

# Investigation of Mammal Arginase Inhibitory Properties of Natural Ubiquitous Polyphenols by Using an Optimized Colorimetric Microplate Assay

## Authors

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## ABSTRACT

Polyphenols are plant secondary metabolites which possess many positive effects on human health. Although these beneficial effects could be mediated through an increase in nitric oxide synthase activity, little is known regarding the inhibitory effect of polyphenols on mammal arginase, an enzyme which competes with nitric oxide synthase for their common substrate, L-arginine. The aim of the present study was to determine the potential of a series of polyphenols as mammalian arginase inhibitors and to identify some structure-activity relationships. For this purpose, we first developed a simple and cost-effective *in vitro* colorimetric microplate method using commercially-available mammal bovine liver arginase (b-ARG 1). Among the ten tested polyphenolic compounds [chlorogenic acid, piceatannol, resveratrol, (-)-epicatechin, taxifolin, quercetin, fisetin, caffeic acid, quinic acid, and kaempferol], chlorogenic acid and piceatannol exhibited the highest inhibitory activities ( $IC_{50} = 10.6$  and  $12.1 \mu\text{M}$ , respectively) but were however less active as (S)-(2-Boronoethyl)-L-cysteine ( $IC_{50} = 3.3 \mu\text{M}$ ), used as reference compound. Enzyme kinetic studies showed that both chlorogenic acid and piceatannol are competitive arginase inhibitors. Structural data identified the importance of the caffeoyl (3,4-dihydroxycinnamoyl)-part and of the catechol function in the inhibitory activity of the tested compounds. These results identified chlorogenic acid and piceatannol as two potential core structures for the design of new arginase inhibitors.

## ABBREVIATIONS

b-ARG 1	bovine arginase 1
BEC	S-(2-boronoethyl)-L-cysteine
CGA	chlorogenic acid
NO	nitric oxide
NOS	nitric oxide synthase
PCT	piceatannol

## Introduction

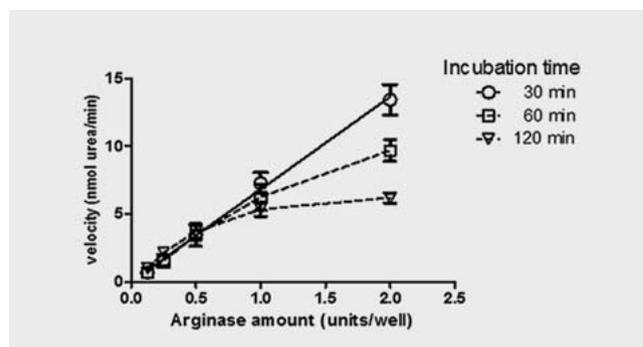
Polyphenols are plant secondary metabolites present in many fruits, vegetables and medicinal plants. Numerous experimental and epidemiological studies strongly suggested their role in the treatment of chronic diseases, including vascular and cardiac diseases, obesity, diabetes and cancer [1]. An abundant literature exists on the mechanisms involved in their positive effects on health and compelling data demonstrated that their effects rely partly on an increase in NO production. Surprisingly, despite the competition between arginase and NOS for NO production is acknowledged [2], is still ill-defined whether polyphenolic compounds exhibit inhibitory activity on mammalian arginase.

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Arginases are metalloenzymes and characterized by an unusual binuclear active site, containing two divalent manganese ions which are responsible for the hydrolysis of L-arginine to L-ornithine and urea [3, 4]. In mammals, arginase has been known for a long time as the final enzyme in the urea cycle. For this reason, the urea cycle arginase, or liver arginase or arginase 1 (L-arginine urea amidino hydrolase, EC 3.5.3.1), is the best characterized mammalian form of arginase. In the 1990s a growing interest in arginase raised after it was demonstrated that arginase might compete with NOS for a common substrate, L-arginine, and therefore regulates NO synthesis. Data from experimental studies provide overwhelming evidence that arginase over-activity is involved in the pathophysiology of various diseases, such as cardiovascular [5], pulmonary [6], and immune diseases [7] or cancer [8], and thereby highlight the promising value of arginase inhibitors for the treatment of various human diseases. It is noteworthy that recent small-scale clinical studies brought “proof-of-concept” for the therapeutic application of arginase inhibition to improve vascular function in patients with hypertension [9], type 2 diabetes associated with coronary artery disease [10], as well as heart failure [2]. Notably, effective synthetic compounds adapted to a long-term clinical use are currently lacking [11]. In addition, various infectious pathogens, including species of *Leishmania*, *Trypanosoma* or *Helicobacter*, were found to express their own arginase. Increased arginase activity is considered to play an important role in the viability and infectivity of these pathogens [12–14], and the use of pathogens arginase inhibitors appears to be also a promising option for the treatment of infectious diseases. Structure comparisons with human and parasite arginase complexes reveal that, although the former are trimer and the latter are hexamer, the active-site clefts of these enzymes are nearly conserved whereas the outer rims are not. Therefore, many recent studies have focused on the interesting differences between these two types of arginase to figure out new isozyme-specific and species-specific arginase inhibitors [15–17].

