Supporting information

Internalization into cancer cells of zwitterionic amino acid polymers via amino acid transporter recognition

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Materials and Methods

**Materials.** Boc-(L)Lys-OH, Boc(D)Lys-OH, Boc-4-amino-(L)Phe-OH, Boc-4-amino-(D)Phe-OH, and Boc-(L)Ser-OtBu were purchased from Watanabe Chemical Industries, Hiroshima, Japan. Methacryloyl chloride, trifluoroacetic acid (TFA), 2-(methacryloyloxy)ethyl 2-(trimethylammonio)ethyl phosphate (MPC), Ethyl 2-bromoisobutyrate, and 2,2’-bipyridyl, and Tris (3-hydropropyl-triazolylmethyl) amine (THPTA) were purchased from Tokyo Chemical Industry, Tokyo, Japan. Propargyl methacrylate, Cy5-azide, sulfoCy5-azide, copper(II) sulfate pentahydrate (CuSO₄·5H₂O), and propargyl 2-bromoisobutyrate were purchased from Sigma-Aldrich Co., Japan. 2,2’-azobis(2-methylpropionitrile) (AIBN), copper(I) bromide (CuBr), 2,2,2-trifluoroethanol (TFE), and sodium ascorbate were purchased from Kanto Chemical Co., Inc., Japan. mPEG-Alkyne (40 kDa) was purchased from Creative PEGWorks, Winston Salem, NC. All chemicals were used as received without further purification.

All chemical reactions were performed under a nitrogen or argon atmosphere. Nuclear magnetic resonance (NMR) spectra were recorded with a 500 MHz JEOL spectrometer using methanol-d₄ or D₂O as a solvent.

**Synthesis of Boc(L)lysine methacrylamide (Boc(L)LysMA).** Boc-(L)Lys-OH (1.0 g, 4.06 mmol) was dissolved in 10 mL of 1M NaOH/THF (50/50 vol%). Methacryloyl chloride (0.467 g, 4.47 mmol) was added dropwise into the reaction mixture over 30 min at 0°C and left to stir at room temperature (RT) for 12 hr. The product was concentrated in vacuo, then dissolved in dichloromethane (DCM). The precipitation of byproduct sodium salts was removed by filtration. The crude product was purified by column chromatography eluted with DCM/methanol (92:8), which afforded white solids (yield: 0.685 g, 2.09 mmol, 52%). ¹H NMR (500 MHz, chloroform-d): δ (ppm) = 5.65 (s, 1H), 5.33 (s, 1H), 4.04 (m, 1H), 3.22 (m, 2H), 1.91 (s, 3H), 1.85–1.50 (m, 6H), 1.40 (s, 9H). ¹³C NMR (125 MHz, methanol-d₄): δ (ppm) = 174.91, 169.97, 156.84, 140.12, 118.87, 79.11, 53.47, 38.95, 31.10, 28.63, 27.40, 27.23, 22.95, 17.50. ESI–MS (M⁻): calcd for C₁₅H₂₅N₂O₅ 313.18, found 313.1.
Synthesis of Boc(D)lysine methacrylamide (Boc(D)LysMA). Boc(D)LysMA was synthesized using a similar procedure described for Boc(L)LysMA. $^1$H NMR (500 MHz, chloroform-d): $\delta$ (ppm) = 5.65 (s, 1H), 5.33 (s, 1H), 4.04 (m, 1H), 3.22 (m, 2H), 1.91 (s, 3H), 1.85–1.50 (m, 6H), 1.40 (s, 9H). $^{13}$C NMR (125 MHz, methanol-d$_4$): $\delta$ (ppm) = 174.92, 169.97, 156.84, 140.10, 118.94, 79.13, 53.47, 38.96, 31.10, 28.63, 27.42, 27.26, 22.95, 17.50. ESI−MS (M$^-$): calcd for C15H25N2O5 313.18, found 313.1.

