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Electronic Supplementary Information

Increase of tyrosinase activity at wound in zebrafish imaged by a new fluorescent probe

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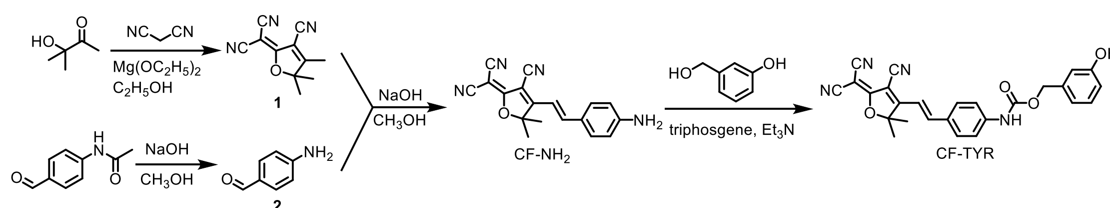
1. Apparatus and reagents

^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker DMX-400 spectrometer in DMSO-d_6. Electrospray ionization mass spectra (ESI-MS) were implemented with a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on an APEX IVFTMS instrument (Bruker, Daltonics). UV-vis absorption spectra were made by a TU-1900 spectrophotometer (Beijing, China). Fluorescence spectra were recorded on an F-4600 spectrophotometer (Hitachi, Japan). MTT analyses were made on a SpectraMax I3 microplate reader (Molecular Devices, USA). Confocal fluorescence images were recorded on an FV 1200-IX83 confocal laser scanning microscope (Olympus, Japan).

3-Hydroxy-3-methyl-2-butanone, malononitrile, magnesium ethoxide, 4-acetamido-benzaldehyde, 3-hydroxybenzyl alcohol, pyridine, triphosgene and triethylamine were obtained from Beijing Innochem Science & Technology Co., Ltd. Tyrosinase, biotinidase (BTD), quinone oxidoreductase (NQO1), nitroreductase (NTR), kojic acid and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. KCl, $CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$ and tert-Butyl hydroperoxide (TBHP) were obtained from Beijing Chemicals, Ltd. Dulbecco's modified Eagle's media (DMEM), B16 and HeLa cell lines were purchased from KeyGEN BioTECH Co., Ltd. Zebrafish and tricaine methanesulfonate were provided by Nanjing EzeRinka Biotechnology Co., Ltd. Reactive oxygen species (ROS) including ·OH, H_2O_2, TBHP, $^{1}O_2$, ClO^-, TBO·, $ONOO^-$, and $\cdot O_2^-$ were prepared following the reported methods.[1] Ultrapure water (over 18 MΩ·cm) produced by a Milli-Q reference system (Millipore) was used throughout the experiments.

2. Synthesis of probe CF-TYR



Scheme S1 Synthesis of probe CF-TYR

The synthetic route of CF-TYR is shown in Scheme S1. Compound **1** and CF-NH_2 were synthesized following the reported procedure.[2] Compound **2** was prepared

according to the previous method.[3]

Synthesis of CF-TYR. CF-NH_2 (150 mg, 0.5 mmol), and triphosgene (222 mg, 0.75 mmol) were dissolved in dry dichloromethane (15 mL), followed by the addition of Et_3N (200 μL, 1.5 mmol) dropwise. The mixture was stirred at 0 °C under Ar atmosphere for 0.5 h. The resulting solution was stirred at room temperature for another 2 h, accompanied by the solution changing from purplish red to light yellow. Then 3-hydroxybenzyl alcohol (124 mg, 1.0 mmol) was added to the mixture with several drops of pyridine. The reaction mixture was stirred at room temperature for 4 h. After removal of the solvent, the residue was extracted with dichloromethane and washed with brine water for 3 times. The product was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH=100/1, v/v) to give CF-TYR as orange powder (90 mg, 40% yield). ^{1}H-NMR (400 MHz, 298 K, DMSO-d_6; Fig. S1): δ 10.27 (s, 1H), 9.49 (s, 1H), 7.89 (t, J=18.0 Hz, 3H), 7.62 (d, J=8.4 Hz, 2H), 7.18 (t, J=15.2 Hz, 1H), 7.09 (d, J=16.4 Hz, 1H), 6.83 (d, J=8.0 Hz, 2H), 6.73 (d, J=7.6 Hz, 1H), 5.10 (s, 2H), 1.79 (s, 6H). ^{13}C-NMR (100 MHz, 298 K, DMSO-d_6; Fig. S2): δ 177.7, 176.0, 157.9, 153.6, 147.9, 143.8, 138.1, 131.5, 130.0, 129.0, 119.0, 118.6, 115.5, 115.3, 113.7, 113.3, 112.5, 111.5, 99.7, 98.3, 66.5, 54.2, 25.7. HR-ESI-MS: m/z calcd for CF-TYR ($C_{26}H_{19}N_4O_4^-$, $[M-H]^-$), 451.1412; found, 451.1412.

