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The Pharmacological Mechanism of Angiotensin-converting Enzyme Inhibition by Green Tea, Rooibos and Enalaprilat – A Study on Enzyme Kinetics

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Green tea (*Camellia sinensis* L.) and Rooibos (*Aspalathus linearis* Dahlg.) inhibit angiotensin-converting enzyme (ACE) *in vitro* and *in vivo*. The ACE inhibitor enalaprilat has been described previously as a competitive inhibitor and sometimes as a non-competitive inhibitor. The aim of this study was to investigate the pharmacological mechanism of ACE inhibition of green tea and Rooibos by enzyme kinetics, and to compare this with enalaprilat. A Michaelis–Menten kinetics and Lineweaver–Burk graph showed mean values of $V_{max} = 3.73$ μM and $K_m = 0.71$ μM for green tea, of $V_{max} = 6.76$ μM and $K_m = 0.78$ μM for Rooibos, of $V_{max} = 12.54$ μM and $K_m = 2.77$ μM for enalaprilat, and of $V_{max} = 51.33$ μM and $K_m = 9.22$ μM for the PBS control. Incubating serum with green tea or Rooibos saturated with zinc chloride did not change the inhibitory effect. Enalaprilat preincubated with zinc chloride showed a decrease in the inhibitory effect. In conclusion, green tea, Rooibos and enalaprilat seem to inhibit ACE activity using a mixed inhibitor mechanism. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Michaelis–Menten; Lineweaver–Burk; flavonoids; tea.

INTRODUCTION

Green tea, *Camellia sinensis* L. (Theaceae), has been shown to inhibit angiotensin-converting enzyme (ACE) *in vitro* (Persson *et al*., 2006) and *in vivo* (Persson *et al*., 2010). Intake of a single dose of green tea lowered ACE activity, with maximum at 30–60 min after intake and the inhibition seems to persist for about 60 min (Persson *et al*., 2010). Rooibos, *Aspalathus linearis* Dahlg. (Leguminosae), significantly inhibits ACE *in vivo* (Persson *et al*., 2010), with maximum at 30–60 min after intake and persisting for about 60 min. Green tea and Rooibos contain high amounts of flavonoids, i.e. catechins (epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate) and other flavonoids (quercetin, aspalathin, nothofagin, isoorientin, orientin, isovitexin, vitexin and rutin) respectively. Since flavonoids are known to be metal-chelators a possible mechanism for green tea and Rooibos is to bind to the Zn^{2+} at the active site of ACE, thereby inhibiting the enzyme.

The function of an enzyme is to catalyse a reaction. Enzymes are known to accelerate the rate of reactions by up to 10^{15} times and to lower the activation energy. Enzyme kinetics deals with factors affecting the rates of enzyme-catalysed reactions. By varying the substrate and product concentrations the kinetic mechanism of the reaction can be summarized. A common use for enzyme inhibitors is as drugs to treat disease, and these inhibitors target a human enzyme to adjust a pathological condition.

Four types of inhibitors are described: competitive, mixed, non-competitive and uncompetitive inhibitors. Competitive inhibitors usually bond at the active site of the enzyme where the substrate also binds. Drugs used as ACE inhibitors are designed to bind to the Zn^{2+} at the active site of ACE (Sturrock *et al*., 2004) and thereby inhibit the ability to convert the inactive angiotensin I to form the active angiotensin II. Angiotensin II functions as a potent vasoconstrictor, causes cell growth, regulates blood volume by release of aldosterone and impairs learning and memory functions. Thus, angiotensin II is involved in the development of cardiovascular disease. The ACE inhibitor enalaprilat has been described previously as a competitive inhibitor (Patchett, 1984; Patchett and Cordes, 1985) and as a non-competitive inhibitor (Baudin and Bénéteau-Burnat, 1999). Mixed-type inhibitors are able to bind both to the active site of the enzyme and at an allosteric site of the enzyme. Non-competitive inhibitors reduce the enzyme activity but do not affect the active site of the enzyme and uncompetitive inhibitors bind to sites available at the substrate–enzyme complex.

