# Analysis of the inhibition of PAI-1 by metal theaflavin complexes and their degradation products

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**Abstract.** The inhibition of elements of the plasminogen activator system [urokinase (uPA), tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)] plays an important role in human diseases. PAI-1 is overexpressed in obesity and diabetes, and the inhibition of this protein has been postulated to alleviate the symptoms of both disorders. We found that two theaflavins (TFs) from black tea inhibit PAI-1 and we suggest that the beneficial effects of drinking tea may be associated with the suppression of PAI-1 activity by theaflavins. Epidemiological studies are controversial; some studies show the beneficial effects of drinking black tea on obesity and diabetes, while others do not. TFs, a family of compounds that can comprise up to 40% the dry weight of black tea, are responsible for the characteristic color, and they are known to chelate metals. We hypothesized that the content/variety of metals present in drinking water may be one of the reasons for such controversies in the population studies. TFs are excellent chelating compounds by trapping metals into complexes; thus, the quality of water used for tea preparation may influence changes in the formation of new products according to TF affinity for different metals, as well as their high molecular weight oxidation products. Our modeling and docking studies suggest that TF/metal complexes have similar affinity to PAI-1 as native TFs. However, analyses using liquid chromatography-mass spectroscopy (LC-MS) revealed the presence of TF degradation products in tea brewed using water containing metal salts. These can further form high molecular weight oxidation products. Thus, metals present in tea could diminish the beneficial effects of black tea by reducing TF concentration via metal-induced degradation and precipitation.

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## Introduction

The inhibition of the elements of plasminogen activator system [urokinase (uPA) and/or tissue plasminogen activator (tPA), as well as plasminogen activator inhibitor-1 (PAI-1)] plays a role in a number of human diseases, including cancer, diabetes, kidney diseases, blood clotting disorders and others (1-5). PAI-1 is overexpressed in obesity and diabetes and the inhibition of this protein has been postulated to alleviate the side-effects of obesity and diabetes (2,6). It has been shown that weight reduction substantially reduces plasma PAI-1 levels in obese individuals (7,8). Moreover, PAI-1 inhibitors have been shown to reduce dietary fat-induced obesity in experimental animals followed by the reduction of circulating active PAI-1 levels in plasma (7,9). It is estimated that the pandemic of diabetes is caused by the rapidly growing prevalence of obesity (10). PAI-1 levels are elevated in type 2 diabetes and these levels correlate with the complications of diabetes (11). Festa *et al* (12) investigated the correlation between the incidence of diabetes and changes in PAI-1 levels over the course of five years. The increase in PAI-1 levels was associated with the rising glucose levels and the development of type 2 diabetes, which was confirmed in 140 out of 843 individuals. Moreover, it has been reported that PAI-1 inhibitors may be used as therapeutic agents in obesity-related diabetes which later results in kidney nephropathy (13,14).

In our previous studies, we found that two theaflavins (TFs) of black tea inhibit PAI-1 and postulated that the beneficial effects of drinking tea may be associated with the inhibition of PAI-1 by TFs (15,16). It has been revealed that drinking black tea, but not green tea, reduces the risk of type 2 diabetes in a clinical study conducted on a large population of 36,908 females and males of Singapore and Chinese origin (17). It was found that four or more cups of coffee per person caused a 30% reduction in risk of type 2 diabetes in comparison with non-daily consumers. Participants consuming one or more cups of black tea/day had a 14% reduction in the risk of diabetes and no reduction in diabetes risk was observed in the group that drank green tea (17). Beresniak *et al* (18) investigated tea consumption in 50 countries, based on 2009 sales data and analyzed World Health Organization data for the same countries on the occurence of diabetes. They reported a strong linear correlation between low rates of diabetes in countries where the consumption of black tea was high. However, these

results were not supported by Boggs *et al* (19) who investigated the effects of coffee, tea and alcohol consumption on diabetes risk in 46,906 African American women over a 12-year period. Their results showed that African American women who drank moderate amounts of coffee or alcohol had a reduced risk of type 2 diabetes, but drinking tea was not associated with the decreased risk of diabetes.

