A novel tumor theranostic strategy based on metabolic glycoengineering and disulfidptosis

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Materials and Methods

Ethanol, methanol, dichloromethane, ethyl acetate, petroleum ether, pyridine, acetonitrile, *N,N*-dimethylformamide, triethylamine, D-mannosamine hydrochloride, cystamine dihydrochloride, trifluoroacetate, acetic anhydride, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), DAPI, bovine serum albumin (BSA), glucose-6-phosphate dehydrogenase enzyme and DTT were obtained from Sigma-Aldrich (USA). Azidoethyl-SS-propionic acid (CAS No: 2228857-32-5), Cy5-DBCO, DBCO-biotin, DBCO-maleamide and Alexa Fluor 488-labeled streptavidin were bought from Ruixi Co. Ltd. (China).

Chemical synthesis of $Ac_4ManNAz$, $Ac_4ManNSSN_3$ and $AcONSSN_3$

$Ac_4ManNAz$: A mixture of D-mannosamine hydrochloride (215.63 mg, 1.00 mmol), EDC (383.40 mg, 2.00 mmol), NHS (172.63 mg, 1.50 mmol) and triethylamine (0.5 mL) was stirred in methanol (10 mL). Azido acetic acid (89.83 μL, 1.20 mmol) was added and the mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (methanol: dichloromethane = 5:1) to yield ManNAz (120.62 mg, 0.46 mmol, 46%) as a white solid. ManNAz (120.62 mg, 0.46 mmol) was added to a flask containing pyridine (5 mL), acetic anhydride (469 μL, 5.00 mmol) and DMAP (6.11 mg, 0.05 mmol). The mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (ethyl acetate: petroleum ether = 1:1) to produce $Ac_4ManNAz$ (189.36 mg, 0.44 mmol, 96%) as a white solid.

$Ac_4ManNSSN_3$: A mixture of D-mannosamine hydrochloride (215.63 mg, 1.00 mmol), EDC (383.40 mg, 2.00 mmol), NHS (172.63 mg, 1.50 mmol) and triethylamine (0.5 mL) was stirred in methanol (10 mL). Azidoethyl-SS-propionic acid (191.35 μL, 1.20 mmol) was added and the mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (methanol: dichloromethane = 5:1) to yield $ManNSSN_3$ (154.73 mg, 0.42 mmol, 42%) as a white solid. $ManNSSN_3$ (154.73 mg, 0.42 mmol) was added to a flask containing pyridine (5 mL), acetic anhydride (469 μL, 5.00 mmol) and DMAP (6.11 mg, 0.05 mmol). The mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (ethyl acetate: petroleum ether = 1:1) to produce $Ac_4ManNSSN_3$ (211.61 mg, 0.39 mmol, 94%) as a white solid.

$AcONSSN_3$: A mixture of 2-aminoethanol (58.12 μL, 1.00 mmol), EDC (383.40 mg, 2.00 mmol), NHS (172.63 mg, 1.50 mmol) and triethylamine (0.5 mL) was stirred in methanol (10 mL). Azidoethyl-SS-propionic acid (191.35 μL, 1.20 mmol) was added and the mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (methanol: dichloromethane = 19:1) to yield $HONSSN_3$ (165.22 mg, 0.66 mmol, 66%) as a slightly yellow solid. $HONSSN_3$ (165.22 mg, 0.66 mmol) was added to a flask containing pyridine (5 mL), acetic anhydride (193 μL, 2.00 mmol) and DMAP (1.21 mg, 0.01 mmol). The mixture was stirred overnight, concentrated by rotary evaporation, and purified by column chromatography (ethyl acetate: petroleum ether = 3:1) to produce $AcONSSN_3$ (189.82 mg, 0.65 mmol, 98%) as a slightly yellow solid.

Cytotoxicity experiments

Cell culture and solution preparation: A549, HeLa, MCF-7 and HUVEC cells were obtained from the Cell Bank of the Chinese Academy of Sciences. A549, HeLa, MCF-7 and HUVEC cells were

cultured by the Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) penicillin/streptomycin while extra VEGF was added to HUVEC culture medium as additive. 100 mM DMSO solution was prepared by mixing 0.1 mmol $Ac_4ManNAz$, $Ac_4ManNSSN_3$ or $AcONSSN_3$ to 1 mL DMSO.

Cell growth inhibitory: The cells were seeded at a density of 40,000 cells per mL in 1 mL culture medium on 18 mm culture dish with addition of pure DMSO, $Ac_4ManNAz$ solution, $Ac_4ManNSSN_3$ solution or $AcONSSN_3$ solution at certain concentration. After appropriate time, number of cells was counted and cell growth inhibitory rate was calculated as the number of cells with $Ac_4ManNAz$, $Ac_4ManNSSN_3$ or $AcONSSN_3$ divided by the number of cells with pure DMSO. Data were collected from 3 parallel tests.

NADP+ and NADPH measurements: The intracellular levels of NADP+/(NADP+ and NADPH) were measured according to reported literature.[21] Cells were washed by extraction buffer (containing nicotinamide, $NaHCO_3$ and Na_2CO_3) for 15 min firstly. The remaining was collected into 1.5 mL microtubes and centrifuged at 3000 rpm. The supernatant in each tube was split into two 150 μL aliquots. For NADPH measurement, one aliquot was incubated at 60 °C for 30 min and then cooled on ice. The other aliquot was for the total NADP measurement. When measuring, 20 μL aliquots from all 150 μL cell-supernatant aliquots (with/without heating) were added into a 96-well plate and mixed with 80 μL NADP-cycling buffer (100 mM Tris–HCl pH 8.0, 0.5 mM thiazolyl blue (MTT), 2 mM phenazine ethosulfate and 5 mM EDTA) containing one unit of glucose-6-phosphate dehydrogenase enzyme. After incubation in the dark, 20 μL of 10 mM glucose-6-phosphate solution was added to each well containing the mixture and the absorbance at 570 nm was measured using a microplate reader. The ratio of NADP+/(NADP+ and NADPH) was calculated subsequently.

