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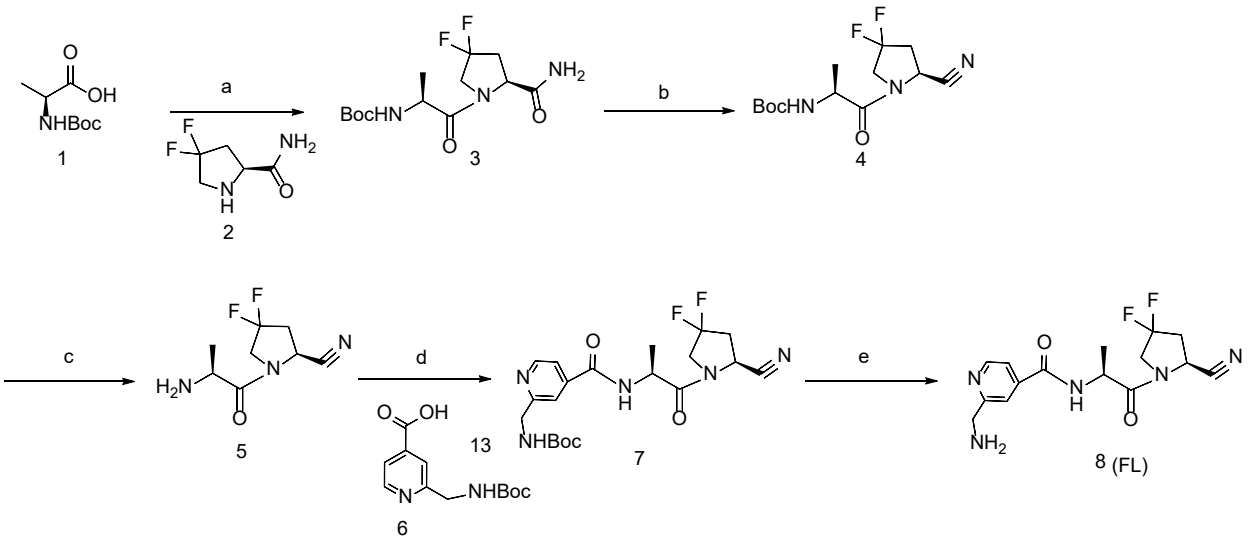


Scheme S1. Synthesis of FAP targeting ligand (FL). Reagents and conditions: a) HATU, Anhy. DIPEA, Anhy. DMF, rt; b) TFAA, Anhy. DCM, Anhy. Pyridine, rt; c) TFA, rt; d) HATU, Anhy. DIPEA, Anhy. DMF, rt; e) TFA, rt.

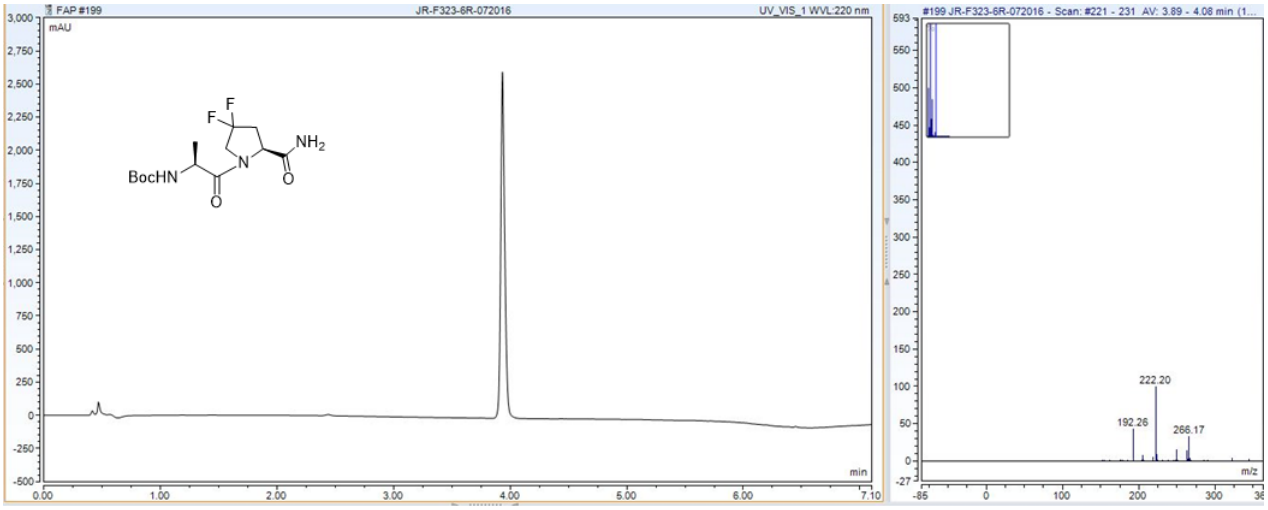


Figure S1. Chemical structure and LC/MS trace of Compound 3.

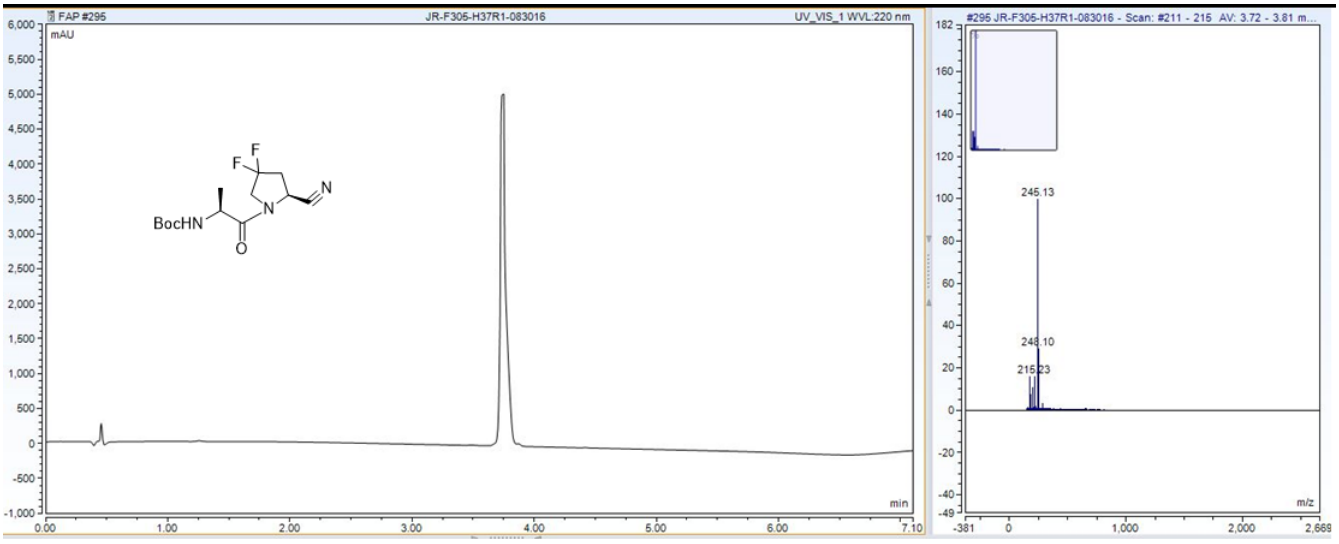


Figure S2. Chemical structure and LC/MS trace of Compound 4.