TFs are a family of compounds that can comprise up to 40% the dry weight of black tea. They are responsible for the characteristic color (20), and they are known to chelate metals (21,22). We hypothesized that one reason for such controversies in the cohort studies may be differences in the presence and type of metals in the drinking water used for tea brewing. The type of metal could influence changes in the affinity for TF/metal formation and could stimulate the creation of new products induced by the presence of metals, or oxidation products of high molecular weight. Our modeling experiments revealed that TF/metal complexes if formed, would have a similar affinity to PAI-1 as native TFs. However, liquid chromatography-mass spectroscopy (LC-MS) revealed the presence of TF degradation products that can form high molecular weight oxidation products rather than metal theaflavin complexes. Thus, metals present in tea or water can reduce the TF concentration by metal-induced precipitation and may thus reduce the health benefits of black tea.

## Materials and methods

*Molecular modeling*. The structure of PAI-1, entry 3r4l, was downloaded from the RCSB Protein Data Bank (23). Two dimensional (2D) TF structures were created by Accelrys Draw version 4.0 in 'SKC' format. 2D structures were converted into three dimensional (3D) and 'PDB' format files by web based programs (http://pasilla.health.unm.edu/tomcat/biocomp/convert or http://www.molecular-networks.com/products). The docking TFs or their products to PAI-1 was performed using AutoDock Vina (24). A search box was set up with following parameters: center: x=-4.1, y=10.2, z=29.3; size: x=50, y=50, z=50 that include approximately a third of the PAI-1 molecule and the entire reactive center loop of PAI-1 with its active site. TF molecules were kept flexible by allowing rotation around single bonds. By default, AutoDock Vina analyzes eight different protein/TF complexes (different conformers) and the one with the lowest free energy is considered the most probable. Free energy can be converted to $K_i$ by the following formula (24-28):

$$K_i = \exp(\Delta G/(R^*T))$$

The final analyses of structures generated by AutoDock and figure generation were performed utilizing PyMOL version 1.4 (29,30).

*Tea brewing*. Black tea was brewed under controlled conditions. Specifically, a Kroger brand (Tetley) value pack of 80 teabags was purchased at a local grocery store. Tea bags were paired and weighed out (typically between 2.50-2.70 g) to possibly equalize the concentration of the tea components. The ions chosen for detection in this experiment were: calcium $(Ca)^{2+}$, iron $(Fe)^{3+}$, magnesium $(Mg)^{2+}$, manganese $(Mn)^{2+}$ and sodium $(Na)^{+}$. The concentrations of stock solutions were: 4 g/1,000 ml of metal chloride salts i.e., 0.4% (w/v), and were further diluted to 0.04, 0.004 and 0.0004% (w/v). Two 500-ml volumetric flasks were filled up to 250 ml, one with distilled water for the control, and the other with the solution of the specified metal concentration, heated to 90˚C and tea bags were immersed into the flasks. Tea was brewed for 5 min and stirred once every minute. Subsequently, the tea bags were removed from the brew and flasks were placed on ice to delay chemical reactions. Tea samples were centrifuged at 300 x g for 5 min, precipitants were dried under a vacuum, and supernatants were diluted 1:50 with distilled water for spectroscopy analysis (250-800 nm).

