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The inhibition of CYP1A2, CYP2C9, and CYP2D6 by pterostilbene in human liver microsomes

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This study used human liver microsomes to assess pterostilbene's effect on the metabolic activity of cytochrome P450 (CYP) 1A2, CYP2C9, and CYP2D6. The metabolism of their substrates (phenacetin, tolbutamide, and dextromethorphan) was assayed by quantifying their relevant metabolites by HPLC. The IC_{50} value was used to express the strength of inhibition, and the value of a volume per dose index (VDI) was used to indicate the metabolic ability of the enzyme. In this study, pterostilbene inhibited CYP1A2, CYP2C9, and CYP2D6's metabolic activities *in vitro*. CYP2C9's activity was most significantly inhibited by pterostilbene; its IC50 value was 0.12±0.04 μM. The IC50 value of CYP1A2 and CYP2D6 was 56.3±10.4 μM and 62.33±11.4 μM, respectively. The finding that suggests that pterostilbene has the potential to interact with CYP2C9 substrates *in vivo*. These results warrant clinical studies to assess the *in vivo* significance of these interactions.

1. Introduction

There is a growing global trend of using herbal remedies and phytochemicals to improve health (Ahad et al. 2020a, b). Around 80% of the world's population consume herbal products (Jagtenberg and Evans 2003; Ekor 2013). Approximately 20% of the U.S. population consumed at least one herbal medicine in the last 12 months (Bardia et al. 2007; Rashrash et al. 2017). The sale of herbal remedies in the United States exceeded $8 billion in 2017 (Ekor 2013). Notably, patients with chronic diseases tend to use herbal remedies more than the general population (Tulunay et al. 2015; Alghamdi et al. 2018). This high rate of herbal remedies intake, especially among patients on prescription medications, warrants further studies on the potential herb-drug interactions.

Cytochrome P450 (CYPs) are a family of enzymes with an important role in phase I xenobiotic metabolism; they metabolize more than 75% of prescribed drugs (Zhou et al. 2009; Zanger and Schwab 2013). Herbal extracts and phytochemicals can induce or inhibit drug-metabolizing enzymes; therefore, they may interfere with the metabolism of co-administered drugs, leading to therapeutic failure or unwanted adverse effects (Wanwimolruk et al. 2014; Rombola et al. 2020).

Pterostilbene is a natural polyphenol (Fig. 1) in blueberries and grapes. In addition to anti-inflammatory and antioxidant characteristics, it has demonstrated beneficial effects on diabetes, hypertension, and cancer (Remsberg et al. 2008; McCormack and McFadden 2013). Also, studies have associated blood pressure reduction with the ingestion of 250 mg daily dose of pterostilbene (Riche et al. 2013).

Since pterostilbene is available in the market as a natural product, a single ingredient, or mixed with other substances, it may interact with other drugs during drug metabolism (Kapetanovic et al. 2010; Kosuru et al. 2016). A previous study used HLM to evaluate pterostilbene's inhibitory effects on UGT1A1, UGT1A6, UGT1A8/10, UGT1A9, CYP2C8, CYP3A and found that pterostilbene inhibited the activity of UGT1A6 and CYP2C8 (Albassam and Frye 2019).

Pterostilbene's effect on CYP1A2, CYP2C9, and CYP2D6 enzyme activities, however, is still unknown. Therefore, this study examines pterostilbene's inhibitory effects on CYP1A2, CYP2C9, and CYP2D6 enzyme activities in human liver microsomes (HLM), using enzyme-specific substrates (Table 1) phenacetin, tolbutamide, and dextromethorphan, respectively.

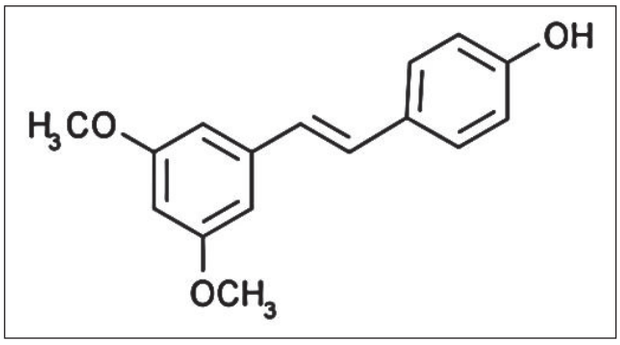

Fig. 1: Chemical structures of pterostilbene.