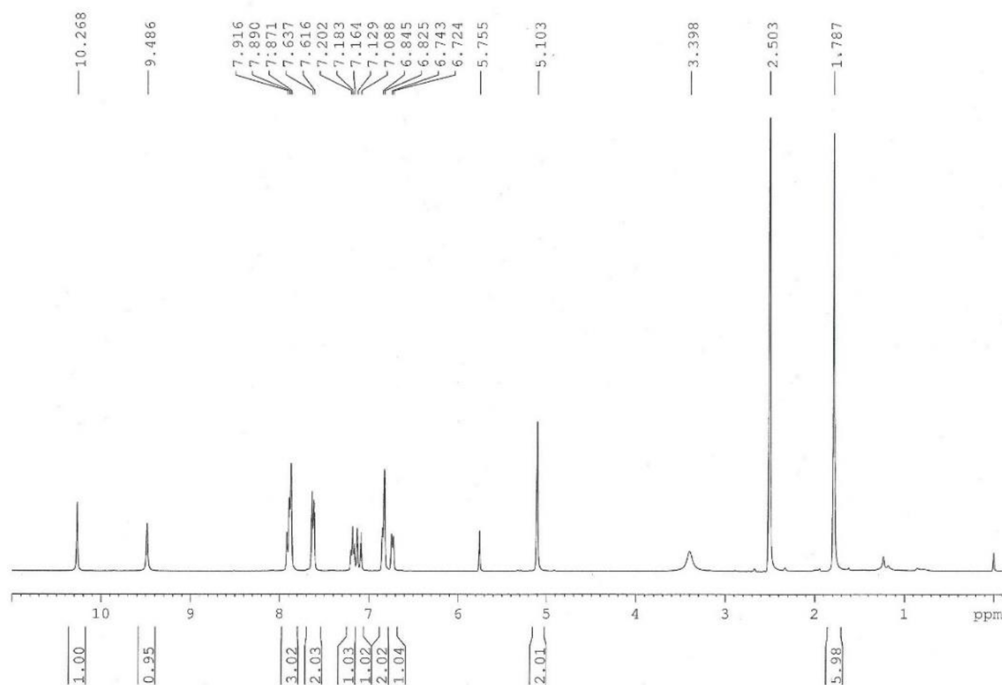


Fig. S1 ^{1}H-NMR spectrum of CF-TYR (400 MHz, 298K, DMSO-d_6).

