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บรรณาธิการแถลง

เรียนสมาชิกทุกท่าน

การประชุมวิชาการประจำปีของชาวเภสัชวิทยาผ่านพ้นไปด้วยความคึกคักเป็นอย่างยิ่ง มีผู้ให้ความสนใจเสนอผลงานเป็นจำนวนมาก ส่งผลให้มีต้นฉบับผลงานวิจัยลงตีพิมพ์ในวารสารหลายเรื่อง ต้องขอขอบพระคุณคณะกรรมการจัดการประชุมวิชาการทุกท่านไว้ ณ ที่นี้

คณะบรรณาธิการวารสาร มีความมุ่งมั่นที่จะพัฒนาวารสารของสมาคมฯ ให้มีคุณภาพมากยิ่งขึ้นและเป็นที่ยอมรับในวงวิชาการ โดยวางเป้าหมายให้วารสารเข้าอยู่ในฐานของ Thai Citation Index ภายในปีนี้ และมีกำหนดออก 3 ฉบับต่อปี การที่จะบรรลุเป้าหมายดังกล่าวได้ ต้องมีระบบการประเมิน (peer reviews) ที่ได้มาตรฐาน และต้นฉบับที่เพียงพอ จึงหวังเป็นอย่างยิ่งว่าจะได้รับความร่วมมือจากทุกท่าน หากมีข้อเสนอแนะใด ๆ คณะบรรณาธิการขอน้อมรับไว้ด้วยความยินดีเป็นอย่างยิ่ง

รองศาสตราจารย์ ดร.ลัดดาวัลย์ ผิวทองงาม

บรรณาธิการวารสาร

RESEARCH ARTICLE

Acute and Chronic Effects of Aspirin on Gastric Ulceration in Rats

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Abstract

Aspirin is the most popular drug in the world. High-dose aspirin was commonly prescribed in the past as an anti-inflammatory drug in several inflammatory diseases. Nowadays, it is usually prescribed in low dose to prevent blood clotting in patients with high cardiovascular risk. A common adverse effect of patients taking either high-dose or low-dose aspirin is gastric ulcer. This study aimed to identify the characteristic differences between acute high-dose and chronic low-dose aspirin on the gastric ulceration in male Wistar rats. In addition, the myeloperoxidase activity of the gastric mucosa was also investigated to evaluate the effects of drugs on neutrophil infiltration. The animals were divided into 3 groups. A high-dose (200 mg/kg) aspirin was given once to the first group, and the gastric ulcer measurement was performed 6 hours later. The second group was given aspirin at a low dose (25 mg/kg) once daily for 28 days and the third group being served as the control was given deionized water. Ulcer lesions were evaluated at 24 hours after the last dose. The results indicate that aspirin at both acute high dose and chronic low dose can cause gastric lesions, although at the high dose can produce more pronounced ulceration. In addition, some characteristic differences of gastric lesions between the two dosages of aspirin were observed. Acute high-dose aspirin caused gastric lesions both in glandular and pyloric parts while chronic low-dose aspirin caused gastric lesions only in glandular part. The severity of gastric ulceration, indicated by the "ulcer index", does not seem to be with neutrophil infiltration. This could probably be partly due to the anti-inflammatory activity of aspirin which can alter the function of neutrophil especially at a high dose.

Keywords aspirin, gastric ulcer, myeloperoxidase activity.

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Introduction

Aspirin is the most popular drug in the world. In the past, it was commonly prescribed in high dose as an anti-inflammatory agent in several inflammatory diseases. Nowadays, it is usually prescribed in low dose to prevent blood clotting in several diseases associated with abnormal blood clotting condition or in patients with high cardiovascular risk (1,2). Gastric ulceration is the most common adverse effect of aspirin and it can occur with both high doses and low doses, although it is more common with high doses (2,3). Whether there will be any differences in the mechanism of ulcer producing effect of aspirin at high and low doses of aspirin is a question. Moreover, it was found that infiltrating neutrophils contribute to the pathogenesis of indomethacin-induced ulceration of the rat gastric antrum, and the myeloperoxidase activity of gastric mucosa was frequently evaluated as the index of neutrophil infiltration (4). The aim of this study was therefore to identify the characteristic differences between acute high dose and chronic low dose of aspirin on the gastric ulceration. Moreover, the myeloperoxidase activity of the gastric mucosa was also investigated to evaluate the effects of the drug on neutrophil infiltration.

Methods

Male Wistar rats (aged 7-8 weeks) obtained from the National Animal Center, Mahidol University at Salaya, were used in this study. The experiment protocol was approved by the Institutional Animal Care and Use Committee. The animals were divided into 3 groups. A high-dose (200 mg/kg) aspirin was given once to the first group, and the gastric ulcer measurement was performed 6 hours later (5). The second group was given aspirin at a low dose (25 mg/kg) once daily for 28 days and the third group being served as the control was given deionized water. Ulcer lesions were evaluated at 24 hours after the last dose.

To investigate the degree of gastric ulcer formation, "the ulcer index" as described below was used. The animals were sacrificed under deep ether anesthesia. The stomachs were removed, opened along greater curvature, and evaluated the severity of the ulcer. The macroscopically apparent damage to the glandular and pyloric part was scored by the following criteria: 0 = normal color with or without very small red spots, 0.5 = red coloration area < 1 mm^2, 1 = red coloration area ≥ 1 mm^2, 1.5 = hemorrhagic streak < 2.0 mm, 2 = hemorrhagic streak ≥ 2.0 mm, 2.5 = ulcer diameter < 1 mm, 3 = ulcer diameter ≥ 1 mm, and, 4 = perforation. Sum of lesion scores for each rat was expressed as ulcer index. At the end of macroscopic investigation, the gastric mucosa was then totally removed, homogenized, extracted and prepared for submitting to the myeloperoxidase enzyme assay using spectrophotometric method as described by Bradley *et al.* (6).

The data were analyzed and expressed as means ± SEM. Statistical analyses were done by ANOVA following by a multiple-comparison. P-values of less than 0.05 were considered statistical significance.

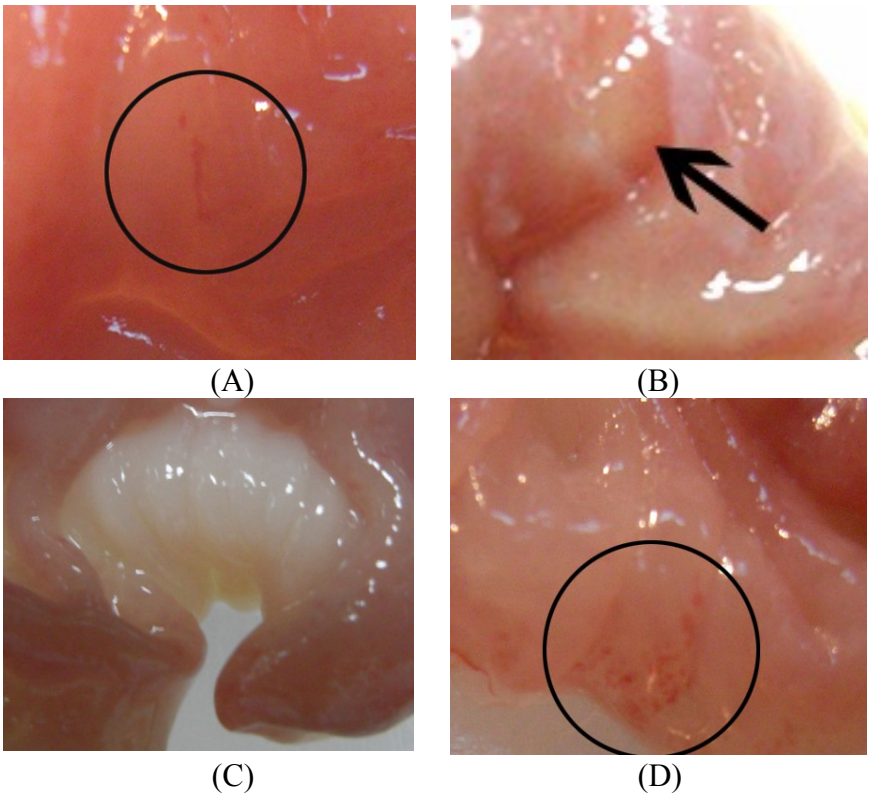

Figure 1 The lesions in the rat stomachs, showing hemorrhagic streak at the glandular part in the low-dose group (A), ulcer lesion at the glandular part in the high-dose group (B), normal gastric mucosa on pyloric part in the low-dose group (C), and hemorrhagic streak at the pyloric part of high-dose group (D).

Results

The gastric lesions, comprised of red coloration, hemorrhagic streak, and ulcer, were determined. In the glandular part of the stomach, the lesions including red coloration and hemorrhagic streak were found in the rats from both aspirin treated groups, *i.e.* acute high dose and chronic low dose, but ulcer lesions were found only in the high-dose group. In the pyloric part, however, the hemorrhagic streaks were detected and found only in the high-dose aspirin treated group (Figure 1).

The severity of ulceration as expressed by the "ulcer index" of the rats treated with acute high-dose aspirin was significantly higher than that of chronic low-dose aspirin or deionized water ($p < 0.001$). The "ulcer index" of the rats given low-dose aspirin was higher than that of the rats given deionized water; however, the difference did not reach statistical significance level ($p > 0.05$). The myeloperoxidase activity of the gastric mucosa in the rats given high-dose and low-dose aspirin did not differ from the activity in the control group ($p > 0.05$) (Figure 2).

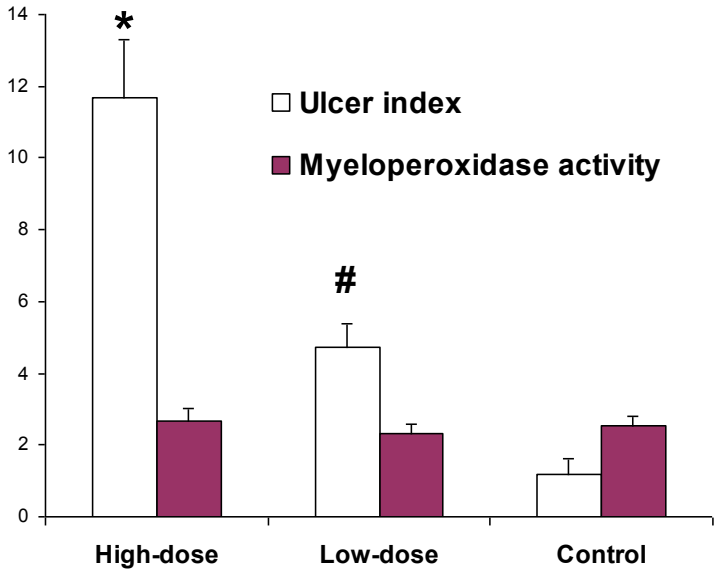

Figure 2 The ulcer index and the myeloperoxidase activity of rats given high-dose aspirin once, low-dose aspirin once daily for 28 days, and deionized water once daily (the control) for 28 days. Values are expressed as means ± SEM. * $p < 0.001$ compared with control and # $p < 0.001$ compared with high-dose aspirin.

Discussion

Gastric ulcer usually occurs in patients taking NSAIDs. They inhibit the synthesis of prostaglandins, e.g. PGE_2 and PGI_2 which both of them are cytoprotectives and inhibitors of acid secretion. The other mechanisms which were described as the mechanisms of aspirin-induced gastric ulcer are induction of lipid peroxidation and increment of gastric acid and pepsin secretion (4,5,7,8). The characteristics of gastric ulcer in those patients include hyperemia, erosions, and/or sub-epithelial hemorrhages (7) as they were found in rats treated with acute high-dose aspirin in the present study. Gastric lesions occurred on the pyloric part of the stomach in high-dose aspirin treated rats, but not in low-dose aspirin treated rats. This may be resulted from direct irritant effect of concentrated aspirin in the gastric lumen (8) particularly in the pyloric part which is the lowest part of the stomach before the drug is moving to the duodenum.

Chronic low-dose aspirin caused less gastric lesions when compared with acute high dose; however, it still caused low-degree lesions on gastric mucosa (3). From the results of neutrophil infiltration in the present study suggested that the mechanism of aspirin-induced gastric ulcer did not correlate with neutrophil infiltration. This could be partly due to the dosage of 200 mg/kg aspirin alone did not reach the level that increases myeloperoxidase activity (5), or could be due to the anti-inflammatory activity of aspirin which could alter neutrophil function (9).

Conclusion

Even though chronic low-dose aspirin causes less gastric lesions when compared with acute high-dose aspirin, it still causes low-degree lesions on gastric mucosa. The characteristic differences of gastric lesions between aspirin two dosages were observed. Acute high-dose aspirin caused gastric lesions both in glandular and pyloric parts while chronic low-dose aspirin caused gastric lesions only in glandular part. The severity of gastric lesions in aspirin-induced gastric ulceration does not correlate with neutrophil infiltration.

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RESEARCH ARTICLE

Distribution and Patterns of Polymorphisms of *Plasmodium vivax* Dihydrofolate Reductase *(Pvdhfr)* and Dihydropteroate Synthase (*Pvdhps*) in Malaria Endemic Areas of Thailand

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Abstract

Malaria is the most important public health problems in several countries. In Thailand, co-infections of *Plasmodium vivax* and *P. falciparum* are common. The prevalence and patterns of mutations of *Pvdhfr* and *Pvdhps* were investigated in a total of thirty-six blood samples collected 9from patients with *P. vivax* infection who attended the malaria clinics in Mae-hongson (n= 9), Ranong (n= 12), Pattani (n= 5), Yala (n=6) and Narathiwat (n= 4) provinces during 2009 and 2010. SNP-haplotypes at positions 13, 33, 57, 58, 61, 117 and 173 of *Pvdhfr* and positions 383 and 553 of the *Pvdhps* were examined by nested PCR-RFLP. . All isolates carried mutations at codons 58 (58R) and 117 (117N, 117T). 17 isolates carried mutations at codons 61 (61M). Mutation at codon 57 consisted of two types, 57I and 57L. For *Pvdhps,* the most prevalent alleles were the mutant 383G (94.4%) and the wild-type A383/A553 (5.5%) alleles. The most common *Pvdhfr* alleles were triple mutants 58R/61M/117N (51.6%), 57I/58R/117T (32.3%), 57L/58R/117T (13.0%), and 57L/58R/117T (3.2%); four isolate carried double mutants 58R/117N/T; only one isolate carried quadruple mutation (57I/58R/61M/117T). The most prevalent combination allele was a triple *Pvdhfr* mutant allele 58R/61M/117N combined with a single mutant *Pvdhps* allele 383G. Two isolates carried wild-type alleles of both genes. The results demonstrated that all *P. vivax* isolates in Thailand carried mutant combination of *Pvdhfr* and *Pvdhps*. The development of new alternative antifolates drugs that are effective against SP-resistant *P. vivax* is required.

Key words malaria, *Plasmodium vivax*, *Plasmodium vivax* dihydrofolate reductase (*Pvdhfr*) and dihydropteroate synthase (*Pvdhps*), PCR-RFLP, sulphadoxine-pyrimethamine (SP).

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Introduction

In Thailand, malaria disease is endemic throughout the country, with the highest incidence reported from Tak Province, the area along the Thai-Myanmar border (Na-Bangchang & Congpuong 2007). The antimalarial combination sulfadoxine-pyrimethamine (SP, Fansidar^{TM}) was introduced to Thailand in 1972 as the first-line treatment of chloroquine resistant *P. falciparum* malaria. Rapidly after its introduction for clinical use, resistance of the parasite to this drug was reported along the Thai-Cambodian border, and by 1982, resistance was widespread throughout the country (Pinichpongse et al. 1982, Thaithong et al. 1992). The molecular targets of action of sulfadoxine and pyrimethamine are dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively. Point mutations within the genes that encode these enzymes are the primary causes of resistance to the drugs (Gregson & Plowe 2005). Although in Thailand, SP has never been used for treatment of *P. vivax* infections, *P. vivax* often co-exists with *P. falciparum* with relative equal frequencies (Snounou & White 2004). Therefore, *P. vivax* has often been exposed unintentionally to SP during treatment of *P. falciparum*, and this has caused a progressive selection of SP-resistant alleles in *P.vivax* (Imwong et al. 2003). In the present study, we determined the prevalence and diversity of *Pvdhfr* and *Pvdhps* mutant alleles in *P. vivax* isolates collected from different malaria endemic areas of Thailand, i.e., Mae-hongson, Ranong, Pattani, Yala and Narathiwat provinces. This information will assist in development of new effective antimalarial drugs with antifolate action.

Materials and Methods

Study areas and sample collection

A total of 36 blood samples with mono-infection of *P. vivax* were collected from patients attending the malaria clinics in Mae-hongson, Ranong, Pattani, Yala and Narathiwat provinces during 2009 and 2010. Approval of the study protocol was obtained from the Ethics Committees of Ministry of Public Health of Thailand. Two-hundred to 300 µl finger-prick blood samples were collected onto filter paper (Whatman No. 3).

Extraction of parasite genomic DNA

Parasite genomic DNA was extracted from individual dried blood spots on filter paper using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification [Qiagen,Valencia, CA].

Amplification of Pvdhfr

The first reaction of *Pvdhfr* was amplified by the primers *Pvdhfr*-OF and *Pvdhfr*-OR [Imwong et al. 2003]. PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec, and then 72°C for 5 min.

Amplification of amino acid codons 13, 33, 58 and 61: The second round amplification of PCR products of the first reaction at amino acid codons 13, 33, 58, and 61 were performed using the *Pvdhfr*-13F and *Pvdhfr*-13R primers.

Amplification of amino acid codons 57 and 173: The second round amplification of PCR products of the first reaction at amino acid codons 57 and 173 were performed using the *Pvdhfr*-F57 and *Pvdhfr*-NR primers.

Amplification of amino acid codons 57 and 117: The second round

amplification of PCR products of the first reaction at amino acid codons 57 and 117 were performed using the *Pvdhfr*-OF and *Pvdhfr*-NR primers. PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec, and then 72°C for 5 min.

Amplification of Pvdhps

The first reaction of *Pvdhps* was amplified by the *Pvdhps*-OF and *Pvdhps*-OR primers. PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 58°C for 2 min, 72°C for 2 min, and then 72°C for 5 min. The second round amplification of PCR products of the first reaction at amino acid codons 383 and 553 were performed using the primers *Pvdhps*-NF and *Pvdhps*-NR, and *Pvdhps*-553OF and *Pvdhps*-NR, respectively. PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 50°C for 2 min, 72°C for 2 min, and then 72°C for 5 min.

Restriction enzyme

To detect mutations at points I13L, P33L, S58R, T61M, F57, I173L, F57I/L andS117T/N, the PCR products were digested with enzymes HaeIII, Cfr42I (SacII), AluI, Tsp45I , XmnI, Eco130I (StyI), BsrGI, PvuII, BsrI, BstNI respectively. And for detect the mutations at positions A383G and A553G, the PCR products were digested with enzymes Msp I (HpaII) and Msc I.

Results

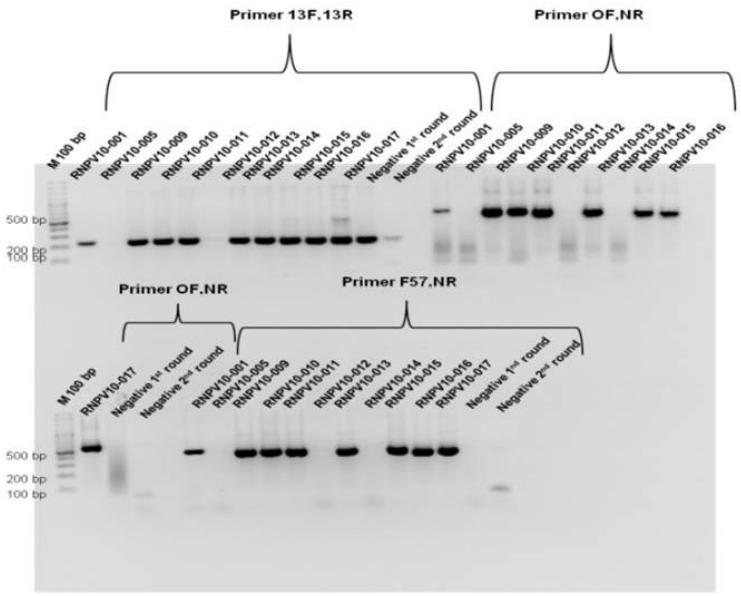

Figure 1 Amplification of *Pvdhfr* at codons *13, 33, 58 and 61*, codons *57 and 173*, codons *57 and 117*

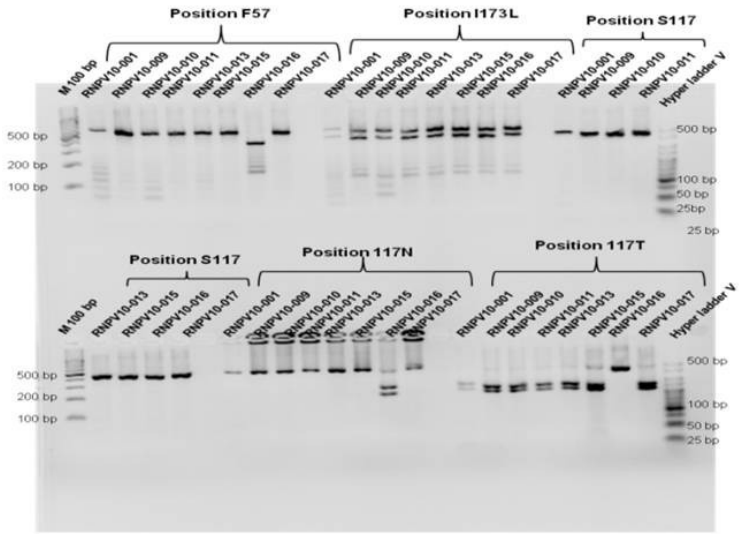

Figure 2 Enzymatic restriction to detect mutations at points F57, I173L, andS117T/N.

Table 1 The frequencies of *Pvdhfr* and *Pvdhps* mutations

Pvdhps				**Pvdhfr**							
A383G	A553G	I13L	P33L	F57I/L	S58R	T61M	S117T/N	I173L	Ranong n=12	Mae-hongson n=9	Pattani ;n=5 Yala; n =6 Narathiwat; n=4
G	A	I	P	F	R	T	N	I	1	-	1
G	A	I	P	I	R	T	T	I	5	5	-
G	A	I	P	L	R	T	T	I	4	-	-
G	A	I	P	F	R	M	N	I	-	2	14
G	A	I	P	I	R	T	T	I	-	2	-
A	A	I	P	L	R	T	T	I	1	-	-
A	A	I	P	I	R	M	T	I	1	-	-
											Total n = 36

Discussion and Conclusion

The frequencies of *Pvdhfr* and *Pvdhps* mutations are summarized in Table 1. All isolates carried mutations at codons 58 (58R) and 117 (117N, 117T). 17 isolates carried mutations at codons 61 (61M). Mutation at codon 57 consisted of two types, 57I and 57L. For *Pvdhps,* the most prevalent alleles were the mutant 383G (94.4%) and the wild-type A383/A553 (5.5%) alleles. The most common *Pvdhfr* alleles were triple mutants 58R/61M/117N (51.6%), 57I/58R/117T (32.3%), 57L/58R/117T (13.0%), and 57L/58R/117T (3.2%); four isolate carried double mutants 58R/117N/T; only one isolate carried quadruple mutation (57I/58R/61M/117T). The most prevalent combination allele was a triple *Pvdhfr* mutant allele 58R/61M/117N combined with a single mutant *Pvdhps* allele 383G. Two isolates carried wild-type alleles of both genes. The data demonstrated that all *P. vivax* isolates in Thailand carried mutant combination of *Pvdhfr* and *Pvdhps*. The development of new alternative antifolates drugs that are effective against SP-resistant *P. vivax* is required.

Acknowledgements

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RESEARCH ARTICLE

The Study of *UGT1A1* Polymorphism in Neonate at Songkhla Hospital

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Abstract

Bilirubin UDP-glucuronosyltransferase or *UGT1A1* gene encodes UGT1A1 enzyme which is responsible for endogenous (bilirubin, steroids, bile acid *etc*.) and xenobiotics glucuronidation to eliminate from the body. Neonatal hyperbilirubinemia (CN1, CN2 and Gilbert's syndrome) and drug toxicity, especially irinotecan caused by *UGT1A1* mutation. *UGT1A1* polymorphism in Asian has been detected such as G71R (mostly found in Japanese), P229Q, F83L, Y486D and $(TA)_7TAA$ (mostly found in Caucasians). For Thai people, no information has been established. In this study, we aimed to study the *UGT1A1* polymorphism in neonate at Songkhla hospital. The 189 cord blood samples were collected and genomic DNA was extracted, then *UGT1A1* exon1 and TATA box region were amplified by PCR. DNA sequencing was performing to detect the mutation. The questionnaires about neonate and family were performed before and after birth. We found 4 known mutations, two in the promoter region [($(TA)_7TAA$ and -64G>C)] and two in the coding region (G71R and P229Q). The orders of variant allele frequency were $(TA)_7TAA$ (0.2037)[6/6= 0.6455, 6/7= 0.3016, 7/7= 0.0529], -64G>C (0.0291)[G/G= 0.9524, G/C= 0.037, C/C= 0.0106], G71R (0.0608)[G/G= 0.8889, G/A= 0.1005, A/A= 0.0106] and P229Q (0.0106)[C/C= 0.9788, C/A= 0.0212]. We found 100 jaundice neonates (55 Male and 45 female) and G71R was the highest relationship with hyperbilirubinemia and prolonged jaundice. We conclude that jaundice after birth may use to be the marker for *UGT1A1* mutation.

Keywords *UGT1A1*, *UGT1A1* polymorphism

Introduction

UDP-glucuronosyltransferase (UGT) is a super-family of phase II biotransformation pathway that produces hydrophilic compounds which readily excreted from body. There are 4 *UGT* gene families *UGT1, UGT2, UGT3* and *UGT8. UGT1A1* gene encodes UGT1A1 enzyme that play a key role in the glucuronidation of bilirubin and some drug, especially irinotecan[1]. To date, more 113 variants of *UGT1A1* have been identified, arising from mutations in the promoter and coding regions. The mutations are important for bilirubin metabolism as cause 'hyperbilirubinemia' (Crigler-Najjar syndrome type I (CN1), CN2 and Gilbert's syndrome).

The polymorphisms of the *UGT1A1* gene have been reported as risk factors for significant hyperbilirubinemia, especially in Asian ethnicities such as G71R (mostly found in Japanese), P229Q, F83L, Y486D and (TA) $_7$TAA (mostly found in Caucasians), these genetic variations cause decreased UGT1A1 activity[2,3]. There is no report in term of *UGT1A1* gene mutation frequency and the relationship between each SNPs and jaundice afterbirth. This study objected to determine the frequency of *UGT1A1* gene mutation and the relationship between each mutation and hyperbilirubinemia in Southern Thai population.

Subjects and Method

Study population

The cord-blood collection of 189 Thai neonates and questionnaire interview were performed during November 2009 and March 2010 at Songkhla hospital. The samples were randomly selected and prospectively studied. The protocol of this study was achieved ethic permission in human experimentation by the human ethic committees of Faculty of Medicine, Prince of Songkla University.

DNA extraction

The genomic DNA was extracted via Illustra^{TM} blood genomic Miniprep Kit protocol. The genomic samples were kept at -80 °C until used.

Analysis of the UGT1A1 gene

The PCR method was applied to detect the variants covering both the promoter and exon 1 coding region. The PCR mixture (20 µl) consisted of 1 µl of genomic DNA, dNTPs 0.5 µl, primers 2 µl, *Taq* buffer 2 µl and $MgCl_2$ 1.4 µl. The PCR reactions were as follows: an initial denaturation for 4 min at 95°C followed by 30 sec at 95°C, 30 sec at 66°C and 1 min at 72°C for 40 cycles. The size of products was proved by 1% agarose gel electrophoresis. The sequences of the amplified DNA fragments were determined by BioDesign Company.

Statistical analysis

The sequencing results were read and all genotype results were analyzed by using the SNPAlyze version 7.0 (trial version, DYNACOM company) to determine Hardy-Weinberg equilibrium, genotype frequency and linkage disequilibrium. The relationship between *UGT1A1* polymorphism and jaundice were calculated by % of each total variant.

Result

Demographic data

A total of 189 Thai neonates, 100 males (52.91%) and 89 females (47.09%), who were full-term with a mean gestational age of 39.83 ± 0.92

Table1 Demographic data of 189 Thai neonates during November 2009- March 2010 at Songkhla hospital

Data	Normal	Jaundice
Male: (n=100)	44 (44%)	56 (56%)
Gestational age ($\bar{x}$ ± S.E.)	39.69 ± 1.41 (32-40 weeks)	39.83 ± 0.85 (35-41 weeks)
Birth weight (g)	3,119.29 ± 445.82	3,118.02 ± 445.53
Female: (n=100)	45 (50.56%)	44 (49.44%)
Gestational age ($\bar{x}$ ± S.E.)	39.90 ± 0.66 (35-40 weeks)	39.90 ± 0.64 (35-40 weeks)
Birth weight (g)	3,063.33 ± 407.07	3,083.18 ± 375.08

Table2 Genotype frequencies of the *UGT1A1* variants in exon1 and promoter region

Genotype		Frequency	Number	Jaundice relationship (%)
-64G>C:	G/G	0.9524	180	
	G/C	0.0370	7	Jaundice 7/9 (78%)
	C/C	0.0106	2	
$(TA)_7TAA$:	6/6	0.6455	122	
	6/7	0.3016	57	Jaundice 37/67 (55%)
	7/7	0.0529	10	
G71R:	G/G	0.8889	168	
(G>A at 211)	G/A	0.1005	19	Jaundice 14/21 (67%)
	A/A	0.0106	2	
P229Q	C/C	0.9788	185	
(C>A at 686)	C/A	0.0212	4	Jaundice 1/4 (25%)
	A/A	-	0	

weeks (32-41 weeks) and mean birth weight of 3,089 ± 424 g (1,640-4,470 g). We found 100 jaundice neonates (male = 56, female = 44), Table 1.

Mutation of UGT1A1 gene and jaundice relationship

We found 4 known SNPs, two in the promoter region [$(TA)_7TAA$ and -64G>C)] and two in the coding region (G71R and P229Q) (Fig.1 and 2). The genotype frequency of each variant is shown as Table 2. We found G71R (coding region) and -64G>R (promoter region) have the highest relationship with jaundice afterbirth.

Linkage disequilibrium analysis

There are two significant close linkage pairs which are [$(TA)_7TAA$ and G71R] and [$(TA)_7TAA$ and P229Q] .