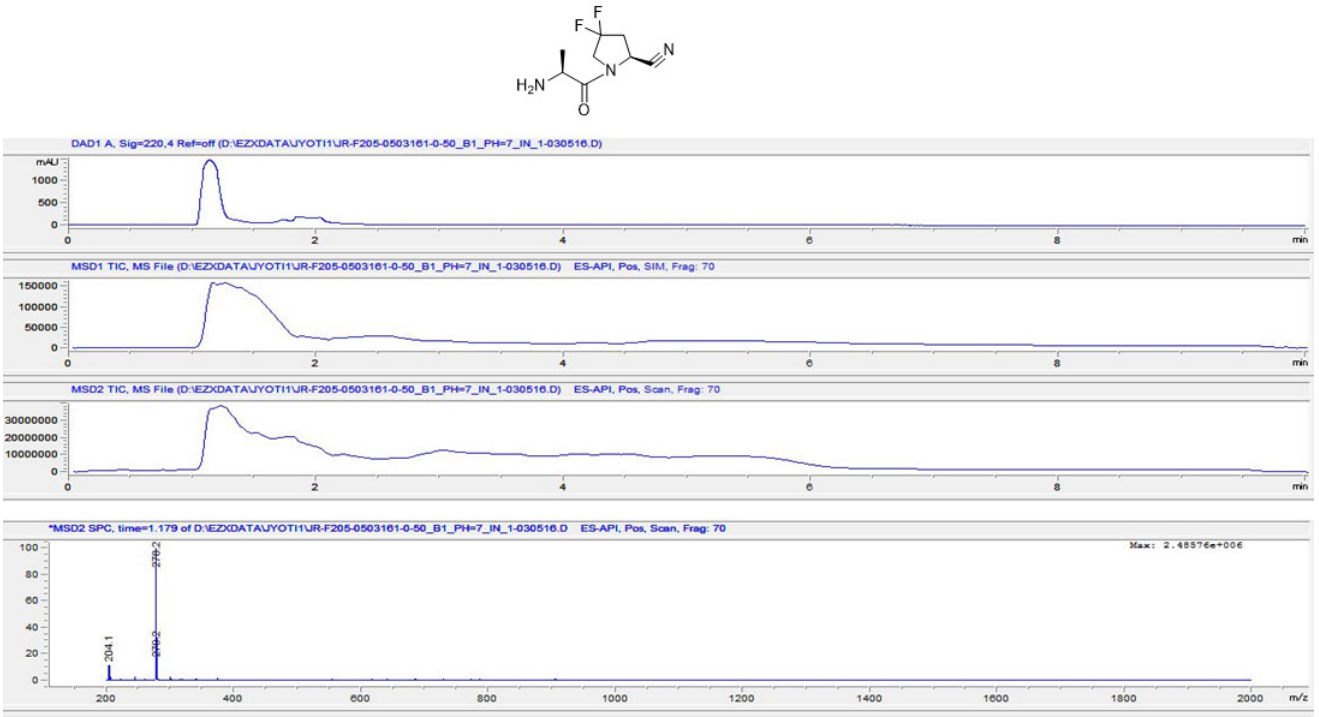


Figure S3. Chemical structure and LC/MS trace of Compound 5.

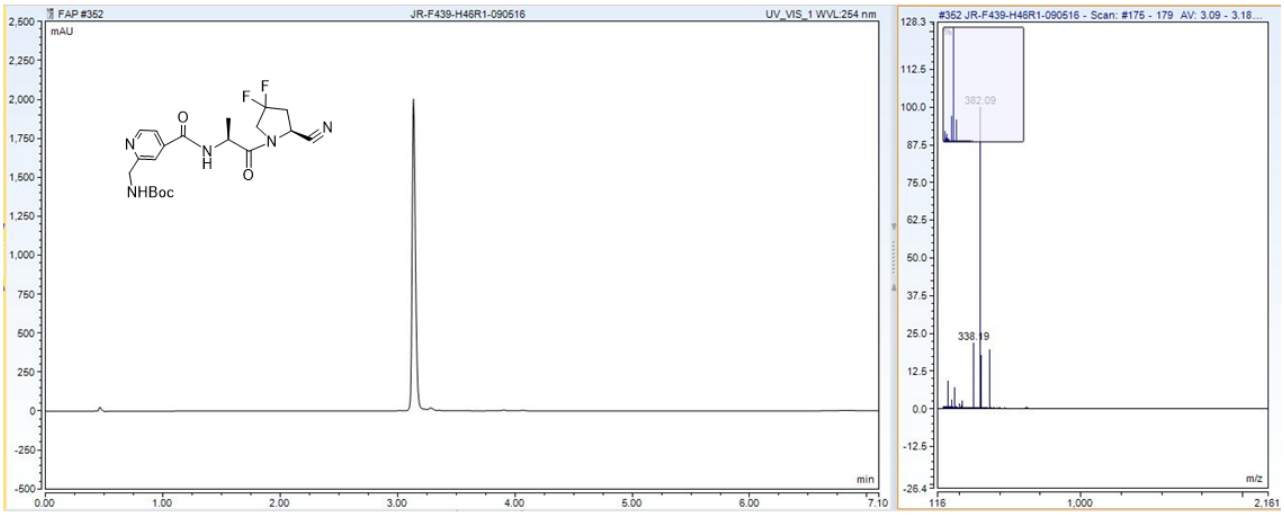


Figure S4. Chemical structure and LC/MS trace of Compound 7.

