GLYCOGEN SYNTHASE KINASE-3β (GSK3β) AS A MOLECULAR TARGET FOR CYTOKINE-MODULATING EFFECT OF CURCUMIN IN A MURINE MODEL OF ACUTE MELIOIDOSIS INFECTION

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Accepted 27 November 2017, Published online 31 December 2017

ABSTRACT

The present study was conducted to investigate the effect of curcumin treatment on survivability, cytokine response, and phosphorylation of glycogen synthase kinase-3β (GSK3β) in Burkholderia pseudomallei-infected mice. Curcumin, a bioactive compound identified in Curcuma longa has been reported to exhibit anti-inflammatory properties via inactivation of nuclear factor-kappa B (NF-κB) and consequent downregulation of pro-inflammatory cytokine production. Glycogen synthase kinase-3β (GSK3β) plays a pivotal role in the regulation of cytokine production. Here we used a murine model of acute melioidosis to investigate the effects of curcumin administration on experimental animal survivability, the phosphorylation state of GSK3β and levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-10 in serum, liver and spleen of B. pseudomallei-infected mice. Intraperitoneal administration of curcumin (60 mg/kg b.w.) significantly (P<0.05) improved survivability (44%) of infected mice compared to non-treated controls. Administration of curcumin resulted in elevated phosphorylation of Ser9 GSK3β (pGSK3β) levels in both liver and spleen of B. pseudomallei-infected mice (1.2-2.6 fold) compared to non-treated infected controls. In addition, curcumin treatment significantly (P<0.05) decreased (30% - 85%) the levels of pro-inflammatory cytokines (TNF-α and IFN-γ) in liver, spleen and serum of mice challenged with B. pseudomallei compared to non-treated infected controls. Increased levels (67%) of anti-inflammatory cytokine (IL-10) was also observed in serum of curcumin-treated infected mice. Our findings demonstrated that curcumin improved survivability of mice experimentally-infected with B. pseudomallei by modulating the pro- and anti-inflammatory cytokine response via inhibition of GSK3β.

Key words: Burkholderia pseudomallei, curcumin, glycogen synthase kinase-3β

INTRODUCTION

Curcumin, (1E,6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione is a polyphenolic compound, extracted from the turmeric rhizome of the herb, Curcuma longa (Family – Zingiberaceae) (Basnet & Skalko-Basnet, 2011). The compound has been shown to be non-toxic to humans (Gupta et al., 2013) and exhibits a multitude of biological activities including anti-inflammatory effects (Aggarwal & Harikumar, 2009; Jurenka, 2009). Molecular targets identified for curcumin include protein kinases, transcription factors and inflammatory cytokines (Lin, 2007; Zhou et al., 2011). The profound anti-inflammatory activities displayed by curcumin has been shown to be attributed to its effects on the inactivation of NF-κB and consequent downregulation of pro-inflammatory cytokine production (Shin et al., 2010). NF-κB activation is regulated by glycogen synthase kinase-3 (GSK3), a serine/threonine kinase and a component of the PI3K/Akt pathway. GSK3, initially identified by Embi et al. (1980) for its role in the regulation of glycogen metabolism, is now recognised to play a central role in the regulations of pathogen-induced inflammatory responses through modulation of pro- and anti-inflammatory cytokine production (Martin et al., 2005; Wang et al., 2014). The kinase is a constitutively active protein kinase existing as two isoforms, GSK3α and...
GSK3β, encoded by two highly conserved genes (Woodgett, 1990). Both isoforms of the enzyme are inhibited by phosphorylation; on Ser21 for GSK3α and Ser9 for GSK3β (Sutherland et al., 1993; Cross et al., 1995). GSK3 is now known to regulate a spectrum of cellular processes such as apoptosis, embryonic development, cell cycle control, cell differentiation, cell mobility, migration and as a pivotal regulator of the inflammatory response to bacterial infections and other insults (Wang et al., 2011; Cortes-Vieyra et al., 2012; Beurel et al., 2015).