Angiotensin-converting enzyme (ACE) inhibitors are the first choice treatment of hypertension. Long-term intake of the ACE inhibitor enalaprilat lowers ACE activity by 16%, with maximum at 4–8 h and persists for 24 h after oral administration (Abrams *et al*., 1984). The ACE is a zinc carboxypeptidase

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(Skeggs *et al.*, 1956) synthesized by the endothelium and present on the luminal surface of the endothelial cell membrane and in plasma (Baudin *et al.*, 1997).

The aim of this study was to investigate the enzyme kinetics of ACE inhibition by green tea and Rooibos, thereby revealing the pharmacological mechanism of these substances. The aim was also to compare the enzyme kinetics of green tea, Rooibos and enalaprilat.

MATERIALS AND METHODS

Tea infusion. Tea infusions were prepared from green tea, Japanese Sencha, imported by Charabang (Stockholm, Sweden), and Rooibos, imported by Norrköping Kolonial (Sweden). Infusions were prepared as previously described by Persson *et al.* (2006). One gram of tea was infused in freshly boiled 20 mL sterile phosphate-buffered saline (PBS) for 5 min for the green tea and 10 min for Rooibos. The infusions were then filtered twice, first through a standard filter 0.45 μm (Munktell, Grycksbo, Sweden) and then through a sterile filter 0.2 μm (Millipore). The concentrations of the filtrates obtained were determined as 1:20 and they were frozen in aliquots at −20°C until use.

Serum collection. Human blood was collected from healthy volunteers. The participants did not use any kind of drugs (medical or herbal) 2 weeks prior to donating blood. They did not use any form of nicotine, and intake of beverage or food containing high amounts of catechins, the flavonol quercetin or anthocyanidins, i.e. chokeberries, aubergine, blackberries, bilberries, elderberries, raspberries, strawberries, coffee, cacao/chocolate, cherries, onions, plums, pears, radishes, red and black currants, red cabbage, black beans, cranberries, tea, wine, grapes and apples were not allowed 48 h before the experiment. Vacutainer tubes without anticoagulantia were used to collect blood for serum-ACE. After 2 h the tubes were centrifugated 1000 × **g** for 20 minutes, at 4°C. Serum was transferred to plastic tubes and ACE activity was analysed the same day. The ACE activity was analysed with a commercial radioenzymatic assay (ACE-direct REA, Bühlmann Laboratories, Allschwil, Switzerland) as described previously by Persson *et al.* (2010).

The study was approved by the ethical review board at the Faculty of Health Sciences, Linköping, Sweden (Dnr M56-07).

Michaelis–Menten kinetics and Lineweaver–Burk equation. The Michaelis-Menten kinetics and Lineweaver-Burk equation was used showing the linear relation between inverse reaction velocity and inverse substrate concentration described in the formula:

$$1/\mathrm{v} = (K_m/V_{max})(1/S) + 1/V_{max}$$

The assay was performed after modification according to Shalaby *et al.* (2006). The substrate used was 0.8 mM N-(3-(2-Furyl) acryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) in 50 mM tris-HCl containing 0.3 M NaCl, pH 7.5. Human serum diluted in PBS to the ACE concentration 0.25 units/mL was freshly prepared each day. The assay was performed in 96-well microtitre plates. 25 μL of serum and 2.8 μL of green tea, Rooibos, enalaprilat, zinc chloride or PBS control, were preincubated for 10 min at 37°C. Then 250 μL substrate solution was added to each well during less than 1 min and the reaction started. The microplate was immediately transferred to the Microplate Scanning Spectrophotometer (VERSAmax™, Molecular Devices, Sunnyvale, CA, USA), 37°C. The absorbance at 340 nm was measured every 30 s for a running time of 5 min. The concentration of green tea and Rooibos used in this study was 1:80, the concentration of enalaprilat was 10^{-8} M, and the concentration of zinc chloride was 10 μM. Three to six replications of each treatment were made, using serum from different individuals for each replication.

