MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ANTI-MALARIAL AND CYTOKINE-MODULATING EFFECTS OF SODIUM TUNGSTATE IN MURINE MODELS OF CEREBRAL AND SEVERE MALARIA INVOLVE INHIBITION OF GSK3B MEDIATED VIA ACTIVATION OF BOTH ERK AND Akt SIGNALING

Nor Nadia Fadiha Aizuddin[1], Izyanti Ibrahim[1], Rusliza Basir[2], Noor Embi [1] & Hasidah Mohd Sidek [1*]

[1] *Faculty of Science and Technology, Universiti Kebangsaan Malaysia*
[2]*Pharmacology Unit, Department of Human Anatomy, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia*

*Corresponding Author: hasidahms@gmail.com

History
Received: 17th October 2019
Accepted: 28th December 2019

Keywords:

Sodium tungstate; GSK3β; ERK; cytokines; Plasmodium berghei

Abstract

Malaria remains a major global health problem, associated with high morbidity and mortality. Dysregulated production of pro-inflammatory mediators and cytokines is one of the factors that contribute to the pathogenesis of severe and cerebral malaria. Glycogen synthase kinase-3 (GSK3) which plays a pivotal role in regulating the production of pro- and anti-inflammatory cytokines is a potential target for anti-malarial therapeutics. Sodium tungstate (Na_2WO_4), which exhibits strong normoglycaemic effects, has been reported to cause phosphorylation of GSK3β. The present study aims to evaluate the anti-malarial and cytokine-modulating effects of Na_2WO_4 using *Plasmodium berghei* ANKA (PbA) and *Plasmodium berghei* NK65 (PbN), established models for cerebral and severe malaria respectively. Intraperitoneal administration of Na_2WO_4 into PbA- or PbN-infected mice resulted in dose-dependent chemo-suppression of parasitaemia. At 50 mg/kg body weight, Na_2WO_4 treatment resulted in suppression exceeding 60% and improved median survival time of infected mice (14 and 17 days for PbA- and PbN-infected animals compared to 7.5 and 12 days in non-treated control respectively). Na_2WO_4 treatment also significantly decreased ($p>0.05$) the serum levels of pro-inflammatory cytokines (TNF-α, IFN-ɤ and IL-18) whilst significantly increasing levels of anti-inflammatory cytokines (IL-10 and IL-4) in mice from both models of malarial infection. In addition, western analysis revealed that in the brain (PbA) and liver (PbN) of infected animals administered with Na_2WO_4, the intensities of pGSK3β (Ser9), pERK1/2 (Thr202/Tyr204), pp90RSK (Ser380) and pAkt (Ser473) were increased significantly whilst pNF-κB (Ser536) was decreased compared to controls. Taken together our results indicate that anti-malarial and cytokine-modulating effects of Na_2WO_4 observed involve at least in part the regulation of NF-κB through inhibition of GSK3β mediated via activation of ERK/p90RSK as well as Akt signaling. Our findings reiterate the importance of GSK3β as a molecular target for anti-malarial therapeutics.

INTRODUCTION

Malaria, a disease caused by the *Plasmodium* parasite and associated with high morbidity and mortality remains a major health problem. Worldwide resistance of the pathogen to current anti-malarials is hampering efforts to eliminate the disease. Dysregulated production of pro-inflammatory mediators and cytokines amongst other factors play important roles in the pathogenesis of severe and cerebral malaria. Host response to combat or limit parasitaemia is also dependent on the balance between pro- and anti-inflammatory cytokine productions. These inflammatory responses need to be regulated accordingly to avoid tissue damage due to overwhelming cytokine responses. The pathophysiology of severe and cerebral malaria is still not fully understood. However, it is known that elevated levels of pro-inflammatory cytokines during infection is a contributing factor responsible for much of the immunopathology seen during symptomatic disease [1]. Overwhelming pro-inflammatory responses can lead not only to organ damage but is also a cause of death in severe [2] and cerebral malaria [3]. As such anti-malarial therapeutics that can mitigate the dysregulation of inflammatory response by regulating the cytokine balance may be developed.

