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Anti-malarial and cytokine-modulating effects of andrographolide in a murine model of malarial infection

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Abstract. Malarial pathogenesis involves among others, uncontrolled or excessive cytokine production arising from dysregulated immune responses mounted by the host to eliminate the plasmodial parasite. The ubiquitous serine/threonine kinase, glycogen synthase kinase-3β (GSK3β) is a crucial regulator of the balance between pro- and anti-inflammatory cytokine productions in the inflammatory response to pathogenic infections. Andrographolide, a bioactive compound in *Andrographis paniculata*, displays GSK3-inhibitory effects. A previous study elsewhere has shown that this compound has anti-malarial activity but the molecular basis of its action is yet to be elucidated. Here we aimed to study the anti-malarial activity of andrographolide in a murine model of malarial infection to investigate whether its mechanism of action involves cytokine modulation and inhibition of GSK3β. Andrographolide showed strong and selective anti-plasmodial activity (IC_{50} = 13.70±0.71 µM; SI = 30.43) when tested against cultures of *P. falciparum* 3D7. Intraperitoneal administration of andrographolide (5 mg/kg body weight (bw)) into *P. berghei* NK65-infected ICR mice resulted in chemo-suppression of 60.17±2.12%, and significantly (P<0.05) improved median survival time of infected mice compared to non-treated control. In addition, andrographolide treatment significantly (P<0.05) decreased the level of serum pro-inflammatory cytokine, IFN-γ (1.4-fold) whilst the anti-inflammatory cytokines, IL-10 and IL-4 were increased 2.3- and 2.6-fold respectively. Western blot analyses revealed that andrographolide treatment of *P. berghei* NK65-infected mice resulted in an increased level of phosphorylated GSK3β (Ser9) in liver of infected mice. Andrographolide administration also decreased the levels of phosphorylated NF-κB p65 (Ser536) and phosphorylated Akt (Ser473) in liver of malaria- infected animals. Taken together, our findings demonstrate that the cytokine-modulating effect of andrographolide in experimental malarial infection involves at least in part inhibition of NF-κB activation as a consequence of GSK3β inhibition. Based on its cytokine-modulating effects, andrographolide is thus a plausible candidate for adjunctive therapy in malaria subject to clinical evaluations.

INTRODUCTION

Malaria continues to be a health threat in tropical and sub-tropical regions with an estimated global incidence of 216 million cases in 2016 (WHO, 2017). Pathogenesis of the disease is due among others to uncontrolled or excessive immune responses mounted by the host in an attempt to eliminate the plasmodial parasite (Lacerda-Queiroz *et al.*, 2011). Overwhelming infection-stimulated cytokine responses can lead to organ damage and is a major cause of mortality in both cerebral (Dieye *et al.*, 2016) and severe malaria (Walther *et al.*, 2009).

A major obstacle in addressing the high mortality in malaria is the lack of specific

therapies to mitigate the dysregulated cytokine production in the host inflammatory response to the parasite. In early animal infection studies using NK65 parasite strain-infected ICR mice, increased serum levels of pro-inflammatory cytokines IL12-p40 (Yoshimoto *et al.*, 1998; Adachi *et al.*, 2001) and IL-18 (Adachi *et al.*, 2001) were observed. In addition, it has also been reported that NK65-infected C57BL/6 and BALB/c mice had excessive increases in serum levels of pro-inflammatory cytokines (TNF-α and IFN-γ) which eventually led to cerebral malaria (CM) (Lacerda-Queiroz *et al.*, 2011). On the other hand, production of anti-inflammatory cytokines in experimental murine malaria has been shown to down-regulate inflammation (Gonçalves *et al.*, 2014) via increased release of IL-10 (Ropert *et al.*, 2008) and IL-4 (Bakir *et al.*, 2011) in preventing detrimental immune reactions.

Anti-malarial drug discovery efforts should not only focus on anti-parasitic effects of potential therapeutic compounds but also on agents that exhibit immunomodulatory activities in the host (Mimche *et al.*, 2011). Fatality rates for severe malaria remain high even in the best clinical settings because anti-malarial drugs act against the parasite without effectively alleviating life-threatening inflammation. Modulation of host immune responses (immunomodulation) using medicinal plant products is thus a potential effective (adjunctive) therapeutic strategy (Chouhan *et al.*, 2015) and a plausible approach to address the overwhelming inflammatory cytokine response during malarial infection.

Protein kinases are among drug targets that have attracted much attention in anti-malarial drug discovery efforts (Doerig *et al.*, 2008). Among these, glycogen synthase kinase-3β (GSK3β) is a molecular therapeutic target that is extensively investigated for Alzheimer's disease, cancer and other inflammation-related diseases (Jope *et al.*, 2017). GSK3β, a central regulator of the cytokine response (Cortes-Vieyra *et al.*, 2012), is a promising therapeutic target to control inflammation (Jope *et al.*, 2017). Although GSK3 was originally discovered as a kinase that phosphorylates (and inactivates) glycogen synthase (Embi *et al.*, 1980), it is now recognised to be involved in numerous other cellular processes (Beurel *et al.*, 2015). Dysregulation of GSK3β is implicated in the pathogenesis of many important diseases including diabetes, Alzheimer's disease, bipolar disorder and cancer (Eldar-Finkelman, 2002; Wang *et al.*, 2011; Beurel *et al.*, 2015; Jope *et al.*, 2017). This kinase exists as α- and β- isoforms encoded by two highly-related GSK3 genes in mammals (Woodgett, 1990); inactivation of GSK3 involves phosphorylation of Ser21 and Ser9 residues for the α- and β- isoforms respectively (Cross *et al.*, 1995).

