CHAPTER V

DISCUSSION AND CONCLUSION

Effect of acute Trikatu treatment in rat

Relationship between short-term treatment (7 days) of trikatu and physical or biochemical effect in rat was studied. The body weight and relative wight of vital organs were monitored. Analysis of body weight and organ weight in pharmacological study is an important endpoint for identification of potentially harmful effects of chemicals (Steven, et al., 2004). Changes in either body weight or vital organs weight are assess to the pharmacological effects of Trikatu in rat. The results showed that body weight of rats treated with 500 mg/kg Trikatu were not changed, but 1,000 mg/kg Trikatu caused a decrease in body weight. A significant increase in liver weight was also found in both acute treatment and control. Interestingly, spleen weight decreased in 1,000 mg/kg treated rats. The administration of Trikatu at 1,000 mg/kg body weight may be not only toxic to liver and spleen but also induced the immunological response in rat.

In this case, histopathological changes in liver of rats fed with various concentrations of Trikatu were studied. Administration of Trikatu at 500 mg/kg showed minimal effect of hydropic degeneration on the liver. No apparent disruptions of the normal liver structure was detected. However, administration at 1,000 mg/kg Trikatu showed a mild hepatic injury. The administration of the Trikatu at 1,000 mg/kg may induce the hepatotoxicity rapidly with unknown mechanism and allow accumulation of water in cytoplasm and between hepatocytes. Serum ALT and AST activity were used to state the hepatocellular membrane permeability (Giboney, 2005). ALT is a cytoplasmic enzyme, and is considered to be liver specific. AST is presented in both the cytoplasm and mitochondria of hepatocytes. No change in activities of both enzymes were detected after treatment with Trikatu. It is indicated that membrane permeability of hepatocytes did not affected by Trikatu. However, necrosis and cirrhosis/fibrosis of the liver are not be excluded.

Effect of subacute Trikatu treatment in rat

Orally administration of 50 and 150 mg/kg Trikatu for 30 days was called as the subacute studies. The body weight of rats after treatment with low concentration of Trikatu for a month were similar to control. Absolute and relative liver weights of rats treated with 150 mg/kg Trikatu increased. A hydropic swelling in liver section was detected. However, changes in liver weight and histopathology of hepatocytes were not found in rats treated with 50 mg/kg Trikatu. Serum ALT and AST did not elevated in both group. Non-significant effect of 50 mg/kg Trikatu on rat livers was similar to Chanda and colleague (2009). Long-term administration of low doses of Trikatu may be considered as relatively safe, as it did not produce severe toxicological effects on body and organs of rats.

Effect of Trikatu on lipid profile in rat

Trikatu reduced serum triglyceride in acute and subacute group, but HDL-cholesterol levels did not changed as compared to control. The effectiveness of reduced triglyceride was found in subacute group. In subacute group, Trikatu decreased triglyceride and cholesterol in rat serum. The cholesterol-lowering abilities of Trikatu appear to be dose-dependent. The obtained results were in agreement with the first report of Valsala and Sivakumar (2004).

Piperine is a major of active compound of long pepper and black pepper. Treatment with piperine significantly reduced not only the serum trigylyceride and total cholesterol levels, but also significantly increased the HDL-c level, which proved its beneficial effect in reducing dyslipidemia (Shreya, et al., 2011). Srinivas (2009) investigated effects of the ethanolic extract of ginger for lipid regulating activities in high-fat diet-fed rat. The result showed that total cholesterol and triglycerides in rat serum were significantly reduced by ginger treatment. However, no significant change in serum HDL-cholesterol. Prakash and Srinivasan (2007) studied effect of dietary test spices on serum lipid profile in normal rats. They showed that piperine and ginger prominently decreased the level of serum triglycerides. The decrease in serum triglyceride was 28.2% and 27% in normal rats, respectively. Taken together, it is suggested that the decreased triglycerides and cholesterol and increased HDL-

cholesterol effect induced by Trikatu may improve dyslipidemia and prevents the risk of atherosclerosis and heart attacks.

Effect of Trikatu on protein expression in liver

Low concentration of Trikatu decreases levels of triglyceride and cholesterol in rat serum. The mechanism underlying this novel finding remained unclear. A proteomic approach is therefore selected to study the proteome change after treatment with Trikatu.