Glycogen synthase kinase-3β (GSK3β) has a pivotal role in regulating the production of pro- and anti-inflammatory cytokines [4] and is a promising therapeutic target to control inflammation [5]. GSK3 is a serine/threonine kinase, initially identified by Embi et al., [6] for its role in the regulation of

glycogen metabolism. GSK3 is a point of convergence for numerous signaling pathways including the PI3K/Akt and MAPK pathways [7-8]. This kinase is now known to be involved in the regulation of many crucial cellular processes such as protein synthesis, transcription factors, embryonic development, apoptosis, cell cycle control, cell differentiation, cell mobility and migration; and in the modulation of cytokine levels during inflammation [9]. GSK3 exists as α- and β- isoforms encoded by two highly-related GSK3 genes in mammals [10]. Inhibition of GSK3 involves phosphorylation of Ser21 and Ser9 residues for the α- and β- isoforms respectively [11]. GSK3β inhibition and consequent inactivation of NF-κB, a transcription factor involved in the immune response, is important in the modulation of cytokine production [12]. Dysregulation of GSK3β is implicated in the pathophysiology of many important diseases including diabetes, Alzheimer's disease, bipolar disorder, cancer [13] and inflammation [5].

GSK3β inhibition is associated with host response to viral, fungal and parasitic infections [9]. Zakaria et al., [14] was the first to report chemo-suppressive effects of the GSK3 inhibitor, LiCl in a murine model of malarial infection. Subsequently, Dai et al., [15] reported that LiCl treatment restored neuro-cognitive function in murine experimental cerebral malaria suggesting GSK3β as a plausible adjunctive therapeutic target for the management of cerebral malaria. Previous studies in our laboratory have also shown that LiCl modulated pro- and anti-inflammatory cytokine productions in mice experimentally-infected with chloroquine-sensitive *P. berghei* NK65 strain, and that this effect involved inhibition of liver GSK3β [16-17]. Another inorganic salt, sodium tungstate (Na_2WO_4) has been reported to induce extracellular signal-regulated kinase (ERK) activation and trigger 90 kDa ribosomal s6 kinase (p90RSK) and GSK3β phosphorylations in diabetic rat hepatocytes and human vascular smooth muscle [18-19]. Na_2WO_4 is known to be a potent insulin-mimetic and anti-diabetic agent in several animal models of diabetes and exhibits low toxicity [20]. Recent studies demonstrated that Na_2WO_4 also regulates the expression and secretion of pro- and anti-inflammatory cytokines [21]. In addition, Na_2WO_4 is able to induce secretion of IL-1Ra, an anti-inflammatory factor that neutralises IL-1 similar to that seen in sera from human Type 2 diabetes patients [22]. Unlike LiCl which can cause phosphorylation of GSK3 directly or through activation of its upstream kinase, Akt of the PI3K/Akt axis, Na_2WO_4 reportedly can cause GSK3β phosphorylation through activation of ERK [23-24]. Here we investigate the cytokine-modulating effects of another inorganic salt, Na_2WO_4 in murine models of cerebral and severe malarial infection to evaluate the involvement of GSK3β in the regulation of pathogen-induced inflammatory responses to reiterate the importance of GSK3β as a molecular therapeutic target.

MATERIALS AND METHODS

Experimental animals

Male ICR mice (18±7 g 4-5 week old juvenile mice; or 25±5 g 6-8 week old adult mice) were obtained from the Animal House Complex, Universiti Kebangsaan Malaysia (UKM) and accommodated at the Malaria Infection Laboratory, UKM. The animals were fed with food pellets and water *ad libitum* in a room maintained at 22°C, 50–70% relative humidity and 12 hour light/dark cycle illumination. Permission and approval for animal studies were obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (reference number: FST/2016/HASIDAH/28-SEPT./793-SEPT.-2016-JAN.-2018).

Animal infection studies

Prior to infection studies, survivability of non-infected experimental animals administered with Na_2WO_4 was recorded for 30 days to ascertain that the dosage of Na_2WO_4 used in further four-day suppressive tests will not affect animal survivability. For this, four groups of non-infected mice (n=6) were injected (ip) with different dosages of Na_2WO_4 (10, 30, 50 or 70 mg/kg bw) for four consecutive days. These dosages were selected based on prior toxicity tests using non-infected mice (data not shown). The control group of animals were administered with 0.2 mL of 0.85% saline solution. Mice were observed for gross behavioral changes while body weights were recorded and survivability monitored for 30 days.

