

Electronic Supplementary Information (ESI)

Targeting lysosomal membrane permeabilization to induce and image apoptosis in cancer cells by multifunctional Au-ZnO hybrid nanoparticles

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1. Experimental procedures

Materials. All chemicals and solvents used were of analytical grade. Water was purified with a Sartorius Arium 611 VF system (Sartorius AG, Germany) to a resistivity of 18.2 M Ω ·cm. oleylamine (>70%), tetralin, tert-butylamine borane, dodecanol, 3-aminopropionic acid, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), HEPES sodium salt hydrate, xanthine, Xanthine oxidase, ethylenediaminetetraacetic acid (EDTA), lysosome isolation kit and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Cathepsin B (active, human), cathepsin B activity fluorometric assay kit and its inhibitor Z-FF-FMK were purchased from BioVision (Milpitas, CA). HAuCl₄·4H₂O and zinc acetate dehydrate were obtained from Sinopharm Chemical Reagent. Co. Ltd. (Shanghai, China). N-hydroxysuccinimide ester-poly(ethylene glycol)-Maleimide (NHS-PEG-Mal; molecular weight of PEG: 5000) were obtained from JenKem Technology Co., Ltd. (Beijing, China). Lyso-Tracker Red and Hoechst 33342 were bought from (Beyotime Inst. Biotech, Haimen, China). Rabbit anti-tBid (p15) cleavage site-specific antibody was bought from (Invitrogen, Carlsbad, CA, USA). Cytochrome c, activated-caspase-3 antibody and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA).

Preparation of Au-ZnO NPs. First, Au NPs were prepared by a previously reported method with some modifications.¹ Typically, HAuCl₄·4H₂O (0.5 mM) were added to the mixed solution of oleylamine (20 mL) and tetralin (20 mL) at the given temperatures and stirred for a few minutes under N₂. Then, tert-butylamine borane complex (1 mM) was dissolved in 2 mL of tetralin and 2 mL of oleylamine and injected to the above solution. After 1 h stirring, ethanol was added and the precipitate was collected by centrifugation. The final product was redispersed in hexane for further use.

Second, the as-prepared Au NPs (60 mg in 10 mL of hexane) to serve as seeds were added under agitation to a mixture of zinc acetate dihydrate (0.5 mM), oleylamine (3 mL), and dodecanol (6 mL). Next, the temperature was slowly raised to 120 °C to remove water and hexane. After that, the above solution was further heated to 180 °C and kept at this temperature for a few minutes. The as-obtained products were washed with ethanol and dispersed in hexane.

Amine Functionalization of Au-ZnO NPs (Au-ZnO-NH₂). On the basis of previous reports,² here we used 3-aminopropionic acid to introduce amine groups onto the surface of Au-ZnO NPs for further functionalization. 3 mg of 3-aminopropionic acid was added to a suspension of 5 mg of Au-ZnO NPs in 2 mL of dichloromethane (DCM). The reaction mixture was stirred at room temperature for 1 h. After evaporation of DCM, the product (Au-ZnO-NH₂) was collected and washed with ethanol three times.

Conjugation of RGD to Amine Functionalized Au-ZnO NPs (Au-ZnO-RGD). To synthesize Au-ZnO-RGD, 10 mg of NHS-PEG-Mal was reacted with 1.5 mg of the c(RGDyC) peptide (cyclic Arg-Gly-Asp-Tyr-Cys, GL Biochem (Shanghai) Ltd.), in 2 mL of PBS (pH = 8.5) for 4 h at room temperature in the presence of TCEP. Subsequently, the pH of the reaction mixture was adjusted to 7.0. 2 mg of Au-ZnO-NH₂ was then added, and the reaction was allowed to proceed for 2 h at room temperature. The purified product was denoted as “Au-ZnO-RGD”.

Conjugation of FITC-RR to Au-ZnO-RGD NPs (FITC-RR-Au-ZnO-RGD). For conjugation of FITC-RR to the Au-ZnO-RGD NPs, a previous established method was used.³ Briefly, FITC-conjugated RR peptide (FITC-Gly-Arg-Arg-Gly-Cys) was purchased from GL Biochem. Ltd. (Shanghai, China). The peptides were immobilized onto Au surfaces *via* the Au-S bond. Briefly, 1.5 mg FITC-RR was added to a suspension of 2 mg of Au-ZnO-RGD NPs in 2 mL of PBS (pH = 8.5). The mixture was left to react for 2 h. Excess FITC-RR were then removed by centrifugation at 10000g for 5 min. The resulting particles were washed with DI water 3 times and resuspended in PBS (pH = 7.4) to form a stock solution of 1 mg/mL for further investigation.

Characterization. The transmission electron micrographs (TEM) were obtained on a JEOL JEM 2010 electron microscope operated at 200 kV. X-ray diffraction (XRD) analysis was carried out on a D/Max 2500 V/PC X-ray diffractometer using Cu (40 kV, 30 mA) radiation. UV-vis spectra were recorded from a Hitachi U-3010 UV-vis spectrometer, which scanned over the range of 400-700 nm. Success of each reaction step was confirmed by monitoring the changes in zeta potential with a Malvern Zeta Sizer Nano (Malvern Instruments). The as-synthesized Au-ZnO and Au-ZnO-NH₂ had zeta potentials of 19.6 ± 1.2 and 22.8 ± 1.6 mV, respectively. The zeta potentials of Au-ZnO-RGD and

FITC-RR-Au-ZnO-RGD were -2.23 ± 0.15 and -5.34 ± 0.31 mV, respectively. On comparison of these values, 3-aminopropionic acid conjugation significantly changed the zeta-potential of Au-ZnO NPs, but RGD peptide and FITC-RR conjugation caused the most pronounced difference. These observations strongly suggested successful surface conjugation of RGD and FITC-RR peptides to the NPs. To further confirm this aspect, Au-ZnO NPs were mixed with the RGD and FITC-RR peptides first and thoroughly washed, which had a zeta potential value of 16.2 ± 3.5 mV, significantly different from that of FITC-RR-Au-ZnO-RGD (-5.34 ± 0.31 mV).

