

# Diverse inhibition of plasminogen activator inhibitor type 1 by theaflavins of black tea

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**Abstract.** Fruits, vegetables, spices and a variety of teas are suggested for the prevention of many diseases. They encompass active, non-nutritional ingredients called nutraceuticals which are defined as food products that provide health benefits. Many nutraceuticals have been tested to identify inhibitors of plasminogen activator inhibitor (PAI-1). PAI-1 is the major and fast acting physiological inhibitor of fibrinolysis. However, preclinical studies of PAI-1 inhibitors have revealed an additional role of PAI-1 in the pathogenesis of vascular remodeling, renal injury, diabetes, obesity, Alzheimer's disease and cancer. Thus PAI-1 is a potential therapeutic target in some of these diseases. Our previous study revealed that a black tea extract (containing mostly theaflavins) inhibits PAI-1. In this study we report results for four pure (>98%) theaflavins. Inactivation of PAI-1 was tested by clot formation and by its lysis using thromboelastometry and measurements of human plasma turbidity. Among four tested theaflavins, theaflavin-3'-gallate was the most potent in PAI-1 inhibition trailed by theaflavin-3,3'-digallate, while the other two i.e., theaflavin and theaflavin-3-gallate did not show inhibitory activity.

## Introduction

Fruits, vegetables, spices and a variety of teas have been widely investigated for the prevention of many diseases because of their many non-nutritional ingredients called nutraceuticals, which are defined as food products providing health benefits.

Examples of well known nutraceuticals include: allicin (garlic), capsaicin (red chili), curcumin (turmeric), catechins (green tea), ellagic acid (pomegranate), genistein (soybean), lycopene (tomato), resveratrol (red grapes, peanuts and berries) (1,2). Many nutraceuticals are strong antioxidants and are therefore, considered as possible modulators of many human diseases related to oxidative stress (for example: neurodegenerative diseases, stroke and others). However, additional mechanisms of their action include direct inhibition of enzyme activity; for example curcumin inhibits cyclooxygenases 1, 2 (COX-1, COX-2), lipoxygenase (LOX) (3) and acts on multiple cell signaling pathways (NF- $\kappa$ B, TNF, IL-6, IL-1, COX-2 and 5-LOX) (4,5). Furthermore, nutraceuticals are usually a mixture of different chemicals, for instance curcumin contains three active major constituents (6). Others could be a mixture of derivatives and isomers and such complexity of the nutraceuticals complicates the understanding of their mechanism of action.

We have been evaluating the inhibition of plasminogen activator inhibitor type 1 (PAI-1) by nutraceuticals. PAI-1 is the major and fast acting physiological inhibitor of fibrinolysis. However, preclinical studies of PAI-1 inhibitors have revealed an additional role of PAI-1 in vascular remodeling, cancer, renal injury, diabetes, obesity and Alzheimer's disease. Inhibition of PAI-1 is a potential therapeutic target in these diseases (7-9). Since PAI-1 quickly converts itself into a latent form (PAI-1,  $t_{1/2} = 2$  h) we have chosen its mutant with an extended half-life for this study (VLHL PAI-1,  $t_{1/2} \geq 700$  h) (10). Using a black tea extract containing 80% of theaflavins including theaflavin-3-3'-digallate and in smaller amounts theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin, we have found that such extract inhibits VLHL PAI-1 (11). Since it has been reported that PAI-1 is implicated in Alzheimer's disease, obesity, diabetes and others, and that drinking black tea can alleviate symptoms of these conditions, understanding which of the theaflavins inhibits PAI-1 might have profound therapeutic implications (12-15). We report that two of the studied black tea theaflavins (theaflavin-3,3'-gallate and theaflavin-3'-gallate) are good inhibitors of PAI-1 while the other two (theaflavin, theaflavin-3-gallate) are not.

