**Effect of Tea Beverages on Aldehyde Oxidase Activity**

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Summary: Aldehyde oxidase (AO) plays an important role in metabolizing antitumor and antiviral drugs, including methotrexate, cyclophosphamide and acyclovir. Green tea and its catechins have been shown to modulate the activities of various xenobiotic-metabolizing cytochrome P450 species, both in vivo and in vitro, but their effect on AO has not been studied. Therefore, we evaluated the effect of tea beverages on AO activity in rat and human liver cytosol. We also investigated the influence of several catechins on AO activity in rat liver cytosol. AO activity was evaluated in terms of oxidation of N-1-methylnicotinamide to N-1-methyl-2-pyridone-5-carboxamide and N-1-methyl-4-pyridone-3-carboxamide. Bottled green tea beverages at 10% (vol/vol) inhibited AO activity by 90.0–93.5%, while at 1.0% (vol/vol), they reduced AO activity by 73.9–90.0%. At 0.1% (vol/vol), green tea II and III, which have high contents of catechins and their derivatives, inhibited AO activity by 24.3% and 38.8%, respectively. Bottled mineral water had no effect. AO activity was inhibited potently by epicatechin and epicatechin gallate. These results indicate that the AO-inhibitory activity of tea beverages is predominantly due to catechins and their derivatives. Thus, consumption of tea beverages may cause a decrease of AO activity, which may result in reduced clearance of drugs that are AO substrates.

Keywords: aldehyde oxidase; tea beverage; drug-drug interaction; catechin

**Introduction**

Aldehyde oxidase (AO, EC 1.2.3.1) catalyzes the oxidation of aldehydes and nitrogenous heterocyclic xenobiotics, including the oxidation of methotrexate to 7-hydroxymethotrexate.1–6 On the other hand, in the presence of its electron donor, AO catalyzes the reduction of sulfoxides, N-oxides, epoxides, aromatic nitro compounds and 1,2-benzisoxazole derivatives, including the reduction of sulindac to sulindac sulfide and the reduction of imipramine N-oxide to imipramine.7,8 AO metabolizes many medical drugs (e.g., methotrexate, acyclovir and cyclophosphamide), as well as physiological compounds such as retinaldehyde and monoamine neurotransmitters.9,10

The introduction of polyethylene terephthalate (PET) bottles has greatly increased the consumption of tea beverages in Japan.11 Green tea has been reported to have health benefits, including antioxidant, anti-inflammatory, ant carcogenic and antimicrobial activity.12–17 In addition, many foods, beverages, and dietary supplements containing high levels of phytochemicals are coadministered with medical drugs, and may lead to drug interactions.18–20 Green tea and its catechin constituents have been shown to modulate various xenobiotic-metabolizing enzymes in animal studies and in vitro systems.21–24 However, there is little information about their effects on AO, which metabolizes a number of drugs, including methotrexate and cyclophosphamide.

In this study, we examined the effect of bottled tea beverages on AO activity. Furthermore, we investigated the influence of catechin derivatives, caffeine and ascorbic acid on AO activity (Fig. 1).

**Materials and Methods**

Materials and chemicals: N-1-Methylnicotinamide (NMN) and N'-methylnicotinamide were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N-1-Methyl-2-pyridone-5-carboxamide (2-PY) and N-1-methyl-
4-pyridone-3-carboxamide (4-PY) were prepared according to Pullman and Colowick\textsuperscript{25} and Shibata et al., respectively.\textsuperscript{26} Ascorbic acid was purchased from Sigma Aldrich Japan Co., Ltd. (Tokyo, Japan). Flavonol was from Kanto Chemical Co., Inc. (Tokyo, Japan), and raloxifene was from LKT Laboratories, Inc. (Minneapolis, USA). Caffeine and catechin derivatives (catechin, epicatechin gallate, epigallocatechin gallate, gallocatechin gallate and epicatechin) were from Nacalai Tesque Co., Ltd. (Kyoto, Japan). All the bottled beverages were obtained commercially: bottled green tea beverages from Ito En Co., Ltd. (Tokyo, Japan) and Kao Co., Ltd. (Tokyo, Japan), blended tea beverage containing nonglutinous brown rice, green tea, Hordeum vulgare, etc.) from Coca-Cola Japan Co., Ltd. (Tokyo, Japan), oolong tea beverage from Suntory Foods Co., Ltd. (Osaka, Japan) and bottled mineral water from House Foods Co., Ltd. (Tokyo, Japan). Pooled human liver cytosols were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All beverages were diluted with distilled water as required. Caffeine and ascorbic acid were dissolved in distilled water and catechin derivatives, flavonol and raloxifene were dissolved in methanol and then diluted as required. The final concentration of methanol did not exceed 10.0%.

