Effects of *Ginkgo biloba* extract and tanshinone on cytochrome P-450 isozymes and glutathione transferase in rats[1]

YANG Xiu-Fen, WANG Nai-Ping[2], LU Wen-Hong[3], ZENG Fan-Dian[4]

Department of Clinical Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030; [2]Department of Pharmacology, Guangxi Traditional Chinese Medical College, Nanning 530001; [3]Institute of Environmental Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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ABSTRACT

AIM: To investigate the effects of *Ginkgo biloba* extract (*Gb*E) and tanshinone (Tan) on cytochrome P450 (CYP) isozymes and glutathione transferase (GT) in rats. **METHODS:** Several CYP-dependent reactions were monitored in liver and kidney microsomes of male rats treated ig with *Gb*E and Tan daily for 10 d. The activity of GT, the levels of malondialdehyde (MDA) and nitric oxide (NO) in the tissues were also determined. **RESULTS:** CYP1A1, 1A2, and 2B1 activities in the liver were all significantly increased (2-9.5 fold) by pretreatment with *Gb*E or Tan ($P<0.01$). An induction (1.4 fold) of CYP 2E1 activity was observed at the higher dose of *Gb*E treatment ($P<0.01$), but a reduction (1.9 fold) after Tan administration ($P<0.01$). Whereas *Gb*E could induce CYP3A (1.6 fold) ($P<0.01$) but Tan had no effects. Furthermore, the activities of CYP 1A1 (5.6-8.9 fold) and 1A2 (2.6 fold) in the kidney were induced by *Gb*E ($P<0.01$). The activity of GT in rat liver receiving Tan was significantly increased ($P<0.05$) and a dramatic reduction in the activity of GT in the kidney was observed in the *Gb*E-treated group ($P<0.01$). In addition, the *Gb*E treatment markedly decreased the levels of MDA and NO in the tissues of rats ($P<0.01$). **CONCLUSION:** The modulation of CYP isozymes by *Gb*E and Tan may result in altered metabolism of coadministered drugs. In addition, *Gb*E is an active antioxidant and nitric oxide inhibitor *in vivo*.

INTRODUCTION

Cytochrome P-450 (CYP: EC 1.14.14.1) enzymes are active in monooxygenation and hydroxylation of various xenobiotics, including drugs, carcinogens, and environmental pollutants, as well as many endogenous substrates such as fatty acids, arachidonic acid, steroids, prostaglandins, leukotrienes[1-4]. Glutathione transferase (GT: EC 2.5.1.18) is a complex multigene family of enzymes that are widely distributed in the animal kingdom[5]. The most important function of GT is detoxification, conjugating reduced glutathione with a large number of electrophilic metabolites derived from a variety of xenobiotics, including carcinogens, toxins, and drugs[5-7]. GT is also involved in the metabolism of endogenous substances such as leukotriene and prostaglandin[5-7].

Ginkgo biloba extract (*Gb*E) and *Salvia miltiorrhiza* extract has been widely used in traditional Chinese medicine for treatment of cerebrovascular or car-

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[4] Correspondence to Prof ZENG Fan-Dian.
Phn 86-27-8363-0652. Fax 86-27-8362-2308.
E-mail xfyangcn@yahoo.com.cn
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diovascular diseases for many years[8,9], but their therapeutic mechanisms are not known completely[8,9]. *Gb*E contains terpenoids and approximately 30 kinds of flavonoids[8]. Tanshinone (Tan), the major active ingredient of *Salvia miltiorrhiza* extract, is a mixture of many kinds of analogue compounds[9]. It was reported that *Gb*E and Tan had antioxidant effects[9-12], *Gb*E had an inhibitory effect on nitric oxide[13,14], and Tan had antitumor potential[15,16].

It is very popular that coadministration of herbal medicines and drugs in the practice of medicine in our country[17,18]. However, concurrent use of herbs may mimic, magnify, or oppose the effect of drugs[17,18]. The bioavailability of many drugs was decreased when combined with *St John's wort*; *Gb*E and *Salvia miltiorrhiza* extract could potentiate the effects of anticoagulants; a serious side effect was observed when *Gb*E was administered concomitantly with paracetamol, ergotamine or caffeine[17,18]. But the mechanism of such herb-drug interactions remains unclear[17,18]. Preliminary data suggested that *St John's wort* induced CYP3A, and therefore, speeded the elimination of concomitant drugs[19].