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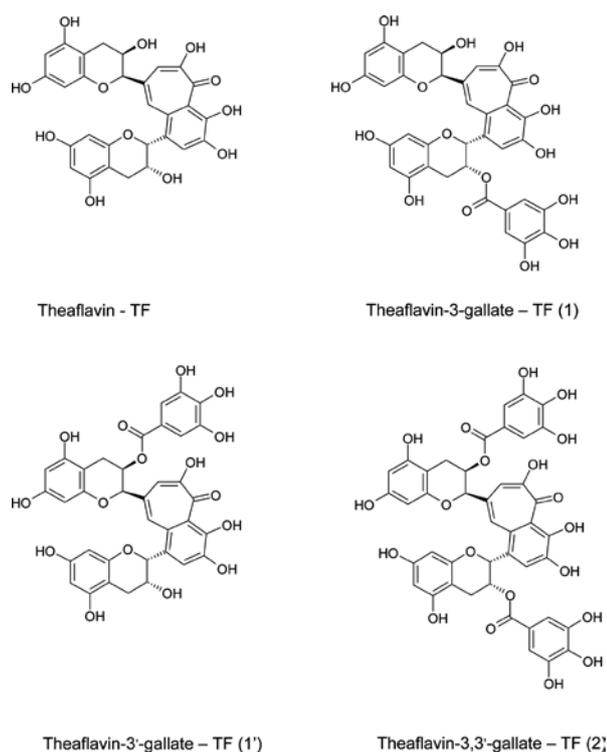


Figure 1. Structure of theaflavins (29).

## Materials and methods

**Chemicals.** Theaflavin (no. F3001), theaflavin-3-gallate (no. F3002), theaflavin-3'-gallate (no. F3003), theaflavin-3-3'-digallate (no. F3004) (all of >98% purity) were purchased from PI & PI Technology, Inc., (Guangzhou, Guangdong, China) (Fig. 1). Theaflavins were dissolved in DMSO and stored at  $-20^{\circ}\text{C}$ .

**Expression and purification of VLHL PAI-1.** The mutation of two amino acids (Gln197→Cys, Gly355→Cys) produced VLHL PAI-1 with a very long half life  $t_{1/2} > 700$  h. To confirm the mutations, the VLHL PAI-1 DNA construct was sequenced (MWG-Biotech Inc., NC). A *Baculovirus* expression system was employed and purification (+95%) of this protein was carried out as previously described (16). Aliquots of the protein were stored at  $-75^{\circ}\text{C}$ .

**Tissue plasminogen activator.** Fully active human tPA (HTPA-TC) was purchased from Molecular Innovations, (Novi, MI). Protein aliquots were stored at  $-75^{\circ}\text{C}$ . Pooled normal human sodium citrated plasma was from American Diagnostica (Stamford, CT, no. 258N) for TEG experiments, and from Helena Laboratories (Beaumont, TX, no. 5301), for spectrophotometric determination of lysis. Lyophilized plasma was stored at  $4^{\circ}\text{C}$ , and the aliquots of plasma were reconstituted in individual vials with 1.0 ml of deionized water before analysis. The vials were allowed to stand at room temperature with periodical swirling for 15-20 min before use.

**Analysis of plasma clot formation with thromboelastography.** Thromboelastography, not only allows for the measurement of a global coagulation profile, but also yields data on the kinetics

Table I. Changes of thromboelastograms and spectrograms as a function of proteolysis status.

	Control	tPA treated	tPA + VLHL PAI-1	tPA + VLHL PAI-1 + Inhibitor
tPA	-	+	+	+
VLHL PAI-1	-	-	-	+
Inhibitor	-	-	-	+
Expected thromboelastogram				
Expected Spectrogram				

and dynamics of clot formation and clot lysis in whole blood or in plasma (17). The critical part of this instrument is a pin hanging on a torsion wire and suspended in a cup holding a sample (360  $\mu\text{l}$ ). This pin oscillates at 6 rpm at a  $4^{\circ} 45'$  angle at  $37^{\circ}\text{C}$ . When plasma changes viscosity during clot formation this pin motion is progressively restrained by the clot and the cup. The strength of the clot determines the degree of the force on the pin. Sodium citrated, reconstituted plasma was used for TEG assays by mixing 1 ml of plasma with 20  $\mu\text{l}$  of kaolin (Haemoscope Co., Neils, IL, USA) to which a constant amount of tPA was added [10  $\mu\text{l}$  of tPA (2.1 mg/ml in 0.4 M HEPES, 0.1 M NaCl; pH 7.4)] as a fibrinolytic agent (18) to measure proteolysis under controlled conditions. Next, 320  $\mu\text{l}$  of the mixture was transferred to each TEG cup containing 20  $\mu\text{l}$  of  $\text{CaCl}_2$  (0.2 M) and an activity assay buffer (50 mM HEPES, 150 mM NaCl, 1% human serum albumin, 0.05% Tween-20 buffer, pH 6.6) with i) VLHL PAI-1 in activity assay buffer to prevent lysis by tPA, or ii) VLHL PAI-1 plus the tested compound in the same buffer to check its inhibitory action demonstrated by lysis of the clot when tPA is unopposed by PAI-1 activity (Table I and Fig. 2). The critical parameters of clotting measured by TEG are as follows: R is the time from the start of the reaction until a measurable clot is detected, K is the time from the R point until a certain clot firmness is achieved, An ( $\alpha$ ) is the maximum angle that represents kinetics of clotting and LY30 (percentage) represents clot lysis 30 min after MA (maximum amplitude) (Table II).

