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Original Article

EFFECT OF STANDARDIZED *EURYCOMA LONGIFOLIA* EXTRACT ON PHASE I ROSIGLITAZONE METABOLISM IN THE OLD NORMAL MALE RAT HEPATOCYTES: A MECHANISM STUDY

PURWANTININGSIH[1]*, ABAS HJ HUSSIN[2,3], KIT LAM CHAN[2]

[1]Department of Pharmacology & Clinical Pharmacy, Faculty of Pharmacy, Gadjah Mada University, 55281 Yogyakarta, Indonesia; [2]School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia; [3]Centre for Drug Research, Universiti Sains Malaysia, 11800 Penang, Malaysia.

Email: purwanti_n004@yahoo.com

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ABSTRACT

Objective: The aim of present study was to evaluate the possible mechanism of *E. longifolia* extract in affecting phase I Rosiglitazone metabolism in the old normal male rat hepatocytes.

Methods: Experiment was performed used ten cellular stimulants/inhibitors. Old normal male rats were divided in ten groups (n = 6) and the hepatocytes were isolated using the perfusion technique. Each group had a negative control (in the presence of vehicle), a positive control (in the presence of cellular inhibitors/stimulants) and 6 test subgroups (in the presence of cellular inhibitors/stimulants and 0.001-100 µg. $.mL^{-1}$ of *E. longifolia* extract). Effect of *E. longifolia* extract on phase I Rosiglitazone metabolism was determined by the colorimetric method of Nash at 415 nm.

Results: The results showed a significant effect of *E. longifolia* extract on phase I Rosiglitazone metabolism was observed after rat hepatocytes were pretreated with okadaic acid and genistein. After pre-incubation with KT5823 and trifluoperazine /TPA, the significant effect was only found in the presence of *E. longifolia* extract at the highest concentration (100 µg. $.mL^{-1}$) and no significant effect was observed in the presence of KT5720, furafyllin, L-ornithine, 3-isobutyl-methylxanthine/IBMX, phorbol-12-myristate-13-acetate/PMA and guanylyl-5′-imidodiphosphate/Gpp.

Conclusion: The data suggested that the possible effect of *E. longifolia* extract on phase I Rosiglitazone metabolism in the old normal male rats was probably mediated through the activation of phosphatase and inhibition of tyrosine kinases.

Keywords: Rosiglitazone, *Eurycoma longifolia*, Old normal male rat, Mechanism study, Stimulant/inhibitor.

INTRODUCTION

The activities of cytochrome P-450 (CYP450) enzymes are affected by endogenous and also by exogenous factors including dietary and environmental factors [1]. Xenobiotics (drugs or herb-drugs and other compounds) are important environmental factors that are able to affect CYP450 activities. Induction or inhibition of CYP450 enzymes by xenobiotics is well known and phosphorylation has been discovered as an important component in the regulation of xenobiotic-metabolizing CYP450s [2, 3]. Many herbs and their natural compounds have been identified as inhibitors or inducers of the CYP450 system [4]. Induction and inhibition may occur through different pathways, depending on their mechanisms, when a substrate (such as drug or hormone) binds to a specific receptor on a target cell, such as the phosphatidyl inositol pathway, cyclic adenosine monophosphate (cAMP) pathway or cyclic guanosine monophosphate (cGMP) pathway. Isolated cells or cultured hepatocytes are often used as *in vitro* models for determining metabolic pathways of drugs [5].

Eurycoma longifolia is a tall tree belonging to the Simaroubaceae family, has been popular in Asia and is distributed in the forests of Burma, southern Myanmar, Thailand, Indochina (Cambodia, Laos and Vietnam), Sumatra, Borneo, Philippines and Malaysia. *E. longfolia* has many local names such as tungat ali, bidara laut or pasak bumi, antong sar, tho nan, plaalai phuenk, Cay ba binh or tongkat ali [6, 7]. In Malaysia, this plant is known as Tongkat Ali and has been reputed as an aphrodisiac. Many results have been published related with the isolation of many active compounds in the *E. longifolia*, aphrodisiac activity or many others activities [8-13] and some interaction studies have been reported [14, 18]. Previous study showed that the standardized extract of *E. longifolia* (TAF-273) affected phase I Rosiglitazone metabolism especially in old normal and diabetic male rat hepatocytes [19]. The present study evaluated the possible mechanism involvement of TAF-273 on phase I Rosiglitazone metabolism in the old normal male rat hepatocytes.