Characterization of ROS Formation in Vitro. The catalytic abilities of ZnO or FITC-RR-Au-ZnO-RGD were evaluated in vitro according to a previously reported method⁴ with some modifications. TEMPO-BDP⁵ synthesized by our group was used as the fluorescent probe for determination of ROS. The NPs (50 $\mu\text{g}/\text{mL}$) were initially incubation in the xanthine/xanthine oxidase (X/XO) superoxide generating system (containing 21 mM EDTA, 7 mM xanthine, 3 milliunits/mL xanthine oxidase) at 37°C for 20 min with vortexing. Then the mixture was centrifuged at 11000g for 5 min, and a clear supernatant solution was discarded. The precipitated NPs were incubated with 80 μL of a solution of H_2O_2 (0.45 mM) and TEMPO-BDP (2 μM in 10 mM HEPES, 0.1M DMSO) with vortexing for an additional 40 min and spun down by centrifugation at 11000g for 5 min. The fluorescence signals of supernatant ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 560/601$ nm) were recorded at the same temperature on an FLS-920 fluorescence spectrophotometer (Edinburg).

Enzymatic Activity of FITC-RR-Au-ZnO-RGD for Cathepsin B. 50 μg of FITC-RR-Au-ZnO-RGD NPs were incubated with 2 μg recombinant cathepsin B in 1.0 mL of assay buffer⁶ (88 mM KH_2PO_4 , 12 mM Na_2HPO_4 , 1.33 mM EDTA- Na_2 , 2.7 mM L-cysteine, pH 6.0) for 2 h at 37 °C. Cathepsin B inhibitor Z-Phe-Phe-FMK (50 μM) was added in the control reaction. Then the detection sensitivity was studied by co-incubating FITC-RR-Au-ZnO-RGD (50 $\mu\text{g}/\text{mL}$) with 0.1, 0.5, 1, 2 and 4 μg of Cathepsin B for 1 h at 37 °C. The fluorescence spectra of the enzymatic reaction solutions ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 490/522$ nm) were measured by FLS-920 fluorescence spectrometer (Edinburg, England) at appropriate time intervals.

Cell Culture. HepG2 and HL7702 cells were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences. Cells were grown in cell culture media and incubated at 37 °C in a 5% CO₂/ 95% air humidified incubator (MCO-15AC, SANYO). The cell culture medium was RPMI-1640 (2000 mg/L D-Glucose, 300 mg/L L-Glutamine, Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA).

Internalization of FITC-RR-Au-ZnO-RGD. HepG2 and HL7702 cells were seeded at a density of 5×10^6 cells/well in a 6 well-plate. After 24 h, the cells were incubated with FITC-RR-Au-ZnO-RGD (50 µg/mL) for 0.5 h and then washed three times to remove unbound NPs. To measure gold content inside lysosome, we used a kit from Sigma-Aldrich for lysosome isolation. Cell fractionation and lysosome isolation were performed according to the manufacturer's instructions. The resulting lysosomal fractions were treated with 0.2 mL of aqua regia (3:1 hydrochloric acid/nitric acid). Following the incubation overnight, the sample was diluted to 5 mL using ultrapure water and then analyzed for total gold content by ICP-AES (Thermo, IRIS Advantage, 242.795 nm). Based on the previous protocol,⁷ the number of Au a cell can uptake was calculated.

Cytotoxicity Assays. Cytotoxicity was measured by using the MTT assay in the logarithmic phase of cell growth. HepG2 and HL7702 cells were seeded at a density of 5×10^4 cells/well in a 96 well-plate and incubated for 24 h before adding the test substance. Then fresh medium containing increasing concentrations of FITC-RR-Au-ZnO-RGD was added to each well. After 24 h incubation, medium was removed and replaced with medium containing MTT (0.5 mg/mL). Cells were incubated at 37 °C for another 4 h after which medium was removed. DMSO (100µL) was added to lyse the cells and dissolve the formazan produced. The absorbance at 570 nm of each well was monitored using a microplate reader. Viability was calculated based on the recorded data.

Characterization of ROS Formation in Cells. HepG2 and HL7702 cells were seeded at a density of 5×10^4 cells/well in a 96 well-plates and incubated for 24 h. Cells were loaded with TEMPO-BDP (2 µM in 10 mM HEPES, 0.10M DMSO) and were incubated at 37 °C for 30 min. The probe was removed, and the cells were washed twice with PBS. Then fresh medium containing FITC-RR-Au-ZnO-

RGD (50 µg/mL) were added to each well. The fluorescence spectrum was recorded every 30 min for a period of 6 h at 37 °C *via* excitation at 560 nm and emission at 601 nm on a fluorescence plate reader (FLUOstar Optima, BMG Labtech, Germany).

Imaging of LMP-Dependent Apoptosis. HepG2 cells were seeded at an initial density of 5×10^4 cells/dish in 35-mm glass bottom dishes (SPL Life Sciences, Seoul, Korea) and placed in the temperature- and CO₂-controlled chamber of a microscope. For visualizing LMP-dependent apoptosis induced by FITC-RR-Au-ZnO-RGD, cells were first incubated in the medium containing Lyso-Tracker Red (50 nM) and Hoechst 33342 (10 µg/mL) to stain lysosomes and the nucleus, respectively. After incubation for 30 min at 37 °C, the cells were washed twice with PBS to remove the excess probe. Then fresh medium containing FITC-RR-Au-ZnO-RGD (50 µg/mL) were added to the dish. As a control, cells were pretreated with 100 µM of ROS scavenger (NAC, N-acetyl cysteine) or Cathepsin B inhibitor Z-Phe-Phe-FMK (50 µM) for 30 min before uptake of FITC-RR-Au-ZnO-RGD. Confocal images were captured at appropriate time intervals using a LTE confocal laser scanning microscope (CLSM, Leica Co. LTD., Germany).

