Supplementary Information

Activatable near-infrared fluorescence probe for real-time imaging of PD-L1 expression in tumors

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Analysis of the dye conjugation site

Peptide digestion

Atezolizumab and Q-Atezol were precipitated using aceton and solubilized in SDS solubilization buffer (5% SDS, 50mM TEAB pH 8.5). Proteins were digested using S-Trap™ spin columns (Protifi, Huntington, NY) with manufacturer's instructions. The samples were reduced by 1,4-dithiothreitol (DTT) and alkylated by iodoacetamide (IAA). After quenching the alkylation reaction, additional SDS and phosphoric acid were added so that the final concentration was 5% SDS and 1.2% phosphoric acid. Acidified samples were mixed with 90% methanol in 100mM TEAB, loaded into the S-Trap micro columns, incubated with mass spec grade trypsin/LysC (Promega) for 3 h at 47°C. Eluted peptides were evaporated using vacuum concentrator and cleaned up using C18 spin columns (Thermo Fisher Scientific, Rockford, IL).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Peptides were resuspended with 0.1% formic acid in water, separated using an Ultimate 3000 RSLCnano system (Thermo Scientific) and analyzed by an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific). Solvents A and B were 0.1% FA in water and 0.1% FA in acetonitrile, respectively. The peptides were loaded onto trap column (Acclaum PepMap™ 100, 75mm x 2cm), separated by the analytical column (Aurora Ultimate TS 25x75 C18 UHPLC column, IonOpticks) with a linear gradient from 3 to 35% solvent B for 90min at a flow rate 0.3 mL/min. The Orbitrap Eclipse Tribrid mass analyzer was operated in a data-dependent method for 3s cycle time. Full MS scans were acquired over the range m/z 375-1500 with mass resolution of 120,000 (at m/z 200). The AGC target value was standard. The most intense peaks with charge state ≥2 was fragmented in the higher-energy collisional dissociation (HCD) collision cell with normalized collision energy of 30 and tandem mass spectra were acquired in the Orbitrap mass analyzer with a mass resolution of 30,000 at m/z 200.

Protein identification and modification

Database searching of all raw data files was performed in Proteome Discoverer 3.1 software (Thermo Fisher Scientific). SEQUEST-HT were used for database searching against

Atezolizumab fasta sequence. Database searching against the corresponding reversed database was also performed to evaluate the false discovery rate (FDR) of peptide identification. The database searching parameters included precursor ion mass tolerance 10 ppm, fragment ion mass tolerance 0.02 Da, static modifications for carbamidomethyl cysteine (+57.021 Da / C) and variable modifications for methionine oxidation (+15.995 Da / M), ATTO655 tags (+510.20 Da / K) and Alexa647 tags (+827.16 Da / K). We obtained FDR of less than 1% on the peptide level and filtered with the high peptide confidence.

Table S1. Analysis of the fluorescence dye-conjugated site in Q-Atezol. The most overlapping fragments were obtained from Q-Atezol digested by trypsin/LysC and analyzed by Q Exactive hybrid quadrupole-orbitrap mass spectrometry. A total of 4.3% and 51.6% of matched sequences were identified in a modified K289 (heavy chain) and K636 (light chain), respectively. Matched mass of b ion and y ion were indicated with red and blue color, respectively.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	148.07569	74.54148	F			18
2	262.11862	131.56295	N	2522.22853	1261.61790	17
3	448.19793	224.60260	W	2408.18560	1204.59644	16
4	611.26126	306.13427	Y	2222.10628	1111.55678	15
5	710.32967	355.66847	V	2059.04296	1030.02512	14
6	825.35662	413.18195	D	1959.97454	980.49091	13
7	882.37808	441.69268	G	1844.94760	922.97744	12
8	981.44649	491.22689	V	1787.92614	894.46671	11
9	1110.48909	555.74818	E	1688.85772	844.93250	10
10	1209.55750	605.28239	V	1559.81513	780.41120	9
11	1346.61641	673.81184	H	1460.74672	730.87700	8
12	1460.65934	730.83331	N	1323.68780	662.34754	7
13	1531.69645	766.35187	A	1209.64488	605.32608	6
14	2168.98986	1084.99857	K289-ATTO655	1138.60776	569.80752	5
15	2270.03754	1135.52241	T	501.31436	251.16082	4
16	2398.13250	1199.56989	K	400.26668	200.63698	3
17	2495.18526	1248.09627	P	272.17172	136.58950	2
18			R	175.11895	88.06311	1

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	72.04439	36.52583	A			7
2	187.07133	94.03930	D	1328.59799	664.80263	6

