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Anti-cancer drug axitinib: A unique tautomerism-induced dual-emissive probe for protein analysis

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1. Materials and methods

1.1 Materials and instruments

All chemicals and solvents were purchased from Energy Chemical China without further purification. Biological analytes including HSA, bovine serum albumin (BSA), lipase, lysosome, globulin, trypsin, glutathione, tyrosinase, creatinine, GSA and RNA were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, 1 mM, pH ~ 7.4) was purchased J&K scientific. UV–Vis absorption and fluorescence spectra were tested by a Thermo-Fisher Evolution 220 and Thermo-Fisher Lumina fluorometer, respectively. Fluorescence lifetimes were obtained by Horiba DeltaFlex with a 359 nm Laser NanoLED.

1.2 Basic test conditions

Stock solutions (10 mM) of axitinib and acetyl-axitinib were prepared in DMSO and stored in the dark environment at room temperature. Stock solutions of HSA and BSA with concentrations of 0.5 mM were prepared in water. Adding axitinib stock solution (2 μL) into PBS buffer (2 mL) could obtain the detection solution. Before conducting any tests, the detection solution of axitinib was exposed to a 365 nm ultraviolet lamp for 5 minutes to complete the tautomerism process. For titration experiments, HSA were added into the detection solution of axitinib and allowed to shake for at least 30 seconds. For indicator displacement experiments, two site-specific drugs, warfarin (DS1) and ibuprofen (DS2) were added proportionally into the axitinib@ALB

complex, and the fluorescence spectra were measured after shaking for 30 seconds. Real serum sample containing 46.0 g/L HSA (certified by BCG method) was added to PBS buffer and diluted to the appropriate concentration. Upon adding the serum sample into the detection solution of axitinib, the mixture was incubated for 1 min at room temperature and then was measured.

1.3 Molecular docking

The 3D geometry of each ligand was energy minimized in Chem3D by working with mm2. The ligand-free crystal structures of HSA (PDB ID: 4K2C) and BSA (PDB ID: 4F5S) were taken from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb) [1]. Flexible ligand docking was performed by AutoDock 4.2 molecular docking program using the implemented empirical free energy function and the Lamarckian Genetic Algorithm . The Autogrid was used to calculate Grids. The grid spacing was 0.375 Å as default. 20 docking runs with 25,000,000 energy evaluations were performed. The output from AutoDock was rendered with PyMol. The 2D diagrams were produced by a Ligplot software.

1.4 Fluorescence lifetime

Fluorescent decay curves were recorded by a Horiba Delta-Flex instrument with a nanoLED with peak wavelength at 359 nm (Pulse duration < 1.4 ns). Lifetime was recorded at peak wavelength. IRF is the instrument response function (prompt). The radiation decay curves were typically fit to the multi-exponential model: $I(t) = A +$

$I_0 \cdot \sum \alpha_i \exp(-t/\tau_i)$. The I_0 represents the original fluorescent intensity; the α_i are pre-exponential factors, which represent the fractional amount of each lifetime component and the $\sum \alpha_i$ is normalized to unity; the τ_i refer to fluorescence lifetimes. The average fluorescence lifetime is calculated by equation: $\tau = \sum \alpha_i \tau_i$.

1.5 Binding constant and stoichiometry

The binding parameters can be calculated using fluorescence data. The calculation of binding constants (k_b) and binding stoichiometry (n) can be conducted based on the previously reported equations.[1]

$$\log\left(\frac{F_0 - F}{F}\right) = \log k_b + n \log[Q]$$

1.6 Limit of detection

The limit of detection (LOD) was calculated by using $3\sigma/k$ rule based on HSA titration experiments. Where σ is the standard deviation of blank measurement for three times and k is the slope of the fluorescence intensity plotted against the HSA concentration.