Chloroquine-sensitive *P. berghei* NK65 (PbN) strain, MRA-268 was obtained from Malaria Research and Reference Reagent Resource Centre (MR4, Manassas, USA); http://www.beiresources.org, a part of BEI Resources, NIAID, NIH: deposited by Miracle Harrington. Chloroquine-sensitive *P. berghei* ANKA (PbA) strain (MRA-311) was a kind gift from Dr. Rusliza Basir, Universiti Putra Malaysia originally purchased from MR4. Both parasite strains were maintained in ICR mice. Mice (juvenile for PbA infection; adult for PbN infection) were randomly divided into seven groups (n=6) and injected intraperitoneally (ip) with 0.2 mL of infected blood (2×10^7 *P. berghei*-parasitised erythrocytes/mL). Starting at three hours post-infection (day 0), the experimental animals were injected (ip) over four consecutive days with either the anti-malarial reference drug (10 mg/kg body weight (bw) chloroquine) or the GSK3 inhibitor reference (100 mg/kg bw LiCl) or 0.85% saline solution [control group A] or different dosages of Na_2WO_4 (10, 30, 50 or 70 mg/kg bw) [test group B] in 0.2 mL volume. The percentage of blood parasitaemia on day 4 post-infection [25] was determined from thin blood smears of tail blood from each animal. The average percentage of chemo-suppression (PC) was calculated by comparing the percentage of blood parasitaemia levels in control group A and test groups B:

$$PC = [(A - B)/A] \times 100$$

Brain or liver sample preparation and protein extraction

A dosage of Na_2WO_4 that caused chemo-suppression of more than 60% (based on the four-day suppressive test described above) was used to determine the phosphorylation state of GSK3β in brain or liver of infected mice. Mice were randomly divided into six groups (n=6) and injected (ip) with PbA- or PbN-infected erythrocytes (inoculum - 2×10^7 *P. berghei*-parasitised erythrocytes/mL). At three hours post-infection [25] a group of mice were treated with 50 mg/kg bw Na_2WO_4 (effective dosage from the four-day suppressive test), while control (non-infected) and infected (non-treated) groups were injected (ip) with 0.85% saline solution. Chloroquine diphosphate (CQ) and LiCl (a GSK3 inhibitor) were each administered at 10 and 100 mg/kg bw dosages respectively. All groups of mice were sacrificed and brain or liver organs harvested for protein analysis. Protein extraction from brain (for PbA infection) or liver (for PbN infection) was carried out according to Dai et al., [15] using a modified lysis buffer as described by Lee [26]. Protein content of brain or liver samples was determined using the Bradford method [27] with bovine serum albumin (BSA) as standard.

Cytokine analysis

From the four-day suppressive test described above (at day 4 post-infection), blood was taken from the experimental animals by cardiac puncture and further processed to obtain serum. The levels of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-18) and anti-inflammatory cytokines (IL-10 and IL-4) in sera of Na_2WO_4 - treated infected mice and control non-treated infected mice were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Genomax Techonologies, Singapore) (QIAGEN, Germany).

SDS-PAGE and western blot analysis

Equal amounts (40 µg) of brain or liver protein samples were loaded into each well of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (30% w/v acrylamide) [28]. Separated proteins were transferred onto nitrocellulose membranes [29] then blocked using 3% BSA in Tris-buffered saline-Tween 20 (TBST) (0.1% w/v Tween-20 in TBS). Membranes were further probed and incubated overnight at 4°C with relevant primary monoclonal antibodies; anti-GSK3β, anti-phosphoSer9-GSK3β, anti-ERK, anti-phosphoThr202/Tyr204-ERK, anti-RSK, anti-phosphoSer380-p90RSK, anti-Akt, anti-phosphoSer473-Akt, anti-NF-κB and anti-phosphoSer536-NF-κB (Cell Signaling, Danvers, MA, USA) prior to a two-hour incubation with the corresponding secondary antibody (anti-rabbit IgG, HRP-linked antibody (Cell Signaling, Danvers, MA, USA) at room temperature. Membranes were stripped with 0.2 M NaOH and then re-probed with anti-β-actin (Santa Cruz Biotechnology, Littleton, USA) to ensure equal protein loading. Immuno-reactive bands were detected using Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Waltham, MA, USA). Band area intensity was then quantified using a densitometer (Vilber Lourmat 302, Sud Marne-la-Vallée Cedex, France).

Statistical analysis

All data were evaluated using the Student's t-test and log rank tests (for Kaplan-Meier survival analysis). All data were analysed and expressed as mean ± SD except for cytokine levels which were expressed as mean ± SEM. P values of <0.05 between groups were considered as significant.