It is well-documented that inhibition of GSK3β modulates the cytokine imbalance in response to infection by bacteria (Wang *et al.*, 2014), viruses (Kehn-Hall *et al.*, 2012), fungi (Spinnler *et al.*, 2010) and parasites (Osolodkin *et al.*, 2011) including malaria. Various extracellular signals may induce rapid phosphorylation of GSK3β (Ser9) mediated via different signalling pathways resulting in its inhibition. Among these upstream regulators of GSK3β is phosphoinositide 3-kinase (PI3K)-Ak thymoma/protein kinase B (Akt/PKB) (Cross *et al.*, 1997). In addition, several other kinases such as the 90 kDa ribosomal protein S6 kinase 1 (p90RSK), serum and glucocorticoid-regulated kinase 1 (SGK1) and MAPK-p38, can phospho-inactivate GSK3β (Beurel *et al.*, 2015). Thus GSK3β is a central downstream target of multiple cell signalling pathways involved in the regulation of cellular processes (Patel & Woodgett, 2016).

We have previously shown that LiCl, an inhibitor of GSK3, suppressed parasitaemia development in a rodent model of malarial infection (Zakaria *et al.*, 2010). Subsequent to our report on the effects of LiCl on malaria-infected mice, Dai *et al.* (2012) reported that LiCl treatment restored neurocognitive function in murine experimental CM suggesting that GSK3 may serve as an adjunctive therapeutic target for the

management of CM. More recently, we showed that curcumin, an immunomodulator of plant origin inhibited GSK3β and modulated the cytokine levels in sera of *P. berghei* NK65-infected mice (Ali *et al.*, 2017).

Andrographolide, a diterpene lactone which is a major constituent of the medicinal plant *Andrographis paniculata*, is frequently used to treat inflammation-related symptoms (Tan *et al.*, 2017). Anti-viral (Wiart *et al.*, 2005), anti-cancer (Shi *et al.*, 2009) and anti-inflammatory properties (Low *et al.*, 2015) of the compound have also been documented. Studies using rat hippocampal neurons showed that andrographolide is an activator of Wnt signalling (Tapia-Rojas *et al.*, 2015). In another study, it is reported that andrographolide inhibits human colorectal carcinoma Lovo cell migration and invasion via down-regulation of MMP-7 expression (Shi *et al.*, 2009). Andrographolide has also been previously reported to exhibit anti-malarial effects against *P. berghei* ANKA parasite in BALB/c mice (Mishra *et al.*, 2011). However, the molecular basis of its action is yet to be elucidated. The immunomodulatory effect of andrographolide in malarial infection has also not been reported. Here we aimed to study the anti-malarial and cytokine-modulating actions of andrographolide in a murine model of malarial infection and to investigate whether these effects involve inhibition of host GSK3β.

MATERIALS & METHODS

In vitro anti-plasmodial assay

Pure grade andrographolide was obtained from Sigma Aldrich, USA (cat no. 365645). The anti-plasmodial activity of andrographolide was evaluated against the chloroquine-sensitive *P. falciparum* 3D7 strain obtained from the Malaria Research and Reference Reagent Resource Center (MR4), Manassas, Virginia, USA. Parasitised and non-parasitised O+ red blood cells; without treatment with andrographolide served as positive and negative controls respectively. *P. falciparum* 3D7 was cultured at 1.5% haematocrit and 2% parasitaemia in the presence of 100 µL of andrographolide (final concentrations 2.9×10^{-5} to 29 µM) or the anti-malarial reference drug, chloroquine diphosphate (final concentrations 2×10^{-5} to 20 µM). The assay is based on the plasmodial lactate dehydrogenase (pLDH) enzymatic reaction (Makler & Hinrichs, 1993). Colour development was monitored colourimetrically at 650 nm (FLUOstar OPTIMA, Allmendgruen, Ortenberg, Germany) after one hour of incubation in the dark. Each concentration was prepared in triplicate and the experiment was performed twice. Data obtained were subjected to regression analyses for IC_{50} values (concentration that inhibited 50% parasite growth).

In vitro cytotoxicity assay

Chang liver cells were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA. Cytotoxicity of andrographolide was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) in Chang liver cells seeded at 2×10^4 cells/mL in complete medium. The final concentrations of andrographolide in test wells ranged from 0.01 to 1×10^4 µg/mL (2.9×10^{-4} to 290 µM). Cell suspensions without test substances were used as positive controls. The culture was incubated in the presence or absence of andrographolide for 48 hours (37ºC, 5% CO_2). MTT-PBS (phosphate buffered saline) was then added to each well and the plates incubated for a further 3 hours (37ºC, 5% CO_2). The medium was then removed and replaced with 100 µL of dimethyl sulphoxide (DMSO) to dissolve the MTT formazon product. The mixture was thoroughly mixed for 15 minutes before measuring absorbance at 540 nm. Each concentration was prepared in triplicate and the experiment was performed twice. The concentration of andrographolide that caused 50% inhibition of cell growth (IC_{50})

was estimated using non-linear regression fitted to the growth curve. Selectivity Index (SI) was then calculated by comparing IC_{50} values from the cytotoxicity and the pLDH assays:

$$SI = IC_{50}\ MTT\ /IC_{50}\ pLDH$$

Experimental animals

Male ICR mice (6–8 weeks old) were obtained from Universiti Kebangsaan Malaysia (UKM) Animal House Complex. Animal experimentations were carried out at the Malaria Infection Laboratory within the same complex. Approval to conduct animal studies was obtained from the university (UKM) Animal Ethics Committee (reference number: FST/2015/HASIDAH/11-FEB./640-FEB.-2015-DEC.-2016).