Cell surface labeling experiments

Preparation for MGE procedure: 100 mM DMSO solution was prepared by mix 0.1 mmol $Ac_4ManNAz$, $Ac_4ManNSSN_3$ or $AcONSSN_3$ with 1 mL DMSO and stored at -20 °C. The solution was warmed to room temperature and added to cell culture medium with a certain concentration before use. The A549 cells were sub-cultured for at least 3 generations before use.

MGE procedure: The cells were seeded at a density of 10,000 cells per mL in 1 mL of the culture medium on 18 mm culture dish with addition of 0.25 mM $Ac_4ManNAz$ solution, $Ac_4ManNSSN_3$ solution or $AcONSSN_3$ solution. After appropriate time, the A549 cells were washed by PBS for at least 3 times and kept alive for subsequent staining.

Specific fluorescent staining of azido groups: The remaining cells with/without azido groups were washed with the labeling buffer (PBS solution with 1% FBS and 1% BSA). The cells were incubated with DBCO-biotin (30 μM) in the labeling buffer for 1 hour at room temperature, washed 3 times with the labeling buffer (each time at least 10 minutes), and then incubated with Alexa Fluor 488-labeled streptavidin (0.9 μg/ml) for 15 minutes in the dark. The cells were fixed by 4% polyformaldehyde for 30 minutes. All the cells were co-stained by DAPI for 3 minutes and the relative fluorescence intensity for each individual cell was monitored by automatic scanning fluorescence microscopy (Axio Imager Z2, Zeiss, Germany). Data points were collected in triplicate from 3 separate experiments.

DTT reducing disulfide bonds to eliminate fluorescence: The cells with fluorescence were treated with 50 mM DTT in PBS for 30 minutes. The remaining cells were washed by PBS for at least 3 times. The fluorescence pictures before/after DTT treatment were taken by fluorescence microscopy (Axio Imager Z2, Zeiss, Germany).

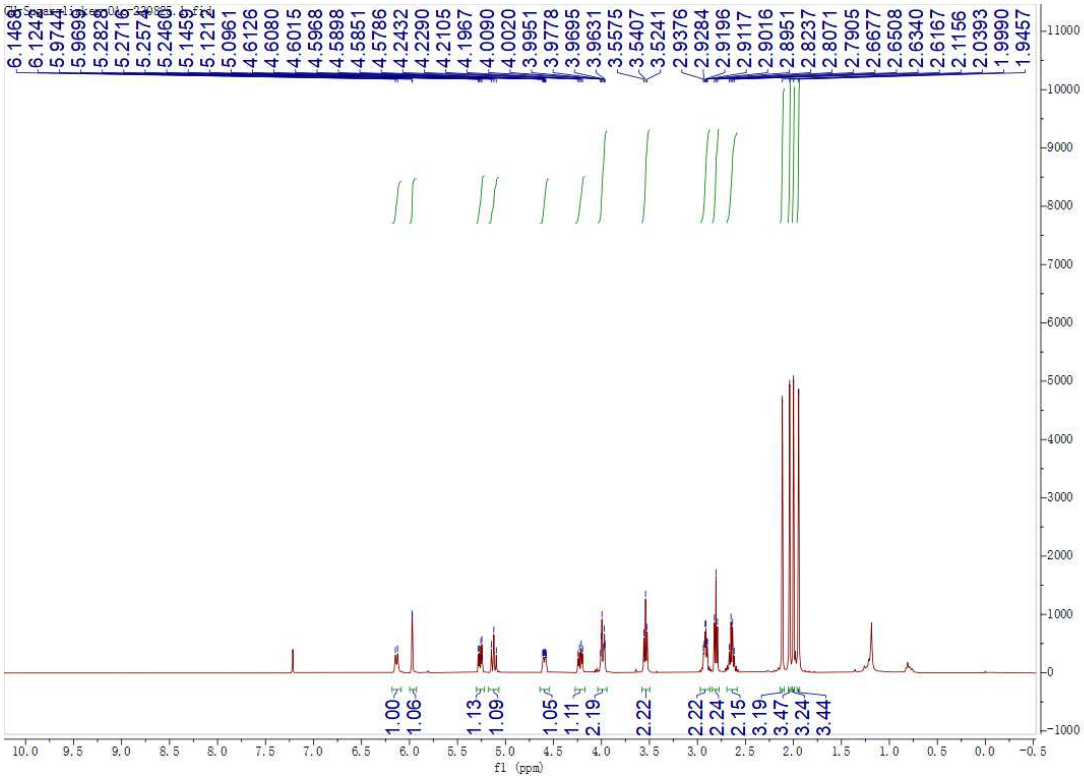
Specific fluorescent staining of thiol groups: The remaining cells were washed by labeling buffer (PBS solution with 1% FBS and 1% BSA). The cells were incubated with DBCO-maleamide (30 μM) in the labeling buffer for 1 hour at room temperature, washed 3 times with the labeling buffer (each time at least 10 minutes), and then incubated with Alexa Fluor 488-labeled streptavidin (0.9 μg/ml) for 15 minutes in the dark. The cells were fixed by 4% polyformaldehyde for 30 minutes. All the cells were co-stained by DAPI for 3 minutes and the relative fluorescence intensity for each individual cell was monitored by automatic scanning fluorescence microscopy (Axio Imager Z2, Zeiss, Germany). Data points were collected in triplicate from 3 separate experiments.

Animal experiments

Inhibitory tumor labeling strategy: For *in vivo* and *ex vivo* experiments, A549 tumors were induced into 5-week-old male athymic nude mice (20 g, Institute of Medical Science, Tokyo, Japan) on both sides of the flank by subcutaneous injection of 1.0×10^7 cells. When the tumor diameters grew to about 10 mm, $Ac_4ManNSSN_3$ (20 μl of 25 mM) were administered into the left tumors once daily for 5 days by intratumoral injection while the same volume of saline was injected into the right tumors. The size of tumor on both sides was recorded. After 24 hours, Cy5-DBCO (20 μl of 25 mM) was intratumoral injected into both sides of A549 tumor-bearing mice. After 4 hours, a 670 nm pulsed laser diode was used to excite Cy5 molecules. NIRF emission at 700 nm was collected and detected. All values were expressed as means±s.e. for groups of five animals.

