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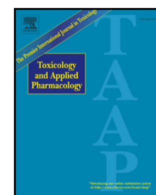

# Bioavailability of andrographolide and protection against carbon tetrachloride-induced oxidative damage in rats

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ABSTRACT

Andrographolide, a bioactive diterpenoid, is identified in *Andrographis paniculata*. In this study, we investigated the pharmacokinetics and bioavailability of andrographolide in rats and studied whether andrographolide enhances antioxidant defense in a variety of tissues and protects against carbon tetrachloride-induced oxidative damage. After a single 50-mg/kg administration, the maximum plasma concentration of andrographolide was 1 μM which peaked at 30 min. The bioavailability of andrographolide was 1.19%. In a hepatoprotection study, rats were intragastrically dosed with 30 or 50 mg/kg andrographolide for 5 consecutive days. The results showed that andrographolide up-regulated glutamate cysteine ligase (GCL) catalytic and modifier subunits, superoxide dismutase (SOD)-1, heme oxygenase (HO)-1, and glutathione (GSH) *S*-transferase (GST) Ya/Yb protein and mRNA expression in the liver, heart, and kidneys. The activity of SOD, GST, and GSH reductase was also increased in rats dosed with andrographolide ($p < 0.05$). Immunoblot analysis and EMSA revealed that andrographolide increased nuclear Nrf2 contents and Nrf2 binding to DNA, respectively. After the 5-day andrographolide treatment, one group of animals was intraperitoneally injected with carbon tetrachloride ($CCl_4$) at day 6. Andrographolide pretreatment suppressed $CCl_4$-induced plasma aminotransferase activity and hepatic lipid peroxidation ($p < 0.05$). These results suggest that andrographolide is quickly absorbed in the intestinal tract in rats with a bioavailability of 1.19%. Andrographolide protects against chemical-induced oxidative damage by up-regulating the gene transcription and activity of antioxidant enzymes in various tissues.

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## Introduction

Oxidative stress is generated when the balance between oxidation and antioxidation is disrupted. Under this condition, reactive oxygen species (ROS) are overproduced, which leads to oxidation of cellular macromolecules and damage to cellular functions. Oxidative stress is known to be associated with the development of chronic human diseases including cardiovascular disease, cancer, cataracts, and neurodegenerative diseases (Cooke et al., 2003). This explains why antioxidant phytocompounds such as flavonoids, organosulfur compounds, terpenoids, and carotenoids in fruits and vegetables display chemoprevention against ROS-related diseases (Hollman and Katan, 1997; Rahman, 2001; Boeing et al., 2012; Tsai et al., 2012). Large cohort studies have demonstrated an inverse correlation between total fruit and vegetable intake and risk of CVD (Hung et al., 2004) and gastric and esophageal cancers (Jeurnink et al., 2012). Similar biological activities of many herbs have also been attributed to their rich contents of flavonoids, terpenoids, and carotenoids (Moon et al., 2006).

To protect against ROS insult, an effective defense mechanism is critical. The inherent antioxidant defense system is composed of antioxidants including vitamin E, glutathione (GSH), and vitamin C and antioxidant enzymes including glutamate cysteine ligase (GCL), GSH peroxidase, GSH reductase, catalase, superoxide dismutase (SOD), heme oxygenase (HO), and GSH *S*-transferase (GST). GSH, a tripeptide, assists in the clearance of ROS and maintains the redox homeostasis (Lu, 2009). GCL catalyzes the rate-limiting step in GSH synthesis. It is a

*Abbreviations:* ARE, antioxidant response element; EMSA, electrophoretic mobility shift assay; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; HO, heme oxygenase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARSs, thiobarbituric acid-reactive substances.