***In vivo* four-day suppressive test**

P. berghei NK65 (chloroquine-sensitive) strain originally purchased from MR4, USA was maintained in ICR mice. Inoculum of 2×10^7 *P. berghei*-parasitised erythrocytes/mL was prepared by dilution with Alserver's solution. Erythrocytes with >50% parasitaemia was taken by cardiac puncture from infected stock mice. Intraperitoneal (ip) injections of infected blood (0.2 mL of 2×10^7 *P. berghei*-parasitised erythrocytes/mL) were given to mice randomly divided into six groups (n = 7). At day 0 (three hours post-infection), the animals were administered (ip) over four consecutive days with either chloroquine (CQ), the anti-malarial reference drug (10 mg/kg bw) or the GSK3 inhibitor (100 mg/kg bw LiCl) as reference or 0.85% saline [control group A] or different dosages of andrographolide (2.5, 5, 15 or 45 mg/kg bw) [test group B] each in 0.2 mL volumes. The percentage of blood parasitaemia on day 4 post-infection (Peters, 1975) was determined from thin blood smears of tail blood from each animal. The percentage (average) of chemo-suppression (PC) was calculated from blood parasitaemia levels (%) in group A (control) and group B (test):

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

Survivability of non-infected mice

Survivability of experimental animals was recorded for 30 days to ascertain that the dosage of andrographolide employed in the suppressive test did not have any effect on animal survival. As such, different dosages of andrographolide (2.5 to 45.0 mg/kg bw) were injected (ip) into four groups of non-infected mice (n = 7) for four consecutive days. A volume of 0.2 mL of 0.85% saline solution was given to the control group of animals. Besides monitoring body weights and survivability, experimental mice were also monitored for gross behavioral changes for 30 days.

Liver sample preparation for protein analyses

A dosage of andrographolide that caused chemo-suppression of more than 60% (effective dose based on the four-day suppressive test) was selected for another infection experimentation to determine liver GSK3β phosphorylation state in infected mice. Four groups of mice containing five mice per group were injected (ip) with *P. berghei* NK65-infected erythrocytes (inoculum: 2×10^7 *P. berghei*-parasitised erythrocytes/mL). At three hours post-infection (Peters, 1975), mice were treated with either 5 mg/kg bw andrographolide or 0.85% saline (control). Non-infected control group of mice were also given (ip) 0.85% saline. Chloroquine diphosphate (CQ) and LiCl (a GSK3 inhibitor) were each administered (ip) at 10 and 100 mg/kg bw dosages respectively in separate control groups. All administrations were carried out for four consecutive days. Experimental mice were euthanised on day 4 post-infection and the liver organs harvested immediately for further analysis.

Extraction of protein from liver was performed as described by Lee (2007). Liver samples obtained from *in vivo* anti-malarial experiments described above were homogenised individually in an extraction buffer (containing protease inhibitors (Sigma, USA) and phosphatase inhibitors (Sigma, USA) at a ratio of 1 gram liver per mL. After centrifugation at 20 000 × g for 30

min at 4ºC, protein content of liver samples were determined (Bradford, 1976) using bovine serum albumin (BSA) as standard.

SDS-PAGE and Western blot analysis

For protein separation using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (30% w/v acrylamide), liver protein samples (40 µg) were loaded into wells of the SDS-PAGE gels (Laemmli, 1970). After separation, proteins were then transferred onto nitrocellulose membranes (Towbin *et al.*, 1979) and blocked using 3% BSA in Tris-buffered saline (TBST) containing 0.1% w/v Tween 20. The membranes were further probed before being incubated at 4°C overnight separately with each of these primary monoclonal antibodies; anti-GSK3β, anti-phosphoSer9-GSK3β, anti-Akt, anti-phosphoSer473-Akt, anti-NF-κB and anti-phosphoSer536-NF-κB (Cell Signaling, Danvers, MA, USA) and subsequently incubated further for two hours with the corresponding secondary antibody (anti-rabbit IgG, HRP-linked antibody (Cell Signaling, Danvers, MA, USA) at room temperature. To ensure equal protein loading, membranes were stripped with 0.2 M NaOH and then re-probed with anti-β-actin (Santa Cruz Biotechnology, Littleton, USA). Immuno-reactive bands were then detected using Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Waltham, MA, USA). The intensity of immune-reactive protein band areas was then quantified using a densitometer (Vilber Lourmat 302, Sud Marne-la-Vallée Cedex, France).

Cytokine analysis

At day 4 post-infection (as described in the four-day suppressive test above), blood was collected from euthanised mice (n = 6) according to Phelan *et al.* (2002). Blood samples were collected via cardiac puncture and processed immediately to obtain serum. Quantification of pro-inflammatory cytokines (TNF-α and IFN-γ) as well as anti-inflammatory cytokines (IL-10 and IL-4) in sera of treated, infected mice and control animals were performed using an enzyme-linked immunosorbent assay (ELISA) (QIAGEN, Hilden, Germany).