Chemicals. The teas were bought at Tebladet (Linköping, Sweden). N-(3-(2-furyl) acryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG), Tris-HCl and zinc chloride were obtained from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA) and enalaprilat (Renitec® 1 mg/mL) was bought from Merck Sharp and Dohme (Haarlem, The Netherlands).

Calculations. Statistical calculations were performed with GraphPad Prism™ 5.0. Linear regression was used, x-axes = $1/S = K_m$ (Michaelis constant), y-axes = $1/v = Vmax$ (the maximum reaction velocity).

RESULTS

Michaelis–Menten kinetics and Lineweaver–Burk equation

The Lineweaver–Burk graph showed mean values for green tea incubation of $V_{max} = 3.73$ μM and $K_m = 0.71$ μM ($n = 5$; Fig. 1a): with $V_{max} = 6.76$ μM and $K_m = 0.78$ μM ($n = 4$; Fig. 1b) for Rooibos; $V_{max} = 12.54$ μM and $K_m = 2.77$ μM ($n = 6$; Fig. 1c) for enalaprilat; and $V_{max} = 51.33$ μM and $K_m = 9.22$ μM ($n = 6$; Fig. 1d) for the control.

The Lineweaver–Burk graph showed mean values for incubating serum with green tea + zinc chloride of $V_{max} = 4.16$ μM and $K_m = 0.91$ μM ($n = 4$; Fig. 2a): with $V_{max} = 4.97$ μM and $K_m = 0.68$ μM ($n = 4$; Fig. 2b) for Rooibos + zinc chloride; $V_{max} = 24.22$ μM and $K_m = 6.24$ μM ($n = 3$; Fig. 2c) for enalaprilat + zinc chloride; and $V_{max} = 27.91$ μM and $K_m = 5.69$ μM ($n = 3$; Fig. 2d) for zinc chloride.

Individual data for all experiments are shown in Table 1.

DISCUSSION

Green tea and Rooibos seem to inhibit ACE using a mixed type of inhibition. This is proposed as a result of the change in V_{max} and K_m seen in this study. Also, enalaprilat does not seem to be a simple competitive inhibitor as proposed by Patchett (1984), but rather a competitive or a non-competitive inhibitor, using a mixed type of

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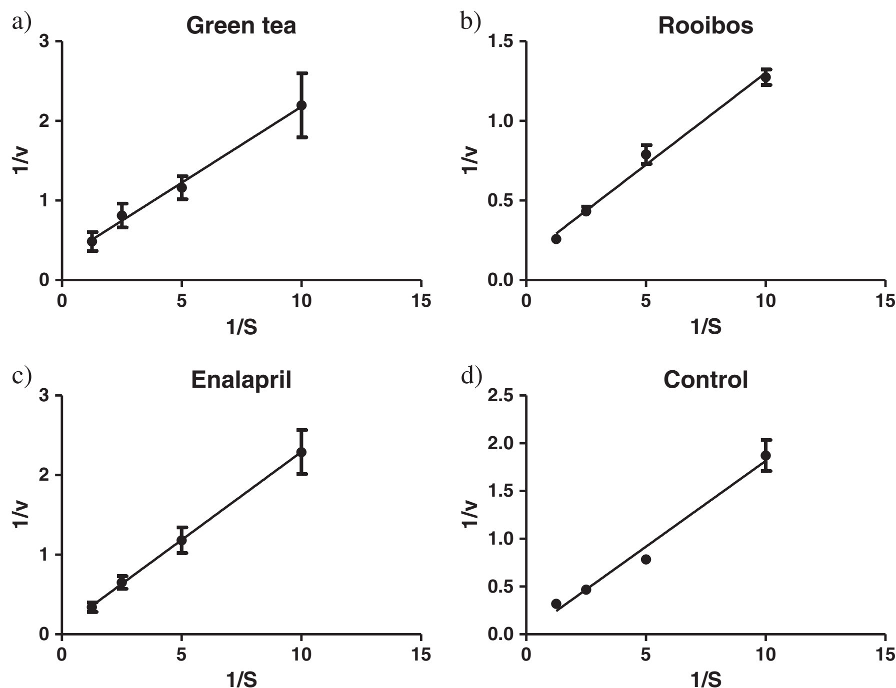

Figure 1. Lineweaver–Burk graph of: (a) green tea, r^2 = 0.70, n = 5; (b) Rooibos, r^2 = 0.96, n = 4; (c) enalaprilat, r^2 = 0.79, n = 6; and (d) PBS control, r^2 = 0.89, n = 6.

