Mechanism of Melanogenesis Inhibition by Keggin-type Polyoxometalates

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Materials and methods

Reagents

$H_5GaMo_{12}O_{40}$[1], $H_4SiMo_{12}O_{40}$[2] and $Na_7PMo_{11}MO_{40}$ (M = Co, Ni, V, Zn)[3,4] were synthesized as previously described[5] with slight modifications. The synthesized POMs were characterized by Fourier transform infrared spectroscopy (FT-IR, Jasco FT/IR-480), UV/Vis absorption spectroscopy (Cary-50) and X-ray diffraction (XRD, Nippon Science Co., Ltd.). PMo_{12} was purchased from Sinopharm Chemical Reagent Co., Ltd, China. Tyrosinase, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethyl-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). L-3,4-dihydroxyphenylalanine (L-DOPA) was bought from Shanghai Baoman Biotechnology Co., Ltd. (China). Trypsin-EDTA solution was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). Other chemicals were local products of analytical grade.

Inhibition of tyrosinase activity assay

The tyrosinase inhibition assay was performed as previously described[6-8]. In this experiment, L-DOPA was used as a substrate to generate dopachrome with catalysis by tyrosinase, and the OD value after the reaction of the system was monitored by the microplate reader (Synergy H1MF) at a wavelength of 475 nm. Briefly, the reaction system was sequentially supplemented with 196 μL L-DOPA dissolved in 50 mM phosphate buffer (pH 6.8), and 7 μL of different concentrations of inhibitors completely dissolved in DMSO solution. Next, 7 μL tyrosinase solution was added and the mixture was incubated at 30 °C for 10 min. The OD value of the 200 μL reaction solution was monitored in a 96-well plate by using the microplate reader at 475 nm. As previously described[6-8], the inhibitory effect (IC_{50}), inhibition mechanism and inhibition type of POMs on tyrosinase were studied.

Molecular simulation study

The 3D homology crystal model structure of *A. bisporus* tyrosinase (PDB ID: 2Y9X) was obtained from the Protein Data Bank (http://www.rcsb.org). Docking calculations were performed using the molecular operating environment (MOE) and Autodock (v4.2) software package[8,9]. The concrete parameters of the MOE used were Placement: Triangle Matcher, Rescoring 1: London dG, Refinement: Induced Fit, Rescoring 2: GBVI/WSA dG, Poses: 1000. Autodock calculations utilized the standard AutoDock force field and the Lamarckian genetic algorithm (LGA) search for the lowest energy docked conformation. Each docking experiment consisted of 500 independent LGA runs.

Fluorescence quenching assays

Fluorescence measurements were carried out with a Cary Eclipse fluorescence spectrophotometer (Agilent, USA). The fluorescence signal (λ_{ex} = 280 nm) was recorded between 290 and 450 nm. Excitation and emission slits for these measurements were set to 5 and 10 nm, respectively. At different temperatures (298, 301 and 310 K), 1 mL of POMs ($GaMo_{12}$, $SiMo_{12}$ and PMo_{12}) of different concentrations were incorporated with tyrosinase solution (2500 U/mL).

Cell culture

The B16 melanoma cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All these cell lines were maintained in a humidified atmosphere of 37 °C in a 5% CO_2 and cultured in RPMI-1640 Medium (SH3080901, Hyclone, USA) with 10% FBS (Sigma) and 1% double antibody. These cells were then used for cell viability, tyrosinase inhibition and melanin content determination.

Cell viability assay (MTT)

B16 melanoma cells (1×10^5 cells/well) were plated in 96-well plates. Each cell lines were treated with POMs at 12.5, 25, 50, 100, and 200 μM for 72 hours. Then, cells were incubated with MTT solution

(10 μL, 0.5 mg/mL) for 4 h. 200 μL dimethyl sulfoxide (DMSO) was added to each well and incubated for 10 min at 37°C with mild shaking. The absorbance was measured at 570 nm[10,11].

Cellular tyrosinase activity assay

Cellular tyrosinase activity was determined by measuring the L-DOPA oxidation rate[11]. Each cell lines were treated with POMs at 0, 12.5, 25, 50, 100, and 200 μM for 72 hours. Then, incubated cells were washed twice with PBS buffer (pH 7.4), and then 90 μL PBS contenting 1% TritonX-100 mixed with 10 μL L-DOPA (1.0 mg/mL) was added to each well. Cellular tyrosinase activity was then determined by measuring the absorbance at 470 nm.

Melanin content determination

Each cell lines were treated with POMs at 12.5, 25, 50, 100, and 200 μM for 72 hours. Cells were harvested by trypsinization and added to the collected medium. After centrifugation at 12000 rpm × 10 min, the separated melanin pigments were solubilized in 1 mL 1 M NaOH containing 10% DMSO was used at 80°C for 2 h. The melanin content was calculated from the absorbance intensity at 405 nm of standard melanin[12].