Statistical analysis

Data for Kaplan-Meier survival analysis were evaluated using the Student's t-test and log rank tests. All data were 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

Andrographolide exhibited good and selective inhibitory activity against *P. falciparum* 3D7

Andrographolide inhibited growth of *P. falciparum* 3D7 in culture (IC_{50} value of 13.70±0.71 µM) and was only slightly toxic toward Chang liver cells *in vitro* (IC_{50} = 416.89±0.86 µM). The selectivity index of 30.43 (SI>10) indicates selective inhibition towards the chloroquine-sensitive parasite strain (Table 1).

Table 1. *In vitro* anti-plasmodial activity of andrographolide against *P. falciparum* 3D7 strain

Compound/ Drugs	Anti-plasmodial Activity, pLDH assay, IC_{50} (µM)±SD	Cytotoxic Effect, MTT assay, IC_{50} (µM)±SD	Selectivity Index (SI) $= \frac{(IC_{50}\ MTT)}{(IC_{50}\ pLDH)}$
Andrographolide	13.70±0.71	416.89±0.86 µM	30.43
Chloroquine	0.05 (0.03 µg/mL)	ND	ND

SD: standard deviation; ND: not determined.

Andrographolide treatment inhibited parasitaemia development in *P. berghei*-infected mice

Andrographolide administration (ip) in mice for four consecutive days following injections with *P. berghei*-infected erythrocytes showed decreased parasitaemia development (Table 2). Andrographolide at the 5 mg/kg bw dose tested, inhibited development of *P. berghei* parasitaemia in mice by 60.17±2.12%. Doses at 15 and 45 mg/kg bw of andrographolide appear ineffective most likely because this compound is toxic at high doses due to saturated detoxification or excretion mechanisms which when fully operative at lower doses do not result in the same toxic response (Watanabe *et al.*, 1977). Doses at 15 and 45 mg/kg bw of andrographolide saturated the host and lead to a dose-independent behavior. The median survival time for andrographolide-treated infected mice was 19 days compared to 15 days for non-treated controls. Animals administered with the reference anti-malarial drug CQ (10 mg/kg bw) exhibited almost 100% suppression of parasitaemia development on day 4 and all the animals survived throughout the 30-day observation period (Table 2) (Figure 1).

Andrographolide treatment increased the level of phosphorylated GSK3β (Ser9) in liver of *P. berghei*-infected mice

In order to further elucidate the involvement of GSK3 in the action of andrographolide, we investigated the effects of andrographolide administration on the phosphorylation state of GSK3β (Ser9). Compared to non-treated controls, administration of andrographolide led to significantly ($P<0.05$) increased levels (6.4-fold) of pGSK3β (Ser9) in liver of *P. berghei*-infected mice (Figure 2). The increased phosphorylation of GSK3β (Ser9) in andrographolide-treated group was also observed in liver of LiCl-treated animals (a significant increase of 2.1-fold in pGSK3β (Ser9)) after four days of treatment.

Treatment with andrographolide decreased the level of phosphorylated NF-κB p65 (Ser536) in liver of *P. berghei*-infected mice

Next, we examined the effect of andrographolide treatment on a possible GSK3 downstream signalling component, NF-κB p65. The level of pNF-kB p65 (Ser536) in andrographolide-treated infected animal liver was significantly ($P<0.05$) lowered

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

Compound/Drugs	Dosage (mg/kg bw)	Average parasitaemia suppression on day 4 (%)	Median survival time (days)
Andrographolide	2.5	54.22±3.35[a,b]	16[b]
	5.0	60.17±2.12[a,b]	19[a,b]
	15.0	58.96±4.75[a,b]	17[b]
	45.0	43.18±6.28[a,b]	14[b]
Chloroquine (anti-malarial reference drug)	10.0	97.78±1.8[a]	>30[a]
LiCl (GSK3 inhibitor reference drug)	100.0	57.32±4.50[a,b]	17[b]
0.85% Saline (negative control)	0.2 mL	–	15

Parasitaemia suppression was calculated on day 4 post-infection and survivability of mice recorded throughout the experimental period (30 days). Data represent mean ± SD for parasitaemia suppression and median survival time (n = 7).
[a] Significantly different from negative control at $P<0.05$.
[b] Significantly different from drug control (chloroquine) at $P<0.05$.