*Mass spectrometry*. Samples were analyzed at the Small Molecule Mass Spectrometry Facility at the Harvard FAS Center for Systems Biology, Cambridge, MA. Precipitants were solubilized in 200 $\mu$l water or 200 $\mu$l water/acetonitrile (50:50 v/v) before being run by LC-MS using a high accuracy Agilent ESI-TOF mass spectometer (Agilent Technologies, Santa Clara, CA) and a 45-min reverse phase gradient elution from 0 to 100% acetonitrile (0.1% formic acid). The data were examined for ions of the target formulas: $C_8H_{10}N_4O_2$, $C_{29}H_{24}O_{12}$, $C_{29}H_{22}MeO_{12}$, $C_{29}H_{20}Me_2O_{12}$, $C_{36}H_{28}O_{16}$, $C_{36}H_{26}MeO_{16}$, $C_{36}H_{24}Me_2O_{16}$, $C_{43}H_{32}O_{20}$, $C_{43}H_{30}MeO_{20}$, $C_{43}H_{28}Me_2O_{20}$, $C_{58}H_{46}O_{24}Me$, $C_{72}H_{54}O_{32}Me$, $C_{86}H_{62}O_{40}Me$, $C_{43}H_{28}Me_2O_{20}$, $C_{29}H_{24}O_{13}$, $C_{29}H_{22}O_{13}$, $C_{28}H_{22}O_{11}$, $C_{29}H_{22}Me_2O_{12}$; where metals can be: Fe, Ca, Mg, Mn and Na.

## Results

*Brewing tea*. Brewing tea in solutions of different salts ($Ca^{2+}$, $Fe^{3+}$, $Mg^{2+}$, $Mn^{2+}$ and $Na^{+}$) at the highest concentrations leads to drastic changes in color and produces precipitates of some of the tea constituents. Changes in color and amount of precipitants were depended on the salt concentration. The most dramatic changes were observed in the case of Fe; other metals produced less precipitation and no precipitation was observed in the case of Na at all concentrations. Examples of spectrograms are shown in Fig. 1.

*Molecular modeling*. We have previously shown that PAI-1 is inhibited by theaflavin-3'-gallate and theaflavin-3,3'-digallate and these bind to PAI-1 in proximity of its active site (15,16,31). Both TFs can react with metal cations as shown in Fig. 2 (22,32). That includes the interaction of TFs with Me(II) and Me(III) forming complexes (22,33,34). The number of possible isomers ranges from two in case 1:1 cation:TF molecule (Fig. 2, structures 1-2 and 2-2) to 81 in case 1:2 cation:TF molecules (Fig. 2, structures 1-4 and 2-4). For this reason, we performed molecular modeling for the limited number of molecules to determine whether any differences can be detected in the binding site or binding affinity to PAI-1. We analyzed total of 32 native and metal/TF molecules. Some TF structures with Mn were not converted into 3D models and these are marked as 'NC' in Table I. Examples of the binding site are shown in Fig. 3, which were binding sites for all tested metal/TF and TF molecules. We were not able to convert dehydrotheaflavins into 3D structures.

The theoretical binding affinity of degradation products of TFs, such as theanaphthoquinone and theabenzoquinone suggested by O'Coinceanainn *et al* (22) and Tanaka *et al* (35) was calculated as: -7.0 and -7.5 kcal/mol, respectively (Fig. 2, structures 3-2 and 3-4). Binding such large molecules as TFs

Table I. Calculated free energies of binding for native and metal complexes of theaflavins.

| Metal | TF(1') kcal/mol | TF(1')Me kcal/mol | TF(1')$Me_2$ kcal/mol | $(TF(1'))_2Me$ kcal/mol | TF(2) kcal/mol | TF(2)Me kcal/mol | TF(2)$Me_2$ kcal/mol | $(TF(2))_2Me$ kcal/mol |
|---|---|---|---|---|---|---|---|---|
| None | -8.2 | - | - | - | -8.9 | - | - | - |
| $Ca^{+2}$ | - | -8.9 | -8.9 | -8.7 | - | -9.1 | -9.3 | -9.3 |
| $Fe^{+2}$ | - | -9.1 | -7.9 | -7.1 | - | -8.5 | -6.9 | -8.3 |
| $Fe^{+3}$ | - | -6.6 | - | - | - | -6.4 | - | - |
| $Mg^{+2}$ | - | -8.2 | -7.9 | -7.6 | - | -8.7 | -8.9 | -6.5 |
| $Mn^{+2}$ | - | -7.5 | NC | -7.8 | - | NC | NC | -6.9 |
| $Na^{+}$ | - | -8.3 | -7.1 | - | - | -7.4 | -7.5 | - |

NC, 2D structures not converted into 3D structures. TF, theaflavin; Me, metal.