During bacterial infections, increased expression of Toll-like receptors (TLRs) results in overwhelming pro-inflammatory response, which may lead to sepsis and fatality (Knapp, 2010). GSK3β has been identified as a point of convergence for host inflammatory response and is able to modulate production of inflammatory cytokines (Martin et al., 2005). Inhibition of GSK3β (Ser9) during bacterial infections has been reported to lower levels of pro-inflammatory cytokines and increase levels of anti-inflammatory cytokines (Woodgett & Ohashi, 2005). The involvement of GSK3β in modulating host innate inflammatory response during bacterial infections has been shown for *Salmonella typhimurium* (Duan et al., 2007), *Francisella tularensis* (Zhang et al., 2009), *Helicobacter pylori* (Nakayama et al., 2009), *Mycobacterium tuberculosis* (Chan et al., 2009), *Escherichia coli* (Ko et al., 2010) and *Burkholderia cenocepacia* (Cremer et al., 2011). In our laboratory, we demonstrated that inhibition of GSK3β by lithium chloride (LiCl) improved survivability of *Burkholderia pseudomallei*-infected mice and modulated host inflammatory cytokine response during melioidosis infection (Tay et al., 2012). Melioidosis, caused by the pathogen, *B. pseudomallei*, is an endemic disease in Southeast Asia and Northern Australia (Currie, 2015). Melioidosis is an emerging infectious disease with diverse clinical manifestations ranging from prolonged asymptomatic infections to rapid fatal outcomes and is a common cause of community-acquired septicemia and pneumonia (Wiersinga et al., 2006; Lazar Adler et al., 2009; Limmathurotsakul et al., 2016). Infection by *B. pseudomallei* induces overwhelming pro-inflammatory response potentially causing multiple major organ damage (sepsis) and death when unchecked (White, 2003; Wiersinga et al., 2012).

Curcumin has been reported to be an activator of Akt (Yu et al., 2012) but thus far there has been no report of its action on GSK3, its downstream target. Although in silico docking simulation studies reported that curcumin possessed GSK3β-inhibitory properties (Bustanji et al., 2009), it is yet to be established whether GSK3 is involved in the underlying mechanism of the effect of curcumin in modulating the inflammatory cytokine response during bacterial infection. Here we investigate the effect of curcumin treatment on the survivability and cytokine response in *B. pseudomallei*-infected mice and on the phosphorylation state of GSK3β in liver of infected mice.

**MATERIALS AND METHODS**

**Bacteria**

Frozen glycerol stocks of *B. pseudomallei* were kindly provided by Prof. Dr. Sheila Nathan from the Pathogen Laboratory, Faculty of Science and Technology (FST), the National University of Malaysia (UKM). Bacterial cultures were grown in Brain Heart Infusion Broth (BHIB) and cultured onto selective medium (Ashdown agar) supplemented with gentamicin.

**Experimental animals**

Male BALB/c mice (6-8 weeks old) were obtained from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. Mice were accommodated in Individual Ventilation Cages (IVC) located at the Infection Studies Laboratory, Animal House Complex, UKM and fed ad libitum and housed in a room with 12 hours dark/light cycles. All studies were performed according to the stipulated guidelines approved by UKM Animal Ethics Committee (UKMAEC) (reference number FST/2014/HASIDAH/20-MAR./581-MAR.-2014.-NOV.-2015).

**Infection studies**

Male BALB/c mice (6-8 weeks old) (*n=9*) were injected intraperitoneally (i.p.) with 7.2 × 10^4 colony forming units (CFU) of *B. pseudomallei* suspended in 200 μL of phosphate buffered-saline (PBS). To investigate effects of curcumin on survivability of *B. pseudomallei*-infected mice (*n=9*), 15, 30 or 60 mg/kg b.w. curcumin was administered (i.p.) into the study animals at 1 h post *B. pseudomallei* infection. For non-treated infected control, *B. pseudomallei*-infected mice were injected (i.p.) with 0.9% sodium chloride (NaCl). The three doses of curcumin selected have been reported to be within the effective dose range in in vivo studies and shown to be able to cause GSK3β inhibition in BALB/c mice (Sharma et al., 2006; Bustanji et al., 2009). Survivability of mice was monitored over a course of 14 days post-infection. Based on the results of the dose response experiment above, all subsequent challenge studies were conducted using a dose of 60 mg/kg b.w.
Bacterial load