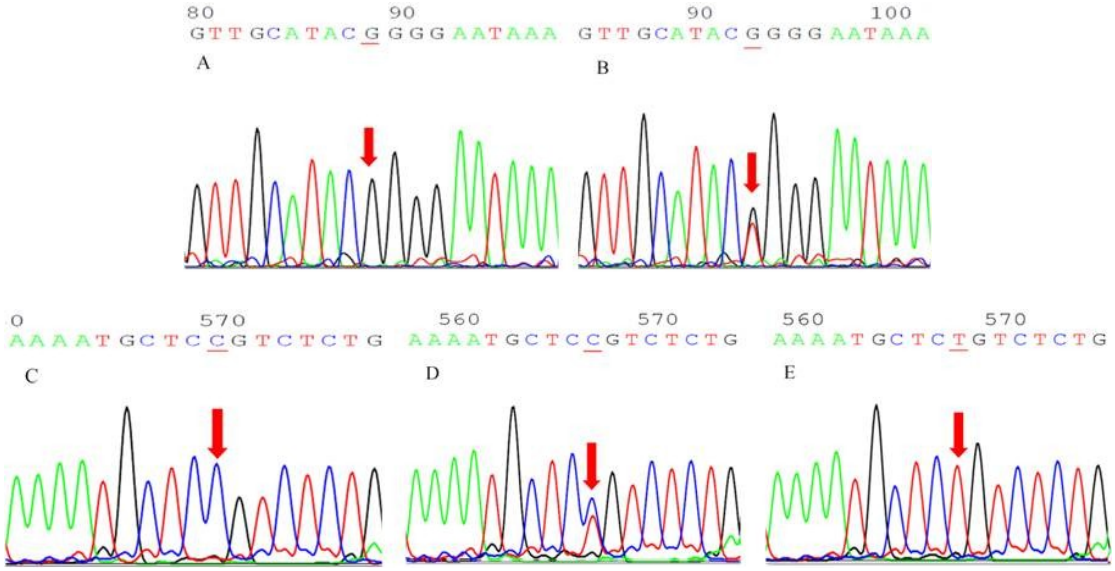

Figure1 Electropherograms for known mutation in the coding region (exon1): A: P229Q (wild type), B: P229Q (C→A at 686) (heterozygous), C: G71R (wild-type), D: G71R (heterozygous), E: G71R (homozygous mutant) (G→A at 211)

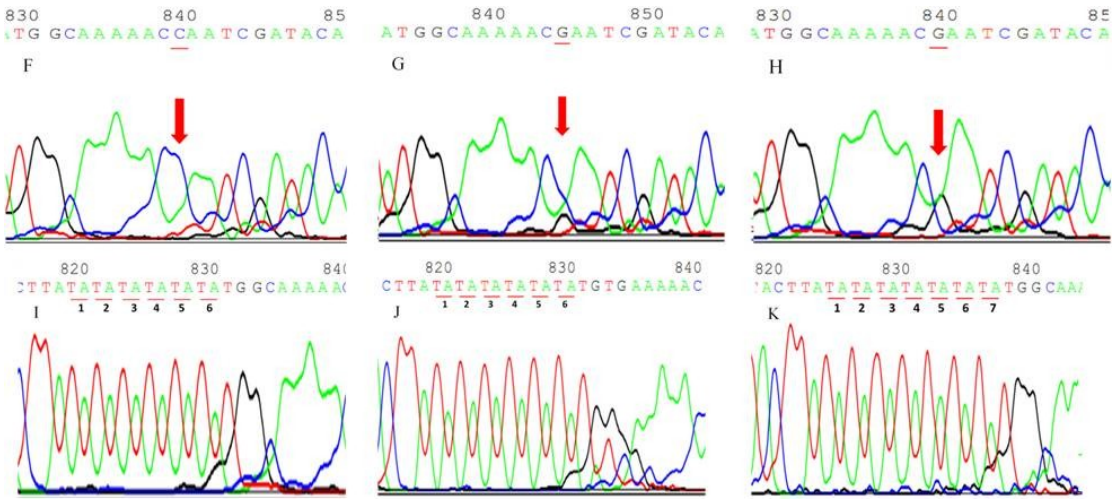

Figure2 Electropherograms for known mutation in the promoter region: F: -64 G>C (wild type),bG: -64 G>C (heterozygous), H: -64 G>C (homozygous mutant), I: $(TA)_{6/6}$TAA (wild type), J: $(TA)_{6/7}$TAA (heterozygous), K: $(TA)_{7/7}$TAA (homozygous mutant)

Discussion

The present study investigated the data of neonates between male and female, we found that sex has no effect to jaundice. There are 4 SNPs, -64G>C, $(TA)_7TAA$, G71R and P229Q, were in Hardy-Weinberg equilibrium, except for -64G>C and P229Q. The factors which have been reported may cause deviation from equilibrium include migration, gene flow, genetic drift and non-random mating which are difficult to detect in practical terms. The frequency of the promoter mutation $(TA)_7TAA$ (0.3545) (heterozygous and homozygous mutants) is higher than G71R (0.1111) in Southern Thai neonate population studies according to the report of Sutomo *et al*. and Yusoff *et al*. that G71R was also found at the lower allele frequency in Malaysians and Javanese (also from south-east Asian regions) whereas in East Asian populations (Japanese, Chinese, Koreans and Taiwanese) have found G71R in higher frequency than $(TA)_7TAA$ [2,4-5].

Conclusion

There are 4 known SNPs: $(TA)_7TAA$, -64G>C, G71R and P229Q. Coding region mutation (G71R) and region (-64G>C) have the highest relationship with jaundice afterbirth. There are 2 pairs close linkage disequilibrium: $(TA)_7TAA$ & G71R and $(TA)_7TAA$ & P229Q.

Acknowledgement

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RESEARCH ARTICLE

The Effect of Standardized Extract of *Centella asiatica* ECa233 on Hemin-Induced LDL Oxidation

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Abstract

Oxidation reaction of low density lipoprotein (LDL) plays a pivotal role in atherogenesis. Hemin (protoporphyrin IX- Fe^{2+}) is oxidative mediator found in plasma of thalassemia patient. *Centella asiatica* have been shown to be antioxidants. The aim of this study was to determine the effect of standardized extract of *Centella asiatica* ECa233 and asiatic acid on hemin-induced LDL oxidation (he-oxLDL). LDL was pre-incubated with either ECa233 or asiatic acid for 30 minutes, and then oxidation was initiated by incubation with hemin for 24 hours. The degree of LDL oxidation was determined by measurement of thiobarbituric acid reactive substances (TBARs) and the relative electrophoretic mobility (REM). The TBARs levels and the REM values were increased in he-oxLDL. However, ECa233 and asiatic acid (20-80 μg/ml) were slightly decreased both TBARs level and the REM value at 24 hr incubation, indicating that ECa233 and asiatic acid could protect LDL oxidation induced by 24 hr- incubation of hemin, though to a lesser extent those of □-tocopherol.

Keywords *Centella asiatica*, LDL oxidation, hemin, TBARs, α-tocopherol

Introduction

Oxidation of low density lipoprotein (LDL) is considered a contributing factor to the development of atherosclerotic lesions (1). Hemin, a product of heme (protoporphyrin IX-Fe^{2+}) oxidation, is one of oxidative mediator found to be elevated in pathological cases like severe hemoglobinopathies, sickle cell anemia and thalassemia. Oxidative stress, occurs from excessive heme in the circulation of thalassemia patient, may lead to complications of vascular diseases such as pulmonary thromboembolism (2) and cerebral thrombosis (3). Antioxidants such as α-tocopherol are often used to prevent atherosclerosis. *Centella asiatica* which have phenolic compounds, triterpenes have been shown to be a potent antioxidant (4) as well as asiatic acid which is a major metabolite of asiaticoside (5).

The aim of this study was to determine the antioxidant effect of *Centella asiatica* extract (ECa233) and asiatic acid on hemin-induced LDL oxidation.

Methods

Standardized extract of *Centella asiatica* ECa233 was prepared by Dr. Chamnan Patarapanich and co-workers, Faculty of Pharmaceutical Sciences, Chulalongkorn University. This study protocol was approved by The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University. Blood was collected from overnight fasting healthy volunteer. Plasma was prepared by centrifugation at 3,250 rpm, 4ºC for 15 minutes then stored at -80ºC. LDL (density = 1.019-1.063 g/ml) was separated from plasma by the sequential density gradient ultracentrifugation method at 50,000 rpm, 4ºC. LDL was dialyzed with PBS pH 7.4 before use. LDL (300 µg protein/ml) was pre-incubated with either ECa233 or asiatic acid at the concentration of 20, 40, 80 µg/ml at 37ºC for 30 minutes. The 80 µg/ml of α-tocopherol (α-TC) was used as positive control. LDL oxidation was initiated by adding 5µM of hemin, further incubated for 24 hours and then stopped reaction with EDTA and BHT. The thiobarbituric acid reactive substances (TBARS) formation and relative electrophoretic mobility (REM) were determined.

Thiobarbituric acid reactive substances (TBARs)

Lipid peroxidation products were determined by spectrofluorometric method with excitation and emission wavelength at 515 and 553 nm, respectively. 1,1,3,3-tetraethoxypropane was used as a standard.

REM by gel electrophoresis

Agarose gel electrophoresis (1%) in barbital buffer was performed with power supply at 70 Volt 400 mAMP for 45 min. and stained LDL bands by coomassie blue.

Data was expressed as mean $\pm$ standard error of means, resulted from five independent experiments. Data between groups were compared by one-way ANOVA. Statistically significant differences were accepted at $P<0.05$ for TBARs formation and at $P<0.1$ for REM.

Results

The results of this study showed that TBARs level in he-oxLDL was dramatically increased at 24 hr incubation (figure 1). α-Tocopherol (80 µg/ml) significantly decreased TBARs formation 68%, while both ECa233 (CA 20-80 µg/ml) and asiatic acid (AA 20-80 µg/ml) decrease only 20% to 30% of those in he-oxLDL (figure 2).

From gel electrophoresis of LDL at 24 hr, the value of REM in he-oxLDL was 1.4. This REM in he-oxLDL was significantly decreased by α-tocopherol but showed a small decrease by ECa233 and asiatic acid (figure 3). The results indicated that both ECa233 and asiatic acid inhibit protein modification in he-oxLDL to a lesser extent than α-tocopherol.

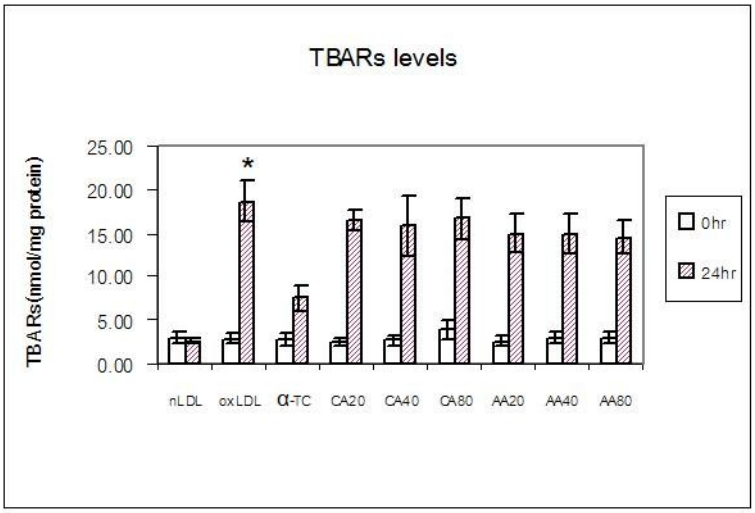

Figure 1 Effect of α-tocopherol, ECa233, and asiatic acid on TBARs levels of oxLDL at 0 and 24 hr of incubation with hemin 5 µM. * $p<0.05$ significantly different compared to the nLDL.

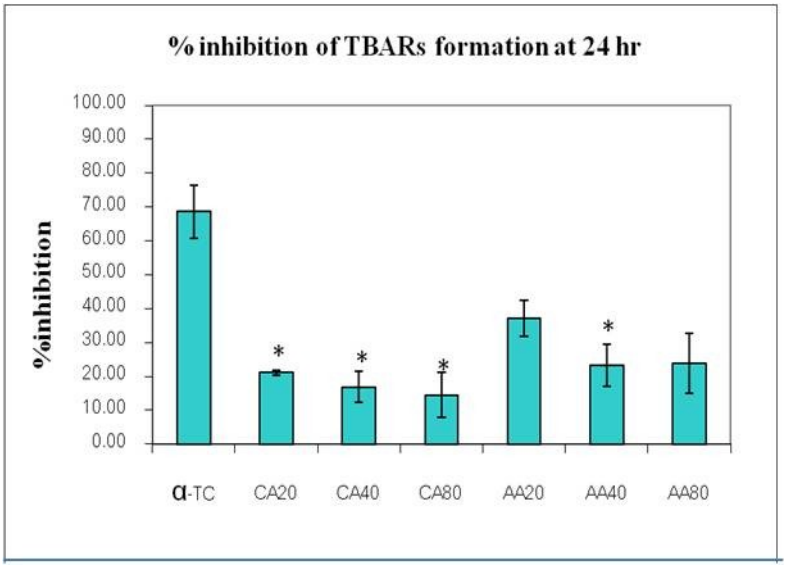

Figure 2 The percent inhibition of α-tocopherol, ECa233, and asiatic acid on TBARs formation at 24 hr of incubation with hemin 5 μM. * $p<0.05$ significantly different compared to the α-TC.

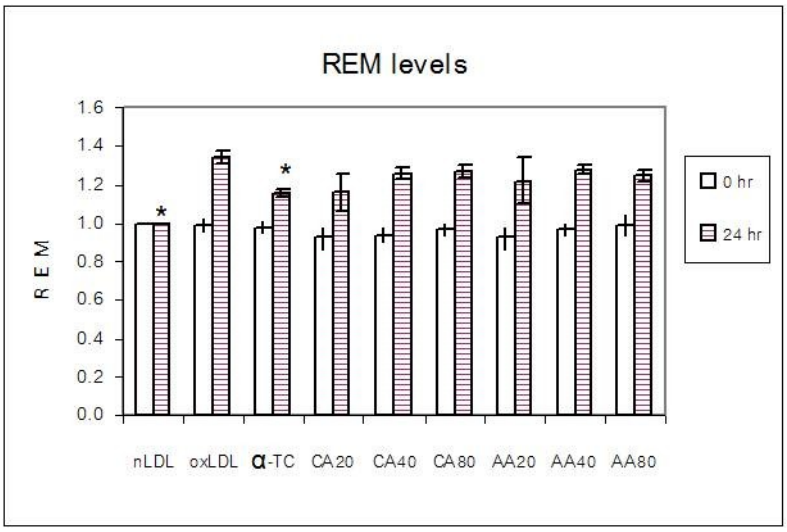

Figure 3 REM levels of oxLDL pre-incubated with α-tocopherol, ECa233, and asiatic acid at 0 and 24 hr of incubation with hemin 5 μM. *$p<0.1$ significantly different compared to the oxLDL.

Discussion and Conclusion

The oxidative state of oxidized LDL was evaluated by measurement the TBARs formation which indicates the lipid peroxidation. Besides the lipid peroxidation, the degree of protein modification was also evaluated by an index of protein peroxidation, the REM of LDL measured by gel electrophoresis. The results demonstrated that hemin can induce oxidation in LDL. The increase of TBARs formation and the increase of REM in he-oxLDL indicate the elevation of lipid peroxidation and protein oxidation, respectively.

In the present study, ECa233 and asiatic acid protect the lipid peroxidation and protein modification on he-oxLDL at 24 hour of incubation less than α-tocopherol. In some studies the antioxidative activity of C. asiatica and the activity of leaf and root of C. asiatica had been reported to be as good as that of α-tocopherol (6). However, the duration of antioxidative action of ECa233 in the present study might not be prolonged to 24 hours. Further study done in shorter time of incubation is highly suggested.

In conclusion, standardized extract of Centella asiatica ECa233 and asiatic acid slightly protect the lipid peroxidation and protein modification on the in vitro LDL oxidation induced by 24-hour incubation of hemin.

Acknowledgements

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RESEARCH ARTICLE

Proteome Analysis of Microglial Secretion Induced by Aβ

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Abstract

Microglia become activated when exposed to amyloid beta peptides (Aβ), a major component of amyloid plaque, one of neuropathological hallmarks in Alzheimer's disease (AD). Key molecules known to be secreted by activated microglia are proinflammatory cytokines, chemokines and reactive oxygen species (ROS). The aim of this study was to identify novel proteins (as compared to the unstimulated control) that were secreted by microglial cells following Aβ stimulation. Here, we reported that microglia stimulated by Aβ for 12 h secreted several proteins, which could be classified by their functions, regulation of gene expression, metabolism, cell communication/signal transduction, protease inhibitor, immune response and protein binding. Many of these proteins, for example biliverdin reductase A, huntingtin interacting protein-2, nascent polypeptide-associated complex, have recently been reported to associate with neurodegenerative processes in AD. Thus, this protein profile may further our understanding of the role of microglia in AD pathogenesis.

Keywords Alzheimer's disease, Amyloid peptide, Activated microglia, Proteomics

Introduction

Microglia are resident immune cells of the brain. They monitor changes in brain microenvironment and respond by becoming activated through morphologic changes with the expression of cell surface markers associated with macrophages. [1] In AD brains, activated microglia are closely associated with core of the amyloid plaque. [2] The plaques compose of Aβ peptides, predominantly a full length Aβ1-42. It has been demonstrated that expose to Aβ1-42 stimulates microglial activation. [3] Aβ induces microglia to secrete proinflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and nitric oxide (NO). [4-6] Although precise mechanisms of neurodegeneration in AD have not yet been elucidated, according to amyloid cascade hypothesis, it is believed that mismetabolism of Aβ precursor protein (APP), subsequently the formation of amyloid plaque and neuroinflammation/neurotoxic mediated by microglia lead to neuronal cell death. [7] Thus, obtaining a profile of proteins secreted by Aβ-stimulated microglia will give us an insight into the role of microglia in AD pathogenesis.

Methods

Mouse microglial BV_2 cells were kindly provided by Professor James R. Connor (The Pensylvania State University, USA). The cells were maintained in 5% FBS-supplemented Dulbeaco's modified eagles's medium (DMEM) at 37^{o}C with 5% CO_2. To begin the experiment, cultured microglia were treated with serum-free medium containing Aβ1-42. The untreated cultures in serum-free medium were served as control. After 12 hours of treatment, cell-free media were collected and processed for protein precipitation using trichloroacetic acid (TCA). After dissolving the protein pellets in a lysis buffer (containing urea, thiourea and CHAPS), protein concentrations in each sample were determined by Bradford assay. The absorbance was measured at 595 nm. After that, protein samples in each condition were separated on 12.5% SDS-PAGE using a constant 100 voltage. Protein bands were visualized by staining with Coomassie blue. Each individual band was cut, tryptic digested and analyzed by LC-MS/MS. Protein sequences were blasted against MASCOT database (http://www.matrixscience.com) to identify protein targets.

Results

We found that Aβ stimulation induced microglia to secrete several novel proteins, that were not detected in the unstimulated control (Table 1). Thus, these proteins are potentially Aβ-inducible proteins, which can be categorized according to their functions: regulation of gene expression, metabolism, cell communication/signal transduction, immune response and protease inhibitor, as shown in Table 2. According to the literatures, several of these proteins have been linked to AD, as discussed below. These findings suggest that Aβ stimulates a broad spectrum response in microglia additional to known inflammatory cytokines, chemokines and ROS.

Table1 Proteins secreted by A□-stimulated microglia identified by LC-MS/MS

No.	Sequences derived from tryptic digestion	Assession Number	Description	MW	pI
1	R.VEELLAEAR.R	gi|93102413	Galactokinase 1	42268	5.17
2	K.WASVVVPLGK.E	gi|160333390	Histocompatibility antigen, H2-Q1	41296	6.52
3	K.FGFPAFSGISR.L	gi|30353884	Biliverdin reductase A	33477	6.53
4	R.QAELVAAS.-	gi|147904547	Postmeiotic segregation increased 2	23401	5.40
5	K.NILFVITKPDVYK.S K.IEDLSQQAQLAAAEK.F	gi|41350312	Nascent polypeptide-associated complex alpha subunit	23370	4.52
6	R.YQLEIK.I R.NAVIVALSSK.S K.IPETYPFNPPK.V K.VDLVDENFTELR.G	gi|2897818	Huntingtin interacting protein-2	22389	5.33

Table2 The association between microglial proteins in table 1 and AD

No.	Description	Postulated functions	Association with AD	Secretion Refs
1	Galactokinase 1	Metabolism	-	-
2	Histocompatibility antigen, H2-Q1	Immune response	-	-
3	Biliverdin reductase A	Cell communication/ signal transduction, regulation of gene expression, metabolism	[8]	[11]
4	Postmeiotic segregation increased 2	Regulation of gene expression	-	-
5	Nascent polypeptide-associated complex alpha subunit	Cell communication/ signal transduction	[9]	[12]
6	Huntingtin interacting protein-2	Protease inhibitor	[10]	-

Discussion

Application of 1D LC-MS/MS to analysis of complex protein mixtures has been previously reported. [13] The same principle was applied in this study. Several novel proteins secreted by microglia after Aβ-stimulation were identified, as compared to that of the unstimulated control. These proteins (shown in Table 1) were considered to be inducible proteins, because their expressions were undetectable in the unstimulated control. The relevance of each protein to AD was discussed below.

First to mention, biliverdin reductase A (BVRA), an abundant enzyme found in the brain as well as in kidney, spleen and liver. BVRA convert biliverdin into bilirubin in heme degradation pathway. [8, 14] An

up-regulation of the BVRA level was found in the serum and hippocampus of the AD patients and in hippocampus of mild cognitive impairment (MCI) suggesting that BVRA may involve in pathological process of AD. [15] It is possible that Aβ-activated microglia may contribute to elevated levels of BVRA observed in AD. Moreover, secretion of BVRA by microglia could also be a direct response of the cells to counteract increased levels of oxidative stress in AD brain. Nascent polypeptide-associated complex alpha (αNAC) was also identified as microglial secretory protein. This protein is found to be associated with ribosome and nascent polypeptide chains. It is postulated that αNAC might prevent newly synthesized proteins from aggregation and play a role in properly folding of nascent proteins. [16] Thus, a substantial decrease in the folding rate could favor protein aggregation. [17] Interestingly, αNAC has been reported to be decreased in AD brain [9]. This is consistent with may affect protein folding rates and stability as previously described. [18, 19] The mechanism by which αNAC was secreted by microglia has not been elucidated, but a previous study has demonstrated that αNAC can be secreted by fibroblasts. [12]

We also identified huntingtin interacting protein-2 (Hip-2), another protein that is highly expressed in the brain. Hip-2 is an ubiquitin conjugating enzyme in ubiquitin-proteasome pathway. [10] The present finding that Aβ induced Hip-2 secretion from microglia was consistent with the previous report showing that Hip-2 was increased around or near the senile plaques where clusters of activated microglia were observed. Moreover, Hip-2 was reported to mediate neurotoxicity of Aβ . [20] Together, these evidences may suggest a close relationship between Hip-2 secreted by microglia and AD pathogenesis. Although galactokinase 1 (GALK1), histocompatibility antigen (H2-Q1), postmeiotic segregation increased 2 (PMS2) have never been reported in AD, the findings that these proteins were secreted by microglia suggest that microglia involve in several aspects of AD pathogenesis such as in metabolism, immune response and regulation of gene expression.

Conclusion

The present study identified novel proteins secreted by microglia in response to Aβ stimulation. These proteins include BVRA, αNAC, Hip-2, GALK1, H2-Q1 and PMS2. The first three proteins identified were previously reported to associate with AD. Thus, it appears that Aβ stimulates a broad spectrum response in microglia in addition to known inflammatory cytokines, chemokines and ROS.

Acknowledgements

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RESEARCH ARTICLE

Screening of microbial-derived products for antimalarial activity

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Abstract

Multidrug resistant *Plasmodium falciparum* is a problem in tropical and subtropical regions particularly Thailand. The aim of study was to evaluate the *in vitro* antimalarial activity of five microbial-derived products against *Plasmodium falciparum* 3D7 (chloroquine-sensitive) and K1 (chloroquine resistant) clones. The microbial derived products were extracted and investigated for their antimalarial activity. Product number 28 was the only extract which exhibited promising antimalarial activity with IC_{50} value (50% and inhibition concentration 50%) of 0.3835 AU/ml against both clones. . Further studies should be performed to investigate the *in vitro* antimalarial activity of this product in *P. falciparum* isolates collected from different malaria endemic areas of Thailand, including its activity in animal model.

Keywords Microbial-derived product, *Plasmodium falciparum*, antimalarial activity

Introduction

Malaria is a disease in tropical and subtropical regions of the world. Major problem which limits the control of the disease is the emergence and spread of multidrug resistant *Plasmodium falciparum*. The parasite has developed resistance to almost all of the available antimalarial drugs including chloroquine, sulfadoxine-pyrimethamine and mefloquine. To cope with the situation, artemisinin based combination therapy (ACT) is currently adopted as first-line treatment of acute uncomplicated falciparum malaria (1). Nevertheless, resistance of the parasite to artemisinins has been reported in areas along the Thai-Cambodian and Thai-Myanmar borders (1-2). Research and development for new promising antimalarials from different sources, *i.e.*, chemical synthesis or natural products is urgently needed (3-5). In the present study, we investigated antimalarial activity of the five microbial-derived products against *Plasmodium falciparum* 3D7 and K1 clones.

Materials and methods

Microbial derived products extraction

Ammonium sulfate (80% saturation) was added to the cell-free culture supernatant while stirring and left overnight. The sample was centrifuged and supernatant was discarded. The precipitate was dissolved in sterile distilled water and dialyzed against sterile distilled water for 16-18 h. The supernatant was used for further analysis.

Culture system and parasite maintenance

P. falciparum 3D7 (chloroquine sensitive) and K1 (chloroquine resistant) clones were cultivated and maintained in O+ human erythrocytes suspended in RPMI 1640 culture medium (at 37ºC under a gas mixture of 5% CO_2, 5% O_2 and 90% N_2) according to the method described by Trager and Jensen (6). The culture medium was supplemented with 25 mM sodium bicarbonate, 10 mg/ml gentamicin sulfate, 25 mM HEPES (pH 7.4), 80 ml/l human B or AB serum. To obtain specific stages of *P. falciparum,* 5% sorbitol treatment was used (7).

Evaluation of antimalarial activity in vitro

Five samples from microbial derived products were screened for their antimalarial activity against *P. falciparum* 3D7 and K1 clones using SYBR Green I based assay (8). Stock solution of the microbial derived products number 6, 11, 16, 26 were prepared at concentrations 25, 5000, 5 and 1000 AU/ml, and further diluted with culture medium to obtain final concentrations 50, 10000, 10 and 200 AU/ml, respectively. The test product number 28 was used at concentration 75 AU/ml in a 96-well microtiter plate. Assays were performed using parasite suspension with 2% parasitemia and 1% hematocrit. Fifty percent inhibitory concentration (IC_{50}) values were calculated based on dose effect analysis (CalcuSyn ^{TM} Software). The antimalarial artesunate was used as a positive control drug.

Table 1 *In vitro* antimalarial activity screening of microbial-derived products against 3D7 and K1 *P. falciparum* clones.

Product Number	**No. 6**		**No. 11**		**No. 16**		**No.26**		**No. 28**
Conc. (AU/ml) / **% Growth**	25	50	5,000	10,000	5	10	1,000	200	75
K1 clone	9%	57%	530%	390%	159%	232%	66%	29%	34%
3D7 clone	13%	64%	728%	527%	166%	419%	120%	70%	44%

Results

Table 1 shows results of screening of antimalarial activity of the five microbial -derived products against 3D7 and K1 strain *P. falciparum in vitro*. Product number 28 was the only one which exhibited promising activity against both *P. falciparum* clones with IC_{50} values of less than 0.3835 AU/ml.

Discussion and Conclusion

The microbial-derived products number 28 exhibited the most potent antimalarial activity with low IC_{50} values against both CQ-resistant (K1) and CQ-sensitive (3D7) *P. falciparum* clones. Further studies should be performed to investigate the *in vitro* antimalarial activity of this product in *P. falciparum* isolates collected from different malaria endemic areas of Thailand, including its activity in animal model.

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RESEARCH ARTICLE

Microglias Stimulated by LPS Secrete Cofilin-1

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Abstract

Microglia are resident cells of the brain. Upon activation (e.g. neurodegenerative and neuroinfectious conditions), microglia become activated. Activated microglia are capable of phagocytosis, secrete pro-inflammatory cytokines, chemokines, ROS and other inflammatory mediators. Recent evidences have provided a link between microglia, inflammation and neurodegeneration. As part of the brain's innate immune system, microglia express several types of pattern recognition receptors (PRRs), including toll-like receptor (TLR) 4, which primarily recognizes bacterial cell-wall lipopolysaccharide (LPS). To give an insight into the earliest stage of microgial response to bacterial brain infection, proteins secreted by LPS-stimulated microglial cells at 6 h were examined using two-dimensional gel electrophoresis (2-DE). As compared to that of the unstimulated control, differentially expressed proteins were determined and identified by LC MS/MS. In addition to known inflammatory cytokines and chemokines, here we reported for the first time that LPS induced secretion of cofilin-1 from microglia. Although the significance of cofilin-1 secretion as part of microglial early response to bacterial infection is not known, it is possible that these cofilins may implicate the roles of microglia in brain Na^+-K^+ homeostasis and neuronal remodeling during bacterial infection.

Keywords Microglia, TLR-4, Brain infection

Introduction

Microglia are the resident macrophages and constitute as many as 12% of cells in the brain. Microglia are the key players in establishing the innate immune reaction within the brain. In the adult normal brain, microglia has a ramified morphology with low expression levels of immunological markers. These "resting" microglia constantly monitor the integrity of the brain microenvironment and react rapidly to any pathological stimulation. Upon activation, "resting" microglia transform into active immune competent cells. Their morphology changes from ramified to rod-like and finally to phagocytic amoeboid (1). Activated microglia migrate through the parenchyma to the sites of lesions or where invading pathogens have been detected. Like other tissue macrophages, activated microglia release pro-inflammatory cytokines such as interleukin (IL)-1, 6, 12, 17, and 18, tumor necrosis factor (TNF)-α, chemokines, reactive oxygen and nitrogen species (ROS/RNS) (2, 3). Moreover, recent findings have linked microglial activation with a diverse range of neurodegenerative disorders.