Synthesis of Boc(L)phenylalanine methacrylamide (Boc(L)PheMA). Boc-4-amino-(L)Phe-OH (1.0 g, 3.57 mmol) was dissolved in 10 mL of 1M NaOH/THF (50/50 vol%). Methacryloyl chloride (0.410 g, 3.92 mmol) was added dropwise into the reaction mixture over 30 min at 0°C and left to stir at room temperature (RT) for 12 h. The product was concentrated in vacuo, then dissolved in dichloromethane (DCM). The precipitation of byproduct sodium salts was removed by filtration. The crude product was purified by recrystallization from hexane, which afforded white solids (yield: 0.685 g, 2.09 mmol, 52%). $^1$H NMR (500 MHz, chloroform-d): $\delta$ (ppm) = 7.49 (d, $J$ = 8.0 Hz, 2H), 7.17 (d, $J$ = 8.5 Hz, 2H), 5.75 (s, 1H), 5.47 (s, 1H), 4.30 (m, 1H), 3.12–2.84 (m, 2H), 1.99 (s, 3H), 1.36 (s, 9H). $^{13}$C NMR (125 MHz, methanol-d$_4$): $\delta$ (ppm) = 174.02, 168.58, 156.49, 140.83, 136.96, 133.46, 129.28, 120.79, 119.25, 79.19, 54.97, 36.79, 27.35, 27.13, 17.60. ESI−MS (M$^-$): calcd for C18H24N2O5 347.17, found 347.1.

Synthesis of Boc(D)phenylalanine methacrylamide (Boc(D)PheMA). Boc(D)PheMA was synthesized using a similar procedure described for Boc(L)PheMA. $^1$H NMR (500 MHz, chloroform-d): $\delta$ (ppm) = 7.49 (d, $J$ = 8.0 Hz, 2H), 7.17 (d, $J$ = 8.5 Hz, 2H), 5.75 (s, 1H), 5.46 (s, 1H), 4.30 (m, 1H), 3.12–2.84 (m, 2H), 1.99 (s, 3H), 1.36 (s, 9H). $^{13}$C NMR (125 MHz, methanol-d$_4$): $\delta$ (ppm) = 174.05, 168.59, 156.51, 140.82, 136.96, 133.47, 129.29, 120.79, 119.27, 79.19, 54.99, 36.78, 27.35, 27.12, 17.61. ESI−MS (M$^-$): calcd for C18H24N2O5 347.17, found 347.1.

Synthesis of Boc(L)serineOtBu methacrylate (Boc(L)SerOtBuMA). Boc-(L)Ser-OtBu (1.5 g, 5.7 mmol) was dissolved in 12 mL of DCM containing 1.2 mL of triethylamine. Methacryloyl chloride
(0.72g, 6.9 mmol) was added dropwise into the reaction mixture over 30 min at 0°C and left to stir at room temperature (RT) for 24 hr. The precipitation of byproducts was removed by washing organic layer with 0.1 M NaOHaq and 0.01M HCl aq. After the washing, the organic layer was dried using saturated NaCl aq and MgSO$_4$ anhydrous. Then, the crude product was purified by recrystallization from cold hexane. which afforded white solids (yield: 1.67 g, 5.1 mmol, 89 %). $^1$H NMR (500 MHz, metahnol-$d_4$): $\delta$ (ppm) = 6.09 (s, 1H), 5.62 (s, 1H), 4.4-4.2 (m, 3H), 1.90 (s, 3H), 1.44 (s, 18H). $^{13}$C NMR (125 MHz, methanol-$d_4$): $\delta$ (ppm) = 169.02, 166.82, 156.42, 135.99, 125.49, 82.05, 79.47, 63.94, 53.72, 27.34, 26.85, 17.02.

**Polymerization of Boc-protected amino acid monomers.** Boc-protected amino acid polymers was synthesized through free radical polymerization using AIBN as the initiator. In a typical example, Boc($L$)LysMA (0.48 g, 1.53 mmol), propargyl methacrylate (9.00 mg, 72.5 $\mu$mol), and AIBN (10.8 mg, 55.1 $\mu$mol) were dissolved in DMF (1.53 mL). After bubbling N$_2$ gas for 30 min at RT, the polymerization was performed at 70°C for 20 h. The reaction was quenched by cooling, and then the polymer solution was precipitated into diethyl ether. The precipitate was collected by dissolving into methanol, then purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against methanol for 3 days (yield: 0.233 g, 49%). $^1$H NMR (500 MHz, methanol-$d_4$): $\delta$ (ppm) = 4.06 (br, 1H), 3.06 (br, 2H), 1.9−0.70 (m, 20H).