Statistical analysis:

All experiments were performed at least in triplicate. SPSS software, version 26 (IBM Company, Armonk, NY) was used for statistical analysis. Data were tested for normality using the Kolmogorov–Smirnov test and described by mean ± standard deviation; the Levene test was used as the homogeneity of variance test. When the data conformed to normal distribution with homogeneity of variance, the groups were compared by ANOVA test or unpaired two-tailed Student's t tests; otherwise, the intergroup comparisons were made by Kruskal–Wallis and Tamhane's T2 test. A difference of $*p < 0.05$ was considered to be significant and that of $***p < 0.01$, $***p < 0.001$ or ****p <0.0001 was considered to be highly significant.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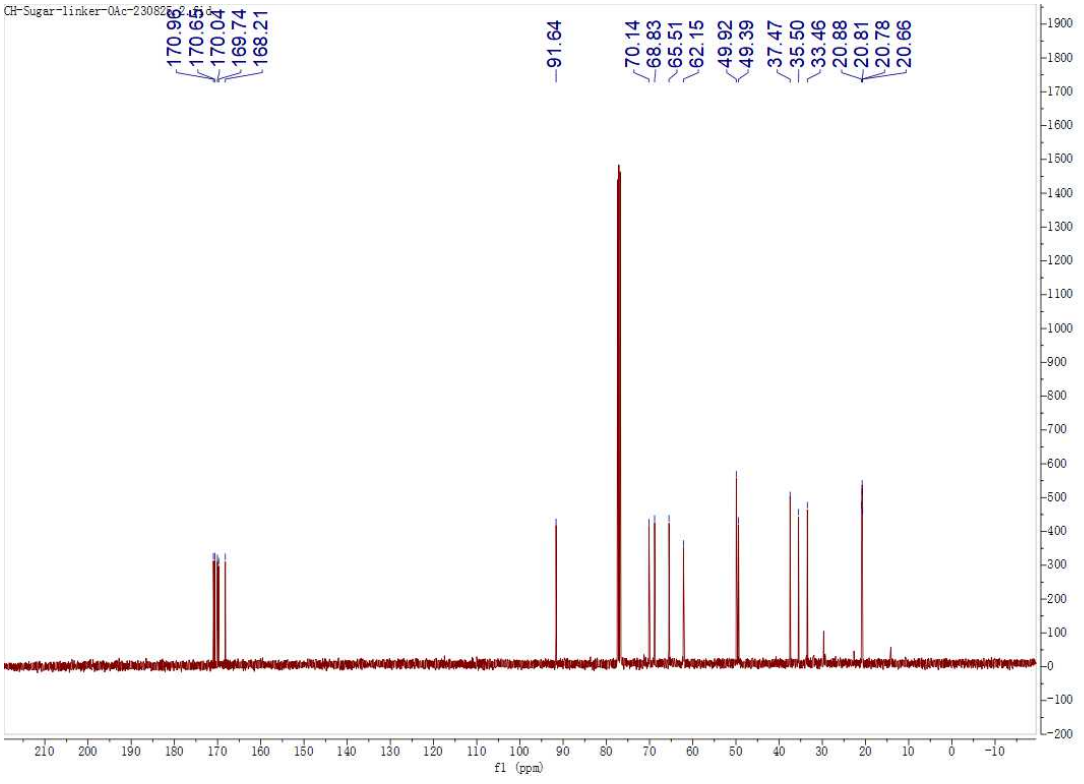
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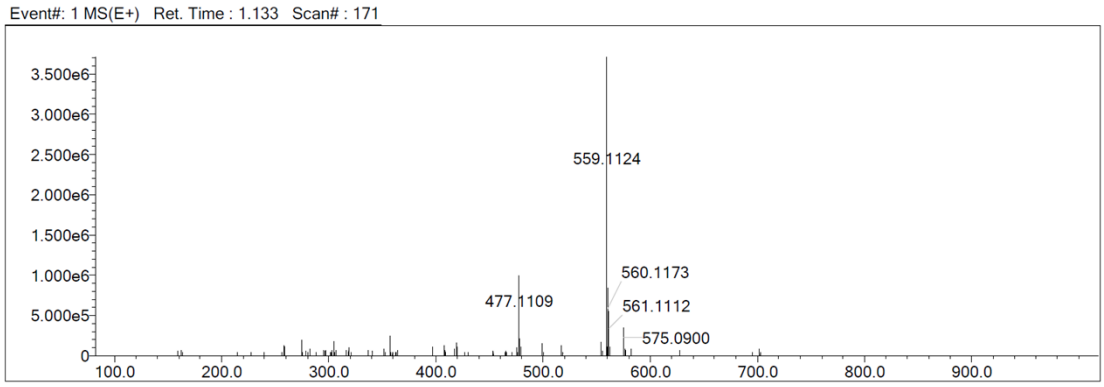
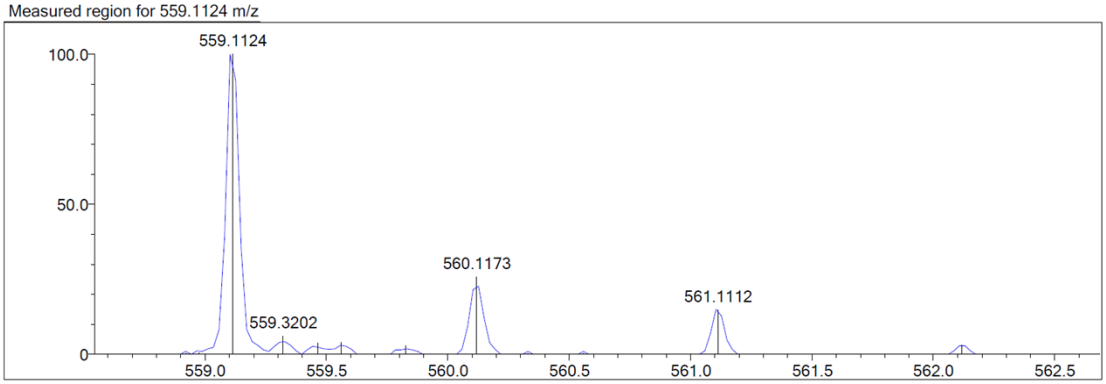
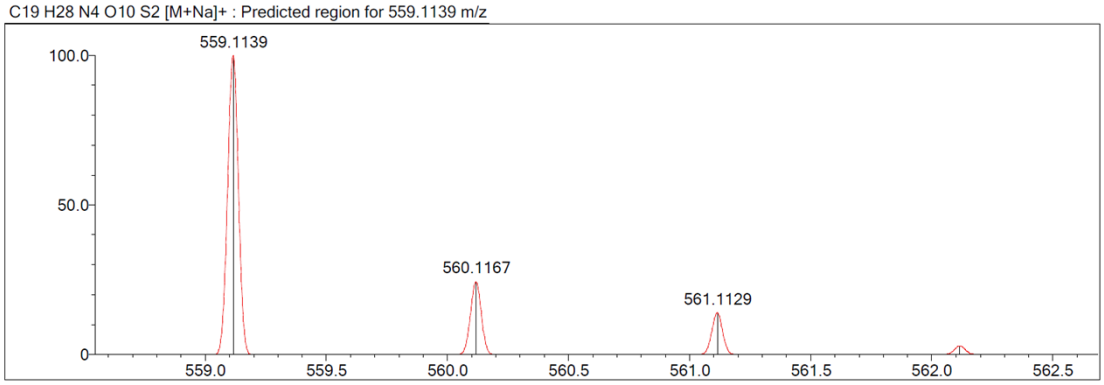
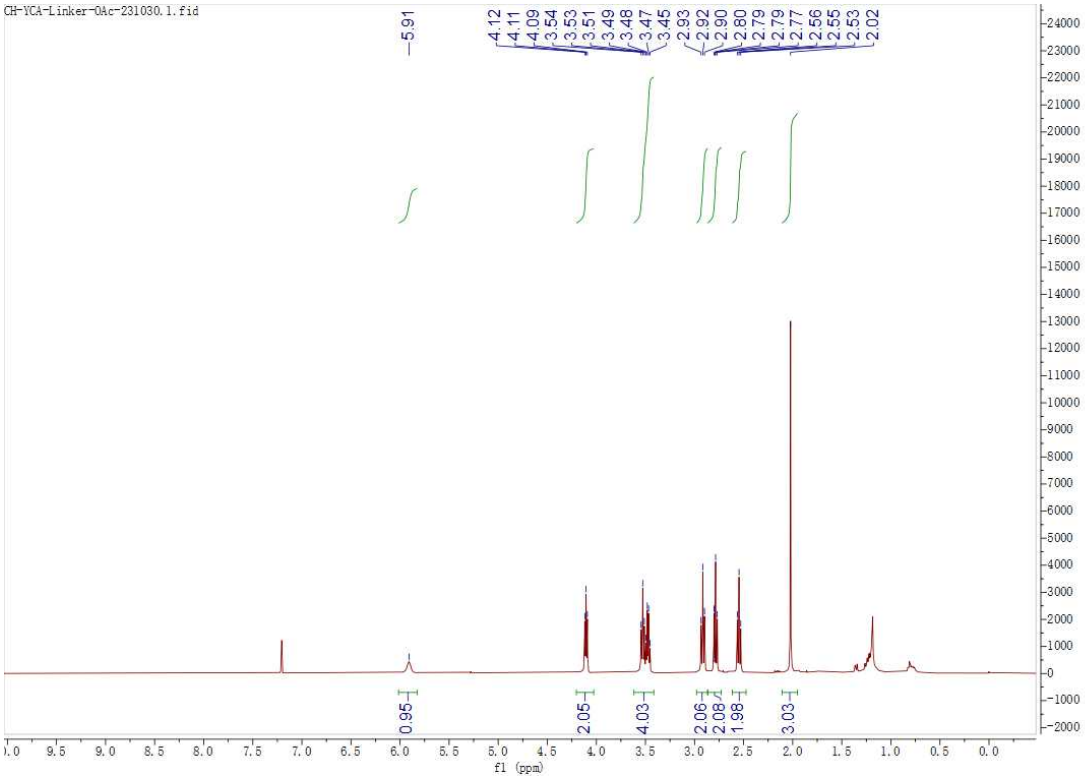






Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Df. (mDa)	Df. (ppm)	Iso	DBE
1	92.87	C19 H28 N4 O10 S2	[M+Na]+	559.1124	559.1139	-1.5	-2.68	96.94	8.0

Figure 1. ^{1}H-NMR, ^{13}C-NMR and HRMS of $Ac_4ManNSSN_3$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.13 (1H, d), 5.97 (1H, s), 5.25 (1H, dd), 5.13 (1H, t), 4.60 (1H, dd), 4.21 (1H, dt), 4.01 (2H, m), 3.54 (2H, t), 2.91 (2H, m), 2.81 (2H, t), 2.63 (2H, m), 2.11 (3H, s), 2.03 (3H, s), 1.99 (3H, s), 1.94 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.96, 170.85, 170.04, 169.74, 168.21, 91.64, 70.14, 68.83, 65.51, 62.15, 49.92, 49.39, 37.47, 35.50, 33.46, 20.88, 20.81, 20.78, 20.66; HRMS, $[M+H]^+$, calculated 559.1139, found 559.1124.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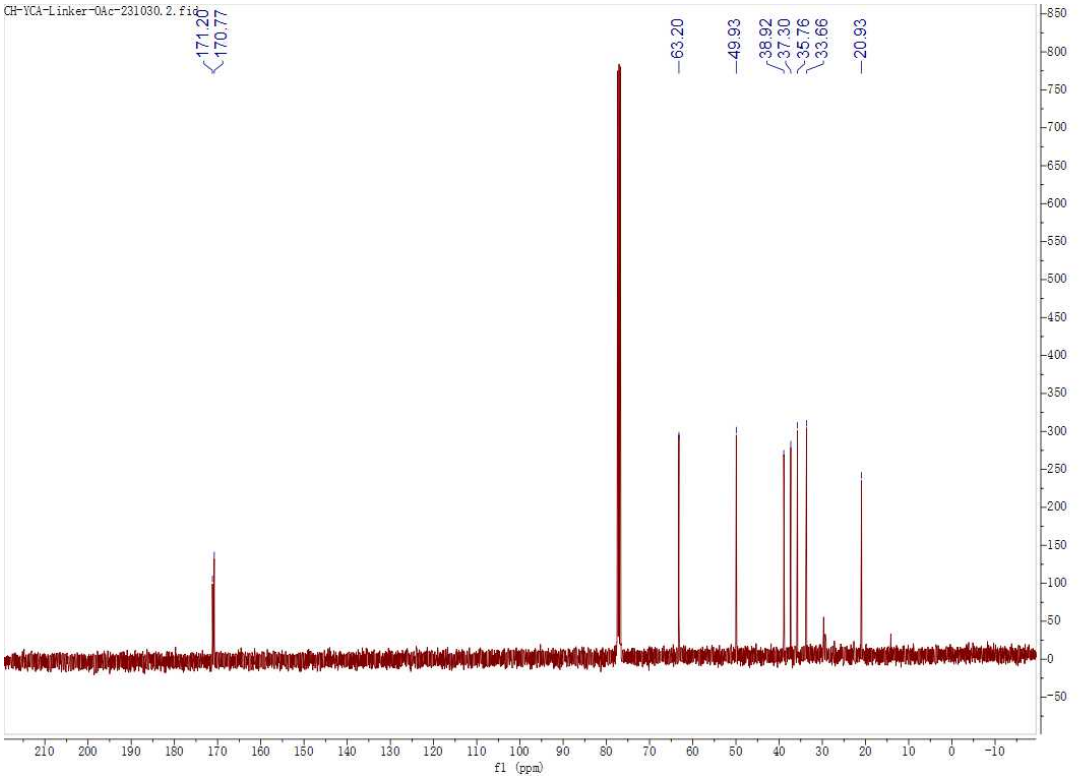
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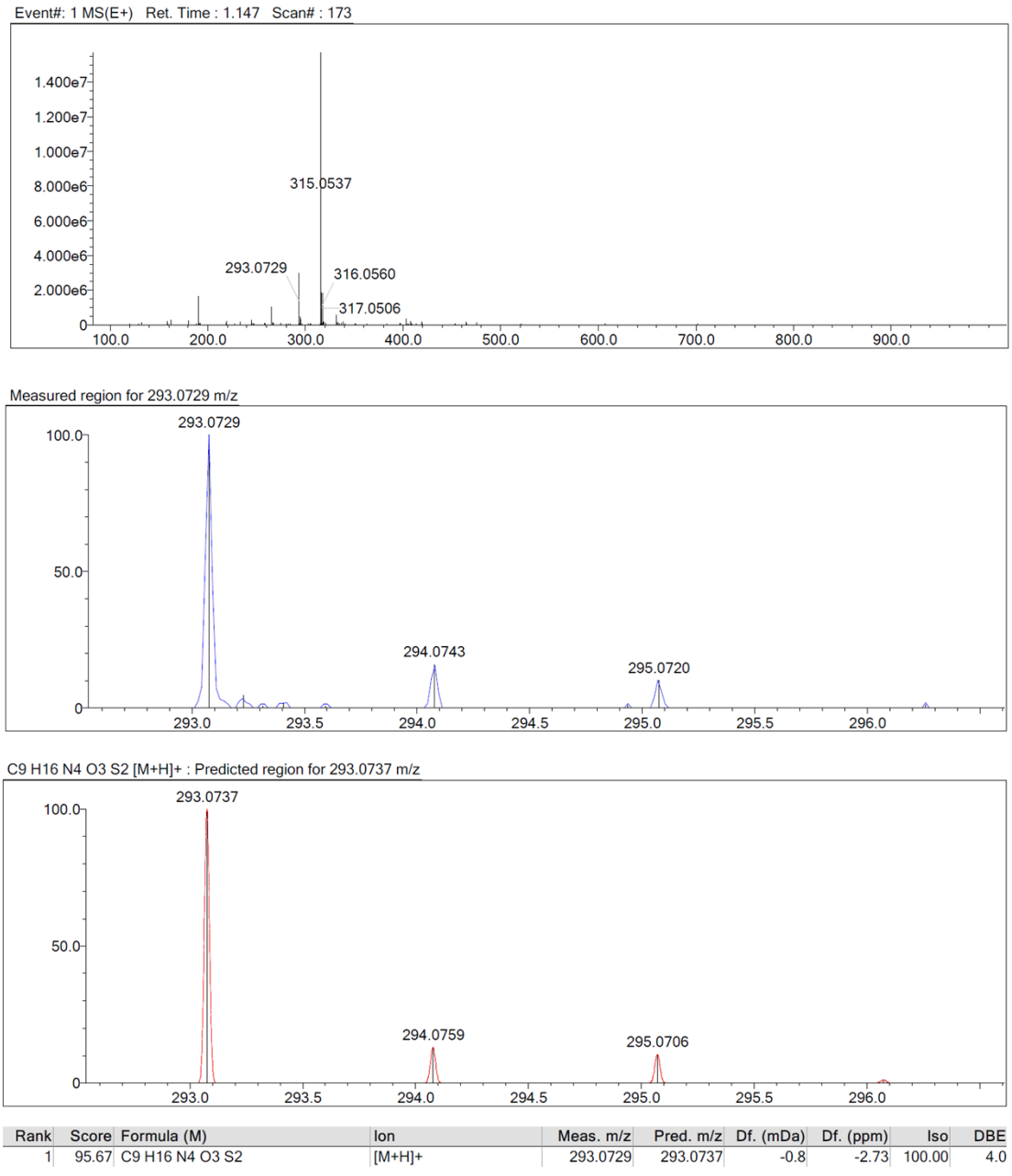

Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Df. (mDa)	Df. (ppm)	Iso	DBE
1	95.67	C9 H16 N4 O3 S2	[M+H]+	293.0729	293.0737	-0.8	-2.73	100.00	4.0

Figure 2. ^{1}H-NMR, ^{13}C-NMR and HRMS of AcONSSN$_3$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.91 (1H, s), 4.12 (2H, t), 3.52 (4H, dt), 2.92 (2H, t), 2.79 (2H, t), 2.54 (2H, t), 2.02 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.20, 170.77, 63.20, 49.93, 38.92, 37.30, 35.76, 33.66, 20.93; HRMS, $[M+H]^+$, calculated 293.0737, found 293.0729.