Proteins related to lipid matabolism

Liver was targeted because it is a primary site of lipid metabolism (Nguyen, et al., 2008). The proteomics data also showed that the level of several proteins changed in response to Trikatu treatment. Of which 5 proteins were related to fatty acid oxidation and 3 proteins were related to triglyceride synthesis and transpot. The carnitinepalmitoyltransferase I (Cpt-1a), short-chain-acyl-CoA dehydrogenase (Acads), long-chain-acyl-CoA dehydrogenase (Acadl), enoyl-CoA hydratase (Echdc) and acetyl-Coenzyme Aacyltransferase 2 (Acaa2) were involved in the fatty acid oxidation within mitochondrial. These proteins were significantly decreased by Trikatu treatment. The Cpt-1a is an essential step in the beta-oxidation of long chain fatty acids (Kerner and Hoppel, 2000), while fatty acids are activated on the outer mitochondrial membrane and mediates the transport of long-chain fatty acids across the membrane by binding them to carnitine, the activated fatty acids are oxidized within the mitochondrial matrix in β-oxidation process (van, et al., 2000; Bonnefont, et al., 2004; Berg, et al., 2007). Cpt-1a is inhibited by malonyl-CoA, although the exact mechanism of inhibition remains to be known (Bonnefont, et al., 2004; Akkaoui, et al., 2009; Song, et al., 2010). The inhibition of Cpt-1a can be a way to prevent simultaneous oxidation of fatty acids within the liver cell. Consequently, a decrease of Cpt-1a after Trikatu treatment may be a good target for future attempts to regulation of fatty acids oxidationin metabolic disorders patient.

The inhibition of lipid transport may affect to a reduces fatty acid oxidation processes in mitochondria. In generally, mitochondrial β-oxidation of fatty acid is a cyclic process by fatty acyl-CoA dehydrogenase such as long-chain (Acadl), medium-chain (Acadm), and short-chain acyl-CoA dehydrogenase (Acads) that work on

catalyzed fatty acid becomes progressively shorter, respectively (Thorpe and Kim, 1995). Enoyl-CoA hydratase (Echdc) catalyzes the second step in the breakdown of β-oxidation. The final step of β-oxidation is the cleavage of 3-ketoacyl CoA by the thiol group of another molecule of CoA. This reaction is catalyzed by Acetyl-CoA acyltransferase (Acaa). The complete breakdown of a fatty acid not only generates these acetyl-CoA molecules, but it also generates a great deal of energy in the form of NADH and $FADH_2$ then feeding electrons into the electron transport system for oxidative phosphorylation to ATP (Nelson, et al.,2005). The result of proteomics showed a decease of enzyme in β-oxidation such as Acadl, Acads, Echdc and Acaa2. The results indicated a decrease in fatty acid oxidation due to Trikatu treatment. Thus, inhibition of fatty acid uptake into mitochondria and reduction of enzymes in fatty acid oxidation simultaneously may occure simultaneously. The expression level of these enzymes may caused a deceases of acetyl-CoA produced from fatty acids within the liver (Figure 13).

Lipogenesis is the process by which acetyl-CoA is converted to fatty acid molecule (Kersten, 2001). The former is an intermediate stage in metabolism of simple sugars, such as glucose, a source of energy of living organisms. Lower expression of acetyl-CoA carboxylase 2 (Acc2) may affect the conversion rate of fatty acyl-CoA to malonyl-CoA, a molecule that is committed to fatty acid synthesis (Abu, et al., 2003). Lower expression of 1-acylglycerol-3-phosphate O-acyltransferase 1 (Agpat1) which is involved in glycerol synthesis from glycolysis was also reported. The construction of fatty acids from fatty acyl-CoA is combined with glycerol 3-phosphate, to form triacylglycerol. Thus, the decreased levels of these two enzymes in Trikatu treatment may lower concentrations of triglyceride. On the other hand, the triglyceride products may be secreted from the liver into the bloodstream in form of very-low-density lipoproteins (VLDL). The proteomics results showed a decrease in microsomal triglyceride transfer protein (Mttp) and apolipoprotein E (ApoE), which are involved in the regulation of synthesis and secretion of VLDL in the liver (Figure 13). Although, the decreased serum triglyceride levels of Trikatu may be consistent with decreased triglyceride synthesis and/or secretion of VLDL from liver.