**Polymerization of Boc($L$)SerOtBuMA.** Poly(Boc($L$)SerOtBuMA) was synthesized through atom transfer radical polymerization (ATRP). Boc($L$)SerOtBuMA (0.28 g, 0.85 mmol), propargyl methacrylate (1.48 mg, 11.9 $\mu$mol), propargyl 2-bromoisobutyrate (1.39 mg, 6.8 $\mu$mol), and 2,2-bipyridyl (12.4 mg, 79.6 $\mu$mol) were dissolved into 0.86 mL of TFE. The mixture was degassed by three freeze-pump-thaw cycles, then transferred into schlenk flask containing CuBr (5.2 mg, 36.2 $\mu$mol). The polymerization was performed at 40°C for 8 h. The reaction was quenched by cooling, and then the polymer solution was dialyzed against methanol using a dialysis membrane with a 3.5 kDa
molecular weight cut-off (Spectrum Laboratories) for 3 days. Then, the polymer was collected by evaporating. (yield: 0.22 g, 78.6 %). ¹H NMR (500 MHz, methanol-d₄): δ (ppm) = 4.5-3.9 (br, 3H), 2.2-1.3 (m, 21H), 1.2-0.8 (m, 2H).

**Deprotection of Boc group and tert-butyl group in Boc(L)SerOtBuMA.** Boc(L)SerOtBuMA were deprotected by TFA treatment. Boc(L)SerOtBuMA (46 mg) was dissolved in TFA (2.0 mL) at RT. The reaction solution was stirred for 5 h at RT, then the polymer solution was dried in vacuo to remove byproducts and unreacted compounds. The polymer was collected by water and purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against water for 3 days. ¹H NMR (500 MHz, methanol-d₄): δ (ppm) = 4.5-3.9 (br, 3H), 2.2-0.3 (m, 5H).

**Deprotection of Boc group in Boc-protected amino acid polymers.** The Boc group in Boc-protected amino acid polymers were deprotected by TFA treatment. In a typical example, pBoc(L)LysMA (50 mg) was dissolved in TFA (1.0 mL) at RT. The reaction solution was stirred for 1 h at RT, then the polymer solution was precipitated into ethyl acetate. The polymer was collected by methanol and purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against methanol for 3 days. ¹H NMR (500 MHz, methanol-d₄): δ (ppm) = 3.84 (br, 1H), 3.06 (br, 2H), 2.2-0.8 (m, 11H).

**Synthesis of poly (2-methacyryloyloxyethyl phosphorylcholine) (pMPC).** Ethyl 2-bromoisobutyrate (1.65 mg, 8.46 μmol), MPC (0.5 g, 1.69 mmol), propargyl methacrylate (11.96 mg, 89.1 μmol), and 2,2-bipyridyl (15.3 mg, 95.9 μmol) were dissolved into TFE. The mixture was degassed by three freeze-pump-thaw cycles, then transferred into schlenk flask containing CuBr (6.4 mg, 44.6 μmol). The polymerization was performed at 40°C for 5 h. The reaction was quenched by cooling, and then the polymer solution was precipitated into acetone. The precipitate was collected by dissolving into water, then purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against water for 3 days. Then, the polymer was collected by lyophilization.
(yield: 183.16 g, 36.7 %). $^1$H NMR (500 MHz, methanol-d4): $\delta$ (ppm) = 4.67 (br, 2H), 4.17 (br, 2H), 4.09 (br, 2H), 3.96 (br, 2H), 3.11 (br, 9H), 1.81 (br, 2H), 1.00 – 0.80 (br, 3H).

**Cy5 modification to amino acid-based polymers and pMPC via click chemistry.** Cy5 was attached to amino acid polymers and pMPC using alkyne-azide click chemistry. In a typical example, pBoc(L)LysMA (50 mg), Cy5-azide (2.46 mg, 4.09 $\mu$mol), CuSO$_4$-5H$_2$O (1.0 mg, 4.00 mol), sodium ascorbate (1.0 mg, 5.05 mmol) was dissolved in DMF (5 mL) at RT. The reaction solution was heated to 60°C and stirred for 12 h. The polymer solution was precipitated into diethyl ether. The precipitate was collected by dissolving into methanol, then purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against methanol for 3 days (yield: 0.233 g, 49%). The Boc group was deprotected using similar procedure described above.