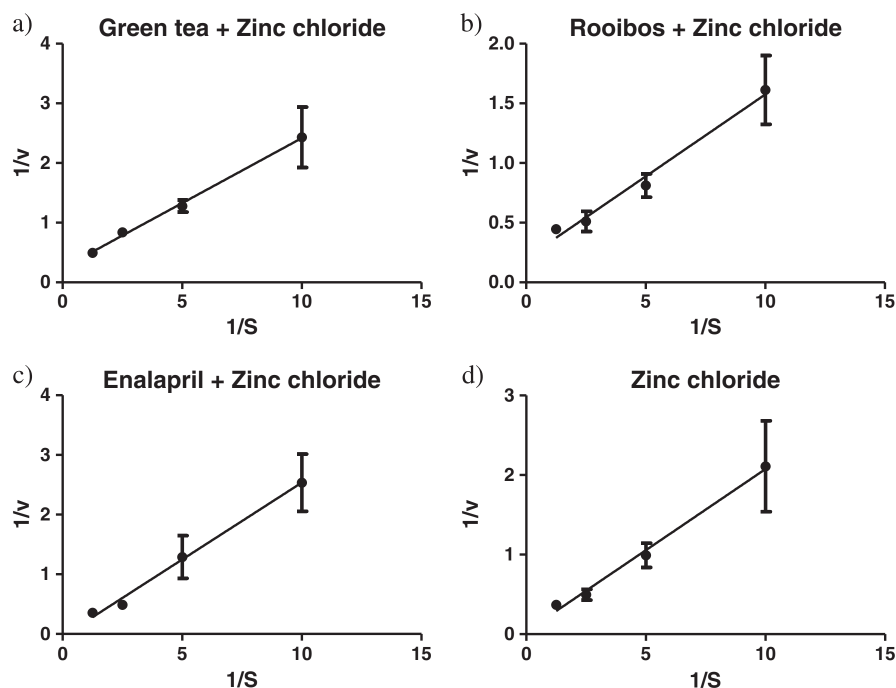

Figure 2. LineweaverBurk graph of: (a) green tea + $ZnCl_2$, r^2 = 0.72, n = 4; (b) Rooibos + $ZnCl_2$, r^2 = 0.73, n = 4; (c) enalaprilat + $ZnCl_2$, r^2 = 0.80, n = 3; and (d) $ZnCl_2$, r^2 = 0.72, n = 3.

inhibition. Focusing on V_{max}, this value is decreased compared with the control. This result is in accordance with Baudin and Bénéteau-Burnat (1999) and Moalli *et al.* (1985). Enalaprilat was designed with an assumed mechanistic similarity of ACE to carboxypeptidase A; the carboxylate group of enalaprilat binds to Zn^{2+} at the active site of ACE, and this assumption would make enalaprilat a competitive inhibitor. This quantitative structure–activity relationship (QSAR; activity = physiochemical properties and/or structural properties) of ACE inhibitors was assumed without insight in the three-dimensional structure of ACE (Natesh *et al.*, 2003). The three-dimensional structure of ACE is probably responsible for the non-competitive inhibition of enalaprilat by enabling it to bind to other parts of the enzyme than the active site and thereby inhibiting the reaction.