A group of mice (n=21) was infected with 7.2 × 10^4 CFU B. pseudomallei and subsequently treated (i.p.) with 60 mg/kg b.w. curcumin at 1 h post-infection. The non-treated infected control group (n=3) consisted of mice infected with B. pseudomallei only. Three mice from each treated group (n=3) were euthanised each day at day 0, 1, 2, 3, 4 and 14 after infection. Organs (liver and spleen) were taken from euthanised mice and processed as described by Leakey et al. (1998). Liver and spleen were homogenised in 10 mL PBS consisting of 0.14 M sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 0.01 M sodium hydrogen phosphate (Na2HPO4) and 1.7 mM potassium dihydrogen phosphate (KH2PO4), pH 7.4. The homogenate was then serially-diluted with PBS and spotted onto Ashdown agar to assess bacterial load in CFUs.

Western blot analysis

To investigate effects of curcumin on phosphorylation state of GSK3 in liver and spleen of B. pseudomallei-infected mice (n=3), 60 mg/kg b.w. curcumin was administered (i.p.) into the study animals at 1 h post B. pseudomallei infection. For non-treated infected control, B. pseudomallei-infected mice were injected (i.p.) with 0.9% NaCl. Liver and spleen samples were obtained from experimental animals (n=3) at 2 and 3 h after infection according to Tay et al. (2012) in order to be able to determine the status of GSK3 phosphorylation at early infection stage in acute infection. Protein extraction was carried out as described by Lee (2007). Liver and spleen were homogenised in 1:1 (w/v) extraction buffer consisting of 1.7 mM Na2HPO4, 9.1 mM NaH2PO4, 0.5% sodium deoxycholate, 150 mM NaCl, pH 7.4, 1% IgepalCA-630 and 0.1% SDS supplemented with phosphatase inhibitors (1 mM sodium orthovanadate (Na2VO4) and 1 mM sodium fluoride (NaF)) and protease inhibitors (1 mM phenylmethylsulphonyl (PMSF) and 50 μg/mL leupeptin) followed by incubation on ice for 30 min. The mixture was then centrifuged at 20 000 ×g for 30 minutes at 4°C. Protein quantification was carried out using bovine serine albumin (BSA) as standard (Bradford, 1976). Protein samples were then diluted with 1:1 (w/v) sample buffer consisting of glycerol, 0.5 M Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue and β-mercaptoethanol for protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% resolving gel (Laemmli, 1970). Separated proteins were electro-transferred onto nitrocellulose membranes and probed with specific primary monoclonal antibodies (IgG) (anti-GSK3β, anti-pospho-GSK3β (Ser9) or anti-β-actin). The membranes were then incubated for 18 h at 4°C (Towbin et al., 1979). After 18 h of incubation, the membranes were probed with HRP-conjugated specific secondary antibodies (IgG) for 2 h before detection of immuno-reactive proteins using enhanced chemiluminescent (ECL) western blotting detection reagents (Thermo Scientific, USA).

Cytokine analysis

Mice were divided into four groups (n=5) comprising of Group I: Normal; Group II: 60 mg/kg b.w curcumin administration only; Group III: B. pseudomallei infection only; Group IV: B. pseudomallei infection + 60 mg/kg b.w curcumin (1-hour post-infection). At one day post-infection, liver, spleen and blood were collected from euthanised mice (n=5) and processed as described by Phelan et al. (2002) in order to assess the effect of LiCl administration on levels of cytokines since it has been reported that pro-inflammatory cytokines are elevated on the first and second day after infection (Ulett et al., 2000). Blood was collected using cardiac puncture and immediately processed to obtain serum. Meanwhile, liver and spleen were homogenised in cytokine extraction buffer consisting of protease-inhibitor combination (1 μg/mL pepstatin A, 1 μg/mL leupeptin and 1 mM PMSF in PBS (pH 7.2)), 0.5% Triton X-100 and 0.05% sodium azide in the ratio of 100 mg tissue per mL). The suspensions were then subjected to three rounds of freeze-thaw cycles prior to incubation at 4°C for 1 h. The suspensions were centrifuged at 12 000 ×g for 30 min. The levels of pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokine (IL-10) in supernatants and serum of treated and non-treated infected mice were determined using an ELISA (enzyme-linked immunosorbent assay) kit (QIAGEN, Germany).