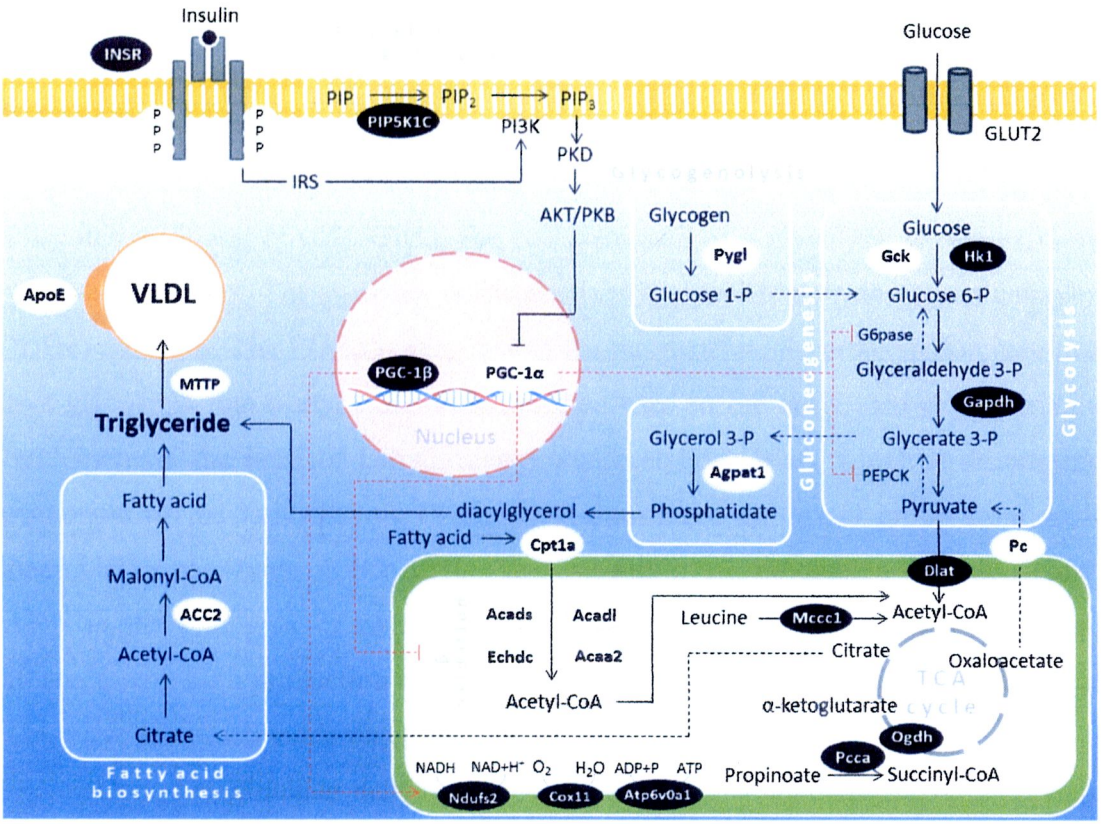

Figure 13 Schematic illustration of proposed mechanism of Trikatu treatment in rat liver. Black circles mean up-regulation and white circles mean down-regulation of protein expression.

Proteins related to carbohydrate metabolism

The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in bloodstream (Postic, et al., 2004). In this study, many up-regulated proteins as a result of Trikatu were associated with glycolysis, gluconeogenesis, tricarboxylic acid cycle and electron transport chain after Trikatu treatment. Glycolysis is the metabolic pathway that converts glucose into pyruvate. A molecule of glucose is performs ten stepwise chemical transformations in cytosol of the liver. Trikatu can induce varius enzymes during this process. Hexokinase (Hk) and Glucokinase (Gck) are an enzyme catalyzed phosphorylation of glucose to yield glucose 6-phosphate (Robey and Hey, 2006). The increased of Hk1 in Trikatu treatment indicated utilizing glucose as energy source.

Glyceraldehyde phosphate dehydrogenase (Gapdh) is dehydrogenated glyceraldehyde 3-phosphate in the first step in the second phase of the breakdown of glucose. Trikatu caused an increase of Gapdh enzyme that affect higher level of cellular 1,3-bisphosphoglycerate. These involved to net gain of the energy-rich molecules ATP and NADH. Then, the complete step of a glycolysis generates more pyruvate molecules. The pyruvate is metabolized bypyruvate dehydrogenase complex (PDHc) enzymes. The PDHc is responsible for the pyruvate decarboxylation step that links glycolysis into acetyl-CoA which is then used in the citric acid cycle. Trikatu could increase the level of Dihydrolipoyl transacetylase (Dlat), which is an enzyme component of the multienzyme PDHc. Dlat transferred the acetyl group from acyl-lipoamide to coenzyme A (CoASH). This results in the production of acetyl CoA, which is the end goal of pyruvate decarboxylation. High Dlat activity may also influence with high production of acetyl CoA.Most of acetyl-CoA molecules enter the TCA cycle in mitochondria and generateenergy by electron transport chain.