**Cy5 modification to poly(L)SerMA and mPEG-alkyne via click chemistry.** Sulfo-Cy5 was attached to the polymers using alkyne-azide click chemistry. In a typical example, poly(L)SerMA (20 mg), Sulfo-Cy5-azide (4.3 mg, 5.6 $\mu$mol), CuBr (2.67 mg, 18.6 $\mu$mol), THPTA (16 mg, 36.8 $\mu$mol) was dissolved in water (5 mL) at RT. The reaction solution was heated to 60 °C and stirred for 24 h. The polymer solution was precipitated into diethyl ether. The precipitate was collected by dissolving into methanol, then purified by dialysis using a dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Laboratories) against water containing EDTA or methanol for poly(L)SerMA$_{Cy5}$ or mPEG$_{Cy5}$, respectively, for 3 days.

**Sample Preparation.** All zwitterionic amino acid polymers (ZAPs) were dissolved in methanol and stored in a refrigerator at 4 °C. The polymer solution was dried in vacuo, and then, a certain amount of buffer solution was added to the desired concentration. Finally, the prepared solutions were filtered through a 0.20 $\mu$m filter membrane before the experiments described below.

**Size exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS) measurements.** To analyze the number-averaged molecular weight ($M_n$) and weight-averaged
molecular weight ($M_0$) of the prepared water-soluble polymers, we used a SEC-MALS-RI system consisting of a Shimadzu LC-20AD pump, SEC columns (SB-806MHQ and SB-802.5HQ), a multi-angle static light scattering (DAWN HELEOS-II) (Wyatt) operating at a wavelength of 658 nm, and a Shodex RI-501 RI detector. The mobile phase was aqueous solution containing 500 mM of acetic acid and 200 mM of sodium nitrate (acetic acid buffer) and eluted at 0.80 ml/min and 40°C. For pBoc(L)SerOtBuMA, we used a SEC-MALS-RI system consisting of a Shodex HPLC pump (DU-H2130), SEC columns (KF-806M and KF-802.5), a multi-angle static light scattering (DAWN HELEOS-II) (Wyatt) operating at a wavelength of 658 nm, and an RI-71S interferometric differential refractive index (RI) detector (Shodex). The mobile phase was THF and eluted at 0.8 ml/min and 40°C. The specific refractive index increments ($\partial n/\partial c$) of p(L)LysMA and p(L)PheMA in acetic acid buffer and pBoc(L)SerOtBuMA in THF were determined using a DRM-1021 differential refractometer (Otsuka Electronics, Osaka, Japan) to be 0.121, 0.111, and 0.054 mg/mL, respectively (Fig. S18).

Cell culture. Mouse colon cancer cells (CT26) and human lung cancer cells (A549) were obtained from ATCC and JCRB cell bank (Japan), respectively. RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin was used as culture medium for CT26. For A549, DMEM high glucose supplemented with 10% FBS and 1% penicillin-streptomycin was used. Both cell lines were cultured in a humidified atmosphere maintaining 5% of CO$_2$ at 37 °C.

Cellular uptake. CT26 and A549 cells were seeded on a 12 well plate ($3.0\times10^5$ cells per well) overnight. The cells were then incubated with fresh medium containing Cy5-labeled polymers (25 μg/mL) for 2 h at 4 or 37°C. The cells were washed with PBS and then harvested by trypsinization. The cellular uptake of Cy5-labeled polymers (ZAP$^{\text{Cy5}}$ and pMPC$^{\text{Cy5}}$) was evaluated by flow cytometry (excitation: 638 nm, emission: 660 nm) using CytoFLEX LX (Beckman Coulter, US). The cellular uptake was quantified by dividing the Cy5 fluorescence intensity from the cells treated with the Cy5-labeled polymers by their fluorescence intensity at the maximum emission wavelength (excitation =
638 nm, emission = 660 nm), which was determined by fluorescence spectroscopy measurements using FP8500 (JASCO, Japan).