As part of the brain's innate immune system, microglia express several types of pattern recognition receptors (PRRs), which are genetically conserved for the detection of pathogen-associated molecular patterns (PAMPs), which are small molecular motifs consistently found on pathogens (e.g., LPS) (2). Example of microglial PRRs are Nod-like receptors and Toll-like receptors (TLR) 2, 3, 4 and 9. In case of bacterial infection, microglial TLR4 recognizes LPS leading to a rapid activation of microglia (4). TLR4 is a well analyzed TLR signaling pathway associated with microglial activation. After binding of LPS, TLR4 via MyD88 activates nuclear factor–kB (NF-kB) and induces massive transcription of IL-1β, IL-6, TNF-α and interferons (5). The implications of these molecules released by microglia include recruitment of leukocytes into the CNS for the purpose of defense, as well as a collateral damage to other brain cells (6). To give an insight into the earliest stage of microgial response to bacterial brain infection, we compared proteins secreted by LPS-stimulated microglia at 6 h with that of the unstimulated control, using 2-DE and mass spectrometry.

Materials and methods

Cell cultures and cell viability

Mouse microglial BV-2 cells were a kind gift of Prof. James R. Connor (The Pennsylvania State University, USA). The cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-Glutamine at 37 ºC and maintained in a CO_2 incubator. To begin the experiments, BV-2 cells with > 95% cell viability determined by tryphan blue dye exclusion method were plated at a density of $1x10^6$cells/flask in a serum-free medium containing LPS (1 µg/ml). Then, cell-free supernatants were collected at 6 h for 2D gel electrophoresis. Supernatants from the untreated cultures were served as control. All cell culture reagents were purchased from Hyclone (USA).

2D gel electrophoresis and mass spectrometry

Proteins in the medium were precipitated using trichloroacetic acid and centrifuged at 15000 x g for 15 min at 4°C. The pellets were collected and dissolved in lysis buffer containing urea, thiourea and CHAPS. Protein concentrations in each condition were determined by Bradford assay and the absorbance was measured at 595 nm. For first-dimension isoelectric focusing (IEF), 200 µg of total protein sample was loaded onto 7 cm Non-linear IPG strip pH 3-10 (GE Healthcare, USA). IEF was performed at 20 ºC on IPGphor (GE Healthcare, USA). For the second dimension, polyacrylamide gels (12.5%) were used for separation. Electrophoresis was run at a constant current of 40 mA until the bromophenol blue front reached the bottom of the gel. After electrophoresis, the gels were stained with Coomassie Blue and scanned by an Image-scanner™ (GE Healthcare, USA). Image spots were initially detected, matched, and manually edited with the ImageMaster™ 2D Platinum 7.0 software (GE Healthcare, USA). Quantification was given as a spot volume percentage (vol %), with each single spot volume normalized with respect to the total spot volume of the gel. The gels were compared between the spot vol % of the stimulated group and the unstimulated group.

Data analysis

This study focused on detecting novel spots that were consistently induced by LPS (n=3) and absent in the unstimulated control (n=3). After protein spots were cut and collected to eppendorf tubes, proteins in each spot were digested by trypsin. The samples were then analyzed by LC MS/MS (Biotech, Thailand). Amino acid sequences obtained from mass spectrometry were searched in the NCBInr database using Mascot software (http://www.matrixscience.com) The criteria for positive identification of proteins were set as follow: the mascot score of a protein matching was higher than 40 ($p < 0.05$).

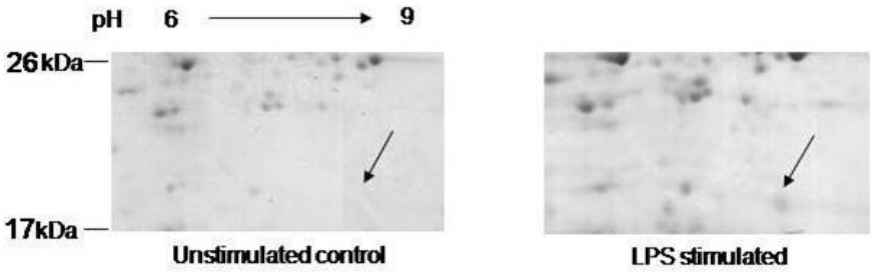

Figure 1 Arrows indicate absence/presence of the target protein in the representative gels.

Table 1 A list of candidate proteins identified by mass spectrometry for a protein spot described in the result section

Sequence[a]	Accession number[b]	Protein name	MW[c] (KDa)	pI[d]	Score[e]	Coverage[f] (%)
K.HELQANCYEEVK.D K.EILVGDVGQTVDDPYTTFVK.M	gi|6680924	Cofilin-1, non-muscle	18.77	8.22	175	19
K.HELQANCYEKVK.D K.EILVGDVGQTVDDPYTTFVK.M	gi|12861068 (Q9CX22)	Putative uncharacterized protein (Actin binding protein encoded by gene cofilin-1[clf1])	25.29	8.63	111	13
K.TVISPR.G K.VKTVISPR.G K.VKTVISPR.G	gi|26354955	Ser/Arg repetitive matrix protein 2	284.04	12.09	41	0
K.TLNGAEMAPIK.I	gi|22095027	Sex comb on midleg1 isoform 1	74.59	9.42	29	1
R.LQLPAPPAPIK.A	gi|74147754 (Q3TLP9)	Putative uncharacterized protein (Encoded by gene zinc finger and BTB domain containing 45 [Zbtb45])	55.74	6.54	29	2
R.SGIVVKDVSIK.V	gi|26334641 (Q8BY76)	Putative uncharacterized protein (Encoded by gene protein tyrosine phosphatase receptor type Q [Ptprq])	121.1	5.42	28	1
R.GEMSGRLGPLK.L	gi|50510447	mKIAA0302 protein	223.80	5.51	27	0

[a] Matched peptides of NCBInr/SWISS-PROT BLAST search
[b] Accession number for NCBInr/SWISS-PROT
[c] Molecular weight
[d] Isoelectric point
[e] Ion score > 40 indicates identity or extensive homology ($p<0.05$) with target sequence
[f] Coverage % = (matched residues/total residues in the entire sequence) x 100

Results

There were several protein spots that were consistently observed in all three gels of LPS-stimulated samples and were undetectable in three gels of unstimulated control samples. This suggested that protein in these spots were LPS inducible proteins. In this preliminary result, a single protein spot of molecular weight (MW) between 17-26 kDa and isoelectric point (pI) in an approximate range of pH 6-9 was selected for mass spectrometry analysis. The location of this spot on the gels was shown in Fig1. Mascot search results identified several candidate proteins, as shown in Table 1. The most likely candidate was identified as cofilin-1. According to Table 1, Its MW and pI based on NCBInr database was consistently with that of protein spot observed on the gel (Figure 1). This result demonstrated for the first time that LPS induced secretion of cofilin-1 from microglia.

Discussion

In this study, cofilin-1 was identified as microglial inducible protein upon LPS stimulation. Cofilin-1 is an actin-binding protein which regulates assembly and disassembly of actin filaments. There are two isoforms of cofilin, cofilin-1 (non-muscle-type) and cofilin-2 (muscle-type), in mammals (7). Although, cofilin-1 does not have signal peptide for secretion, they can be found as secreted proteins. Similar to cyclophilins, SF20/IL-25 and galectin-1, cofilin-1 has been postulated to be secreted through an ER/Golgi-independent pathway (8, 9). Little is known about molecular and cellular targets of cofilin-1 secreted during early microglial response to bacterial infection. A future study to identify cofilin-interacting targets may advance our understanding of brain response to bacterial infection. However it has been reported that cofilin-1 binds to itself, actin interacting protein-1 (AIP-1) (10), cyclase associated protein (CAP) (11), testicular protein kinase 1/2 (TESK 1/2) (12), Na,K-ATPase α subunit (13), LIM kinase 1 (14), neutrophil kinase (15), serine/threonine phosphatases of type 1 and type 2A (PP1 & PP2A) (16) and slingshot (17). Among these proteins, Na,K-ATPase and LIMKinase-1 are found to be expressed in the brain (18, 19). Na,K-ATPase is a channel that exchanges intracellular sodium for extracellular potassium (13), whereas LIMKinase has been reported to regulate formation of actin based structures in growth cones and postsynaptic densities of neurons (14).

Conclusion

We reported for the first time that microglia secreted cofilin-1 in response bacterial infection. Thus, it is possible that microglia may be involved in brain Na^+-K^+ homeostasis and neuronal remodeling during bacterial infection through the secretion of cofilin-1. This study suggests that cofilin-1, which contribute to understanding of brain response to bacterial infection during the early state.

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RESEARCH ARTICLE

Association between *in vitro* Sensitivity of *Plasmodium vivax* Isolates and Polymorphisms of Dihydrofolate Reductase (*Pvdhfr*) and Dihydropteroate Synthase (*Pvdhps*)

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Abstract

In Thailand, the proportion of *Plasmodium vivax* infection currently becomes equal to *P. falciparum*. The study was performed to investigate the association between the polymorphisms of *P. vivax* dihydrofolate redutase (*Pvdhfr*) and *P. vivax* dihydropteroate synthase (*Pvdhps*) and *in vitro* sensitivity of *P. vivax* isolates in Thailand to chloroquine and WR99210 (the experimental inhibitor of *dhfr* enzyme). A total of 32 *P. vivax* isolates were collected from Mae Sot District, Tak Province, the malaria endemic area of Thailand with highest annual malaria incidence. *In vitro* sensitivity test was performed by schizont maturation inhibition test and mutations of *Pvdhfr* and *Pvdhps* were detected by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). Median (95% CI) values for IC_{50} (drug concentration which produces parasite's growth inhibition by 50%) of chloroquine and WR99210 were 134.7 (1.17-264.99) and 139.95 (0.21-523.08) nM, respectively. Mutant alleles of *Pvdhfr* were observed in 20 isolates; 15, 2, 2 and 1 isolates carried 57I/58R/117T, 58R/61M/117N, 57L/58R/117T and 57L/58R/117R, respectively. Nineteen, and 1 isolates carried a single 383G and wild-type A383 of *Pvdhps*, respectively. There was no association between *in vitro* sensitivity of *P. vivax* isolates and mutations of *Pvdhfr* and *Pvdhps.*

Key words *Plasmodium vivax, In vitro* sensitivity, Chloroquine, WR99210, *Plasmodium vivax dihydrofolate reductase (Pvdhfr), dihydropteroate synthase (Pvdhps)* and PCR-RFLP

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Introduction

In Thailand, the proportion of *Plasmodium vivax* infection currently becomes equal to *P. falciparum*. The blood schizontocide chloroquine and tissue schizontocide primaquine have remained the mainstay chemotherapeutics for treatment of *P. vivax* infection in Thailand for more than 60 years with reserved clinical efficacy of virtually 100% (1). Nevertheless, accumulating reports of chloroquine resistance *P. vivax* in other parts of the world during the past three decades (2) emphasize the need for closely and continuously monitoring of therapeutic efficacy of chloroquine and investigate novel antimalarial against chloroquine resistance *P. vivax.* WR99210 is a novel inhibitor of enzyme dihydrofolate reductase (DHFR) in malaria parasites, it shows activity against the most pyrimethamine-resistant *P. falciparum* strains and extremely effective inhibitor of the *P.vivax* DHFR including mutations that confer high-level resistance to pyrimethamine(3). Due to chloroquine resistance *P. falciparum* occurred in Thailand, antifolate drugs, sulphadoxine (S) and pyrimethamine (P) combination known as Fansidar , has been introduced for treatment of falciparum malaria. Using SP is caused of drug accidental expose to *P. vivax* and leads to *P. vivax* antifolate resistance (4). The study was performed to investigate the association between the polymorphisms of *P. vivax* dihydrofolate reductase (*Pvdhfr*) and *P. vivax* dihydropteroate synthase (*Pvdhps*) and *in vitro* sensitivity of *P. vivax* isolates in Thailand to chloroquine and WR99210 (the experimental inhibitor of *dhfr* enzyme).

Materials and Methods

Sample collection

The study was conducted at Mae Tao clinic for migrant workers, Tak Province during April 2009 - July 2010. The study was approved by the Ethics Committee of Ministry of Public Health of Thailand. A total of 32 blood samples (1 ml blood collected into sodium heparinized plastic tube and blood dried spots on filter paper) were collected from patients with mono-infection with *P. vivax* malaria prior to treatment with standard regimen of a 3-day chloroquine. Written informed consent for study participation was obtained from all patients.

In vitro drug sensitivity assay

Antimalarial compounds: P. vivax field isolates were tested for their sensitivities against chloroquine and WR99210. The concentration ranges for each drug used were 0-10,000 nM for chloroquine and 0–2,560 nM for WR99210. The drug was prepared to obtain desired concentration and 50 µl of each concentration was pipetted into 96 well plate.

Schizont maturation inhibition assay: The schizont maturation inhibition was performed with all *P. vivax* field isolates collected from all patients using a modified method of Russell and colleagues (5). Briefly, The pack red cells was washed by RPMI 1640 medium. The pellet was then resuspended in human AB serum to obtain a haematocrit of 40%. The blood–serum suspension was mixed with McCoy's 5A medium at the ratio of 1:10. Fifty microliters of this mixture were added to each well of a 96-well microtiter plates pre-dosed with drug. The tested plate was incubated at 37.5°C in a candle jar containing 5% CO_2 for 24–36 hours

depending on the stage of the parasite before culturing. After incubation, a thick blood film was prepared from each well and the number of normal schizonts (containing > 8 nuclei) *per* 200 asexual stage parasites was counted.

Data analysis: The number of schizonts in each well that contained drug was compared with that in the control well (drug free) and expressed as a percentage of the control. The dose response curves obtained from both assays were analyzed by nonlinear regression analysis using CalcuSyn™ software (Biosoft™, Cambridge, UK). The log-transformed concentration and probit-transformed inhibition data were processed as linear regressions. The results were expressed as 50% inhibition of parasite development as compared to the control.

Detection of mutations in the Pvdhfr and Pvdhps genes

Extraction of parasite genomic DNA: 20 individual blood dried spots on filter paper was was extracted to obtain parasite genomic DNA using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification.

Nested PCR-RFLP: total of 20 *P. vivax* genomic DNA was used for study of *Pvdhfr* and *Pvdhps* point mutations by nested PCR-RFLP. For *Pvdhfr,* mutation at 7 amino acid codons: 13, 33, 57, 58, 61, 117 and 173 were investigated according to the previously described methods of Imwong and Snounou (6-8) and point mutations of *Pvdhps* at 2 amino acid codons (383 and 553) were investigated according to method of Rungsihirunrat and Imwong (6,9)

Results

Table 1 Median(95% CI) IC_{50} values of WR99210 and Chloroquine by schizont maturation inhibition assay (*n=32*)

Drugs	Median IC_{50} (95% CI) (nM)
Chloroquine (CQ)	134.70 (1.17-264.99)
WR99210	139.95 (0.21-523.08)

Table 2 *Pvdhfr* and *Pvdhps* genotypes and IC_{50} of WR99210

Genotype	IC_{50} (nM)	
	CQ	**WR99210**
pvdhfr 57I/58R/117T, *pvdhps*383G (*n*=15)	34.57- 239.82	32.77-413.16
pvdhfr 58R/61M/117N, *pvdhps* 383G (*n*=2)	234.60-248.86	151.23-285.58
pvdhfr 57L/58R/117T *pvdhps*383G (*n*=2)	141.99-193.98	84.55-153.30
pvdhfr 57L/58R/117T, *pvdhps* A383 (*n*=1)	25.86	414.01

Discussion

An *in vitro* assessment of drug response of *P. vivax* is useful for monitoring of drug resistance since clinical studies is sometimes impracticable by long time follow up (10). The sensitivity of *P. vivax* as indicated by IC_{50} values for chloroquine and WR99210 were 134.70 (1.17-264.99) and 139.95 (0.21-523.08) nM, respectively. It was noted that the IC_{50} of chloroquine in *P. vivax* was about 2-4 fold of that of *P. falciparum*, but the variation is probably higher with *P. vivax*. The *in vitro* cut-off value defining clinically relevant chloroquine resistance in *P. vivax* malaria has yet to be clearly defined. WR99210, a novel antifolate, even it never be introduced for clinical usage but the 50% inhibition activity to *P. vivax in vitro* is lower than 600 nM (139.95 (0.21-523.08) nM) and most effective when compared with other antifolate drugs; pyrimethamine, sulfadoxine and dapsone (4). The molecular targets of action of antifolate drugs are dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). Point mutations within the genes that encode these enzymes are the primary causes of resistance to the drugs (11). Our molecular analysis revealed that all parasite isolates carrying triple mutation of *pvdhfr* and 383G point mutation of *pvdhps* was found in 19 of 20 isolates. Interestingly, the same *pvdhfr* and *pvdhps* point mutations still showed high variation of their IC_{50} values for WR99210; 15 out of 20 isolates carried 57I/58R/117T of *pvdhfr* and 383G of *pvdhps*, with IC_{50} of WR99210 ranging from 32.77 to 413.16 nM.

Conclusion

With limited sample size, in was concluded for the lack of association between *in vitro* sensitivity of *P. vivax* isolates and mutations of *pvdhfr* and *pvdhps.*

Acknowledgements

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RESEARCH ARTICLE

Regulation of Cholesterol Transporter Protein by Black Pepper and Piperine

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Abstract

Black pepper extract *(Piper nigrum L.)* and piperine, the active compound of black pepper, were previously shown to block the uptake and absorption of cholesterol into Caco-2 cells. The present study was then aimed to determine the effect of black pepper extract and piperine on the expression of certain proteins functioning in the regulation of cholesterol transport in Caco-2 cells. The expression of proteins was determined by western blotting. The results showed that there was no change in the expression of ABCG5, ABCG8 and ACAT2 in cells treated with black pepper extract and piperine. The expression of cholesterol transporter, NPC1L1, in membrane fraction of Caco-2 cells was lower than that of control. This reduction was not observed in whole cell lysate. The disappearance of NPC1L1 in the membrane fraction may indicate that black pepper extract and piperine could regulate the translocation of NPC1L1 between cell membrane and cytoplasmic compartment.

Keywords Cholesterol, Black pepper, Piperine, NPC1L1.

Introduction

According to our previous study of twelve plant extracts, black pepper extract showed the best inhibition of cholesterol uptake in differentiated Caco-2 cells [1]. From our recent study, not only black pepper extract but also piperine effectively decreased cholesterol uptake and absorption in Caco-2 cells (unpublished data). To investigate their mechanism of action, we tested their effect on the expression of certain proteins functioning in cholesterol absorption. These proteins include Niemann-Pick C1-Like 1 (NPC1L1); a cholesterol transporter, acyl-coenzyme A cholesterol acyltransferase (ACAT2); a cholesterol esterification enzyme, ATP binding cassette proteins G5 (ABCG5) and G8 (ABCG8); cholesterol efflux proteins.

Method

Cell preparation

Caco-2 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in DMEM/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 37 °C in CO_2 incubator containing 95% air and 5% CO_2. Cells were propagated in culture flasks and subsequently plated in 6-well plates. Caco-2 cells were cultured for 14- 21 days allowing cell differentiation.

Western blotting

Differentiated Caco-2 cells were treated with black pepper extract and piperine for 24 h. The cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 0.5% SDS and 1% of protease inhibitor cocktail) and stored at -20°C before use. The protein concentrations were determined by BCA protein assay kit. Proteins in cell lysate were separated on SDS-polyacrylamide gel and transferred onto PVDF membrane. The membrane was blocked with PBS containing 5% skim milk at room temperature. Then, membrane was incubated with primary antibodies recognized NPC1L1, ABCG5, ABCG8 or ACAT2 overnight at 4°C. Proteins were detected using secondary antibodies IgG conjugated with horseradish peroxidase and then visualized by enhanced chemiluminescence.

Results

The result show that NPC1L1 level in membrane fraction was lower than that control, whereas in whole cell lysate was not altered after treating cells with black pepper extract and piperine for 24 h. (Fig. 1). The levels of ABCG5, ABCG8 and ACAT2 proteins did not change following such treatment (Fig. 2).

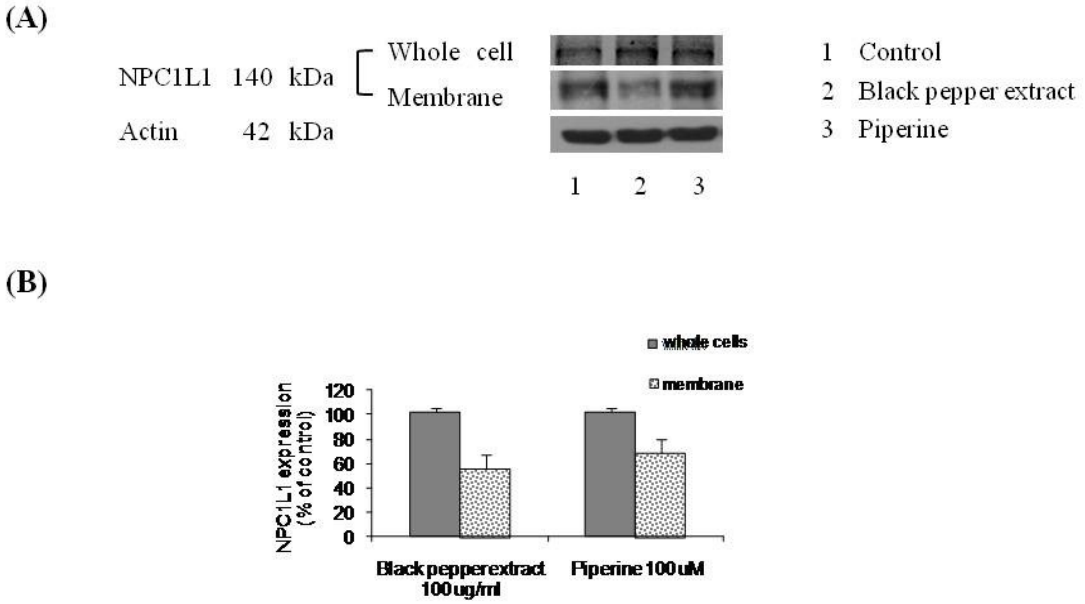

Figure 1 NPC1L1 protein expression in whole cell lysate and membrane fraction of differentiated Caco-2 cells incubated with black pepper extract and piperine for 24 h (A). Protein band densities were averaged from 3-4 experiments (B). Expression values ware normalized by actin. * $p<0.05$ compared to untreated cells (control).

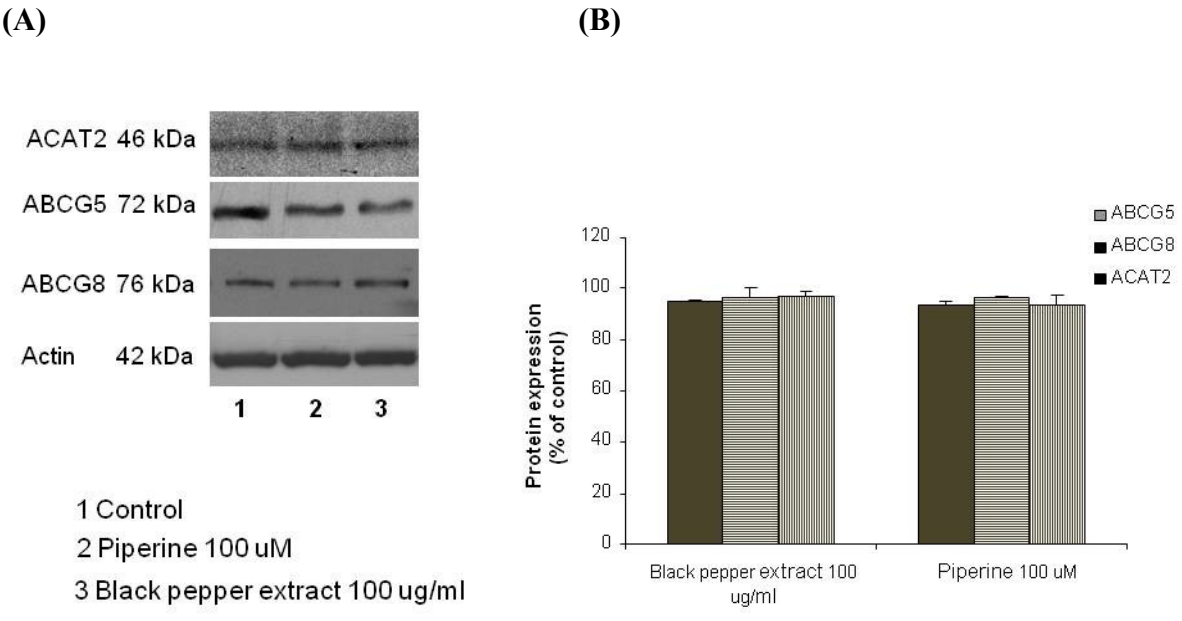

Figure 2 ABCG5, ABCG8 and ACAT2 protein expression in differentiated Caco-2 cells incubated with black pepper extract and piperine for 24 h (A). Protein band densities were averaged from 3 experiments (B). Expression values were normalized by actin.

Discussion

NPC1L1 is a cholesterol transporter protein found in both brush border membrane and the intracellular compartment of intestinal cells [2]. The present study showed that black pepper extract and piperine reduced NPC1L1 levels only in the membrane fraction. There was no change of NPC1L1 in whole cells lysate representing the total level of cellular NPC1L1 protein. This can be suggested that black pepper extract and piperine do not affect the overall expression of NPC1L1 but regulate the movement of NPC1L1 between cell membrane and cytoplasmic compartment. In this case, they promote the internalization of membrane NPC1L1 to the cytoplasm. There was evidence showing that cholesterol and ezetimibe (a cholesterol absorption inhibitor) can inhibit the internalization of NPC1L1 [3]. Although black pepper extract and piperine did not affect the expression of ACAT2 responsible for cholesterol esterification in this study, it was previously reported to inhibit the activity of ACAT1 and ACAT2 in mouse macrophages [4]. Possibly, black pepper extract or piperine alter only the activity but not the expression of ACAT2. In addition, no change of ABCG5 and ABCG8 expressions was observed in this study. Taken together with our previous study (unpublished data), black pepper extract and piperine do not have any effect on the cholesterol efflux.

Conclusion

One possible mechanism of the cholesterol absorption inhibitory activity of black pepper extract and piperine could be through the regulation of NPC1L1 translocation between cell membrane and cytoplasmic compartment. However, further study is required to verify this assumption.

Acknowledgements

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RESEARCH ARTICLE

Effect of Vitamin C on Aortic Elasticity of Mice After Cadmium Exposure

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Abstract

Cadmium (Cd) is a toxic heavy metal that is associated with cardiovascular diseases, especially hypertension. Cd-induced toxicity appears to be mediated via oxidative stress. This study was aimed to investigate the effect of vitamin C on aortic elasticity of mice with Cd–induced hypertension. Male ICR mice were received $CdCl_2$ (100 mg/L) in their drinking water whereas normal controls received deionized water. The other two groups of animals treated with $CdCl_2$ were concurrently administered with vitamin C at doses of 50 and 100 mg/kg/day for eight weeks. It was found that Cd administration elevated arterial blood pressure, increased oxidative stress and decreased aortic elasticity. Vitamin C dose-dependently decreased blood pressure and oxidative stress. Moreover, vitamin C at dose of 100 mg/kg/day largely improved aortic stiffness. These findings provide the evidence for the role of antioxidant vitamin C in alleviation of oxidative stress and blood pressure reduction of mice with sub-chronic exposure to CdCl*2*.

Keyword cadmium, hypertension, elastic properties, oxidative stress

Introduction

Cadmium (Cd) is an important environmental metal that has a close linkage with pathogenesis of cardiovascular disease. Cadmium exposure also cause oxidative stress, impaired vascular functions and increased blood pressure (Sompamit et al., 2010). It was suggested that high blood pressure exerts influence on the vascular structure mechanical properties (Hayashi and Naiki, 2009). Antioxidant vitamin C, is widely distributed in natural products. Epidemiological studies proposed the possible beneficial effects of vitamin C supplementation for the prevention of cardiovascular disease (Duarte and Lunec, 2005). Therefore, the aim of this study was investigated effect of vitamin C on the aorta elastic properties in cadmium chloride ($CdCl_2$) –induced hypertensive mice.

Methods

In these experiments, adult male ICR mice weighing 25–30 g were randomly divided into four groups of eight animals each. Mice were received $CdCl_2$ (100 mg/L) in their drinking water for eight weeks whereas normal control mice received deionized water. The other two groups of animals treated with $CdCl_2$ were orally administered with vitamin C at doses of 50 and 100 mg/kg/day for 8 weeks. The experimental protocols and treatments were reviewed and approved by the Animal Ethics Committee of Khon Kaen University. After 8 weeks, each group was further divided into two subgroups of four animals. The first subgroup for assessment of blood pressure (BP), heart rate (HR) and oxidative stress markers including aortic superoxide production ($O_2^{\cdot-}$) and plasma malondialdehyde (MDA). Oxidative stress markers were measured by previously described method (Sompamit et al., 2009). The second subgroup for measurement of aortic elastic property. The mouse thoracic aorta was cannulated and connected barium sulphate to perfusion apparatus for elastic property evaluation. The pressurized pressures were varying from 0-200 mmHg, the arterial image of each pressure was captured and integrated with image analysis software (ImagePro Plus).

Results

This study found that systolic blood pressure (SPB), diastolic blood pressure (DBP) and mean arterial blood pressure (MBP) were significantly increased in mice exposed to $CdCl_2$ when compared with the control group. Interestingly, vitamin C at doses of 50 and 100 mg/kg/day significantly decreased arterial blood pressure of $CdCl_2$-treated mice (Fig. 1A; $P<0.05$). However, there was no significant difference in heart rate (HR) among all experimental groups (Fig. 1B). Increases in aortic $O_2^{\cdot-}$ production and plasma MDA level were also found in $CdCl_2$ -treated mice (Fig. 2; $P<0.05$). Vitamin C significantly reduced $O_2^{\cdot-}$ production and lipid peroxidation in mice exposed to $CdCl_2$ as shown in Figure 2 ($P<0.05$). Figure 3 shows the incremental modulus and the relative radius relationships which are the indicators for arterial elastic property. It was found that the aortic elasticity of mice treated with $CdCl_2$ was shifted to the left and moved upward when compared to the control mice,

suggesting that the arterial wall was stiffer than normal. Interestingly, supplementation with vitamin C, especially at high dose alleviated the arterial stiffness (Fig. 3, P<0.05).