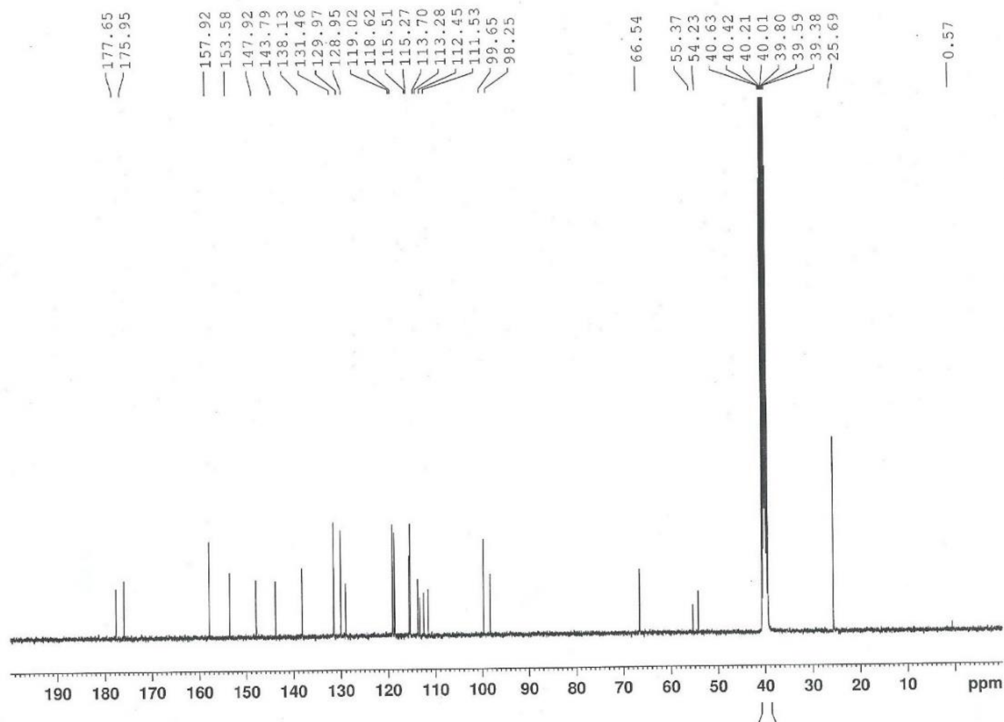
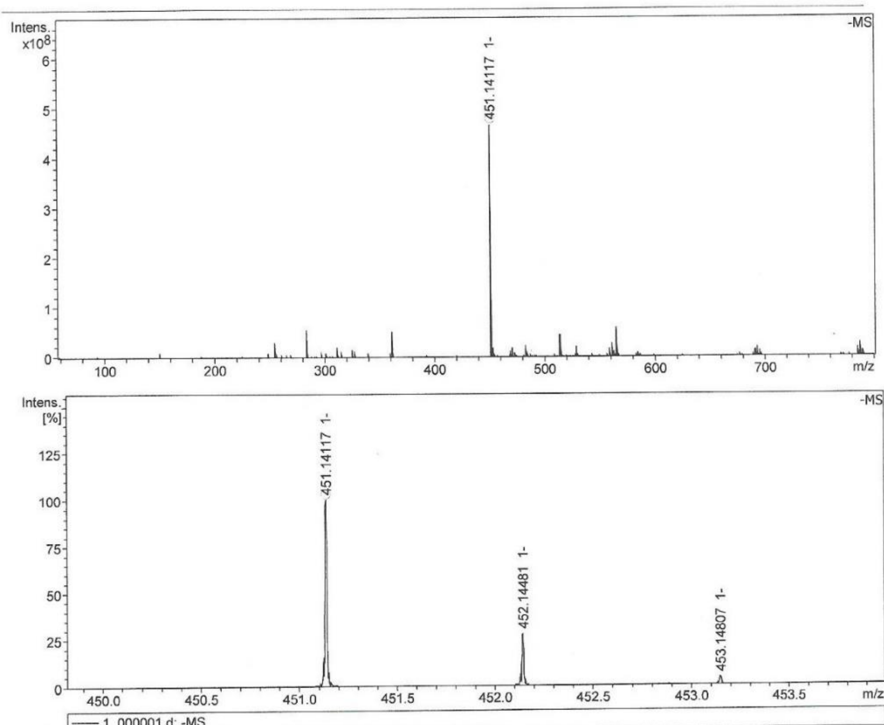


Fig. S2 ^{13}C-NMR spectrum of CF-TYR (100 MHz, 298K, DMSO-d_6).

Acquisition Parameter

Acquisition Mode	Single MS	Acquired Scans	8
Polarity	Negative	Broadband High Mass	800.0 m/z
Broadband Low Mass	57.7 m/z		



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
451.141165	1	C26H19N4O4	100.00	451.141179	0.0	-0.3	12.2	19.5	even	ok

Fig. S3 HR-ESI-MS spectrum of CF-TYR.

3. Supplementary methods

General procedure for fluorescence assay

Unless otherwise noted, the fluorescence of CF-TYR (10 μM) reacting with tyrosinase was measured in phosphate buffer saline (PBS, pH 7.4). In a test tube, 0.8 mL of PBS and 10 μL of 1 mM stock solution of CF-TYR in DMSO were mixed, followed by addition of tyrosinase solution. The final volume was adjusted to 1 mL with PBS. After reaction at 37 °C for 3 h in a shaker incubator, the reaction solution was transferred to a 1 cm quartz cell to measure fluorescence with $\lambda_{ex/em}$ = 560/612 nm. Meanwhile, a solution without tyrosinase was made and determined for comparison under the same conditions. Data are expressed as mean ± standard deviation (n = 3).