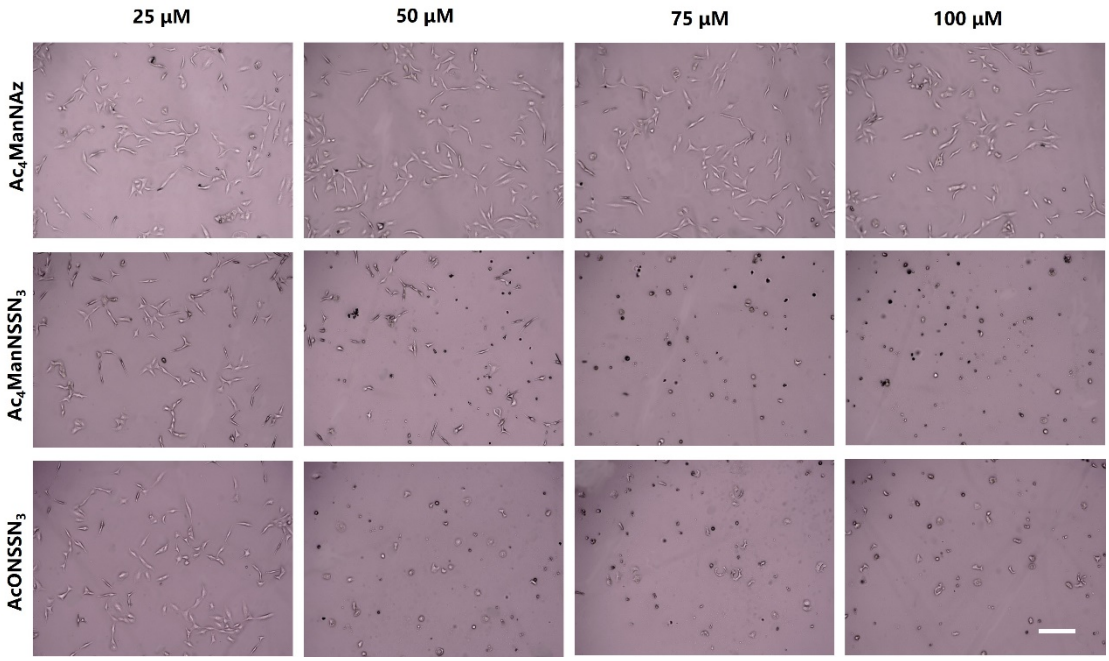

Figure 3. Picture of A549 cultured under different condition after 72 hours, scale bar = 20 μm.

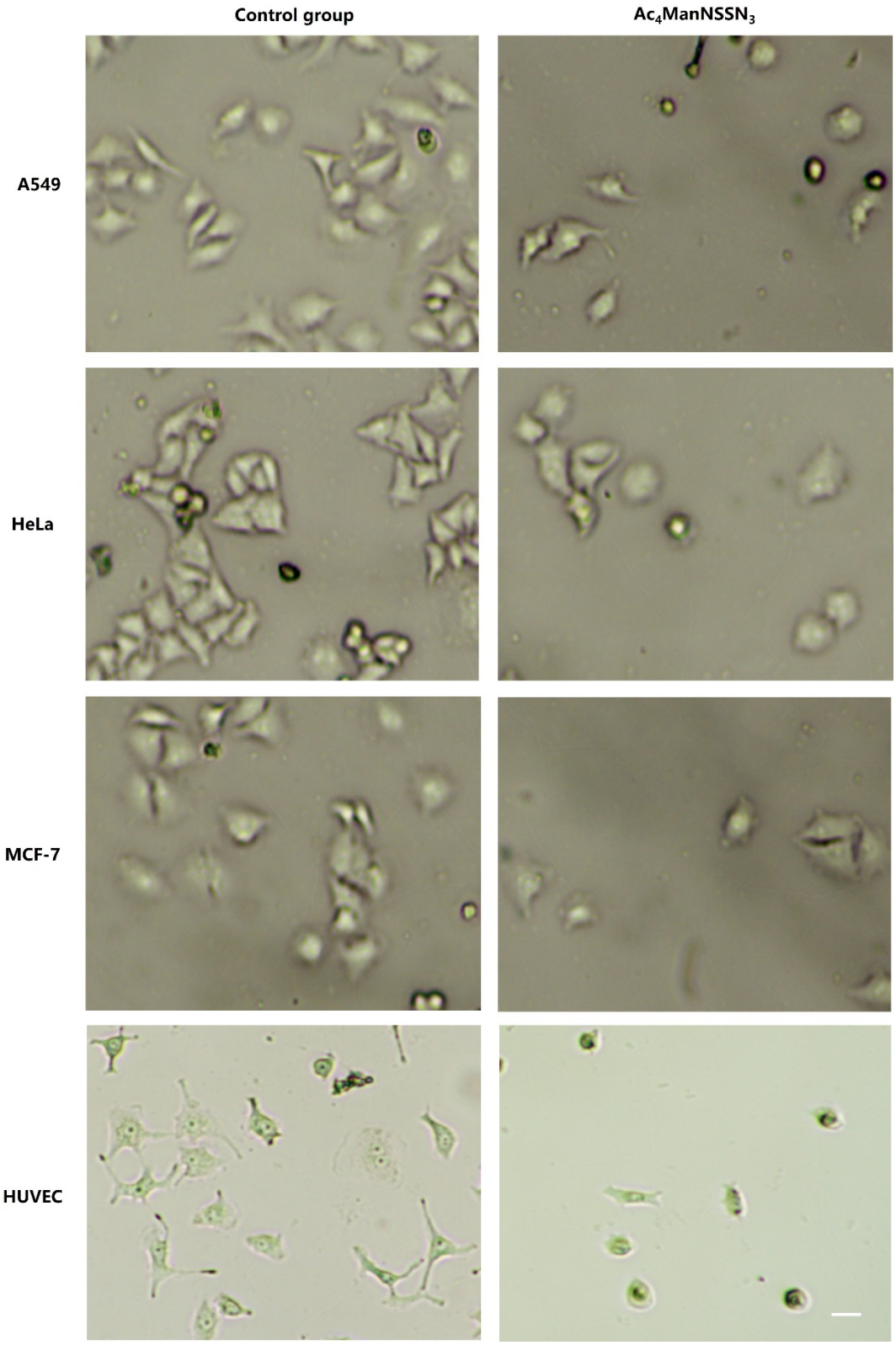

Figure 4. Picture of different cell lines cultured by **Ac_4ManNSSN$_3$** and control group, scale bar = 10 μm.