RESULTS

Na_2WO_4 INHIBITED PARASITAEMIA DEVELOPMENT IN *P. berghei*-INFECTED MICE

Mice administered (ip) with Na_2WO_4 for four consecutive days following injections with *P. berghei*-infected erythrocytes showed dosage-dependent suppression of parasitaemia development **(Table 1 and 2**). At 50 mg/kg bw, Na_2WO_4 inhibited PbA or PbN parasitaemia development in mice by 62.21±3.29% and 61.19±1.24% respectively. Mice administered with the reference anti-malarial drug CQ (10 mg/kg bw) showed nearly 100% suppression of parasitaemia development on day 4 and the animals survived throughout the observation period of 30 days for both PbA-(**Table 1**) and PbN-(**Table 2**) infected mice. The median survival time for Na_2WO_4-treated infected mice was 14 and 17 days for PbA- (**Table 1**) and PbN- (**Table 2**) infected animals compared to 7.5 and 12 days for non-treated controls respectively. We have employed the Kaplan-Meier survival curves (not shown here) to determine the median survival time described earlier for the 50 mg/kg bw Na_2WO_4 treatment. For subsequent studies involving cytokine and western analyses, we employed the dosage of 50 mg/kg bw Na_2WO_4 for treatment as the optimal dose tested based on effective (>60%) chemo-suppression results.

Table 1. Suppressive action of Na_2WO_4 on *P. berghei* ANKA-infected mice

Compound/Drugs	Dosage (mg/kg bw)	Average parasitaemia suppression on day 4 (%)	Median survival time (days)
Na_2WO_4	10	40.14 ± 1.27*	9.5
	30	49.14 ± 0.88*	11*
	50	62.21 ± 3.29*	14*
	70	70.93 ± 6.62*	16.5*
Chloroquine (anti-malarial reference drug)	10	96.70 ± 0.7*	>30*
LiCl (GSK3 inhibitor reference)	100	72.70 ± 4.25*	22.5*
0.85% Saline (control)	0.2 mL	-	7.5

Parasitaemia suppression was calculated on day 4 post-infection and survivability of mice recorded throughout the experimental period (30 days). Data represent mean ± SEM for parasitaemia suppression and median survival time (n = 6).* Significantly different from negative control at P<0.05

Table 2. Suppressive action of Na_2WO_4 on *P. berghei* NK65-infected mice

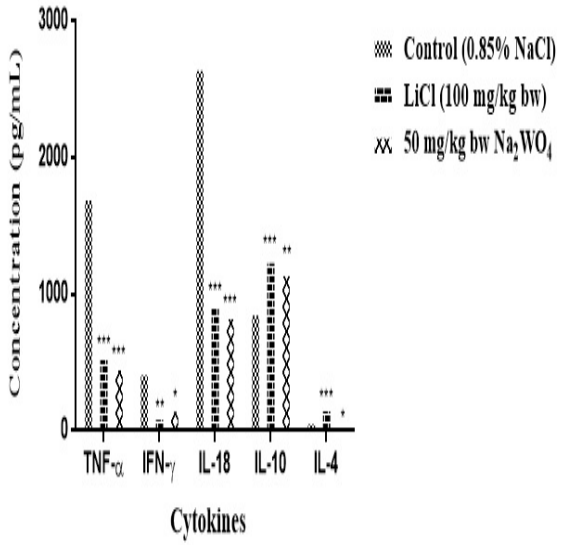
Compound/Drugs	Dosage (mg/kg bw)	Average parasitaemia suppression on day 4 (%)	Median survival time (days)
Na_2WO_4	10	39.67 ± 2.23*	13.5*
	30	47.55 ± 0.86*	15.5*
	50	61.19 ± 1.24*	17*
	70	67.81 ± 3.52*	19.5*
Chloroquine (anti-malarial reference drug)	10	93.70 ± 1.67*	>30*
LiCl (GSK3 inhibitor reference)	100	69.78 ± 4.53*	27*
0.85% Saline (control)	0.2 mL	-	12

Parasitaemia suppression was calculated on day 4 post-infection and survivability of mice recorded throughout the experimental period (30 days). Data represent mean ± SEM for parasitaemia suppression and median survival time (n = 6). * Significantly different from negative control at P<0.05