**Inhibitor studies for endocytic pathway.** CT26 cells were pre-incubated with chlorpromazine (10 μM), genistein (200 μM), amiloride (200 μM), and cytochalasin D (0.5 μM) for 2 h. ZAP\(^{Cy5}\) (25 μg/mL) were added and incubated for 4 h at 37°C. The cells were washed with PBS and then harvested by trypsinization. The cellular uptake of ZAP\(^{Cy5}\) were evaluated by flow cytometry (excitation: 638 nm, emission: 660 nm) using CytoFLEX LX (Beckman Coulter, US) to analyze the positive rate.

**Analysis of subcellular distribution using structured illumination microscopy.** CT26 cells were seeded on glass bottom 8-well plate (LAB-TEK II, Thermo Fisher Scientific) (2×10\(^4\) cells per well) overnight. The cells were then incubated with fresh medium ZAP\(^{Cy5}\) (25 μg/mL) for 1 h at 37°C. After being stained with LysoTracker Red (100 nM) for 30 min, the cells were washed with PBS and fixed using 4% paraformaldehyde for 10 min at room temperature. The cell nucleus was stained at room temperature (10 min) using 4’,6-diamidino-2-phenylindole (DAPI). Images were taken in PBS solution using BZ-X800 (Keyence, Osaka, Japan).

**Inhibitor studies for amino acid transporter recognition.** CT26 cells were incubated with fresh medium containing ZAP\(^{Cy5}\) (25 μg/mL) with inhibitors such as phenylalanine and leucine at different concentration for 4 h at 37°C. The cells were washed with PBS and then harvested by trypsinization. The cellular uptake of ZAP\(^{Cy5}\) were evaluated by flow cytometry (excitation: 638 nm, emission: 660 nm) using CytoFLEX LX (Beckman Coulter, US) to analyze the positive rate.
Scheme S1. Synthesis scheme of a zwitterionic amino acid polymer: p(L)LysMA. p(D)LysMA was also synthesized via same procedure described as that of p(L)LysMA.
Scheme S2. Synthesis scheme of a zwitterionic amino acid polymer: p(L)PheMA. p(D)PheMA was also synthesized via same procedure described as that of p(L)PheMA.
Scheme S3. Synthesis scheme of a zwitterionic amino acid polymer: p(L)SerMA.
NMR spectra (Fig. S1-S21)

**Fig. S1** $^1$H-NMR spectrum of Boc(L)LysMA.

**Fig. S2** $^{13}$C-NMR spectrum of Boc(L)LysMA.
Fig. S3 $^1$H-NMR spectrum of Boc(D)LysMA.

Fig. S4 $^{13}$C-NMR spectrum of Boc(D)LysMA.
Fig. S5 $^1$H-NMR spectrum of Boc(L)PheMA.

Fig. S6 $^{13}$C-NMR spectrum of Boc(L)PheMA.
**Fig. S7** $^1$H-NMR spectrum of Boc(D)PheMA.

**Fig. S8** $^{13}$C-NMR spectrum of Boc(D)PheMA.
Fig. S9 $^1$H-NMR spectrum of Boc(L)SerOtBuMA.

Fig. S10 $^{13}$C-NMR spectrum of Boc(L)SerOtBuMA.
Fig. S11 $^1$H-NMR spectrum of pBoc(L)lysMA.

Fig. S12 $^1$H-NMR spectrum of pBoc(D)lysMA.
Fig. S13 $^1$H-NMR spectrum of pBoc(L)PheMA.

Fig. S14 $^1$H-NMR spectrum of pBoc(D)PheMA.
Fig. S15 $^1$H-NMR spectrum of pBoc(L)SerOtBuMA.

Fig. S16 $^1$H-NMR spectrum of p(L)LysMA.
Fig. S17 $^1$H-NMR spectrum of p(D)LysMA.

Fig. S18 $^1$H-NMR spectrum of p(L)PheMA.
Fig. S19 $^1$H-NMR spectrum of p(D)PheMA.