Previous studies identified the inhibitory effect of several polyphenols such as flavonoid type compounds and resveratrol on *Leishmania* arginase [18, 19]. As regards mammalian arginase, a few studies reported that plant extracts known to be rich in polyphenols such as cocoa beans extract [20] or *Scutellaria indica* extract [21] exhibited an arginase inhibitory effect. In addition, several phenolic compounds isolated from plants were found to inhibit arginase, such as piceatannol-3'-O- $\beta$ -D-glucopyranoside [22] or salvianolic acid [23]. One limitation of the available data on mammalian arginase inhibitors from natural sources is the great heterogeneity of the *in vitro* assays, making comparisons between studies difficult [18]. Indeed, the available assays for arginase activity include assays using different colorimetric methods [24, 25] and biological materials, such as isolated human erythrocytes [25] or animal organ homogenates [18], as sources of arginase. The limits of these methods are their cost, and the fact that biological materials from humans or animals can hamper feasibility and reproducibility, thereby making them not really adapted to a routine screening.

In the present study, we aimed to determine the potential of a series of polyphenols as mammalian arginase inhibitors in order to identify some structure-activity relationships (SARs). For this pur-



► **Fig. 1** Effects of arginase amount and incubation time on urea production. A fixed amount of L-arginine (14.3 mM/well) was incubated with a range of enzyme amounts during 30, 60, or 120 min at 37 °C. Only for an incubation time of 30 min, the urea production is linear over the whole range of arginase amounts tested (black line). Values are means  $\pm$  SD from 3 independent replicates.

pose, we developed a cost-effective *in vitro* colorimetric microplate method using commercially-available mammal liver arginase. This assay was adapted to the screening of natural compounds as new arginase inhibitors and was also used for the determination of enzyme kinetic constants.

## Results and Discussion

Among the colorimetric arginase assays, which are cheap and straightforward to implement, we chose to optimize the widely-used arginase assay from Corraliza et al. [24] based on urea production measurement. We used a commercially-available and low cost purified liver bovine arginase instead of cell lysates or tissue homogenates from animals and miniaturized all steps of the assay in a single 96-well microplate.

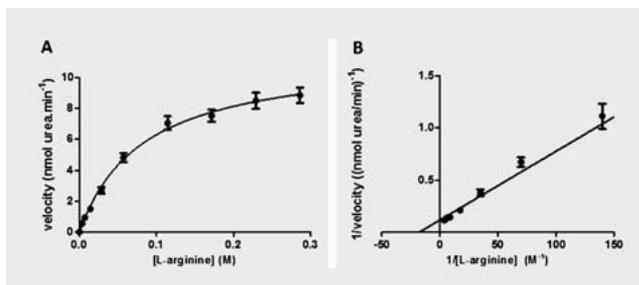
In a first step, we aimed to determine the adequate quantity of enzyme to use in the assay and measured urea production in the presence of several combinations of enzyme amounts (from 0.125 to 2 units per well) and incubation times (30, 60, or 120 minutes). As shown in ► **Fig. 1**, the relationship between urea production and enzyme amounts was linear up to 0.5 U of arginase, regardless of the incubation time. However, with higher amounts of arginase (1 and 2 units per well) the velocity of urea production was linear when the enzyme was incubated for 30 min (black line on ► **Fig. 1**) but decreased for longer incubation times (60 and 120 min). In these latter cases, the higher consumption of L-arginine over 60 or 120 min must have resulted in a higher production of L-ornithine which is known to inhibit mammalian liver arginase activity [26]. In addition, the decrease of L-arginine concentration over time during long incubations could also contribute to the reduced velocity of arginase according to Michaelis-Menten kinetics. On the basis of these data, we chose to use 0.25 U of arginase/well for further experiments and an incubation time of 60 min in order (1) to use a minimum amount of enzyme that (2) could produce enough urea to be quantified, and (3) to be able to distinguish between various inhibitor poten-