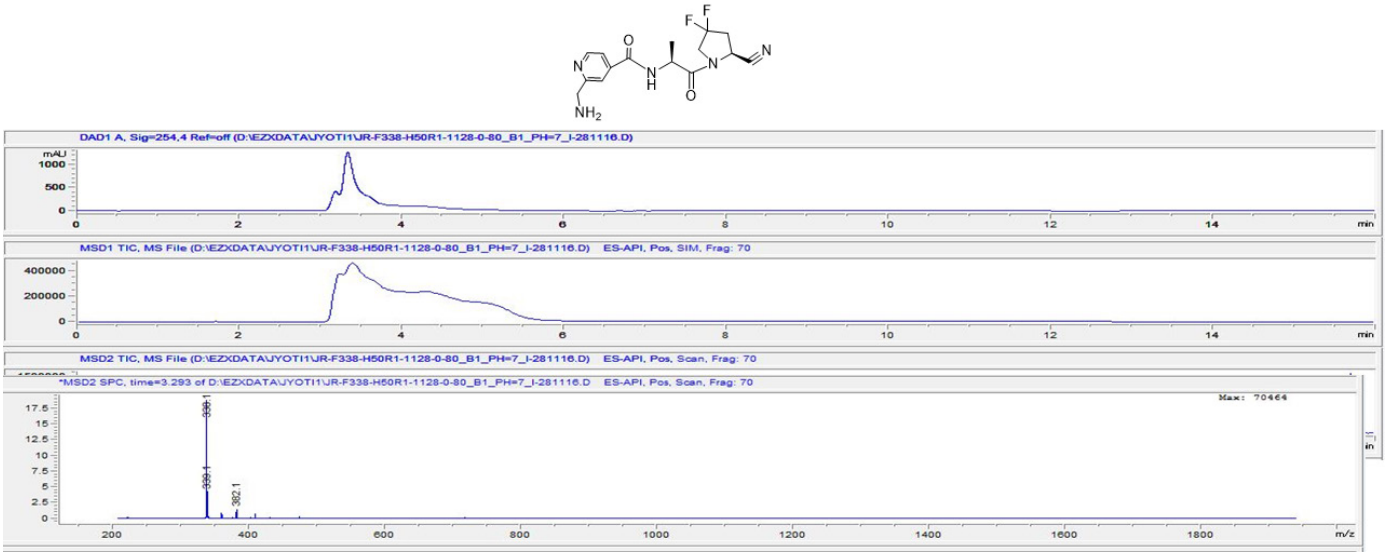
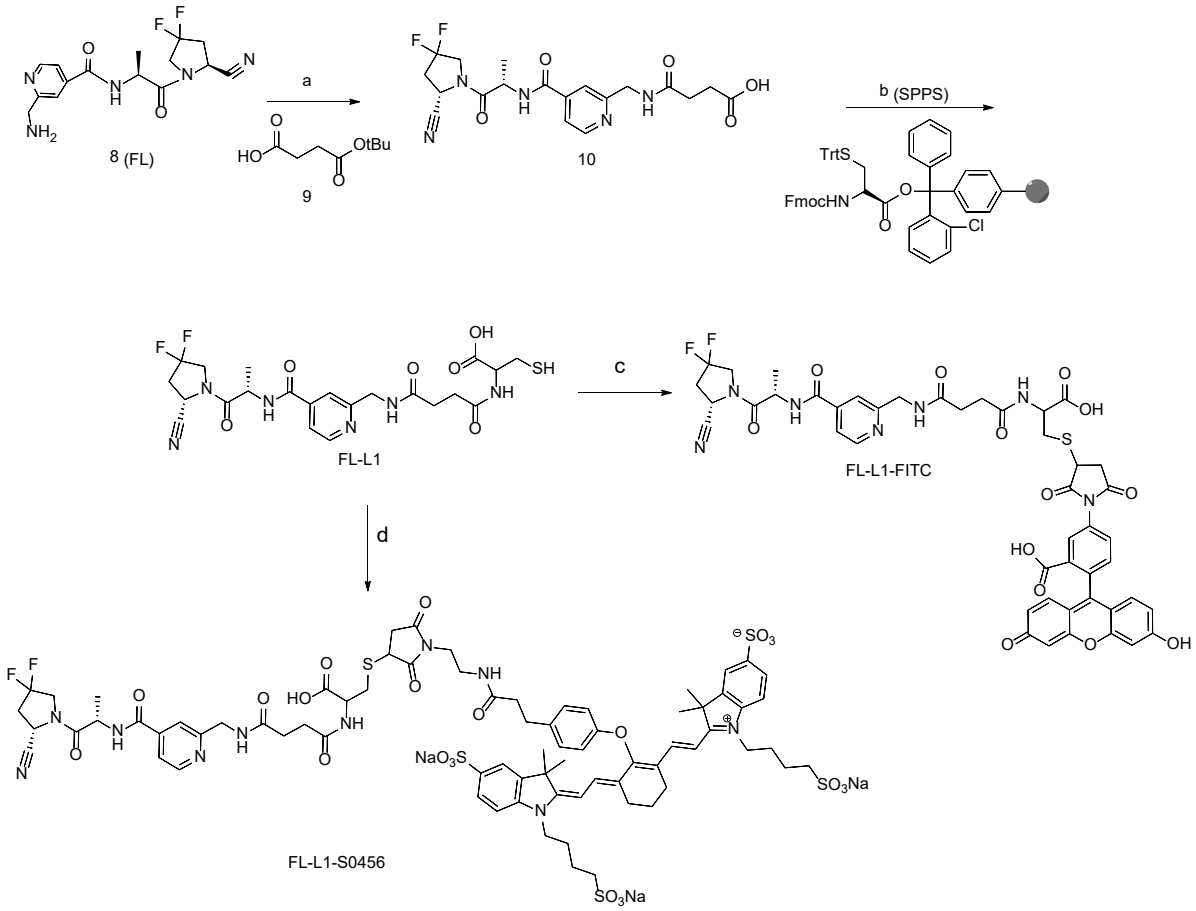


Figure S5. Chemical structure and LC/MS trace of Compound 8 (FL).



Scheme S2. Synthesis of FL-L1-S0456 and FL-L1-FITC. Reagents and conditions: a) (i) HATU (2.5 eq), Anhy. DIPEA (5 eq), Anhy. DMF, rt. (ii) TFA, rt b) PyBop (2.5 eq), Anhy. DMF, Any. DIPEA (5 eq), rt. (ii) TFA/H_2O/TIPS/EDT (92.5:2.5:2.5:2.5), 1 h. c) FITC maleimide (1 eq), DMSO, DIPEA (5 eq), rt. d) S0456 maleimide (1 eq), DMSO, DIPEA (5 eq), rt.