Fig. S20 $^1$H-NMR spectrum of p(L)SerMA.
Fig. S21 $^1$H-NMR spectrum of pMPC.
Fig. S22 Mass spectra of (a) Boc(L)LysMA, (b) Boc(D)LysMA, (c) Boc(L)PheMA, and (d) Boc(D)PheMA.
Fig. S23 Concentration dependence of refractive index increment for (a) p(L)LysMA and (b) p(L)PheMA in aqueous solution containing 500 mM of acetic acid and 200 mM of sodium nitrate. (c) Concentration dependence of refractive index increment for pBoc(L)SerOtBuMA in THF.
Fig. S24 SEC chromatograms of (a) p(L)LysMA, (b) p(D)LysMA, (c) p(L)PheMA, (d) p(D)PheMA, (e) p(L)PheMA_{large}, (f) pMPC, and (g) pBoc(L)SerOtBuMA measured with LS (Black lines), RI (gray lines) detectors. The SEC was performed in acetic acid buffer and THF for water-soluble polymers (a-f) and pBoc(L)SerOtBuMA (g), respectively.
Fig. S25 Berry plots [i.e., \((K_c/R_0)^{1/2}\) vs. \(\sin^2(\theta/2)\)] at around the maximum in the SEC-MALS fractogram of p(L)LysMA, p(D)LysMA, p(L)PheMA, p(D)PheMA, p(L)PheMA_{large}, pBoc(L)SerOtBuMA, and pMPC.
Fig. S26 UV spectra of the synthesized polymers in phosphate buffered saline (PBS).
Figure S27

(a) Figure S27 Fluorescence spectra of the synthesized polymers in (a) RPMI-1680 growth medium, (b) DMEM growth medium, and (c) PBS(−) containing 10% FBS (excited at 638 nm).
Fig. S28 Cellular uptake of the ZAPs against human lung cancer cell (A549). A549 cells were incubated in growth media containing 25 μg/mL of ZAP for 2 h at 37 °C, and the fluorescence of the cells was evaluated using a flow cytometer. The fluorescence intensities were normalized by dividing by the fluorescence intensity of the Cy5-labeled polymers at the maximum emission wavelength (excitation = 638 nm, emission = 660 nm). The results are indicated as means ± standard deviation (n = 3 biological replicates).
Fig. S29 Time-dependence of ZAPs internalization against CT26. CT26 cells were incubated in PBS(-)/10 % FBS containing 25 μg/mL of ZAP for 2 h at 37 °C, and the fluorescence of the cells was evaluated using a flow cytometer. The fluorescence intensities were normalized by dividing by the fluorescence intensity of the Cy5-labeled polymers at the maximum emission wavelength (excitation = 638 nm, emission = 660 nm). The results are indicated as means ± standard deviation (n = 3 biological replicates).
The colocalization analysis of emdosomes/lysosomes and ZAP distribution (Fig. 2). This analysis was performed using “ImageJ Fuji”. The Pearson’s coefficients of the colocalization on p(L)LysMA_{Cy5} and p(L)PheMA_{Cy5} experiment were calculated as 0.89 and 0.86, respectively.
Cellular uptake of p(L)LysMA<sub>Cy5</sub>, p(D)LysMA<sub>Cy5</sub>, p(L)PheMA<sub>Cy5</sub>, p(L)PheMA<sub>large,Cy5</sub>, and p(D)PheMA<sub>Cy5</sub>, p(L)SerMA<sub>Cy5</sub>, pMPC<sub>Cy5</sub>, and PEG<sub>Cy5</sub>. CT26 cells were incubated in growth media containing 25 μg/mL of ZAP for 2 h at 37 °C, and the fluorescence of the cells was evaluated using a flow cytometer. The fluorescence intensities were normalized by dividing by the fluorescence intensity of the Cy5-labeled polymers at the maximum emission wavelength (excitation = 638 nm, emission = 660 nm). The results are indicated as means ± standard deviation (n = 3 biological replicates).
Fig. S32 Cell viability of CT26 cells after 24 h of exposure to the zwitterionic amino acid polymers p(L)LysMA and p(L)PheMA at concentrations of 0, 31.3, 62.5, 125, 250, and 500 μg/mL. The results are indicated as means ± standard deviation (n = 3 biological replicates).