Animals: Jcl:SD rats (6 weeks old, male) were obtained from Clea Japan Co., Ltd. (Tokyo, Japan). The animals were housed in cages at 22 ± 2°C with a 12-h light/dark cycle and given free access to tap water and a standard pellet diet CE-2 (Clea Japan Co., Ltd., Tokyo, Japan).

The animal protocol was approved by the Animal Care and Use Committee of Hiroshima International University.

Liver preparations: Male rats were killed and their livers were excised and homogenized in four volumes of 1.15% KCl. The cytosolic fraction was obtained from the homogenate by successive centrifugation at 9,000 × g for 20 min and 105,000 × g for 60 min. Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as the standard protein.\textsuperscript{27}

Assay for aldehyde oxidase activity: AO activity was measured in terms of NMN oxidase activity.\textsuperscript{28} The amounts of 4-PY and 2-PY formed were measured by HPLC. Briefly, the incubation mixture consisted of 0.2 µmol of NMN with or without tea beverages or catechin derivatives and included liver cytosol equivalent to 50–100 mg of liver wet weight in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). Incubation was performed at 37°C for 40 min, 10 µg of N'-methylnicotinamide (internal standard) was added, and the mixture was extracted with five volumes of ethyl acetate. The extract was evaporated to dryness. The residue was redissolved in 0.1 ml of methanol, and an aliquot was subjected to analysis by HPLC with a Capcell pak C18 UG120 column (25 cm × 4.6 mm, Shiseido Co. Ltd., Tokyo, Japan) to separate 2-PY and 4-PY. The mobile phase was acetonitrile-water (3:97, vol/vol), the flow rate was 0.8 ml/min, and the detection wavelength was 254 nm. The elution times of 4-PY, 2-PY and N'-
methylnicotinamide (internal standard) were 8.0, 9.0 and 19.2 min, respectively.

**Assay for xanthine oxidase activity:** The assay was performed by measuring the oxidation of 1-methylxanthine to 1-methyluric acid. The incubation mixture consisted of 0.2 µmol of 1-methylxanthine with or without tea beverages or catechin derivatives and included liver cytosol equivalent to 50–100 mg of liver wet weight in a final volume of 0.5 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). Incubation was performed at 37°C for 40 min. After incubation, the reaction was stopped by addition of 500 µl of methanol, and 50 µg of acetaminophen (internal standard) was added to the mixture. After mixing, the solution was centrifuged at 3,000 × g for 5 min, and an aliquot of the supernatant was subjected to HPLC. 1-Methyluric acid concentration in the mixture was determined by HPLC on a Mightysil RP-18 (4.6 mm × 150 mm) column (Kanto Chemical Co., Inc., Tokyo, Japan) with ultraviolet detection at 280 nm. The elution times of 1-methyluric acid, 1-methylxanthine and acetaminophen (internal standard) were 15.0, 19.6 and 23.0 min, respectively.

**Statistical analysis:** Data are presented as mean ± standard deviation (S.D.). The statistical significance of differences was evaluated by using analysis of variance (ANOVA) followed by the Tukey honestly significant difference test. A value of \( P < 0.05 \) was considered significant.

**Results**

**Effect of green tea beverages on aldehyde oxidase activity in rat liver cytosol:** AO activity was significantly inhibited by 10.0% (vol/vol) bottled tea beverages (Fig. 2). The extent of inhibition by green tea I was 90.0 ± 4.1%, green tea II 92.8 ± 6.2%, green tea III 93.5 ± 5.3%, oolong tea 93.9 ± 2.7%, and blended tea 74.2 ± 3.7%. These beverages at 1.0% (vol/vol) also significantly inhibited AO activity; the extent of inhibition by green tea I was 73.9 ± 1.0%, green tea II 89.3 ± 3.7%, green tea III 90.0 ± 1.9%, oolong tea 65.9 ± 3.5%, and blended tea 26.5 ± 4.1%. Bottled mineral water used as a control had no effect. At a concentration of only 0.1% (vol/vol), green tea II reduced AO activity by 24.3% ± 7.0%, and green tea III reduced it by 38.8 ± 15.8%. The extent of inhibition of AO activity by the teas appeared to be concentration-dependent. For 0.1% (vol/vol) bottled tea beverages, the amount of 4-PY formation was equal to that of 2-PY formation. However, for 10.0% (vol/vol) bottled tea beverages, the amount of 4-PY was lower than that of 2-PY, i.e., 4-PY formation was inhibited more strongly than 2-PY formation (Fig. 2).