MATERIALS AND METHODS

Plant materials

The standardized extract of E. longifolia (TAF-273) was provided by Prof. Chan Kit Lam from the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The extract was made according to Chan et al. (2004) [11] and a voucher specimen of the plant have been deposited, with Reference No. 785-117, at the Penang Botanical Garden. The E. longifolia extract was standardized with euryomanone as a marker compound, using a MICROTOF-QII LC-MS (Bruker ®, USA) and the eurycomanone content in the extract was found to be 15.89 % ± 0.95 SD [19].

Chemicals

Ten selular inhibitors/inductors was used in this study namely: genistein (purity 97.3%), L-N5-(1-iminoethyl)-ornithine [L-ornithine] (purity >98%), 9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epxoy-1H,8H,11H-2,7b-11a-triazadibenzo (a,g) cycloocta(cde)-trinden-1-one [KT5823] (purity >99.0%), 9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg: 3′,2′,1′-kl] pyrrolo[3,4-i] [1,6]benzodiazocine-10-carboxylic acid hexyl ester [KT5720] (purity 98%), 3-isobutyl-1-methylxanthine [IBMX] (purity >99%), phorbol-12-myristate-13-acetate [PMA] (purity 98.08%), and guanylyl-5′-imidodiphosphate tetralithium salt [Gpp] (purity 95.4%), okadaic acid potassium salt [OKA] (purity >98%) were purchased from Calbiochem, Germany, while trifluoperazine dihydrochloride [TPA] (purity 98%) and furafylline (purity ≥98%) that were purchased from Sigma Chemicals Co, USA. Diethyl ether, magnesium sulphate and magnesium chloride were obtained from BDH Laboratory Supplies, UK, while acetyl acetone and trypan blue were purchased from Sigma Chemicals Co, USA. Glucose monohydrate and calcium chloride were purchased from Riedel-deHaen, France while disodium hydrogen phosphate, ammonium acetate, barium hydroxide, zinc sulphate heptahydrate were obtained from R & M

Chemicals, UK and formaldehyde solution 37% from Merck, Germany. Rosiglitazone was purchased from Wuhan Sunrise Technology, China. Reagents used in this study were from sources described previously or were of the highest qualities commercially available.

Experimental animals

The rats for this experiment was bred in the Animal Research and Service Centre (ARASC) of the Universiti Sains Malaysia, Penang, Malaysia. All the male Sprague Dawley (SD) rats had access to standard food pellets (Gold Coin®, Penang, Malaysia) and tap water ad libitum. The study protocol was approved by the Animal Ethics Committee, University Sains Malaysia, Penang, Malaysia with approval no. USM/PPSF/50 (066) Jld.2.

Experimental procedures: mechanism study of TAF-273 in affecting phase I Rosiglitazone metabolism in adult normal male rat hepatocytes

In this study, ten cellular stimulants or inhibitors were used to examine the possible effect of TAF-273 on Rosiglitazone N-demethylase activity. The concentrations of ten cellular stimulants/inhibitors used in this experiment were based on the IC50, EC50 or Ki according to the previous studies: genistein (IC50 = 10 µM [20]), KT5823 (Ki = 234 nM [21]), KT5720 (Ki = 56 nM [22]), IBMX (IC50 = 50 µM [23]), PMA (EC50 = 1 µM [24]), Gpp (EC50 = 1 µM [25]), OKA (IC50 = 15 nM [26]), TPA (IC50 = 100 µM [27], furafylline (IC50 = 70 nM [28] and L-ornithine (IC50 = 0.5 µM [29].