cies in further experiments. These experimental conditions regarding arginase amount and incubation time allowed us to fulfill these criteria and led to absorbance values between 0 (with inhibitor) and 1 (without inhibitor, i.e. corresponding to 100% of arginase activity; raw data not shown). Given that most if not all spectrophotometers give a linear range for absorbance between 0 and 1 a. u., our experimental conditions could be used in most laboratories.

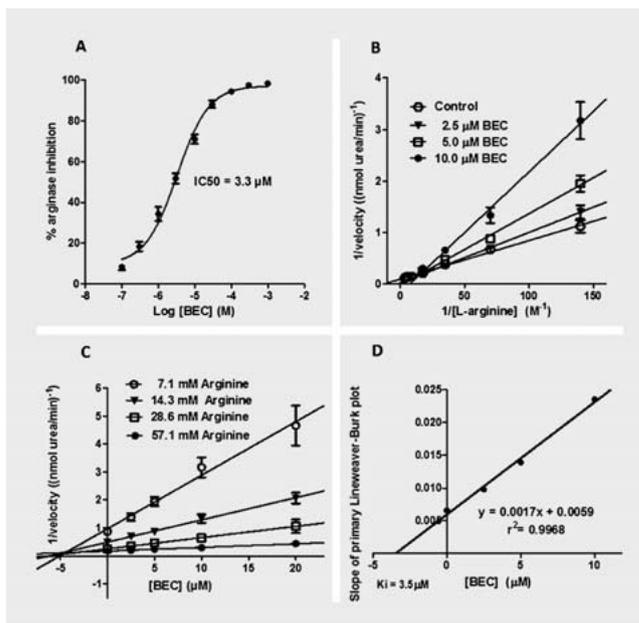
In a second step, we studied the velocity of bovine arginase as a function of substrate amounts (► Fig. 2A). The linearity of the Lineweaver-Burk double plots (► Fig. 2B) is in agreement with the already known Michaelis-Menten kinetics for this enzyme [25]. This plot also allowed us to determine the values of  $K_M$  ( $55.5 \pm 10.5$  mM) and  $V_{max}$  ( $11.5 \pm 0.5$  nmol urea/min) in our experimental conditions. The  $K_M$  value is in accordance with previous data showing a  $K_M$  of  $36 \pm 6$  mM for beef liver arginase [27].

S-(2-boronoethyl)-L-cysteine (BEC) is a commercially-available arginase inhibitor, widely-used in *in vitro* and *in vivo* studies [28, 29]. In order to use it as reference inhibitor in the newly-developed screening assay, we determined its half-maximal inhibitory concentration ( $IC_{50}$ ) and its maximum percentage of inhibition corresponding to the upper plateau of the sigmoid curve ( $E_{max}$ ); these two parameters represent the activity and efficacy of a compound, respectively. The calculated values from the sigmoidal curves were  $3.3 \mu\text{M}$  and 97.3%, respectively (► Fig. 3A). To further characterize the inhibition profile of BEC on liver arginase, enzyme inhibition experiments were carried out with various substrate concentrations in presence or absence of various inhibitor concentrations. As shown in ► Fig. 3B, the primary Lineweaver-Burk plot (reciprocal velocities vs. reciprocal of substrate concentrations) shows that the straight lines intersected on the common point in the first quadrant (counterclockwise). The Dixon plot linear transformation (reciprocal enzyme reaction velocity vs. inhibitor concentrations) indicated that the straight lines intersected on the second quadrant (► Fig. 3C). Taken together, these results argued for a competitive inhibition of BEC on the commercially-available bovine liver arginase, in agreement with data obtained in rat or human arginase [30,31]. The  $K_i$  value of BEC obtained from the secondary Lineweaver-Burk plot (► Fig. 3D) was  $3.5 \mu\text{M}$ , which is a slightly higher than the value obtained in recombinant rat arginase 1 ( $0.4\text{--}0.6 \mu\text{M}$ ) but in agreement with the dissociation constant of  $2.22 \mu\text{M}$  measured by isothermal titration calorimetry [31–33].