**Effect of green tea beverages on xanthine oxidase activity in rat liver cytosol:** Xanthine oxidase (XO) activity was moderately inhibited by 10.0% (vol/vol) bottled tea beverages in some cases (Fig. 3). The extent of inhibition by green tea I was 36.0 ± 4.31%, by green
tea II was 42.7 ± 3.70%, and by green tea III was 47.2 ± 3.21%. However, 10% vol/vol oolong tea, blended tea and mineral water were ineffective.

Effect of catechin derivatives on aldehyde oxidase activity:  
We examined the effect of catechin derivatives (catechin, epigallocatechin gallate, epigallocatechin, gallocatechin gallate and epicatechin), flavonol and caffeine, which are contained in tea beverages, on AO activity in rat liver cytosol.

AO activity was not inhibited by 100 µM caffeine. On the other hand, 100 µM catechin reduced AO activity by 77.4 ± 3.6%, 100 µM epicatechin by 73.7 ± 8.5% and 100 µM epigallocatechin by 60.1 ± 9.7%. The inhibition by these compounds appeared to be dose-dependent (Fig. 4).

Most catechin derivatives inhibited the formation of both 4-PY and 2-PY; however, epigallocatechin gallate and gallocatechin gallate were more potent inhibitors of the oxidation leading to 4-PY formation.

Effect of green tea beverages on aldehyde oxidase activity in human liver cytosol:  
At a concentration of 10.0% (vol/vol), the tea beverages inhibited human liver AO activity by 58.2–75.8% (Fig. 5). However, there was no significant difference among them. These results are consistent with those for rat liver AO.

Discussion

Aldehyde oxidase, a cytosolic enzyme, contains flavin adenine dinucleotide (FAD), molybdenum and iron-sulfur centers, and is closely related to xanthine oxidase. The two enzymes have a very close evolutionary relationship, based on recent cloning of the genes, and show a high degree of amino acid sequence homology. In this study, we evaluated the effect of bottled tea beverages and some of their constituents on AO activity.

The tea beverages, supplied in PET bottles, showed potent inhibition of AO activity (Fig. 2). It has been reported that the extract from PET has estrogenic activity. Moreover, Obach et al. observed that estrogen analogs tamoxifen and raloxifene are potent inhibitors of AO. The possibility that extract from PET bottles might have inhibited AO activity in our case seems to be ruled out by the observation that bottled mineral water had no effect.

On average, the bottled tea beverages contained 1466 ± 683 µM ascorbic acid. It has been reported that ascorbic acid affects drug-metabolizing enzymes such as cytochrome P450. However, the concentration of ascorbic acid in 10% vol/vol bottled tea beverages, estimated to be in the range of 100–200 µM, had no effect on AO activity.

We observed that green teas II and III were more potent AO inhibitors than the other beverages examined in this study. Furthermore, green tea III inhibited XO more potently than the other beverages (Fig. 3). The beverage companies claim that these drinks contain high levels of catechins and their derivatives. The main catechin component of green tea is epigallocatechin gallate. Therefore, we hypothesized that catechin derivatives or caffeine might affect AO activity.
Fig. 4. Inhibition of metabolism of NMN to total 4-PY and 2-PY (representing aldehyde oxidase activity) and inhibition of metabolism of NMN to 4-PY or 2-PY in rat liver cytosol by various catechins

Catechin was added to the mixture to give a final concentration of 0.5, 1, 5, 10, 50 or 100 µM. Each point represents the mean ± S.D. of 3 experiments. AO activity **p < 0.01, *p < 0.05 vs control (means ± S.D., n = 3).
As shown in Figure 4, catechin derivatives inhibited AO activity at concentrations of 5–50 μM. Nakamura et al. reported that bottled green tea beverages contain caffeine (531.5 μM; 103.1 mg/L), epigallocatechin gallate (427.9 μM; 196.0 mg/L), epigallocatechin (255.1 μM; 78.1 mg/L), galloccatechin gallate (153.9 μM; 70.5 mg/L), epicatechin (98.3 μM; 28.5 mg/L), catechin (88.3 μM; 25.6 mg/L) and epicatechin gallate (44.4 μM; 19.6 mg/L).³⁸ According to Nakamura et al.,³⁸ the concentrations of catechin derivatives in 10% (vol/vol) bottled green tea beverages were calculated as falling in the range 5–50 μM. Therefore, AO may be inhibited by the combination of catechin derivatives present in the bottled tea beverages.