Statistical analysis

Statistical significance between groups was evaluated using Student’s t-test and log rank test (for Kaplan-Meier survival analysis). Data obtained were expressed as mean ± SD (n=9). P value of < 0.05 between groups was considered as significant.

RESULTS

Curcumin increased survivability of mice challenged with B. pseudomallei

BALB/c mice infected (i.p.) with 7.2 × 10^4 CFU B. pseudomallei died within two to five days post-infection indicating that acute B. pseudomallei infection (melioidosis) was successfully established (Leakey et al., 1998). Administration (i.p.) of 60 mg/kg b.w. curcumin to B. pseudomallei-infected mice at 1 h after infection resulted in improved survivability (44%) compared to non-treated infected
control (P<0.05) (Figure 1a). Mice administered with 15 or 30 mg/kg b.w. curcumin at 1 h after infection failed to result in a significant infected animal survival advantage over the infection-only control group (P>0.05). A repeat of the survivability experiment using 60 mg/kg b.w. curcumin showed prolonged survivability (44%) of *B. pseudomallei*-infected mice compared to non-treated infected control (P<0.05) (Figure 1b). Results from the challenge studies therefore showed that administration of curcumin conferred protection to *B. pseudomallei*-infected mice.

Curcumin reduced bacterial counts in liver and spleen of *B. pseudomallei*

We next sought to examine the bacterial load in liver and spleen as these two are the most affected organs during melioidosis infection (Bast et al., 2011). Figure 2a showed that bacterial loads in liver and spleen from non-treated as well as curcumin-treated infected mice increased drastically (up to 10⁹ CFU) until day 4 post-infection. Results obtained showed that bacterial load in organs of *B. pseudomallei*-infected BALB/c mice increased to >10⁶ CFU within three days of bacterial infection.

![Kaplan-Meier survival curve of *B. pseudomallei*-infected mice (7.2 x 10⁴ CFU) (a) without (Bp) or with curcumin treatment (15, 30 or 60 mg/kg b.w.) at 1 h post-infection (n=9). Non-treated (non-infected) mice were used as control. Experimental animals were monitored for 14 days for survivability. (b) without (Bp) or with curcumin treatment (60 mg/kg b.w.) at 1 h post-infection (n=9). Significant difference between treated (Bp + curcumin) and non-treated infected control group (Bp) was evaluated at P < 0.05 (*).](image1)

![Bacterial load in 1 mL sample prepared from (a) liver and (b) spleen of *B. pseudomallei*-infected mice without (Bp) or with curcumin treatment (Bp + 60 mg/kg b.w. curcumin) at day 1, 2, 3, 4 and 14 after infection. Data for day 14 for *B. pseudomallei*-infected mice was not obtained since all animals in this group died within 5 days post infection. No significant difference between treated and non-treated infected mice was observed (P>0.05) (n=9).](image2)
(i.p.) similar to that reported by Leakey et al. (1998) indicating acute melioidosis infection.

There was no significant difference (P>0.05) between curcumin-treated and non-treated infected mice in terms of bacterial load in these organs (Figure 2b). These findings suggest that the improved survivability conferred by curcumin (observed and described earlier) was not attributed to the reduction of bacterial load in liver and spleen of curcumin-treated infected mice.

**Curcumin administration resulted in increased phosphorylation of GSK3β (Ser9) in both liver and spleen of *B. pseudomallei*-infected mice.**

Administration of curcumin (i.p.) resulted in significant increase of GSK3β (Ser9) phosphorylation by 1.2-2.6 fold in both liver (Figure 3a) and spleen (Figure 3b) of *B. pseudomallei*-infected mice. The effects were detected as early as 2 h after infection and persisted until 3 h after infection compared to non-treated infected group. These findings demonstrate that curcumin could induce inhibition of GSK3β (Ser9) in both liver and spleen of *B. pseudomallei*-infected mice during the early phase of bacterial infections. It is interesting to note that LiCl used here as a reference GSK3 inhibitor similarly resulted in increased GSK3β (Ser9) phosphorylation in both liver and spleen of *B. pseudomallei*-infected mice.