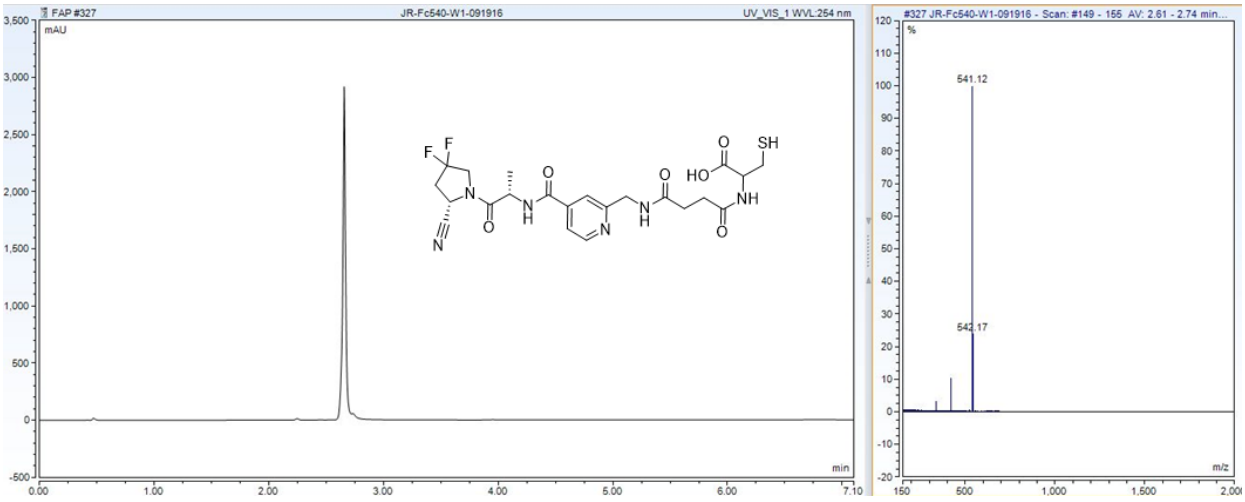


Figure S6. Chemical structure and LC/MS trace of FL-L1.

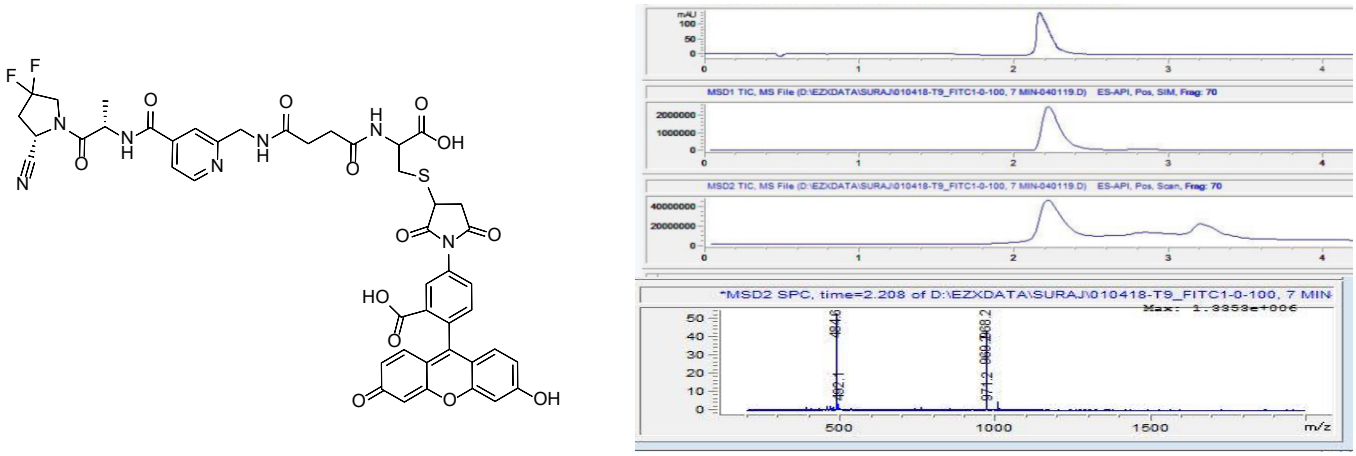


Figure S7. Chemical structure and LC/MS trace of FL-L1-FITC.