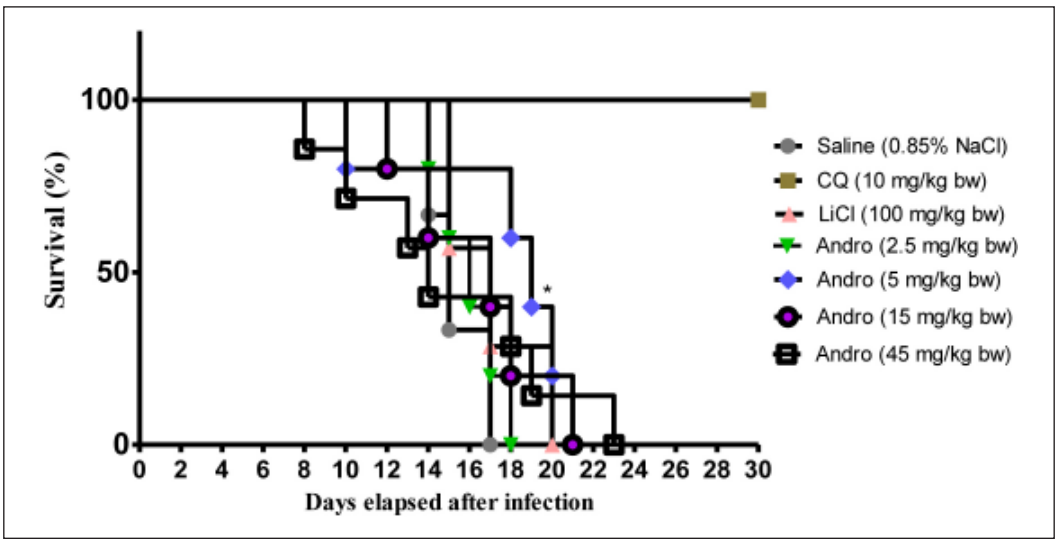

Figure 1. Representative Kaplan-Meier survival curve of mice infected with *P. berghei* NK65 with and without andrographolide treatment in 4-day suppressive test. Data represent survival of negative control (non-treated *P. berghei* NK65-infected mice) (n = 7) and treatments; andrographolide-treated (n = 7), CQ-treated (n = 7) and LiCl-treated (n = 7) groups of experimental mice. Significant differences between tested and control groups were evaluated at $P<0.05$ (*).

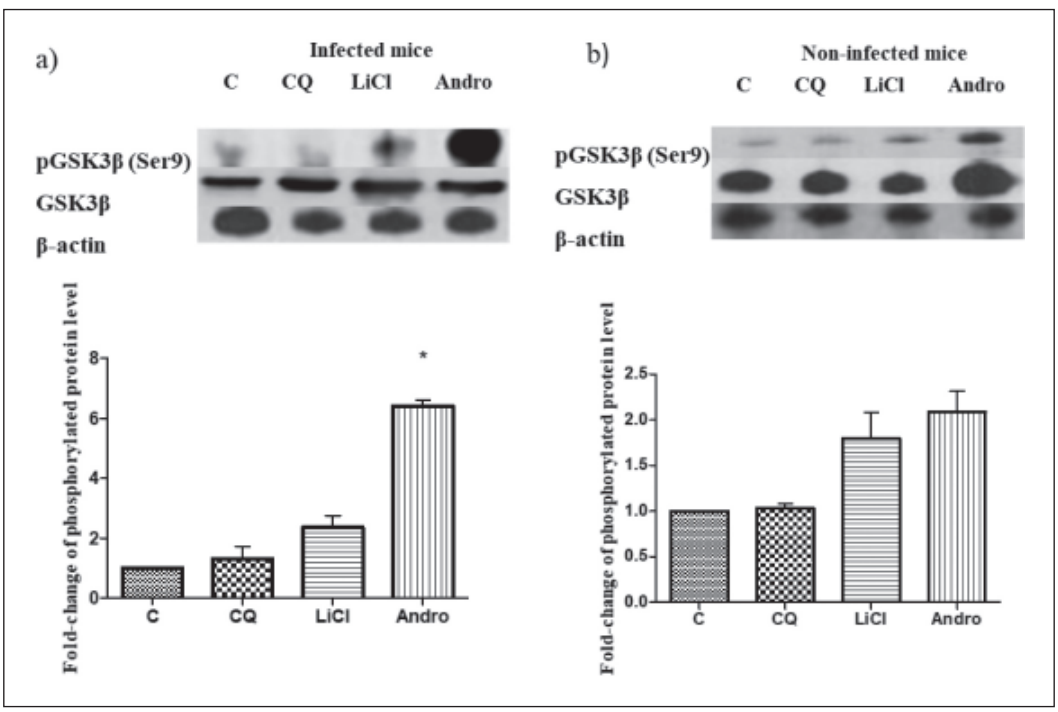

Figure 2. (a) GSK3β phosphorylation levels in liver of *P. berghei*-infected mice administered with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. (b) GSK3β phosphorylation levels in livers of non-infected mice administered with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. Total GSK3β and pGSK3β (Ser-9) from liver were measured using a densitometer. 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 $P<0.05$ (*).