Analysis of Cathepsin B Activity. To measure cathepsin activity in the cytosol, cytosolic fractions were prepared as described by Nakayama⁸ with minor modifications. Briefly, HepG2 cells in the logarithmic growth phase were plated in 6-well cell culture plates at initial densities of 5×10^6 /well and incubated for 24 h. Fresh medium containing FITC-RR-Au-ZnO-RGD (50 µg/mL) was added to the cells and incubated for 0.5, 1, 3, or 6 h. After incubation, cells were washed with PBS, centrifuged at 600g for 5 min and resuspended in an appropriate extraction buffer (200 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM pefablock, 10 µg/mL leupeptin). After 30 minutes on ice, cells were lysed with a glass dounce homogenizer with 100 strokes of the B pestle, followed by centrifugation at 750g for 10 minutes at 4°C to remove the nuclei and unbroken cells. Supernatants (enucleated whole-cell lysates) were then centrifuged at 15 000g for 30 minutes at 4°C to remove lysosomes and mitochondria. The resultant supernatants were further centrifuged at 100 000g for 1 hour at 4°C, and the final supernatants (cytosolic fractions) were collected. Cathepsin B activity was detected using a cathepsin B activity

fluorometric assay kit. For each well in a 96 well microplate, cytosolic fractions (50 μ L), reaction buffer (50 μ L), and cathepsin B substrate (Ac-RR-AFC, 2 μ L) were combined. The samples were then incubated at 37°C for 2 h and then characterized with a microplate reader operating at a wavelength of 405 nm.

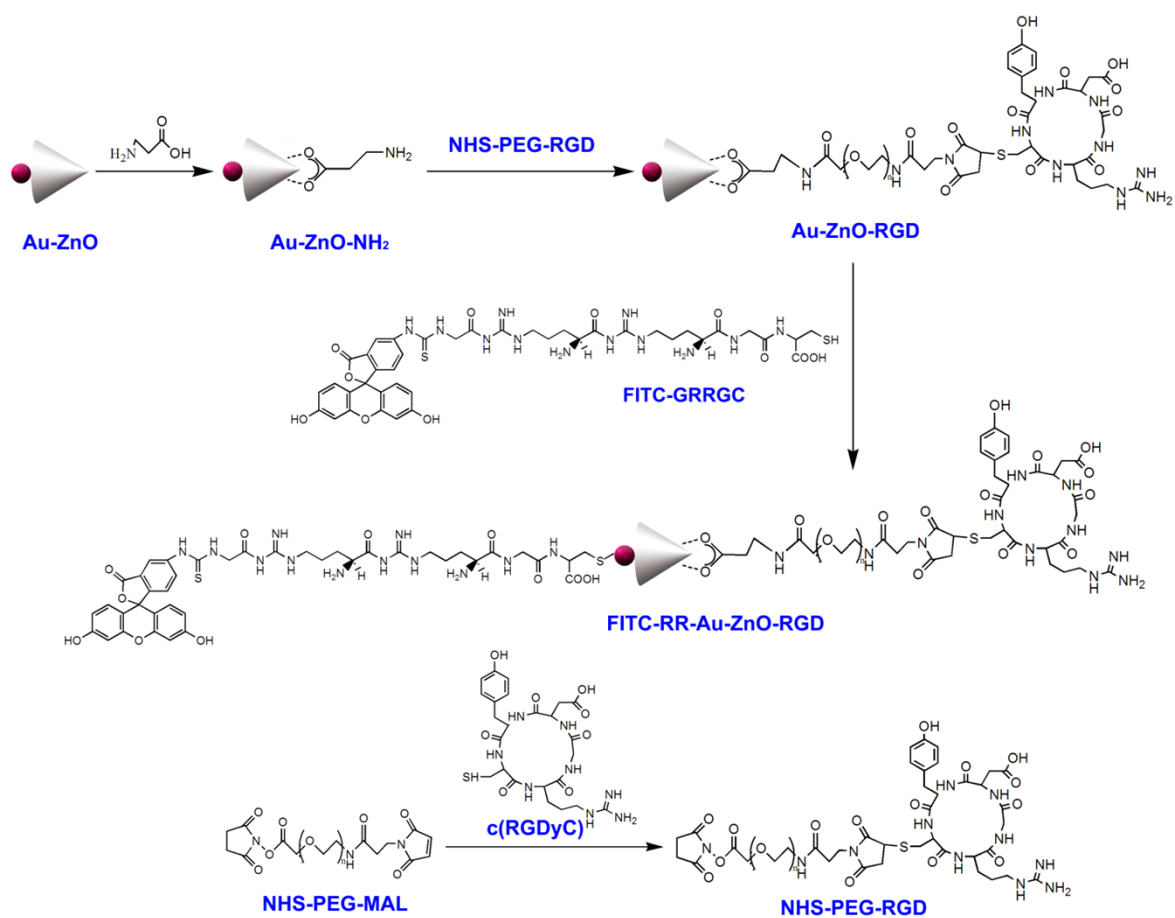
Western Blotting. HepG2 cells in the logarithmic growth phase were plated in 6-well cell culture plates at initial densities of 5×10^6 /well and incubated for 24 h. Fresh medium containing FITC-RR-Au-ZnO-RGD (50 μ g/mL) was added to the cells and incubated for 0.5, 1, 3 or 6 h. After incubation, cells were collected, washed with cold PBS twice and centrifuged at 1200g for 5 min. The cell pellets were permeabilized with 100 μ g/mL digitonin at 4 °C for 10 min. The supernatant representing the cytosol and the mitochondria-containing pellet fraction were separated by centrifugation and denatured based on the previous protocol.⁹ The amount of protein was measured using a protein assay kit (DC protein assay reagent, Bio-Rad, Hercules, CA, USA). Equal amounts of protein (50 μ g) were resolved on 12.5% SDS-PAGE gels and electro-blotted onto a nitrocellulose membrane (BioRad) in 20% methanol, 25 mM Tris and 192 mM glycine. Membranes were then blocked with 5% non-fat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) overnight at 4 °C. Next, membranes were incubated for 1 h with different primary antibodies against tBid, cytochrome c and activated caspase-3, respectively, followed by HRP-conjugated secondary antibody (1:1000) for 1 h. Detection was carried out by incubating membranes for 5 min with the enhanced chemiluminescence reagent, followed by exposure to ECL X-ray film (Amersham Biosciences, Piscataway, NJ).