Table 1: Enzyme-specific substrates for CYP1A2, CYP2C9, and CYP2D6, with the corresponding metabolites and positive control inhibitors

Enzyme	Substrate	Metabolite
CYP1A2	Phenacetin	Acetaminophen
CYP2C9	Tolbutamide	4-Hydroxytolbutamide
CYP2D6	Dextromethorphan	Dextrorphan-D-tartrate

2. Investigations, results and discussion

The effect of pterostilbene at three concentrations on the activity of CYP1A2, CYP2C9, and CYP2D6 was examined using enzyme-specific substrates. The resultant enzyme activity data

after the incubation with pterostilbene were used to calculate the initial values of IC50 and volume /dose index (VDI).

The interaction between pterostilbene and the different drug-metabolizing enzyme was considered based on the initial VDI value. The VDI cut-off value was established according to the anticipated interaction between pterostilbene and the enzyme; the VDI cut-off value was defined to be two liters for enzymes expressed in the intestine, i.e., CYP2C9 and CYP2D6, and five liters for enzymes exist in the liver, i.e., CYP1A2 (Zanger and Schwab 2013). The coefficient of determination (*r2*) was used to determine the appropriateness of the inhibition curves fit, which was more than 0.9. A pterostilbene-enzyme interaction that suggested potential inhibition had a VDI value beyond the defined cut-off value and the lowest IC50 during preliminary screening (Table 2). Such interaction would be investigated more rigorously with a more extensive range of pterostilbene concentrations.

Table 2: Rough IC50 and volume/dose index (VDI) values for the inhibition of enzyme-specific metabolites formation by pterostilbene

Enzyme	RDI of Pterostilbene (mg)	IC50 (μM)	VDI (L/dose)
CYP1A2	250	56.3 ± 10.4	17.4
CYP2C9	250	0.7 ± 0.3	>50
CYP2D6	250	62.33 ± 11.4	15.7

Enzyme selective substrates and pooled HLM were incubated with at least three different concentrations of pterostilbene. All incubations were performed in triplicate. Nonlinear regression was applied and rough IC50 values were calculated by applying the IC_{50} equation. Values are reported as best fit IC_{50} (mean±standard error). All resulting values had r^2 value for goodness of fit of at least 0.9. The recommended daily intake (RDI) values were determined based on the commercially offered products and a previous clinical study.

Phenacetin, tolbutamide, and dextromethorphan were used as substrates to evaluate the inhibitory effect of pterostilbene on the enzyme activity of CYP1A2, CYP2C9, and CYP2D6, respectively. The substrate and pterostilbene were incubated with the HLM. The formation of acetaminophen, 4-hydroxytolbutamide, and dextrorphan-D-tartrate, which were the metabolites of phenacetin, tolbutamide, and dextromethorphan, respectively, were used as indicators for CYP1A2, CYP2C9, and CYP2D6 enzyme activities, respectively (Table 1).

The metabolite formation for acetaminophen and dextrorphan was inhibited by more the 50% (Fig. 2), with the rough IC50 values of pterostilbene at 56.3±10.4 μM and 62.33±11.4, respectively.