Among the natural substances from plants constituting a valuable source of new arginase inhibitors, polyphenol-type compounds are of particular interest [18]. In the present study, we investigated the potential as mammalian arginase inhibitors of ten ubiquitous polyphenols by using the new arginase inhibition assay. The evaluated polyphenols were chosen regarding their ubiquity in the plant kingdom and particularly in our diet. Furthermore, we selected structures allowing us to draw SARs. We first evaluated each compound at an initial concentration of  $100 \mu\text{M}$  in order to obtain a rapid estimation of their inhibitory potential. Excepted for quinic acid, all tested compounds exhibited an inhibitory activity greater than 40% at this concentration. Then,  $IC_{50}$  and  $E_{max}$  values of the compounds were assessed. In terms of inhibitory effect ( $IC_{50}$ ) the compounds could be ranked in the fol-



► Fig. 2 Michaelis-Menten kinetic profile (A) and Lineweaver-Burk representation (B). A fixed amount of arginase (0.25 U/well) was incubated with a range of L-arginine concentrations (0.0125 to 1 M) for 15 min at 37 °C. Lineweaver-Burk plots the reciprocal of the initial rate vs. the reciprocal of substrate concentration allowing the determination of kinetic parameters  $K_M$  ( $55.5 \pm 10.5$  mM) and  $V_{max}$  ( $11.5 \pm 0.5$  nmol urea/min) from the y and x intercept and the slope of the line, using Prism (v 5.0.3, GraphPad Software). Values are means  $\pm$  SD from 4 independent replicates (i.e. in separate experiments).

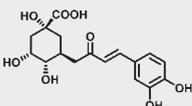
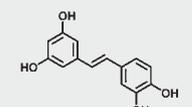
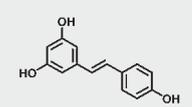
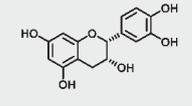
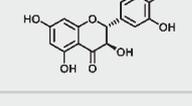
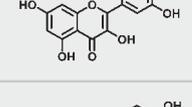
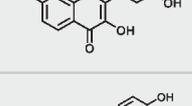
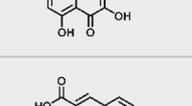
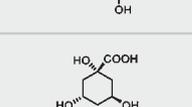
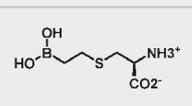
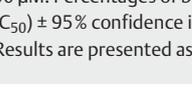


► Fig. 3 Concentrations-response curves of BEC. Percentage of arginase inhibition at different concentrations of BEC (A) allowing the determination of  $IC_{50}$  ( $3.3 \mu\text{M}$ ). Dixon (C) and Lineweaver-Burk (B, D) plots allowing the determination of the inhibition type (competitive) and the  $K_i$  ( $3.5 \mu\text{M}$ ). Values are means  $\pm$  SD from 3 separate experiments.

lowing order: chlorogenic acid > piceatannol > resveratrol  $\geq$  (-)-epicatechin > taxifolin > quercetin > fisetin > caffeic acid > kaempferol > quinic acid (► Table 1).

The two most active polyphenols exhibited  $IC_{50}$  values of  $10.6 \mu\text{M}$  for chlorogenic acid (CGA; ► Fig. 4A) and  $12.1 \mu\text{M}$  for piceatannol (PCT; ► Fig. 5A). These levels of activity, close to  $10 \mu\text{M}$ , could be qualified as marginal, according to the definition of White [34]. Although their inhibitory activities remained