### Analysis of plasma clot formation measured by turbidity.

For plasma turbidity measurements (19) reconstituted human plasma (1 ml) was added to a vial to which a constant amount of tPA was added [10  $\mu\text{l}$  of tPA (2.1 mg/ml in 0.4 M HEPES, 0.1 M NaCl; pH 7.4)] as a fibrinolytic agent. The mixture was divided into two parts and the following was added to each part: i) VLHL PAI-1 to prevent lysis by tPA, or ii) VLHL PAI-1 plus tested compound to check its inhibitory action demonstrated by lysis of the clot when tPA is unopposed by PAI-1 activity. Prior to that VLHL PAI-1 was incubated for 15 min with either DMSO or different compounds in DMSO. Clot formation and dissolution was monitored at 405 nm every 60 sec at  $37^{\circ}\text{C}$  using a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). In this assay, clot formation is indicated by an increase in OD. Over time, the OD decreases due to clot dissolution by fibrinolytic processes, which reaches the initial OD

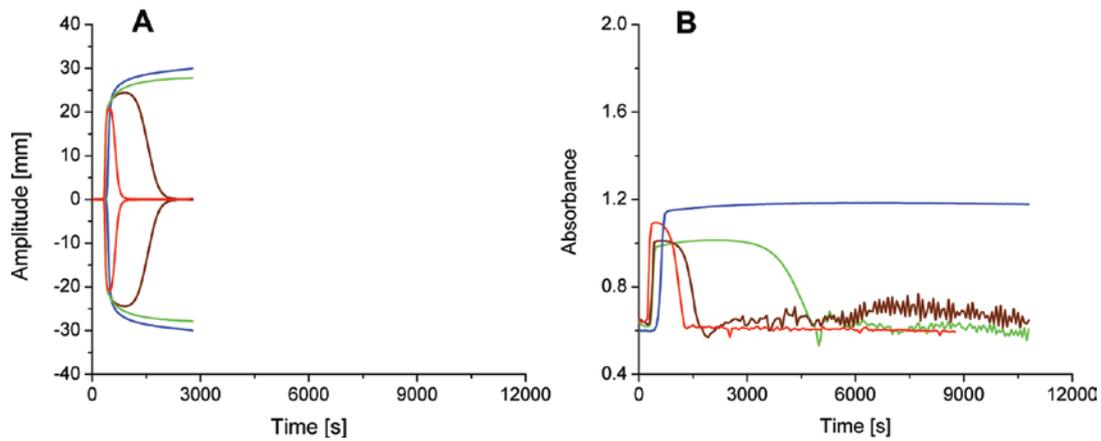


Figure 2. Example of (A) thromboelastogram and (B) turbidity, of clotted plasma not treated (control, blue); treated with tPA (red); treated with tPA and VLHL PAI-1 (green); and treated with tPA, VLHL PAI-1 and TF(1') (brown).

when the clot is completely dissolved. The critical parameters of clotting measured by this method are as follows:  $R_T$  is the time from the start of the reaction until a measurable OD is detected, and  $LY_{T30}$  (percentage) represents clot lysis 30 min after maximum OD (Table III).

**Results**

Initial assessment of PAI-1 inactivation by theaflavins was done by thromboelastometry and standardized to control of pooled normal human plasma. In general we observed differences between parameters of control plasma and treated samples as can be seen in Table II. There are no normal values

of TEG parameters for plasma in the literature. However,  $R$ , 120-480 sec;  $K$ , 60-180 sec;  $An$ , 55-78°C (this is  $\alpha$ -angle);  $MA$ , 51-69 mm and  $LY_{30}$ , 0-8% for blood samples are considered normal (20). Presented values in Table II for plasma are within this normal range. Only,  $MA$  was consistently lower in all plasma samples than in normal blood presumably due to an absence of platelets which are critical for clotting but absent in plasma.

Two of the black tea theaflavins inactivate PAI-1 as showed in Table II (theaflavin-3,3'-gallate and theaflavin-3'-gallate) while the other two (theaflavin, theaflavin-3-gallate) do not show inhibitory activity. Most potent was theaflavin-3'-gallate while theaflavin-3,3'-gallate was less potent.