Focusing on the K_m value, a competitive inhibitor increases the apparent K_m for a given substrate meaning that in the presence of a competitive inhibitor more substrate is needed to achieve half V_{max}. The K_m values in this study were increased in ascending order: green tea, Rooibos and enalaprilat. This could imply that

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Table 1. Individual data of each experiment in this study

Experiment	1/S	1/v					
Control, n = 6	10.000	1.613	2.091	1.649	2.004	1.391	2.483
	5.000	0.725	0.775	0.719	0.817	0.711	0.960
	2.500	0.571	0.457	0.458	0.438	0.474	0.414
	1.250	0.457	0.263	0.268	0.202	0.363	0.364
Green tea, n = 5	10.000	2.926	3.143	1.884	0.896	2.132	
	5.000	1.028	1.567	1.065	0.749	1.399	
	2.500	0.706	1.368	0.597	0.532	0.853	
	1.250	0.513	0.516	0.187	0.322	0.892	
Rooibos, n = 4	10.000	1.355	1.150	1.348	1.243		
	5.000	0.923	0.718	0.851	0.667		
	2.500	0.410	0.467	0.492	0.360		
	1.250	0.251	0.274	0.273	0.235		
Enalaprilat, n = 6	10.000	1.797	1.754	3.343	2.037	1.871	2.933
	5.000	1.002	0.748	1.620	0.759	1.423	1.543
	2.500	0.709	0.487	0.936	0.380	0.645	0.750
	1.250	0.307	0.278	0.428	0.198	0.242	0.597
Green tea + Zinc chloride, n = 4	10.000	3.583	1.236	2.865	2.041		
	5.000	1.333	1.147	1.095	1.545		
	2.500	0.697	0.891	1.019	0.744		
	1.250	0.426	0.436	0.718	0.401		
Rooibos + Zinc chloride, n = 4	10.000	2.444	1.120	1.382	1.503		
	5.000	0.972	0.641	0.647	0.987		
	2.500	0.763	0.443	0.403	0.435		
	1.250	0.476	0.459	0.422	0.425		
Enalaprilat + Zinc chloride, n = 3	10.000	3.437	2.376	1.792			
	5.000	2.004	0.926	0.940			
	2.500	0.580	0.432	0.454			
	1.250	0.297	0.418	0.350			
Zinc chloride, n = 3	10.000	3.245	1.647	1.436			
	5.000	1.267	0.742	0.967			
	2.500	0.567	0.361	0.562			
	1.250	0.393	0.392	0.322			

enalaprilat is more competitive using less of a mixed inhibition mechanism than Rooibos and green tea.

Compared with the control, green tea and Rooibos showed the same inhibitory pattern as enalaprilat, i.e. non-competitive inhibition, using a mixed type inhibition seen as a decrease in V_{max}.

The decreased V_{max} seen with green tea and Rooibos compared with enalaprilat may indicate that green tea and Rooibos bind to other alternative sites rather than the catalytic site, and this might be due to the number of OH groups present in the catechins in green tea, and to the different flavonoids in Rooibos (Persson *et al.*, 2006). Chemical structures of the catechins present in green tea, and of quercetin as an example of the flavonoids present in Rooibos and enalapril, are shown in Fig. 3. The flavonoids, e.g. catechins and quercetin, present more possible binding sites on the enzyme than enalaprilat.

Zinc chloride was used to chelate the flavonoids in green tea and Rooibos prior to incubation to make it impossible for the flavonoids to bind to the active site at the ACE. This was done to investigate if there was any change in the inhibition pattern of green tea and Rooibos when metal chelators in the infusions were already bound to Zn^{2+}. Using zinc chloride as control, enalaprilat did not show any inhibitory effect, which confirms the effect of enalaprilat as a competitive inhibitor.

Compared with zinc chloride, green tea and Rooibos showed a mixed inhibitory effect. Either a higher concentration of zinc is needed to saturate the metal-chelating flavonoids, or the binding to sites other than the catalytic site is important for the inhibitory effect. Flavonoids saturated with zinc did not affect the inhibitory effect of ACE activity in human endothelial cell either (unpublished data).