In this investigation we attempted to examine: (i) whether *Gb*E or Tan were able to affect some CYP isoforms in liver and kidney in male rats by using highly selected substrates to different CYP isozymes; (ii) the effects of *Gb*E and Tan on GT in rat liver, kindney, and lung; (iii) in addition, the effects of *Gb*E and Tan on malondialdehyde (MDA) and nitric oxide (NO) *in vivo*.

MATERIALS AND METHODS

Chemicals and drugs β-Nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$), glucose 6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (reduced form) tetrasodium salt (NADPH), glucose-6-phosphate dehydrogenase (G6PDH), dimethyl sulfoxide, methoxyresorufin, ethoxyresorufin, pentoxyresorufin, and resorufin were purchased from Sigma Chemical Co (St Louis MO, USA). All other chemicals and solvents were of highest purity analytical grade and obtained from Hubei Province Chemicals Co Ltd (Wuhan, China).

The powder form of *Gb*E was kindly donated from Shutai Biochemical Products Factory of Deyang City (Sichuan, China). The *Gb*E contained 25.2 % flavonoids and 6.2 % terpenoids, and <5 parts per million of ginkgolic acids .The composition of the flavonoids and terpenoids in *Gb*E was similar to that of Egb 761[8]. Tan (a mixture of 15 kinds of analogue compounds, containing tanshinone II A 20 %, a powder form) was obtained from Guangzhou SHR Biotechnology Co Ltd.

Treatment and administration of *Gb*E and Tan Principles in good laboratory animal care were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in Huazhong University of Science and Technology. Male Sprague-Dawley rats (Grade II, Certificate No 19-053) weighing 200-250 g, were obtained from Experimental Animal Center of Tongji Medical College, and housed at a constant room temperature of 22 ℃ under a 12-h light/dark cycle. The animals were allowed free access to food and drinking water. Rats were randomly assorted into the following groups (each group consisting of 6 rats): group I (control) rats were treated with corn oil ig daily for 10 d; group II rats were treated with of tanshinone (Tan) 100 mg/kg; group III or IV rats were treated with *Gb*E 100 mg/kg or 200 mg/kg. Tan and *Gb*E were all dissolved in corn oil and administered ig daily for 10 d.

Preparation of tissues subcellular fractions The subcellular fractions of rat tissues were separated by a standard differential centrifugation procedure described previously[20].

Assays of aminopyrine *N*-demethylase (APND), aniline hydroxylase (ANHD), methoxyresorufin *O*-demethylase (MROD), ethoxyresorufin *O*-deethylase (EROD), and pentoxyresorufin *O*-dealkylase (PROD) activities APND was determined by estimation of formaldehyde production by use of Nash reagent[21]. ANHD activity was measured by following the formation of the *p*-amiophenol from aniline, according to the method described previously[22]. MROD, EROD, and PROD activities were determined as described in our previous study[20].

Assay of nitrite and nitrate The amount of NO in liver tissues was determined using an NO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Briefly, the method involved measuring the amount of NO metabolites (nitrite and nitrate), which were more stable than NO. Nitrate in the tissues was reduced first to nitrite by the action of nitrate reductase. Then the reaction was initiated by the addition of Griess reagent, and absorbance of the mixture at 550 nm was determined[23].

Other biochemical index assays The rat liver MDA levels were evaluated in order to estimate the ex-

tent of lipid peroxidation in the tissues, the assay was carried out using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). GT activity in the subcellular fractions was determined using 1-chloro-2,4-dinitrobenzene as a substrate in the presence of glutathione[24], the assay was also carried out using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein content was estimated by the dye binding assay of Bradford[25], with bovine serum albumin used as a standard.

Statistics Data were expressed as mean±SD and analyzed with Microsoft Excel. Statistical analyses were performed by Student's *t*-test. *P* value of less than 0.05 was considered statistically significant.

RESULTS

Behaviour of liver, kidney, and lung weights of *Gb*E and Tan treated rats No significant differences in either absolute or relative organ (liver, lung, and kidney) weight under the different treatment schedules were observed, only in the animals treatment with *Gb*E 200 mg/kg, the absolute weights of liver and kidney were increased slightly (Tab 1).