3	350.13466	175.57097	Y	1213.57104	607.28916	5
4	479.17725	240.09227	E	1050.50771	525.75750	4
5	1116.47066	558.73897	K636-ATTO655	921.46512	461.23620	3
6	1253.52957	627.26842	H	284.17172	142.58950	2
7			K	147.11280	74.06004	1

Table S2. Analysis of the same part as Q-Atezol in Atezolizumab. Atezolizumab was digested with trypsin/LysC and analyzed by Q Exactive hybrid quadrupole-orbitrap mass spectrometry. Mass modified with ATTO655 was not indicated for both K289 and K636 from Atezolizumab.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	148.07569	74.54148	F			18
2	262.11862	131.56295	N	2013.03008	1007.01868	17
3	448.19793	224.60260	W	1898.98716	949.99722	16
4	611.26126	306.13427	Y	1712.90784	856.95756	15
5	710.32967	355.66847	V	1549.84451	775.42590	14
6	825.35662	413.18195	D	1450.77610	725.89169	13
7	882.37808	441.69268	G	1335.74916	668.37822	12
8	981.44649	491.22689	V	1278.72769	639.86749	11
9	1110.48909	555.74818	E	1179.65928	590.33328	10
10	1209.55750	605.28239	V	1050.61669	525.81198	9
11	1346.61641	673.81184	H	951.54827	476.27777	8
12	1460.65934	730.83331	N	814.48936	407.74832	7
13	1531.69645	766.35187	A	700.44643	350.72686	6
14	1659.79142	830.39935	K289	629.40932	315.20830	5
15	1760.83909	880.92319	T	501.31436	251.16082	4
16	1888.93406	944.97067	K	400.26668	200.63698	3
17	1985.98682	993.49705	P	272.17172	136.58950	2
18			R	175.11895	88.06311	1

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	72.04439	36.52583	A			7
2	187.07133	94.03930	D	819.39954	410.20341	6
3	350.13466	175.57097	Y	704.37260	352.68994	5
4	479.17725	240.09227	E	541.30927	271.15827	4
5	607.27222	304.13975	K636	412.26668	206.63698	3
6	744.33113	372.66920	H	284.17172	142.58950	2
7			K	147.11280	74.06004	1

