB in the inflammatory response following various neurological insults, including TBI, this evidence provides a rationale for exploring the potential of MLC601 to modulate NF-κB activity in the context of TBI as well. In addition to its effects on NF-κB signaling, previous research has also shown that MLC901 can influence other key components of the inflammatory cascade. Specifically, MLC901 has been shown to inhibit the upregulation of Prx6 and to suppress TLR4 signaling. Prx6, or peroxiredoxin 6, is an enzyme that has been implicated in post-ischemic inflammation. Its upregulation contributes to the inflammatory processes that occur following ischemic stroke. TLR4, or Toll-like receptor 4, is a transmembrane protein that plays a crucial role in initiating inflammatory responses and activating NF-κB. TLR4 is a pattern recognition receptor that recognizes specific molecules associated with pathogens or tissue damage, triggering a signaling cascade that leads to the activation of NF-κB and the subsequent production of pro-inflammatory mediators. By targeting these upstream components of the inflammatory cascade, namely Prx6 and TLR4, MLC901, and potentially MLC601, may exert a broader and more comprehensive anti-inflammatory effect. This broader anti-inflammatory action could be particularly beneficial in the context of TBI, where the inflammatory response is complex and involves multiple interacting pathways. The ability to modulate different aspects of the inflammatory cascade could translate to a more effective therapeutic intervention. It is also important to consider the evidence regarding the effects of MLC601 and related formulations in the specific context of traumatic brain injury. While the current study focuses on the effects of MLC601 on NF-κB expression in an experimental TBI model, previous research has explored its effects in TBI patients and in preclinical models. This prior research provides valuable context for interpreting the current findings and suggests potential mechanisms through which MLC601 might exert its therapeutic effects in TBI. Previous research has indicated that MLC601 can promote neuronal plasticity and regeneration in patients with moderate TBI who received non-surgical treatment. This observation suggests that MLC601 might have the capacity to support neurological recovery following TBI through mechanisms that extend beyond simply reducing inflammation. While inflammation plays a significant role in secondary injury, TBI also involves other processes that contribute to long-term deficits, such as neuronal damage and impaired repair mechanisms. The ability of MLC601 to promote neuronal plasticity and regeneration suggests that it might address these other aspects of TBI pathophysiology, potentially

leading to improved functional outcomes. In addition to clinical studies, preclinical studies using animal models of TBI have also provided insights into the potential therapeutic effects of MLC601. These studies have shown that MLC601 can attenuate neurological motor deficits, which are common and debilitating consequences of TBI. Furthermore, MLC601 has been shown to reduce brain apoptosis, the programmed cell death that contributes to neuronal loss following TBI. Moreover, preclinical research has demonstrated that MLC601 can modulate microglial activation associated with cerebral contusion caused by TBI. Microglia are the resident immune cells of the central nervous system. They play a complex and often dual role in TBI pathophysiology. On one hand, microglia can contribute to clearing cellular debris and promoting tissue repair. On the other hand, excessive or prolonged microglial activation can exacerbate inflammation and neuronal damage. The ability of MLC601 to modulate microglial activation suggests that it can influence the inflammatory environment in the brain following TBI, potentially promoting a more beneficial balance between neuroprotection and neuroinflammation. The current study aimed to build upon this existing body of evidence by specifically investigating the effect of MLC601 on NF-κB expression in a controlled experimental model of TBI in rats. This focus on NF-κB modulation allows for a more targeted assessment of the drug's potential anti-inflammatory effects in the context of TBI. While the results of this study, as presented earlier, did not demonstrate a statistically significant reduction in NF-κB expression, it is crucial to emphasize that the observed trend aligns with previous findings. The trend towards lower NF-κB expression in the NeuroAid™-treated group is consistent with the evidence from previous studies suggesting that MLC601 can indeed modulate inflammatory pathways. It is important to acknowledge that discrepancies in statistical significance can arise from a variety of factors. These factors include differences in experimental design, variations in the specific TBI models employed, or differences in the outcome measures used to assess the effects of the treatment. Each of these methodological variables can influence the results of a study and contribute to variations in the observed effects of a drug. These discrepancies highlight the need for further research to fully elucidate the complex effects of MLC601 in TBI and to determine the optimal conditions for its therapeutic application.[14-17]

Several potential factors could have influenced the outcome of this study and contributed to the lack of statistical significance in the observed reduction of NF-κB expression. These factors warrant careful consideration as they can provide valuable insights for future research directions and the interpretation of the current findings. One crucial factor is the sample size used in the study. With nine animals per group, the study might have been underpowered to detect a smaller, yet potentially meaningful, effect of NeuroAid™ (MLC601) on NF-κB expression. Statistical power refers to the probability of correctly rejecting the null hypothesis when it is false, meaning the ability to detect a real effect if it exists. A small sample size can lead to lower statistical power, increasing the risk of a Type II error, which is failing to detect a true effect. In preclinical research, determining the appropriate sample size is a critical step in the study design. It involves considering factors such as the expected effect size, the desired level of statistical power, and the variability within the experimental groups. Post-hoc power analyses can be performed to estimate the achieved power of a study after the data has been collected. However, it is generally preferable to conduct power analyses a priori, during the study design phase, to ensure that the sample size is adequate to detect the anticipated effect. Increasing the sample size in future studies could potentially increase the statistical power and improve the ability to detect a significant effect of NeuroAid™ (MLC601) on NF-κB expression. However, it is also important to consider the ethical implications of using larger numbers of animals in research and to carefully weigh the potential benefits of increased statistical power against the welfare of the animals. Another critical factor that