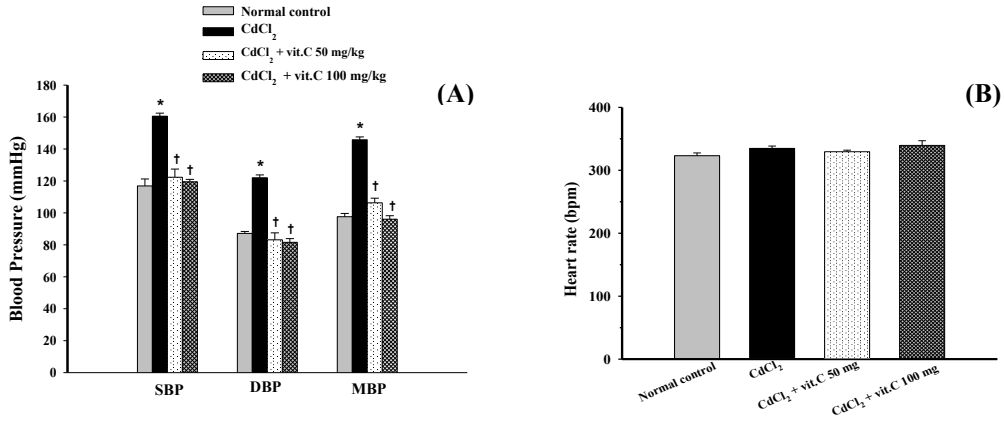

Figure 1 Effect of vitamin C on blood pressure (A) and heart rate (B) in mice exposed to $CdCl_2$l.
* $P < 0.05$ vs. control; † $P < 0.05$ vs. $CdCl_2$ control, n = 4/group

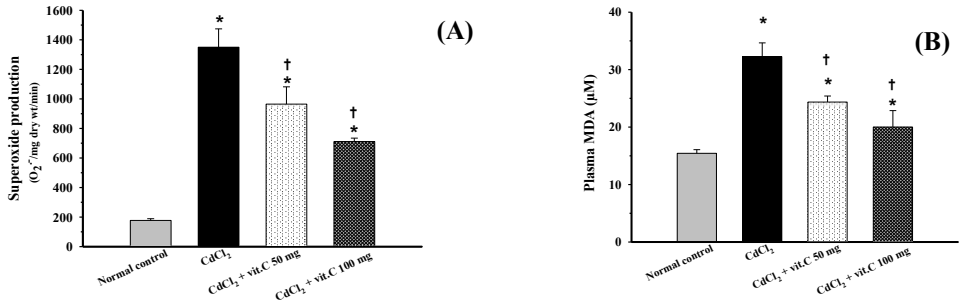

Figure 2 Effect of vitamin C on $O_2^{\bullet-}$ production (A) and plasma MDA (B) in mice exposed to $CdCl_2$.
* $P < 0.05$ vs. control; †$P < 0.05$ vs. $CdCl_2$ control , n = 4/group

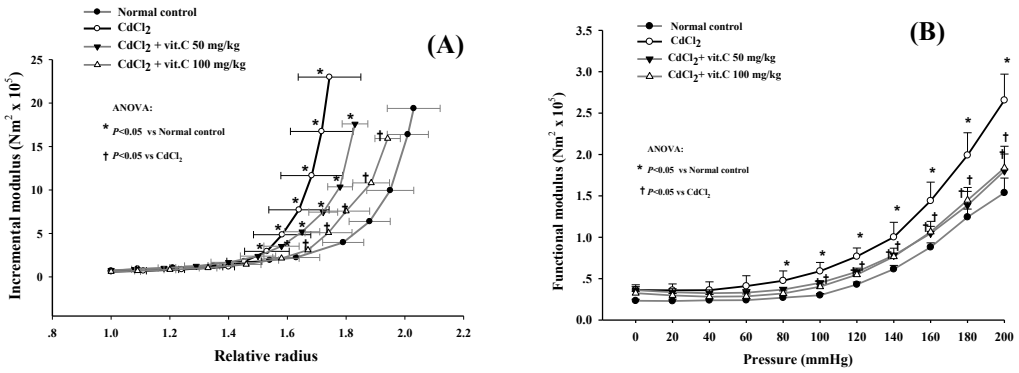

Figure 3 The relationship of material incremental modulus and relative radius (A) and the relationship of functional stiffness and blood pressure (B). * $P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. $CdCl_2$ control, n = 4/group

Discussion

It was found that mice with subchronic exposure to Cd had an increase in blood pressure and decreased aortic elasticity when compared with normal controls, suggesting that vascular dysfunction occurs in these animals. It has been demonstrated that the vascular wall is one of the target organs of Cd deposition, the accumulation of Cd in the aorta facilitates the weakening of the aorta through the adverse effects on smooth muscle cell metabolism (Abu-Hayyeh et al., 2001). Based on our observation, the novel finding of this study is that the subchronic exposure to Cd results in increased blood pressure, increased oxidative stress and decreased arterial elasticity. We also have been demonstrated that vitamin C especially 100 mg/mg can decrease oxidative stress markers, decrease blood pressure and improves aortic elastic property. The beneficial effect of vitamin C might be due to its strong anti-oxidative effects.

Conclusion

Vitamin C supplementation protected the increase in oxidative stress, blood pressure and the blunted aortic elasticity in mice with sub-chronic exposure to $CdCl_2$. The beneficial effect of vitamin C might be due to its metal chelating and strong anti-oxidative effects. Vitamin C might provide as preventive and therapeutic values in the treatment of Cd-induced cardiovascular disease.

Acknowledgements

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RESEARCH ARTICLE

Identification of Potential Biomarkers in Plasma of Cholangiocarcinoma Patients by Using Gel-LC-MS/MS

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Abstract

Cholangiocarcinoma (CAA), a cancer of bile ducts is the cause of severe health problems especially in the northeastern part of Thailand. At present, there is no effective diagnosis or specific biomarkers that can detect the early stage or prognosis of CCA. The aim of the study was to identify new specific biomarkers for CCA using GEL-LC-MS/MS technique. The proteins (30 μg/well) from plasma samples of healthy subjects and patients with CCA were resolved by 12.5% SDS-PAGE and detected by silver staining. Each gel sample lane was cut and single fragments of proteins with molecular weights of less than 14 kDa were digested with trypsin and subjected to LC-MS/MS analysis. The obtained LC-MS/MS data were analyzed by DeCyder^{TM}, Mascot^{TM} and MeV^{TM} softwares (t-test, $\alpha = 0.05$) to discriminate significantly expressed proteins from plasma samples of CCA patients from healthy subjects. Six proteins were identified, namely selenocysteine insertion sequence-binding protein 2, class A basic helix-loop-helix protein 15, C-type lectin-like receptor-2 , HBF-3, contactin-2 precursor, and hypothetical protein LOC9813. Real-time PCR and Western blot analyses and real-time RT-PCR will be performed to confirm the differentially expressed mRNA and proteins in both samples. Analysis of higher molecular weight proteins in plasma samples from both groups is under way to identify additional potential biomarkers. Efficiency (sensitivity, specificity) of these proteins to be exploited as potential biomarkers for diagnosis of CCA will also be evaluated by ELISA and/or Western blot analysis.

Keywords Biomarker, Cholangiocarcinoma, GEL-LC-MS/MS.

Introduction

Cholangiocarcinoma (CAA), a cancer of bile ducts is the cause of severe health problems especially in the northeastern part of Thailand. Infection with the liver fluke *Opisthorchis viverrini* (OV) and consumption of nitrosamine containing fermented food has been identified as one significant factor associated with the risk of development of CCA in Thai patients. Chronic parasite infection, reinfection, chemotherapeutic treatment with praziquantel, and eating habits may promote the development of this cancer. At present, there is no effective tool or specific biomarkers that can detect the early stage or monitor status of CCA. Established serum tumor markers include carbohydrate antigen 19.9 (CA19-9) and carcinoembryonic antigen (CEA). However, these markers are not always helpful, with sensitivities of approximately 70 and 50%, respectively. The aim of the study was to identify new specific biomarkers for CCA using GEL-LC-MS/MS technique.

Materials and Methods

SDS-PAGE and staining

Plasma samples obtained from healthy Thai subjects (control) and patients with CCA (20 samples each) were used in the study. Protein concentration of each sample was measured according to the method of Lowry and colleagues[1]. Plasma proteins in each sample were resolved by 12.5% SDS-PAGE at 20 mA for 80 min. The gel was then silver stained and stored in 0.1% acetic acid until use. Each gel sample lane was divided into seven major sections using the six standard protein bands (Low Molecular Weight Marker, GE healthcare) as landmarks for cutting. Single gel fragments (molecular weights of less than 14 kDa) of each sample lane were selected for further analysis by LC-MS/MS.

In gel digestion

The gel fragments were transferred into separate wells of a 96-well microplate and washed with water. Gel was incubated with 100% acetonitrile at room temperature for 5 min with agitation, and the gel fragments were air-dried at room temperature for 5-10 min, and then treated with denaturing buffer I (10 mM dithiothreitol, 10 mM ammonium bicarbonate) and carbamidomethyl buffer (100 mM iodoacetamide, 10 mM ammonium bicarbonate). Protein digestion was performed using a mixture of 10 ng trypsin in 50 acetonitrile/10 mM ammonium bicarbonate (20 min incubation at room temperature). Acetonitrile (30%) was added into each well and the plate was incubated at room temperature for 3 h. The supernatant was collected and the gel was further extracted with 50% acetonitrile (room temperature with agitation, twice, 10 min each). Finally, the acetonitrile extracts were pooled and allowed to be dry at 40°C (3-4 h or overnight) and stored at -80°C until use.

Nano ESI-MS/MS analysis

The protein digests were injected into µ-precolumn (Monolithic Trap Column, 200 µm i.d. x 5 mm) coupled with Ultimate 3000 LC system (Dionex), and ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray at a flow rate of 20 µL/min. Proteins were separated on a nano column (Monolithic Nano Column, 100 µm i.d. x 5 cm) with a solvent gradient mobile phase consisting of solvent A (0.1%

formic acid) and solvent B (50% water, 50% acetonitrile, 0.1% formic acid) running at a flow rate of 1 μL/min with 20 min run time.

Analysis of LC-MS/MS data

The data from LC-MS/MS were formatted, edited and analyzed by the DeCyder™ 2-D Differential Analysis Software (GE Healthcare) to discriminate proteins separated from plasma samples from CCA patients and control samples. Mascot software™ (Matrix Science Ltd.) was used to analyze the obtained peptide fingerprints. The putative function of each identified protein was predicted by the gene ontology categorization software GOCat (http://eagl.unige.ch/GOCat/). Significantly difference of proteins level in plasma samples of CCA patients compared with those of control samples were analyzed by t-test using MultiExperiment Viewer (MeV)™ of the TM4 microarray software suite[2] at a statistical significance level of $\alpha = 0.05$.

Results and Discussion

Proteins of plasma samples from CCA patients and control samples (20 samples each) were resolved by 12.5% SDS-PAGE and stained with silver (Figure 1). Proteins having molecular weights of less than 14 kDa were selected for further analysis by LC-MS/MS. Preliminary analysis of the separated proteins with molecular weights of less than 14 kDa by GEL-LC-MS/MS revealed 78 proteins, but only 6 proteins showed significant differences in their abundance between plasma samples from healthy subjects and CCA patients (Figure 2). These included selenocysteine insertion sequence-binding protein 2, class A basic helix-loop-helix protein 15, C-type lectin-like receptor-2 , HBF-3, contactin-2 precursor, and hypothetical protein LOC9813. Analysis of proteins of different molecular weights (> 14 kDa) is under way to identify additional potential biomarkers for CCA. Confirmation of protein expression by real time RT-PCR and/or Western blot analysis will be performed only with proteins which have been reported to be involved in carcinogenesis. Analysis of higher molecular weight proteins in plasma samples from both groups is under way to identify additional potential biomarkers. Efficiency (sensitivity, specificity) of these proteins to be exploited as potential biomarkers for diagnosis of CCA will also be evaluated by ELISA and/or Western blot analysis.

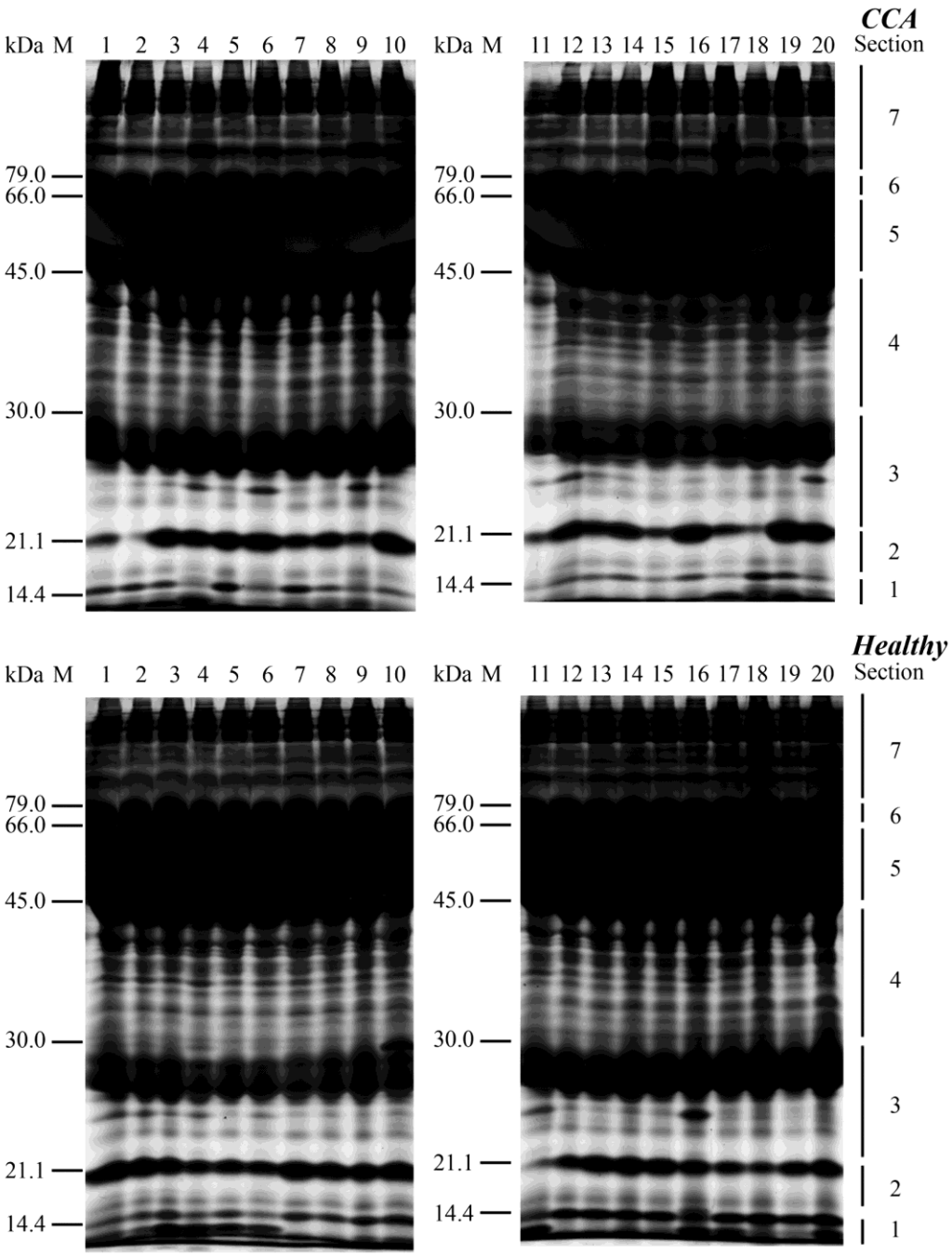

Figure 1 The 12.5% SDS-PAGE of CCA patients and healthy samples (20 samples each). The standard protein size was separated parallel with the serum protein and used as a landmark for cutting. The number (1-7) indicates the seven major sections. A single piece from section 1 was subjected for LC-MS/MS.

Figure 2 Analysis of LC-MS/MS data by the MeV^{TM} software. 1DR and 1NR indicate CCA patients and healthy subjects (control), respectively.

Conclusion

Six candidate biomarkers of CCA have been identified in plasma samples from patients with CCA by GEL-LC-MS/MS. The efficiency of these proteins as potential biomarkers for CCA will be further evaluated.

Acknowledgements

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RESEARCH ARTICLE

Associations of *CYP2C9*, *CYP2C19* Genetic Variants and Non-genetic Variants with Phenytoin Blood Concentrations in Thai Epileptic Patients

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Abstract

Purpose: This study aims to investigate the association of genetic variants in *CYP2C9, CYP2C19* and *ABCB1* genes along with non-genetic variants with phenytoin steady state blood concentrations in Thai patients with epilepsy.

Method: One hundred and eight Thai epileptic patients on phenytoin maintenance therapy were included in this study. Four candidate SNPs including *CYP2C9*3*(c.1075A>C), *CYP2C19*2* (c.681G>A), *CYP2C19*3*(c.636G>A), and ATP binding cassette subfamily B (*ABCB1* c.3435C>T) were genotyped. Stepwise multiple linear regression statistics was used to identify the association of phenytoin steady state blood concentrations with genetic and non-genetic variants.

Results: A stepwise multiple linear regression model revealed significant association of phenytoin blood concentrations with the presence of *CYP2C9*3* or *CYP2C19*2* allele, gender and body weight. The model explain 25.5% of the variability in phenytoin blood concentrations per dose (R^2=0.255, *P*=0.028).

Conclusion: This study suggests that genetic variants in *CYP2C9* and *CYP2C19* together with non-genetic variants (i.e. gender and body weight) influence variability in phenytoin steady state blood concentrations in Thai patients with epilepsy. This finding could be to use to evaluate the efficacy of phenytoin in treating epileptic patients.

Keywords *CYP2C9, CYP2C19*, non-genetic variants, phenytoin blood concentrations, Thai epileptic patients

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Introduction

Phenytoin is an effective and inexpensive first-line antiepileptic drug widely used for the treatment of partial and generalized epilepsy in Thailand. However, a wide range of phenytoin blood concentration was found in each individual. High phenytoin blood concentrations have been shown to be associated with several adverse drug reactions. Whereas, low blood concentrations might, in part, give rise to an ineffective treatment. Given its narrow therapeutic index and the danger of uncontrolled epilepsy, phenytoin blood concentration is one of an important determinant for the safety and efficacious uses of phenytoin.

There might be several genetic and non-genetic factors influencing phenytoin serum concentrations. Phenytoin is mainly metabolized by CYP2C9 (90%) and CYP2C19 (10%) [1] and also a substrate of an efflux transporter, P-glycoprotein (P-gp) [2]. Therefore, genetic variations in genes encoding these enzymes and transporter might play a crucial role in the pharmacokinetic processes of phenytoin and therefore give rise to its wide variations in blood concentrations.

In addition to genetic variations, non-genetic variations including patients' characteristics, inter-individual variations in phenytoin maintenance doses and co-medications which are common in clinical treatment of epilepsy might also affect phenytoin pharmacokinetics and therefore its blood concentrations.

Therefore, the purpose of the present study was to investigate the association of genetic variants in *CYP2C9, CYP2C19* and *ABCB1* genes along with non-genetic variants with phenytoin steady state blood concentrations in Thai patients with epilepsy and to quantify the association by using stepwise multiple linear regression models.

Methods

Subjects

Epileptic patients who have been treated and followed up at neurology unit of three public tertiary care hospitals in Thailand were recruited for this study. The study was approved by the ethic committees of the hospitals: Phramongkutklao Hospital, Police General Hospital, and Surin Hospital. A total of 120 patients with epilepsy were recruited after written informed consents were obtained. These patients had routinely taken oral phenytoin to control seizure and had a stable phenytoin dose, without change in co-medications for at least 2 months. Patients were excluded if they had hepatic or renal dysfunction and a history of non-compliance. Patients' medications were collected from OPD cards and by interviewing the patients or their caregivers. Blood samples were drawn from their cubital vein.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by using QIAamp DNA blood mini kit® (Qiagen, German). Four candidate SNPs including *CYP2C9*3* (c.1075A>C), *CYP2C19*2* (c.681G>A), *CYP2C19*3* (c.636G>A), and a SNP in ATP binding cassette subfamily B (*ABCB1* c.3435C>T) were genotyped by using Taqman SNP genotyping assay (Applied biosystems, USA).

Phenytoin blood concentrations determination

Serum phenytoinconcentrations were measured by using fluorescence polarize immunoassay (AxSYM System, Abbott, USA).

Statistical Analysis:

Bivariate correlation between phenytoin blood concentration and demographic data were evaluated by using non parametric Spearman-rank correlation. Comparison of blood concention across the different subgroups were performed by using non-parametric, Mann-Whitney or Kruskal Wallis test. Multivariate correlation was evaluated by using stepwise multiple linear regression to identify the association of phenytoin blood concentration with genetic and non-genetic variants. A *P* value of less than 0.05 was considered to be statistically significant. All analyses were performed with the SPSS program version 17.

Results

A total of 120 patients consented to participate in this study but only 108 patients met inclusion criteria and were included for the analysis. The patients' demographic and clinical characteristics are shown in Table 1. About half of the patients (62 patients [57.4%]) had co-medication as show in Table 2.

Table 1 Demographic and clinical characteristics of study populations

Characteristics	No., (Mean ± SD.)	%, (Range)
Total	108	
Gender		
Male	63	58.30
Female	45	41.70
Age (years)	(43.55 ± 14.57)	(19 - 77)
Weight (kgs)	(60.43 ± 11.47)	(40 - 94)
Phenytoin dose (mg/day)	(295.14 ± 47.42)	(100 - 400)
Phenytoin dose/weight (mg/day/kg)	(5.04 ± 1.17)	(1.67 - 7.50)
Phenytoin blood level (ug/ml)	(14.31 ± 8.20)	(1.23 - 46.28)

Table 2 The list of Co-medications

Comedications	No.	%
Folic	17	12.50
Valproate	13	9.56
Phenobarbital	12	8.82
Carbamazepine	10	7.35
Topiramate	6	4.41
Clonazepam	6	4.41
Aspirin	6	4.41
TCA	4	2.94
Clobazam	3	2.21
Other non-interacting drugs	59	43.38
Total	136	100.00

Table 3 Genotypes distribution of *CYP2C9, CYP2C19* and *ABCB1* genetic variants

SNPs	Genotypes	No.	%
***CYP2C9*3* (c.1075A>C)**	AA (*1/*1)	114	95.00
	AC (*1/*3)	6	5.00
	CC (*3/*3)	0	0.00
	total	120	
***CYP2C19*2* (c.681 G>A)**	GG (*1/*1)	62	51.67
	GA (*1/*2)	52	43.33
	AA (*2/*2)	6	5.00
	total	120	

SNPs	Genotypes	No.	%
***CYP2C19*3* (c.636 G>A)**	GG (*1/*1)	119	99.17
	GA (*1/*3)	1	0.83
	AA (*3/*3)	0	0.00
	total	120	
***ABCB1* (c.3435C>T)**	CC	39	32.50
	CT	57	47.50
	TT	24	20.00
	total	120	

Genotyping results of *CYP2C9*, *CYP2C19* and *ABCB1* genetic variants are shown in Table 3. All genotype frequencies were consistent with Hardy-Weinberg equilibrium (P>0.05, Chi-square test). The minor allele frequencies of *CYP2C9*3, CYP2C19*2, CYP2C19*3* and *ABCB1* 3435C>T were 0.025, 0.267, 0.004 and 0.44 respectively.

By using bivariate analysis, the Spearman-rank correlation revealed that phenytoin blood concentrations per dose was negatively correlated with body weight (r = -0.417, P <0.001). But there was no significant correlation between phenytoin blood concentrations per dose and age. There was a significant different in phenytoin blood concentrations per dose between male and female (P <0.001, Mann-Whitney Test) and between *CYP2C9*3* genotype (P = 0.018, Mann-Whitney Test). There was no significant different in phenytoin blood concentrations per dose between *CYP2C19*2* genotype (P = 0.173, Kruskal Wallis-Test) and *ABCB1* 3435C>T genotypes (P = 0.452, Kruskal Wallis-Test) (Figure 1 and Figure 2).

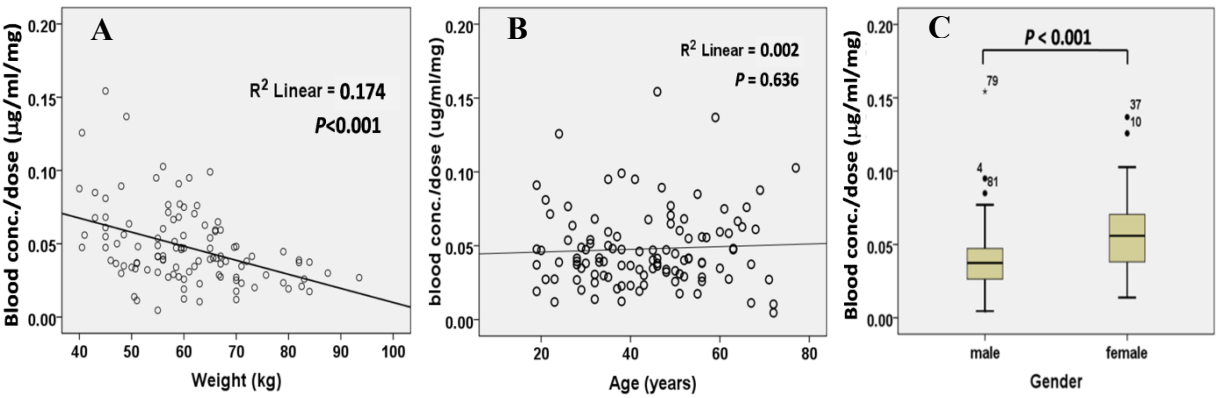

Figure 1 The bivariate relationship between phenytoin steady state blood concentrations per dose and Body weight (A), Age (B), and Gender (C)

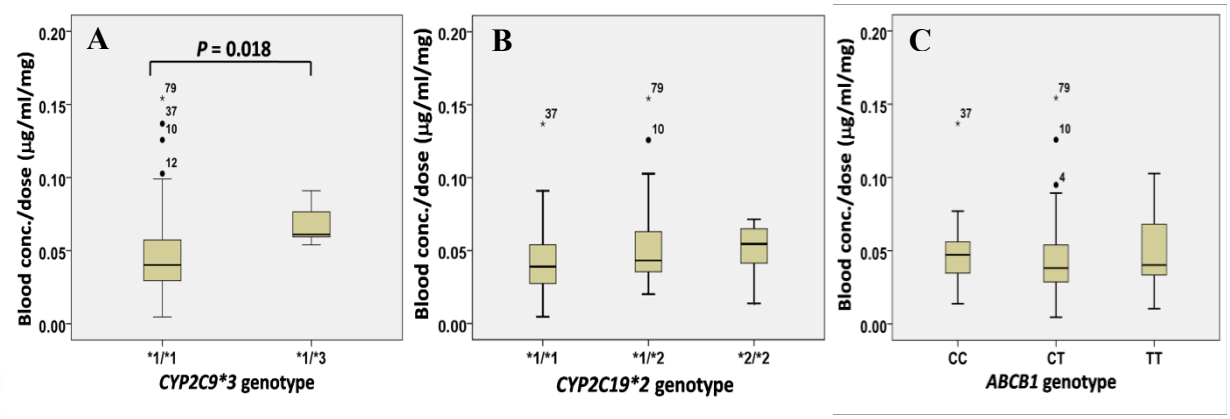

Figure 2 The relationship between phenytoin steady state blood concentrations per dose and the genotypes of *CYP2C9*3* (A), *CYP2C19*2* (B), and *ABCB1* (C)

To perform multivariate analysis, phenytoin steady state blood concentrations per dose were logarhithmically transformed to achieved normal distribution. A multiple linear regression models revealed a significant association of log phenytoin steady state blood concentrations per dose with gender , body weight, and *CYP2C19*1/*2* and *CYP2C9*1/*3* genotypes as shown in Table 4. The model explains 25.5% of the variability in log phenytoin steady state blood concentrations per dose (R^2 = 0.255, *P* = 0.028).

Table 4 Stepwise multiple linear regression model for the association of log phenytoin steady state blood concentrations per dose with genetic and non-genetic variants

Factor	Unstandardized Coefficient (B)	*P*-value	95%CI	R^2 for model	*P*-value
(Constant)	-1.176	0	[-1.444 ~ -0.908]	0.255	0.028
Gender (female)	0.143	0.003	[0.050 ~ 0.236]		
*CYP2C19*1/*2*	0.12	0.005	[0.037 ~ 0.203]		
Weight (kg)	-0.005	0.008	[-0.10 ~ -0.001]		
*CYP2C9*1/*3*	0.219	0.028	[0.024 ~ 0.415]		

Discussion

By using stepwise multiple linear regression models, we found that carriers of *CYP2C19*2* and *CYP2C9*3* allele were associated with higher log phenytoin blood concentrations per dose than its corresponding reference wild-type group. In contrast to previous studies [3-4], female gender was associated with increase log phenytoin blood concentrations per dose. This might be, in part, the result of the use of oral contraceptive drugs which significantly inhibit CYP2C19 activity [5-6]. Whereas, the negative correlation of log phenytoin steady state blood concentrations per dose with body weight is predictable because of the increase of the apparent volume of distribution of drug in proportion to body weight. Co-medication was not significantly associated with log phenytoin steady state blood concentrations per dose in this study.

Though blood samples were taken during the daily routine at the variable time points, 2-25 hours after last dose, the peak to trough level fluctuations of phenytoin during steady state are low due to its slow absorption and long elimination half life [7-8]. In addition, the Spearman-rank correlation and stepwise multiple linear regression analysis of the data revealed that phenytoin blood concentrations per dose was not significantly associated with time interval from last dose to blood sampling (P>0.05).

Conclusion

Our study suggests the contribution of *CYP2C9* and *CYP2C19* genotypes and non-genetic factors including gender and body weight to interindividual variability in phenytoin steady state blood concentrations per dose in Thai epileptic patients. However, these variables could explain about 25.5% of the variability in phenytoin steady state blood concentrations per dose.

Acknowledgements

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RESEARCH ARTICLE

Screening of antimalarial activities of Thai medicinal plants

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Abstract

Malaria remains one of the most serious causes of mortality and morbidity in the world. The problem of multidrug resistant *Plasmodium falciparum* has been aggravating particularly in Southeast Asia. Therefore, development of new potential antimalarial drugs is urgently required. In the present study, we assessed the *in vitro* antimalarial activity of the ethanolic extracts of the thirty Thai medicinal plants/herbal formulations against chloroquine (CQ)-resistant (K1) and CQ-sensitive (3D7) clones of *P. falciparum.* Seventeen ethanolic extracts showed promising antimalarial activity against both clones with parasite survival of less than 50% at the concentration of 50 µg/ml. The extracts from the eight plants (DiMEt, DrLEt, KaGEt, MyFEt, PiCEt, PlIEt, BJK1R and GaMEt) were found to possess potent antimalarial activity with IC_{50} of less than 10 µg/ml for both K1 and 3D7 clones.