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heterodimeric protein composed of catalytic (GCLC) and modifier (GCLM) subunits that are expressed by distinct genes (Franklin et al., 2009). SOD, both Cu/Zn- and Mn-SOD, quenches the superoxide anion and generates $H_2O_2$, and the $H_2O_2$ is then decomposed to $H_2O$ by catalase and GSH peroxidase. HO is responsible for degrading free heme into $Fe^{2+}$, carbon monoxide, and biliverdin, the latter being subsequently catabolized into bilirubin by biliverdin reductase (Ryter et al., 2006). GST catalyzes the conjugation of GSH with a variety of electrophilic xenobiotics and also displays selenium-independent GSH peroxidase activity (Reddy et al., 1981). In fact, both HO and GST are recognized as not only antioxidant enzymes but also phase II drug metabolizing enzymes. Higher antioxidant enzyme activity promises better protection of animals against oxidative injury.

Most antioxidant enzymes are inducible, and the nuclear factor erythroid 2-related 2 (Nrf2) plays a key role in up-regulating their transcription (Baird and Dinkova-Kostova, 2011). The transcription factor Nrf2 positively regulates the basal and inducible expression of a large battery of genes including not only the familiar antioxidant and phase II detoxification enzymes, but also the genes that control seemingly disparate processes such as immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis and metastasis, and even cognitive dysfunction and addictive behavior (Baird and Dinkova-Kostova, 2011; Hybertson et al., 2011). Under unstressed conditions, Nrf2 is retained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), which is constantly ubiquitinated and rapidly degraded through the proteasome pathway (Katoh et al., 2005). In response to oxidative and electrophilic stress, Nrf2 is released from Keap1 and quickly translocates into the nucleus, where the free Nrf2 binds to the antioxidant response element (ARE). The ARE is found in many antioxidant enzyme genes including GCLC, GCLM, SOD, cytosolic GSH peroxidase, gastrointestinal GSH peroxidase, GSH reductase, GST, and HO-1 (Taguchi et al., 2011). An increase of GSH content and antioxidant enzyme expression and activity ameliorate oxidative insults and prevent the incidence of oxidative-related diseases (Dai et al., 2007; Kumar et al., 2012; Venkateshappa et al., 2012).

Andrographolide, a diterpene lactone, is the most active and abundant terpenoid of *Andrographis paniculata* (Burm. f) (Pholphana et al., 2004). *A. paniculata*, a popular medicinal herb in Asia, is used to treat infections, colds, fever, inflammation, and diarrhea. In vivo and in vitro studies indicate that *A. paniculata* and andrographolide have diverse physiological activities, including antioxidant, anti-inflammatory, anti-atherosclerosis, anti-cancer, and hypoglycemic actions (Chao and Lin, 2010). The anticancer activity of andrographolide in a variety of cancer cells is attributed to its potency at inhibiting proliferation, inducing apoptosis and cell-cycle arrest, and modulating the immune response against these cells (Varma et al., 2011). In streptozotocin-induced diabetic rats, andrographolide and aqueous and ethanolic extracts of *A. paniculata* decrease the blood glucose level (Zhang and Tan, 2000; Husen et al., 2004) and induce glucose transporter 4 activity (Yu et al., 2003). Andrographolide suppresses intracellular adhesion molecule 1 expression in tumor necrosis factor α-activated vascular endothelial cells and leads to an inhibition of monocyte adhesion to the endothelial cells (Chen et al., 2011). Recent works have also indicated that andrographolide pretreatment inhibits carbon tetrachloride ($CCl_4$)- and cigarette smoke-induced mouse liver and lung injuries by suppressing inflammatory responses and increasing the GSH level and GSH peroxidase, GSH reductase, and HO-1 activity (Ye et al., 2011a; Guan et al., 2013). The modulatory effect of andrographolide on the antioxidation defense of tissues other than the liver and lung, however, is limited.

In this study, we firstly determined the pharmacokinetics and bioavailability of andrographolide in rats. Thereafter, we examined the modulation by andrographolide of the antioxidant defense in red blood cells and tissues including the liver, kidneys, and heart in rats. Finally, we investigated whether this modulation of antioxidant defense protects against $CCl_4$-induced damage.