Cytotoxicity

The cytotoxicity of CF-TYR on B16 cells and HeLa cells were evaluated by the MTT assay, as described previously.[1]

Cell culture and fluorescence imaging in cells

B16 cells and HeLa cells were cultured in DMEM containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. For fluorescence imaging, the cells were incubated with 5 μM CF-TYR in DMEM at 37 °C. After incubated with probe, cells were washed twice with DMEM to remove the free probe.

Fluorescence imaging in zebrafish

Zebrafish grew in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, and 0.7 mM $NaHCO_3$; without methylene blue). For fluorescence imaging, 3-day-old zebrafish were incubated with CF-TYR for 3 h, and then subjected to fluorescence imaging after washing with PBS. Tail-cutting zebrafish were prepared by using a blade under anesthetization with tricaine methanesulfonate.

Fluorescence imaging was conducted with an excitation wavelength of 559 nm. The fluorescence was collected in the range of 600-700 nm. Imaging processing and analysis was performed on Olympus software (FV10-ASW). Data are expressed as mean ± standard deviation.

4. **Supplementary figures**

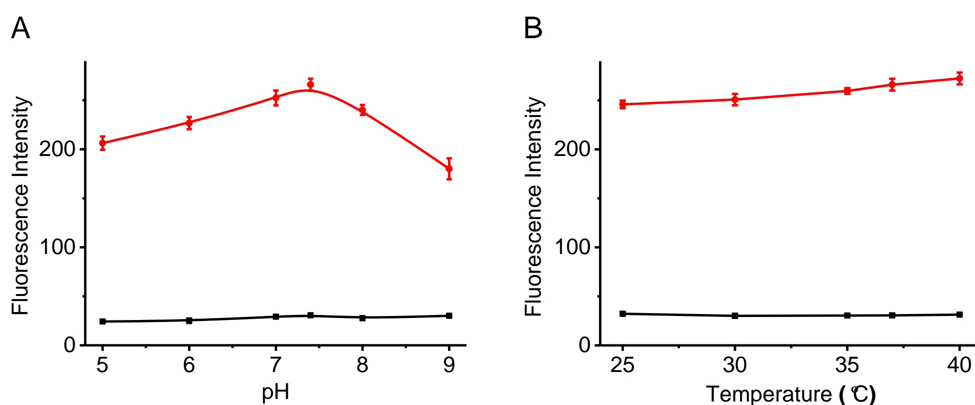


Fig. S4 Effects of (A) pH and (B) temperature on the fluorescence intensity of CF-TYR (10 μM) with (red) and without (black) tyrosinase (100 U/mL). $\lambda_{ex/em}$ = 560/612 nm.

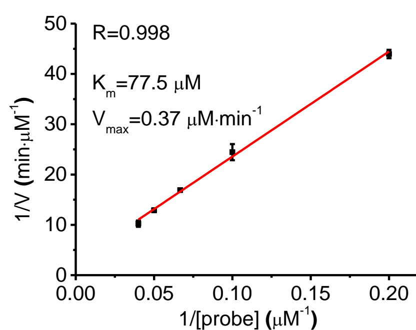


Fig. S5 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe]/(K_m + [probe]), where V is the reaction rate, [probe] is the probe concentration, and K_m is the Michaelis constant. Conditions: 50 U/mL tyrosinase, 5-25 μM of CF-TYR. $\lambda_{ex/em}$ = 560/612 nm.