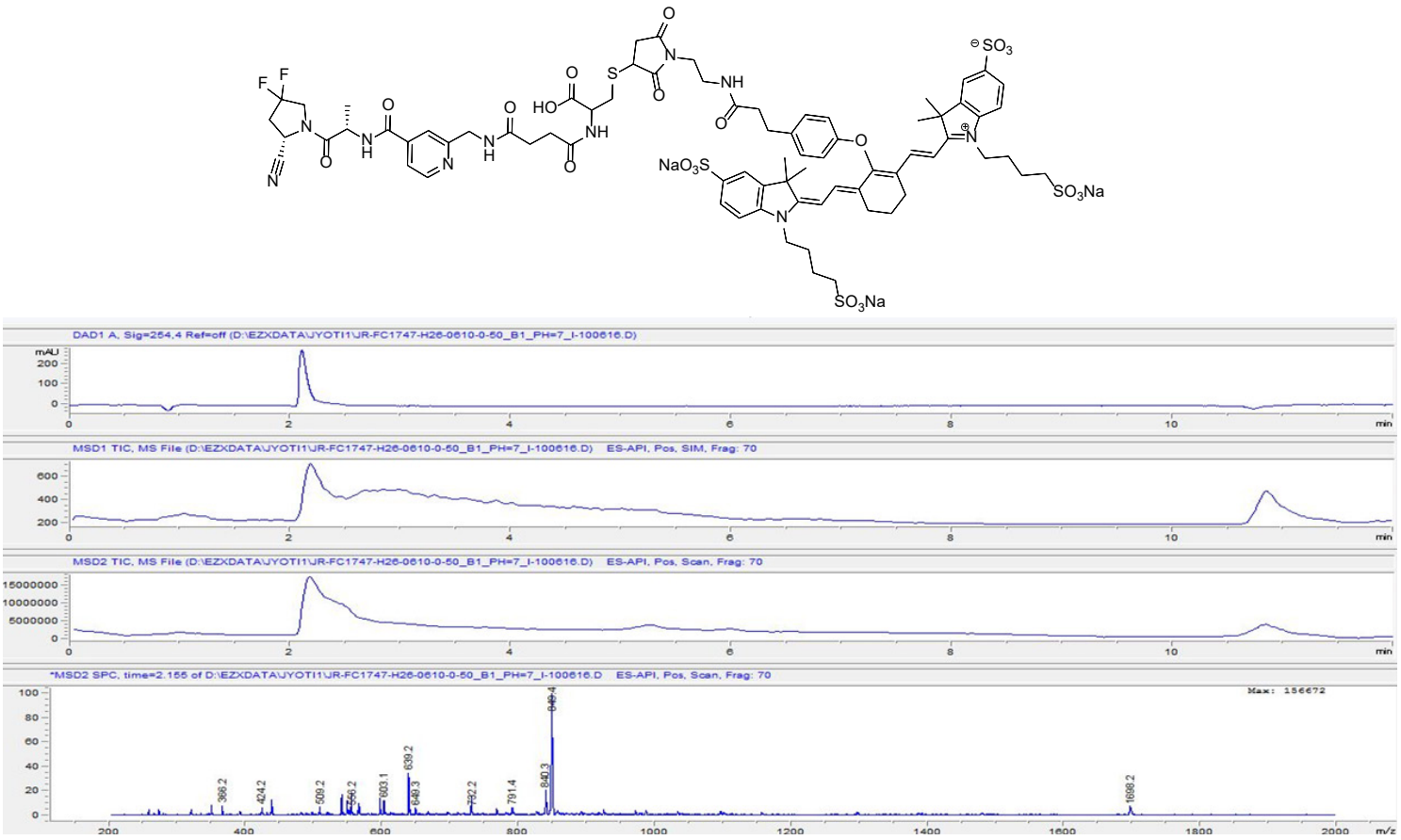
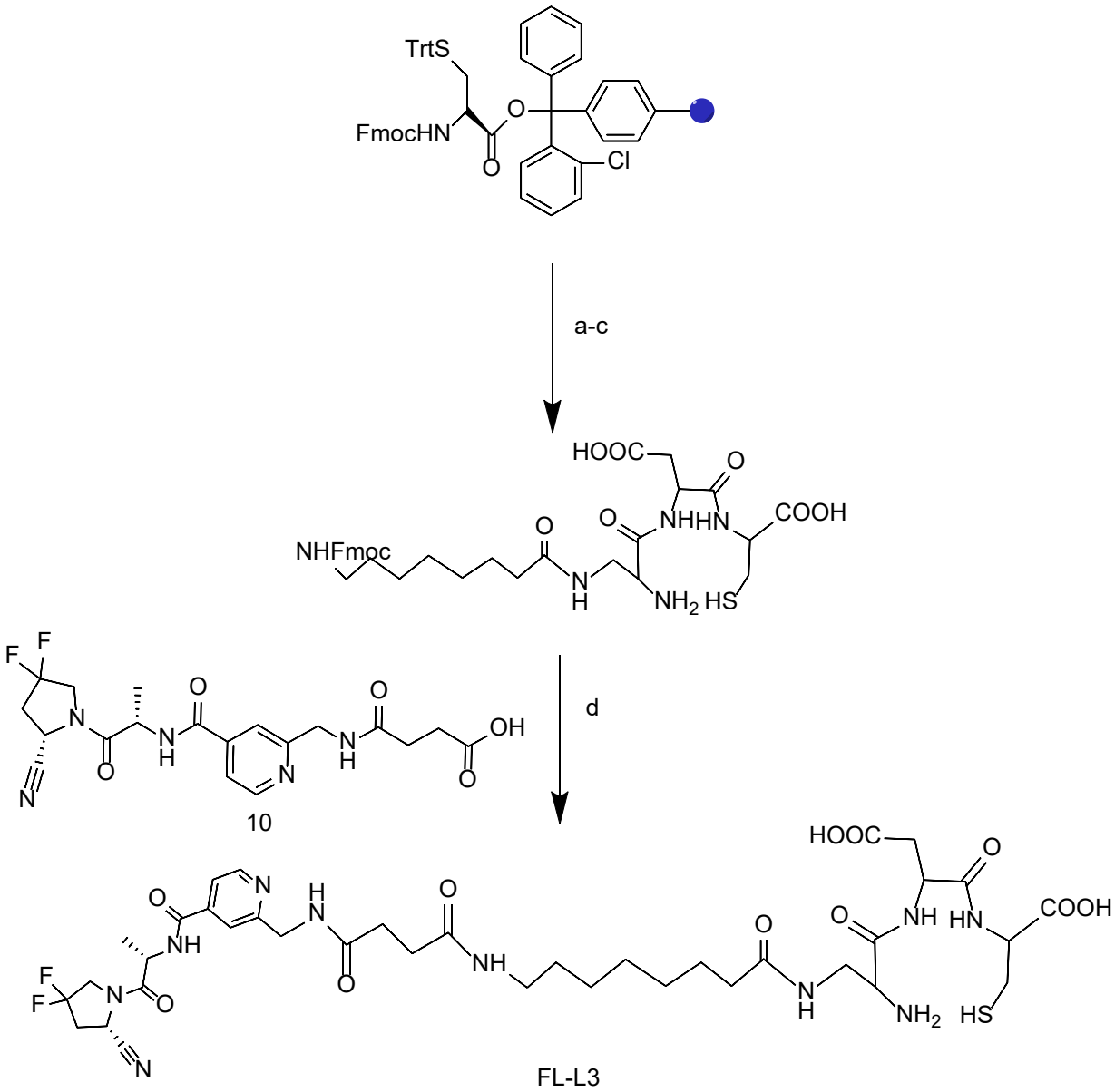
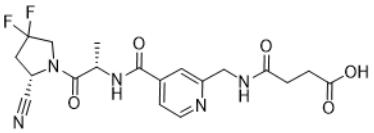


Figure S8. Chemical structure and LC/MS trace of FL-L1-S0456.



FL-L3

Scheme S3. Synthesis of FL-L3. Reagents and conditions: (a) (i) 20% piperidine/DMF, rt, (ii) Fmoc-Asp(OtBu), PyBop, DMF, DIPEA, (b) (i) 20% piperidine/DMF, rt,10 min (ii) Fmoc-diaminopropionic (DAP) acid, PyBop, DMF, DIPEA, (c) (i) 20% piperidine/DMF, rt, (ii) Fmoc-8-amino-octanoic acid, PyBop, DMF, DIPEA, (d) (i) 20% piperidine/DMF, rt,10 min (ii) Compound 10, PyBop, DMF, DIPEA, (iii) TFA/H_2O/TIPS/EDT (92.5:2.5:2.5:2.5), 1 h. The crude product was purified by using HPLC and characterized by using LC/MS.