Interestingly, AO activity was strongly inhibited by catechin, epicatechin and epicatechin gallate. These compounds have two hydroxyl groups at C-3′ and C-4′ of the flavonol skeleton. It is noteworthy that catechin, epicatechin and epicatechin gallate (with two hydroxyl groups at C-3′ and C-4′ of the flavonol skeleton) inhibited the oxidation processes leading to both 2-PY and 4-PY formation. On the other hand, galloyl gallate and epigallolochalcone galate (with a galloyl group at C-3 of the flavonol skeleton) potently inhibited only the oxidation process leading to 4-PY formation. Further study of the mechanisms of inhibition of AO activity would be of interest. In addition, further work is needed to investigate the inhibitory kinetics and the structure–AO-inhibitory activity relationship of catechin derivatives and tea beverage constituents.

AO is similar to XO in structure and amino acid sequence,³⁰–³² so that the mode of inhibition of AO by catechin derivatives might be similar to that for XO. Nagao et al. reported that epicatechin gallate and epigallolochalcone galate inhibited XO, whereas catechin and epicatechin did not.³⁹ On the other hand, in this study we found that catechin, epicatechin and epicatechin gallate inhibited AO, whereas galloyl gallate, epigallolochalcone and epigallo-

catechin gallate did not. Thus, the inhibition pattern of AO activity at concentrations of 5 μM; 196.0 mg/L; 50 μM. Therefore, AO may be inhibited by the combination of catechin derivatives present in the bottled tea beverages.

Moreover, we have reported that the oxidation of NMN is not inhibited by oxypurinol, an inhibitor of XO.⁴⁰ The activity was not enhanced by addition of NAD⁺/NADPH, the cofactors of aldehyde dehydrogenase. Therefore, the oxidation activity towards NMN may be due mainly to AO.

Rajagopalan and Handler reported that there were two mechanisms for the inhibition of AO activity.⁴¹,⁴² One is decreased affinity for the substrate-binding site,⁴¹ and the other is inhibition of the electron transport chain (molybdenum, FAD, coenzyme Q10, and iron) of AO.⁴² Catechins and their derivatives are antioxidants, and serve as electron acceptors. Therefore, they might inhibit AO activity via the electron transport chain.

Marked interspecies variation of AO activity has been reported.⁴³,⁴⁴ Therefore, we evaluated the effect of tea beverages on AO activity in human liver cytosol. The tea beverages inhibited human AO activity to 24.2–41.8% of the control (Fig. 5). The order of inhibitory potency of tea beverages for rat liver cytosol was green tea beverage III ≥ II ≥ I ≥ oolong tea, while that for human was green tea beverage I = oolong tea ≥ II ≥ III. Our results confirm a species difference between humans and rats, as suggested by Schofield et al.⁴⁴

AO has been detected in liver, small intestine, stomach, vagina, penis, oral and nasal cavities, esophagus, tongue and uterus.⁷ Tea beverages and catechins, administered orally might inhibit AO in small intestine and influence the first-pass effect for AO substrates.

In addition, Donovan et al. reported that the maximum concentration of epigallocatechin gallate in plasma was 6.1 μM when green tea extract was administered for 2 weeks to humans.⁴⁵ The maximum concentrations of catechins in plasma after single dose administration of green tea catechin derivatives mixture Polyphenon E (comprising 1200.0 mg epigallocatechin gallate, 291.0 mg epigallocatechin, 205.2 mg epicatechin, 120.0 mg epicatechin gallate, and other tea catechins) were 8.7 μM (epigallocatechin
gallate), 1.1 µM (epigallocatechin), 0.9 µM (epicatechin) and 1.0 µM (epicatechin gallate). Considering the concentrations of catechin derivatives in bottled tea beverages, general drinking of tea beverages in moderate amounts may not significantly affect AO activity. However, AO activity might well be inhibited in people taking dietary supplements with high levels of catechin derivatives, potentially leading to increased risk of severe side effects, including leukopenia, thrombocytopenia, liver failure, renal failure and nephropathy, if these people are also being treated with drugs such as methotrexate and cyclophosphamide.

In conclusion, the tea beverages examined inhibit aldehyde oxidase activity in vitro. It appears that this effect is predominantly due to catechin constituents. Further studies of the effect of tea beverages on aldehyde oxidase activity in vitro seem worthwhile.

References

32) Terao, M., Kurosaki, M., Saltini, G., Demontis, S., Marini, M.,