Alpha-ketoglutarate dehydrogenase (Ogdh), one subunit of the 2-oxoglutarate dehydrogenase complex increased after Trikatu treatment. This complex catalyzes the overall conversion of alpha-ketoglutarate to succinyl-CoA and CO_2 during the citric acid cycle. Interestingly, Trikatu can increase several enzyme complexes in electron transport chain such as NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (Ndufs2), cytochrome c oxidase assembly protein 11 (COX11), cytochrome c oxidase 7A2 (COX7A2), cytochrome c oxidase subunit 4 (COX4) and ATPase, H+ transporting, lysosomal V0 subunit A1 (Atp6v0a1). Higher electrochemical proton gradient may generate more energy in the form of ATP which powers most cellular reactions (Figure13).

Regulation of hepatic lipid and glucose metabolism

The liver plays a key role in glucose homeostasis, lipid and energy metabolism. The result of proteomics showed that Trikatu increased insulin receptor (Insr), it responses to many pathways including the stimulation of lipid synthesis and storage, glycolysis and glucose storage, and the inhibition of ketogenesis and gluconeogenesis (Shaham, et al., 2008). The activation of insulin receptor can activate the intermediated proteins in PI3K-PDK-Akt/PKB cascade (Fritsche, et al., 2008). Trikatu activated phosphatidylinositol-4-phosphate 5-kinase (PIP5K1C) that is an enzyme

catalyzes phosphatidylinositol-4-phosphate (PIP) to phosphatidylinositol-4,5-bisphosphate (PIP2). PIP2 act as substrates for enzymes PI3K, that produced phosphatidylinositol-3,4,5-trisphosphate (PIP3) in plasma membrane. PIP3 regulates main classes of signalling molecules as phosphoinositide-dependent kinase 1 (PDK1), one of the serine kinases that phosphorylates and activates the serine/threonine kinase Akt/PKB (Alessi, et al., 1997). This influenced the increased glucose transports into the liver cells (Cheng, et al., 2010). Moreover, Trikatu may modulate gene transcription by activating various transcription factor such as peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) and beta (PGC-1β) in the liver.

Trikatu repressed PGC-1α by AKT cascade that controls the transcription of genes involved in gluconeogenesis, fatty acid β-oxidation and ketogenesis (Yoon, et al., 2001; Lin, et al., 2005; Finck, et al., 2006; Liang, et al., 2006). PGC-1α expression is relatively low in liver that rely in fed conditions (Daitoku, et al., 2003). The essential role of PGC-1α is induced of hepatic gluconeogenesis enzymes expression such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (Leone, et al., 2005). Thus, the reduction of PGC-1α may inhibit gluconeogenic metabolism. PGC-1β is a recently identified homologue of PGC-1α. It appears that the expression levels of PGC-1β correspond to the mitochondrial content (Meirhaeghe, et al., 2003). When overexpressed in mice, increase the activity of mitochondria, the intracellular organelles that turn sugars and fats into heat or the cellular fuel ATP (Lelliott, et al., 2006; Junichiro, et al., 2007). PGC-1β coactivated with FOX2 that regulates hepatic lipid homeostasis by affecting the clearance rate of fatty acids through oxidation and/or secretion of lipids in response to insulin (Christian and Markus, 2006; Wolfrum, et al., 2006). Therefore, the activation of PGC-1β in Trikatu treatment may increase the energy in form of ATP and reduce serum lipid by stored its precursor in hepatocytes.

Conclusion

Trikatu had an antihyperlipidemic effect. It is capability in reducing triglyceride and cholesterol levels. The best model of this observed study, a oral administration of Trikatu at dose 50 mg/kg for 30 day, which might be a better consumed dose as a reducing of lipid levels and no obvious toxic found in the liver.

This is the first proteomics study of rat liver after orally administration of Trikatu. Several proteins related to carbohydrate and lipid metabolism were altered during treatment. Up-regulation of proteins related to glycolysis and oxidative phosphorylation were recorded. Conversely, down-regulation of proteins involved in lipid oxidation and triglyceride synthesis were found. These Trikatu responsive pathwaymay be regulated through insulin receptor system. Higher activity of level if insulin receptor accelerate glucose transport and simultaneously increase the activity of the regulatory enzymes in glycolysis. In addition, Trikatu may regulate transcription factor that leads to a decrease of fatty acid oxidation and triglyceride synthesis.

Several interesting proteins may be benefit to study the effect and toxicity of drugsor natural products on lipid controlling. The expression level of these proteins are necessary to be validated and the knowledge obtained will allow a better understanding of the biological pathway underlying the pharmacological of Trikatu.