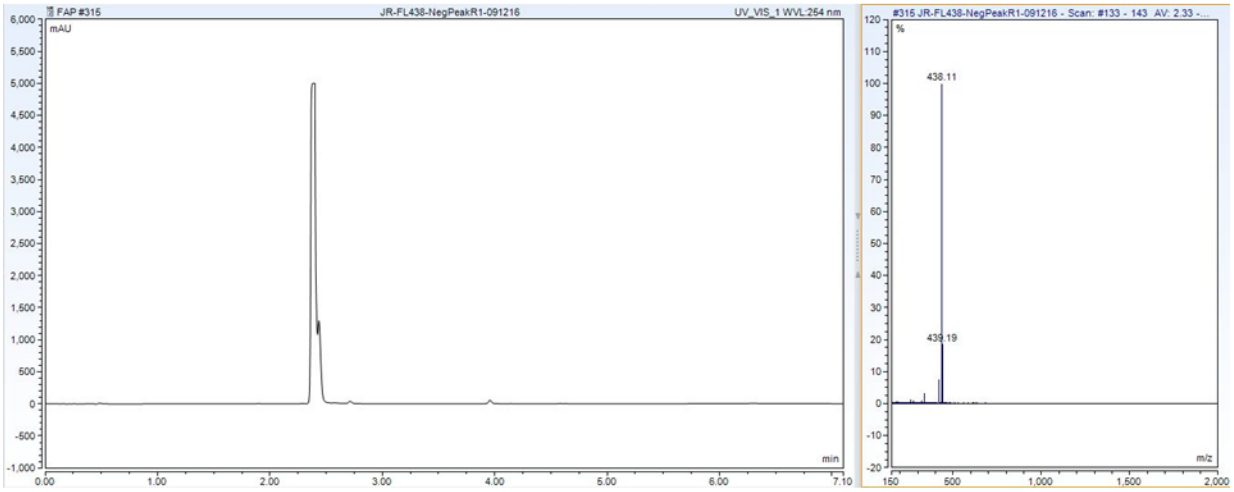
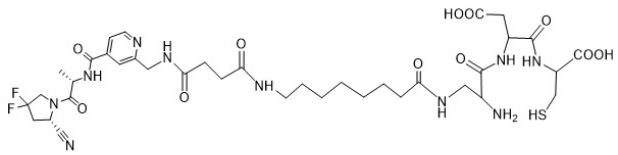


Figure S9. Chemical structure, LC/MS trace, and ^{1}H NMR of Compound 10.

^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.58 – 8.47 (d, J = 4.8 Hz, 1H), 7.67 – 7.40 (m, 2H), 5.10 – 5.02 (dd, J = 9.1, 4.3 Hz, 1H), 4.64 – 4.54 (q, J = 7.2 Hz, 1H), 4.45 (s, 2H), 4.22 - 4.13 (m, 2H), 3.05 – 2.70 (m, 2H), 2.55 (s, 4H), 1.43 – 1.33 (d, J = 7.1 Hz, 3H).



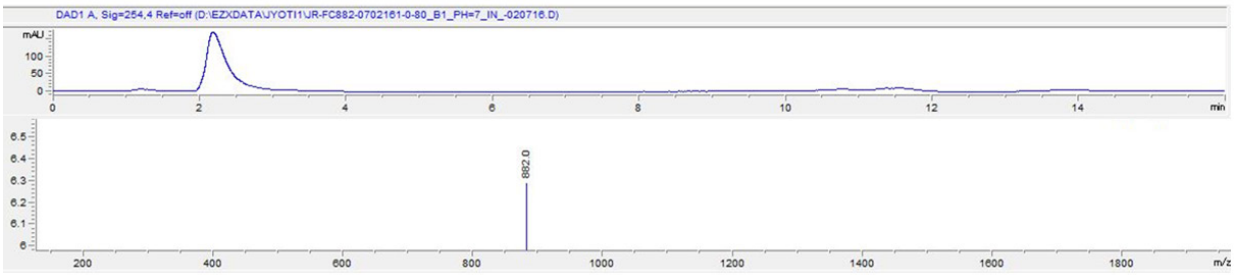
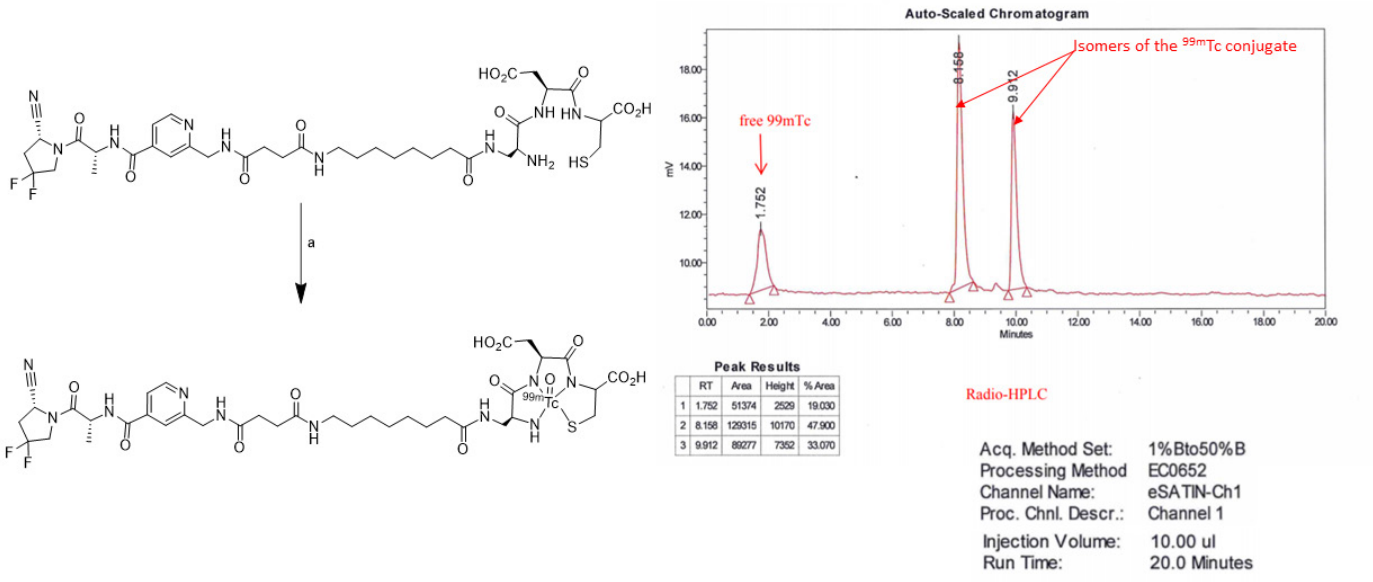
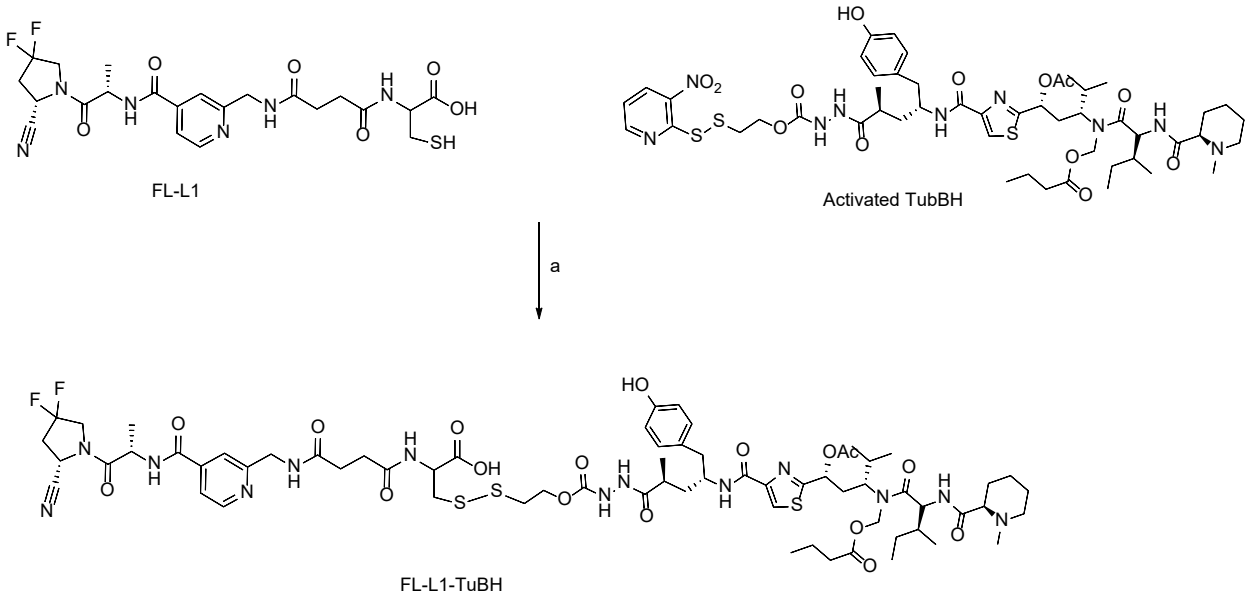