Hepatocytes were isolated from adult normal male SD rats by perfusion technique [30]. Peristaltic pump (Watson Marlow®, England) was used to perfuse the liver sample from the animal test. Perfusion was done in situ through the portal vein at speed 15 mL. .min-1. Isolation of hepatocytes from the sample was done by centrifugation using centrifuge (Centrifuge 5403) [Eppendorf®, Germany]. Microscope (Olympus®, Optical works, ERMA, Tokyo) was used to account the hepatocytes and to check the cell viability. The cell viability was determined using trypan blue solution and hepatocytes with cell viability above 85% were used in this experiment. Experiments were divided into ten groups (ten stimulants/inhibitors were used in this experiment) and each group had eight subgroups (n=6). Group I was negative control [C(-)] (in the absence of TAF-273 and stimulants/inhibitors) and group II was positive control [C(+)] (in the absence of TAF-273 but in the presence of stimulants/inhibitors), whereas groups III to VIII [E1-E6] were test groups (in the presence of TAF-273 and stimulants/inhibitors).

Petri dishes containing isolated hepatocytes ($75x10^3$ cells. .mL-1) in the incubation medium were pre-incubated with stimulants/inhibitors or vehicle (for negative control group) for 15 min. Then an equal volume (1.0 mL) of TAF-273 serial dilution (ranging from 0.001 to 100 µg. mL^{-1}) in distilled water were added and followed by Rosiglitazone (0.75 mM). The final volume was 10 mL. Samples were incubated for 12 min at 27 ºC on a shaker (Bellly Dancer®, Stovall, Life Science, INC. USA). The reaction was stopped by 25% (w/v) ZnSO4 (0.5 mL) and followed by saturated Ba(OH)2 (0.5 mL) after 5 min, then were centrifuged at 1000 r. .p. .m. for 10 min and 1 mL of supernatant was taken out and added to 2 ml of Nash reagent. The mixture was incubated at 60 ºC for 30 min in a water-bath with shaking. Nash reagent is a mixture of ammonium acetate 30% (w/v) and acetyl acetone 0.4% (v/v) [31]. Rosiglitazone N-demethylase activity was determined by measuring of absorbance at 415 nm by microplate reader (Powerwave X-340®, Bio-TEK Instruments, INC. .USA) according to the colorimetric method of Nash (1953) [32]. The absorbance was converted to the formaldehyde concentration by plotted the absorbance in the formaldehyde standard curve.

Data analysis

The results were observed as the mean ± standard deviation (mean ± SD) of formaldehyde formation as a result in the Rosiglitazone metabolism process and were presented in the percentage of activity as compared to the positive control. Results were analyzed using Analysis of Variance (ANOVA) and Tukey test. The level of significance was set at $P<0.05$ for all the statistical tests.

RESULTS AND DISCUSSION

The cytochrome P-450s [P-450] enzymes are responsible for a major of phase I drug metabolism. In phase I metabolism, many reactions such as hydroxylation, reduction or dealkylation can occur. Dealkylation reaction occurs with drugs or xenobiotics that have the functional groups such as an alkoxy group or an alkyl substituted thiol, a secondary and tertiary amine and will produce the formaldehyde (HCHO) [33]. Depending on the type of atom that binds the alkyl group, these reactions are divided to the N-, O- or S-dealkylations [34, 33]. Rosiglitazone is example of drugs that is metabolized through N-demethylation. The reaction occurs in two stages, the first is attachment of the hydroxyl group to the methyl group next to the nitrogen and then the decomposition process of this intermediate with the loss of formaldehyde.

The CYP450 enzymes can be affected by the other compounds both as inhibitors or stimulants. This study used ten cellular stimulants/inhibitors that were targeted on the protein kinases (protein kinase A [PK_A], protein kinase C [PK_C] and protein kinase G [PK_G]), G-protein, either in cAMP, cGMP, phosphodiesterase, phosphatase or phosphatidylinositol pathway. The results showed significant increases in Rosiglitazone metabolism ($P<0.05$) in the presence of KT5823 (fig. 1), TPA (fig. 2), OKA (fig. 3) and genistein (fig. 4) when compared to levels in the positive control. In the KT5823 and TPA groups, unfortunately, the significant changes ($P<0.05$) were only found in the presence of TAF-273 at the highest concentration tested (100 µg/mL) after rat hepatocytes were pre-incubated with KT5823 and trifluoperazine. KT5823 is a specific PK_G inhibitor, thus will affect drug metabolism through the inhibition of PK_G [21]. Inhibition of PK_G increases the P-450 enzymes activities and P-450 phosphorylation will lead to alteration of P-450 from the active form to the inactive state [35], and leading to elevation of formaldehyde formation. The presence of KT5823 in the positive control predictably increased Rosiglitazone metabolism significantly ($P<0.05$) as compared to negative control. Addition of TAF-273 in rat hepatocytes after treatment with KT5823 did not increase Rosiglitazone metabolism at concentrations ranging from 0.001 to 10 µgmL^{-1} ($P>0.05$) if compared to the positive control group.