References

- 1 P. Li, Zhe Wei, T. Wu, Q. Peng and Y. Li, *J. Am. Chem. Soc.*, 2011, **133**, 5660.
- 2 S. Mitra, S. B., P. Patra, S. Chandra, N. Debnath, S. Das, R. Banerjee, S. C. Kundu, P. Pramanik and A. Goswami, *J. Mater. Chem.*, 2012, **22**, 24145; H. Hong, J. Shi, Y. Yang, Y. Zhang, J. W. Engle, R. J. Nickles, X. Wang and W. Cai, *Nano Lett.*, 2011, **11**, 3744.
- 3 B. Kang, M. A. Mackey and M. A. El-Sayed, *J. Am. Chem. Soc.*, 2010, **132**, 1517.

- 4 M. A. Voinov, J. O. S. Paga'n, E. Morrison, T. I. Smirnova and A. I. Smirnov, *J. Am. Chem. Soc.*, 2011, **133**, 35.
- 5 P. Li, T. Xie, X. Duan, F. Yu, X. Wang and B. Tang, *Chem. Eur. J.*, 2010, **16**, 1834.
- 6 Y. Zhang, E. Spiess, M. H. Groschup and A. B. rkle, *J Gen Virol.* , 2003, **84**, 2279.
- 7 M. Domenech, I. Marrero-Berrios, M. Torres-Lugo and C. Rinaldi, *ACS Nano*, 2013, **7**, 5091.
- 8 M. Nakayama, K. Ishidoh, N. Kayagaki, Y. Kojima, N. Yamaguchi, H. Nakano, E. Kominami, K. Okumura and H. Yagita, *J Immunol*, 2002, **168**, 734.
- 9 M. W. Ward, M. Rehm, H. Duessmann, S. Kacmar, C. G. Concannon and J. H. M. Prehn, *J Biol Chem.*, 2006, **281**, 5837.

2. Supplementary Figures



Scheme S1. Schematic illustration of the synthesis of FITC-RR-Au-ZnO-RGD.

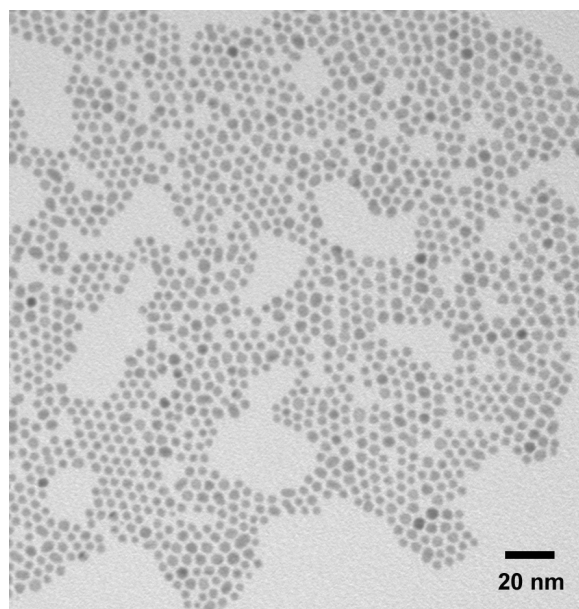


Fig. S1 TEM image of 6 nm Au NPs. Scale bar = 20 nm.

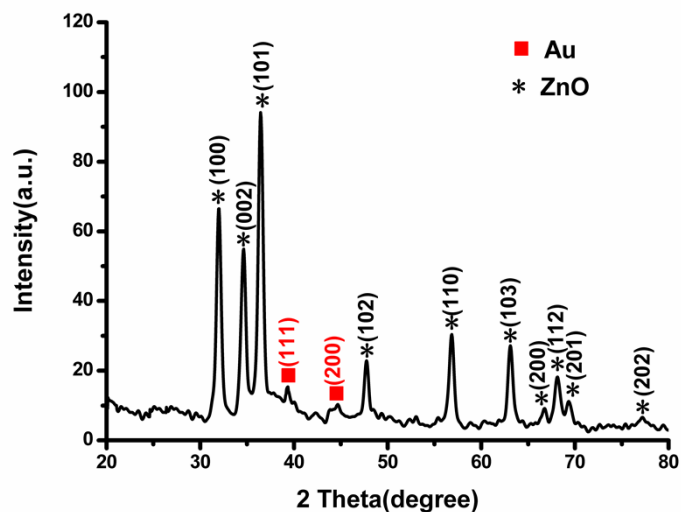


Fig. S2 The XRD pattern of Au-ZnO hybrid NPs. The diffraction peaks and relative intensity matched with standard Au and ZnO powder diffraction data.

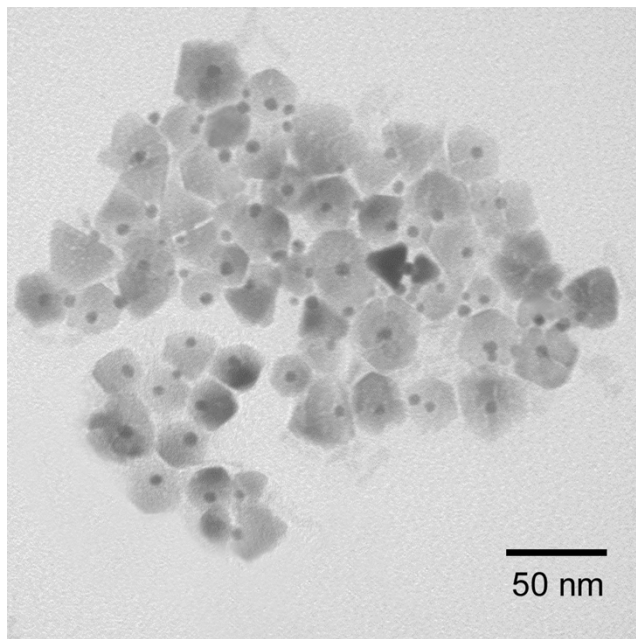


Fig. S3 TEM image of the FITC-RR-Au-ZnO-RGD NPs dispersed in PBS buffer. Scale bar = 50 nm.