Keywords *Plasmodium falciparum*, Thai herbal medicine, drug resistance

Introduction

Malaria is widespread in tropical and subtropical regions (1). The emergence and spread of multidrug-resistant *Plasmodium falciparum* has become problematic in undermining malaria control programs in most endemic regions of the world. Since antimalarial drug resistance compromises the effective treatment of the disease, there is a pressing need for ongoing drug discovery research that will provide effective and affordable antimalarial agents. Several approaches have been employed in searching for new antimalarial drugs. Research on natural-product-derived compounds is now being a focus as the key features of the natural products are their enormous structural and chemical diversities. Thailand encompasses a wide range of tropical habitats, and is a country rich in bio-resources. The objective of the research proposal was to search for new promising antimalarial drugs from natural. A total of 30 Thai medicinal plants/formulations were initially screened for their antimalarial activity *in vitro*. The extracts which showed promising activity were further investigated for their possible protein targets of action by proteomics approach.

Methods

Plant materials

Plant materials from a total of 25 medicinal plants and 5 herbal formulations (Table 1) were collected from various parts of Thailand and some were purchased from the city market. Stock solutions of the ethanolic extracts were prepared at concentration of 1 mg/ml.

Parasite culture

CQ-sensitive (3D7) and CQ-resistant (K1) clones of *P. falciparum* were maintained in continuous culture in O^+ human erythrocytes suspended in RPMI 1640 culture medium (at 37°C under a gas mixture of 5% CO_2, 5% O_2, and 90% N_2) according to the standard method described by Trager and Jensen (2). The parasite culture was treated with 5% sorbitol in order to obtain the ring stage of *P. falciparum* (3).

Assessment of antimalrial activity *in vitro*

Antimalarial activity screening of the twenty-five medicinal plants and five herbal formulations were performed in a 96-well microtiter plate using a SYBR green-I-based assay (4-5). All assays were performed using 2% parasitemia and 1% hematocrit. Each extract was initially screened for antimalarial activity against 3D7 and K1 clones of *P. falciparum* at the concentration of 50 µg/ml. Antimalarial activity was expressed as percentage of parasite survival as follow:

$$\text{Parasite survival (\%)} = \frac{\text{OD of treated wells}}{\text{OD of control wells}} \times 100$$

Table 1 Percentage survival of *Plasmodium falciparum* 3D7 and K1 clones following exposure to the crude ethanolic extracts of a total of 25 plants and 5 herbal formulations at the concentration of 50 µg/ml

		Median (range) percentage values of parasite survival	
	Ethanolic extracts	3D7	K1
1	*Amomum testaceum*	75.4 (85.0-54.1)	91.5 (98.0-78.3)
2	*Anethum graveolens*	100.9 (101.6-91.4)	106.1 (116.2-83.5)
3	*Angelica dahurica*	70.0 (85.5-63.7)	84.8 (90.0-80.1)
4	ArAEt	1.5 (5.4-0.3)	3.1 (4.2-0.0)
5	*Asclepias curassavica*	95.4 (108.4-92.0)	101.4 (103.7-98.1)
6	AtLEt	5.5 (6.9-5.4)	2.9 (4.3-0.5)
7	BJK1R	3.8 (6.8-0.5)	2.8 (5.3-0.3)
8	Ben-ja-Kul 2 Recipe	1.5 (75.7-0.0)	68.9 (89.8-11.6)
9	CuCEt	21.7 (59.8-8.0)	28.9 (42.8-14.1)
10	DiMEt	0.0 (3.9-0.0)	0.0 (0.1-0.0)
11	DiMEt & SMCEt	53.1 (59.7-49.2)	39.6 (46.2-24.7)
12	DrLEt	7.5 (10.6-0.4)	4.0 (9.2-2.2)
13	*Foeniculum vulgare*	106.4 (109.8-91.2)	98.5 (109.1-96.4)
14	GaMEt	10.6 (18.4-7.3)	10.1 (11.1-3.9)
15	KaGEt	0.0 (4.1-0.0)	1.7 (4.3-1.5)
16	*Mammea siamensis*	78.9 (87.1-63.6)	73.5 (73.9-68.9)
17	MeFEt	0.0 (3.7-0.0)	1.6 (3.3-0.0)
18	*Mimusops elengi*	67.6 (79.6-56.6)	67.9 (70.4-45.4)
19	MyFEt	0.0(0.7- 0.0)	0.8 (3.4-0.6)
20	*Nigella sativa*	103.2 (133.4-73.5)	100.2 (108.0-86.5)
21	PiCEt	0.0 (0.4-0.0)	0.0 (0.1-0.0)
22	PiPEt	6.4 (13.6-6.0)	12.7 (14.6-8.4)
23	*Piper sarmentosum*	77.7 (90.0-74.1)	86.3 (88.2-78.5)
24	PlIEt	0.0 (0.1-0.0)	0.0 (0.1-0.0)
25	PSPYR	19.7 (21.8-5.6)	6.5 (44.16-6.03)
26	*Smilax corbularia*	127.1 (157.4-110.7)	113.8 (115.7-87.8)
27	*Syzygium aromaticum*	69.9 (74.4-59.8)	68.4 (71.5-65.0)
28	Tein-5 Recipe	107.1 (117.2-97.5)	104.2 (111.8-95.8)
29	ZiLEt	15.3 (23.1-12.0)	9.0 (20.3-7.7)
30	ZiOEt	13.7 (16.3-11.0)	11.0 (30.3-7.9)

Data are presented as median (range) from 3 independent experiments (triplicate each)

Results and Discussion

Results from the initial screening demonstrated that the extracts of seventeen out of thirty medicinal plants/herbal formulations showed promising activity against both K1 and 3D7 clones of *P. falciparum* with parasite survival of less than 50% at the concentration of 50 µg/ml (Table 1). Among these, the six plants showed potent antimalarial activity with IC_{50} of less than 10 µg/ml for both K1 and 3D7 clones (DiMEt, DrLEt, KaGEt, MyFEt, PiCEt and PlIEt). The extract from BJK1R and GaMEt showed IC_{50} of less than 10 µg/ml against only K1 strain, but the IC_{50} in 3D7 clone exceeded 10 µg/ml. Further investigation of all the potential candidates for their cytotoxic activity in normal human cells is underway.

Conclusion

The eight ethanolic extracts (DiMEt, DrLEt, KaGEt, MyFEt, PiCEt, PlIEt, BJK1R and GaMEt) of Thai medicine plants/herbal formulations exhibited promising antimalarial activity against both CQ-resistant (K1) and CQ-sensitive (3D7) *P. falciparum* strains.

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RESEARCH ARTICLE

Association between *pfatp6* and *pfmdr1* Polymorphisms and Treatment Response to Artesunate-Mefloquine Combination Regimen in Patients with Acute Uncomplicated *Plasmodium falciparum* Malaria in Thailand

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Abstract

The aim of the study was to investigate the association between genetic polymorphisms of multi-drug resistance 1 (*pfmdr1)* and sarco/endoplasmic reticulum Ca^{2+}- ATPase (*pfatp6*) of *Plasmodium falciparum*, and *in vitro* sensitivity in 226 *P. falciparum* isolates. Polymorphism of *pfatp6* at codons R37K, G639D, S769N and I898I, and of *pfmdr1* at codons N86Y, N1042D, D1246Y were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Copy numbers of *pfatp6* and *pfmdr1* gene of all isolates were analyzed by quantitative real time-polymerase chain reaction (qRT-PCR). Marked decline in sensitivity to artesunate (mean $\pm$ SD IC_{50} 3.66$\pm$0.46 nM) was observed in nine isolates. All carried wild-type alleles of *pfatp6* and *pfmdr1*; only one isolate carried mutation at codon 1042. Analysis of *pfmdr1* copy number showed a single gene copy in 74 out of 102 isolates, whereas 12, 5, 8 and 3 isolates carried 2, 3, 4, and $\geq$ 5 gene copies, respectively. All of the 14 isolates selected based on clinical response to artesunate and mefloquine combination carried only a single copy of the *pfatp6* gene. Interestingly, the increase of *pfmdr1* copy number was markedly present in isolates collected from all (13/13 cases) patients with treatment failure. No association between SNP and amplification of *pfatp6* gene and *in vitro* sensitivity of *P. falciparum* isolates to artesunate and mefloquine was found.

Keywords *Plasmodium falciparum*, *pfmdr1, pfatp6*, single-nucleotide polymorphism, gene amplification, artemisinin, drug resistance, PCR-RFLP

Introduction

Multidrug resistance is still the one of the major obstacles to effective malaria treatment and control (1). Due to the worldwide spread of *Plasmodium falciparum* resistant to chloroquine and sulphadoxine-pyrimethamine, the World Health Organization (WHO) recommends the use of artemisinin-based combination therapy (ACT) as first-line malaria treatment. Artemisinin and its derivatives rapidly reduce clinical symptoms and parasite burden. A 3-day artesunate (ARS)-mefloquine (MQ) combination is currently being used as the first-line treatment of uncomplicated falciparum malaria to cope with the situation of multi-drug resistance (1). Recently however, reports from Cambodia identified parasites with slightly reduced in vitro sensitivity, but also showed significant prolongation of parasite clearance times after treatment with artesunate (2). The mechanism of decreased susceptibility of malaria parasites to artemisinins remains unclear. Gene amplification or specific point mutations in codons 86, 184, 1034, 1042 and 1246 of the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) have been associated with alterations in artemisinin sensitivity and also lead to diminished response to some other antimalarials, including mefloquine and lumefantrine. The alteration of artemisinin sensitivity has been proposed to involved with the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA), PfATP6. Recent evidences reveal that polymorphisms (single nucleotide polymorphism: SNP and gene amplification) in this gene at codon S769N have led to decrease *in vitro* sensitivity to artemisinins. In addition, amplification of this gene has also been linked with both artesunate and mefloquine resistance (3). The assessment of clinical response, *in vitro* sensitivity test together with molecular surveillance system can help target *in vivo* studies to where they are needed the most. Polymorphisms of genes involved in vial process of malaria parasite are suggested as the key factor contributing to drug resistance. In the present study, we investigated the association between *in vitro* sensitivity to artesunate and mefloquine, and the polymorphisms of *pfatp6* and *pfmdr1* gene in a total of 226 *P. falciparum* isolates collected from an area along the Thai-Myanmar border during 2007-2009.

Methods

Venous blood samples (3 ml) were collected from patients attending malaria clinics in areas along the Thai-Myanmar border after microscopically confirmation of *P. falciparum* mono-infection. Approval of the study protocol was obtained from the Ethics Committee of Ministry of Public Health of Thailand. Written informed consents were obtained from all patients prior to blood collection. *P. falciparum* isolates were adapted to culture *in vitro* (4) for sensitivity testing to artesunate and mefloquine, as well as for investigation of single nucleotide polymorphism (SNP) and amplification of the *pfatp6* and *pfmdr1* gene. *In vitro* sensitivity testing (3 independent experiments, triplicate each) of all isolates to artesunate (concentration range 0.39-50 nM), and mefloquine (concentration range 1.56-200 nM) was performed in a 96-well microtiter pate based on SYBR green I-based Assay (5), in order to obtain the IC_{50} values (concentrations that produce 50% inhibition of parasite

growth). SNPs of *pfatp6* gene at codon S769N, R37K, G639D and I898I and *pfmdr1* at codon N86Y, N1042D and D1246Y were examined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (6-8). *Pfatp6* and *pfmdr1* gene copy numbers of all isolates were analyzed by quantitative real time-polymerase chain reaction (qRT-PCR) (9).

Results

In vitro Sensitivity Testing

In vitro sensitivity test was successful in a total of 50 isolates out of 226 *P. falciparum* . Mean ± SD IC_{50} values for artesunate and mefloquine were 2.21±0.99 and 43.12±26.12 nM, respectively. All were classified as sensitive to artesunate and 68% (34/50) were classified as resistance to mefloquine (IC_{50} >24 nM) (14). There were 9 isolates with marked decline in sensitivity to artesunate (mean $\pm$ SD IC_{50} =3.66$\pm$0.46 nM); mean$\pm$SD IC_{50} of 26 and 15 isolates were between 1.42$\pm$0.27, and 2.50$\pm$0.28 nM, respectively.

SNPs and Amplification of pfatp6 and pfmdr1:

All isolates carried wild-type allele *pfatp6* (Table 1) and *pfmdr1*(Table 2), there are two isolates carried mutation at codon 86 (86Y) and only one isolate carried mutation at codon 1042 (1042D). All of the fourteen isolates selected based on clinical response to artesunate and mefloquine combination carried only a single copy of the *pfatp6* gene. Interestingly, the analysis of *pfmdr1* copy number showed a single gene copy in 74 (72.5%) out of 102 isolates, whereas 12 (11.8%), 5(4.9%), 8(7.8%) and 3(2.9%) isolates carried 2, 3, 4, and $\geq$ 5 gene copies, respectively. Moreover, the increase of *pfmdr1* copy number was markedly present in isolates collected from all (13/13 cases) patients with treatment failure.

Table 1 Prevalence of SNP in *pfatp6* gene

Gene	***pfatp6* Codon**							
Target allele	**37R**	**37K**	**693G**	**693D**	**769S**	**769N**	**898I**	**898I**
%Prevalence (*N*)	100(63)	-	100(214)	-	100(93)	-	100(143)	-

Table 2 Prevalence of SNP in *pfmdr1* gene

Gene	***Pfmdr1* Codon**					
Target allele	**86N**	**86Y**	**1042N**	**1042D**	**1246D**	**1246Y**
%Prevalence (*N*)	98.9(184)	1.1(2)	99.5(220)	0.5(1)	100(210)	-

Table 3 Association between IC_{50} values of antimalarial drugs and *pfmdr1* copy number

Drug	Single *pfmdr1* copy number	Increased *pfmdr1* copy number
	IC_{50} (nM) (*N*)	IC_{50} (nM) (*N*)
Mefloquine	27.61(9.05-65.87) (*14*)	51.41(12.09-100.48) (*21*)
Artesunate	1.9(1.29-5.32) (*14*)	2.27(1.01-4.33) (*20*)

Pfmdr1 copy number and in-vitro drug sensitivity

Increased *pfmdr1* copy number was associated with reduced antimalarial drug susceptibility; parasites with an increased copy number show higher median IC_{50} values for mefloquine and artesunate (Table 3).

Discussion

Results of the present study indicate the increase in prevalence of mefloquine resistant *P. falciparum* isolates (68%) collected from the multi-drug resistance area of Thailand compared with the isolates collected during 1998-2005 (32%) (10) and 2007 (46%) (11). Sensitivity to artesunate is also gradually declining with about 14.3% (9 isolates) exhibiting IC_{50} of greater than 3 nM. The IC_{50} range observed for artesunate in this study is similar to that reported in Africa (12). Nevertheless, at the molecular level, no genetic changes (SNP and amplification) in the *pfatp6* and *pfmdr1* gene which is proposed to be linked with resistance of artemisinins or mefloquine were found. Interestingly, increased *pfmdr1* copy number was associated with the reduced of susceptibility to mefloquine and linked to the result of treatment failure to artesunate-mefloquine combination regimen (13). It is not clear that the association between *pfmdr1* copy number and artesunate-mefloquine combination treatment failure is mediated by resistance to mefloquine alone or in combination with decreased sensitivity to artesunate.

Acknowledgements

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RESEARCH ARTICLE

Cytotoxic, Anti-oxidant, Apoptosis and Multi-drug Resistant Gene Inducing Activities of *Zingiber officinale* Roscoe

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Abstract

The aim of the study was to investigate cytotoxicity, anti-oxidant, apoptosis and multi-drug resistant gene inducing activities of the crude ethanolic extract of ginger (*Zingiber officinale* Roscoe) against cholangiocarcinoma (CCA). Cytotoxic activity against CCA cell line (CL-6) was assessed by calcein-AM and Hoechst 33342 assay and its anti-oxidant activity was evaluated by DPPH assay. Median IC_{50} values for cytotoxicity and anti-oxidant activities were 10.95, 53.15 and 27.86 μg/ml, respectively. Investigation of the apoptotic activity was performed by DNA fragmentation assay and induction of genes that may be involved in resistance of CCA to anticancer drugs (MDR-1, MRP-1, MRP-2 and MRP-3) were characterized by real-time PCR. More than ten DNA fragments were visualized and up to 7-9 fold up-regulation of MDR-1 and MRP-3 genes were observed following exposure to ethanolic extract of ginger. Results from *in vitro* studies indicate promising anticancer activity of the crude ethanolic extract of ginger against CCA. Nevertheless, MDR1 and MRP3 may be involved in conferring resistance of CCA to the ginger extract.

Keywords cholangiocarcinoma, cytotoxicity, The ethanolic extract of ginger

Introduction

Cholangiocarcinoma (CCA) is an uncommon adenocarcinoma which arises from the epithelial cells of bile ducts anywhere along intrahepatic and extrahepatic biliary tree excluding the papilla of Vater and the gall bladder (1). *Opisthorchis viverrini* (OV) infection is a high risk factor of cholangiocarcinoma (CCA) (2). Thai medicinal plants have been increasingly applied as an alternative treatment for various diseases particularly cancer. The rhizome of *Zingiber offinale* Roscoe is widely used as a dietary condiment throughout the world, especially, in traditional oriental medicine to ameliorate such symptoms as inflammation, rheumatic disorders, gastrointestinal discomforts, loss of appetite, travel sickness, hypercholesteremia and high level of triglyceride (3). Several lines of evidence suggest that 6-gingerol, which is one of the active ingredient of ginger, is effective in the suppression of the transformation, hyperproliferation, and inflammatory processes that initiate and promote carcinogenesis, as well as the later steps of carcinogenesis, the angiogenesis and metastasis (4-8). Our previous study has demonstrated promising cytotoxic activity of the ethanolic extract of ginger against CL-6 (CCA cell line obtained from human), HepG2 (hepatocarcinoma) and Hep-2 (laryngeal carcinoma) cell lines *in vitro* with IC_{50} (concentration that inhibits cell growth by 50%) of less than 50 µg/ml (9). The aim of the present study was to further investigate the cytotoxic activity of crude ethanolic extract of ginger in other *in vitro* models (calcein-AM release and Hoeschst 33342 assays), as well as its anti-oxidant activity, apoptotic activity, and activity on inducing the expression of multidrug resistance genes.

Methods

Cytotoxicity assays

CL-6, HepG2 and HRE cells were plated in 96-well culture plates (1×10^4 cells/well). Calcein-AM release and Hoechst assays were performed according to the methods of Neri *et al* (10) and Schoonen *et al* (11).

Anti-oxidant activity

The method was evaluated using the colorimetric free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (12). Vitamin C (ascorbic acid) was used as a positive control reagent.

Apoptosis activity

DNA fragmentation was used to determine apoptosis by electrophoresis of DNA (13). Negative control wells consisted of untreated and cells similarly treated with 50% ethanol. 100 bp plus DNA ladder was used as a marker.

Induction of the expression of multidrug resistant genes

CL-6, HepG2 and HRE cells (1×10^6) were treated with the ethanolic extract of ginger at concentrations of 25, 50 and 100 µg/µl for 24 and 48 hours. Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified spectrophotometrically by Nanodrop machine. First-strand cDNA was synthesized from 100 ng total RNA by reverse transcription using oligo-dT primers and reverse transcriptase (Superscript III; Invitrogen, USA) according to the manufacturer's instructions. Relative quantitation of gene expression was measured by real-time PCR. Five sets of primers were used in all reactions to obtain

amplification of a house keeping genes control, GAPDH and a specific target gene of interest, multidrug resistance 1 (MDR1), multidrug resistance protein (MRP1, MRP2 and MRP3) genes. The fluorescence threshold (Ct) was calculated from $2^{-\Delta\Delta CT}$. The absence of non-specific products was confirmed by the analysis of the melting-point curves.

Results

In vitro models for assessing cytotoxic, anti-oxidant and apoptotic activities

Cytotoxic activity

The cytotoxic activity of the ethanolic extract of ginger was investigated against the human CCA cell line CL-6, in comparison with HepG2 and HRE cell lines. The IC_{50} [median (range)] and selectivity index (SI) values of the extract in three cell lines based on the calcein-AM and Hoechst assays are presented in Table 1.

Anti-oxidant activity

The *in vitro* DPPH assay demonstrated comparable anti-oxidant activity of the crude ethanolic extracts of ginger with ascorbic acid (positive control), with IC_{50} [median (range)] values of 27.86 (27.05-28.03) and 21.38 (21.25-21.49) µg/ml, respectively.

Apoptotic activity

Agarose gel electrophoresis showed more than ten DNA fragments were visualized and the activity was concentration dependent as the proportion of apoptotic cells increased with higher concentrations of the extract.

Table 1 *In vitro* cytotoxic activity [median (range) values] and selectivity index (SI) of the crude ethanolic extract compared with 5-FU against CL-6, HepG2 and HRE cell lines. Data are presented as median (range) values.

Cell line	Cytotoxicity assay	Potency/selectivity	Ethanolic extract of ginger	5-FU
CL-6	Calcein-AM	IC_{50} (µg/ml)	10.95 (10.87-11.12)	89.87 (89.57-90.84)
		SI	18.09	3.19
	Hoechst33342	IC_{50} (µg/ml)	53.13 (48.25-55.13)	95.29 (92.84-98.24)
		SI	4.63	3.12
HepG2	Calcein-AM	IC_{50} (µg/ml)	71.89 (69.88-73.14)	74.86 (73.42-77.96)
		SI	2.76	3.83
	Hoechst33342	IC_{50} (µg/ml)	92.88 (87.15-94.26)	118.60 (115.67-120.19)
		SI	2.68	2.51
HRE	Calcein-AM	IC_{50} (µg/ml)	198.15 (196.99-205.67)	286.74 (275.78-286.74)
		SI	1	1
	Hoechst33342	IC_{50} (µg/ml)	245.91 (234.87-250.17)	297.39 (289.57-311.87)
		SI	1	1

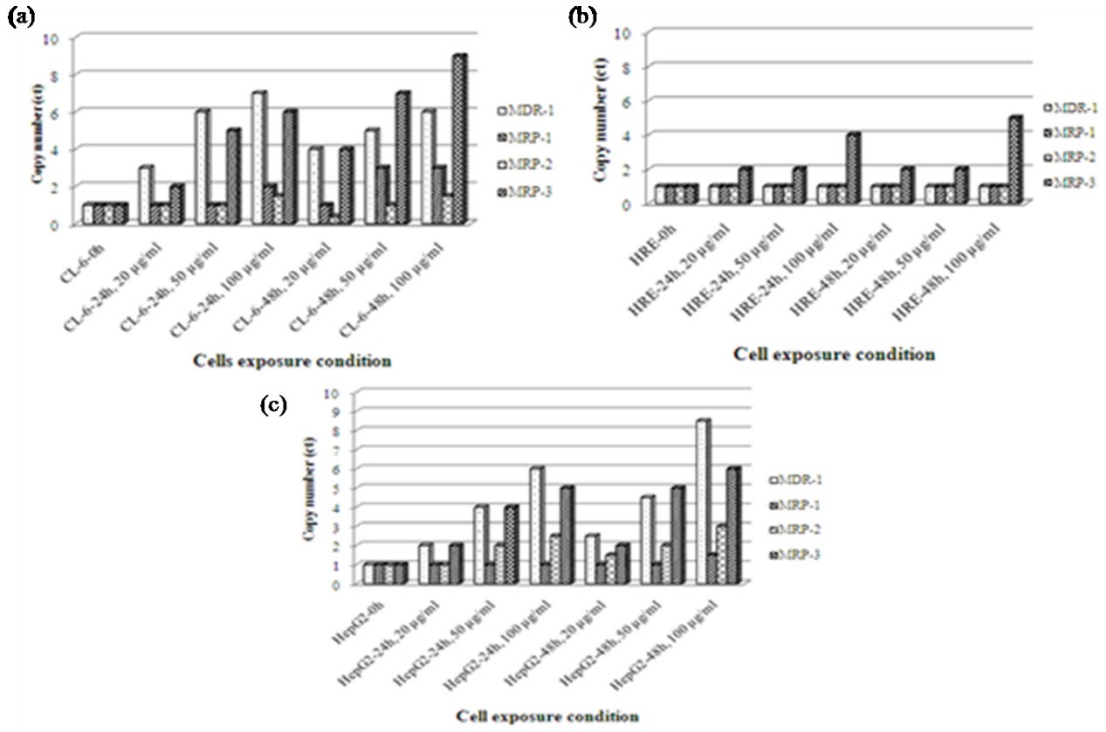

Figure 1 Expression (gene copy number compared with baseline) of multidrug resistance genes MDR1, MRP1, MRP2 and MRP3 following 24 and 48 hours exposure of (a) CL-6, (b) Hep-G2 and (c) HRE cell lines, to the ethanolic extract of ginger at concentrations of 20, 50 and 100 µg/ml.

Induction of the expression of multidrug resistant genes

A quantitative analysis showed that the copy number of MDR-1 and MRP-3 genes were found to be 7-9 fold up-regulated following exposure to the extract compared with the non-exposed CL-6 cell at time zero (Figure 1). Compared with the non-exposed cells at time zero, the expression of MDR1 and MRP3 in HepG2 cells was 5-8 fold up-regulated following exposure to the extract, whereas for HRE cells, only MRP3 was found to be about 5 fold up-regulated.

Discussion and Conclusion

The results from the present study suggest that the crude ethanolic extract of ginger exhibited *in vitro* cytotoxic, anti-oxidant and apoptotic activities. Nevertheless, the inducing activity of the extract on the expression of drug resistant genes is of major concern. The molecular and cellular mechanisms of action and resistance of the active principles 6-gingerol should be further investigated.

Acknowledgements

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RESEARCH ARTICLE

Analgesic Efficacy of Diclofenac and Ibuprofen in Patients with Pain after Third Molar Surgery

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Abstract

This study aimed to collect the information on the efficacy, onset of action, and duration of action of a single dose of 100 mg of diclofenac compared to 400 mg of ibuprofen for pain relief in patients after impacted third molar surgery. This randomized double-blind clinical trial was performed in sixty Laotian patients undergoing lower third molar removal. Patients were divided into two groups and randomly assigned to treat with either 100 mg of diclofenac or 400mg of ibuprofen. Patients rated their pain intensity (PI) by using visual analog scale (VAS) for 12 h. They started to take medication when they had at least moderate to severe pain. The onset of action was determined by means of a 2-stopwatch method. The duration of action was determined by the time that patients requested the rescue medication. The onset of analgesia for meaningful pain relief (MPR) for ibuprofen (64 ± 26.1minutes) was faster than diclofenac (83 ± 21.1minutes) ($p<.05$). The duration of analgesia for diclofenac and ibuprofen were 406 ± 54.19 minutes and 422 ±44.3 minutes respectively. There were no statistically significant differences in duration of analgesia between diclofenac and ibuprofen groups ($p>.05$). Patients reported no significant differences in analgesic efficacy between 100 mg of diclofenac and 400 mg of ibuprofen. The duration and pain relief of 100 mg of diclofenac and 400 mg of ibuprofen to control pain after impacted third molar surgery were found to be comparable. The onset of analgesia for ibuprofen was faster than diclofenac.

Key Words Pain, Diclofenac, Ibuprofen, Third Molar Surgery

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Introduction

Postoperative pain management after removal of third molar teeth may be achieved by the use of non-steroidal anti-inflammatory drugs (NSAIDS), opioids, or a combination thereof (1,2,3). Ibuprofen and diclofenac are commonly used to control pain in various countries. In Laos, we use diclofenac, but Thai dentists prescribe ibuprofen for patients after third molar surgery. The postoperative period is characterized by pain, trismus and inflammation as the most frequent complications. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective for the management of mild to moderate postoperative pain , and are often used in combination with opioids for the treatment of more severe pain .

This study aimed to assess the analgesic efficacy, onset of action and duration of action of a single dose of 100 mg of diclofenac compared to 400 mg of ibuprofen for pain relief in patients having removal of third molar teeth with moderate to severe pain.

Materials & methods

Sixty adult patients in Laos who needed to have third molar teeth removed under local anesthesia were invited to participate in the study. This study was performed at the Department of Oral Surgery, Faculty of Dentistry, Health Science University, Vientiane Laos P.D.R .The approval of the Regional Ethics committee for the Research, Faculty of Dentistry, Health Science University, was obtained and all patients gave written informed consent to participate in this study. They were randomly assigned to be given either 400 mg of ibuprofen or 100 mg of diclofenac to control pain after surgery. Before the operation, each patient was shown a visual analogue scale (VAS) and given an explanation about how this would be used to measure pain at various times after surgery. The VAS was a 100 mm horizontal scale (0 = no pain; 100 = worst pain possible) and the assessment was made for 12 hours postoperatively. They started to take medication when they had at least moderate to severe pain. The onset of action was determined by means of a 2-stopwatch method. The duration of action was determined by the time that patients requested rescue medication. We used the Statistical Package for the Social Sciences (SPSS Inc., version 16) to analyze the data obtained in this study. The onset and duration were compared between the two groups using the t- test. A significance level of 0.05 was used in all analyses.

Results

Of the 60 patients divided into two groups, 30 (50%) were in the ibuprofen group, 30 (50%) were in the diclofenac group. There was no significant difference in the baseline pain score, operation time, age, height or weight among the two groups. Details of the data were showed in Table 1 and Table 2. The onset of action and the duration of action of the medication are shown in Table 3, Fig 1 and Table 4, Fig 2 respectively. The onset of analgesia for meaningful pain relief (MPR) for ibuprofen (64 ± 26.1minutes) was faster than for diclofenac (83 ± 21.1minutes) ($p<.05$). The duration of analgesia for diclofenac and ibuprofen were 406 ± 54.19 minutes and 422 ±44.3minutes respectively. There were no statistically significant differences in duration of analgesia between the diclofenac and ibuprofen groups ($p>.05$).

Table 1 Show the characteristic of impacted lower third molar teeth in each group.

Characteristic of Impacted Teeth	**Ibuprofen (n=30)**	**Diclofenac (n=30)**
Impacted teeth		
38	14	19
48	16	11
Position		
A	0	1
B	29	29
C	1	0
Classification		
Class I	0	0
Class II	30	30
Class III	0	0
Angulation		
Vertical angulation	2	5
Mesio-angulation	17	16
Disto-angulation	0	3
Horizontal angulation	11	6

Table 2 Show demographic and baseline characteristic of the 2 groups of patients

Demographic Characteristics	Ibuprofen n=30	Diclofenac n=30
Age		
Mean ± SD	26 ± 5	25 ± 4.61
Sex		
male	9 (30%)	12 (40%)
female	21 (70%)	18 (60%)
Weight ± SD (kg)	54 ± 9.22	59 ± 8.69
Height ± SD (cm)	159 ± 6.44	161 ± 8.35
Baseline pain scores	35.8± 5,6	34.5± 4.9
Operation time (min)	25 ± 6.83	24 ± 6.22

Table 3 The onset of analgesia for meaningful pain relief (MPR) of patients in each group.

