Synthesis of natural Urolithin M6, a Galloflavin mimetic as potential inhibitor of Lactate dehydrogenase A

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Electronic Supporting Information

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Fig. 1 Gut microbiota metabolism of ellagitannins and ellagic acid (adapted from R. Garcia-Villalba, et al., *J Agric Food Chem*, 2013, 61, 8797).
Detailed biological protocols

Enzymatic assays on purified human LDH-A

Purified LDH-A (from human liver) was obtained from Lee Biosolutions (St Louis, Missouri, USA). 20 mM stock solutions of compound was prepared in DMSO. It was added in scalar amounts (0 – 200 \( \mu \)M final concentrations) to a reaction mix containing 100 mM phosphate buffer pH 7.5, 0.015 U LDH / ml, 1 mM pyruvate and 150 \( \mu \)M NADH. For all the determinations (including those without the compounds), DMSO in the reaction mix was always kept to 0.6%. The enzymatic activity was measured by monitoring NADH oxidation for a period of 3 min. To avoid interference of the compounds with the UV reading of NADH oxidation, we adopted the procedure reported by Moran\(^7\), which measured LDH activity by recording the decrease of NADH fluorescence. The assay was performed in 96-well white body plates, using a Fluoroskan Ascent FL reader (Labsystems). The concentration of compounds causing 50% inhibition of LDH activity (IC\(_{50}\)) was calculated from the second order polynomial regression of the experimental data, using the Prism 5 GraphPad software.

Cell culture conditions

Raji cells were grown as a suspension culture in RPMI 1640 containing 10% FBS, 100 U/ml penicillin/streptomycin, 4 mM glutamine and were maintained at a concentration of 1-2 x 10\(^5\) viable cells/ml. All media and supplements were from Sigma. In all experiments, inhibitors were added to the culture media in the presence of 0.6% DMSO. The same amount of DMSO was always added to the control, untreated cultures.

Effects of the inhibitors on lactate production
5 x 10^5 cells in 1 mL of culture medium were seeded in each well of a 6-well Nunclon plate. Scalar amounts of inhibitor (0-200 μM, tested in duplicate) were then added to the cultures. Lactate was measured in 3 untreated wells at the start of experiment (baseline value) and 3 h after incubation at 37°C. In each well, we simultaneously measured released in medium and intracellular lactate: at the end of incubation 100 μl of 100% trichloroacetic acid (TCA) solution was added; the cell lysate was collected and the well was washed with 1 mL 10% TCA. After centrifugation, lactate in the supernatant was measured according to the method of Barker and Summerson. The amount of metabolite formed during the 3 h incubation with or without the inhibitor was calculated by subtracting the baseline value. The dose of compound causing 50% inhibition of lactate production (IC50) was calculated from the second order polynomial regression of experimental data, using the Prism 5 GraphPad software.

**Effects of the inhibitors on cell growth or viability**

To study the effect of the inhibitor on cell proliferation, 1 x 10^5 cells were seeded in 24-multiwell plates and treated for 24 h at 37°C with scalar doses of inhibitors (0-200 μM, tested in duplicate). After incubation, cells were counted under a light microscope using a Neubauer chamber and their viability was determined by Trypan blue exclusion. The cell growth was calculated from the difference between the number of viable cells counted at 24 h and that at the beginning of experiment. Data were plotted as cell growth vs dose of compound. The dose causing 50% inhibition of cell growth (cell growth IC50) was calculated by applying the second order polynomial regression to the experimental data.
$^1$H and $^{13}$C NMR spectra (graphs) of compounds

$^1$H NMR (400 MHz)
Impurities from commercially available 4-methoxyphenyl boronic acid
Impurities from commercially available 4-methoxyphenyl boronic acid
CDCl₃

6

CH₂Cl₂ traces

"grease" traces

f₁ (ppm)

0 1000 2000 3000 4000 5000 6000 7000 8000 9000 10000 11000

6 CDCl₃

O

O

O

O

O

CDCl₃

3.87 3.95 3.97 4.03 6.84 6.85 6.87 6.89 6.90 7.26 7.70 8.71 8.73

f₁ (ppm)

10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
Hydrogen-Deuterium exchange

DMSO-d6

UM6
$^{13}$C NMR (100 MHz)

![NMR Spectrum](image-url)
CDCl$_3$