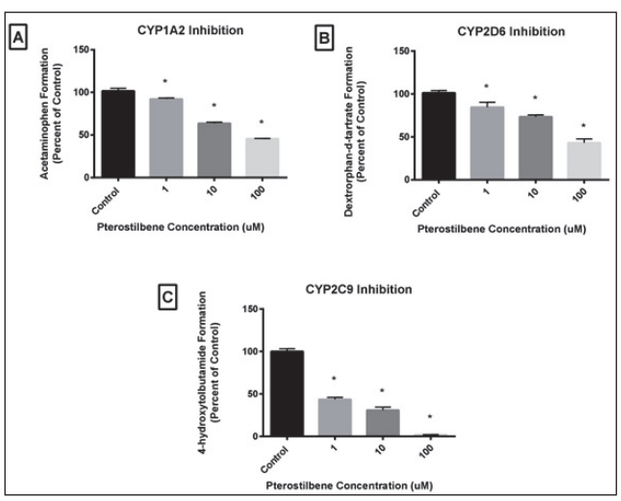

Fig. 2: Effects of pterostilbene on metabolite formation are shown as indexes of CYP1A2, CYP2C9, and CYP2D6 activity in HLM. Each enzyme-selective substrate, phenacetin, tolbutamide, and dextromethorphan for CYP1A2, CYP2C9, and CYP2D6, respectively, were incubated with HLM and three concentrations of pterostilbene at 1, 10, and 100 μM (n=3, mean±SE). *p < .05 compared to control.

Pterostilbene at 0.70 μM inhibited the CYP2C9-mediated metabolism by <50%. The VDI values were 17.4 L, >50 L, and 15.7 L for pterostilbene after the incubation with CYP1A2, CYP2C9, and CYP2D6, respectively (Table 2).

CYP2C9 was the most inhibited enzyme with an approximate IC50 value of 0.7 μM. Its VDI was significantly greater than the pre-identified cut-off at 50 L. Accordingly, CYP2C9 was chosen for further investigation. Its precise IC50 value of pterostilbene was determined to be 0.12±0.04 μM (Table 3 and Fig. 3). Moreover, the VDI value was confirmed to be more than 50 L.

Table 3: The precise IC50 and volume/dose index values for pterostilbene showing potent inhibition of 4- hydroxy tolbutamide formation from tolbutamide

Enzyme	IC50 (μM)	VDI (L/dose)
CYP2C9	0.12 ± 0.04	>50

Tolbutamide and pooled HLM were incubated with different concentrations of pterostilbene. All incubations were done in triplicate, and the data represent the best fit IC50 values±standard error. The r^2 values for the fit were above 0.9. The RDI values (250 mg) were decided based on commercially offered products and a previous clinical study.

Pterostilbene is abundant in blueberries and many trees, such as the Indian kino tree (*Pterocarpus marsupium*). It has been used for its potential astringent, antioxidant, and anti-inflammatory effects (Manickam et al. 1997; Maurya et al. 2004). Lately, in animal models, pterostilbene's anti-cancerous, anti-hypercholesterolemic, anti-hypertensive, and anti-diabetic activity have been reported (McCormack and McFadden 2013). In a recent clinical study, a 250 mg daily dose of pterostilbene was considered safe (Riche et al. 2013). In addition, investigators have found this dose is connected with reducing both systolic and diastolic blood pressure by 7.8 mmHg and 7.3 mmHg, respectively (Riche et al. 2014).

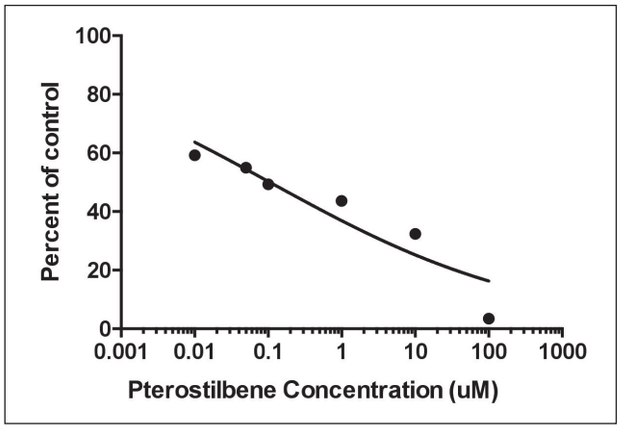

Fig. 3: Inhibition of 4-hydroxytolbutamide formation by pterostilbene. Tolbutamide, the substrate of CYP2C9, was incubated with HLM and multiple concentrations of pterostilbene. The data points represented the remaining CYP2C9 activity as a percent of the control (untreated) group. Data analysis was done through nonlinear regressions (n=3, mean±SE).