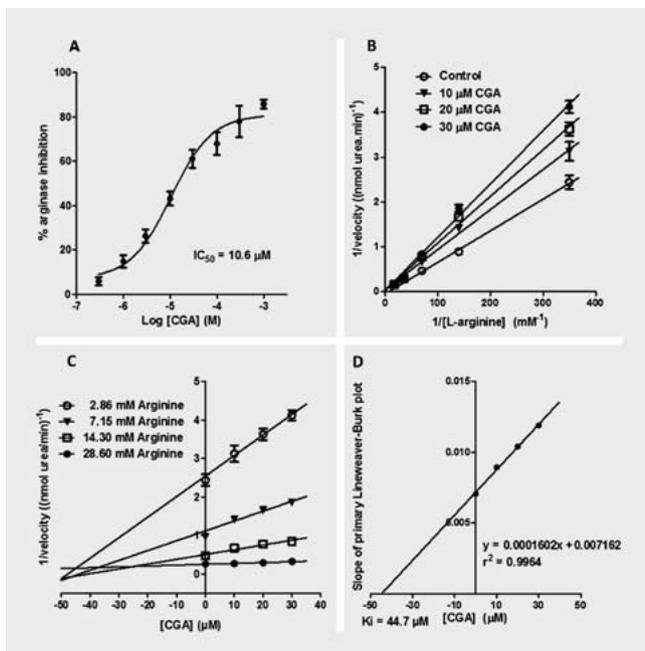
► **Table 1** b-ARG 1 inhibition percentages, IC<sub>50</sub> and E<sub>max</sub> values of polyphenols evaluated for their bovine arginase inhibitory capacities.

Compound	Structure	Screening (%) <sup>a</sup>	IC <sub>50</sub> (μM) <sup>b</sup>	E <sub>max</sub> (%) <sup>c</sup>
Chlorogenic acid		70.7 ± 0.7	10.6 (6.4–17.3)	81.0 ± 2.7
Piceatannol		75.9 ± 2.1	12.1 (8.8–16.5)	98.1 ± 2.1
Resveratrol		57.5 ± 2.1	18.2 (8.5–38.9)	86.0 ± 4.1
(-)-Epicatechin		56.8 ± 4.7	19.9 (12.5–31.7)	87.6 ± 3.1
Taxifolin		57.2 ± 5.9	23.2 (15.5–34.6)	96.5 ± 2.1
Quercetin		64.4 ± 2.5	31.2 (12.4–78.5)	90.3 ± 8.3
Fisetin		50.6 ± 4.2	82.9 (46.2–138.7)	102.4 ± 5.6
Kaempferol		41.1 ± 2.9	179.1 (110.6–290.1)	107.1 ± 7.9
Caffeic acid		61.3 ± 4.1	86.7 (63.2–118.9)	96.1 ± 2.4
Quinic acid		18.3 ± 5.9	3060.0 (2030–4614)	111.9 ± 4.9
BEC (reference)		93.6 ± 1.1	3.3 (2.6–4.1)	97.3 ± 1.3

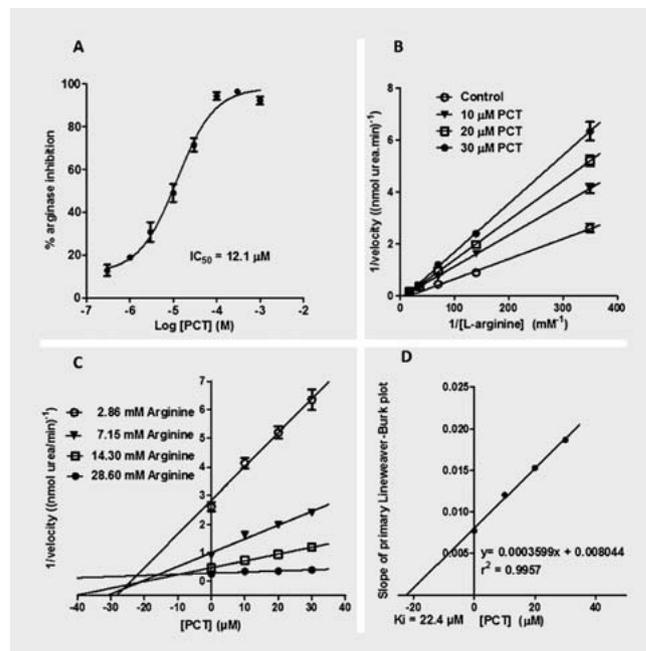
<sup>a</sup> All compounds were screened at 100 μM. Percentages of b-ARG 1 inhibition are presented as mean ± SD (n = 3); <sup>b</sup> results are presented as mean of half-maximal inhibitory concentrations (IC<sub>50</sub>) ± 95% confidence interval (n = 3); <sup>c</sup> E<sub>max</sub>: Maximum percentage of b-ARG 1 inhibition obtained when the sigmoid curve reaches to the plateau phase. Results are presented as mean ± SD (n = 3).