Onset of action (min)	Ibuprofen (400mg)	Diclofenac (100mg)
Mean± SD	64 ± 26.1	83 ± 21.1
Minimum	30	45
Maximum	120	120

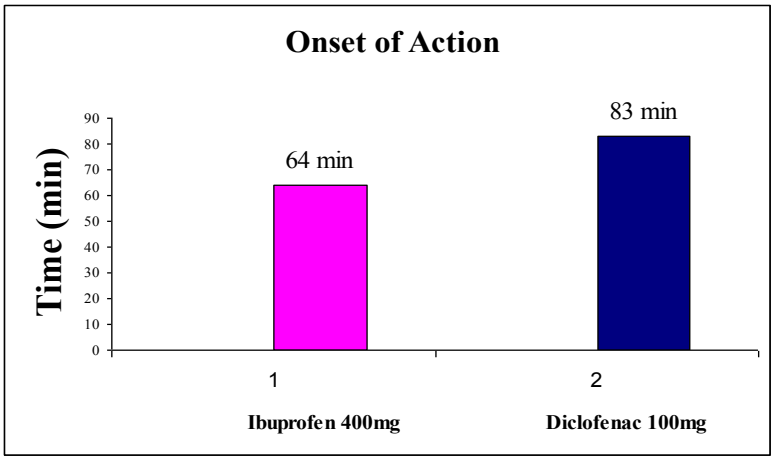

Figure 1 Show onset of action of two analgesics.

Table 4 Show the duration of action for ibuprofen and diclofenac for control pain after impacted third molar removal.

Duration of action	Ibuprofen 400mg (min)	Diclofenac100mg (min)
Mean ± SD Minimum Maximum	422 ± 44.3 310 530	406 ± 54.19 330 505

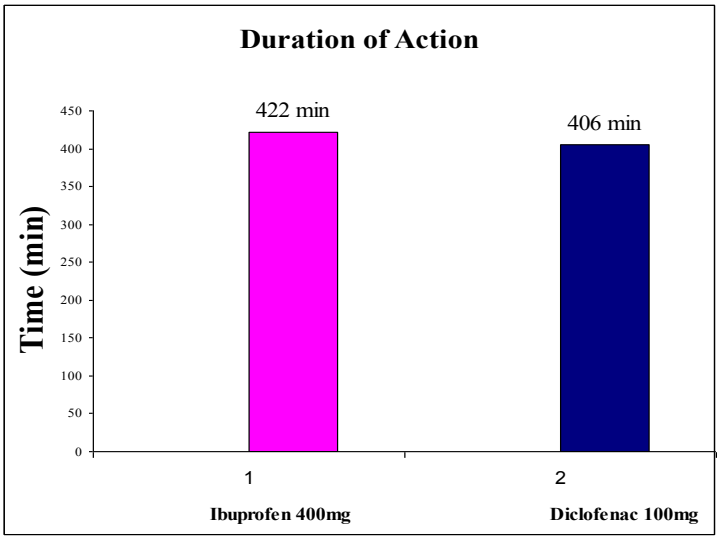

Figure 2 Show the duration of action of ibuprofen and diclofenac for control pain after impacted third molar removal.

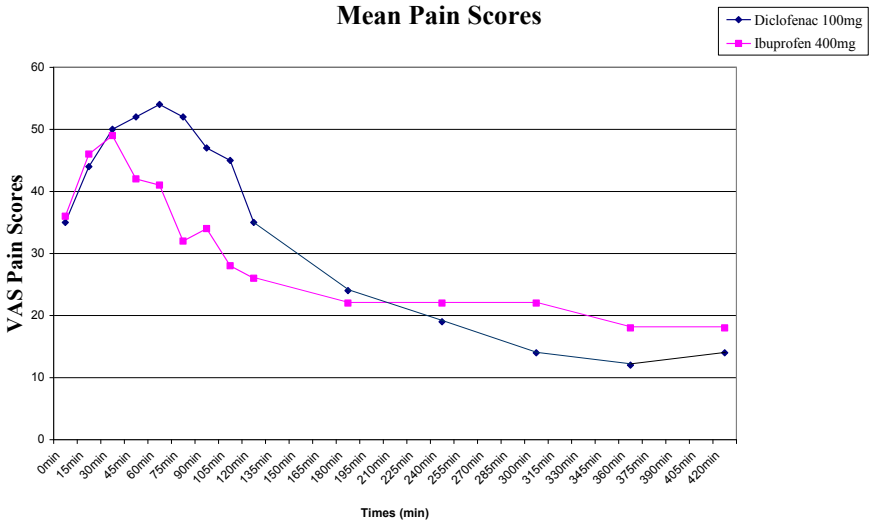

Figure 3 Time effect curves of mean pain intensity scores after the patients had taken ibuprofen or diclofenac for control of pain after impacted third molars surgery.

Discussion

Postoperative pain after removal of impacted third molars is widely used as a model for evaluating analgesic efficacy (4,5). Findings from many studies suggest that analgesics with an anti-inflammatory action are effective for controlling postoperative dental pain. The results of this study demonstrate that 400 mg of ibuprofen and 100 mg of diclofenac are effective and potent analgesics in the treatment of moderate-to-severe acute pain resulting from third molar impacted surgery. While time to onset of analgesia for ibuprofen (64±26.1min) was faster than for diclofenac (83±21.1min).Therefore the analgesic should be given immediately after third molar surgery before the patients feel pain. The peak analgesic effect of 400mg of ibuprofen (422±44.3min) had a longer duration of action than the 100mg diclofenac (406±54.19min). These data suggest that in the great majority of patients who undergo oral surgery, a single oral dose of 400 mg of ibuprofen or 100 mg of diclofenac was sufficient to provide satisfactory analgesia over a 6-9 hour period.

Conclusion

The duration and pain relief of 100 mg of diclofenac and 400 mg of ibuprofen to control pain after impacted third molar surgery were found to be comparable. The onset of analgesia for ibuprofen was faster than for diclofenac.

Acknowledgements

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RESEARCH ARTICLE

Mercury in Saliva of Subjects with Amalgam Restorations

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Abstract

The adverse effect of mercury from amalgam restoration is today a matter of debate in several countries. The aim of this study was to evaluate the mercury concentrations in saliva in patients who have had amalgam restorations compared with non-amalgam restoration subjects. The saliva was obtained from twenty-three subjects who had amalgam restorations, and fourteen subjects without amalgam restorations. We used cold vapor atomic absorption spectrometry to quantify mercury concentrations in saliva. The saliva mercury concentrations in the amalgam group and non-amalgam group were 0.37±0.18 micrograms/liter and 0.47±0.23 micrograms/liter respectively. There was no statistically significant difference in the saliva mercury concentrations between the groups (t-test P>.05). Statistical analysis showed a low non- significant negative correlation between saliva mercury concentrations and the number of amalgam restorations (Pearson Correlation r = -0.20, P>.05). Mercury in the saliva of subjects who had amalgam restorations was not higher than those of non-amalgam restoration subjects. No significant difference could be found in the saliva mercury levels between subjects with and without amalgam restoration. Mercury in saliva did not correlate with the amount of amalgam restorations.

Keywords Mercury, Saliva, Amalgam Restoration

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Introduction

Since the environmental disaster in Minamata in the 1950s, it is well established that excessive exposure to organic mercury can cause dramatic health effects including neurological, immune, motor, sensory, and behavioral dysfunctions(1). Several authors have reported correlations between the number of amalgam fillings and mercury (Hg) concentration in blood plasma, urine, feces, saliva, oral air, pituitary gland, the occipital cortex of the brain, the renal cortex, and liver of patients (2,3). However, there is very little evidence of direct health effects related to amalgam restorations. While a few examples of relief from significant symptoms following removal of amalgams seems to have been established, there is very little general information on the effects of long term exposure to significant but sub toxic levels of Hg., whereas some European governments have banned the use of amalgam in children, women of childbearing age, and renal patients. If mercury is released from dental amalgams, measurements in the saliva should be of interest. In the study on dental amalgams, Hg concentration in the saliva of subjects was found to correlate linearly with the number of amalgam restorations, suggesting that the Hg burden is directly related to the number of amalgams (4). However, Hg concentrations from other body liquids were not available, and thus no information on Hg uptake could be given. Also, quantitative analysis of mercury in saliva is difficult because measurements display a poor reproducibility (5). The purposes of this study were to compare the saliva mercury concentrations in subjects with and without amalgam restorations and to analyze the correlation between the number of amalgam restorations and mercury in saliva.

Material & methods

This research proposal was approved by the Ethic Research Committee of Mahidol University. Total mercury in saliva was performed on thirty three subjects aged 20-22. Twenty-three of them have 7.35±5.69 surfaces of amalgam restoration in their mouths and fourteen subjects were without amalgam restoration. The subjects brushed their teeth prior to collecting the unstimulated whole saliva for ten minutes. Determination of saliva Hg concentration was carried out by microwave digestion and by cold vapor atomic absorption spectrometry using reduction by tin chloride in a Perkin Elmer Fims 400 Atomic Absorption Spectrophotometer. A 1 ml sample of saliva was mineralized with 2.5 ml HNO_3 65% , 2.5 ml HCl 30% , and 1 ml H_2O_2 30% for 30 minutes in closed quartz vessels. The clear solution was cooled down and ready for use in Atomic Absorption Spectrophotometer analysis under standard conditions. For calibration a certified aqueous mercury atomic absorption calibrator from Perkin Elmer (1 g Hg/l 10% HNO_3) was used in appropriate dilutions (6).

Table 1 Show the mean mercury in saliva of amalgam and non amalgam group.

Subjects	Mean ±S.D. (Mercury: microgram / liter)
Amalgam group (n=23)	0.37±0.18
Non amalgam group (n=14)	0.47±0.23

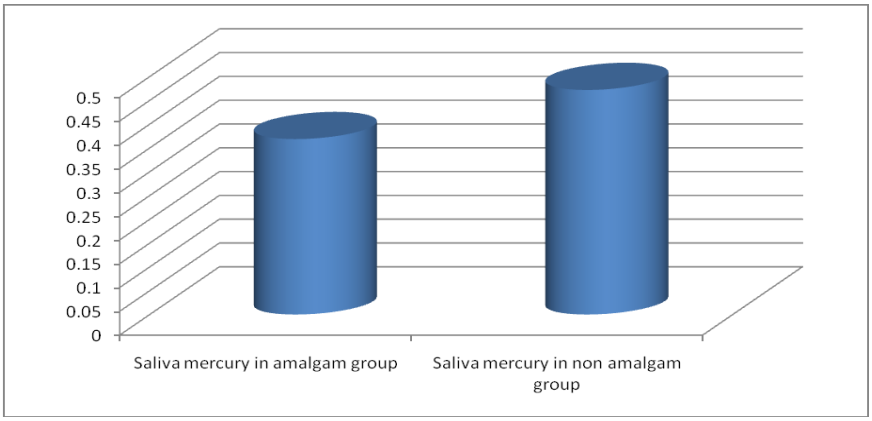

Figure 1 Show mercury level in saliva of amalgam group and non amalgam group.

Results

The mean saliva mercury of amalgam restoration group and non amalgam restoration group was showed in Table 1 and Fig.1. There was no statistically significant difference in saliva mercury concentrations between the amalgam restoration group and the non-amalgam restoration group (t-test P>.05). The correlation between the number of amalgam restorations and the mercury content in saliva was showed in Fig.2. Statistical analysis showed a low non- significant negative correlation between the mercury level in saliva and the number of amalgam restorations (Pearson Correlation r = -0.20, P>.05).

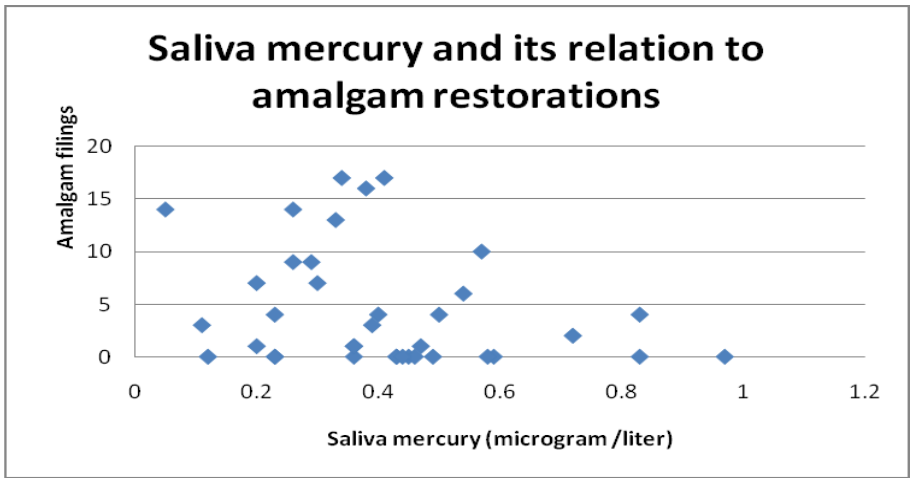

Figure 2 Show correlation between mercury level in saliva and number of amalgam restoration. (Pearson Correlation r = -0.20, P>0.05).

Discussion

In order to evaluate the impact of Hg release from amalgams, we compared the saliva mercury in subjects with amalgam restorations and those without amalgam restoration. The previous study of Leistevuo J and colleagues reported in 2001 that the amount of organic and inorganic mercury in paraffin-stimulated saliva was significantly higher ($p<0.001$) in subjects with dental amalgam restorations (n = 88) compared to the nonamalgam study groups (n = 43 and n = 56) (3). Spearman correlation coefficients of inorganic and organic mercury concentrations with the number of amalgam-filled tooth surfaces were 0.46 and 0.27, respectively.[3] The data obtained from this study found a weak negative correlation but it was not statistically significant between the number of amalgams and the Hg concentration in saliva (Pearson Correlation r = -0.20, P>.05). The mercury content in saliva of amalgam restoration group was also not higher than the non amalgam restoration group (t-test P>.05). But the study of Leistevuo J and associate reported in 2002 that the risk of exceeding the limit increased 2-fold for every 10 additional amalgam-filled surfaces (7). Ganss C and colleagues determined the relationship between mercury content of resting and stimulated saliva, blood and urine in eighty subjects with amalgam restorations. They found a weak correlation between mobilized mercury in saliva and serum ($r = 0.27$; $P < 0.05$) or urine ($r = 0.47$; $P < 0.001$). For resting saliva, the respective values were $r = 0.45$ ($P < 0.001$) and $r = 0.60$ ($P < 0.001$). They stated that saliva testing was not an appropriate measure for estimating the mercury burden derived from dental amalgam (2). The results obtained from this study revealed that mercury released from amalgam into saliva was probably not the main source of saliva mercury.

Conclusion

Mercury released from amalgam into saliva of amalgam restoration subjects was not higher than non amalgam restoration subjects.

No significant difference could be found in saliva mercury between the two groups. Mercury in saliva did not correlate with the amount of amalgam restorations.

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RESEARCH ARTICLE

Inhibition of Human CYP1A2 Activity by Thai Medicinal Plants with Promising Activities against Cholangiocarcinoma

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Abstract

Traditional medicine is commonly used as an alternative treatment for cancer. Several Thai traditional folklores have been shown to possess anticancer activities in various human cancerous cell lines with some promising candidates. The cytochrome P450 (CYP) superfamily of enzymes play crucial roles in the metabolism of drugs and thus have a significant impact on the occurrence of drug-drug interactions. Herbal preparations consist of multiple and often unidentified biological constituents. There is a greater possibility that drug-drug interactions could occur between a complex herbal product and administration of single drug than during the co-administration of two individual drugs. The aim of the present study was to investigate the propensity to inhibit CYP1A2-mediated hepatic drug metabolism *in vitro*, of four crude ethanolic extracts of Thai medicinal plants with promising anticancer activity against cholangiocarcinoma, *i.e.,* extracts of *Atractylodes lancea*, *Zingiber officinal*, *Piper chaba* and *Pra-Sa-Prao-Yhai* formulation, using human liver microsomes. The ethanolic extracts from Pra-Sa-Prao-Yhai herbal formulation inhibited CYP1A2 activities in dose dependent manner with potent activity similarly to that of α-napthoflavaone (selective inhibitor of CYP1A2), with IC_{50} (concentration causing 50% inhibition of enzyme activity) of 0.03 μg/ml. Results showed that metabolic drug interaction may be of concern for clinical use of Pra-Sa-Prao-Yhai herbal formulation in cholangiocarcinoma.

Keywords Thai medicinal plants, cholangiocarcinoma, Cytochrome P4501A2, herb-drug interaction

Introduction

Cholangiocarcinoma (CCA), also known as gall bladder cancer, is a malignancy of the biliary duct system and may originate either in the liver or the extrahepatic bile ducts. Chemotherapeutic treatment of CCA is largely ineffective. The standard chemotherapeutic agent, 5-fluorouracil (5-FU) when used alone or in combination with other drugs always produces low clinical response rate (0-40%) and survival rate (less than 12 month) (1). Investigation of new drugs from chemical synthesis or herbal medicines are being a research area of focus. The cytochrome P450 enzymes (CYP) represent a large family of proteins involved in the metabolism of drugs and other xenobiotics, as well as some endogenous substrates (2). Drug interactions can frequently arise when drugs are coadministered and one drug inhibits the metabolic clearance of the second drug by inhibition of a specific CYP enzyme (3). Such an interaction has resulted in the observations of severe adverse drug interactions, inhibition of CYP enzymes can also be affected by natural products (4). Thus, it is possible that many constituents in herbal preparations could also possess capacities to inhibit drug-metabolizing enzymes. Although new drug candidates are now routinely examined for the potential to cause drug interactions *via* inhibition of drug-metabolizing enzymes, herbal preparations are not subject to such examination. Thus, the potency exists that herbal preparations could cause drug interactions with concomitantly administered medications. The objective of these experiments was to determine the propensity to inhibit CYP1A2-mediated hepatic drug metabolism *in vitro*, of the four crude ethanolic extracts from Thai medicinal plants with promising anticancer activity against cholangiocarcinoma, *i.e.,* extracts of *Atractylodes lancea*, *Zingiber officinal*, *Piper chaba* and *Pra-Sa-Prao-Yhai* herbal formulation, using human liver microsomes.

Methods

Plant materials were collected from various parts of Thailand and some were purchased from the city markets. Ethanolic extracts of all plant materials were prepared. CYP1A2 activity was assessed through measurement of the formation of paracetamol from the substrate drug phenacetin (5). The incubation mixture (final volume of 500 µl in 0.1 M phosphate buffer, pH 7.4) consisted of 0.2 mg/ml human liver microsomes, ethanolic extracts of each plant, a selective CYP1A2 substrate (phenacetin). α-naphthoflavone was used as a selective inhibitor of CYP1A2 and water was added in control wells. The reaction was initiated by the addition of β-NADPH and then incubated at 37°C for 60 min with shaking in a Thermomixer at 800 rpm. Reaction was stopped by the addition of 250 µl of acetonitrile, and 50 µM of caffeine was added as an internal standard. The concentration of metabolite (paracetamol) was measured by HPLC with UV detection (240 nm). The IC_{50} values (concentrations causing 50% inhibition of enzyme activity) were calculated using probit analysis software (CalcySyn™, USA).

Table 1 IC_{50} values of four ethanolic extracts of herbal plants for human CYP1A2 activities compared with selective CYP1A2 inhibitor.

Plant Name	**IC_{50} values (μg/ml incubation)**
Atractylodes lancea	0.20 ± 0.07
Piper chaba	0.20 ± 0.07
Zingiber officinal	0.68 ± 0.02
Pra-Sa-Prao-Yhai herbal formulation	0.03 ± 0.00
α-naphthoflavone (positive control)	0.01 ± 0.00

Data presented as mean ± SD from four microsomal preparations.

Results

The ethanolic extracts from *Atractylodes lancea, Zingiber officinal, Piper chaba* and *Pra-Sa-Prao-Yhai* herbal formulations inhibited CYP1A2 activities in dose dependent-manner with varied IC_{50} values ranging from 0.03-0.68 (μg/ml incubation (Table 1) and *Pra-* Pra-Sa-Prao-Yhai herbal formulation had the most potent activity similarly to that of α-napthoflavaone (selective inhibitor of CYP1A2), with IC_{50} of 0.03 μg/ml.

Discussion and Conclusion

Among the four crude ethanolic extracts, Pra-Sa-Prao-Yhai herbal formulation showed the most potent inhibitory activity of CYP1A2 activity. Several other factors are necessary to be considered for definitive conclusion on the interaction, including the comparative disposition of the individual constituents responsible for inhibition, as well as the locations of the affected CYP (intestine, liver, etc.). It is not known whether hepatic or intestinal intracellular concentrations of Pra-Sa-Prao-Yhai herbal formulation constituents achieve values proximate to the *in vitro* IC_{50} values observed. Concurrent administration of Pra-Sa-Prao-Yhai herbal formulation with drugs that are metabolized by CYP1A2 may result in untoward effects from metabolic enzyme inhibition. Until further clinical investigation in healthy subjects are confirmed, the potential of this plant for use in treatment of CCA may be limited.

Acknowledgements

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RESEARCH ARTICLE

Antioxidant Activity and Phenolic Contents of Thai White Mulberry (*Morus alba* L.) and Butterfly Pea (*Clitoria ternatea* L.) Teas

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Abstract

In this study, two commercially available herbal teas: white mulberry (*Morus alba* L.) and butterfly pea (*Clitoria ternatea* L.) teas were evaluated for their polyphenolic content, and antioxidant activity. Antioxidant activity of aqueous tea infusions derived from five brands of two herbal teas was evaluated using three anti-oxidative methods: the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, the ferrous ion chelating ability, and soybean lipoxygenase inhibition assay. The polyphenol component of herbal tea infusions, key to their healthful qualities, was determined as Gallic acid equivalents (GAE). In addition, anthocyanin content in butterfly pea tea was measured by pH-different method. The low variations in the contents of total polyphenols were observed among tea infusion from different brands of white mulberry tea and butterfly pea tea. Whereas, the high variations in the contents of total anthocyanin was found among infusions from different brands of butterfly pea tea and this may be related to the color intensity of butterfly pea flowers. White mulberry tea infusion showed stronger antioxidant activity, while butterfly pea infusion showed weaker antioxidant activity using two methods (DPPH scavenging method and ferrous ion chelating method). However, both tea infusions showed high and relatively similar antioxidant activity using soybean lipoxygenase inhibition method. Moreover, the amount of total polyphenols found in white mulberry tea was 1.7 times higher than butterfly pea tea. A correlation was observed between DPPH scavenging activity, ferrous ion chelating activity and the total polyphenols contents. Therefore, both herbal teas can be considered to be a good source of water-soluble antioxidants and phenolic compounds.

Keywords white mulberry (*Morus alba)* tea, Butterfly pea *(Clitoria ternatea)* tea, Antioxidant, Total polyphenols.

Introduction

Public interest in herbal teas has grown recently due to the potential health benefits from their consumption. Herbal infusions become popular because of their simple uses and inexpensive costs. The uses of herbal teas are primarily based on tradition, they often have been thoroughly tested for their efficacy in human and most of the consumers are convinced that natural products are non-toxic because of their natural origin. Due to their popular use as antioxidant herbal teas, with increased consumption by the Thai, white mulberry (*Morus alba* L.) and butterfly pea (*Clitoria ternatea* L.) teas were selected in this study to evaluate their antioxidant activity and total polyphenol contents, which play important role in preventing the development of chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis and cataracts (Ames et al., 1993).

In most mulberry-growing countries, in particular in India and China, white mulberry is used for its foliage, to feed the silkworm (*Bombyx mori* L.). There are evidences shows that the leaves contain therapeutic agents such as Moran 20K and 1-deoxynojirimycin (DNJ) against diabetes mellitus while Isoprene-substituted flavanone (kuwanon G and kuwanon C) as antimicrobial agent (Kimura et al., 2007 and Kim et al., 1999).

Clitoria ternatea L. (Family: Fabaceae) commonly known as 'Butterfly pea', a traditional Ayurvedic medicine, has been used for centuries as a memory enhancer, nootropic, anti-stress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent. The flower contains anthocyanin (Ternatin A1, A2, B1, B2, D1, and D2) possess blood platelet aggregation-inhibiting and vascular smooth muscle relaxing activities, while its ethanol extract shows anti-diabetic activity and strong antioxidant activity (Mukherjee et al., 2008 and Kamkaen and Wilkinson, 2009).

The information from this study will provide the knowledge of the antioxidant profiles, which will be helpful in medicine and health care leading to integrate traditional medicine to the mainstream health care system and to foster its use for health promotion.

Materials and Methods

The selected herbal teas were based on their frequency of health promotion use and commercial importance. Five commercial brands of white mulberry and butterfly pea teas were randomly selected for investigation. One sack of herbal tea was infused in 120 ml of hot water (80 ºC) for 3 min. The cool sample was filtered and dried by lyophilize.

Determination of the phenolics content

The amount of total polyphenols of herbal tea infusion was determined with the Folin-Ciocalteu's reagent and Na_2CO_3 (Singleton and Rossi, 1965). The absorbance of sample was measured at 765 nm.

The amount of total anthocyanin of butterfly pea tea infusion was determined using pH-different method. Two sets of sample (pH 1 and pH 4.5) were prepared for determination. The extract was scanned at the wavelengths 520 nm to 700 nm and calculated as cyanidin-3-glucoside equivalents (C3GE).

Determination of antioxidant activity

The 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of herbal tea infusion was determined by spectrophotometer in terms of hydrogen donating or radical scavenging ability. The change in color was measured at 510 nm on an automated micro-plate reader.

The ferrous ion (Fe^{2+}) chelating activity, the herbal extract was reacted with ferrozine in the present of $FeSO_4$. The Fe^{2+}-ferrozine complex was measured at 562 nm.

Lipoxygenase is known to catalyze the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid in the present of herbal tea infusion was followed spectrophotometer by the appearance of a conjugate diene at 234 nm on a UV/visible spectrophotometer.

Statistical analysis

Statistical analysis of the data was done with SPSS statistical analysis software (version 11.5). Results were expressed as mean $\pm$ SD. Data was tested by one-way-ANOVA followed by Tukey's test for multiple comparisons.

Results and Discussion

In this study, white mulberry tea is made from the leaf part and butterfly pea is made from flower part. There was a significant difference regarding the tea bag weights of white mulberry tea among brands (1.17-1.84 g). The amount of crude extract was varying upon the weight of tea bag; the percentage yield of crude extract calculated per bag was not significant difference among the brands of white mulberry tea. Tea infusion from two grams of different brands of butterfly pea dried flowers showed no significant difference regarding the amount of crude extract and the percentage yield. Researches indicate that phytophenolics are potent antioxidants in scavenging free radicals and inhibiting lipid peroxidation in human tissues. They play roles as reducing agents, metal cheaters, singlet oxygen quenchers, and hydrogen donors. Furthermore, studies show that these phenolic compounds are linked with lower occurrence of and lower mortality rates from various human diseases (Geleijnse et al., 2002). There was no significant difference regarding the total polyphenols contents among brands of white mulberry tea and butterfly pea tea, the values were 80.26-106.36 and 47.07-54.69 mg GAE / g of crude extract, respectively. From this study the total polyphenols content of white mulberry tea infusion was found to be 1.7 times higher than butterfly pea tea infusion. However, the amount of anthocyanin that involved in antioxidant activity of butterfly pea flower was significant difference among brands and this may related to the intensity of the flower color.

White mulberry tea infusions showed high antioxidant activity while; butterfly pea tea showed a weak antioxidant effect as determined by DPPH radical scavenging method and ferrous ion chelating method. The DPPH radical scavenging activity and ferrous chelating capacity of white mulberry tea infusion were 5.7 and 9 times higher than the butterfly pea tea infusion, respectively. Phenolic compounds also possess an array of potentially beneficial lipoxygenase inhibitory and anti-oxidant properties; they have been used for the treatment of inflammatory diseases (Sreejayan and Rao, 1996). In 1991, Gardner

showed that the plant lipoxygenase pathway is in many respects the equivalent of the 'arachidonic acid cascades' in animals. For this reason, the in vitro inhibition of soybean lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential (Abad et al., 1995). A combination of this assay with an evaluation of the radical scavenging activity by the DPPH method constitutes a good indication on the potential anti-inflammatory activity of a drug (Alitonou et al., 2006). Interestingly, our study found that both white mulberry and butterfly pea tea infusions showed comparable high antioxidant activity as determined by soybean lipoxygenase inhibition method. It can be observed that the content of phenolics in the mulberry tea infusions correlate with their iron chelating and radical scavenging and iron activities.

Conclusion

Our study showed the differences in antioxidant capacity among the two herbal tea infusions, which due to the diversity and complexity of the natural mixtures of phenolic compounds in the herbs. Each herb generally contained different phenolic compounds, and each of these compounds possessed differing amounts of antioxidant activity. There were also some antioxidant activities in both herbs that may be attributable to other unidentified substances or to synergistic interactions as high anti-inflammatory activity. This information may help in the identification of the herbal teas that could contribute to sustain antioxidant status that protect against oxidative damage and for the development of safe food products and additives with appropriate antioxidant properties.

Acknowledgements

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RESEARCH ARTICLE

Toxicity of Silver Nanoparticles in A549 Cells

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Abstract

Introduction: Silver nanoparticles (AgNPs) are presently incorporated in textiles, cosmetics, medical devices, personal care and house hold products. Despite increasing production and application of AgNPs, large knowledge gaps regarding their possible toxic effects to human health and environment are awaiting to be elucidated.

Objective: To determine toxic effects of silver nanoparticles in terms of mitochondrial-related cytotoxicity and change of cell cycle in human lung carcinoma (A549) cells.

Materials and Methods: A549 cells were cultured in F-12K media containing 10% FBS and incubated in 95% humidified atmosphere and 5% CO_2 at 37°C. AgNPs suspended in the MilliQ water were treated to the cells at final concentrations of 25, 50, 100 and 200 µg/mL for 24 and 48 h. Cytotoxicity was determined by MTT assay. The cell cycle was determined by FACS analysis.

Results: The results demonstrated that AgNPs not only affected cell viability, as indicated by decrease of mitochondrial function, but also disturbed normal cell cycle of A549 cells in time- and concentration-dependent manner.

Discussion and Conclusion: The results from this study suggest potential cytotoxic effects of AgNPs in A549 cells.