could have influenced the study's outcome is the treatment regimen employed, including the dosage of NeuroAid™ (MLC601) and the timing of its administration. In this study, NeuroAid™ (MLC601) was administered three times within the first 16 hours following the induction of TBI, and NF-κB expression was assessed 1 hour after the final dose. This relatively short treatment duration and early assessment time point might not have been optimal for observing the full extent of NF-κB modulation by MLC601. The inflammatory response following TBI is a dynamic process that evolves over time. NF-κB activation and the subsequent upregulation of pro-inflammatory mediators occur in a temporal pattern, with different phases of inflammation dominating at different time points. It is possible that the peak effect of MLC601 on NF-κB modulation occurs at a later time point than the one assessed in this study. Optimizing the treatment regimen, including exploring different dosages, administration frequencies, and treatment durations, could potentially reveal a more pronounced effect of NeuroAid™ (MLC601) on NF-κB expression. Future studies could investigate the time course of NF-κB modulation by MLC601, assessing its expression at multiple time points following TBI and treatment. This would provide a more comprehensive understanding of the temporal dynamics of the drug's effect and potentially identify the optimal window for therapeutic intervention. The use of an animal model of TBI introduces inherent variability, which can also influence the study's outcome. While the weight-drop method employed in this study is a commonly used technique to induce TBI in rodents, it can still result in variations in the severity and pattern of injury. Factors such as the precise point of impact, the angle of impact, and individual differences in the animals' response to the injury can all contribute to variability in the extent of brain damage and the subsequent inflammatory response. This variability can make it more challenging to detect a consistent effect of the treatment, especially with a relatively small sample size. Future studies could consider employing more refined TBI models or techniques to reduce variability and ensure a more consistent injury across animals. Additionally, careful monitoring and characterization of the TBI in each animal, including assessments of neurological function and imaging studies, could provide valuable information for interpreting the study's findings. The primary outcome measure in this study was the immunoreactivity score for NF-κB expression, assessed using immunohistochemistry. While immunohistochemistry is a widely used technique for visualizing and quantifying protein expression in tissue samples, it has some limitations. The quantification of staining intensity and the determination of immunoreactivity scores can be subjective and may vary depending on the observer. Furthermore, the localization of NF-κB within the cells can provide important information about its activation state. Under normal conditions, NF-κB is primarily located in the cytoplasm, bound to inhibitory proteins. Upon activation, it translocates to the nucleus, where it initiates gene transcription. The presence of NF-κB in both the cytoplasm and the nucleus, as observed in this study, suggests an ongoing inflammatory process in both the control and treated animals following the traumatic brain injury. Future studies could consider using more quantitative and objective methods for assessing NF-κB activation, such as Western blotting or ELISA, which can measure protein levels with greater precision. Additionally, techniques such as immunofluorescence or confocal microscopy can provide more detailed information about the subcellular localization of NF-κB, which could help to better understand the effects of NeuroAid™ (MLC601) on its activation and translocation.[18-20]

5. Conclusion

This preclinical study aimed to investigate the efficacy of NeuroAid™ (MLC601) in modulating NF-κB expression in a rat model of TBI. While the results indicated a trend towards lower NF-κB expression in the treatment group compared to the control group, this difference was not statistically significant. The study acknowledges several limitations that may have influenced the outcome, including the relatively small

sample size, the treatment regimen, the inherent variability of the animal model, and the potential subjectivity of the immunohistochemistry analysis. These factors highlight the complexities of studying TBI and the challenges in definitively establishing the therapeutic efficacy of interventions in preclinical models. Despite the lack of statistical significance, the observed trend aligns with previous research suggesting that NeuroAid™ (MLC601) can modulate inflammatory pathways. Furthermore, previous studies have demonstrated NeuroAid™'s neuroprotective and neurorestorative properties in other neurological conditions, including stroke, by reducing phosphorylated NF-κB levels and inhibiting neutrophil recruitment. These findings provide a rationale for continued investigation into the potential therapeutic benefits of NeuroAid™ in TBI. Future research should focus on addressing the identified limitations to provide a more comprehensive understanding of NeuroAid™'s effects on NF-κB modulation and its potential to improve outcomes following TBI. Studies with larger sample sizes, optimized treatment regimens, refined TBI models, and more quantitative outcome measures are warranted to further elucidate the therapeutic potential of NeuroAid™ in mitigating the detrimental effects of neuroinflammation and promoting recovery after TBI.

6. References

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