2. Supporting Figures

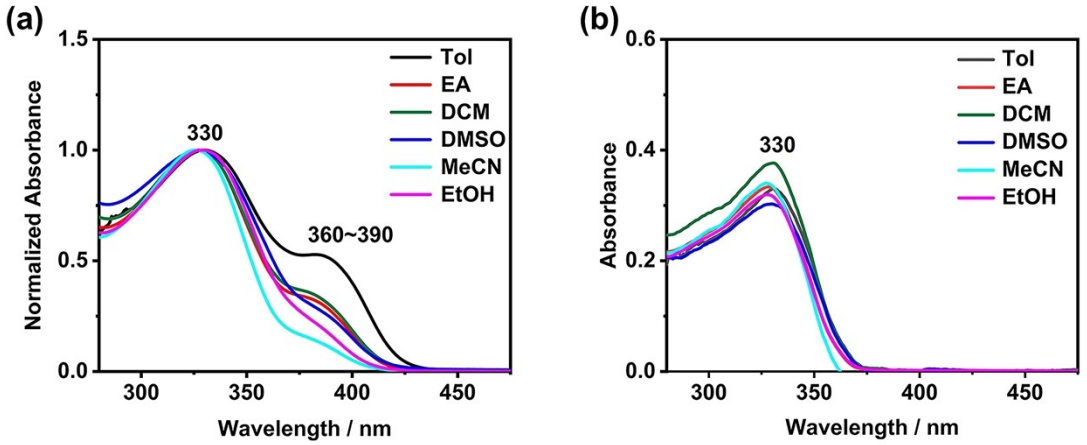

Fig. S1 Absorbance spectra of (a) axitinib and (b) acetyl-axitinib in different solvents, respectively. [axitinib] = [acetyl-axitinib] = 10 μM.

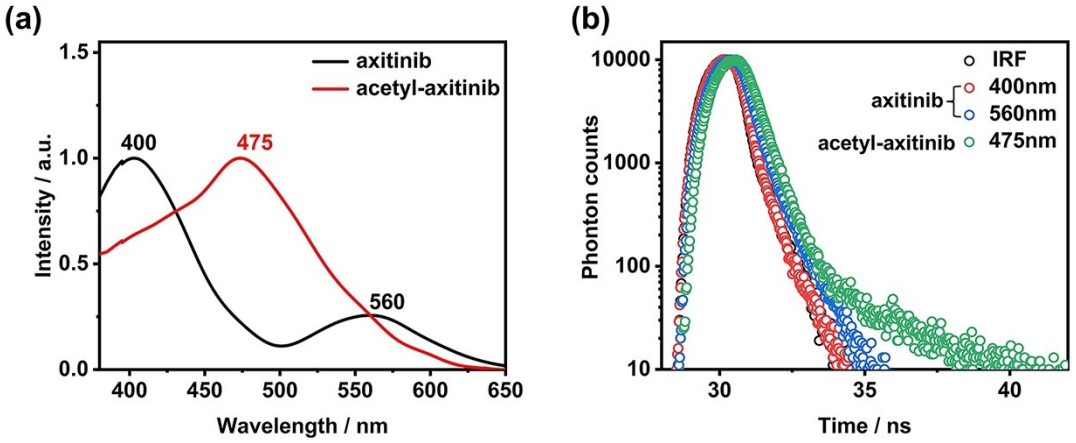

Fig. S2 (a) Fluorescence spectra of axitinib and acetyl-axitinib in EtOH. (b) Fluorescence decay curves of axitinib and acetyl-axitinib in EtOH. IRF: instrument response function (prompt). Laser source is a 359 nm NanoLED. [axitinib] = [acetyl-

axitinib] = 10 μM.

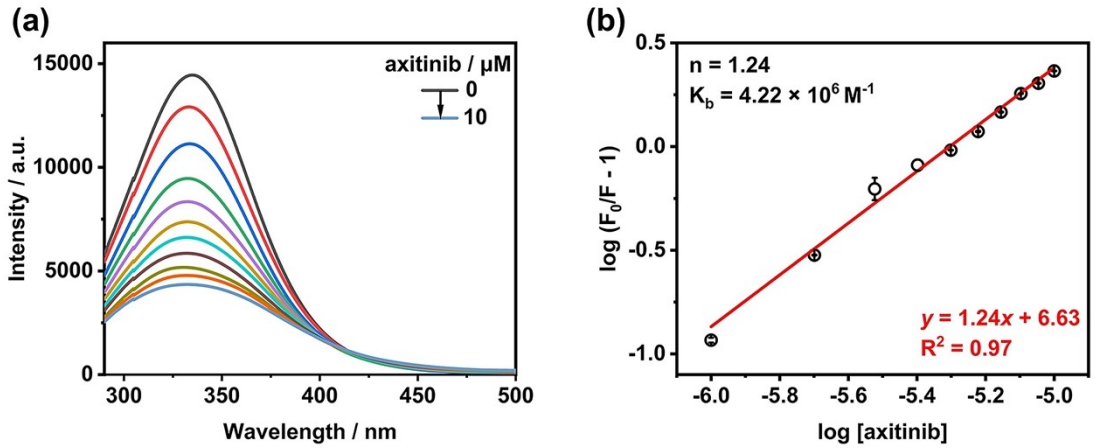

Fig. S3 (a) Fluorescent spectra of HSA (10 μM) in the presence of axitinib (0-10 μM) in PBS buffer (1 mM, pH ~ 7.4). (b) The log (F_0/F - 1) *vs*. log [axitinib] for the complex-HSA interaction corresponds, F_0 and F are the fluorescence intensities of HSA in the absence and presence of axitinib. λ_{ex} = 280 nm.