Figure S10. Structure and LC/MS of FAP conjugate (FL-L3).



Scheme S4. Radioactive labeling of FL-L3 with ^{99m}Tc and radio HPLC. Reagents and conditions: (a) ^{99m}Tc sodium pertechnetate (15 mCi, 1 ml), 100 ^{o}C, 18 min. Chelation efficiency was confirmed by radio HPLC.



Scheme S5. Synthesis of FL-L1-TubBH. Reagents and conditions: a. (i) FL-L1, H_2O/$NaHCO_3$ (pH= 7.0-7.2), Argon, r.t. (ii) activated TubBH, anhydrous THF, argon, r.t.

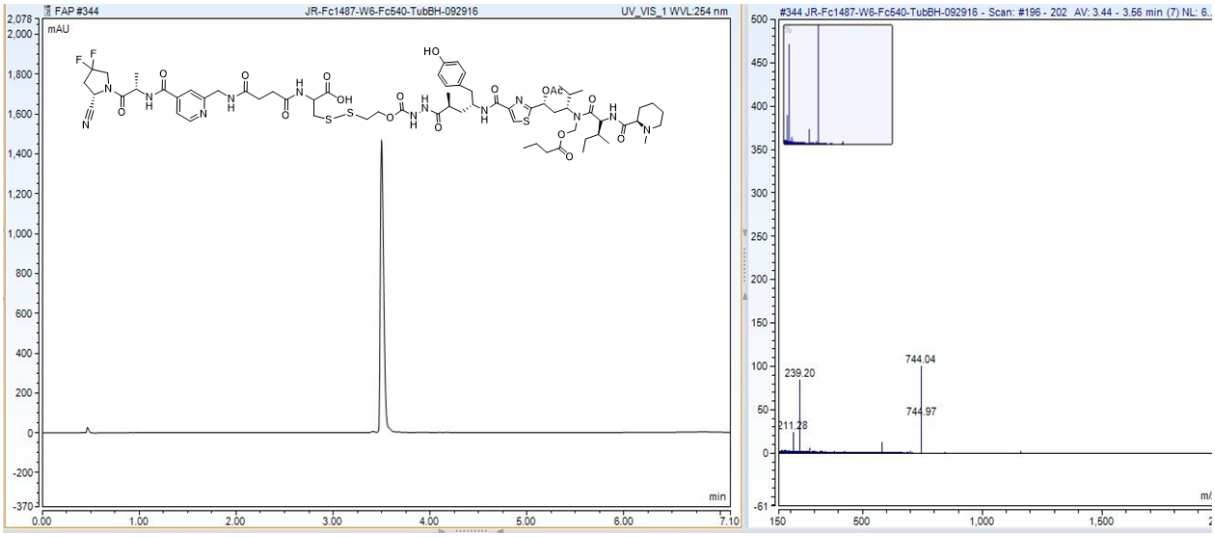


Figure S11. Structure and LC/MS of FAP tubulysin B hydrazide conjugate (FL-L1-TubBH).