Na_2WO_4 ADMINISTRATION DECREASED LEVELS OF PRO-INFLAMMATORY CYTOKINES AND INCREASED LEVELS OF ANTI-INFLAMMATORY CYTOKINES IN *P. berghei*- INFECTED MICE

Cytokine assays were carried out to determine the levels of TNF-α, IFN-γ, IL-18, IL-10 and IL-4 in serum from PbA- or PbN-infected mice at day 4 post-infection. Administration of 50 mg/kg bw Na_2WO and 100 mg/kg bw LiCl each showed significantly lowered levels of pro-inflammatory cytokines; TNF-α, IFN-γ and Il-18, and increased significantly levels of anti-inflammatory cytokines; IL-10 and IL-4 in comparison to levels in PbA- or PbN-infected mice (**Figure 1**).

a) PbA



b) PbN

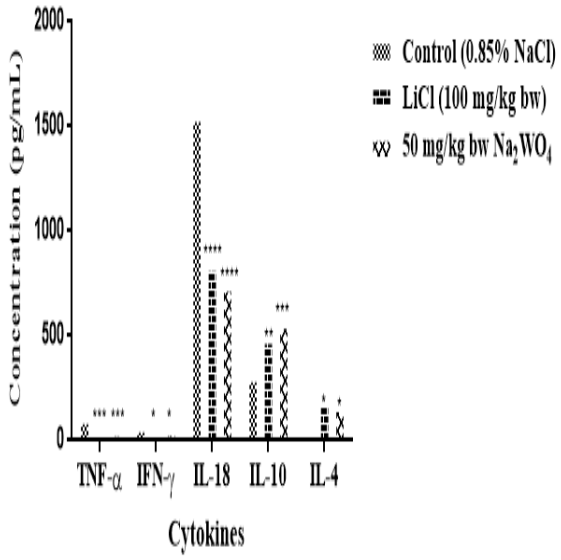


Figure 1. The levels of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-18) and anti-inflammatory cytokines (IL-10 and IL-4) in serum of mice infected with PbA (a) and PbN (b) administered with Na_2WO_4 or LiCl. Data are expressed as mean ± SEM; n = 6 ICR mice per group. Significant difference as compared with control infected group was evaluated at P < 0.05 (*), P < 0.001 (***), P < 0.0001 (****).

Na_2WO_4 ADMINISTRATION INCREASED LEVEL OF PHOSPHORYLATED GSK3β (SER9) IN BRAIN or LIVER OF *P. berghei*- INFECTED MICE

In order to further explain the involvement of GSK3β in the action of Na_2WO_4, we investigated the effects of Na_2WO_4 treatment on the phosphorylation states of GSK3β (Ser9). As compared to the non-treated infected controls, Na_2WO_4 administration resulted in significant ($p<0.05$) 3.3 fold and 2.6 fold increase in brain (**Figure 2** (a)) and liver pGSK3β (Ser9) (Figure (b)) for PbA- and PbN-infected mice respectively. Similarly, LiCl (100 mg/kg bw) also resulted in significant increase of pGSK3β (Ser9). In non-infected animals, we also observed significant increase in pGSK3β (Ser9) albeit with lower fold-change (Data not shown). The results obtained shows that Na_2WO_4 administration caused inhibition of GSK3β in both brain and liver of malaria-infected mice.

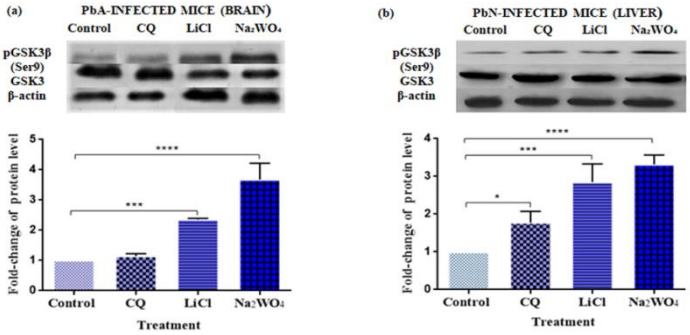


Figure 2. (a) GSK3β phosphorylation levels in brain of PbA-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw) (b) GSK3β phosphorylation levels in liver of PbN-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw). Total GSK3β and pGSK3β (Ser9) from brain or brain were measured and levels of phosphorylated GSK3β were normalised to total levels of GSK3β. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images are shown. Significant differences between tested and control groups were evaluated at P<0.05 (*).