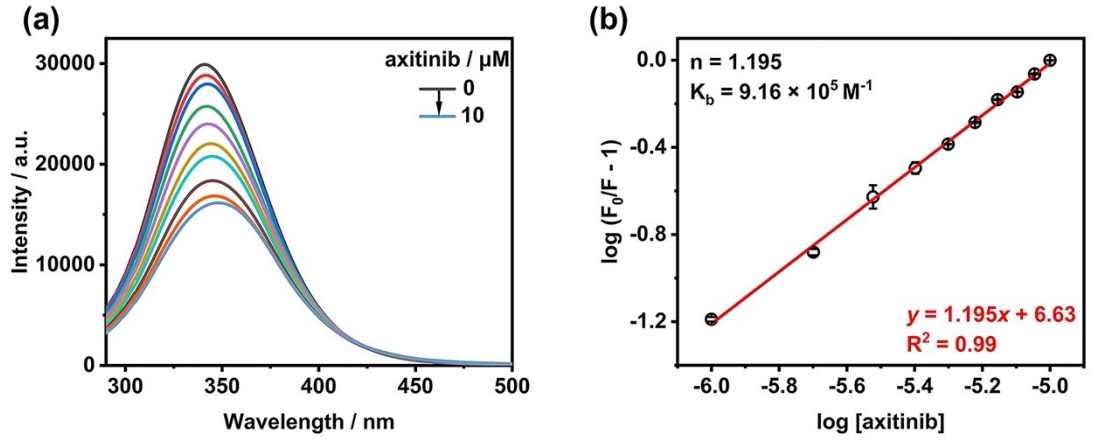

Fig. S4 (a) Fluorescent spectra of BSA (10 μM) in the presence of axitinib (0-10 μM) in PBS buffer (1 mM, pH ~ 7.4). (b) The log (F_0/F - 1) *vs*. log [axitinib] for the

complex-BSA interaction corresponds, F_0 and F are the fluorescence intensities of BSA in the absence and presence of axitinib. λ_{ex} = 280 nm.

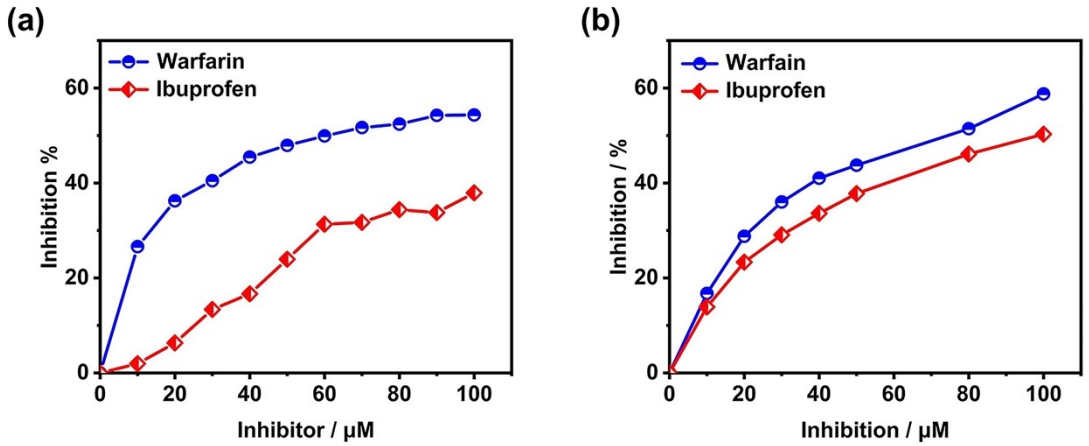

Fig. S5 Fluorescence quenching degrees of complex (a) axitinib@HSA and (b) axitinib@BSA with addition of site-specific indicators (warfarin for DS1 and ibuprofen for DS2), respectively. [axitinib@HSA] = [axitinib@BSA] = 10 μM.