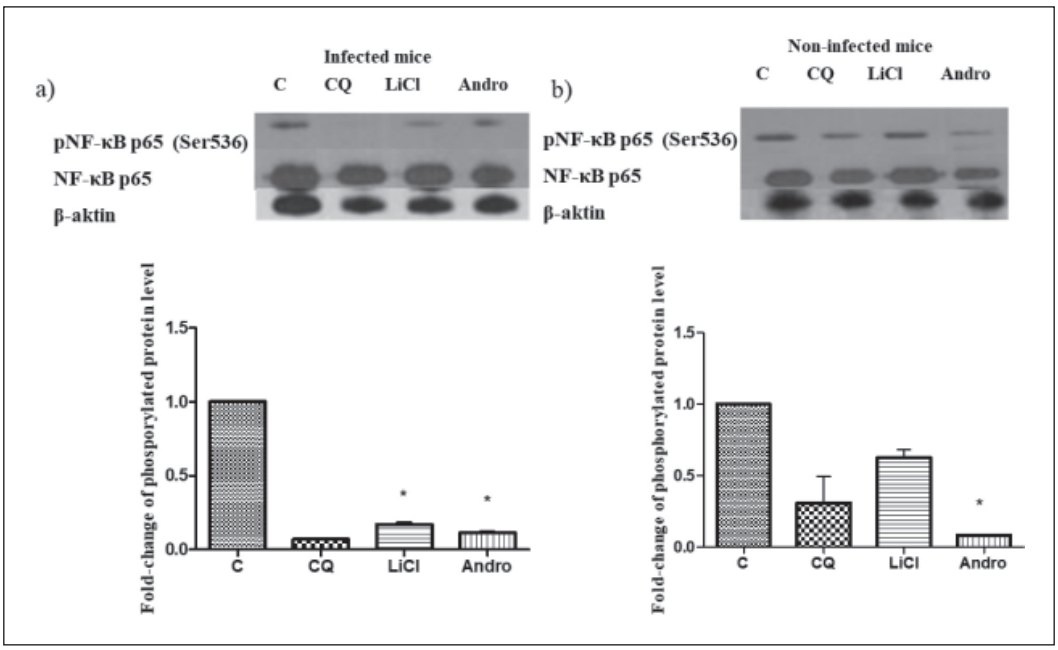

Figure 3. (a) NF-κB phosphorylation levels in liver of *P. berghei*-infected mice administered with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. (b) NF-κB phosphorylation levels in livers of non-infected mice administered with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. Total NF-κB and pNF-κB (Ser536) from liver were measured using a densitometer. 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 $P<0.05$ (*).

10.0-fold from the level in control animals. A similar pattern of the effect on phosphorylated NF-κB p65 (Ser536) level was observed in liver of mice treated with LiCl (lowered 5.0-fold from the level in control) (Figure 3).

Treatment with andrographolide decreased the level of phosphorylated Akt (Ser473) in liver of *P. berghei*-infected mice

We then proceeded to investigate the effect of andrographolide administration on a possible GSK3 upstream signalling component, Akt. Treatment with andrographolide induced a significant ($P<0.05$) decrease in the level of phosphorylated Akt (Ser473) in liver to 10.0-fold from the level in control (Figure 4) indicating that Akt is not activated due to andrographolide treatment. This shows that the inhibition of host liver GSK3β (phosphorylation on Ser9) described above by andrographolide during malarial infection was not mediated via Akt. The findings suggest that GSK3β inhibition resulting from andrographolide administration into malaria-infected mice explained earlier could be mediated through a signalling pathway other than PI3K/Akt.

Treatment with andrographolide modulated pro- and anti-inflammatory cytokine levels in sera of *P. berghei*-infected mice

We also investigated whether andrographolide administration modulated serum levels of the cytokines in *P. berghei*-infected mice. The results obtained (Figure 5) showed that there were increases in both pro- and anti-inflammatory cytokine levels on day 4 following *P. berghei* NK65 infection. Malarial infection significantly ($P<0.05$) increased the serum levels of pro- and anti-inflammatory cytokines TNF-

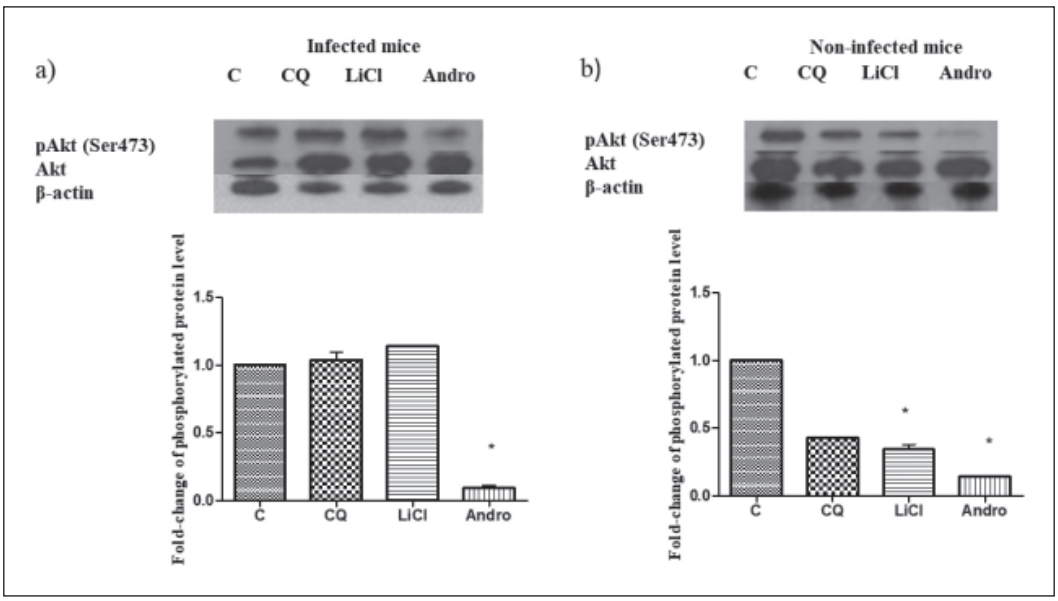

Figure 4. (a) Akt phosphorylation levels in liver of *P. berghei*-infected mice treated with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. (b) Akt phosphorylation levels in livers of non-infected mice administered with chloroquine (CQ), lithium chloride (LiCl), or andrographolide. Total Akt and pAkt (Ser473) from liver were measured using a densitometer. 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 $P<0.05$ (*).