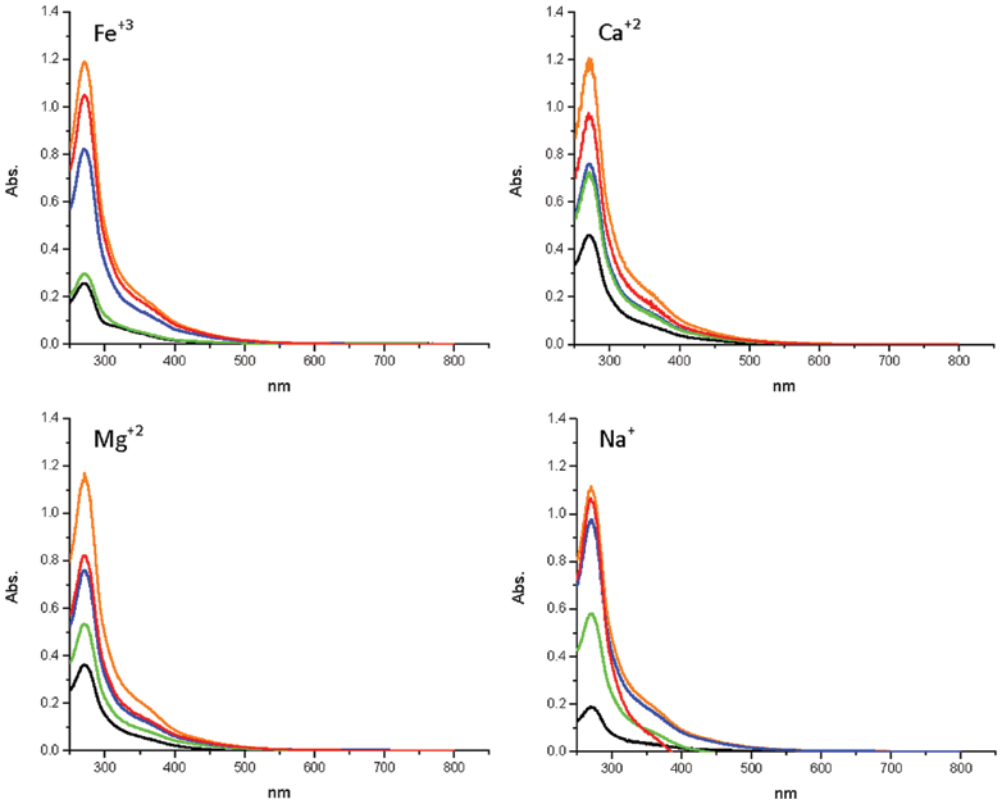


Figure 1. Examples of tea brewed in: distilled water (orange); and metal chloride salts, 0.4% (black), 0.04% (green), 0.004% (blue), or 0.0004% (w/v) (red).

in the proximity of the PAI-1 reactive loop can interfere with its binding to uPA or tPA, inactivating PAI-1 (36). As shown in Table I, the affinity for metal/TF was very similar to the affinity for binding native TFs to PAI-1. The authors of the Vina program state that the prediction of the binding site can be modeled with high precision; however, results of affinity calculation (free energies of binding) within the range ±2.5 kcal/mol are practically the same (24). As shown in Table I, some differences in the free energy of binding can be observed between native TFs and TF/$Fe^{3+}$; these cannot provide evidence of changes in affinity binding after TF reaction with metals.

*Mass spectrometry*. In the samples of precipitants dissolved in water we were able to identify caffeine, TFs, theaflavin-3-galate and theaflavin-3,3'-galate (Fig. 4). These compounds were also detected in all samples dissolved in water/acetonitrile. No TF ionized by protonation or its adduct ions with metals (Ca, Fe, K, Mg, Mn and Na) was detected. Potassium and Na TF derivatives are naturally found in black tea (20). In precipitants treated with water/acetonitrile we identified the following ions (Fig. 2, structures 3-2, 3-3, 3-3a and 3-4): (m/z 535.1142, $C_{28}H_{22}O_{11}$-theanaphthoquinone; m/z 579.112, $C_{29}H_{22}O_{13}$-dehydrotheaflavin; and m/z 717.1432, $C_{36}H_{28}O_{16}$-theabenzoquinone) (22,35,37). The assignments related to the absolute configuration (3 or 3') have to be viewed with skepticism, since using mass spectroscopy, we can deduce the formula of the polyphenolic compound but not the exact orientation.