A large number of polyphenols are rapidly conjugated in the intestine after oral administration. Consequently, a small fraction of the free polyphenols is absorbed for systemic circulation. According to several animal studies, the bioavailability of pterostilbene is greater than that of resveratrol (Kapetanovic et al. 2011). Dellinger et al. (2014) identify UGT1A1 and UGT1A3 as the main enzymes for the metabolism of pterostilbene. Polyphenol metabolites are mostly excreted in the urine; however, few are excreted in the bile (Gao and Hu 2010; Asensi et al. 2011).

The bioavailability of pterostilbene is superior to resveratrol, yet it has a low water solubility and stability, which will affect its bioavailability and action. Lately, investigators try to solve

the problem through dosage modification, such as nanoemulsion formulations and aqueous solutions (Yeo et al. 2013; Zhang et al. 2014). These methods have been shown to improve pterostilbene's bioavailability and, therefore, increase its potential interactions with medications metabolized by the same enzymes.

The activity of CYP1A2, CYP2C9, and CYP2D6 enzymes was tested after the incubation with pterostilbene in HLM. Selective substrates were used to assess the activities of the enzymes in an *in vitro* model. Phenacetin was used to assess the activity of CYP1A2, tolbutamide for CYP2C9, and dextromethorphan for CYP2D6. In this study, the maximum inhibitory effect of pterostilbene was found to be on CYP2C9 activity because of the lowest IC50 and highest VDL values. Therefore, pterostilbene might have the ability to reduce the metabolism of CYP2C9 *in vivo*. The IC50 value of pterostilbene on tolbutamide (CYP2C9 substrates) was 0.12±0.04 μM. Pterostilbene's calculated VDI value passed 50L per dose unit with tolbutamide.

Of the tested enzymes, the *in vitro* results indicate that pterostilbene can significantly inhibit CYP2C9 activity, leading to potential interactions with medications metabolized by CYP2C9. Clinical evaluation is necessary to confirm the interaction between pterostilbene and CYP2C9 *in vivo*.

One fifth of all dispensed drugs is metabolized by the subfamily CYP2C, representing about 18% of the total microsomal CYP proportion in the liver (Paine et al. 2006; Van Booven et al. 2010; Daly et al. 2018). CYP2C9 is the most abundant enzyme in this subfamily and involve in the metabolism of angiotensin II receptor blockers, anti-inflammatory drugs (NSAIDs), oral anti-diabetic agents, and anticoagulant drugs, such as warfarin (Miners and Birkett 1998; Evans and Relling 2004). *In vitro* models have studied pterostilbene's effect on drug-metabolizing enzymes. Pterostilbene's inhibitory effects on CYP and UGT enzymes were evaluated using HLM. Pterostilbene was discovered to significantly inhibit CYP2C8 and UGT1A6 with IC50 values of 3.0±0.4 μM and 15.1±2.8 μM, respectively (Albassam and Frye 2019). Additionally, CYP2C19 activity was inhibited in HLM by pterostilbene at the IC50 value of 12 μM (Hyrsova et al. 2019).

In an earlier study using mouse liver microsomes, pterostilbene was found to be an inhibitor of CYP1A2 with K*i* = 390 μM (Mikstacka et al. 2006) suggesting that such inhibition is not likely to be observed *in vivo* since most of the polyphenols go through extensive and rapid metabolism in the intestinal tract in both human and animals (Kapetanovic et al. 2011; Wang and Sang 2018). In another study, Hyrsova et al. have examined the impact of various stilbenes including pterostilbene on the activities (and expression) of all major CYP isoforms. These study found only a mild inhibitory effect of pterostilbene on the activity of CYP2C9 (using diclofenac as probe) (Hyrsova et al. 2019). However, in our study we found that pterostilbene inhibited the CYP activity strongly. This difference in the results could be explained by the use of different substrates and that could influence the inhibitory effect of the inhibitor as it was reported by Kumar et al. (2006).