α, IFN-γ, IL-10 and IL-4 by 34.0-, 45.2-, 2.6- and 1.4-fold respectively in serum (Figure 5). However, treatment of infected mice with andrographolide decreased the serum levels of pro-inflammatory cytokines TNF-α and IFN-γ (by 1.1- and 1.4-fold from the levels in control respectively) but increased the levels of anti-inflammatory cytokines IL-10 and IL-4 (by 2.3- and 2.6-fold respectively) (Figure 5). These findings suggest that cytokine modulation by andrographolide observed was likely mediated by GSK3β inhibition. In mice treated with LiCl, serum levels of TNF-α, IFN-γ and IL-10 decreased (by 1.7-, 5.0- and 1.1-fold from the levels in control respectively) whilst levels of IL-4 increased (by 6.0-fold) compared to infected controls. Pro- and anti-inflammatory cytokines TNF-α, IFN-γ and IL-10 levels were also decreased significantly ($P<0.05$) by chloroquine treatment after infection.

DISCUSSION

In the present study, we showed that andrographolide treatment *in vitro* exhibited good and selective inhibitory activity against *P. falciparum* 3D7 corroborating previous findings on suppression of plasmodial development by the compound (Zaid *et al.*, 2015). *P. falciparum* GSK3 (*Pf*GSK3) is a potential drug target for anti-malarials (Droucheau *et al.*, 2004). GSK3 inhibitors have been shown to act synergistically with p38 MAPK inhibitors on growth of *P. falciparum ex vivo* (Marhalim *et al.*, 2014).

GSK3β is central in the regulation of cytokine response in pathogenic infections (Wang *et al.*, 2011). Findings from this study demonstrated that GSK3β inhibition is at least in part involved in the cytokine-modulating and consequent anti-malarial

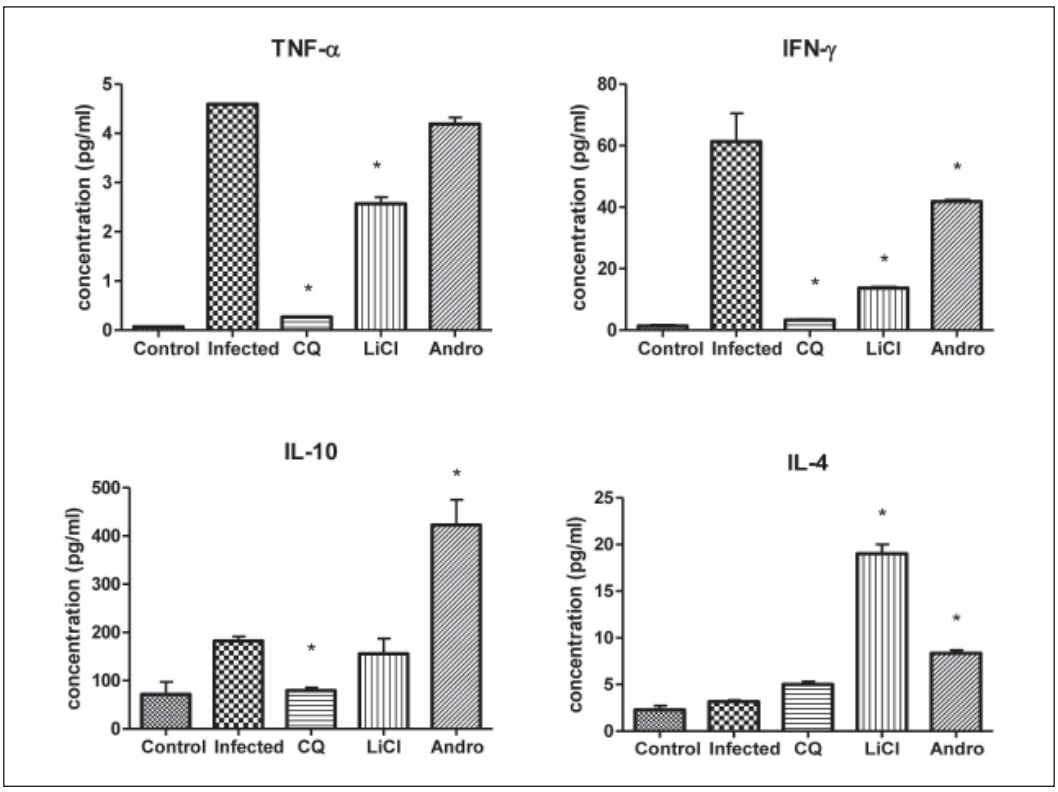

Figure 5. Pro-inflammatory cytokine (TNF-α and IFN-γ) levels and anti-inflammatory cytokine (IL-10 and IL-4) levels of serum in mice administered with chloroquine (CQ), lithium chloride (LiCl) or andrographolide in a 4-day suppressive test. Data represent mean ± SEM of controls, infected and CQ/LiCl/andrographolide-treated mice. Significant differences between infected and CQ/LiCl/andrographolide-treated group were determined at $P<0.05$.

effects of andrographolide. Although the anti-malarial effect of this compound has been previously reported elsewhere based on the chemo-suppressive activity *in vivo* (Mishra *et al.*, 2011), the mechanism of its action was not investigated. We were first to report the involvement of GSK3β in our studies on the chemo-suppressive effects of LiCl (a GSK3 inhibitor) treatment in malaria-infected mice (Zakaria *et al.*, 2010). Here, we provide evidence on the involvement of GSK3β on the cytokine-modulating effects of andrographolide in addition to its chemo-suppressive effect. This finding represents another example of a plant-derived compound displaying immunomodulatory effects in addition to our recent report on the cytokine-modulating property of curcumin (Ali *et al.*, 2017) mediated via GSK3β inhibition in *P. berghei* NK65-infected mice.