slightly lower than that of the reference arginase inhibitor BEC (IC<sub>50</sub> = 3.3 μM), these two polyphenolic compounds also exhibited a good efficacy with E<sub>max</sub> values reaching 81% for chlorogenic acid and 98% for piceatannol, i.e. an E<sub>max</sub> value similar to that of BEC (97 ± 1%) for the latter compound. Then we carried out enzyme

kinetic experiments to gain further insight in the inhibitory profile of these two phenolic compounds. The primary Lineweaver-Burk plot and the Dixon plot linear transformation led us to identify a competitive inhibition for both chlorogenic acid (► Fig. 4B, C) and piceatannol (► Fig. 5B, C). The dissociation constant (K<sub>i</sub>) val-



► **Fig. 4** Concentrations-response curves of CGA. Percentage of arginase inhibition at different concentrations of CGA (A) allowing the determination of  $IC_{50}$  (10.6  $\mu$ M). Dixon (C) and Lineweaver-Burk (B, D) plots allowing the determination of the inhibition type (competitive) and the  $K_i$  (44.7  $\mu$ M). Values are means  $\pm$  SD from 3 separate experiments.



► **Fig. 5** Concentrations-response curves of PCT. Percentage of arginase inhibition at different concentrations of PCT (A) allowing the determination of  $IC_{50}$  (12.1  $\mu$ M). Dixon (C) and Lineweaver-Burk (B, D) plots allowing the determination of the inhibition type (competitive) and the  $K_i$  (22.4  $\mu$ M). Values are means  $\pm$  SD from 3 separate experiments.

ues obtained from the secondary Lineweaver-Burk plots were 44.7  $\mu$ M for chlorogenic acid (► **Fig. 4D**) and 22.4  $\mu$ M for piceatannol (► **Fig. 5D**). Thus, despite a close inhibitory activity ( $IC_{50}$ ), it is likely that piceatannol exhibits a greater affinity for the enzyme than chlorogenic acid. Our results on the inhibitory effects of piceatannol are consistent with the previously reported arginase inhibitory activity of one of its glucoside isolated from Rhubarb (*Rheum undulatum* L.), piceatannol-3'-O- $\beta$ -D-glucopyranoside, studied in rat liver homogenate ( $IC_{50}$  = 11  $\mu$ M) [22]. The present study reports a significant mammalian arginase inhibitory effect for chlorogenic acid, an ubiquitous polyphenol contained in several dietary and medicinal plants [33, 35]. Of interest, the two building parts of chlorogenic acid, namely caffeic and quinic acids, were found to exhibit much less inhibitory activity ( $IC_{50}$  = 86.7 and 3060  $\mu$ M, respectively), as compared to the whole molecule ( $IC_{50}$  = 10.6  $\mu$ M). Moreover, caffeoyl (3,4-dihydroxycinnamoyl) may be important for the inhibitory activity as both piceatannol and chlorogenic acid share this moiety. The results showed that the caffeoyl derivatives, which possess an additional side chain, exhibit a higher inhibitory activity than caffeic acid. How the side chain participates to the inhibitory activity needs further investigation. As compared to piceatannol and chlorogenic acid, resveratrol was slightly less active suggesting that the presence of a catechol group could enhance the inhibitory activity. To confirm the importance of the catechol group, we compared the inhibitory activity of a series of flavonoids with (epicatechin, taxifolin, quercetin, fisetin) or without the catechol group (kaempferol). Our results (► **Table 1**) demonstrated that the lack

of the catechol group in kaempferol is associated with a dramatic decrease in inhibitory activity ( $IC_{50}$  kaempferol = 179  $\mu$ M vs.  $IC_{50}$  ranging from 20 to 83  $\mu$ M for the other flavonoids). Actually, strong arginase inhibitors like boronic acid derivatives, e.g. BEC, tend to bind to the OH group coordinated to two Mn (II) ions in the active site, and then disturb the enzymatic reaction of arginase [4]. Therefore, it is likely that the catechol function of polyphenolic compounds links to the Mn (II) ions or to Mn (II) and OH group to display the inhibitory activity. Further studies such as docking simulations or crystallographic experiments would be needed to clarify the precise binding mode of this type of molecule to arginase. In this series, the presence of a carbonyl group at position 4 did not change the inhibitory activity ( $IC_{50}$  epicatechin 19.9  $\mu$ M vs. taxifolin 23.2  $\mu$ M) whereas the unsaturation of the 2,3-bond slightly reduced inhibitory activity ( $IC_{50}$  taxifolin 23.2  $\mu$ M vs.  $IC_{50}$  quercetin 31.2  $\mu$ M). Interestingly, the absence of a phenol-functional group at position 5 induced a reduction of inhibitory activity (quercetin 31.2  $\mu$ M vs. fisetin 82.9  $\mu$ M).