Na_2WO_4 ADMINISTRATION INCREASED LEVEL OF PHOSPHORYLATED Akt (SER473) IN BRAIN or LIVER OF *P. berghei*-INFECTED MICE

We then proceeded to investigate the effect of Na_2WO_4 administration on the phosphorylation of Akt, an upstream component of GSK3β signaling. As compared to the controls, Na_2WO_4 administration resulted in significant ($p<0.05$) 4.5 fold and 1.8 fold increase in brain (**Figure 3** (a)) and liver pAkt (Ser473) (Figure 3 (b)) for PbA- and PbN-infected mice respectively. Similarly, LiCl (100 mg/kg bw) also resulted in significant increase of pAkt (Ser473). In non-infected animals, we also observed significant increase in pAkt (Ser473) albeit with lower fold-change (Data not shown). The results obtained shows that Na_2WO_4 administration caused inhibition of GSK3β in both brain and liver of infected mice mediated through the PI3K/Akt pathway.

Na_2WO_4 ADMINISTRATION INCREASED LEVEL OF PHOSPHORYLATED ERK1/2 (THR202/TYR204) IN BRAIN or LIVER OF *P. berghei*-INFECTED MICE

We next investigated the effects of Na_2WO_4 treatment on the phosphorylation state of ERK1/2 (Thr202/Tyr204) which is also a

possible upstream component of GSK3β signaling. As compared to the controls, Na_2WO_4 administration resulted in significant ($p<0.05$) 8.1 fold and 7.4 fold increase in brain (**Figure 4** (a)) and liver pERK (Thr202/Tyr204) (**Figure 4** (b)) for PbA- and PbN-infected mice respectively when compared to controls. Similarly, LiCl (100 mg/kg bw) significantly increased pERK1/2 (Thr202/Tyr204). In non-infected animals, we also observed significant increase in pERK1/2 (Thr202/Tyr204) with lower fold-change (Data not shown). The results obtained shows that Na_2WO_4 administration induced ERK1/2 activation which led to inhibition of GSK3β in both brain and liver of infected mice.

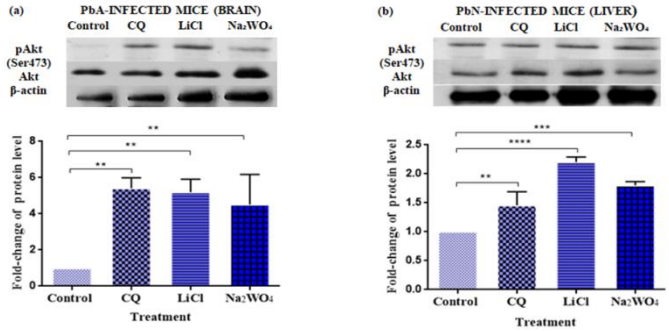


Figure 3. (a) Akt phosphorylation levels in brain of PbA-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw) **(b)** Akt phosphorylation levels in liver of PbN-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw). Total Akt and pAkt (Ser473) from brain or liver were measured and levels of phosphorylated Akt were normalised to total levels of Akt. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images are shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

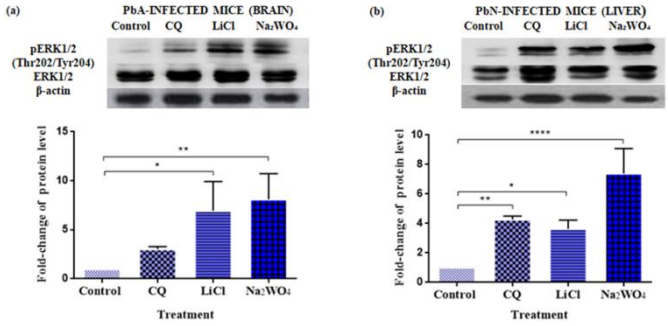


Figure 4. (a) ERK1/2 phosphorylation levels in brain of PbA-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw) (b) ERK1/2 phosphorylation levels in brain of PbN-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw). Total ERK1/2 and pERK1/2 (Thr202/Tyr204) from brain or liver were measured and levels of phosphorylated ERK1/2 were normalised to total levels of ERK1/2. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images are shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

Na_2WO_4 ADMINISTRATION INCREASED LEVEL OF PHOSPHORYLATED p90RSK (SER380) IN BRAIN or LIVER OF *P. berghei*-INFECTED MICE