Our current investigation revealed that treatment with andrographolide increased pGSK3β (Ser9) in host liver but significantly decreased pAkt (Ser473) indicating that this upstream kinase of GSK3β was not activated by andrographolide and that the observed GSK3β inhibition was not elicited through the PI3K/Akt axis. This is not unexpected since andrographolide is an activator of Wnt signalling (Tapia-Rojas *et al.*, 2015). Another study has also shown that andrographolide inhibited growth of human T-cell acute lymphoblastic leukaemia Jurkat cells by the down-regulation of PI3K/Akt (Yang *et al.*, 2016) suggesting that the phosphorylation and inhibition of GSK3β in these leukaemic cells could not have

been mediated by activation of Akt (phosphorylation of Ser473). Thus, the increased phosphorylation of GSK3β after treatment with andrographolide observed in our study could be due to either direct inhibition by andrographolide or mediated via activation of Wnt signalling (Tapia-Rojas *et al.*, 2015). The Wnt/β-catenin signalling pathway is also known to control inflammatory responses during bacterial infections (Silva-Garcia *et al.*, 2014) but this has yet to be reported in malarial infection. Nevertheless, canonical Wnt signalling plays an important role in modulating inflammatory responses; for example, in the inhibition of NF-κB as a result of β-catenin activation in inflammation (Silva-Garcia *et al.*, 2014). Our results also revealed that andrographolide treatment in malaria-infected mice resulted in the inactivation of NF-κB, thus a possible explanation of the cytokine-modulating effect of the compound.

In deciphering andrographolide mechanism of action, Tan *et al.* (2017) revealed that the inhibition of NF-κB activity is the prevailing anti-inflammatory mechanism elicited by this compound among all the signalling pathways investigated. Inactivation of NF-κB could be seen in many disease models such as chronic obstructive pulmonary disease (COPD) (Zhu *et al.*, 2013a), idiopathic pulmonary fibrosis (IPF) (Zhu *et al.*, 2013b; Yin *et al.*, 2015), multiple sclerosis (Iruretagoyena *et al.*, 2006) and systemic lupus erythematosus (SLE) (Kalergis *et al.*, 2009). There is also increasing evidence supporting endogenous anti-oxidant defense enhancement through activation of Nrf2 by andrographolide for e.g. in asthma steroid resistance (Liao *et al.*, 2016) and COPD exacerbation (Tan *et al.*, 2016). In a study on liver injury, andrographolide exhibited inhibitory effects on GSK3β (Lu *et al.*, 2011). Rivera *et al.* (2016), in a study using a model of Alzheimer's disease also reported phosphorylation of GSK3β (Ser9) by andrographolide reiterating that andrographolide can cause GSK3β inhibition (Tapia-Rojas *et al.*, 2015). Although the inactivation of NF-κB and the inhibition of GSK3β have been reported in many disease models, findings from the present study, to our knowledge, represent the first report on the inactivation of NF-κB mediated through inhibition of GSK3β by andrographolide in a murine model of malarial infection.

Dysregulation of cytokine production is a key factor contributing to pathogenesis and severity of malaria (Angulo & Fresno, 2002; Wynn *et al.*, 2016). Overwhelming infection-stimulated cytokine responses mounted by the host results in damage to vital organs leading to death in cerebral malaria patients (Dieye *et al.*, 2016). Our present study demonstrated that treatment of malaria-infected mice with andrographolide significantly decreased the serum level of pro-inflammatory cytokine (IFN-γ) whilst increasing the levels of anti-inflammatory cytokines (IL-10 and IL-4). This suggests that andrographolide modulated pro- and anti-inflammatory cytokines in malaria-infected mice and this could be in part mediated through inhibition of GSK3β. This corroborate many other reports on the effects of inhibition of GSK3β in reducing inflammation and mortality in other infectious disease models (Chang *et al.*, 2013; Gui *et al.*, 2012; Zhang *et al.*, 2009). GSK3β is thus a novel target for the cytokine-modulating effects of andrographolide which is potentially useful for adjunctive therapy in malarial infection.

Andrographolide, a major bioactive compound from *A. paniculata* is among several plant-derived immunomodulators undergoing clinical trials for various chronic inflammatory conditions such as brain atrophy, colorectal cancer and rheumatoid arthritis (Jantan *et al.*, 2015). Andrographolide has a good safety profile both in pre-clinical and clinical toxicological studies (Tan *et al.*, 2017) and is reported to be well-tolerated in a clinical trial (Bertoglio *et al.*, 2016). Andrographolide also has potent cytotoxic effects against MDA-MB-231 breast cancer cell line and is a potential anti-cancer agent (Banerjee *et al.*, 2016).

CONCLUSION

Findings described above on host response of andrographolide treatment demonstrate that inhibition of host GSK3β is in part involved in the underlying anti-malarial action of andrographolide. Hence, andrographolide is a potential immunomodulator for adjunctive therapy in malaria and this warrants further studies to investigate the usefulness of the compound for the treatment of malaria.

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