In conclusion, the present study reports the inhibitory potential of several natural polyphenols on mammal arginase, including chlorogenic acid, piceatannol, resveratrol, and epicatechin. The evaluation was performed using a new, simple and cost-effective *in vitro* assay which uses small amounts of a commercially-available purified bovine arginase 1 and requires material present in most laboratories. Our data identified the importance of the caffeoyl (3,4-dihydroxycinnamoyl) part and the catechol function in the inhibitory activity of the tested compounds, giving features for the development of new arginase inhibitors via a rational drug design.

## Materials and Methods

### Materials

The tested polyphenols were obtained from commercial suppliers: chlorogenic acid (98%), (-)-epicatechin ( $\geq 90\%$ ), taxifolin ( $\geq 90\%$ ), quercetin ( $\geq 95\%$ ), fisetin ( $\geq 98\%$ ), kaempferol ( $\geq 97\%$ ), and caffeic acid ( $\geq 98\%$ ) from Sigma-Aldrich, piceatannol ( $> 98\%$ ) from TCI Chemicals, resveratrol ( $\geq 98\%$ ) from Alexis Biochemicals, and quinic acid (98%) from Alfa Aesar. The reference inhibitor S-(2-boronoethyl)-L-cysteine (BEC, purity  $\geq 97\%$ ) was purchased from Calbiochem (EMD Millipore) and the purified liver bovine arginase 1 from MP Biomedicals. One unit (1 U) of bovine arginase is defined by this manufacturer as the amount of enzyme that converted 1  $\mu\text{mole}$  of L-arginine to urea and L-ornithine per minute at pH 9.5 and 37 °C. 1 U/ $\mu\text{L}$  stock solution of this enzyme was prepared in the following buffer: Tris/HCl (50 mM, pH = 7.5) with 0.1% Bovine Serum Albumin (referred as TBSA buffer thereafter) containing NaCl 0.1 M and 20% glycerol. This stock solution was stored at  $-26\text{ }^{\circ}\text{C}$  until use.

### Arginase assay

We adapted the colorimetric method to measure arginase activity developed by Corraliza et al. [24]. As compared to the original method, all steps of the method were performed in a microplate, and the method uses a commercially-available purified bovine liver arginase instead of cell or tissue lysates. In each well of a microplate the following solutions were added in this order: (1) 10  $\mu\text{L}$  of TBSA buffer with or without (control) arginase at 0.025 U/ $\mu\text{L}$  unless otherwise stated, (2) 30  $\mu\text{L}$  of Tris-HCl solution (50 mM, pH 7.5) containing  $\text{MnCl}_2$  10 mM as a co-factor, (3) 10  $\mu\text{L}$  of a solution containing an inhibitor or its solvent (as a control), (4) 20  $\mu\text{L}$  of L-arginine (pH 9.7, 0.05 M, unless otherwise stated). The microplate was covered with a plastic sealing film and incubated for 60 min (unless otherwise stated) in a 37 °C water bath. The reaction was stopped by adding 120  $\mu\text{L}$  of  $\text{H}_2\text{SO}_4/\text{H}_3\text{PO}_4/\text{H}_2\text{O}$  (1:3:7) after placing the microplate on ice. Thereafter, 10  $\mu\text{L}$  of alpha-isonitrosopropiophenone (5% in absolute ethanol) was added and the microplate was covered with an aluminium sealing film and heated in a 100 °C oven for 45 min. The microplate was kept in the dark until reading since the reaction between urea and alpha-isonitrosopropiophenone is light-sensitive. After 5 min of centrifugation and cooling for another 10 min, the microplate was shaken for 2 min and the absorbance was read at 550 nm and 25 °C with a spectrophotometer (Synergy HT BioTeck). The level of arginase activity was either expressed as the amount of urea produced per min, calculated from a standard curve of urea or relative to the "100% arginase activity". For the standard curve, urea solutions (70  $\mu\text{L}$ ) of increasing concentrations were added to each well and the same procedure as described above, from the 37 °C step (not included) up to the absorbance reading was carried out. All experiments were performed at least in triplicate in three independent experiments.