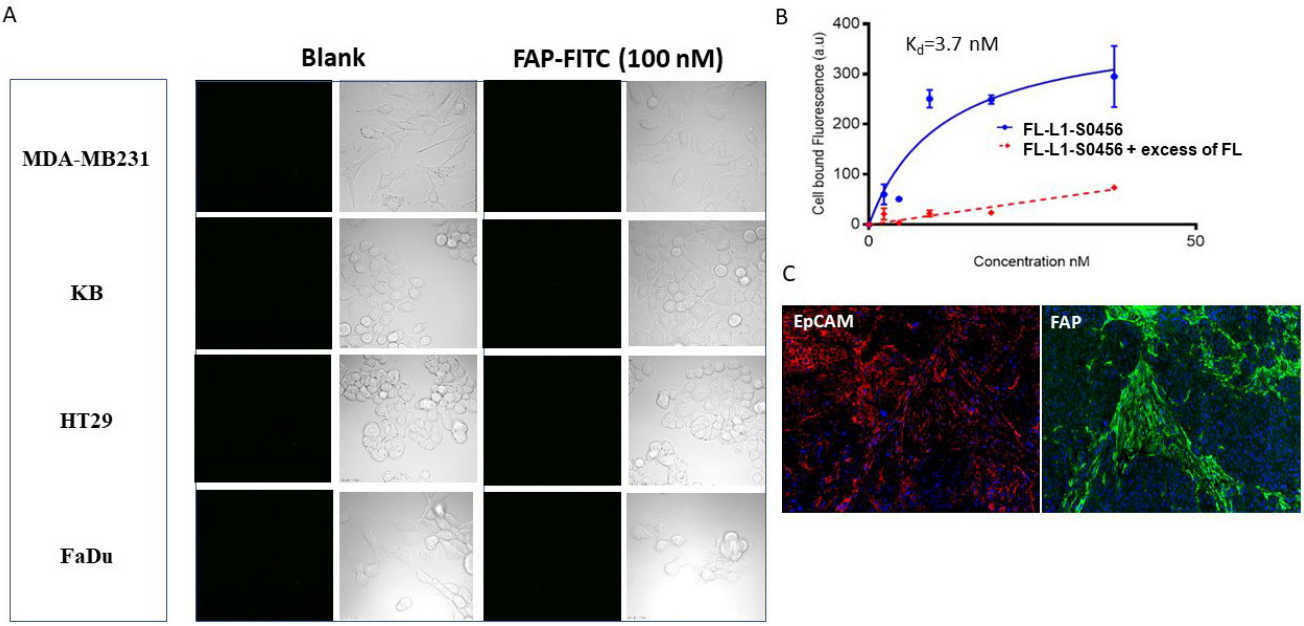


Figure S12. Confocal Imaging and in vitro binding affinity of FL-L1-FITC and FL-L1-S0456. A. The cancer cells were incubated with FL-L1-FITC (100 nM) at 37°C. After incubating for 1 h the cells were washed with medium to remove unbound dye conjugate and observed under confocal microscopy. B. HEK293-FAP cancer cells were incubated with FL-L1-S0456 in the presence or absence of 100-fold excess of FL at 37°C. After incubating for 1 h the cells were washed with medium to remove unbound dye conjugate. Cells were dissolved with 1% SDS and the cell bound fluorescence was measured using a fluorimeter. The K_d represents the specific binding of FL-L1-S0456 for FAP. C. MDA-MB231

tumor- bearing mice were injected with FL-L1-S0456 (green), the tumors were excised, sectioned and stained for cancer epithelial cell marker (EpCAM, red) and nuclei (DAPI, blue).

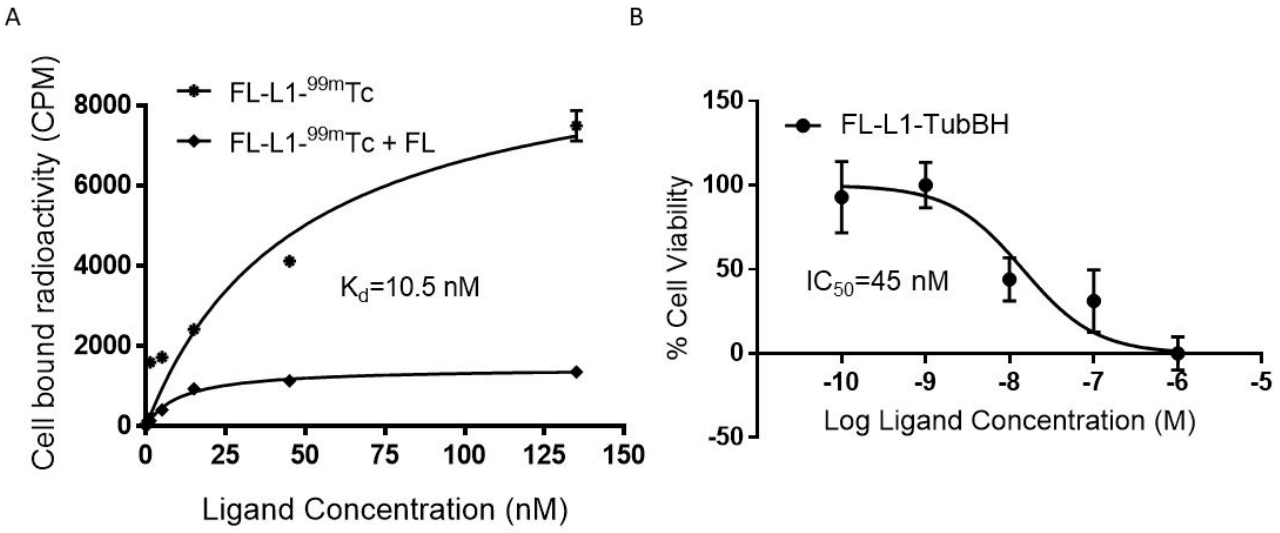


Figure S13. In vitro binding affinity and cytotoxicity plots. A. In vitro binding of FL-L1-^{99m}Tc. hFAP transfected HEK293 cells were incubated with various concentrations of FL-L3-^{99m}Tc either in the presence or absence of 100-fold excess of FL. The cell bound radioactivity was determined by gamma counting. K_d value was determined by using GraphPad Prism 7. B. In vitro cytotoxicity of FL-L1-TubBH. HLF1 cells transfected with hFAP were incubated with various concentrations of FL-L1-TubBH and the cell viability was determined by using "*Cell Titer-Glo® Luminescent Cell Viability Assay kit".* The cell luminescence was determined and plotted in GraphPad Prism 7 to determine the IC_{50}.

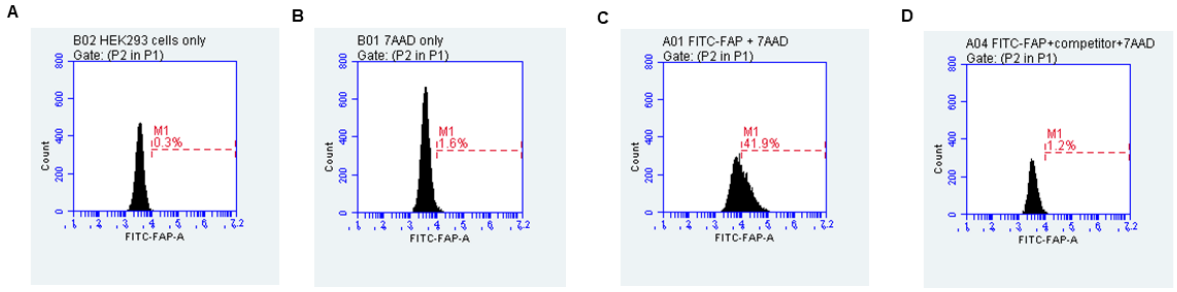


Figure S14. Representative flow cytometry histograms to determine FAP expression on HEK293-hFAP cells and FAP mediated specific binding of FL-L1-FITC. Histograms represent A) unstained control cells B) viable cells determined by 7AAD staining C) binding of cells to FL-L1-FITC dye conjugate (FITC-FAP+7AAD) D) binding of cells to FL-L1-FITC in the presence of 100-fold excess of FL to determine specificity (FITC-FAP+competition+7AAD).