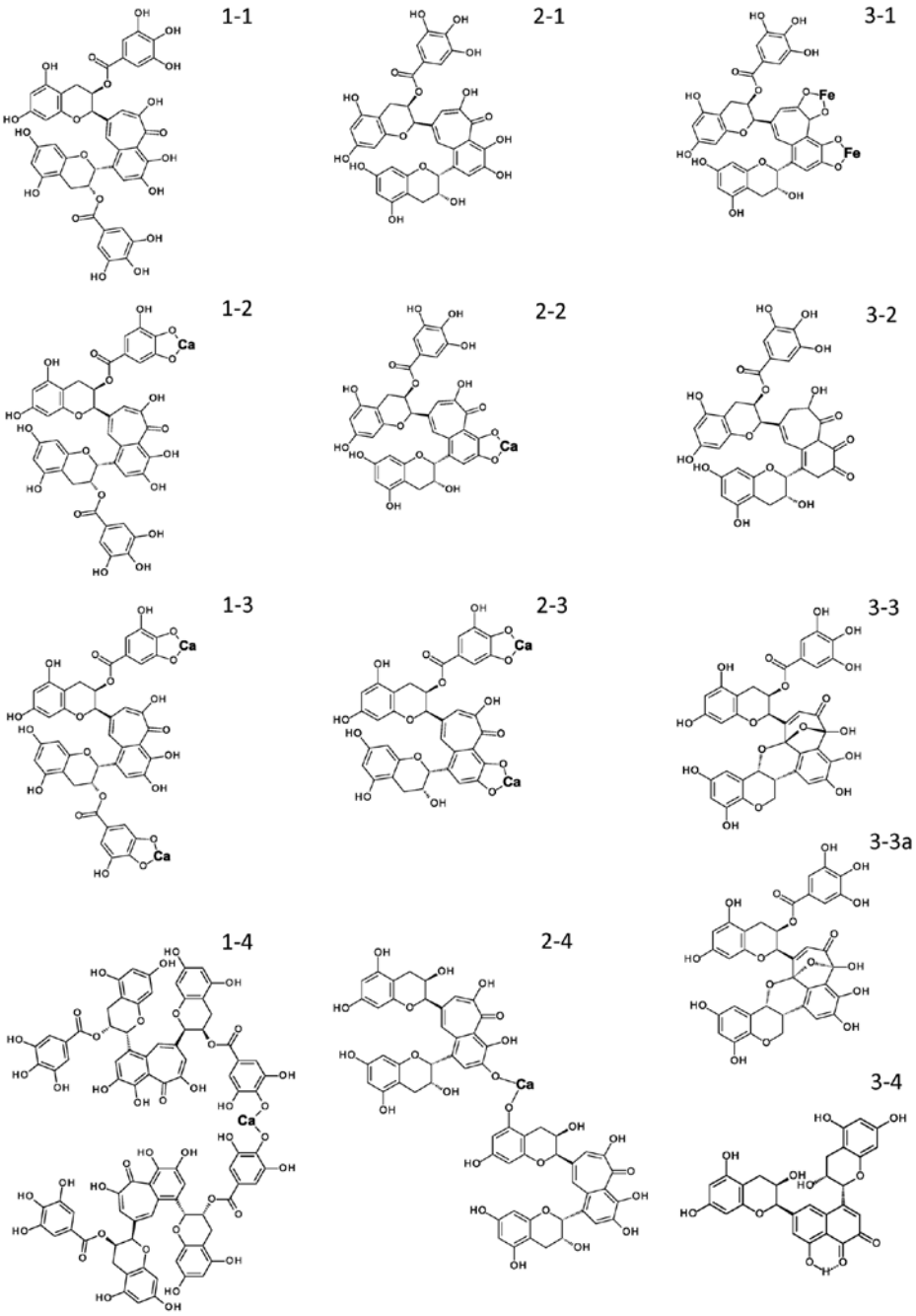


Figure 2. Structures of theaflavins, metal theaflavin complexes and degradation products.