Angiotensin-converting enzyme is known as an unspecific enzyme and K_m varies according to the substrate. A small K_m value means that the enzyme binds strongly to its substrate and the lower the K_m the lower concentration of substrate is needed. The lowest K_m is usually seen with the ‘natural’ substrate of the enzyme. The natural substrate of ACE is angiotensin I and/or bradykinin. The K_m value of Ang I is 16–90 μM and for bradykinin is 0.18–1.0 μM (Dorer *et al.*, 1974; Jaspard *et al.*, 1993; Campbell, 1995).

As V_{max} is the rate at which a substrate will be converted to a product once bound to the enzyme and K_m is how effectively the enzyme binds to the substrate, these two values are a reflection of affinity. Both V_{max} and K_m show the quantity value of the enzyme inhibition. The quantity value seen in this study is in ascending order; green tea, Rooibos and enalaprilat.

The V_{max} /K_m values of enalaprilat, green tea and Rooibos represent the catalytic ability/affinity. For the

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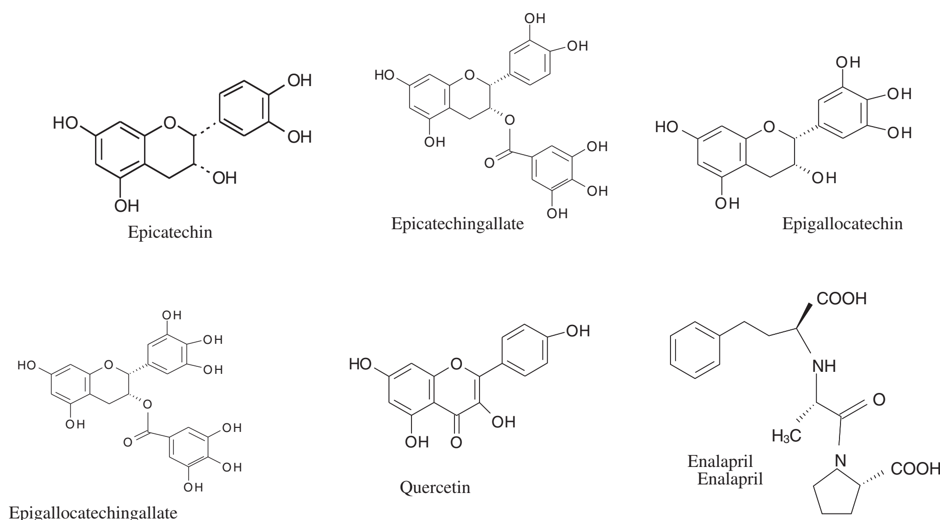

Figure 3. Chemical structures of the flavanol compounds in green tea: epicatechin, epicatechingallate, epigallocatechin, epigallocatechingallate; the flavonol quercetin found in Rooibos; and enalaprilat.

substances tested in this study, V_{max}/K_m was: 4.53 μM for enalaprilat; 5.25 μM for green tea; and 8.67 μM for Rooibos. Values of K_m for green tea and Rooibos showed small differences, but Rooibos had increased V_{max} and V_{max}/K_m values compared with green tea, indicating no difference in the enzyme–substrate affinity but a difference in the catalytic ability. The V_{max} and K_m values of enalaprilat were lower compared with green tea and Rooibos, and the effect on the enzyme–substrate affinity and the catalytic ability for enalaprilat was lower compared with Rooibos and green tea. All drugs tested showed a reduction in catalytic ability when pretreated with Zn^{2+}.

CONCLUSIONS

According to results for V_{max} and K_m seen in this study, green tea and Rooibos are proposed to inhibit ACE activity using a mixed inhibitor mechanism. It also seems that green tea and Rooibos use the same enzyme kinetic mechanism as the ACE inhibitor enalaprilat, i.e. mixed type inhibitors. However, enalaprilat is more competitive than Rooibos and green tea. The quantity value of inhibition is in ascending order: green tea, Rooibos and enalaprilat. The quality of the inhibition have the opposite order: enalaprilat, green tea and Rooibos.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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