In another investigation, after being incubated with tolbutamide, the reported Ki (inhibitory constant) values of sulfaphenazole and amiodarone (well-known inhibitors of CYP2C9) were 0.22 μM and 0.81 μM, respectively (Kumar et al. 2006). The IC50 value of sulfaphenazole after incubated with tolbutamide in HLM was found to be 0.17 μM (von Moltke et al. 1998). Moreover, fluconazole, an antifungal and robust CYP2C9 inhibitor, inhibited CYP2C9 activity with the reported IC50 value of 18 μM (Giancarlo et al. 2001).

In this study, pterostilbene was found to inhibit tolbutamide metabolism by CYP2C9 at the IC50 value of 0.12 μM, which is smaller than those of sulfaphenazole and fluconazole. Furthermore, the VDI value was over 50 L; theoretically, this means a 250-mg dose of pterostilbene, even when diluted in more than 50 L, could still reach a concentration sufficient to reduce CYP2C9 by 50%. Thus, when pterostilbene is co-administered with CYP2C9 substrates, pterostilbene likely inhibits CYP2C9 activity, leading to undesirable effects.

Pterostilbene was found to be a potent inhibitor of CYP2C9 activity *in vitro* and increase CYP2C9's estimated VDI to over 50 L, indicating a possible interaction with the metabolism of CYP2C9 substrates *in vivo*. This warrants clinical studies to determine whether pterostilbene effect drugs metabolize through CYP2C9.

3. Experimental

3.1. Materials

Acetonitrile was received from Winlab (Leicestershire, UK). Phenacetin, dextrorphan-D-tartrate, and dextromethorphan hydrobromide (ICN Biomedicals, Inc., Eschwege, Germany) were acquired. Reduced β-nicotinamide adenine dinucleotide 2′-phosphate (NADPH) tetrasodium salt hydrate (Chem-Implex Int' l Inc., Wood Dale, IL, USA) and HLM (BD UltraPool HLM 150, protein concentration: 20 mg/mL) were purchased from BD Biosciences (Bedford, MA, USA). Potassium dihydrogen phosphate (Fisher Scientific, Leicestershire, UK), caffeine (Alfa Aesar, Ward Hill, MA, USA), and tolbutamide (Sigma–Aldrich, MO, USA), 4-hydroxytolbutamide (Cayman Chemical, MI, USA), and pterostilbene (Carbosynth Compton Laboratories, Berkshire, UK) were also purchased.

3.2. Pterostilbene preparation

The stock solution of pterostilbene was prepared in methanol. The stock solutions were diluted with water to have less than 10% organic solvent in the working solutions. Consequently, incubation samples and controls had less than 1% methanol. The initial investigation tested three concentrations of pterostilbene; later, an extensive series of concentrations across the rough IC50 were used for validation tests.

3.3. CYP1A2 incubation

A volume of 10 μL of pterostilbene at 1, 10, or 100 μM was mixed with 100 μM phenacetin in an Eppendorf tube. After HLM at 0.5 mg/mL and 0.1 M phosphate buffer at pH 7.4 were added, the samples were incubated in a 37 °C water bath for 5 minutes. Next, 1.0 mM NADPH was added to initiate the reaction at the final total volume of 500 μL. After incubating for 30 minutes, the reaction was stopped with 10μL of 70% perchloric acid and shaken for 2.0 minutes (Eagling et al. 1998; Kobayashi et al. 1998). The internal standard of 25 μL of 10 μM caffeine was added to all samples. The reaction was then vortexed for 2 min and centrifuged for 10 min at 12,000 rpm. A small amount of the supernatant was removed and injected into HPLC (Albassam et al. 2018; AlRabiah et al. 2018).

3.4. Acetaminophen analysis in HLM

As described previously, acetaminophen was analyzed using HPLC (Kobayashi et al. 1998; AlRabiah et al. 2018). Concentrations ranging from 1.0 to 15 μM of acetaminophen were measured at 245 nm to obtain the calibration curve. Next, the stocks of substrate, metabolite, and internal standard were dissolved in methanol. Finally, the chromatographic separation was then performed using a Nucleodur C18, 5.0 μm (4.6 × 250 mm) column. The mobile phase contained acetonitrile and 50 mM phosphate buffer at a ratio of 15:85 with a flow rate of 1.3 mL/min.