**Curcumin administration modulated the levels of pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokine (IL-10) in liver, spleen and serum of *B. pseudomallei*-infected mice**

To address the immunomodulatory role of curcumin in *B. pseudomallei*-infected mice, we examined levels of inflammatory cytokines (TNF-α, IFN-γ and IL-10) in liver, spleen and serum of curcumin-treated and non-treated infected mice. Our findings revealed that infection by *B. pseudomallei* elevated levels of TNF-α, IFN-γ and IL-10 in liver, spleen and serum of non-infected mice (Figure 4a, 4b and 4c). However, upon curcumin treatment, levels of TNF-α were significantly (P<0.05) decreased in liver (85%), spleen (68%) and serum (30%) of *B. pseudomallei*-infected mice compared to non-treated infected control (Figure 4a). Levels of IFN-γ were also significantly (P<0.05) decreased in liver (39%) of curcumin-treated infected mice compared to non-treated infected control (Figure 4b) but, no significant changes in IFN-γ levels were detected in spleen and serum. Curcumin administration resulted in significant (P<0.05) increase of anti-inflammatory cytokine (IL-10) only in serum (67%) of *B. pseudomallei*-infected mice compared to non-treated infected control (Figure 4c).

**DISCUSSION**

Curcumin is known to exhibit potent anti-inflammatory activities and thus potentially useful as an adjunctive therapy for the management of inflammation-related conditions such as sepsis (Xiao et al., 2012), diabetes (Kuroda et al., 2005; Yu et al., 2012), arthritis (Shakinaei et al., 2005), inflammatory bowel disease (IBD) (Holt et al., 2005), gastritis (Kim et al., 2005; Swarnakar et al., 2005) and fever (Shao et al., 2004). Anti-inflammatory activities displayed by curcumin are attributed to its effects on the inactivation of NF-κB and consequent downregulation of pro-inflammatory cytokine production (Shin et al., 2010).

During infections, pathogen-associated molecular patterns (PAMPs) and microbial products such as lipopolysaccharide (LPS), flagella, endotoxin and capsule, are often recognised as external intimidation by host-specialised receptors, Toll-like receptors (TLRs) (Cavaillon & Adib-Conquy, 2006). These host-pathogen interactions result in production of stress proteins including inflammatory cytokines to combat the infection. However, dysregulated production of pro-inflammatory cytokines such as TNF-α and IFN-γ, can lead to overwhelming inflammatory response in the host. LPS has been implicated as an important pathogenic factor in the induction of sepsis, which is characterised by an inflammatory cytokine storm consequently causing multiple organ failure and eventually death (White, 2003; Wiersinga et al., 2007; Chantratita et al., 2013). It is noteworthy that LPS has a central role in the innate immune response to *B. pseudomallei* infection (Chantratita et al., 2013).

A crucial mediator in the regulation of host innate inflammatory response to bacterial infections is GSK3β, a kinase known to act by modulating production of pro- and anti-inflammatory cytokines (Wang et al., 2014). In the case of melioidosis infection, we were the first to demonstrate that GSK3β is implicated in the modulation of cytokine production in *B. pseudomallei*-infected mice reiterating the important role of GSK3β in the inflammatory response caused by bacterial pathogens (Tay et al., 2012). Previous studies have shown that mice administered with GSK3 inhibitor peptide and subsequently challenged with *E. coli* LPS were protected from endotoxic shock (Ko et al., 2010). Inhibition of GSK3β also conferred distinct survival advantage to *F. tularensis*-infected mice (Zhang et al., 2009) through modulation of inflammatory cytokines production.
Fig. 3. Fold-change of GSK3β phosphorylation levels in both (a) liver and (b) spleen of non-treated non-infected mice (N), non-infected treated mice (N+C), infected mice (Bp), curcumin-treated infected mice (Bp+C) and LiCl-treated infected mice (Bp+LiCl) at 2 h and 3 h after infection. Levels of phosphorylated GSK3β (Ser9) were normalised to total levels of GSK3β. Significant difference between infected group (Bp) and treated infected group (Bp+C) was evaluated at P<0.05 (*).
Fig. 4. Inflammatory cytokine levels of (a) TNF-α, (b) IFN-γ and (c) IL-10 in liver, spleen and serum of non-treated non-infected mice (N), non-infected treated mice (N+C), infected mice (Bp) and curcumin-treated infected mice (Bp+C) at one day post-infection. Liver, spleen and serum samples were taken one day after infection. Significant difference between infected group (Bp) and curcumin-treated infected group (Bp+C) was determined at P<0.05 (*).