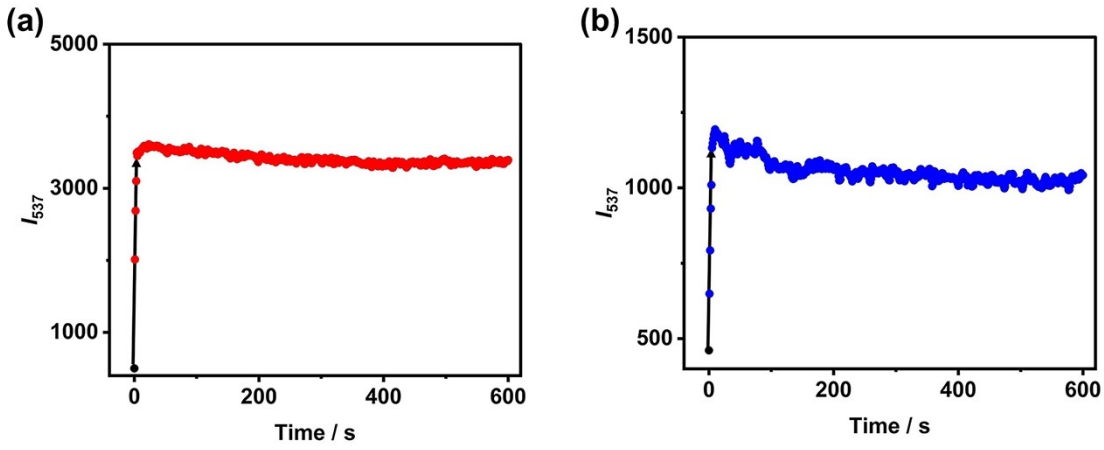

Fig. S6 Time-dependent peak intensity of axitinib (10 μM) in the presence of 1 equivalent (a) HSA and (b) BSA in PBS buffer (1 mM, PH ~ 7.4), respectively.

[AXT@HSA] = [AXT@BSA] = 10 μM.

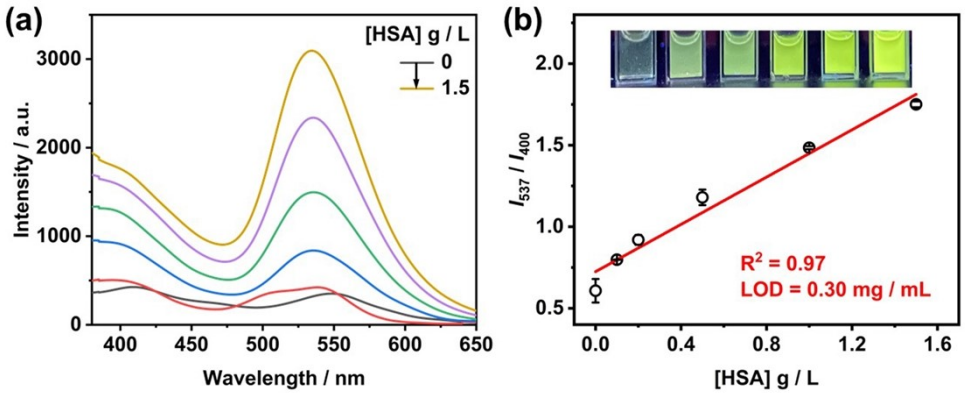

Fig. S7 (a) Fluorescence spectra of axitinib (10 μM) with addition of real serum containing the different concentration of HSA in PBS buffer (1 mM, pH ~ 7.4). (b) Linear relationship between intensity ratios (I_{537} / I_{400}) of axitinib (10 μM) and concentration of HSA. Error bars = ±SD for three measurements. λ_{ex} = 360 nm.

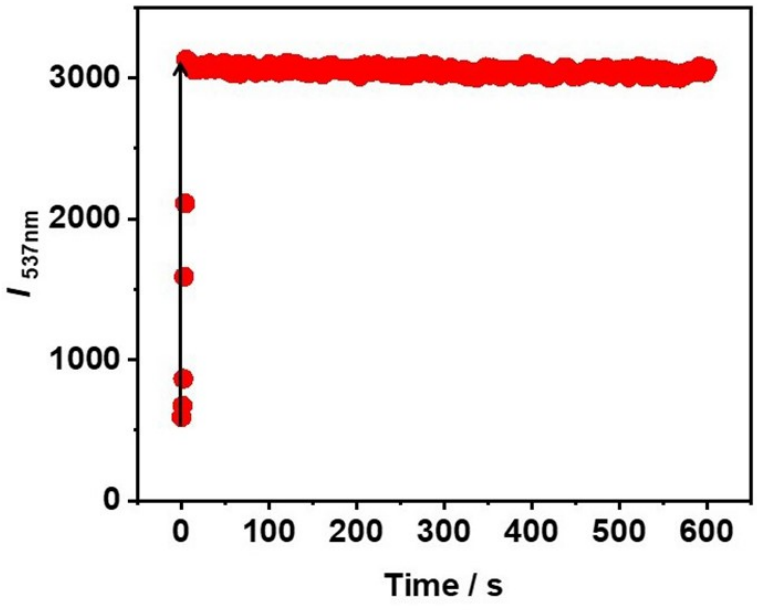

Fig. S8 Time-dependent peak intensity of axitinib (10 μM) with addition of real serum containing the HSA (1.5 g/L) in the PBS buffer (1mM, pH ~ 7.4). λ_{ex} = 360 nm.

Table S1 Detection of HSA in serum by using axitinib

Added (g / L)	Detected (g / L)	Recovery (%)	RSD (%)
0.10	0.11	108.27	1.40
0.50	0.52	103.70	8.49
1.00	1.06	106.15	1.30

Reference:

1. Y. Wang, H. Yu, X. Shi, Z. Luo, D. Lin and M. Huang, *Journal of Biological Chemistry*, 2013, **288**, 15980-15987.