CHAPTER IV

DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that mainly affects the synovial joint lead to destruction of cartilage and bone. One predominant of this disease is fibroblast- like synovial cells (FLS) excessively synthesize synovial joint fluid and a major component of synovial joint fluid is hyaluronan (HA) (89, 90). Recently, it has been reporting that elevated serum levels of HA correlate with joint inflammatory in RA (91). HA is synthesized by a class of membrane-bound haluronan synthase (HAS) protein. Three human haluronan synthase (HAS1, HAS2 and HAS3) genes were cloned and characterized as synthesizing HA of different molecular weights (45). In addition, different cell type or phenotypes expressed different HAS enzyme (92, 93). The expression of HASs gene were induced by pro-inflammatory cytokines especially interleukin (IL)-1β lead to HA release whereas the elevation of IL-1β production was increased by Lipopolysaccharide (LPS) via NF-κB and/or MAP kinase signaling pathway (24, 69, 70, 71, 72, 73). Possibly, LPS stimulation might enhance HASs gene expression and HA synthesis. Hence, this study proposed to investigate the roles of LPS on HASs gene expression and HA synthesis in human synovial fibroblast SW982 cell line and FLS.

Primary mammalian cells in culture have a finite replicative life span, eventually enter a state of senescence, and routinely obtaining RA-derived synovial

tissue samples is difficult (101). In the previous study, they used human synovial fibroblast SW982 cell line as a likely tool to study the effect of cytokines on matrix metalloproteinases (MMPs) gene expression that involved RA condition. Nevertheless, the effect of LPS on HAS1, 2 and 3 gene expressions in SW982 cell line has never been studied (13).

FLS, mediate inflammation and autoimmunity, respond to inflammatory mediators such as IL-1β (94), Tumor necrosis factor (TNF)-α (95) and/or LPS (96). In response to LPS, Toll-like receptor (TLR)-4 or LPS receptor, initiates a cascade of serine/threonine kinases that eventually lead to the transcription of genes involved in inflammatory such as IL-1β and TNF-α (97). In previous report indicated that IL-1β markedly enhanced the gene expression of HAS2 and HAS3 in cultured human orbital fibroblasts (98), human periodontal ligament (99), and rabbit knee synovial membrane fibroblasts (100). These agreed with results from the present study showed LPS significantly increased HAS2 and HAS3 gene expression in human synovial fibroblast cell. Furthermore, LPS also induced the gene expression of TLR-4 that is mediator of LPS signals and increased IL-1β and ICE gene expression (96).

In this presence, LPS increased production of inflammatory cytokine molecules that may involve in up regulation of TLR-4, TLR-4 was regulated by MD-2, through MyD88-dependent pathway, then induced MAPK or NF-κB contributed to enhanced the inflammatory cytokines molecules particularly IL-1β and/or interleukin-1 converting enzyme (ICE) (43, 67, 102). It has been reported that the expression of HAS genes were induced by IL-1β lead to HA release (24, 71, 72, 73). It was well know that pro-IL-1β was cleaved into bioactive form (IL-1β) by ICE

(68). In addition, ICE plays a key role in regulation of Toll-like receptor (TLR)-4 signaling pathway via an effect on MyD88 adaptor-like (Mal). Mal was cleaved by ICE and that inhibitor of ICE activity blocked TLR4-mediated NF-κB and p38 MAP kinase activation (103).

Our experiment interestingly investigated the effect of LPS on TLR-4, IL-1β, ICE, HAS1, HAS2 and HAS3 gene expression in SW982 cell line. In result suggested that LPS elevated TLR-4, IL-1β, ICE, HAS2 and HAS3 gene expression in SW982 cell line similar to human synovial fibroblast cell, whereas gene expression of HAS1 could not be detected by RT-PCR even stimulated by LPS in the both cell. These results were in agreement with the previous studies reporting that HAS1 mRNA could not be detected in human periodontal ligament cell and rabbit synovial cells (99, 100). Thus, SW982 cell lines are a useful tool for the study of gene expression of TLR-4, IL-1β, ICE, HAS1, HAS2 and HAS3. Moreover, in this experiment found that the synthesis of HA was enhanced by the stimulation of LPS in SW982 cell line and human synovial fibroblast. Concurrently, HAS2 and HAS3 gene expression were remarkably up-regulated by LPS stimulation. These finding suggest that the HA synthesis is caused by the up-regulation of HAS gene expression. Thus, an elevation of HAS gene expression caused by pathological conditions contributed to the high level of HA accumulation in synovial joint fluid that one hallmark of RA (4).

Normally, HAS2 produced significantly more high-molecular-weight hyaluronan (HMW-HA) which is an indispensable component in the maintenance of joint homeostasis (35, 48). Up-regulated HAS2 gene during rheumatic inflammation resulted in increasing of HA synthesis. Altogether with this event, hyaluronidase; HA

breaking enzyme also up-regulated. By the action of hyaluronidase, long chain HA was cropped to be small size HA in rang of 200 Da (42,104). As reported by previous study, fragmented HA, that the size small than 250 kDa, trigger inflammation gene expression through TLR-4 signaling pathway (42,105). Hence, over-production of HA by HAS2 mRNA co-incident with up-regulation of hyaluronidase contributed to generating HA fragment. However, we should further investigate the effect of LPS on hyaluronidase genes expression in primary human synovial fibroblast compared with SW982 cell line to use SW982 cell line as a likely model to study the effect of LPS on hyaluronidase gene expression that involved RA condition.