Findings from the present study revealed that the improved survivability of *B. pseudomallei*-infected mice with curcumin treatment involved inhibition of host GSK3β. Liver and spleen are involved in host innate immune response to bacterial stimuli (Jia & Pamer, 2009; Swirski et al., 2009). During acute *B. pseudomallei* infection, the hepatic and splenic organs are among the most affected organs (Kuroda et al., 2005; Apisarnthanarak et al., 2006). Here we report that there was no significant difference...
between the *B. pseudomallei* loads in liver and spleen of curcumin-treated and non-treated mice (*P*>0.05). As such, we postulate that the improved survivability of *B. pseudomallei*-infected mice conferred by curcumin was more likely attributed to the consequent modulation of the inflammatory (cytokine) response to infection.

With respect to this response, in this study we also demonstrated that curcumin modulated the cytokine response. Cytokines play a pivotal role in immunomodulation, and curcumin is known to regulate cytokine balance through inhibiting production of pro-inflammatory cytokines (Yadav *et al.*, 2005). We found that administration of curcumin lowered production of pro-inflammatory cytokines (TNF-α and IFN-γ) in most organs of *B. pseudomallei*-infected mice tested. We also detected higher levels of anti-inflammatory cytokine (IL-10) in serum of curcumin-treated infected mice. In a previous study, Jagetia and Aggarwal (2007) reported that curcumin inhibited expression of TNF-α, IL-6 and other chemokines. It was also reported that curcumin inhibited production of TNF-α, MIP-1α, MCP-1, IL-1β and IL-8 induced by microbial products in alveolar macrophages and human peripheral blood monocytes (Abe *et al.*, 1999). Curcumin binds and suppresses the activity of COX-2 and 5-LOX in arachidonic acid metabolism (Hong *et al.*, 2004).

Moreover, curcumin is a potent inhibitor of STAT 3, a transcription factor that mediates the effects of pro-inflammatory cytokines (Bharti *et al.*, 2003). Treatment with curcumin inhibited the mRNA transcripts of IL-1α, IL-β, IL-6, IL-10 and TNF-α, suggesting the protective effects of curcumin against hemorrhagic liver injury (Gaddipati *et al.*, 2003). In addition, curcumin protects rat liver from CC14-caused injury by suppressing hepatic inflammation (Fu *et al.*, 2008). Curcumin has been shown to suppress activation of NF-κB (Weber *et al.*, 2006; Aggarwal & Harikumar, 2009) and downregulate expression of various inflammatory cytokines including TNF-α, IFN-γ, IL-8 and IL-12 (Kang *et al.*, 1999; Jang *et al.*, 2001; Jurrmann *et al.*, 2005; Yeh *et al.*, 2005; Fu *et al.*, 2008). It is known that inhibition of GSK3β can cause inactivation of the transcription factor, NF-κB and consequent modulation of pro- and anti-inflammatory cytokines levels (Wang *et al.*, 2014).

The above findings suggest that curcumin suppresses inflammation through various pathways and GSK3β, being a point of convergence for multiple signaling pathways may be one of the cellular targets for the anti-inflammatory effects of curcumin in pathogen-induced inflammation.

In conclusion, we have provided evidence that GSK3β is a molecular target for the cytokine-modulating effect of curcumin in mice experimentally-infected with *B. pseudomallei*. Our research findings represent further scientific evidence for the traditional use of curcumin as remedy for inflammation-related diseases. Preclinical studies and clinical trials are certainly required to further validate the benefits of curcumin in pathogen-induced inflammations.

**ACKNOWLEDGEMENT**

This work was supported by a research grant from the Centre for Collaborative Innovation, Universiti Kebangsaan Malaysia (UKM ETP-2013-052).

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GSK3β as a molecular target for cytokine-modulating effect of curcumin


