In Vitro Studies on the Mechanism of Action of Two Compounds with Antiplasmodial Activity: Ellagic Acid and 3,4,5-Trimethoxyphenyl (6′-*O*-Galloyl)-β-D-glucopyranoside

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Abstract

To investigate the mechanism of action of two antiplasmodial compounds, ellagic acid and 3,4,5-trimethoxyphenyl (6′-*O*-galloyl)-β-D-glucopyranoside (TMPGG), we studied *in vitro* two metabolic reactions of intraerythrocytic parasites: the activity of recombinant plasmepsin II, one of the haemoglobin proteases, and the detoxification of haematin into β-haematin. Both compounds inhibited plasmepsin II activity, but at concentrations ten-fold higher than those needed for inhibiting parasite growth. Moreover, ellagic acid inhibited the formation of β-haematin, with an IC_{50} only 3-fold higher than that of chloroquine. These data suggest that the antiplasmodial activity of ellagic acid could be related to the inhibition of β-haematin formation, whereas plasmepsin II does not represent the main target of the two compounds.

The search for new antimalarial compounds is essential due to the increase in parasite resistance to available drugs [1]. The pharmacological targets in the intraerythrocytic parasites include the proteolytic cleavage of haemoglobin and the detoxification of haem leading to the formation of haemozoin [1]. *Plasmodium falciparum* degrades haemoglobin in a digestive vacuole (DV) by means of aspartic proteinases (plasmepsins I – X) [2] and one cysteine proteinase (falcipain) [1]. Plasmepsins are presently considered good targets for the search for new drugs [2].

The digestion of haemoglobin releases haem that is oxidised to haematin and detoxified as a crystalline pigment, haemozoin [3]. A synthetic compound, called β-haematin, spectroscopically identical to the native pigment, can be obtained *in vitro*. Quinoline antimalarials, as well as compounds able to form π-π interactions with haematin, have been shown to inhibit β-haematin formation (for a review see [3]).

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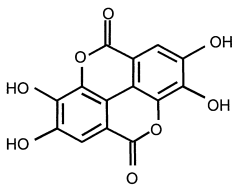
Received: May 31, 2002 · **Accepted:** September 22, 2002

Bibliography: Planta Med 2003; 69: 162–164 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

It was previously shown that the methanolic extract of the bark of *Tristaniopsis calobuxus* Brongniart & Gris (Myrtaceae) inhibited parasite growth *in vitro*. Two compounds with antiplasmodial activity were isolated and identified as ellagic acid and 3,4,5-trimethoxyphenyl (6′-*O*-galloyl)-β-D-glucopyranoside (TMPGG) (Fig. **1**) [4]. Since *T. calobuxus* bark extract was shown to inhibit other proteases [5], we investigated whether the compounds inhibited plasmepsins, using recombinant plasmepsin II as target [6]. In addition, the ability of ellagic acid to inhibit β-haematin formation *in vitro* was evaluated [7].

Both ellagic acid and TMPGG inhibited recombinant plasmepsin II (Fig. **2**): the IC_{50} values were 4.02 ± 1.26 and 35 ± 8.74 μM for ellagic acid and TMPGG, respectively (mean ± sd). These doses are 10-fold higher than those needed to inhibit the parasite growth *in vitro* (0.5 μM and 3.2 μM for ellagic acid and TMPGG, respectively) [4]. The potency ratio between the two compounds remained similar, equal to 10. These observations suggest that the inhibition of plasmepsin II may not be the preferential mechanism of action of the two compounds, assuming that they can enter the parasite DV where proteases act [1]. Alternatively, ellagic acid and TMPGG could be more active on other DV enzymes, e. g., plasmepsin I rather than II [8], or interact with plasmepsin II in the erythrocyte cytoplasm where the enzyme digests the cytoskeleton proteins at pH 6.8 [9].

Ellagic acid inhibited β-haematin formation dose dependently (Fig. **3**). At the highest dose the inhibition of ellagic was 65.6% versus 100% by chloroquine, the reference compound. The IC_{50} s were 1.67 ± 0.81 and 5.42 ± 1.3 molar equivalents for chloroquine and ellagic acid, respectively. A factor which could strongly contribute to the inhibition of β-haematin by ellagic acid is its high electronic density dispersed in a flat molecular structure. Due to its ability to form π-π complexes, it may strongly bind haematin monomers, thus inhibiting their association. Alternatively, the presence of free aryl hydroxy groups, may contribute towards the formation of coordination bonds between the OH groups of ellagic acid and the Fe(III) centre of Fe(III)PPIX. The process of β-haematin formation, in principle, could be inhibited by acting on haem monomers to coordinate and/or avoid their oxidation [3],



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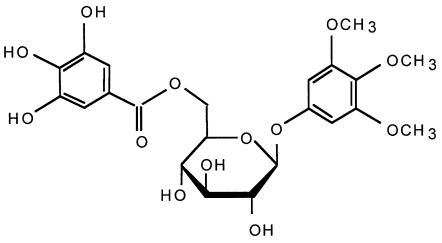

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Fig. **1** Structures of ellagic acid (**1**) and TMPGG (**2**).