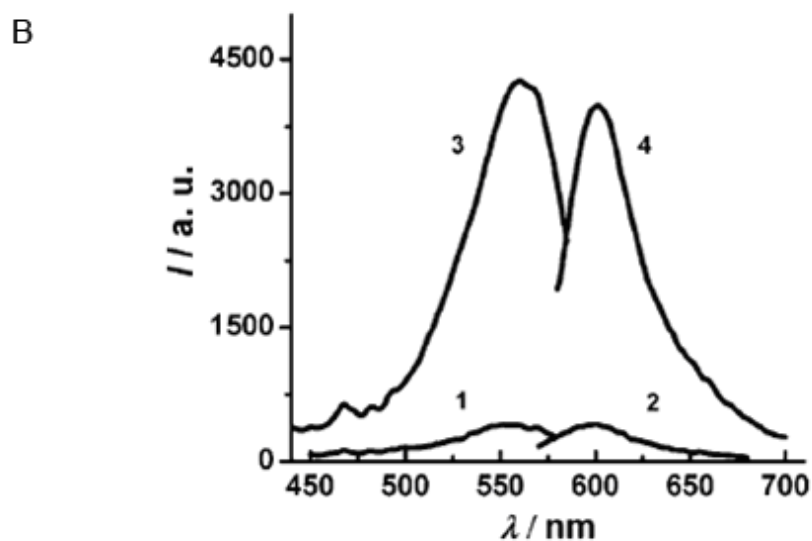
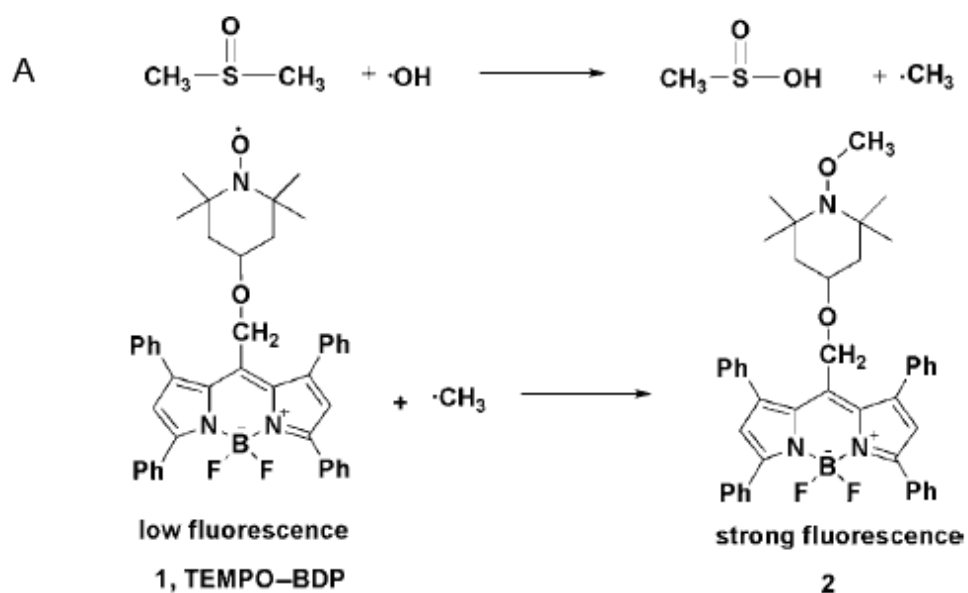


Fig. S4 (A) Chemical structure and reaction mechanism of TEMPO-BDP. (B) Excitation spectra (lines 1 and 3) and the fluorescence intensity (lines 2 and 4; excitation at 560 nm) of TEMPO-BDP (2.0 μM , 0.10 M DMSO) either with (upper lines) or without (lower lines) 1.0 μM Fe^{2+} /EDTA and 6.0 μM H_2O_2 . The spectra were acquired in 10 mM HEPES buffer (pH 7.4).

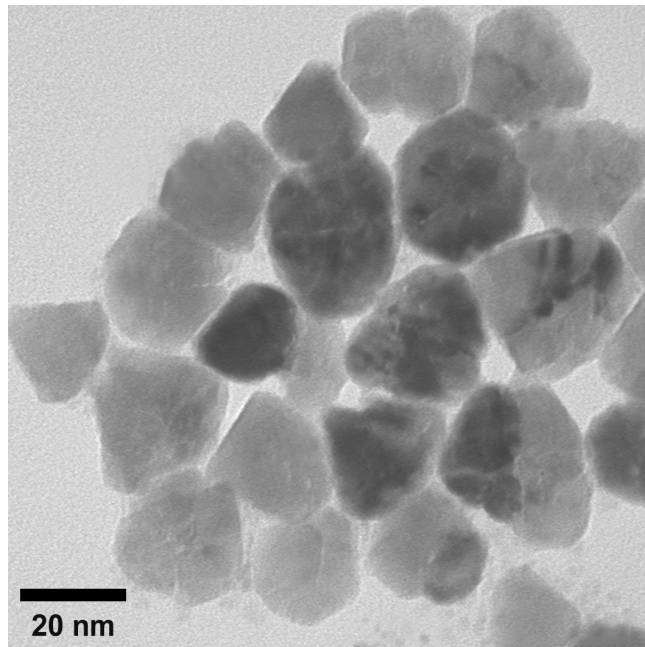


Fig. S5 TEM image of the 25 nm ZnO NPs obtained by etching Au away from the Au- ZnO NPs. Scale bar=20 nm.

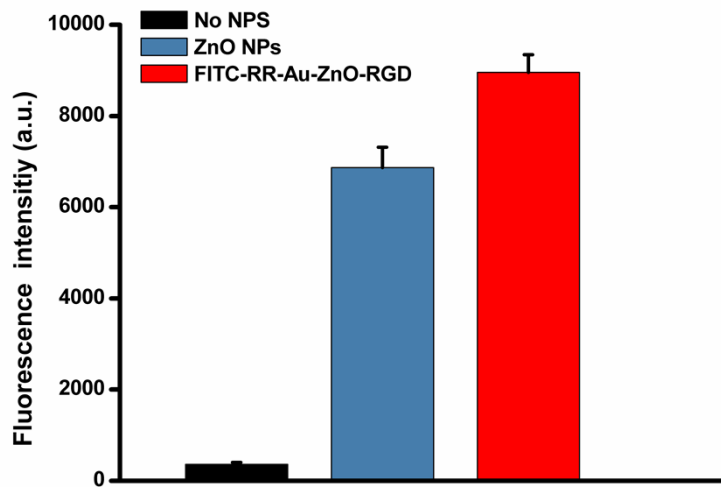


Fig. S6 Test of the capability of FITC-RR-Au-ZnO-RGD to catalyze ROS generation. The ROS levels were expressed as fluorescence intensity. Data are shown as mean \pm S.D. of three independent experiments.