3.5. CYP2C9 incubation method

A 0.15 mM tolbutamide solution and pterostilbene at 1, 10, or 100 μM were mixed, followed by HLM at 0.25 mg/ml and a 0.1 M phosphate buffer pH 7.4. Then, samples were mixed, vortexed, and incubated for 5 min in a 37 °C water bath. The reaction was initiated with the addition of 1.0 mM NADPH to the final total volume of 0.5 mL. Next, the samples were incubated for 30 min and stopped with 250 μL of cold methanol (Al-Jenoobi 2010). Then, 25 μL of 1.0 μg/mL nitrazepam was added as an internal standard for each sample. Afterward, the samples were centrifuged for 10 min at 12,000 rpm. Finally, an aliquot of the supernatant was transferred to an HPCL vial and injected for analysis.

3.6. 4-Hydroxytolbutamide analysis in HLM

Symmetry C18, 5.0 μm (4.6 × 150 mm) was used to analyze the level of 4-hydroxytolbutamide in HLM. The mobile phase contained acetonitrile and 0.02 M potassium dihydrogen orthophosphate buffered at pH 3.4 in a ratio of 25:75 with the flow rate at 1.5 mL/min. Tolbutamide and 4-hydroxytolbutamide stock solutions were dissolved in acetonitrile and methanol, retrospectively. Concentrations of 4-hydroxytolbutamide, ranging from 0.05 to 5.0 μM, were measured at 230 nm to obtain the calibration curve (Korashy et al. 2015).

3.7. CYP2D6 incubation method

Dextromethorphan at 25 μM and pterostilbene at 1, 10, or 100 μM were added to tubes. Next, HLM at 0.5 mg/mL and 0.1 M phosphate buffer at pH 7.4 were mixed in and incubated in a 37°C water bath for 5 minutes. Then, 1 mM NADPH and 6 mM magnesium chloride were added to initiate the reaction and bring the final total volume to 500 μL. The samples were then incubated in a 37 °C water bath for 30 mins, stopped with 10 μL of 70% perchloric acid, vortexed for 2 min, and centrifuged at 12,000 rpm for 10 min. Finally, a portion of the supernatant was used for HPLC analysis (Al-Jenoobi et al. 2010, 2014).

3.8. Dextrorphan-D-tartrate analysis in HLM

Samples were analyzed by HPLC-fluorescence with a Nucleodur C18, 5.0 µm, 4.6× 250 mm column (Bendriss et al. 2001). The mobile phase contained acetonitrile and water with 0.1% trimethylamine and 1.5% glacial acetic acid, pH 3, in a ratio of 25:75. The flow rate was 1 mL/min. Dextrorphan-D-tartrate solutions with concentrations ranging from 0.5 to 5.0 µM were measured using a fluorescence detector set at λEX = 280 nm and λEM = 330 nm to obtain a calibration curve.

3.9. Statistical analysis

The enzyme activity was denoted as a control percentage. Equation (1) was used to predict IC50 values by fitting both the inhibitor pterostilbene data and the data of remaining enzyme activity in a sigmoidal concentration-response model. The VDI, or the volume in which one single dose should dissolve to get the equivalent IC50 concentration, was estimated to find a way to approximate the *in vivo* IC50 concentration (Eq. 2), according to Strandell et al. (2004).

Eq. (1) :

$$Y = 100 - \frac{100 \cdot [I]^H}{IC_{50}^H + [I]^H}$$

Y is the remaining enzyme activity (percent of control). H is the Hill coefficient. [I] is the concentration of pterostilbene.

Eq. (2)

$$VDI(L) = \frac{RDI}{IC50}$$

RDI is the recommended daily intake.

GraphPad Prism 6 (GraphPad, Software Inc., San Diego, CA, USA) was used to calculate the IC50 values. Statistical tests, such as the ANOVA test and then Dunnett's test were applied to evaluate the data, and a P-value equal to or smaller than 0.05 was considered significant.

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