DPPH radical scavenging assay

DPPH was used for determination of free radical-scavenging activity[13,14]. Briefly, 2 mL of POMs solution was mixed with 2 mL of 0.1 mM DPPH ethanol solution and incubated at room temperature for 30 min in the dark. The absorbance was recorded at 517 nm.

ABTS radical scavenging assay

The ABTS mixture (2.45 mM potassium persulfate with 7 mM ABTS salt) was combined with ethanol until an absorbance of 0.7 (at 734 nm). Then, POMs solution (0.1 mL) was mixed with 1 mL ABTS reagent and incubated for 6 min at room temperature. After that, the absorbance was measured at 734

nm wavelength[14,15].

Supplementary figures

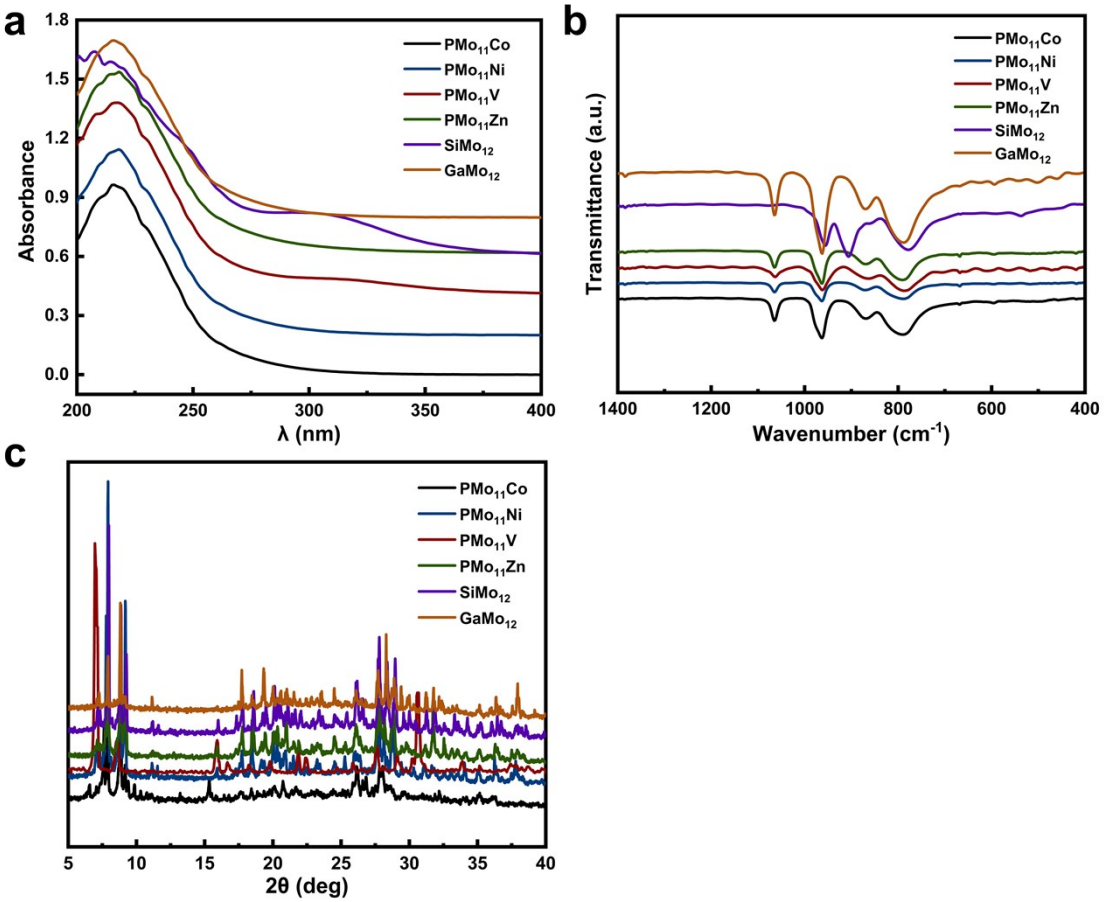

Fig. S1. Structural characterization of the Keggin-type POMs ($GaMo_{12}$, $SiMo_{12}$ and $PMo_{11}M$ (M = Co, Ni, V, Zn)). (a) UV/Vis absorption spectrum, (b) FT-IR spectrum, and (c) X-ray powder diffraction (XRD) spectra.

Supplementary tables

Table S1. Reaction parameters calculated according to the Stern-Volmer equations of $SiMo_{12}$.

T (K)	K_{sv} (M^{-1})	K_q (M^{-1} S^{-1})	R^2	K (M^{-1})	n	R^2	△H (kJ mol^{-1})	△G (kJ mol^{-1})	△S (J mol^{-1})
298	1.15×10^4	1.15×10^{12}	0.983	5.93×10^3	0.93	0.966		-380.6	
304	1.18×10^4	1.18×10^{12}	0.992	2.87×10^2	0.64	0.990	-381.6	-380.6	-3.29
310	5.43×10^4	5.43×10^{12}	0.992	1.14×10^4	0.85	0.988		-380.5	

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