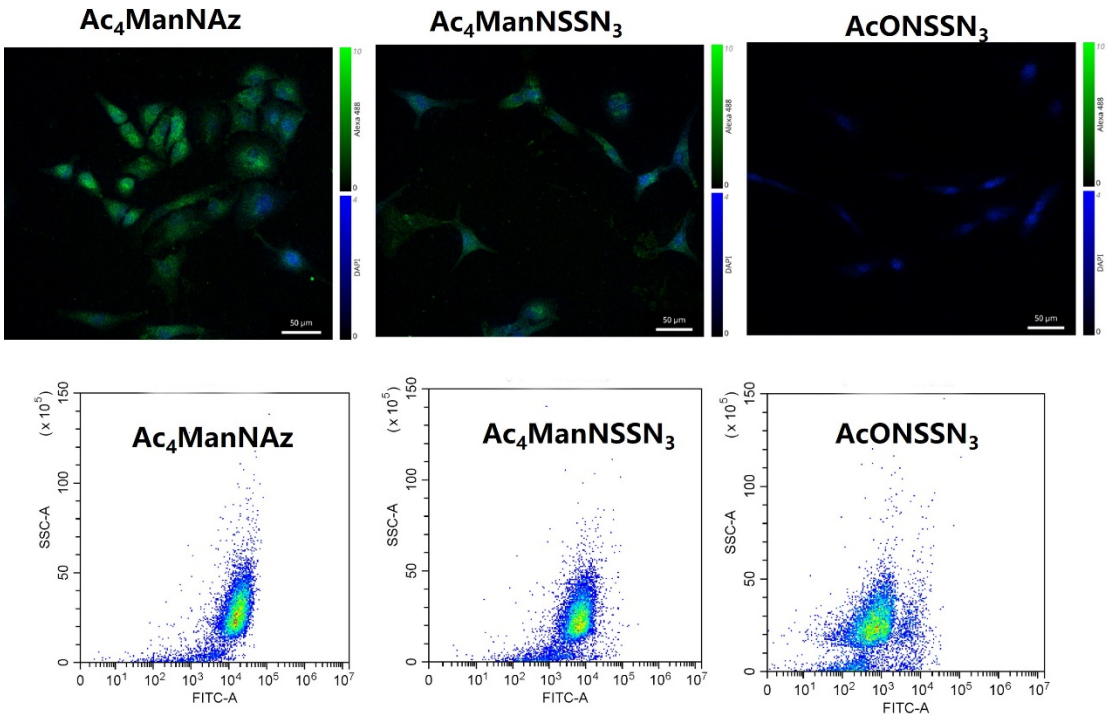

Figure 5. Fluorescence picture and flow cytometry analysis of A549 cultured under different condition after 72 hours.

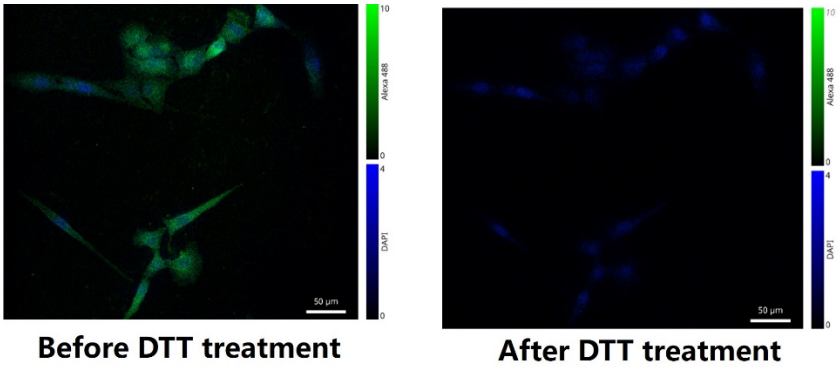

Figure 6. Fluorescence picture of labeled A549 before and after treatment of DTT.