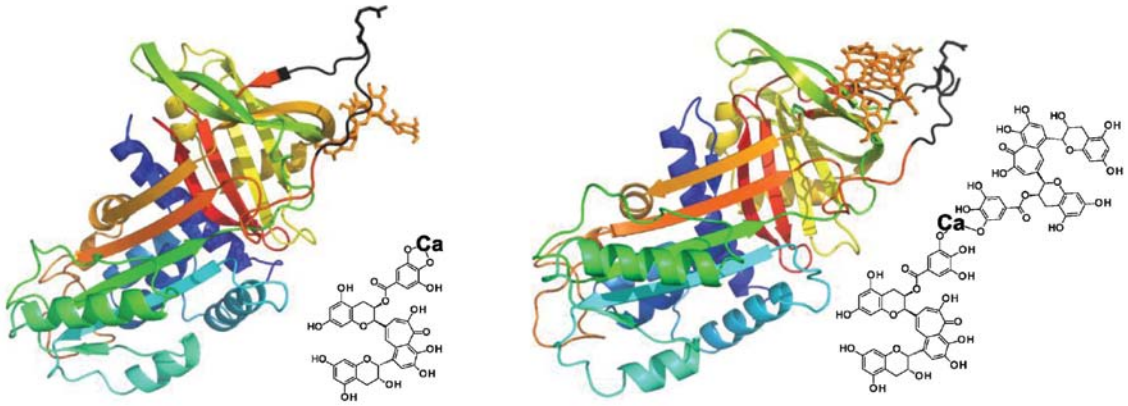


Figure 3. Ribbon model of PAI-1 and stick models of theaflavin (TF):Ca and $(TF)_2Ca$ complexes. Protein is colored as 'rainbow', and residues of reactive center loop 363-374 that serve as bait to draw uPA onto the top of the PAI-1 molecule are colored in black; Arg369 is shown as a stick model.

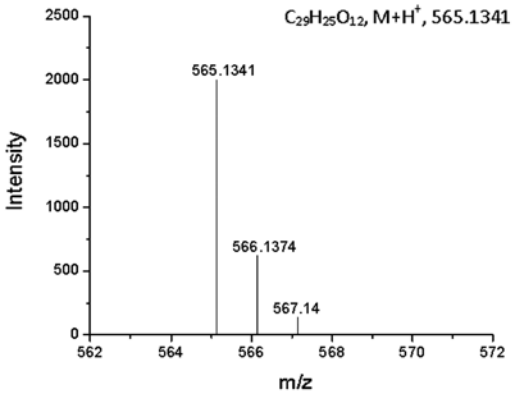


Figure 4. Mass spectrometry of theaflavin, $C_{29}H_{24}O_{12}H^+$.

## Discussion

A number of factors can affect the health benefits of tea, such as the brand of black tea, duration of brewing and temperature, and these factors can significantly affect the content and concentration of TFs in a cup of tea (38-40). However, TFs can often have metal adduct ions associated with them instead of protonation and hydrogen (41-48) and these can influence PAI-1 inhibitory activity as well. We can envision three distinct possibilities: i) metal/TF adducts can have different affinity to PAI-1; ii) metal/TF adducts can precipitate and would not react with PAI-1; iii) metal/TF adducts degrade to smaller compounds or form non-water soluble products.