Effects of *Gb*E and Tan on various CYP isozymes in rat liver and kidney microsomes Tan was able to significantly induce MROD (CYP1A2) (7.6 fold increase), EROD (CYP1A1) (7.1 fold), and PROD (CYP2B1) (2.1 fold) activities in liver microsomes ($P<0.01$, Tab 2). But a significant reduction (a 1.9 fold decrease) of ANHD activity (CYP2E1) ($P<0.01$) and no change of APND activity (CYP3A) were observed in this group (Tab 2). However, the MROD and EROD activities in the kidney microsomes did not change (Tab 3). Treatment with *Gb*E at a higher dose (200 mg/kg) was able to significantly ($P<0.01$) induce MROD (2.3-fold increase), EROD (9.4 fold), PROD (8.3 fold), APND (1.6 fold), ANHD (1.4 fold) in liver microsomes (Tab 2). But at a lower dose (100 mg/kg), no significant alterations of EROD and ANHD activities were observed, and MROD, PROD, and APND activities only demonstrated a 1.4-fold, 1.8-fold, and 1.3-fold increase, respectively (Tab 2). Furthermore, *Gb*E could significantly induce MROD (2.6-fold increase) and EROD (8.9 fold) activities in kidney microsomes at a higher dose ($P<0.01$). EROD activity demonstrated a 5.6-fold increase ($P<0.01$) and MORD activity did not change at

Tab 1. Behaviour of liver, kidney, and lung weights of *Gb*E and Tan treated rats. *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control group.

Group	Liver weight		Kidney weight		Lung weight	
	Absolute/g	Liver/body (%)	Absolute/g	Kidney/body (%)	Absolute/g	Lung/body (%)
Control (corn oil)	7.3±0.6	3.37±0.22	1.68±0.15	0.78±0.07	1.10±0.15	0.51±0.07
Tan 100 mg/kg	7.8±0.5	3.46±0.19	1.62±0.22	0.72±0.09	1.00±0.15	0.44±0.06
*Gb*E 100 mg/kg	7.6±0.4	3.30±0.09	1.72±0.13	0.75±0.04	1.05±0.10	0.457±0.023
200 mg/kg	7.87±0.18 ^{b}	3.28±0.13	2.02±0.21^{b}	0.84±0.06	1.03±0.08	0.43±0.04

Tab 2. Effects of *Gb*E and Tan on various CYP isozymes activities in rat liver microsomes. *n*=5-6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* *Gb*E 100 mg/kg.

Group	Activities of different enzymes/nmol·min^{-1}·g^{-1} protein				
	MROD	EROD	PROD	APND*	ANHD*
Control	52±6	35±9	22±4	33±4	15±4
Tan 100 mg/kg	397±39^{c}	248±93^{c}	45±7^{c}	34±6	8.1±2.3^{c}
*Gb*E 100 mg/kg	74±8^{c}	38±6	40±7^{c}	43±7^{b}	15±4
200 mg/kg	119±31^{cf}	328±170^{cf}	181±66^{cf}	52±4^{ce}	21.8±2.6^{cf}

MROD: methoxyresorufin *O*-demethyl ase; EROD: ethoxyresorufin *O*-deethylase;
PROD: pentoxyresorufin *O*-dealkylase; APND: aminopyrine *N*-demethylase;
ANHD: aniline hydroxylase. * nmol·mg^{-1} protein·30 min^{-1}

Tab 3. Effects of *Gb*E and Tan on various CYP isozymes activities in rat kidney microsome. *n*=5. Mean±SD. $^{c}P<0.01$ *vs* control group.

Group	Activities of different enzymes/ nmol·min^{-1}·g^{-1} protein	
	MROD	EROD
Control	1.4±0.3	2.0±0.5
Tan 100 mg/kg	1.47±0.16	1.9±0.3
*Gb*E 100 mg/kg	2.3±1.1	11±5^{c}
200 mg/kg	3.7±1.0^{c}	18±6^{c}

MROD: methoxyresorufin *O*-demethyl ase; EROD: ethoxyresorufin *O*-deethylase.

a lower dose in this group (Tab 3).