It was well known that responsibility of HAS3 was produced-low molecular weight HA (LMW-HA) about 200 kDa and HA, the size small than 250 kDa, induced inflammatory genes via TLR-4 signaling pathway (42, 105). Therefore, HAS3 play essential role to induce inflammation. Our experiment found that LPS significantly increased HAS3 gene expression lead to elevated LMW-HA.

Rheumatoid arthritis drugs aim to reduce joint inflammation, however drugs treatment have many side effects (75-77). Therefore, the several previous reports studied alternative therapies to reduce side effects. In the present study indicatd that compound C, extracted from *Z. cassumunar* Roxb., which is a medicinal herb, exhibited anti-inflammatory. In experiment to estimate the effect of compound C on cell viability found that the range of 0-200 μM compound C did not affect SW982 cell line viability. From this result, compound C significantly decreased the expression of HAS2 and HAS3 mRNA and HA release in SW982 which were treated with or without LPS. This data were confirmed by the reduction of HA synthesis which

represented activity of HASs. Interestingly, compound C also suppressed TLR-4, IL-1β and ICE in present and absence LPS. It was found that compound C inhibited expression of TLR-4, IL-1β, ICE and HASs mRNA including HA release bring about decreased the over-accumulated HA in join that cause rheumatoid inflammation. To support the effect of compound C that act as anti-rheumatoid, so our experiment investigated the mechanism of compound C compared with dexamethasone, anti-rheumatoid drug. In result found that compound C incubated with SW982 cell line inhibited the expression of TLR-4, IL-1β, ICE and HASs mRNA and HA synthesis in absence and presence LPS stimulation similar to dexamethasone. Thus, compound C strongly relieve to rheumatoid inflammation.

However, we should study the direct binding between LPS with compound C by pre-incubate of LPS with SW982 cell line, and then wash out LPS before SW982 cell line is incubated with compound C or by BIAcore analysis.

From the all results can conclude that LPS induced the TLR-4, IL-1β, ICE and HASs mRNA, ultimately leading to the high level of HA accumulation however the effect of LPS-induced TLR-4, IL-1β, ICE and HASs mRNA expression were suppressed by compound C. These can be summarized in Figure 32.