Keywords Silver, Nanoparticles, Toxicity, Cell cycle, A549

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Introduction

The rapid advancement of nanotechnology leads to producing of a vast array of engineered nanoparticles (NPs) which are defined as materials measured in length or diameter within the range of 1-100 nm in at least one dimension. With reduction in size, specific surface area and reactivity of NPs dramatically increase. It has been reported that these novel physicochemical properties bring NPs to be bioreactive species when accessing into living organisms (1).

Silver nanoparticles (AgNPs) have gained considerable attention as compared to other metal nanomaterials because of their attractive physicochemical properties. AgNPs are nowadays incorporated in a large number of consumer and medical products (2). Several experiments demonstrated that nano-sized silver can be toxic to human's vital organs especially lung which is found to be the least protective exposure route. AgNPs not only bind proteins contained –SH groups with strong affinity but also persistently accumulate in the lung for many years (3). Although one of the toxicities of AgNPs is to mediate oxidative stress (4), mechanism of AgNPs toxicity at the cellular and molecular level has not yet been fully explained. Thus, the aim of this study was to investigate cell viability and change of cell cycle in response to AgNPs treatment in order to elucidate a possible mechanism of AgNPs-induced pulmonary toxicity.

Materials and Methods

Materials

AgNPs, in an amorphous form, were purchased from Sigma (St. Louis, MO). The product information indicates that particle sizes were less than 100 nm and specific surface areas were 5.0 m^2/g.

Cell lines and cell culture

Human lung carcinoma (A549) cells were obtained from ATCC (Manassas, VA) and grown in F-12K media supplemented with 10% fetal bovine serum, in a 95% humidified atmosphere and 5% CO_2 at 37°C.

Particle Characterization

The particles were characterized by transmission electron microscope (TEM; JEM-2010, Jeol).

In vitro cytotoxicity assay

Cytotoxicity was assessed by using MTT, a mitochondrial-based cell viability assay. A549 cells ($5x10^3$cells/well) were seeded in 96-well plates. After 24 h, the cells were treated with 0, 25, 50, 100, 200 µg/ml of AgNPs for 24 and 48 h. Subsequently, MTT reagent was added to the cells and further incubated for 1 h. The resulted formazan crystal was dissolved with DMSO. Absorbance of the solution was measured at a wavelength of 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Cell cycle analysis

The cells were cultured in media containing 0, 50 and 100 µg/ml of AgNPs for 24 and 48 h. At the end of treatment, the cells were harvested, fixed with 80% ethanol for 30 min at 4°C, and stained with 50 µg/ml propidium iodide in the presence of 0.2 mg/ml RNaseA for 30 min at room temperature. The cell suspension was filtered by a nylon mesh filter, and the filtrate was subjected to FACS analysis using a MoFlo cell sorter analyzer (Dako Cytomation, Kyoto, Japan).

Statistical analysis

All data are presented as mean ± SD. Statistical difference among groups was determined by ANOVA followed by the Dunnett's test. The level of statistical significance was set at p value < 0.05.

Results

From TEM analysis, the morphology of agglomerated micro-sized particles was shown in Figure 1. In fact, each particle was in the range of nano-size. As shown in Figure 2, cell viability decreased in relation to concentration and time exposure of AgNPs. Flow cytometric analysis showed that ratio of cells in sub G1 phase, which is the indicator of apoptosis, increased in the concentration-dependent manner after cells exposed to AgNPs.

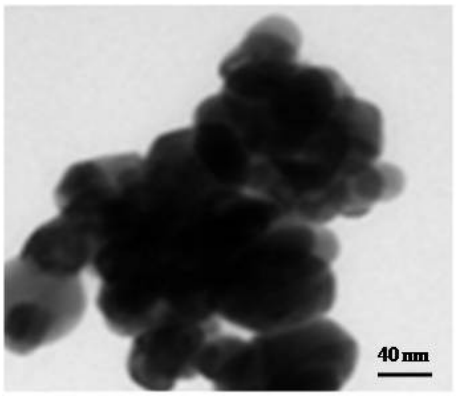

Figure 1 Primary Silver nanoparticles

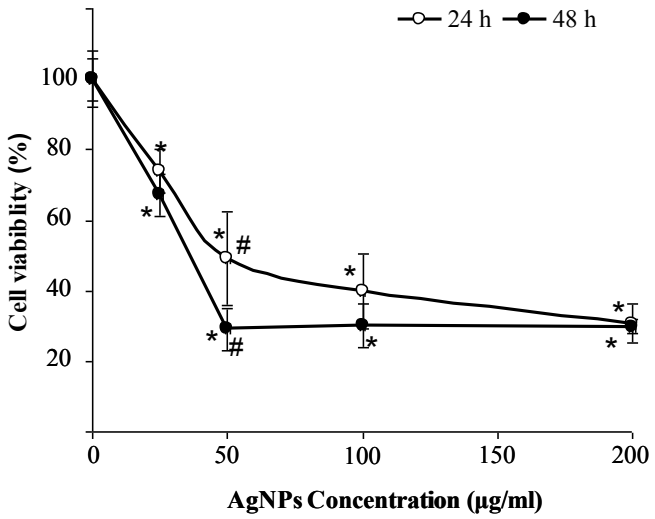

Figure 2 Percent cell viability of A549 cells after treatment with different concentrations of silver nanoparticles for 24 and 48 h
* p < 0.05; AgNPs treatment vs control
\# p < 0.05; 24 h vs 48 h

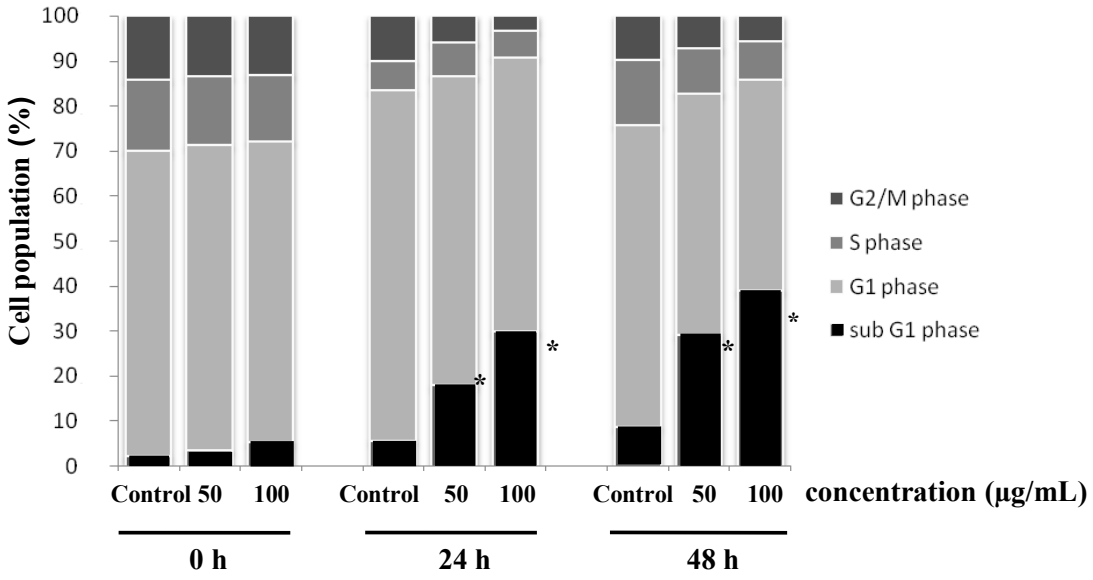

Figure 3 A concentration-dependent sub G1 arrest after A549 cells exposed to AgNPs for 24 and 48 h
* $p < 0.05$; AgNPs treatment vs control

Discussion and Conclusion

Results of this study demonstrated the toxicological effect of AgNPs on A549 cells which included interfering of mitochondrial function, disturbing of normal cell cycle. Therefore, this study demonstrated that AgNPs have a potential to initiate pulmonary toxicity.

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RESEARCH ARTICLE

*HLA-B*15:02* Allele is not a Genetic Marker for Steven Johnson Syndrome and Toxic Epidermal Necrolysis Induced by Phenytoin.

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Abstract

Steven Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe cutaneous adverse drug reactions (SCAR) which associated with several aromatic antiepileptic drugs (AEDs) including carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PNB) and lamotrigine (LMG). Cross reactivity of SCAR among these AEDs has been reported. Recently, a strong association between CBZ-induced SJS or TEN has been described with *HLA-B*15:02* in Han Chinese and Thais as well as other Asian populations. Although an association of *HLA-B*15:02* and PHT-induced SJS/TEN has been noted in Thai and Chinese patients, the number of patients in these studies were too small to draw any definite conclusion. A case-control study was therefore carried out in order to determine relationship between *HLA-B*15:02* and SJS/TEN induced by PHT in a Thai population. In this study, 25 patients who have been diagnosed as PHT-induced SJS/TEN and 74 patients who were tolerant to PHT were enrolled as cases and controls. Peripheral blood or buccal swab samples were collected for genomic DNA extraction. The presence of *HLA-B*15:02* in these patients were analyzed using a PG1502 DNA detection kit.

Among the PHT-induced SJS/TEN patients, 20.00 % carried the *HLA-B*15:02* while 14.86 % of the PHT -tolerant controls carried this allele. The prevalence *HLA-B*15:02* in the cases and controls was not significantly different among the case and the control groups suggest that *HLA-B*15:02* may not be a valid genetic marker for screening Thai patients who at higher risk of PHT-induced SJS/TEN.

Keywords *HLA-B*15:02*, phenytoin, Steven Johnson syndrome and toxic epidermal necrolysis

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Introduction

Severe cutaneous adverse drug reactions (SCAR) including Steven Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and hypersensitivity syndrome (HSS) (Figure 1) are associated with several aromatic antiepileptic drugs (AEDs) including carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PNB) and lamotrigine (LMG)[1].Cross reactivity of AEDs-induced SCAR has particularly been reported[2]. Recently, a strong association between CBZ-induced SJS or TEN has been described with *HLA-B*15:02* in a Thai and other Asian populations[3-6]. Although an association of *HLA-B*15:02* and PHT-induced SJS/TEN have been noted in Thai and Chinese patients, the number of patients enrolled in these studies were rather small and only weak association were observed [7-10]. In 2008, the US-FDA has made an announcement to healthcare providers that screening for *HLA-B*15:02* before initiating treatment with CBZ are necessary for Asian ancestry patients in order to reduce the risk of SJS/TEN. Moreover, healthcare providers should also consider avoiding phenytoin and fosphenytoin as alternatives for CBZ in patients who test positive for *HLA-B*15:02* [11].

Due to the fact that the association between PHT-induced SJS/TEN and *HLA-B*15:02* is still unclear, more study to evaluate the possible risk of SJS/TEN from PHT in patients with *HLA-B*15:02* need to be performed.

Thus, the present study was aimed to investigate the relationship between PHT-induced SJS/TEN and *HLA-B*15:02* in a Thai population in order to identify whether the *HLA-B*15:02* allele is a valid marker for screening patients who are at higher risk of PHT-induced SJS/TEN.

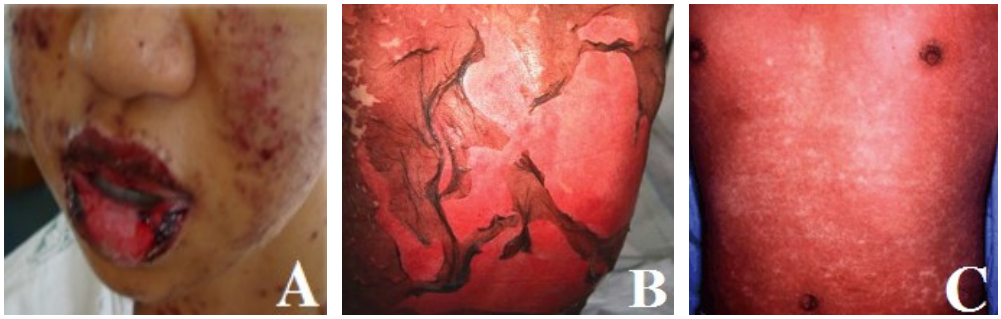

Figure 1 Steven Johnson syndrome (A), toxic epidermal necrolysis (B) and hypersensitivity syndrome (C).

Materials and Methods

Study design

The case–control study was conducted.

Patients

25 subjects who had PHT-induced SJS/TEN were identified from their medical records. The diagnoses of SJS/TEN were confirmed by either a dermatologist or an internist based on the clinical morphology of the patients'skin according to Roujeau *et al.*[12]. PHT was identified as the culprit drug if the cutaneous symptoms occurred within the first 3 months of exposure and the symptoms resolved upon withdrawal of this drug. 74 subjects who had used PHT for ≥ 6 months without evidence of any cutaneous reactions were recruited as controls. Subjects were informed both verbally and in writing about the experimental procedures and the purpose of the study. The study protocol was approved by the institutional review boards.

*HLA-B*15:02 genotyping*

Genomic DNA of each patient was extraction from peripheral blood or buccal cells and used as template for *HLA-B*15:02* detection. The presence of the *HLA-B*15:02* allele was analyzed using a PG1502 DNA detection kit (Pharm*i*Gene, Inc., Taipei, Taiwan).

Results

Twenty-five patients who had been diagnosed with PHT-induced SJS/TEN and 74 patients who received PHT for at least 6 months without any evidence of adverse drug reactions were enrolled as cases and controls.

Among, the PHT-induced SJS/TEN patients, the *HLA-B*15:02* allele was present in 5/25 (20.00%) patients, whereas only 11/74 (14.86 %) of the PHT -tolerant controls carried this allele. The risk of PHT-induced SJS/TEN was not significantly higher in the patients with *HLA-B*15:02.*

Table 1 Prevalence of the *HLA-B*15:02* carrier among PHT-induced SJS/TEN patients and the PHT-tolerant controls

HLA-B*15:02	**Number of patient**	
	SJS/TEN	**Control**
Positive	5 (20.00 %)	11 (14.86 %)
Negative	20 (80.00 %)	63(85.14%)
Total number	25	74

Conclusion

Our result revealed that the frequency of the *HLA-B*15:02* carriers among the PHT-induced SJS/TEN patients and the PHT-tolerant controls were not statistically significant different. Lack of association between *HLA-B*15:02* and PHT-induced SJS/TEN clearly suggest that the *HLA-B*15:02* screening test is not a valid genetic marker for PHT-induced SJS/TEN in Thai population. Other allele of HLA or other genes may involve with these SCAR and warrant further study.

Acknowledgements

This study was supported by the national Science and Technology Development Agency (NSTDA), Thailand and Khon Kaen University, Thailand and the Office of the Higher Education Commission, National Research Council of Thailand, and Khon Kaen University, Thailand. We would like to thank Parinya Konyoung and Usanee Khunarkornsiri, Pharmacy Unit, Udonthani Hospital, Udonthani; Supinya Tirasilawet, Mahasarakham Hospital, Mahasarakham. We also thank all patients who participated in the study.

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RESEARCH ARTICLE

Antioxidant and Antiglycation Activities of Anthocyanin-Enriched Red Grape Skin Extract

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Abstracts

This study was to determine antiglycation and antioxidant effect of anthocyanin-enriched red grape skin extract (RGSE). The phytochemical analysis revealed that the total anthocyanin content of RGSE was 36.733 ± 0.8 mg cyanidins-3-glucoside/g dried extract. The results show that RGSE significantly inhibited the formation of advanced glycation end products (AGEs), decreased the content of N^{ε}-(carboxymethyl) lysine (CML) and increased free thiol group of fructose-modified BSA in concentration-dependent manner. In addition, RGSE was the effective DPPH (1,1-diphenyl 2-picrylhydrazyl) radical scavenger with IC_{50} value of 0.024 ± 0.001 mg/ml. Taken together, the results from this study show that RGSE had antiglycation and antioxidant activities, which may be beneficial for prevention of diabetic complications.

Keywords red grape skin extract, anthocyanin, antioxidant, glycation

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Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia, dyslipidemia, and protein metabolism that results from defects in both insulin secretion and/or insulin action. The long-term hyperglycemia exerts an important role in the development and progression of diabetic complications such as neuropathy, nephropathy, cardiovascular and cerebrovascular diseases (1). Recent findings report that these complications are associated with the glycation process by reaction between the reducing sugars and the amino groups of proteins, consequently, formation of advanced glycation end products (AGEs) such as N^{ε}-(carboxymethyl) lysine or CML (2). In addition, the glycation process also generates reactive oxygen species (ROS) which cause extensive damage to the cells. Thus, inhibition of AGEs and ROS formation would be a potential for the prevention and treatment of diabetic complications (3).

The pharmacological effects of anthocyanin, a natural plant pigment, include anti-cancer, anti-diabetes and anti-glycation (4). Red grape skin extract (RGSE) contains various functional compounds such as vitamins, minerals and polyphenols, especially, anthocyanin (5). Although anti-hyperglycemic activities of RGSE were well documented, studies regarding its ability in prevention of diabetic complication by inhibiting the formation of AGEs and ROS of red grape skin have not been undertaken. Therefore, the aim of the study was to determine the inhibitory effects of RGSE on the formation of AGEs and ROS. Moreover, the total content of anthocyanin in the extract was also investigated.

Materials and methods

Plant material

Dried red grape skin extract was purchased from Breko GmbH Co. (Bremen, Germany).

Total Anthocyanin Content (TAC)

Total anthocyanin content (TAC) in the extract was determined by using pH differential method (4). RGSE was added to two buffer systems including 0.025M potassium chloride at pH 1.0, and 0.4 M sodium acetate at pH 4.5, respectively. The calculated absorption was determined by following the equation: $A = (A_{510} - A_{700})$ pH1.0 - $(A_{510} - A_{700})$ pH4.5 and the TAC in the testing solution was calculated as cyanidin-3-glucoside equivalents (mg/L).

In vitro glycation of BSA

Bovine serum albumin (BSA) at 10 mg/ml was incubated with 1.1 M fructose in 0.1M sodium phosphate buffer (PBS), pH 7.4 with various concentrations of RGSE, ranging 0.25 – 1 mg/ml (final concentration), and aminoguinidine (AG) at 1 mg/ml as a positive control. All treatments were incubated at 37 C° for 7, 14, 21 and 28 days with 3 replications. Total AGEs was quantified by using a fluorescence spectrophotometer at excitation and emission wavelengths of 355 nm and 460 nm, respectively. The inhibitory effect of AGEs was evaluated by calculating the percentage of fluorescence intensity in each sample.

Determination of CML

N^{ε}-(carboxymethyl) lysine (CML) content was measured by ELISA test kit (Cell Biolabs, Inc., USA). The absorbance was measured at 450 nm. The CML concentration of sample was calculated based on the

standard curve prepared by using various concentration of BSA-CML.

Determination of Thiol groups

Thiol groups of BSA were measured by the method of Ellman's assay. (6) Briefly, BSA (3.5 mg/ml) was incubated with 2.5 mM DTNB in 0.1M PBS pH 7.4 for 15 min. The absorbance was measured at 410 nm. The free thiol groups were calculated based on the standard curve prepared by using various concentration of L-cysteine.

DPPH radical scavenging activity

DPPH (1,1-diphenyl 2-picrylhydrazyl) radical scavenging activity was measured by the method of Blois (7). Briefly, sample was added with 0.2mM DPPH as the free radical source and incubated for 30 min at room temperature. The decrease in the solution absorbance was measured at 515 nm. The IC_{50} values were calculated from standard curve prepared by using ascorbic acid.

Statistical analysis

Data were presented as the mean ± standard error of mean (S.E.M). The results were determined by one-way ANOVA followed by Turkey's HSD test. *P*-value < 0.05 was considered to be statistically significant.

Results

The total anthocyanin content was 36.733 ± 0.8 mg cyanidins -3-glucoside/g dried extract. After 4-week of incubation, RGSE (0.25 to 1 mg/ml) significantly decreased the fluorescence intensity when compared to the control group (Figure 1A). The percent inhibition of AGEs formation of RGSE was 62.93% (0.25 mg/ml), 63.52% (0.5 mg/ml), and 71.60% (1 mg/ml), respectively (Figure 1B), which was higher than AG at 1 mg/ml (73.33%).

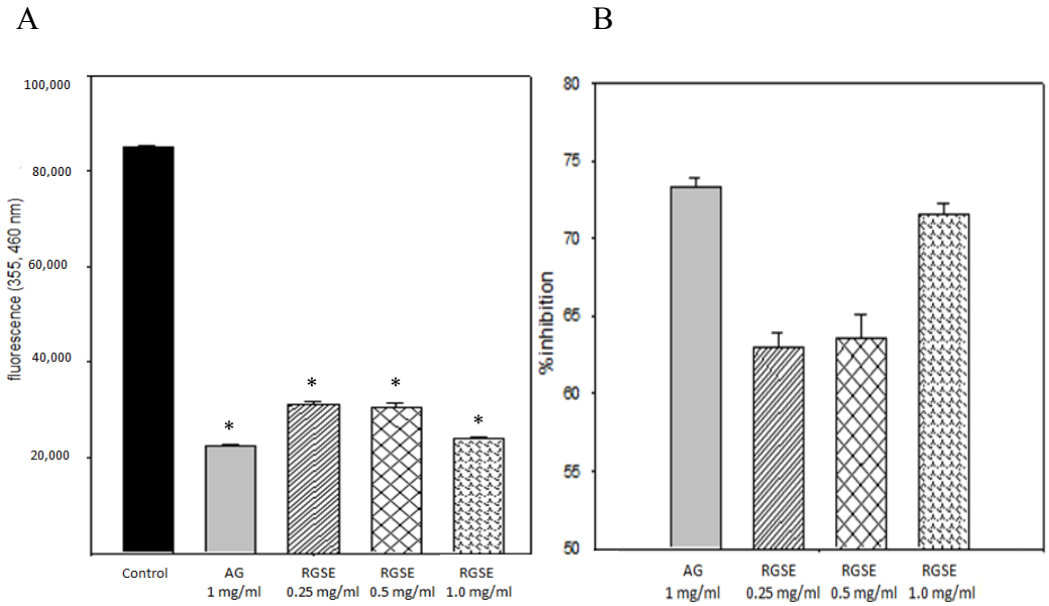

Figure 1 (A) The inhibitory effect of red grape skin extract (RGSE) and aminoguinidine (AG) on formation of AGEs. (B) The percent inhibition of RGSE and AG on formation of AGEs at week 4 (n=3). * $p < 0.05$ compared to control group.

Table 1 The effect of red grape skin extract (RGSE) and aminoguinidine (AG) on the content of N^{ε}-(carboxymethyl) lysine (CML) and free thiol group in fructose-modified BSA.

Treatment	CML (ng/ml)	Protein thiol (pmol/mg protein)
Control	3.636 ± 0.300	0.69 ± 0.141
AG (1 mg/ml)	0.429 ± 0.131*	1.40 ± 0.125*
RGSE (0.25 mg/ml)	2.118 ± 0.686*	0.94 ± 0.069
RGSE (0.5 mg/ml)	1.523 ± 0.227*	1.04 ± 0.124
RGSE (1.0 mg/ml)	0.898 ± 0.210*	1.31 ± 0.176*

Values are mean ± SEM, * $p < 0.05$ compared to control group.

Table 2 IC_{50} values of red grape skin extract (RGSE) on DPPH radical scavenging activity

Antioxidant activities	IC_{50} (mg/ml)
DPPH	0.024 ± 0.001
Ascorbic acid (positive control)	0.011 ± 0.001

Conclusion

The data in this study show that RGSE markedly inhibited formation of AGEs in fructose-mediated non-enzymatic glycation. In addition, it also decreased the content of CML and increased free thiol group of fructose-modified BSA. Moreover, RGSE shows an antioxidant activity. These beneficial effects would be helpful for prevention of diabetic complications.

Acknowledgement

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RESEARCH ARTICLE

Preliminary Study on Pharmacokinetic Profiles of Standardized Extract of *Centella asiatica* ECa 233 in Rats

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Abstract

ECa 233 is a standardized extract of *Centella asiatica* containing mainly madecassoside and asiaticoside. Pharmacological evaluation of ECa 233 in animals demonstrated positive effect on learning and memory deficit induced by β-amyloid as well as wound healing effects. The aim of this study was to investigate the pharmacokinetic profiles of ECa 233 after oral administration in rats. Male Sprague-Dawley rat received ECa 233 at the doses of 30 mg/kg by oral administration and blood sample were collected from tail's vein at time 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hour after administration. Madecassic acid levels in plasma were determined by high performance liquid chromatography (HPLC). The main pharmacokinetic parameters obtained were: T_{max}, 1.67±0.33 hour; C_{max}, 1.92±0.39 μg/ml; AUC_{0-24}, 6.64±0.97 μg h/ml, respectively.

Keywords Pharmacokinetics, ECa233, Standardized extract of *Centella asiatica*, Madecassic acid

Introduction

Centella asiatica (L.) Urban (Umbelliferae) commonly used as medicinal herb all over Asia, has been claimed to possess a wide range of pharmacological effects e.g. wound healing effect, anti-inflammatory effect, brain stimulating effect and being used as a treatment of venous hypertension and microangiopathy (1-3). The active compounds of *Centella asiatica* are triterpenoids including asiatic acid, madecassic acid, asiaticoside and madecassoside (4).

ECa 233 is a standardized extract of *Centella asiatica* defined as a white to off-white extracted powder of *C. asiatica* containing triterpenoids not less than 80% and the ratio between madecassoside and asiaticoside is 1.5±0.5 : 1. Pharmacological evaluation of ECa 233 in animals demonstrated positive effect on learning and memory deficit induced by β-amyloid as well as wound healing effects (5-6). In acute and sub-chronic toxicity testing, ECa 233 has been found to exhibit a favorable safety profile (7). However, no information on pharmacokinetics of ECa 233 is currently available. Therefore, the present study was aimed to investigate the pharmacokinetic profiles of ECa 233 in rats.

Materials and Methods

Chemicals and reagents

ECa 233 containing Madecassoside 42.90% and asiaticoside 38.8% was kindly provided by Dr. Ekarin Saifah and co-workers, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. It was suspended in distilled water at concentration of 20 mg/ml. Madecassic acid (98.4% purity) were purchased from LKT Laboratories,Inc. Glibenclamide (internal standard) and tetrabutyl ammonium bisulfate (97%) were purchased from Sigma, USA. Ammonium chloride (99%) of analytical grade was purchased from Fluka, Germany. 85% phosphoric acid, acetonitrile (HPLC grade), methanol (HPLC grade) and ethyl acetate were purchased from RCI Labscan Limited, Thailand. The ultra pure water was used for HPLC analysis.

Experimental animal and study design

Male Sprague-Dawley rats weighing between 350-450 g were obtained from the Laboratory Animal Center, Mahidol University, Salaya,Nakornpathom,Thailand were housed under controlled environmental conditions of a 12-h light/dark cycle and 25±2°C.

Three rats received ECa 233 30 mg/kg by oral administration. Blood samples were collected from the tail's vein at time 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hour after dosing. Samples were centrifuged immediately at 10,000 rpm for 10 minutes. Plasma was separated and stored at -20°C until analysis.

The study protocol was approved by the Ethic Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Assay method

This method was modified from P. Thongnopnua (8). Plasma (150 µl) was added to 10 µl of 5 µg/ml methanolic glibenclamide solution (internal standard). Liquid extraction was preceded by adding H_3PO_4(42.5%), 0.01 M tetrabutyl ammonium bisulfate solution, 6.0 M aqueous ammonium

chloride solution and ethyl acetate, respectively and vortex mixing. Separation of the organic phases by centrifugation at 4,500 rpm for 10 minutes and dried under nitrogen gas. The residue was dissolved in 120 µl of methanol and injected 50 µl into the HPLC system.

The chromatographic separation was made on a Phenomenex 250 x 4.6 mm C18, 5 µm column using gradient elution. The mobile phase was a mixture of ultra pure water and acetonitrile at the ratio 44.5:55.5. The flow rate was 0.60 ml/min and monitored by UV detector at wavelength of 217 nm.

Pharmacokinetic analysis

The pharmacokinetic parameters of madecassic acid; time to peak plasma concentration (T_{max}) and peak plasma concentration(C_{max}) were determined by visual inspection of the observed plasma concentration-time data. The area under the concentration-time curve from time 0-24 h(AUC_{0-24}) was estimated by the linear trapezoidal rule.

Result

The mean concentration-time curve of madecassic acid was shown in Figure 1. and the pharmacokinetic parameters was shown in Table 1. Madecassic acid was absorbed very fast, and approximate T_{max} were observed 1 hour after administration of ECa 233. This data shown the pharmacokinetic profiles : T_{max}, 1.67±0.33 hour; C_{max}, 1.92±0.39 µg/ml; AUC_{0-24}, 6.64±0.97 µg h/ml, respectively.

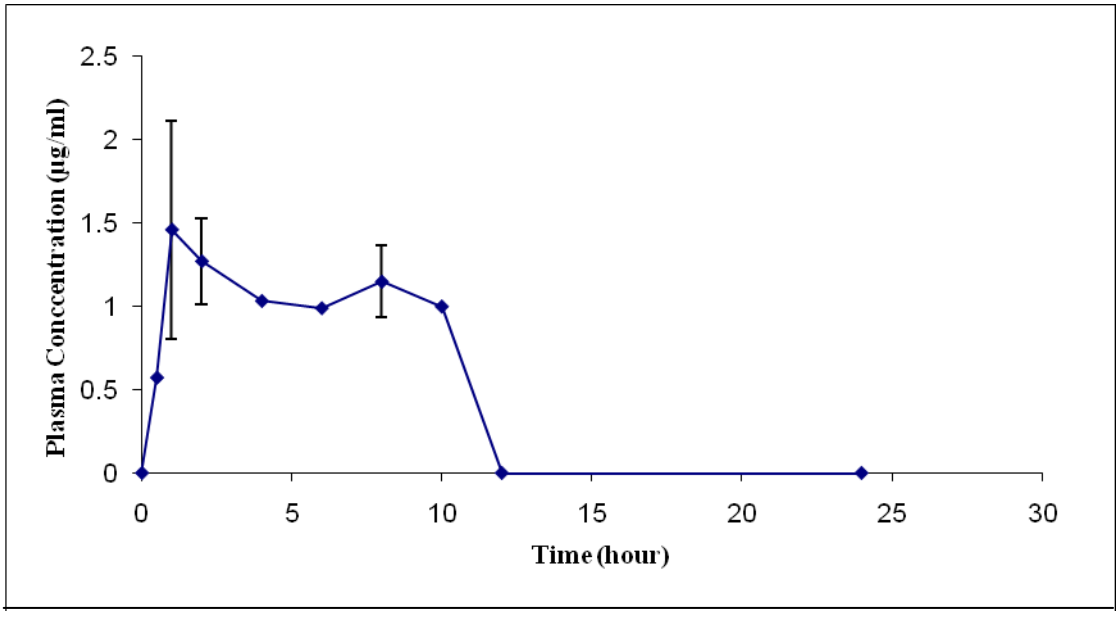

Figure 1 Mean concentration-time curves of madecassic acid in rat plasma after oral administration of ECa 233 (n=3)(at time 0.5, 4, 6, 10; n=1).