## Materials and methods

*Chemicals and reagents.* Andrographolide, NADPH, GSH, GSH disulfide (GSSG), Ellman's reagent, 1-chloro-2,4-dinitrobenzene, pyrogallol, 2-thiobarbituric acid, 5,5′-dithiobis(2-nitrobenzoic acid), and methyl cellulose were obtained from Sigma (St. Louis, MO). TRIzol was purchased from Invitrogen (Carlsbad, CA). Carbon tetrachloride and acetonitrile were from Merck (Darmstadt, Germany). Fresh whole plants of *A. paniculata* were procured from Hualien, Taiwan. All other chemicals and reagents were of analytical grade and were obtained commercially.

*Animals and treatments.* Seven-week-old Sprague–Dawley rats were purchased from the Bio LASCO Experimental Animal Center (Taipei, Taiwan). The animals were fed a standard pelleted diet and were randomly assigned to the control, 30-mg/kg/day andrographolide, or 50-mg/kg/day andrographolide group ($n = 6$ per group). Rats were housed in plastic cages in a room kept at $23 \pm 1$ °C and $60 \pm 5\%$ relative humidity with a 12-hour light and dark cycle. Food and drinking water were available ad libitum. Andrographolide was suspended in 0.5% methyl cellulose and was intragastrically given (10 mL/kg) for 5 consecutive days. At the end of the experimental period, rats were fasted overnight and were then killed by exsanguination via the abdominal aorta while under carbon dioxide ($CO_2/O_2$, 70%/30%) anesthesia. Heparin was used as the anticoagulant.

Plasma and red blood cells were separated from the blood by centrifugation ($1750 \times g$) at 4 °C for 20 min. The liver, heart, and kidneys from each animal were excised, weighed, freeze-clamped in liquid nitrogen, and stored at −80 °C. Animals in this study were treated on the basis of the animal ethics guidelines of the Institutional Animal Ethics Committee.

For $CCl_4$ treatment, rats were intraperitoneally injected with 1 mL/kg $CCl_4$ (50% in olive oil, v/v) after being intragastrically dosed with 0, 30, or 50 mg/kg/day andrographolide for 5 days ($n = 8$ per group). Blood was drawn 24 and 48 h after $CCl_4$ treatment with heparin as an anticoagulant and the plasma was prepared for transaminase activity assay. The rats were then sacrificed as described above and the liver was removed for lipid peroxide determination.

For the andrographolide pharmacokinetic study, 7-week-old male Sprague–Dawley rats cannulated in the jugular vein were purchased from the Bio LASCO Experimental Animal Center (Taipei, Taiwan). The animals were fed a standard rat diet and were randomly assigned to a group treated with the ethanolic extract of *A. paniculata* (APE-treated, $n = 4$) and an andrographolide-treated group ($n = 3$). APE was prepared as described previously (Chen et al., 2013). Food and drinking water were available ad libitum. A single dose of 50 mg/kg of andrographolide or 940 mg/kg APE (equivalent to 50 mg/kg andrographolide), which was suspended in 0.5% aqueous methyl cellulose, was orally administered (10 mL/kg) to each rat. Serial blood samples with EDTA as an anticoagulant were collected up to 12 h after dosing from each rat. To determine the bioavailability of andrographolide, a group of animal ($n = 3$) were intravenously injected with andrographolide at a dose of 10 mg/kg.

*Preparation of cellular subfractions.* The frozen liver, heart, and kidneys were thawed and then homogenized (1:4, w/v) in ice-cold 100 mM phosphate buffer (pH 7.4) containing 1.5% KCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 10,000 $\times g$ for 30 min at 4 °C. The supernatant was further ultracentrifuged at 105,000 $\times g$ for 1 h and the final cytosol and microsome fractions were used for enzyme activity and immunoblotting assays. The frozen red blood cells were thawed and then hemolyzed (1:40, v/v) with hypotonic 5 mM Tris–HCl buffer, pH 7.4. After centrifugation at 10,000 $\times g$ for 10 min, the supernatant was used for enzyme activity determination. The protein content of the cytosolic and

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