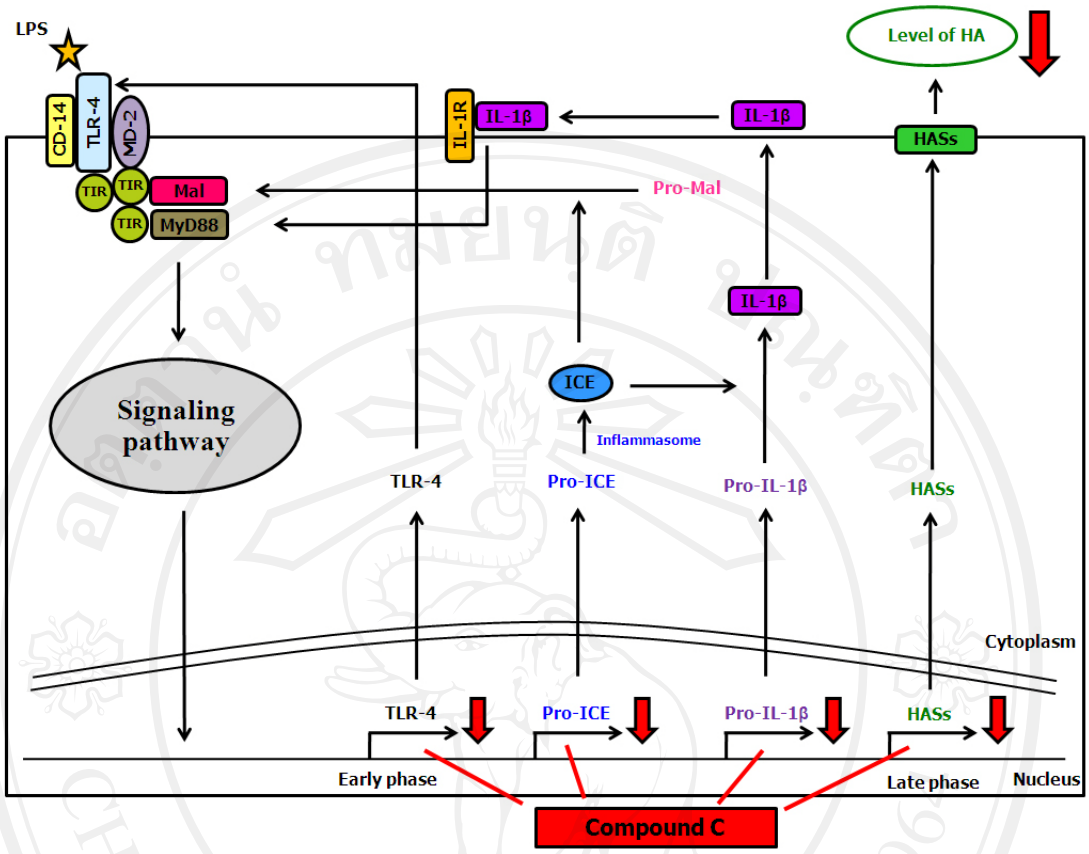

Figure 32. Model of propose the propose mechanism of compound C inhibit LPS-induce TLR-4, IL-1β, ICE and HASs genes expression contribute to reduce HA synthesis in SW982 cell line. This figure was modified from Watters M. *et al.* (43).

The further studies

We will investigate the molecular mechanisms, in which *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-{(E)-2''',4''',5'''-trimethoxylstyryl}-cyclohex-1-ene (compound C) inhibits transcription of HAS genes via signaling pathways such as Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or Mitogen-activated protein kinase pathway (MAPK) cascades as shown in Figure 33.

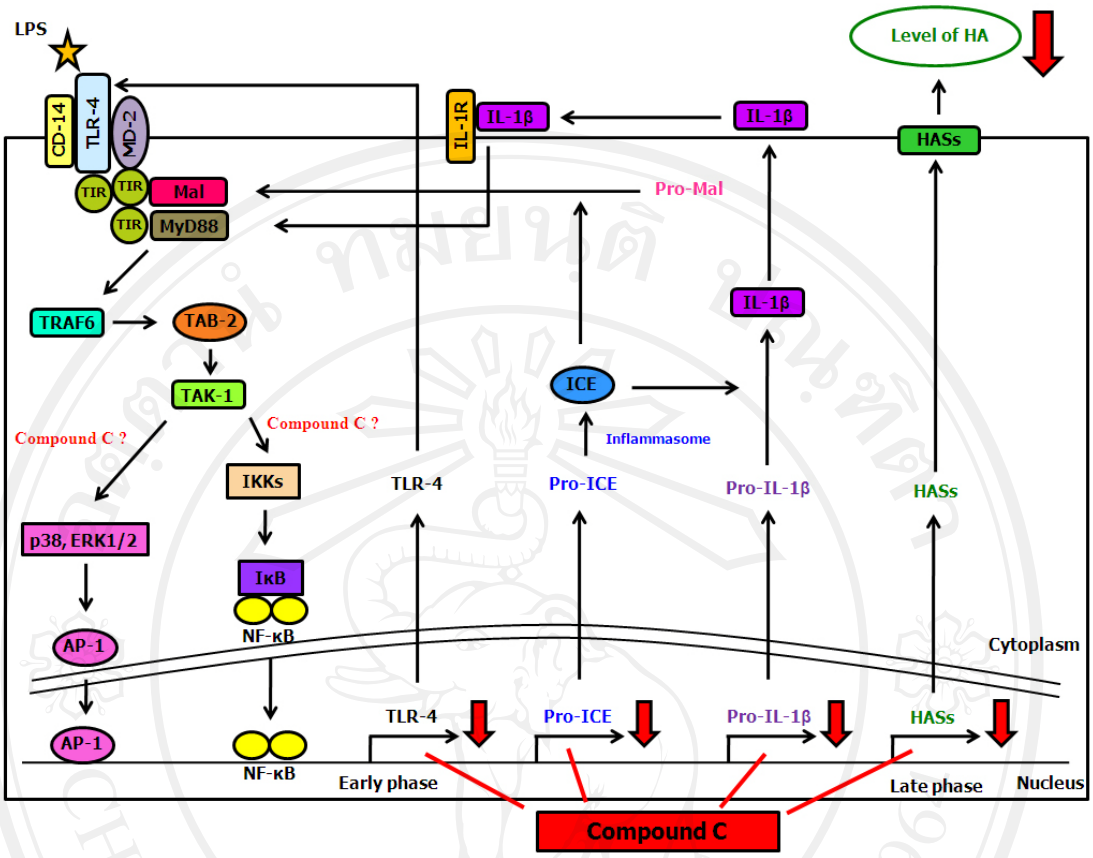

Figure 33. Further study model of propose the propose the molecular mechanisms, in which compound C inhibits transcription of HAS genes via signaling pathways such as Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or Mitogen-activated protein kinase pathway (MAPK) cascades. This figure was modified from Watters M. *et al.* (43).

CONCLUSION

In conclusion, LPS was found to induce TLR-4, IL-1β, ICE, HAS2 and HAS3 gene expression, and hyaluronan synthesis in a human synovial fibroblast SW982 cell line, which were similar to those of human synovial fibroblast cell. These effects were inhibited by compound C, one of the active compounds which were isolated from *Z. cassumunar* Roxb.

Therefore, it might be possible to use this compound as new pharmacological agent for the management of rheumatoid arthritis and other arthritis.