Effects of *Gb*E and Tan on GT activity in microsomes or S_9 fractions in rats GT activity in liver microsomes was significantly ($P<0.05$) induced when treated with Tan, but in kidney microsomes and lung S_9 fraction, this enzyme activity did not change. When treated with *Gb*E, GT activity in kidney microsomes was down-regulated significantly ($P<0.01$). But in liver microsomes and lung S_9 fraction, no change of GT activity was observed (Tab 4).

Tab 4. Effects of *Gb*E and Tan on glutathione transferase (GT) activity in microsomes or S_9 fraction in rats. *n*=5-6. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group.

Group	GT activity/μmol·min^{-1}·g^{-1} protein		
	Liver	Kidney	Lung*
Control	1115±280	578±199	318±89
Tan 100 mg/kg	1559±314^{b}	576±123	302±42
*Gb*E 100 mg/kg	1218±213	346±122^{c}	343±38

* S_9 fraction

Effects of *Gb*E and Tan on the lipid peroxidation in microsomes or S_9 fraction in rats The MDA levels in kidney microsomes were decreased significantly with *Gb*E treatment ($P<0.01$). At a higher dose, a significant reduction of MDA levels was also observed in liver microsomes and lung S_9 fractions ($P<0.01$). Tan treatment did not affect the levels of MDA in the liver, kidney, and lung (Tab 5).

Tab 5. Effects of *Gb*E and Tan on lipid peroxidation (MDA level) in microsomes or S_9 fractions in rats. *n*=5-6. Mean±SD. $^{c}P<0.01$ *vs* control group. $^{f}P<0.01$*vs Gb*E 100 mg/kg.

Group	MDA level/μmol·min^{-1}·per g protein		
	Liver	Kidney	Lung*
Control	4.7±1.8	3.9±1.0	1.00±0.14
Tan 100 mg/kg	5.3±0.8	3.2±0.3	1.09±0.16
*Gb*E 100 mg/kg	4.4±0.5	2.6±0.4^{c}	1.10±0.19
200 mg/kg	2.2±0.2^{cf}	2.5±0.4^{c}	0.71±0.15^{cf}

*S_9 fraction. MDA: malondialdehyde

rat liver tissues The amount of NO in the liver tissues was determined by measuring the amount of NO metabolites (nitrite and nitrate). The total nitrite and nitrate levels were decreased significantly after treatment with *Gb*E ($P<0.01$), but Tan had no effect on NO (Fig 1).

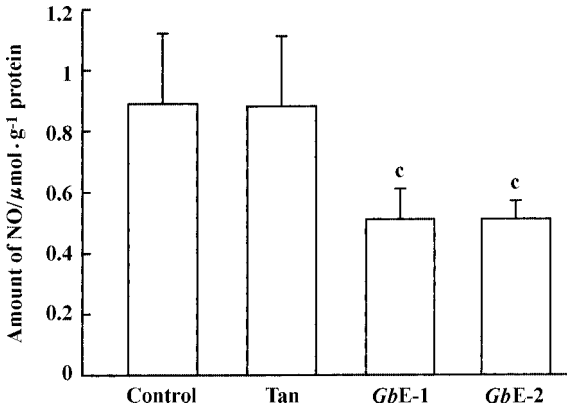

Fig 1. Effect of *Gb*E and Tan on NO in rat liver tissues. *n*=5. Mean±SD. Tan: 100 mg/kg; *GbE*-1: 100 mg/kg; *GbE*-2: 200 mg/kg. $^{c}P<0.01$ *vs* control group.

DISCUSSION

CYP1A1/A2 is primarily involved in the metabolism of various food components such as caffeine, and drugs such as paracetamol, theophylline, mexiletine, and quinolones[3,4]. The CYP3A is the major one expressing in the liver and predominantly implicated in the metabolism of a vast variety of drugs[3,4]. Furthermore, CYP1A1/A2 also plays a key role in arachidonic acid (AA) hydroxylation to produce 16-, 17-, 18-, and 19-OH-AA[2,26]. CYP2B1 is a main AA epoxygenase which

metabolizes AA to produce 5,6-,8,9-,11,12-, and 14,15-EET[2,26]. In this study, a marked enhancement of MROD (CYP1A2), EROD (CYP1A1), PROD (CYP2B1) activities in liver microsomes was observed in rats given *Gb*E and Tan, and the APND activity (CYP3A) was also found to be induced by *Gb*E. Although species differences make it difficult to extrapolate the induction from the rats study to human directly, caution should be paid to the possible drug interaction in patients who concurrently use *Gb*E or Tan and these CYP substrates. Moreover, the hydroxylation and epoxidation of AA *in vivo* would be affected by *Gb*E or Tan treatment.