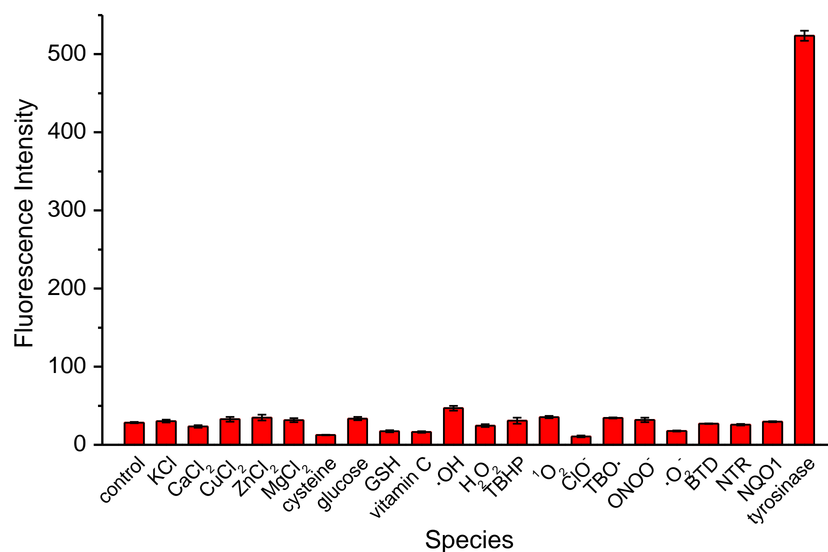


Fig. S6 Fluorescence responses of CF-TYR (10 μM) to various species: (1) control; (2)

KCl (150 mM); (3) $CaCl_2$ (1 mM); (4) $CuCl_2$ (10 μM); (5) $ZnCl_2$ (100 μM); (6) $MgCl_2$ (1 mM); (7) cysteine (1 mM); (8) glucose (10 mM); (9) GSH (1 mM); (10) vitamin C (1 mM); (11) ·OH (100 μM); (12) H_2O_2 (100 μM); (13) TBHP (100 μM); (14) $^1O_2$ (100 μM); (15) ClO^- (100 μM); (16) TBO· (100 μM); (17) $ONOO^-$ (100 μM); (18) $\cdot O_2^-$ (100 μM); (19) BTD (0.5 μg/mL); (20) NTR (5 μg/mL); (21) NQO1 (0.5 μg/mL); (22) tyrosinase (200 U/mL). The results are expressed as the mean ± standard deviation of three separate measurements. $\lambda_{ex/em}$ = 560/612 nm.

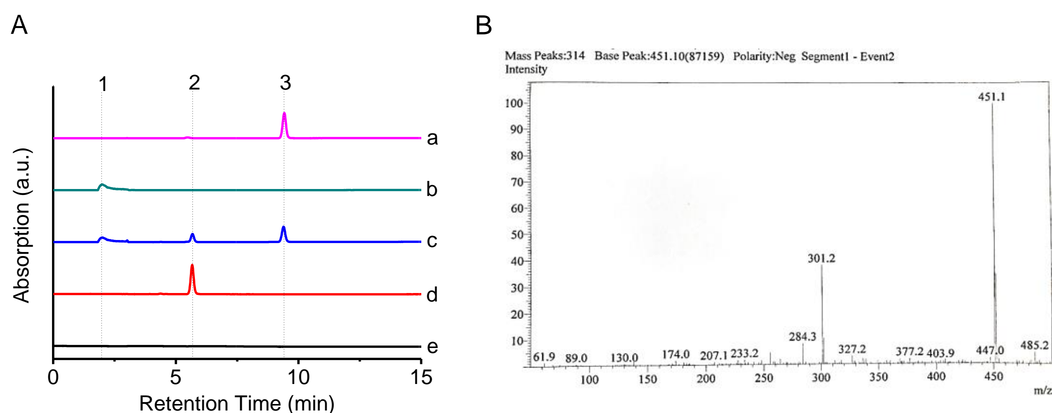


Fig. S7 (A) Chromatograms of different reaction systems: a) CF-TYR (50 μM); b) tyrosinase (500 U/mL); c) reaction solution (50 μM CF-TYR + 500 U/mL tyrosinase); d) $CF-NH_2$ (50 μM). The assignments of the peaks: (1) t_R = 2.0 min, tyrosinase; (2) t_R = 5.6 min, $CF-NH_2$; (3) t_R = 9.4 min, CF-TYR. HPLC analyses were made using two LC-20AT pumps, SPD-M20A diode array detector (Shimadzu, Japan) and Inertsil ODS-SP column (5 μm, 4.6 mm×250 mm, GL Sciences Inc.) with methanol and water as eluents [elution program: 0-20 min, 70% to 100% methanol (flow rate, 1.0 mL/min)]. The chromatography peaks were monitored by the absorbance at 510 nm. (B) ESI mass spectrum of the reaction solution of CF-TYR (10 μM) with tyrosinase (200 U/mL). The peak at m/z =301.2 $[M-H]^-$ indicates the generation of fluorophore $CF-NH_2$.