Table 1 Pharmacokinetic parameters of madecassic acid in rat plasma after oral administration of ECa 233 (n=3).

Parameters	Single dose of ECa 233 30 mg/kg
T_{max} (hour)	1.67±0.33
C_{max} (μg/ml)	1.92±0.39
AUC_{0-24} (μg h/ml)	6.64±0.97

Value are means + S.E.M.

Discussion and conclusion

The present study is the first report of pharmacokinetic profiles of ECa 233 in experimental animals. Though, a few investigation to determine asiatic acid in plasma has been successfully carried out, no report on plasma concentration of madecassic acid has ever been reported. Similar to asiaticoside which has been transformed to asiatic acid after being ingested (9-10), madecassoside in ECa 233 were transformed into madecassic acid which was detected in rat plasma. Madecassoside seemed to be rapidly absorbed giving T_{max} of 1.67±0.33 hours and the plasma concentration was rather stable over a period of about 10 hours before rapidly declined below detection limit at 12 hours. Unfortunately, the assay method developed in the present study was not concurrently able to determine asiatic acid in animal plasma. Studies to give complete pharmacokinetic profiles of single and repeated dose(s) of ECa 233 would be further accomplished.

Acknowledgements

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RESEARCH ARTICLE

Study on *in vitro* Antimalarial Activity of Cyclooxygenase Inhibitors against *Plasmodium falciparum*

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Abstract

Multi-drugs resistance of *Plasmodium falciparum* is a major problem for controlling malaria in Thailand. To deal with this problem, development of novel antimalarial drugs and the use of drug combination have been proposed. The objective of the present study was to assess *in vitro* antimalarial activity of the combination of artesunate and cyclooxygenase inhibitors (aspirin, naproxen and piroxicam), against chloroquine resistant (K1) and chloroquine sensitive (3D7) *P. falciparum*. The test was performed based on fluorescent-based technique. The combination of artesunate and aspirin was observed based on the ratios: 10:0, 7:3, 5:5, 3:7 and 0:10, respectively. The highest final concentrations of artesunate and aspirin used were 50 and 10,000 nM, respectively. Results showed that the concentration that inhibited parasite growth to 50% (IC_{50}) of artesunate against K1 and 3D7 were 2.5 (1.6-3.4) and 2.2 (1.2-3.2) nM, respectively. The IC_{50} values of aspirin, naproxen and piroxicam were greater than 100,000 nM for both strains. The SUM-FIC values of drug combination between artesunate and aspirin against K1 and 3D7 were 0.60 and 0.64, respectively. Results indicated marked synergism of the combination of artesunate and aspirin for both *P. falciparum* strains.

Keywords *Plasmodium falciparum;* cyclooxygenase (COX) inhibitor, drug resistance

Introduction

The problem of multi-drug resistance *Plasmodium falciparum* is a major problem for controlling malaria in Thailand. Several approaches have being introduced to overcome drug resistance in *P. falciparum* including the development of new antimalarials (chemical synthesis, natural products), *de novo* drug design, chemical modification of existing drugs, screening drugs currently in clinical use to treat other diseases, and chemical reversal of drug resistance (1, 2). Combination of artesunate with other drugs that could reverse drug resistance would also be one promising approach. Cyclooxygnase (COX) enzyme is one promising antimalarial drug target. The present study investigated antimalarial activities of various known COX inhibitors, *i.e.,* aspirin, naproxen and piroxicam including their effects on artesunate activity.

Methods

In vitro drug susceptibility testing

P. falciparum laboratory clones (K1 and 3D7) were cultured according to the methods of Trager and Jensen (3) with modifications. Antimalarial activities of artesunate, aspirin, naproxen and piroxicam against the *P. falciparum* clones were assessed using the SYBR Green I assay (4). IC_{50} value (drug concentration that inhibits the parasite growth by 50%) was used as an indicator for antimalarial activity of the tested drugs and was determined from a log-dose response curve plotted using the Grafit™ computer program (Erithacus Software Ltd., U.K).

Antimalarial interactions between artesunate and aspirin

The interaction between artesunate and aspirin was determined *in vitro* based on the method described by Fivelman et al. (5). The test wells consisted of varying concentrations of artesunate and aspirin at the ratios of 10:0, 7:3, 5:5, 3:7 and 0:10, respectively. The control wells (row H) consisted of drug-free parasitized erythrocytes. All experiments were performed in triplicate and data analysed as described above. Two IC_{50} values for each of the five combination curves were calculated separately by using the known concentration ratios of artesunate and aspirin. The fractional inhibitory concentration of artesunate (FIC-artesunate) and aspirin (FIC-aspirin) were calculated for each point and isobolograms were plotted. To obtain numeric values for the type of interaction, results were expressed as the sum of the fractional inhibitory concentrations (sum FIC) at the given IC (inhibitory concentration) by the formula (ICx of agent A in the mixture/ICx of agent A alone) + (ICx of agent B in the mixture/ICx of agent B alone) (6). Sum FIC values indicate the type of antimalarial interactions as follows: 'synergistic' if sum FIC < 1; 'fully additive' if sum FIC = 1; partially additive if sum FIC < 2 provided that both contributory FICs < 1; antagonistic if any FIC>1.

Results

Antimalarial activity of artesuante and COX inhibitors

Antimalarial activities of artesunate, aspirin, naproxen and piroxicam against K1 chloroquine-resistan and 3D7 chloroquine-sensitive

P. falciparum clones are presented in Table 1. The potency of each drug was indicated by IC_{50} value calculated from concentration–response curve. The IC_{50} values of aspirin, naproxen and piroxicam for both clones were greater than 100 μM. The median (range) IC_{50} values of artesunate for both clones (K1 *vs.* 3D7) were 2.5 (1.6-3.4) *vs.* 2.2 (1.2-3.2) nM, respectively.

Antimalarial interactions between artesunate and aspirin

The antimalarial activity of the combination of artesunate and aspirin was investigated against K1 and 3D7 *P. falciparum* clones. The median (range) sums of the FICs for the antimalarial interaction for the K1 and 3D7 *P. falciparum* clones were 2.5 (1.6-3.4) and 2.2 (1.2-3.2) nM, respectively. The sums of the FICs of less than 1.0 in both clones indicated synergistic interaction between artesunate and aspirin. These synergistic interactions were supported by the isobolograms representing the interactions between the partner drugs in both parasite clones (Figure 1).

Table 1 The median (range) IC_{50} values of artesunate, aspirin, naproxen and piroxicam against K1 and 3D7 *P. falciparum* clones

Drugs	The median (range) IC_{50} values (nM)	
	K1	3D7
Artesunate	2.5 (1.6-3.4)	2.2 (1.2-3.2)
Aspirin	> 100,000	> 100,000
Naproxen	> 100,000	> 100,000
Piroxicam	> 100,000	> 100,000

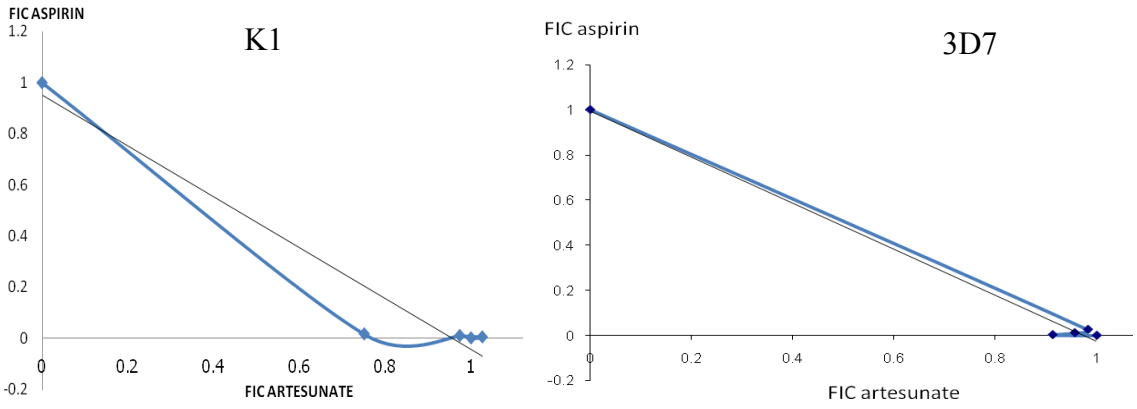

Figure 1 Isobologram of Artesunate-Aspirin Combination

Discussion

Kubata B et al., (7) have reported the production of prostaglandins (PGs) by *P. falciparum* following the incubation of *P. falciparum* cell homogenates with arachidonic acid. The produced PGs was not affected by NSAIDs like aspirin and indomethacin and was partially heat resistant. In contrast, PG biosyntesis by mammalian (human) COX was completely inhibited by these chemicals and by heat treatment. Results observed from the present study suggest no inhibition of PG production in both *P. falciparum* clones. It is possible that COX enzyme structure in malaria parasite may be different from mammalian COX.

Conclusion

The present study preliminarily investigated *in vitro* antimalarial activity of various COX inhibitors, i.e., aspirin, naproxen and piroxicam. None of them showed promising antimalarial activity (IC_{50} values > 100 μM). Combination of artesunate with aspirin showed synergistic effects against both K1and 3D7 clones.

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RESEARCH ARTICLE

The Effects of *Andrographis paniculata* on the Pharmacokinetics of Midazolam in Normal Healthy Volunteers

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Abstract

Andrographis paniculata has been widely used for centuries in Asia. Previous *in vitro* studies have shown that *A. paniculata* extracts and its major component, andrographolide, inhibited activity and mRNA expression of human cytochrome P450 3A4 enzyme (CYP3A4). The purpose of this study was to evaluate the effect of *A. paniculata* on the pharmacokinetics of midazolam, a CYP3A4 probe drug. The study was an open-label, randomized, two-phase crossover design. Twelve healthy male volunteers received 4 capsules of 250 mg *A. paniculata* three times a day orally for 7 days. Midazolam plasma concentration time profiles were characterized after a single oral dose of 7.5 mg midazolam on the day before and after *A. paniculata* medication. The results demonstrated that treatment with *A. paniculata* did not change mean pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0\text{-}12h}$, $AUC_{0\text{-}\infty}$, $T_{1/2}$, Cl/F) of oral midazolam. Thus, no clinically relevant CYP3A4 inhibition after *A. paniculata* in healthy volunteers was suggested.

Keywords *Andrographis paniculata*, Andrographolide, Midazolam, Pharmacokinetics, Drug interaction

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Introduction

During the past decade, the use of herbal medicines among the general public has been increased dramatically. Many reports indicated that herbal medicines were often taken concurrently with conventional therapy (1). Although herbal medicines are generally perceived as safe when used alone at the recommended dose and duration, there are increasing evidences of herb-drug interaction which may lead to serious adverse reactions or failure of therapy with conventional medicines, such as bleeding tendency caused by ginkgo and aspirin interaction or graft rejection in patient taking immunosuppressant, cyclosporine, with St John's wort (2).

Andrographis paniculata (Burm. F.) Wall. ex Nees (Fa-ta-lai-jone) belongs to Family Acantheceae. It has been widely used for centuries in Asia to treat common cold, diarrhea, and fever. The three major active components in *A. paniculata* are andrographolide, neoandrographolide and 14-deoxy-11,12-didehydro-andrographolide. Many studies have shown that *A. paniculata* and its major components, andrographolide, have various pharmacological activities, such as anti-inflammatory, anticancer, anti-platelet aggregation, anti-viral and hepatoprotective effects (3). Previous *in vitro* studies demonstrated that *A. paniculata* extracts and andrographolide inhibited activity and mRNA expression of various CYP isozymes including CYP3A4, CYP2D6 and CYP2C in human and rat liver microsomes (4,5), while CYP1A inducing activity was also reported (6). However, an *in vitro* or even *in vivo* experiment in animal cannot be precisely extrapolated to a clinical situation. To our knowledge, there are still lack of *in vivo* studies relating to the interaction of *A. paniculata* on CYP enzymes in human. The purpose of this study was to evaluate the effect of *A. paniculata* on pharmacokinetics of midazolam to assess its safety through its potential to interact with co-administered drugs which are the substrate of the most abundant cytochrome P450, CYP3A4.

Materials and methods

Subjects

Twelve healthy male non-smoking and non-alcoholic volunteers with a mean age of 27.5±6.1 years, a height of 164.6±5.0 cm, a weight of 56.8±7.0 kg, and a body mass index of 20.9±1.9 kg/m^2 participated in the study. Participants were determined to be healthy on the basis of medical history, physical examination, clinical chemistry, and hematologic screenings. One month before the start of the study and during the study, all subjects were not allowed to drink alcohol or taking any medicines. Participants provided their written informed consents before participation in the study. The study was approved by the ethics committee of the Faculty of Science, Prince of Songkla University.

Study design

The study was an open-label, randomized, two-phase crossover design with a 2-weeks washout period, of single-dose (midazolam) and multiple-dose (*A. paniculata*) drug-drug interaction study. In the first phase, after an overnight fast, volunteers received a single oral dose of 7.5 mg midazolam (Dormicum®, Hoffmann-La Roche Ltd, Basel, Switzerland) with 200 ml of water. Baseline pharmacokinetics of

midazolam was evaluated. The second phase, a second midazolam pharmacokinetic characterization was performed on day 8th of the *A. paniculata* medication period with the regimen presented as below, both midazolam pharmacokinetic study phases were conducted identically. Blood samples were taken through a venous catheter before administration of midazolam and at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, and 12h after midazolam ingestion. The plasma was collected and stored at -20 ºC until analysis.

A. paniculata medication

Volunteers received 4 capsules of 250 mg *A. paniculata* three times a day orally for 7 days and 4 capsules once in the morning on day 8th. The capsules (Lot no. CAP030) were manufactured by Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Analysis of the *A. paniculata* main constituent (andrographolide) in the capsules was performed as described previously (7). The content of andrographolide per capsule was 8.36±0.06 mg.

Midazolam analysis

Midazolam plasma levels were determined by HPLC. The analysis method was modified from Lehmann and Boulieu (8). The 500 µl of sample was added with 1 ml of diethyl ether. The mixture was vortexed for 5 minutes and centrifuged at 1,000×g for 10 minutes. The 800 µl of upper organic phase was separated and evaporated at 35 ºC under air flow. The residue was reconstituted in 100 µl of the mobile phase and 50 µl was injected into the HPLC system (Agilent Technologies). The mobile phase consisted of 0.05M KH_2PO_4 (pH 4.0): acetonitrile: methanol (55:30:15 v/v/v), at a flow rate 1.2 $ml.min^{-1}$. The peak was detected using a UV detector set at 210 nm. The calibration curves were prepared in a concentration range of 5-250 $ng.ml^{-1}$ of midazolam and the lower limit of quantification was 5 $ng.ml^{-1}$.

Pharmacokinetic and statistical analysis

All pharmacokinetic parameters were analyzed by non-compartment model with the use of WinNonlin Professional Software Version 1.1. Results were expressed as mean±SD and statistical comparisons were made using Paired *t-test*. The significance level was set at *p*-value of less than 0.05.

Results

Mean plasma concentration-time profiles of midazolam before and after treatment with *A. paniculata* were shown in Fig.1. The mean $T_{max,}$ $T_{1/2}$ and Cl/F of midazolam were not changed. Although the mean C_{max} was decreased by 19.49% (75.2±5.2 from 93.4±56.1 ng/ml), $AUC_{0\text{-}12h}$ was increased by 9.93% (191.5±114.4 from 174.2±83.9 ng/ml·h) and $AUC_{0\text{-}\infty}$ was increased by 9.81% (226.1±172.2 from 205.9±139.6 ng/ml·h), but the differences were not statistically significant (p>0.05) as shown in Table 1.

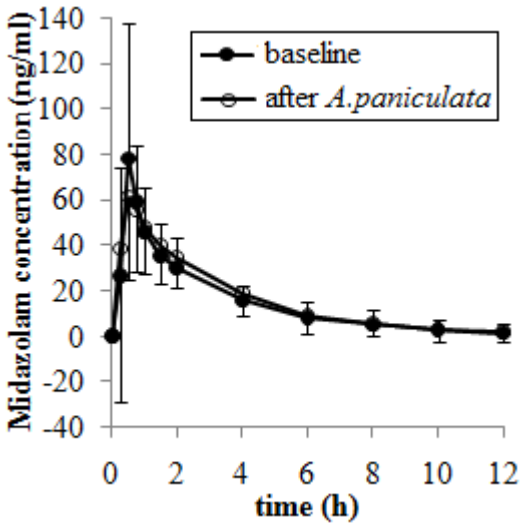

Figure 1 Mean plasma concentration-time profiles after a single oral dose of 7.5 mg midazolam before (baseline, *solid circles*) and after *A. paniculata* (*open circles*)

Table 1 Pharmacokinetic parameter (mean±SD) of midazolam before and after *A. paniculata*

Parameter	Baseline of midazolam	Midazolam after *A. paniculata*
C_{max} (ng/ml)	93.42±56.12	75.15±52.03^{NS}
AUC_{0-12} (ng/ml·h)	174.18±83.89	191.50±114.38^{NS}
$AUC_{0-\infty}$(ng/ml·h)	205.88±139.61	226.11±172.19^{NS}
T_{max} (h)	0.56±0.16	0.56±0.22^{NS}
$T_{1/2}$ (h)	2.63±2.51	3.01±3.05^{NS}
Cl/F (L/h)	0.048±0.02	0.046±0.02^{NS}

NS; no significant difference compared to baseline of midazolam values with Pair *t-test*

Discussion

In Thailand, *A. paniculata* is one of the most popular herbal medicines. It has been placed in the National List of Essential Medicines for treatment of non-infectious diarrhea and common cold since 1999 (9). Although it is a herbal medicine developed from a single plant with an evidence indicating its safety for use in humans. Data concerning interaction of *A. paniculata* with other drugs used in conventional therapy are very limited. In the present study, we demonstrated that treatment with 4 capsules of 250 mg *A. paniculata* three times a day for 7 days in 12 normal volunteers did not significantly change the mean pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-12h}, $AUC_{0-\infty}$, $T_{1/2}$, Cl/F) of oral midazolam. Each capsule of *A. paniculata* used in the study contained about 8.36±0.06 mg of andrographolide (unpublished data). The dose regimen of *A. paniculata* used in this study was corresponds to about 1.8 mg/kg/day of the active principle andrographolide (12 capsules of 8.36 mg of andrographolide per day and average weight of the subjects was 56.8 kg). The dose was close to that recommended for treatment of respiratory diseases (4 capsules of 250 mg tid-qid orally for 7 days) (9). According to Panossian *et al* (10), the calculated steady state plasma concentration of andrographolide for multiple doses of *A. paniculata* after the normal therapeutic dose regimen (3x4 tablets/day, about 1 mg andrographolide/kg/day) was approximately 1.9 μM (660 ng/ml), which is less than the concentration affecting CYP activities *in vitro* (12.5-50 μM) as reported previously (4-6). This could be the explanation of the negligible effect of *A. paniculata* on pharmacokinetics of midazolam in our study. Oral midazolam is used as a CYP3A probe drug since it is the most sensitive substrate for both intestinal and hepatic CYP3A. Regulatory authorities suggest that in case of a negative drug interaction with the most sensitive substrate, it can be presumed that less sensitive substrates would also be unaffected (11).

In conclusion, no significant changes in pharmacokinetics of oral midazolam was found after medication with *A. paniculata* at the recommended dose regimen. It is suggested that there is no clinically relevant CYP3A4 inhibition after *A. paniculata* treatment in healthy volunteers. Thus, herb-drug interaction between *A. paniculata* and CYP3A4 substrate is considered clinically insignificant.

Acknowledgements

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RESEARCH ARTICLE

Cytotoxic Activity of the Ethyl Acetate Extract from *Glycosmis parva* Leaves on Human B- Lymphoma Cells

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Abstract

This study aimed to investigate cytotoxicity of the ethyl acetate extract from the leaves of *Glycosmis parva* (Criab), a tradition thai herbal medicine, on human B-lymphoma cells or Ramos cells. The extract demonstrated cytotoxic activity against Ramos cells with its IC50 15.68 µg/ml at 24 hours exposure by rezasurin staining assay. It mainly induced apoptosis of these cancer cells after 8 and 16 h of exposure by annexin V-FICT and propidium iodide staining assay. This apoptotic induction activity of the extract was markedly dependent on caspase activation. These results demonstrated that the ethyl acetate extract from leaves of *G. parva* potentially has anti-tumor activity against B- lymphoma cells.

Key words *Glycosmis parva*, cytotoxic, apoptosis, cell cycle regulation

Introduction

Glycosmis parva Criab (Rutaceae) is a small shrub wildly distributes in thailand (Chansriniyom C. *et al.* 2009). It is used as a traditional medicine for treatments of abscess, scabies, and snakebite. Plants in the genus *Glycosmis* contain two main chemical compounds including acridone alkaloids and sulfur containing propanamides in their branches and leaves. Several studied have been reported that acridone alkaloids demonstrated antiviral activities against Epstein bar virus, herpes simplex virus, human immunodeficiency virus-1, antiprotozoa against *Plasmodium* species, anti-allergic activity and mouse skin tumor inhibitory activity. Sulfur containing propanamides had been reported to inhibit bacterial and fungal growth, anti- Epstein barr- virus (Chansriniyom C. *et al.* 2009). The hexane and ethyl acetate extracts from leaves of *G. parva* inhibited expression of proinflammatory cytokines, cyclooxygenase 2 (COX2), and nutric oxide synthase (iNOS) in lipopolysaccharide activated-macrophages (Chumsang S. 2010). COX2 overexpression has been demonstrated in a variety of tumors (Khuri FR. *et al*. 2001). Sulfur containing propanamide containing furanone ring from leaves of *G. parva* has also been reported to inhibit cancer cell growth (Hofer O. *et al.* 1998). This study intended to further investigate cytotoxic and apoptotic induction activities of *G. parva* on human B-lymphoma cells.

Materials and methods

Plants extract

The ethyl acetate extract from the leaves of G. parva was prepared by solvent partitioning technique. Ethyl acetate was totally removed and the extract was in the solid phase stored at -20^{o}C until being used

Cells

Human B lymphoma cells, Ramos cells were obtained from ATCC. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37 ˚C in a humidified atmosphear of 5% CO_2

Chemicals

Anexin V apoptosis detection kit was obtained from Santa Cruz Biotechnology, USA. The caspase inhibitor Z-VAD-FMK was from Promega, USA. Etoposide was optained from Ebewe Pharma, Austria. Other chemicals were optained from Sigma, Germany.

Cytotoxicity test

$1x10^6$ cells/ml Ramos cells were treated with 25-100 µg/ml ethyl acetate extract of *G. parva* for 24 h. Viability of these cell were determined by resazurin staining assay. Cytotoxicity activity of the extract and IC50 was evaluated compared to untreated Ramos cells.

Apoptotic induction determination

$1x10^6$ cells/ml Ramos cells were treated with 7.5, 15 and 30 µg/ml extract for 8 h. The treated cells were stained with annexin V-FITC and propidium iodine (PI). The pattern of cells death were identified by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells.

The caspase dependence of apoptosis induction activity of the extract was also determined. Ramos cells were pre-treated 50 µM pan

caspase inhibitor (Z-VAD-FMK) for 1 h befor adding the extract.

Stastistical analysis

All assay were perform in an at least three independent experiment (n=3). The data were presented as mean ± S.E data analysis was performed on SPSS 17.0. Statistical significance was determined by one-way ANOVA followed by Turkey's post hoc test. The p-value < 0.05 was considered statistically significance.

Result

Cytotoxicity of the ethyl acetate extract from leaves of G. parva

The extract had cytotoxic activity on Ramos cells in a concentration dependent manner with IC_{50} at 15.68 µg/ml after 24 h exposure (Fig. 1).

Apoptotic induction activity of the extract

The extract induced Ramos cell death mainly by apoptosis after 8 h exposure. Ethyl extract from leaves of *G. parva* at the concentration 7.5, 15 and 30 µg/ml caused Ramos cell death mainly by apoptosis after incubated 8 hr. It induced apoptosis in a concentration and time dependent manner (Fig. 2).

Caspase dependence of apoptotic induction activity of the extract

The apoptotic activity of the extract on Ramos cells was markedly mediated by caspase activation. The pan caspase inhibitor Z-VAD-FMK could almost tatally inhibit this activity at both concentrations of the extract used in the study (Fig. 3).

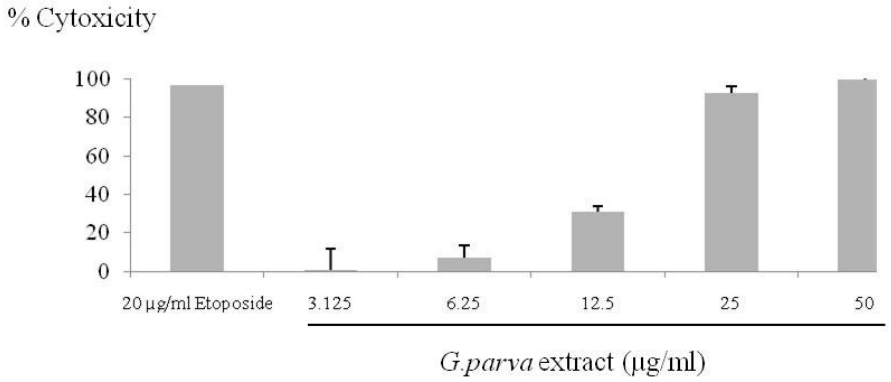

Figure 1 Cytotoxic activity of the ethyl acetate extract from leaves of *G. parva* on on Ramos cells. Data are presented as means± S.E of three independent experiment (n=3).

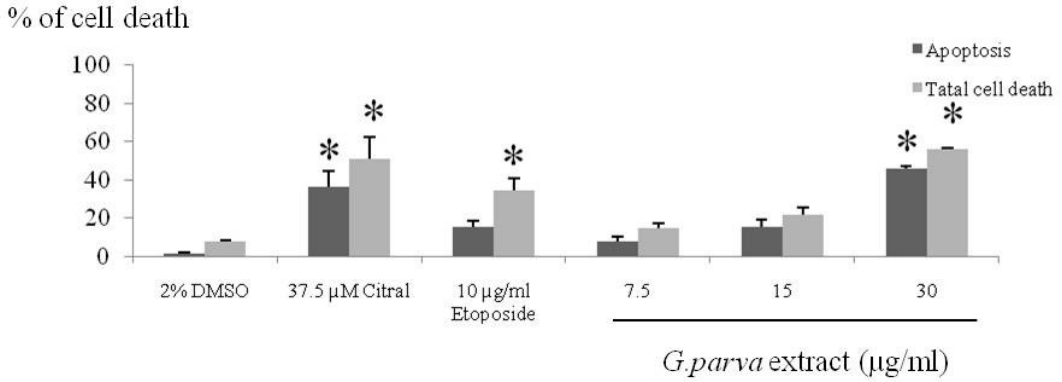

Figure 2 Apoptotic induction effect of the ethyl acetate extract from leaves of *G.parva* on Ramos cell by Annexin V-FITC/PI staining assy. The cells were treated with 7.5, 15, 30 µg/ml extract for 8 h and then stained with annexin V-FITC and PI. The patterns of cell death were determined by fluorescence flow cytometer. Anexin V-FITC positive cells were identified as apoptotic cells. The data are represented as mean ± S.E. of three independent experiment (n=3). * $p<0.05$ compared to 0.2% DMS

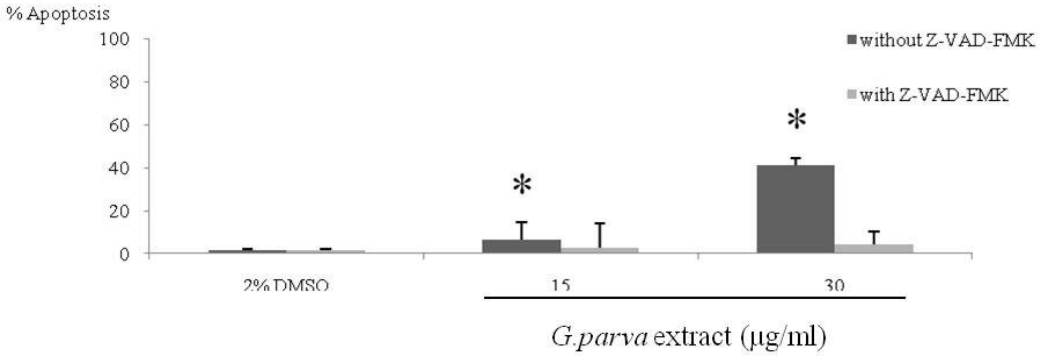

Figure 3 Caspase dendence of apoptotic induction activity of the ethyl acetate extract from the leaves of G. parva. Ramos cells were pre-treated with Z-VAD-FMK for 1 hour and then treated with 15 and 30 µg/ml extract for 8 h. Apoptotic cells were determinded by staining with annexin V-FITC and detecting with fluorescence flow cytometer. Anexin V-FITC positive cells were determind as apoptotic cells. The data expressed as mean ± S.E. of two independent experiments (n=2). * $p<0.05$ compared between with and without Z-VAD-FMK.

Discussion and Conclusion

It has been reported that acridone alkaloids and sulfur containing propanamides are main componds found in the branches and the leaves of this plant (Chansriniyom C. *et al.* 2009). A sulfur containing propanamide containing furanone ring from the leaves of *G. parva* could inhibit cancer cell growth (Hofer O. et all. 1998).We demonstrated in this study that the ethyl acetate extract from the leaves of *G. parva* induced human B-lymphoma cell death mainly by apoptosis induction. It apoptotic induction activity is dependent on caspase activation because a pan-caspase inhibitor Z-VAD-FMK could totally inhibit this activity. This extract also inhibited the expression of many inflammatory mediators in LPS-activated macrophage J774A.1 cells. It decreased expression of TNF-α, IL-1, IL-6, iNOS as well as COX-2 in these cells (Chumsang S. 2010). COX-2 overexpression is observed in many of tumor cells (Khuri FR. *et al*. 2001). COX-2 is one of target molecules for target-based anticancer drugs because its overexpression is involeved in metastasis of cancer cells (Subbaramaiah K. and Dannenberg AJ. 2003). It is possible that the leaves of *G. parva* may contain active compounds which are candidates for new anti tumor agents that have both anticancer as well as anti-inflammatory activities.

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