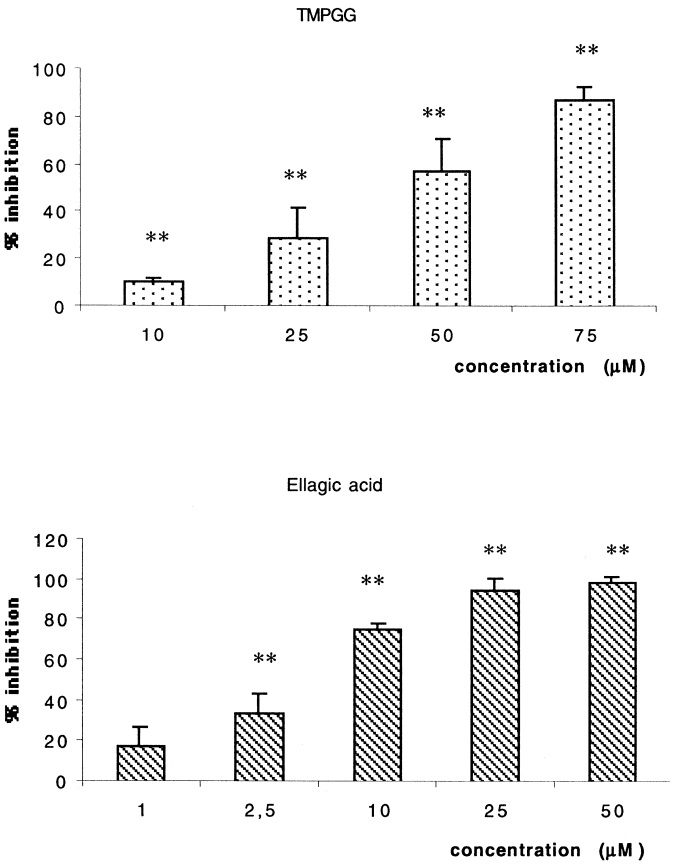

Fig. **2** Effect of ellagic acid and TMPGG on plasmepsin II. The results are expressed as % inhibition of enzyme activity, in the presence of increasing concentration of the compounds (mean ± sd, n = 3).

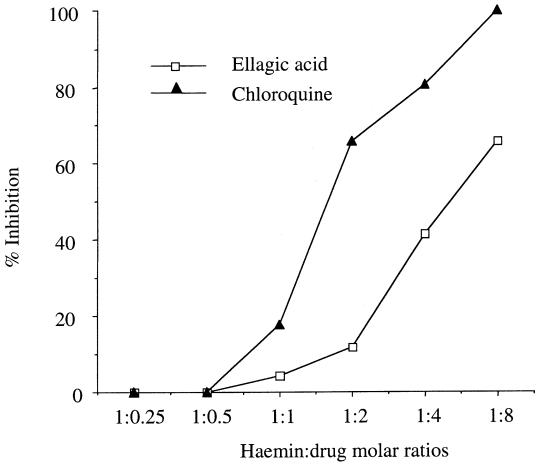

Fig. **3** Inhibition of β-haematin formation by ellagic acid and chloroquine. The results are expressed as percent inhibition in the presence of different drugs compared to haemin alone. Note that the error bars fall within the boundaries of the symbol.

[10]. A phenol, and more likely a doubly hydroxylated benzene ring, like a catechol, acts on the whole as a strong antioxidant agent [11], by scavenging any oxidant with a suitable electrochemical potential, present in the medium.

The inhibition of β-haematin formation by ellagic acid may thus explain its antiplasmodial activity, whereas the inhibition of plasmepsin II by both compounds seems to occur at concentrations too high to account for their antiplasmodial activity.

Materials and Methods

All chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Haematin (ferriprotoporphyrin IX hydroxide) (H-3505), haemin chloride (H-5533), chloroquine diphosphate (C-6628) were obtained from Sigma, Milan, Italy. Lys-Glu-Phe-Val-Phe*NPhe-Ala-Leu-Lys (where NPhe is *para*-nitrophenylalanine) was synthesised by Alta Bioscience, University of Birmingham (UK). Ellagic acid (purity > 98%) was obtained from Fluka (Sigma-Aldrich, Italy), TMPGG (purity > 95% as determined by NMR analysis) was isolated from *T.c.* bark extract [4].

For plasmepsin II preparation and evaluation the method of Hill et al. [6] was followed. The enzyme activity was evaluated spectrophotometrically at 37 °C, by following the cleavage of the chromogenic substrate Lys-Glu-Phe-Val-Phe*NPhe-Ala-Leu-Lys at 300 nm for 5 min on a Jasco Uvidec 610 UV-Vis spectrophotometer. 2.5 μg of protein were incubated with 50 μg of substrate in Na-acetate buffer 100 mM, pH 4.7, final incubation volume was 0.8 mL. Ellagic acid (1 – 50 μM) and TMPGG (10 – 75 μM) were added in DMSO and methanol, respectively. The amount of solvents in the incubation sample was 1%.

β-Haematin formation was assayed by a spectrophotometric microassay BHIA (β-haematin inhibitory assay) previously reported [7]. Haemin in DMSO (0.4 μmol/well) was distributed in 96-well microplates. Chloroquine or ellagic acid, dissolved in water or DMSO, respectively, were added in doses ranging from 0.25 to 8 molar equivalents to haemin. In control wells, water or DMSO were added maintaining in each well the same DMSO:water ratio of 1 : 1 in a final volume of 100 μL. The drug concentration required to inhibit β-haematin formation by 50% (IC_{50}) was determined.

All tests were performed three times in triplicate and the results analysed by using the one way analysis of variance. IC_{50} values were calculated from the dose-response curves. Linear regression for the equation Y = a (± sd) logX + b (± sd) was calculated by the least squares methods.

Acknowledgements

The authors are indebted to Dr. D. Monti for skillful discussion. The research was funded by the Ministry of University and Scientific and Technological Research (MURST) COFIN 2001 and The Royal Society (CB).

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