On the other hand, TPA affected drug metabolism by inhibiting of calmodulin activation [36]. Releasing of Ca^{2+} from the store and increasing extracellular entry will induce in-active Calmodulin to the active form which had the function of stimulating P-450 phosphorylation. Thus would decreased Rosiglitazone metabolism. The presence of TPA in positive control group increased formaldehyde formation from Rosiglitazone as compared to negative control ($P<0.05$). After pre-incubation with TPA and in the presence of TAF-273, the significant increase was not observed in the Rosiglitazone metabolism in male rat hepatocytes at TAF-273 concentrations ranging from 0.001 to 10 µg. mL^{-1} ($P>0.05$). So, it was difficult to conclude whether the effect of TAF 273 on Rosiglitazone metabolism was mediated through activation of the PK_G and Calmodulin or not.

The presence of OKA in the positive control group caused significantly decreased of formaldehyde formation as compared to the negative control group ($P<0.05$). Phosphatase enzyme has responsibility to keep P450 still in the active state, so the inhibition of phosphatase by OKA will lead the P450 to the in-active form [26]. However, the significant increase of formaldehyde formation occurred in all of the test groups that were treated with TAF-273 at concentrations ranging from 0.001 to 100 µg. $.mL^{-1}$ as compared to the positive control, respectively ($P<0.05$). TAF-273 may induce the phosphatase activity.

Genistein influenced drug metabolism by inhibiting tyrosine kinases which had the function to stimulate P-450 phosphorylation [37]. Activation of tyrosine kinases would change the P450 in the active form to the in-active form, so will reduce the formaldehyde formation. Addition of genistein should increase Rosiglitazone metabolism and elevated the formaldehyde formation. The unexpected result was occurred, because there was significantly decreased in the positive control group ($P<0.05$). However, treatment of male rat hepatocytes with TAF-273 at concentrations 0.1 to 100 µg. $.mL^{-1}$ significantly increased Rosiglitazone metabolism

(P<0.05). This may be occurred if TAF-273 acted as an inhibitor of tyrosine kinases. The discussion above suggested that the effect of TAF-273 in increasing Rosiglitazone metabolism in adult male rat hepatocytes was probably mediated through the inhibition of tyrosine kinases.

The effect was not significantly different in rat hepatocytes that were pre-incubated with KT5720, L-ornithine, IBMX, PMA, Gpp or furafylline (P>0.05), data were not shown. The study suggested that the effect of TAF-273 on hepatic phase I Rosiglitazone metabolism in the old normal male rats it was probably mediated through inhibition of protein phosphatase and tyrosine kinases.

Interaction study the effect of some herbal medicines on the N-demethylase activity has been reported. Abas *et al.* (1998) [38] reported about the effect of seven traditional medicines on aminopyrine N-demethylase in rat hepatocytes. The results showed that some of these preparations affected significantly aminopyrine N-demethylase activity. Han & Hussin (2007) [39] had studied about the effect of *O. stamineus* Benth on aminopyrine metabolism. *O. stamineus* showed a significant increase in aminopyrine N-demethylase activity. The effects of *O. stamineus* and *M. citrifolia* on aminopyrine N-demethylase in rats have been reported by Chin *et al.* (2009) [40].

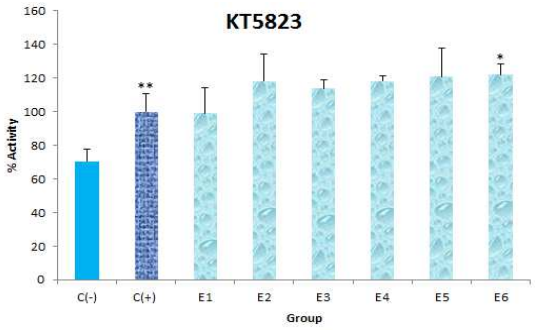

Fig. 1: Percentage of the Rosiglitazone N-demethylase activity in the normal male rat hepatocytes after pretreated with KT5823. Results were analyzed by ANOVA and followed by Tukey Test, values = mean ± SD, n=6, **P < 0.05 significantly different between positive and negative control, *P < 0.05 significantly different as compared to positive control, [C(-)] = in the absence of TAF-273 and cellular stimulants/inhibitor, [C(+)] = in the absence of TAF-273 but in the presence of cellular stimulants/inhibitor, [E1-E6] = test groups, in the presence of TAF-273 and stimulants/inhibitors)