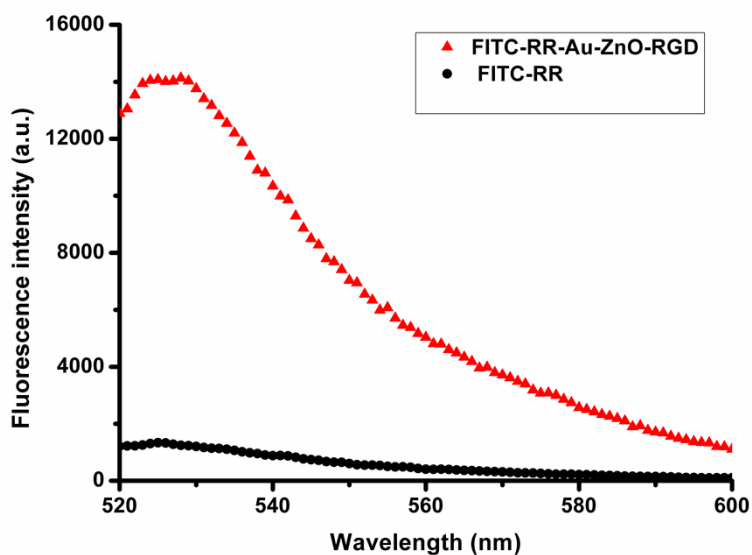


Fig. S7 Fluorescence spectra and images of FITC-RR and FITC-RR-Au-ZnO-RGD.

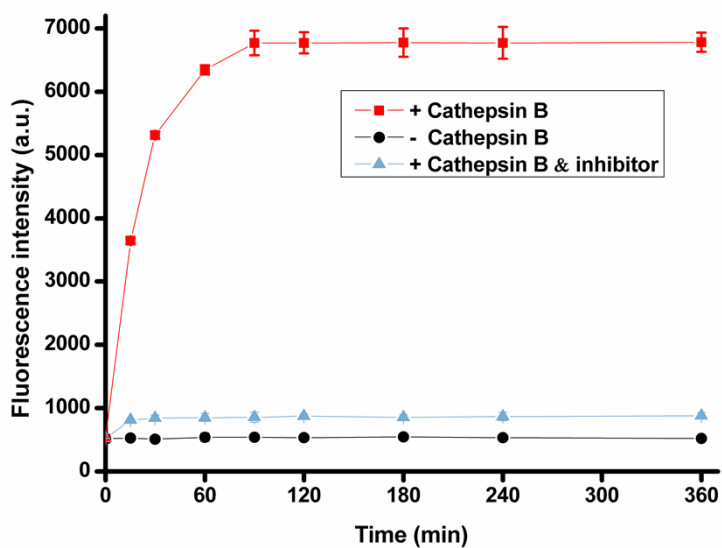


Fig. S8 Fluorescence intensity of the FITC-RR-Au-ZnO-RGD in the presence of cathepsin B and cathepsin B with inhibitor as function of incubation time at 37 °C. Data are shown as mean \pm S.D. of three independent experiments.

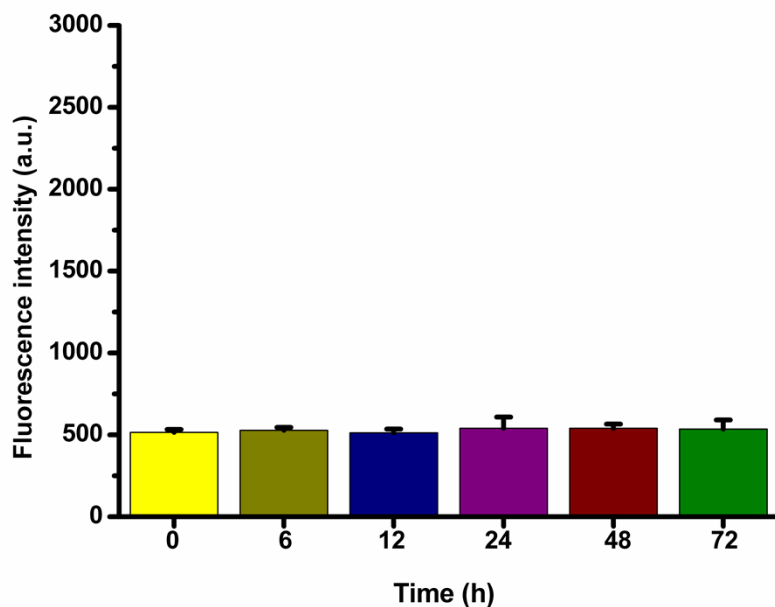


Fig. S9 Fluorescence intensity change of FITC-RR-Au-ZnO-RGD (50 µg/mL) in buffer solution during the 72 h incubation period at 37 °C. Data are shown as mean ± S.D.

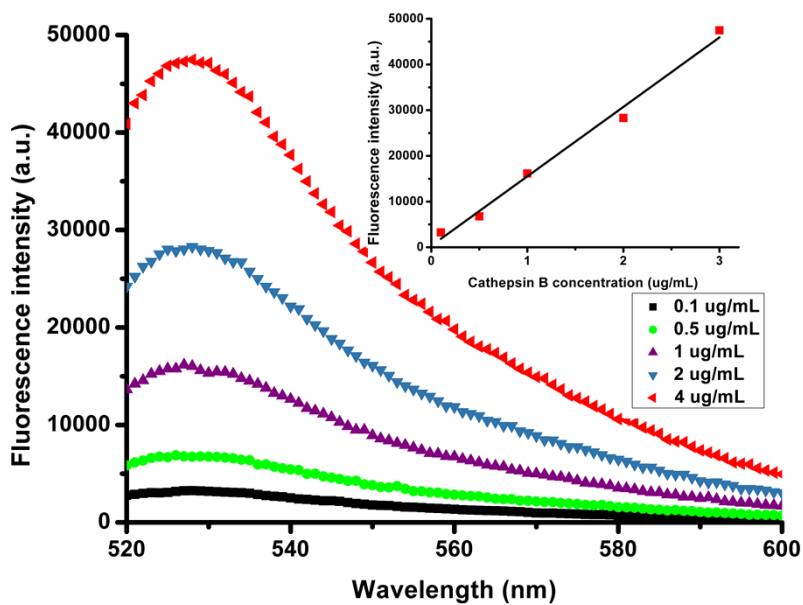


Fig. S10 Fluorescence spectra of the FITC-RR-Au-ZnO-RGD in the presence of various concentrations of cathepsin B following incubation for 30 min at 37 °C. Inset: cathepsin B standard curve.