Next, we investigated the effects of Na_2WO_4 treatment on the phosphorylation state of p90RSK (Ser380), a component of the ERK pathway upstream of GSK3β. As compared to the controls, Na_2WO_4 administration resulted in significant ($p<0.05$) 3.3 fold and 3.4 fold increase in brain (**Figure 5** (a)) and liver p90RSK (Ser380) (**Figure 5** (b)) for PbA- and PbN-infected mice respectively. Similarly, LiCl (100 mg/kg bw) also resulted in significant increase of pp90RSK (Ser380). In non-infected animals, we also observed significant increase in pp90RSK (Ser380) with lower fold-change (Data not shown). The findings indicate that Na_2WO_4 administration induced ERK activation which further resulted in inhibition of GSK3β and the phosphorylation of p90RSK in both brain and liver of infected mice.

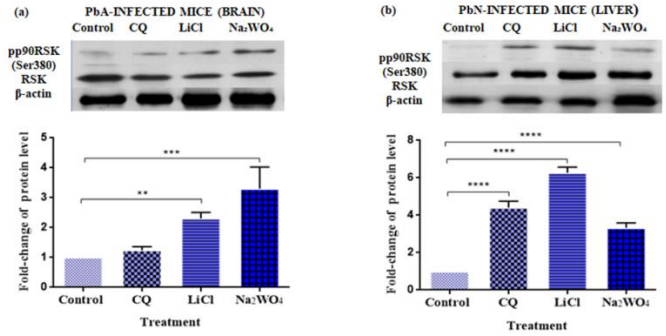


Figure 5. (a) p90RSK phosphorylation levels in brain of PbA-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw) **(b)** p90RSK phosphorylation levels in liver of PbN-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw). Total RSK and pp90RSK (Ser380) from brain or liver were measured and levels of phosphorylated p90RSK were normalised to total levels of p90RSK. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images are shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

Na_2WO_4 ADMINISTRATION DECREASED LEVEL OF PHOSPHORYLATED NF-κB p65 (SER536) IN BRAIN or LIVER OF *P. berghei*-INFECTED MICE

We then studied the effects of Na_2WO_4 administration on the phosphorylation state of NF-κB p65 (Ser536), a possible downstream component of GSK3β upon activation of PI3K/Akt or ERK pathways. As compared to the controls, Na_2WO_4 administration resulted in significant ($p<0.05$) decrease in brain (**Figure 6** (a)) and liver pNF-κB p65 (Ser536) (**Figure 6** (b)) for PbA- and PbN-infected mice to levels that are 0.1 to 0.2 times the level of the phosphorylated protein in controls respectively. Similarly, LiCl (100 mg/kg bw) also resulted in a significant decreased of pNF-κB p65 (Ser536) level. In non-infected animals, we also observed a significant decrease in brain pNF-κB (Ser536) with lower fold-change but not in liver (Data not shown). The

results obtained shows that Na_2WO_4 administration caused inhibition of the activation of NF-κB p65 in both brain and liver of malaria-infected mice.

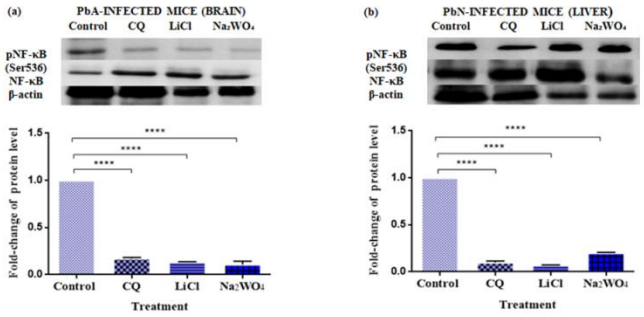


Figure 6. (a) NF-κB phosphorylation levels in brain of PbA-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw) **(b)** NF-κB phosphorylation levels in liver of PbN-infected mice administered with chloroquine (CQ; 10 mg/kg bw), lithium chloride (LiCl; 100 mg/kg bw), or sodium tungstate (Na_2WO_4; 50 mg/kg bw). Total NF-κB and pNF-κB (Ser536) from brain or liver were measured and levels of phosphorylated NF-κB were normalised to total levels of NF-κB. Densitometric measurements are illustrated as mean ± SD of treated group compared to non-treated control. β-actin was used as internal loading control. Representative western blot images are shown. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