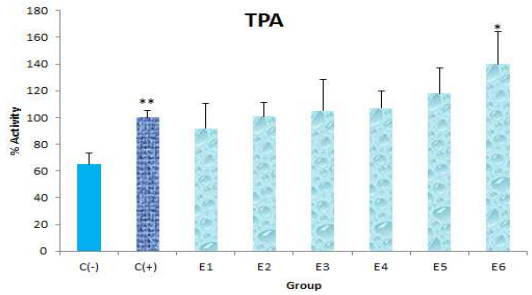

Fig. 2: Percentage of the Rosiglitazone N-demethylase activity in the normal male rat hepatocytes after pretreated with trifluoperazine/TPA. Results were analyzed by ANOVA and followed by Tukey Test, values = mean ± SD, n=6, **P < 0.05 significantly different between positive and negative control, *P < 0.05 significantly different as compared to positive control, [C(-)] = in the absence of TAF-273 and cellular stimulants/inhibitor, [C(+)] = in the absence of TAF-273 but in the presence of cellular stimulants/inhibitor, [E1-E6] = test groups, in the presence of TAF-273 and stimulants/inhibitors)

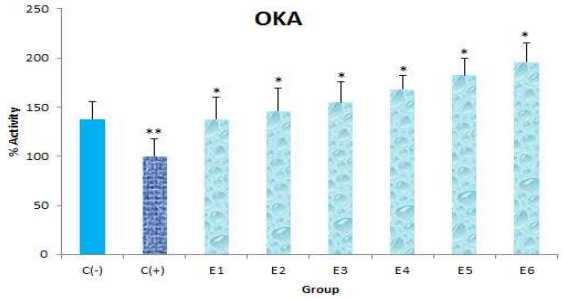

Fig. 3: Percentage of the Rosiglitazone N-demethylase activity in the normal male rat hepatocytes after pretreated with okadaic acid (OKA). Results were analyzed by ANOVA and followed by Tukey Test, values = mean ± SD, n=6, **P < 0.05 significantly different between positive and negative control, *P < 0.05 significantly different as compared to positive control, [C(-)] = in the absence of TAF-273 and cellular stimulants/inhibitor, [C(+)] = in the absence of TAF-273 but in the presence of cellular stimulants/inhibitor, [E1-E6] = test groups, in the presence of TAF-273 and stimulants/inhibitors)

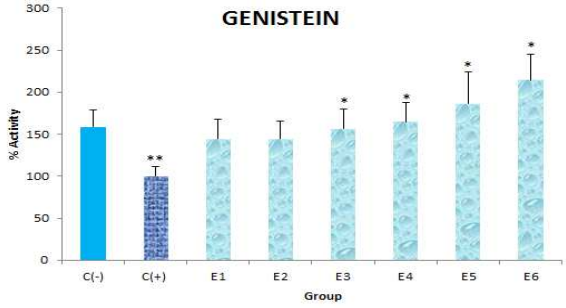

Fig. 4: Percentage of the Rosiglitazone N-demethylase activity in the normal male rat hepatocytes after pretreated with genistein. Results were analyzed by ANOVA and followed by Tukey Test, values = mean ± SD, n=6, **P < 0.05 significantly different between positive and negative control, *P < 0.05 significantly different as compared to positive control, [C(-)] = in the absence of TAF-273 and cellular stimulants/inhibitor, [C(+)] = in the absence of TAF-273 but in the presence of cellular stimulants/inhibitor, [E1-E6] = test groups, in the presence of TAF-273 and stimulants/inhibitors)

CONCLUSION

The study suggested that the effect of TAF-273 on hepatic phase I Rosiglitazone metabolism in the old normal male rats was probably mediated through inhibition of protein phosphatase and tyrosine kinases.

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CONFLICT OF INTERESTS

Declared None

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