Figures

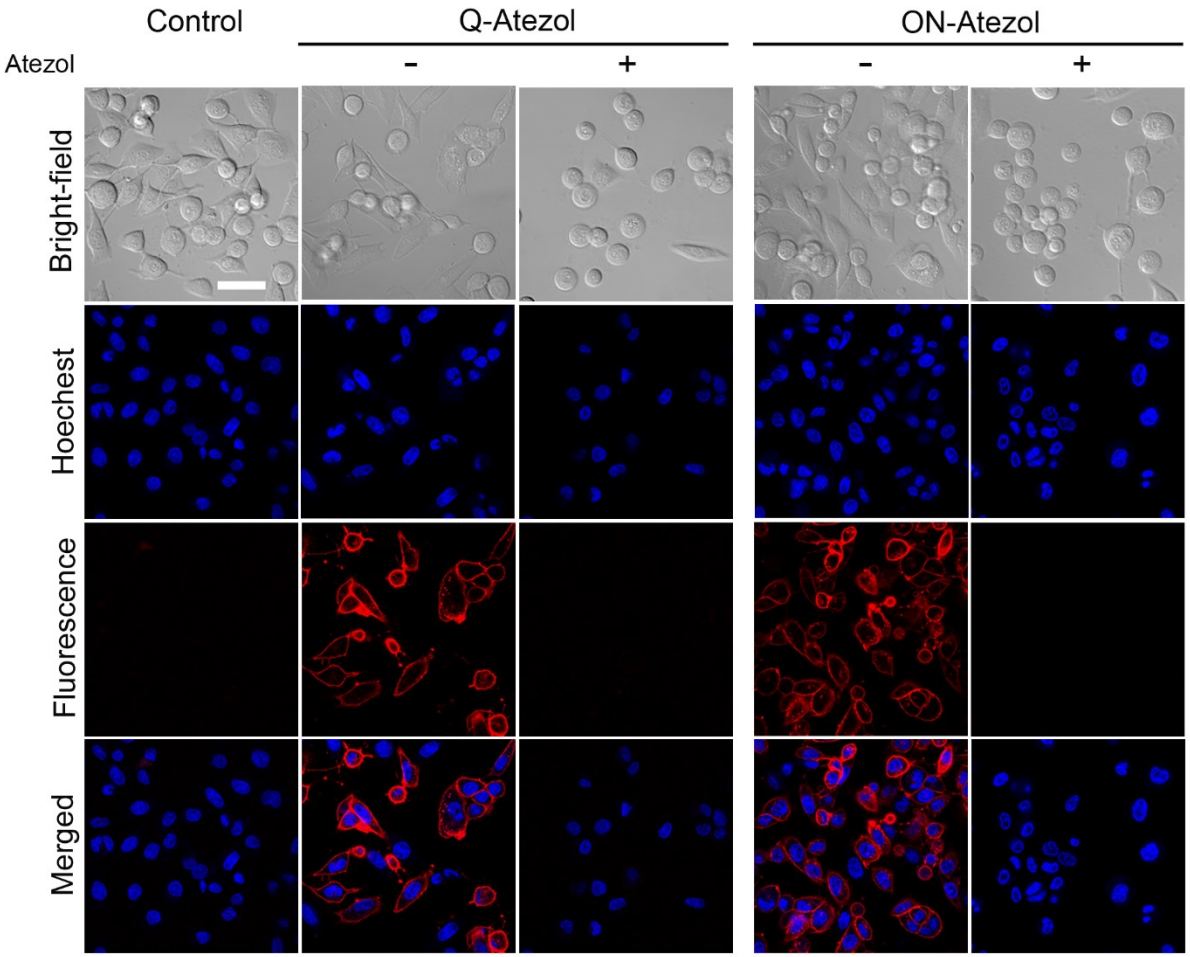

Fig. S1. Confocal images ($\lambda_{ex.}$633 nm, $\lambda_{em.}$ 638–759 nm) of H1975 cells treated with Q- and ON-Atezols (1 μM dye equivalent). For the competitive PD-L1 binding assay, H1975 cells were pre-treated with an excess of unlabelled Atezolizumab (250 μg) for 30 min, and then treated with Q-Atezol and ON-Atezol for 30 min. After washing the cells three times, confocal images of the cells were obtained. Untreated cells were used as controls. Scale bar = 50 μm.

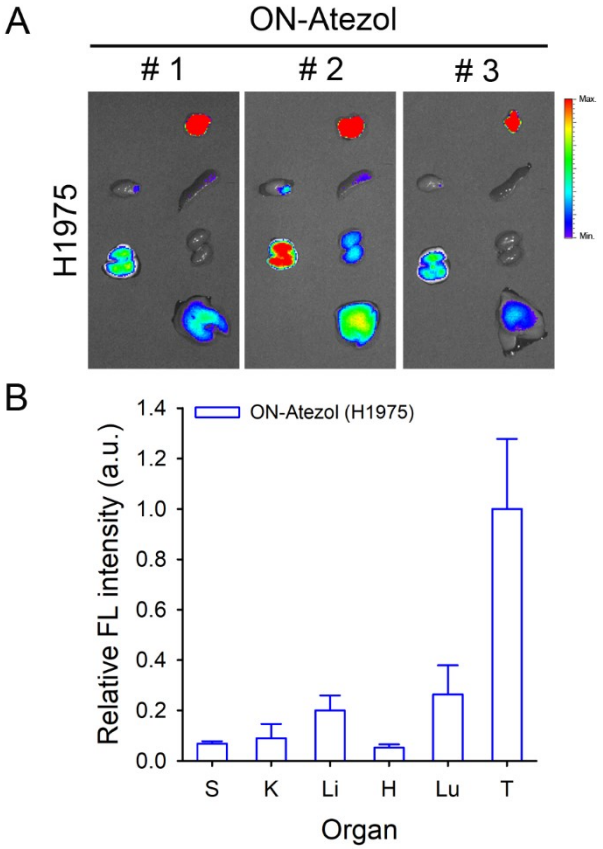

Fig. S2. (A) Representative *ex vivo* NIR fluorescence images of tumors and major organs from ON-Atezol-treated H1975 tumor-bearing mice. ON-Atezol was intravenously injected and *ex vivo* NIR fluorescence images of tumors and organs ($\lambda_{ex.}$ 660/20 nm, $\lambda_{em.}$ 710/40 nm) were obtained 24 h post-injection. (B) Comparison of relative fluorescence (FL) intensities in tumors and organs.