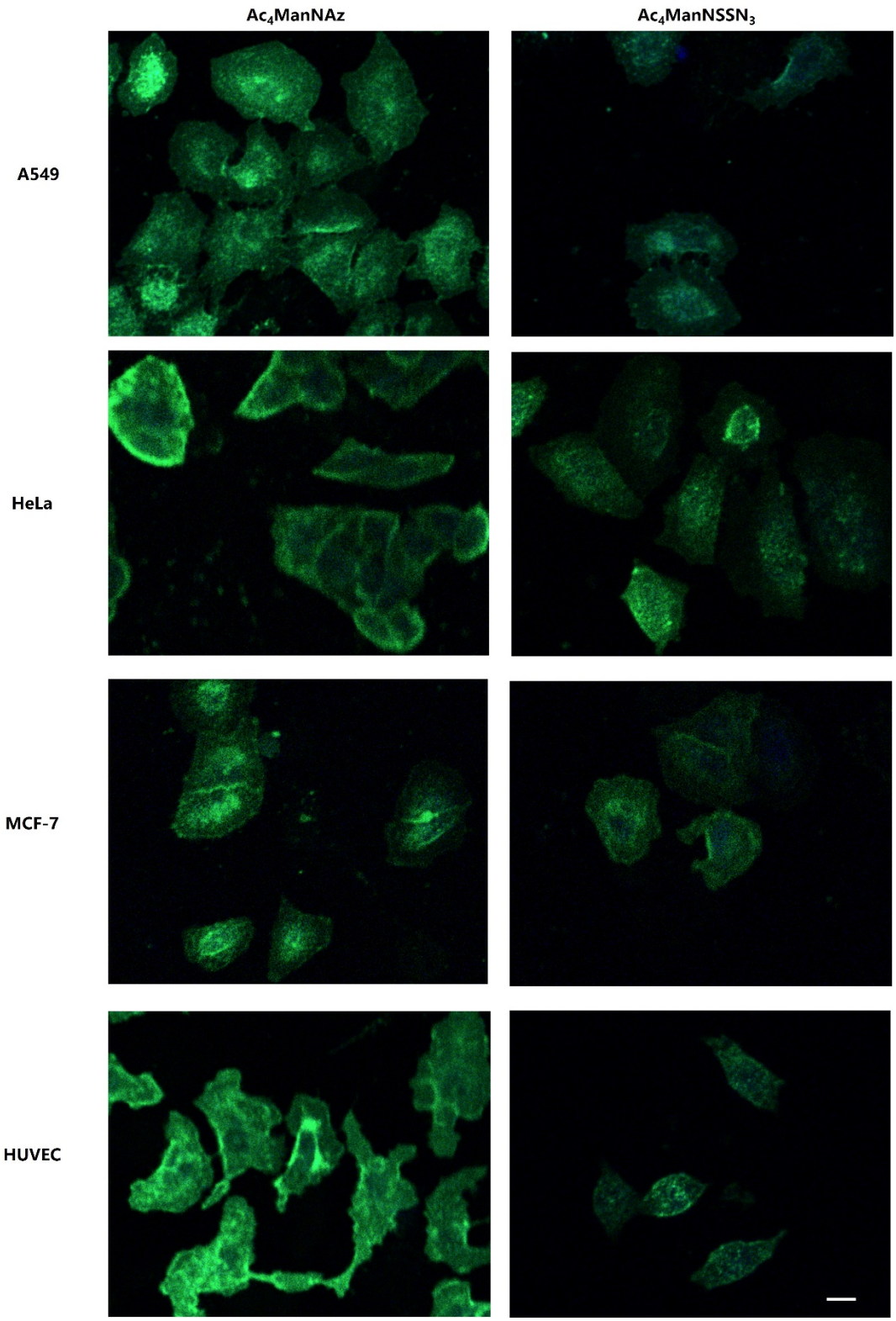

Figure 7. Fluorescence picture of different cell lines, scale bar = 5 μm.

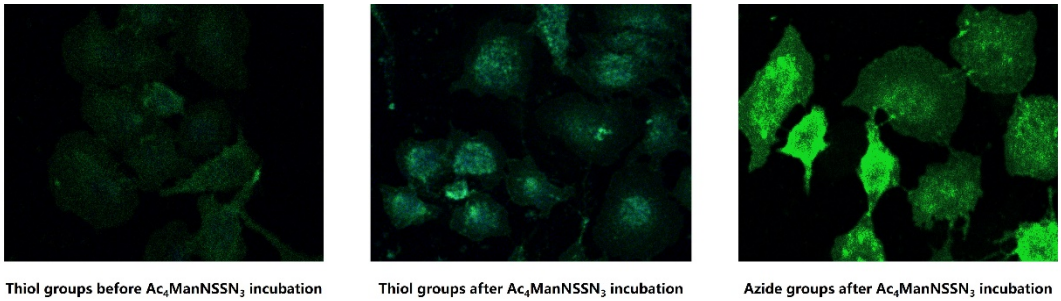

Figure 8. Fluorescence picture of azide and thiol group on A549 cell surface, scale bar = 5 μm.

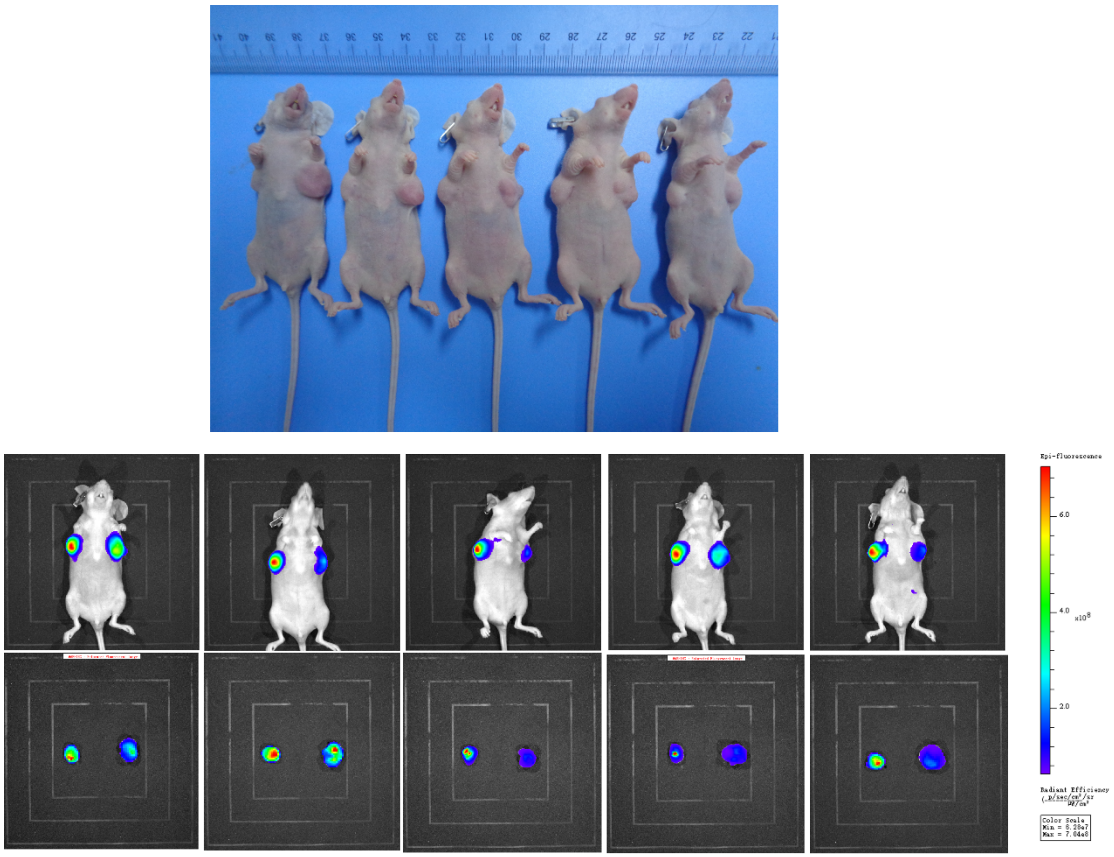

Figure 9. Animal experiment results of inhibitory tumor labeling strategy. The left side tumor is the experimental group, and the right side tumor is the control group.