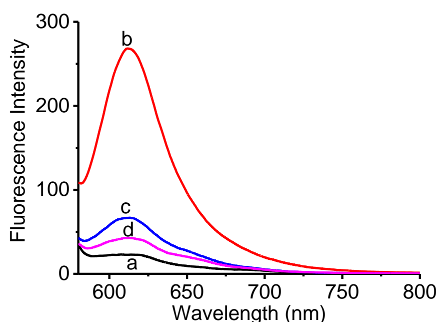


Fig. S8 The effects of inhibitor, kojic acid, on the fluorescence spectra of the reaction

systems. (a) CF-TYR (10 μM); (b) CF-TYR (10 μM) + tyrosinase (100 U/mL); (c) system (b) + kojic acid (100 μM); (d) system (b) +kojic acid (200 μM). $\lambda_{ex/em}$ = 560/612 nm.

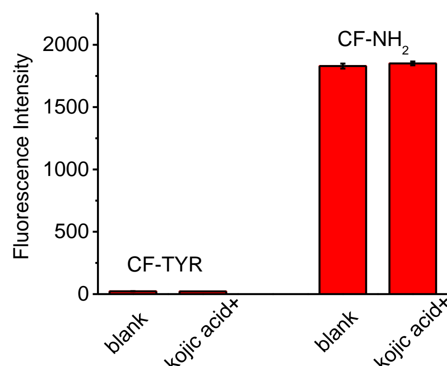


Fig. S9 The effects of kojic acid on the fluorescence of CF-TYR (left) and CF-NH_2 (right). CF-TYR: 10 μM; CF-NH_2:10 μM; kojic acid: 200 μM. $\lambda_{ex/em}$ = 560/612 nm.

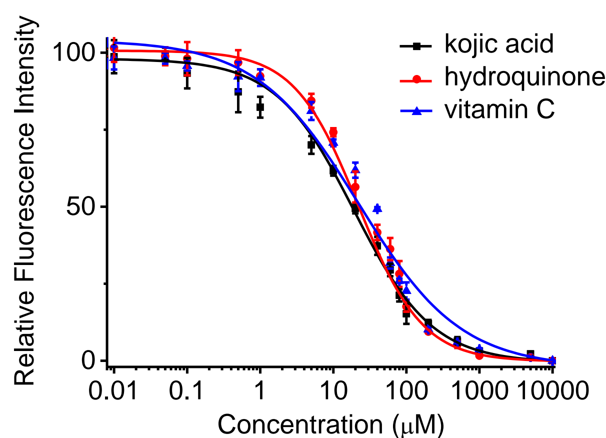


Fig. S10 The inhibition curves of kojic acid, hydroquinone and vitamin C. Testing system: CF-TYR: 10 μM; tyrosinase: 200 U/mL. $\lambda_{ex/em}$ = 560/612 nm.

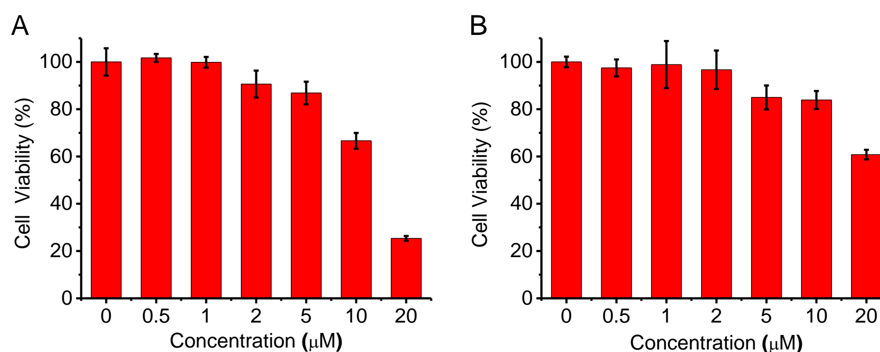


Fig. S11 Effects of CF-TYR on the viability of (A) B16 cells and (B) HeLa cells. The viability of cells without CF-TYR is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements at each probe concentration.