DISCUSSION

We demonstrate in the present study for the first time that Na_2WO_4, possessed anti-malarial activity (*in vivo*) in both *P. berghei* ANKA (PbA) and *P. berghei* NK65 (PbN) malarial infection models. The chemo-suppressive effect of Na_2WO_4 is good and comparable to the GSK3 inhibitor, LiCl [14, 16, 30] albeit its potency is much lower than that of the anti-malarial reference drug, chloroquine. More importantly, the present findings show that Na_2WO_4 treatment modulated the cytokine production in both cerebral and severe malarial animal models. Treatment of malaria-infected mice with Na_2WO_4 significantly decreased the serum levels of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-18) whilst increasing the levels of anti-inflammatory cytokines (IL-10 and IL-4) in both malarial infection models. Overwhelming cytokine production is a key factor contributing to pathogenesis and severity of malaria [3]. It is known that dysregulation of GSK3β may result in the overproduction of pro-inflammatory cytokines (and low production of anti-inflammatory cytokines) consequently leading to overwhelming inflammatory response in the host [12].

Western analysis of brain and liver samples from animals infected with PbA and PbN demonstrated that Na_2WO_4 administration increased the levels of phosphorylated GSK3β (Ser9) indicating that Na_2WO_4 administration caused inhibition of GSK3β. Thus, this suggests that Na_2WO_4 modulated the levels of pro- and anti-inflammatory cytokines in malaria-infected mice at least in part via GSK3β inhibition. The findings corroborate many previous reports on the consequence GSK3β inhibition in reducing inflammation and mortality in malaria following treatment with known inhibitors of the kinase such as LiCl [15], curcumin [16], kaempferol [31] and andrographolide [17].

Next, we attempted to provide an explanation of a possible molecular mechanism on the cytokine-modulating effect of Na_2WO_4 that may have contributed to its anti-malarial activity. Our results demonstrate that Na_2WO_4 treatment caused increased phosphorylation of Akt (Ser473), ERK1/2 (Thr202/Tyr204) and p90RSK (Ser380) in brain or liver of PbA- of PbN-infected mice, which may have led to inhibition of GSK3β. Akt plays a critical role in regulating and controlling cell growth and survival. GSK3β is one of the many downstream components in PI3K/Akt signalling and is inhibited among others by Akt-mediated phosphorylation at Ser9 [32]. One of the properties of Na_2WO_4 is its capacity to increase ERK phosphorylation in several cell lines [33]. ERK is one of the upstream kinase of GSK3β in the MAPK signaling [34]. Na_2WO_4 is reported to induce ERK1/2 activation and trigger phosphorylation of its downstream kinase, 90 kDa ribosomal S6 kinase (p90RSK) and GSK3β [20]. Taken together, our findings suggest that treatment with Na_2WO_4 resulted in phosphorylation and consequent inhibition of GSK3β involving both MAPK and PI3K/Akt pathways.

In this study, we also investigated how GSK3β influenced the phosphorylation of NF-κB in *P. berghei*-infected mice treated with Na_2WO_4. Our results indicate that Na_2WO_4 treatment decreased phosphorylation of pNF-κB p65 (Ser536) in both brain of PbA- or liver of PbN-infected mice implying that Na_2WO_4 caused inhibition of NF-κB activation as a consequence of GSK3β inhibition. Given that ERK1/2 contributes to the activation of NF-kB, which in turn commands the gene expression of various cytokines [35], the anti-malarial activity and survival advantage observed with Na_2WO_4 administration in our study may be a consequence of the modulation of inflammatory cytokines mediated via GSK3β inhibition and inhibition of NF-κB activation.

CONCLUSION

Our findings show that Na_2WO_4 treatment resulted in GSK3 (Ser9) phosphorylation in both cerebral and severe malaria infection models through PI3K/Akt and ERK/p90RSK pathways suggesting the involvement of GSK3β inhibition in the modulation of cytokine levels during malarial infection. Thus the anti-malarial and cytokine-modulating effects of Na_2WO_4 in murine models of cerebral and severe malaria involve at least in part the regulation of pNF-κB through inhibition of GSK3β. Our findings reiterate the importance of GSK3β as a molecular target for anti-malarial therapeutics which may act through either MAPK or PI3K pathways. In conclusion, GSK3β is a plausible target for immunomodulation in the development of adjunctive therapeutics against cerebral and severe malaria.

ACKNOWLEDGEMENT

This research was funded by a postgraduate research allocation from the Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

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