Table II. TEG clotting parameters of control plasma and plasma treated with tPA+VLHL PAI-1 and theaflavins (N=4).

Compound	R (sec)	K (sec)	An (°)	MA (mm)	LY30 (%)
Control plasma	408±6	84±6	70.6±0.3	27.2±0.6	0
TF 44 μM	336±24	76±6	75.2±2.1	25.1±0.4	0
TF(1) 34 μM	354±18	72±12	76.4±0.3	26.5±1.1	0
TF(1') 34 μM	330±12	72±6	75.7±1.0	24.3±0.6	36.6±30.9
TF(2) 28 μM	348±10	72±18	75.7±1.0	24.7±0.8	30.0±21.0

Table III. Turbidity measurements of clotting parameters control plasma and plasma treated with tPA+VLHL PAI-1 and theaflavins in different concentrations (N=4).

Compound	Concentration (μM)	$R_T$ (sec)	$LY_{T30}$ (%)
TF	44	340±81	0
TF(1)	34	360±60	0
TF(1')	34	240±60	100
TF(1')	17	260±80	100
TF(1')	8	300±60	64.2±2.1
TF(2)	28	300±60	46.3±5.8
TF(2)	14	240±60	13.8±3.8
TF(2)	7	300±60	1.7±0.6

Evaluation of PAI-1 inhibition was repeated by analysis of clot formation and lysis measured by turbidity. In this method comparison of K, An and MA to TEG analysis is meaningless. However R and R<sub>T</sub> as well as LY<sub>30</sub> and LY<sub>T30</sub> can be judged against each other. Within experimental errors the values of R and R<sub>T</sub> were the same. PAI-1 inhibition by theaflavins was similar as observed in another method, but turbidity analysis showed higher inhibitory activity of theaflavins as manifested by LY<sub>T30</sub> values (Table III). That should not be considered as a surprise since mechanical stress on fibrin during TEG measurement confers proteolytic resistance to fibrin, which is related to poor tPA binding and penetration in the fibrin network and consequently weaker plasminogen driven proteolysis (21). The pattern of inactivation of PAI-1 by theaflavins based on turbidity measurements was the same as in the TEG method.

## Discussion

Nutraceuticals including plant extracts are gaining popularity in disease prevention, but are also frequently utilized in uncontrolled therapy. For example, it is estimated that over 50% of patients diagnosed with cancer explore herbal medicine or extracts of different plants (22). Thus, understanding the possible effects of a common beverage such as black tea is important. PAI-1 could have acute effects in the management of PAI-1 affected ailments.

There is very limited literature on PAI-1 and black tea and the findings are controversial. Vorster *et al* investigated the antigen level of PAI-1 in a group of volunteers given 6 mugs of tea daily (23). They did not observe differences between people that drank tea and those drinking hot water. However, compliance with tea intake was measured by adding p-aminobenzoic acid to the tea bags and measuring its recovery in 24 h urine collections. This acid is an inhibitor of tPA and uPA and could influence measurements of PAI-1 (23,24). Contrary to that Loktionov *et al* found that tea caused a significant decrease of PAI-1 activity in the subjects with E2/E3 genotype (mean placebo 7.21 U/ml vs. mean tea 5.88 U/ml, P=0.007) (25). ApoE has six common isoforms: E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4. These are products of the ε2, ε3 and ε4 alleles of the ApoE gene (26). Estimated human genotype frequency of ApoE E2/E3 is in the range of ~15% of the general population (26). These results provide evidence that dietary intervention may be very effective in population groups with specific genetic makeup rather than in the general population. Moreover, it is difficult to compare the effects of black tea consumption in the absence of basic information on the concentration of theaflavins in the cup, and form of the tea, for example with lemon (changes in pH) or milk (high in Ca<sup>2+</sup>) vs. without any of them. Also, the content of total theaflavins as well as individual theaflavins can vary depending on the brand of tea and the brewing method making it difficult to draw conclusions from human studies of tea drinkers. But, assuming good bioavailability of theaflavins and conversion of other components of black tea (for instance thearubigins) to theaflavins in the acidic environment of the stomach, it is possible to reach their PAI-1 inhibitory concentration in the blood of the black tea heavy drinkers (27,28). In conclusion, among four tested theaflavins theaflavin-3'-gallate and theaflavin-3,3'-gallate inhibit PAI-1

while theaflavin and theaflavin-3-gallate do not. Theaflavin-3'-gallate was most potent while theaflavin-3,3'-gallate showed lower inhibitory activity.

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