Two are better than one: a case study of the biferrocenyl-thymidine “click” conjugate biFcT as novel ferroptosis-inducing agent in lung cancer cells

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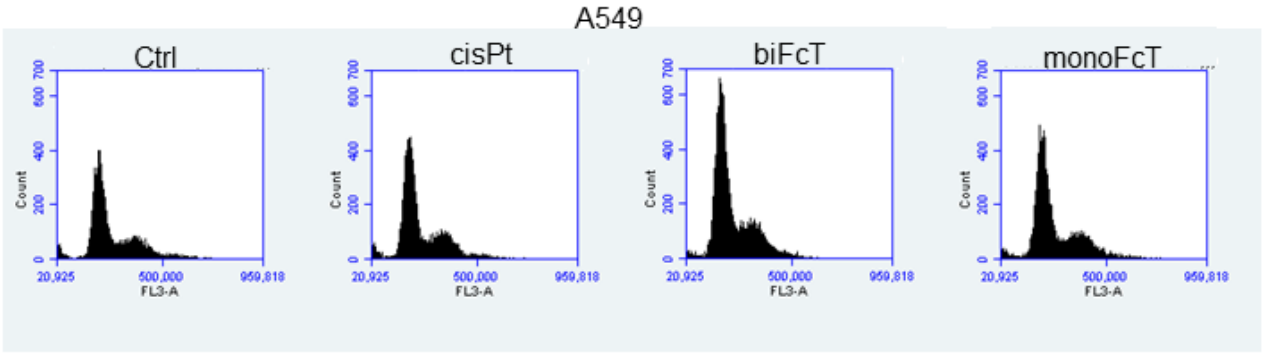
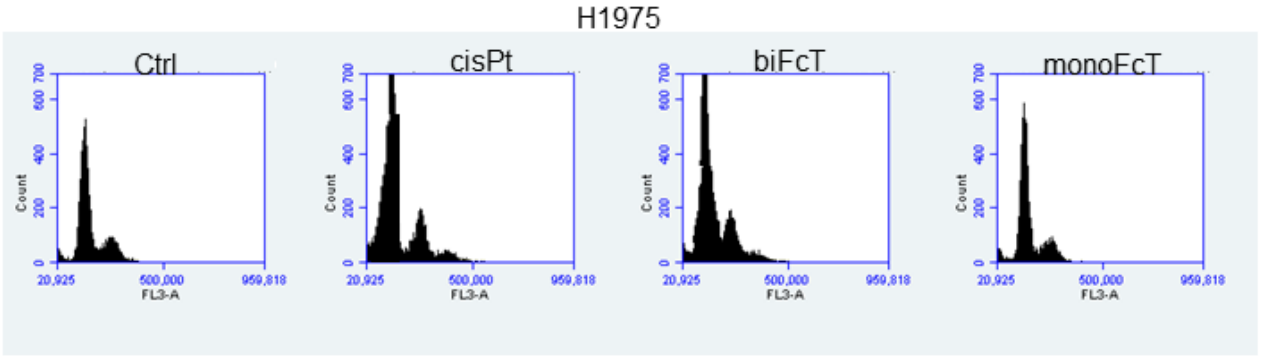
Contents



A549	sub-G1		G0/G1		S		G2/M	
	mean	SD	mean	SD	mean	SD	mean	SD
Ctrl	5,145578	0,727193	66,72003	1,015414	9,332875	0,96481	18,80152	2,401996
Pt	9,379236	6,067956	50,74361	5,061937	21,3792	2,184706	18,49795	2,489881
biFcT	5,23	0,44456	59,83333	7,949493	18,71083	3,333359	16,15	1,818653
monoFcT	3,1	0,57735	53,83333	3,767552	18,9	1,732051	24,85	2,396004



H1975	sub-G1		G0/G1		S		G2/M	
	mean	SD	mean	SD	mean	SD	mean	SD
Ctrl	2,793898	0,564117	64,19453	3,17723	10,98637	2,474033	22,0252	1,511016
Pt	11,21916	1,893795	71,13457	2,374644	8,315864	0,666911	9,330402	0,082359
biFcT	7,548242	1,916108	68,24179	0,788368	9,36324	0,802069	14,84673	1,321448
monoFcT	3,551827	0,327353	60,79606	2,113971	8,695086	0,302471	26,95703	1,589575

Fig. S1 Effect of **biFcT** (20 µM), **monoFcT** (20 µM) and **cisPt** (50 µM) on the cell cycle progression of A549 and H1975 cells after 24 of treatment. Data shows representative histograms obtained from flowcytometry and means ± SD (*n* = 3).