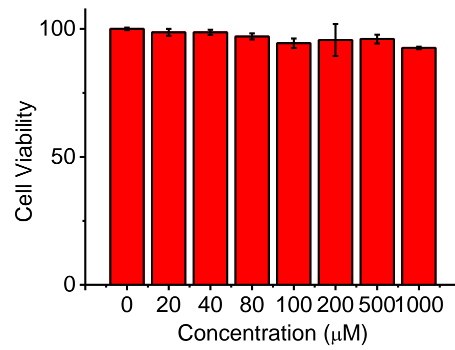


Fig. S12 Effects of kojic acid on the viability of B16 cells. The viability of cells without kojic acid is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements.

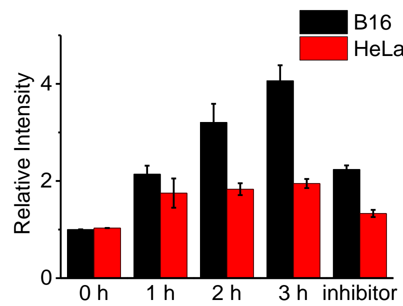


Fig. S13 Relative fluorescence intensity of cells in Fig. 2. The intensity at least from ten cells in the fluorescence image was measured and averaged.

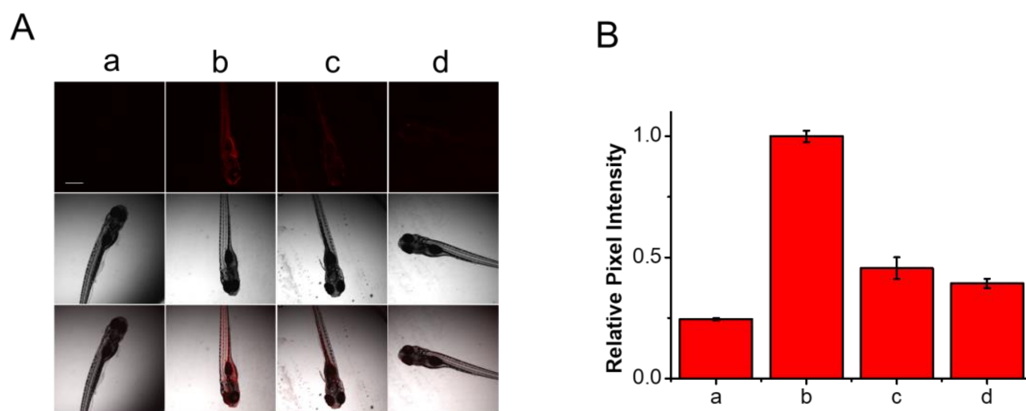


Fig. S14 (A) Representative fluorescence images of zebrafish: (a) zebrafish only; (b) zebrafish incubated with CF-TYR for 3 h; zebrafish pretreated with (c) 200 μM and (d) 500 μM kojic acid for 2h, and then incubated with CF-TYR for 3 h. Scale bar, 500 μm. (B) Relative fluorescence intensity of zebrafish (n = 5 fishes in each group) in panel (A).

5. References

1. (a) X. Y. Zhu, M. Y. Xiong, H. W. Liu, G. J. Mao, L. Y. Zhou, J. Zhang, X. X. Hu, X. B. Zhang and W. H. Tan, *Chem. Commun.*, 2016, **52**, 733-736; (b) R. M. Uppu, *Anal. Biochem.*, 2006, **354**, 165-168; (c) J. Zhou, L. H. Li, W. Shi, X. H. Gao, X. H. Li and H. M. Ma, *Chem. Sci.*, 2015, **6**, 4884-4888.
2. P. Gopalan, H. E. Katz, D. J. McGee, C. Erben, T. Zielinski, D. Bousquet, D. Muller, J. Grazul, and Y. Olsson, *J. Am. Chem. Soc.*, 2004, **126**, 1741-1747.
3. I. C. Pintre, J. L. Serrano, M. B. Ros, J. M. Perdiguero, I. Alonso, J. Ortega, C. L. Folcia, J. Etxebarria, R. Alicante and B. Villacampa, *J. Mater. Chem.*, 2010, **20**, 2965-2971.