The significant inhibitory effect on aniline hydroxylation (CYP2E1) exhibited by Tan is of interest. CYP2E1 is mainly known to be associated with the metabolism of a wide range of compounds[22], such as aromatic compounds (eg, benzene, phenol, acetaminophen, chlorzoxazone, pyrazole), halogenated alkanes and alkene (eg, chloroform, halothane), alcohols/ketones/nitriles (eg, ethanol, propanol, butanol), and nitrosamines/azocompounds. Most of these compounds are toxins or carcinogens; some of them are drugs. Therefore, inhibition of this particular CYP isoform could indicate the usefulness of Tan as chemopreventive agent and Tan may potentiate the effects of CYP2E1 substrates when given concomitantly.

The increase in the GT activity observed in the rat liver micrsomes after pretreatment with Tan could also be an important effect. GT in the liver plays an important role in conjugating the metablites resulting from the action of cytochrome P-450, favouring their elimination from the body[5-7]. It has already been reported that induction in GT in the liver is implicated in protection against various cytotoxic, mutagenic and carcinogenic chemicals[5-7]. However, GT in the kidney was also found to mediate cell damage[27]. We used 1-chloro-2,4-dinitrobenzene as a non-specific substrate in our study to determine the GT activity. Thus the specific activity of the enzyme measured was the sum of all its isoforms. But Tan had no effects on the activity of GT in lung S_9 fraction and kidney microsomes, which indicated that the effect of Tan on GT was different in different tissues. Similar results were observed in the animals with *Gb*E treatment. However, *Gb*E did not affect the GT activity in liver microsomes and lung S_9 fraction, but significantly deceased this enzyme activity in kidney microsomes.

Many kinds of flavonoids and their derivatives were reported to induce or inhibit GT and numbers of cytochrome P450 members such as CYP1A1, 1A2, 2B1, 2E1, and 3A[4]. *Gb*E contains approximately 30 kinds of flavonoids (eg, bilobetin, ginkgetin, sciadopitisin, quercetin, isorhamnetin, kaempferol) and their derivatives and terpenoids such as ginkgolide (A, B, C, M) and bilobalide[8]. The major active ingredient, Tan, purified from *Salvia miltiorrhiza* extract, is a mixture of many kinds of analogue compounds such as tanshinone I-VI, cryptotanshinone, isotanshinone, isocryptotanshinone , hydroytanshinone[9]. The different modulations of CYP and GT between *Gb*E and Tan may contribute to their different ingredients. Since both *Gb*E and Tan are a complex mixture, we also need to study whether the observed modulation is due to a single "active component" in the mixture.

MDA, a product of membrane lipid peroxidation, has been shown to react with critical biomolecules such as nucleic acids, thus damaging the cells[28]. Our results showed that the MDA levels in lung S_9, liver, and kidney microsomes were all decreased after *Gb*E treatment. This suggests *Gb*E can protect the cells in these tissues from injury by inhibiting membrane lipid peroxidation. Furthermore, our data also demonstrated that the NO level in rat liver was decreased after *Gb*E treatment. These results are consistent with the previous studies[10,11,13,14] by using other tissues or cell lines. Our findings support that *Gb*E is an active antioxidant and NO inhibitor.

A complex pattern of CYP or GT modulation has been recorded in this investigation. The effects of *Gb*E and Tan on GT or CYP were different in different tissues of the rats. The CYP isozymes in this study were found to be induced by *Gb*E, but an induction, a reduction or no effect on different CYP isoforms was observed in the animals with Tan treatment. Furthermore, the effects of *Gb*E and Tan on GT were also different. This may contribute to the different components in these two herbs, however, further study needs to be carried out in this field to elucidate clearly the modulatory mechanism.

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