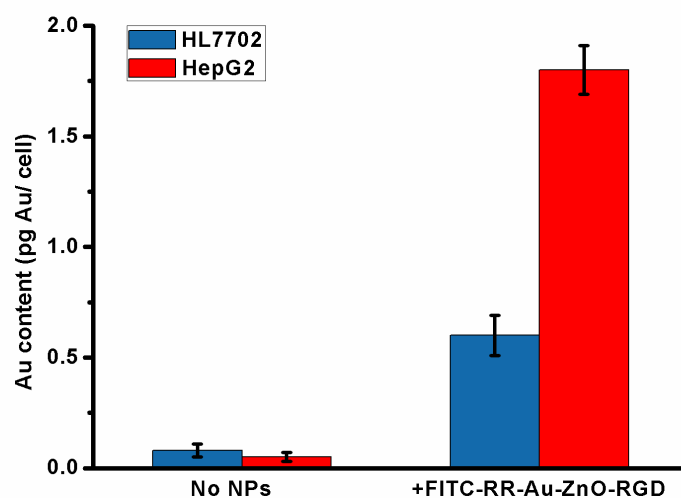


Fig. S11 Cellular uptake of FITC-RR-Au-ZnO-RGD NPs in lysosomes of HepG2 and HL7702 cells. Attached cells were incubated with NPs (50 $\mu\text{g}/\text{mL}$) for 0.5 h. Cell samples were washed three times to remove unbound NPs and digested in aqua regia. HepG2 cells had higher uptake than HL7702 cells. Bars show the average gold content per cell. Data are shown as mean \pm S.D. of three independent experiments.

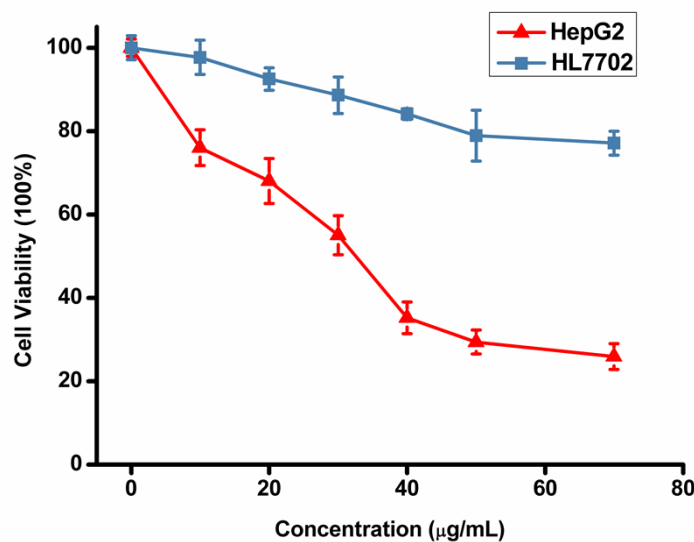


Fig. S12 MTT viability assay of HepG2 and HL7702 cells incubated with the FITC-RR-Au-ZnO-RGD NPs at 37°C for 24 h. HepG2 cells showed a lower viability compared to HL7702 cells. The IC₅₀ value of FITC-RR-Au-ZnO-RGD for HepG2 cells was only 32 $\mu\text{g}/\text{mL}$. Data are shown as mean \pm S.D. of three independent experiments.

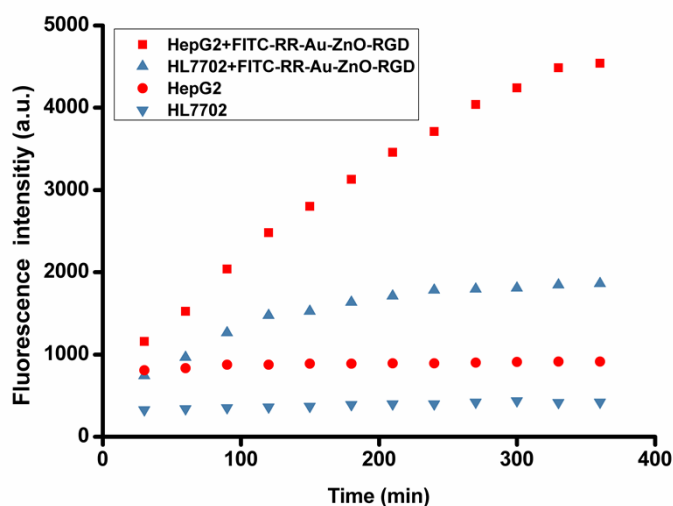


Fig. S13 ROS generation in HepG2 and HL7702 cells incubated with or without the FITC-RR-Au-ZnO-RGD NPs. After the cells were loaded with TEMPO-BDP, FITC-RR-Au-ZnO-RGD (50 $\mu\text{g}/\text{mL}$) were treated and the fluorescence signals were recorded every 30 min over a 6 h period. The fluorescence intensity was increased in RGD-targeted HepG2 cells relative to the HL7702 cells. The FITC-RR-Au-ZnO-RGD NPs exhibited a 5-fold higher intensity after 6 h in HepG2 cells. Data are shown as mean \pm S.D. of three independent experiments.