### Determination of the optimal quantity of arginase and incubation time for the assay

In order to determine the adequate quantity of enzyme to use in the assay, the urea production was measured in presence of sev-

eral combinations of amount of enzyme (0.125, 0.25, 0.50, 1, and 2 units per well) and incubation times (30, 60, or 120 min; ► Fig. 1) according to the protocol described in the above Arginase assay section.

### Determination of $V_{\text{max}}$ and $K_{\text{M}}$ of commercially-available bovine liver arginase

The activity of purified arginase was assayed at different concentrations of L-arginine at 37 °C for 15 min. We used serial dilutions of a 0.5 M stock solution of L-arginine, giving a concentration of this substrate in each well that ranged from 0 to 286 mM. Using the urea standard curve mentioned above, we converted the absorbances at 550 nm into nmol of urea produced per min. The values of kinetic parameters  $K_{\text{M}}$  and  $V_{\text{max}}$  were inferred from Lineweaver-Burk (► Fig. 2B; Prism; v 5.0.3, GraphPad Software). This experiment was carried out four times independently.

### Determination of $E_{\text{max}}$ , $\text{IC}_{50}$ and the constant $K_{\text{i}}$ of inhibitors

To validate the use of the adapted assay for discovering new arginase inhibitor, we first characterized the inhibitory effect of commercially-available synthetic arginase inhibitor, 2-boronoethyl)-L-cysteine (BEC).  $\text{IC}_{50}$  value was determined by rate measurement for nine concentrations of BEC ( $10^{-7}$  to  $10^{-3}$  M) incubated with 14.3 mM of L-arginine in TBSA buffer (50 mM), final pH 8. The reaction mixture was then incubated with arginase (0.25 unit) for one hour, as described in the Arginase assay section above. For each inhibitory concentration the resulting absorbance was converted into percentage of arginase inhibition, i.e. relative to the absorbance of controls with no inhibitor ("100% arginase activity"). The mathematical sigmoidal model ( $\log \text{IC}_{50}$ ) was used to calculate the median inhibitory concentration ( $\text{IC}_{50}$ ) and the maximal inhibitory effect ( $E_{\text{max}}$ ) values using GraphPad Prism v 5.0.3 (► Fig. 3A). The type of inhibition and  $K_{\text{i}}$  value was determined with the same experimental approach, with three concentrations of BEC (2.5, 5, and 10  $\mu\text{M}$ ) and a control under increasing L-arginine concentrations (7.1, 14.3, 28.6, and 57.1 mM). The kinetics data were analyzed using a Lineweaver-Burk plot (obtained by reciprocal reaction velocities vs. reciprocal of substrate concentrations; ► Fig. 3B) and Dixon Plots (obtained by reciprocal reaction velocities vs inhibitor concentrations; ► Fig. 3C). The  $K_{\text{i}}$  value was obtained by a secondary plot of the Lineweaver-Burk plot (obtained by the slopes of the regression lines in the Lineweaver-Burk plot vs inhibitor concentrations; ► Fig. 3D). These plots were established by using Prism (v 5.0.3, GraphPad Software).

The inhibitory effect of chlorogenic acid (CGA), piceatannol (PCT), resveratrol, (-)-epicatechin, taxifolin, quercetin, fisetin, caffeic acid, quinic acid, and kaempferol against bovine liver arginase was first evaluated at a concentration of 100  $\mu\text{M}$ . Then the  $E_{\text{max}}$  and  $\text{IC}_{50}$  values of these compounds were determined for at least nine concentrations ( $10^{-7}$  to  $10^{-3}$  M) as described above for BEC (► Table 1). The type of inhibition and  $K_{\text{i}}$  values of CGA and PCT were determined as described for BEC with three concentrations (10, 20 and 30  $\mu\text{M}$ ) and a control under increasing L-arginine concentrations (2.86, 7.15, 14.3 and 28.6 mM) (► Figs. 4 and 5).

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## Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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