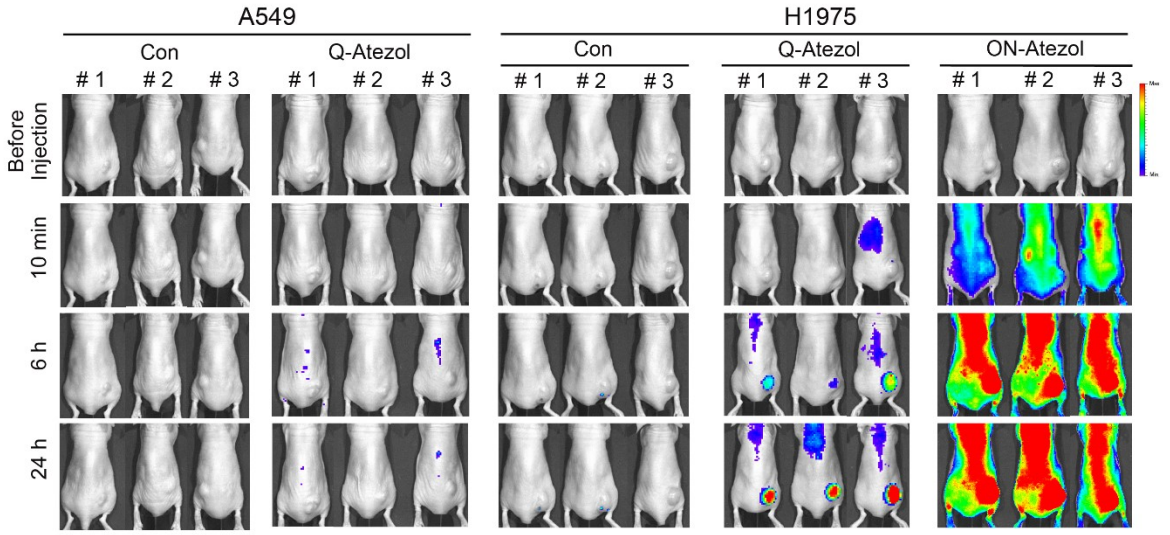

Fig. S3. NIR fluorescence images of A549 and H1975 tumor-bearing mice. Q- and ON-Atezol were intravenously injected into mice, and NIR fluorescence images ($\lambda_{ex.}$ 660/20 nm, $\lambda_{em.}$ 710/40 nm) were obtained at various time points.

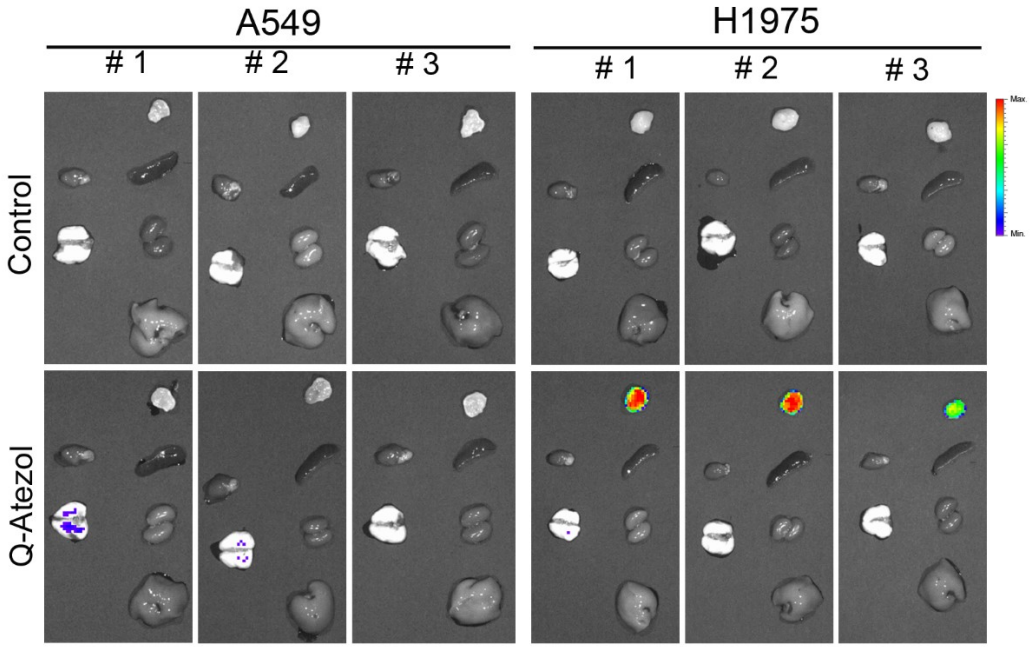

Fig. S4. E*x vivo* NIR fluorescence images of tumors and major organs ($\lambda_{ex.}$ 660/20 nm, $\lambda_{em.}$ 710/40 nm) 24 h post-injection. (H, heart; Lu, lung; T, tumor; S, spleen; K, kidney; Li, liver).

Movies

Movie S1. Confocal fluorescence images of H1975 tumor spheroids without probe treatment.

Movie S2. Confocal fluorescence images of A549 tumor spheroids without probe treatment.

Movie S3. Confocal fluorescence images of H1975 tumor spheroids treated with ON-Atezol (red).

Movie S4. Confocal fluorescence images of A549 tumor spheroids treated with ON-Atezol (red).

Movie S5. Confocal fluorescence images of H1975 tumor spheroids treated with Q-Atezol (red).

Movie S6. Confocal fluorescence images of A549 tumor spheroids treated with Q-Atezol (red).