i) All investigated metals are not considered as a health hazard; however, they can alter the taste and appearance of brewed tea. Metals are present in drinking water or in tea itself. Recommendations of the maximum concentration of metals in drinking water can vary depending on the country, but in general, these levels are not higher than: 0.3 mg/l for Fe, 0.5 mg/l for Mg, 0.3 mg/l for Mn and 20 mg/l for Na. Ca levels are mostly not regulated. However, sometimes these values are much higher in drinking water, and in extreme situations can reach as high as 25 mg/l Fe (49). All of these scenarios were within concentrations we investigated. Fernandez-Caceres *et al* (42) analyzed the metal content of 46 tea samples, including Ca, Fe, Mg, Mn and Na. The average content of the metals was as follows: Ca, Fe, Mg, Mn and Na: 4,252 (range, 474-6,324), 810 (range, 74-2,036), 1978 (range, 1,489-4,272), 825 (range, 148-1,595), and 458 (range, 100-1,760) mg/kg, respectively. They found that teas from Africa and Asia differed significantly in their metal content (42). These concentrations are within the same limits that we investigated. Spectrophotometric data clearly indicate that teas brewed in water containing metals show a reaction of metal cations with the components of black tea. Since TFs can constitute up to 40% the dry weight of black tea, are responsible for the characteristic color (20), and are known to chelate metals (21,22), it is highly likely that these chemicals react with metals. We hypothesized that metals are chelated as shown in Fig. 2. The molecular modeling of the binding of metal/TF and their decomposition products (Fig. 2, structures 3-2, 3-3, 3-3a and 3-4) to PAI-1 has revealed that all these chemicals bind (Fig. 3) close to Arg369 and Met370 (an active site of PAI-1) on the reactive center loop. The crystal structure of the PAI-1-uPA Michaelis complex (PDB entry 3pb1) has revealed that the PAI-1 reactive center loop serves as bait to draw uPA onto the top of the PAI-1 molecule. The 365-374 residues of the reactive center loop interact with the uPA catalytic site, inactivating urokinase (36). Anything binding into/near this site creates an obstruction that would definitely interfere with the formation of the uPA/PAI-1 complex. Theoretical binding affinity for the aforementioned compounds was in the range of ~$2x10^{-4}$-$2x10^{-7}$ (6.5-9.3 kcal/mol). Since the binding affinity of native TFs was calculated as ~$2x10^{-7}$, we cannot conclude that the chelation of metals or their decomposition will significantly affect the inhibition of PAI-1.

ii) The other possibility includes the precipitation of metal/TF complexes that consequently will not react with PAI-1. We analyzed these by mass spectrometry. In the initial approach we analyzed precipitants treated with water. We identified caffeine, TFs, theaflavin-3-galate and theaflavin-3,3'-galate. No metal/TF molecules were found. In the following experiments, precipitants were treated with 50/50 water/acetonitrile. In these experiments we did not detect metal/TF complexes. They may not be soluble at these conditions or are not charged to be easily detected. However, in addition to compounds detected in the previous experiment we found theanaphthoquinone, dehydrotheaflavin and theabenzoquinone. The structures of these compounds can only be postulated based on the m/z spectral data; however, supporting literature data allowed us to be confident in the assignment of these structures (34,35). Furthermore, the cited authors suggest that theabenzoquinone can be further oxidized to form presumably not water soluble high molecular weight oxidation products.

iii) The coordination of aluminium, Ca, Fe, Mg, Mn, Na with TFs has been reported by a number of studies (33,41-49). However, O'Coinceanainn *et al* (22) and Tanaka *et al* (35) suggested that the reaction between TFs involves the formation of a metal complex, followed by subsequent decomposition or formation of the non-metallic intermediates that generate high molecular weight oxidized TF products. More specifically, they suggested that metal/TFs decompose to form theabenzoquinone (Fig. 2, structure 3-2) or derivatives that form unspecified high molecular weight oxidation products. They also suggested that theabenzoquinone can form dehydrotheaflavins (Fig. 2, structures 3-3 and 3-3a) which can further degrade or form other unspecified products (22,35,37).

In conclusion, we can conclude that metals present in water used for brewing tea, as well as metals naturally occuring in black tea leaves, induce a cascade of reactions during which TFs undergo significant changes, including degradation to form products of various molecular weights, or some of them can precipitate. Thus, future epidemiological studies on the health benefits of black tea should take these factors into consideration.

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