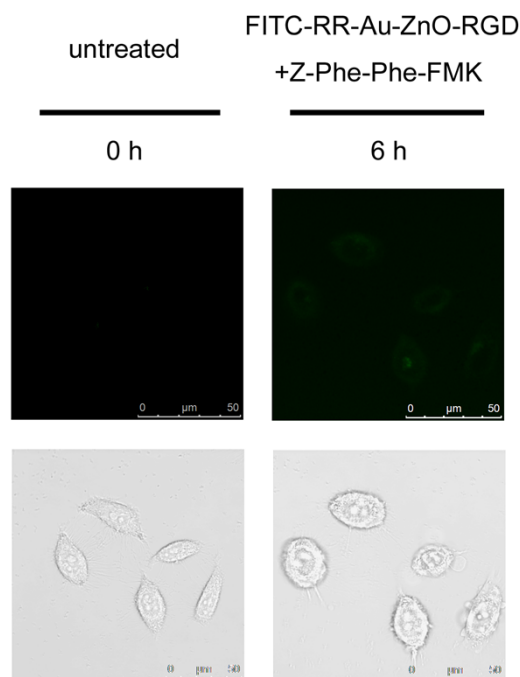


Fig. S14 Confocal images of HepG2 cells incubated with FITC-RR-Au-ZnO-RGD plus cathepsin B inhibitor (Z-Phe-Phe-FMK) for 6 h.

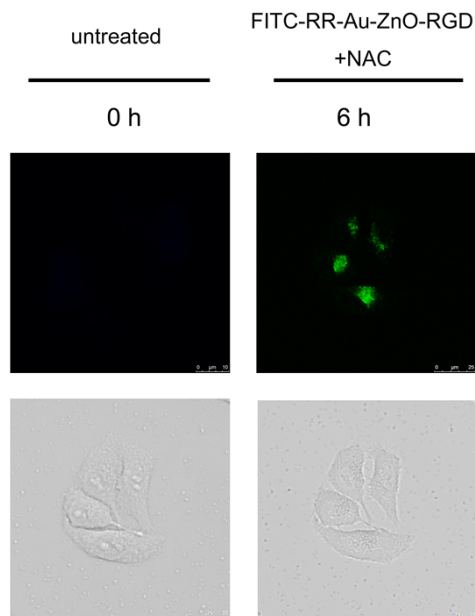


Fig. S15 Confocal images of HepG2 cells incubated with FITC-RR-Au-ZnO-RGD plus ROS scavenger (NAC) for 6 h.

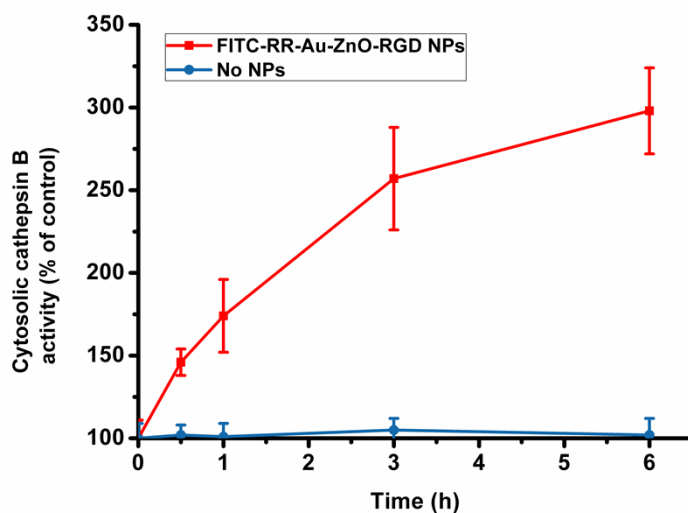


Fig. S16 Cathepsin B is released and active in the cytosol during LMP-dependent apoptosis induced by FITC-RR-Au-ZnO-RGD. Cells were treated with FITC-RR-Au-ZnO-RGD (50 $\mu\text{g}/\text{mL}$), and cytosolic extracts were prepared at indicated times. Equal amounts of proteins were analyzed for cathepsin B activity using the fluorogenic protease substrate Ac-RR-AFC. Cytosolic cathepsin B activity was detected as soon as 0.5 h, and it reached a maximum after 6 h of treatment with FITC-RR-Au-ZnO-RGD, whereas no significant change was observed with in the control cells. Data are expressed as mean \pm S.D. of three independent experiments.

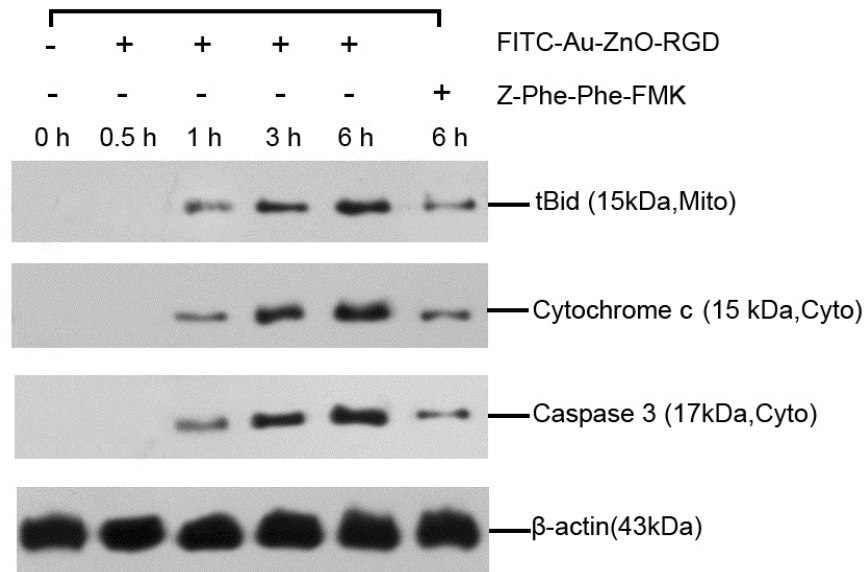


Fig. S17 tBid translocation, cytochrome c release and caspase-3 activation following FITC-RR-Au-ZnO-RGD-induced LMP. HepG2 cells were treated with FITC-RR-Au-ZnO-RGD for the indicated times, with or without pretreatment with the cathepsin B inhibitor (Z-Phe-Phe-FMK, 100 uM). Mitochondrial and cytosolic fractions were prepared and subjected to western blot analysis for tBid, cytochrome c and caspase 3. β-actin was used as the loading control.