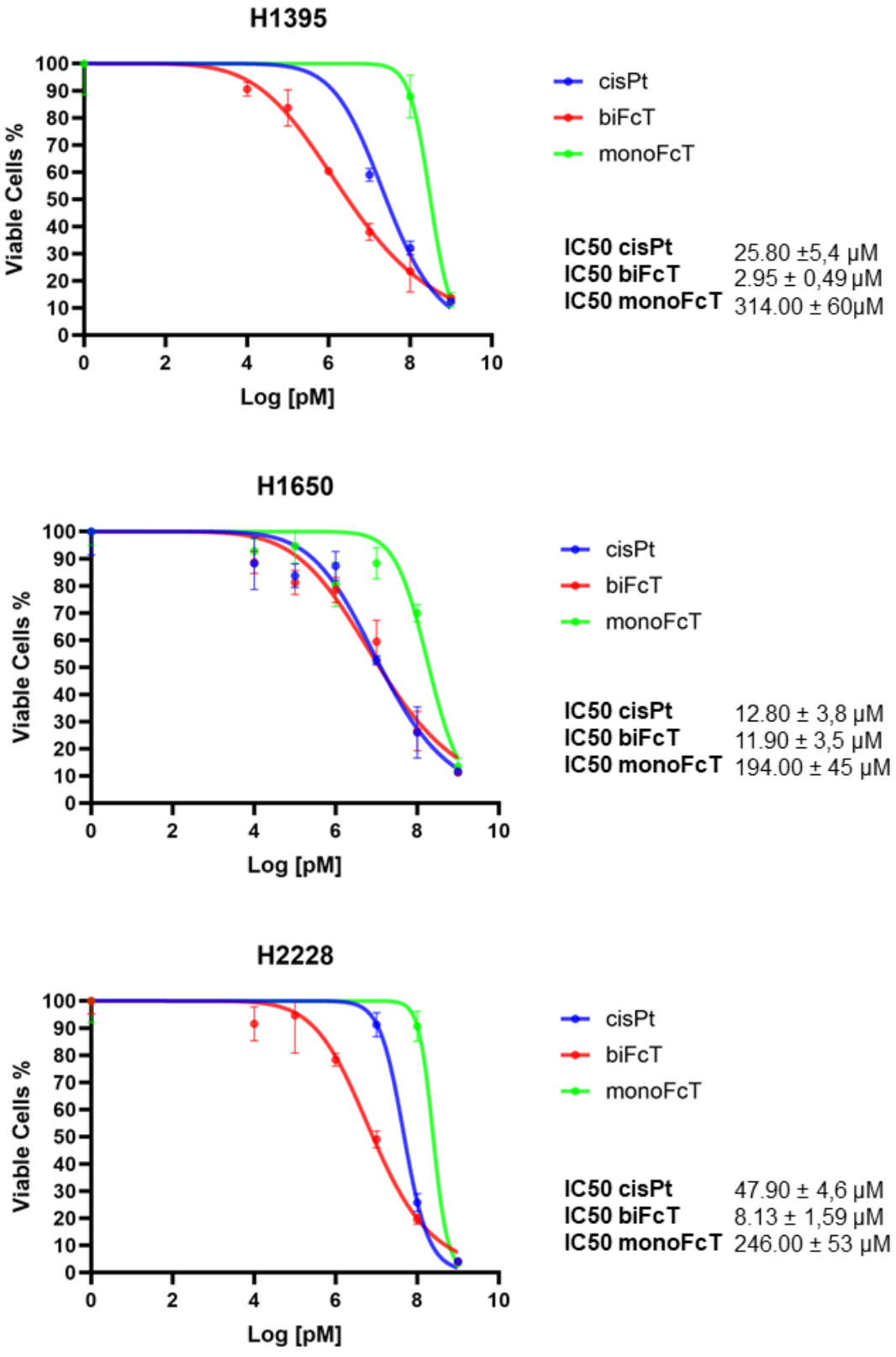


Fig. S2 Human lung cancer cell lines (H1650, H1395, H2228) were incubated 48 h with increasing concentrations (10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1mM) of **biFcT**, **monoFcT** and **cisPt**. Cell viability was measured using a WST-1 assay after 48 h in quadruplicates. IC_{50}

were defined as the concentrations of each drug that reduced viability to 50% compared to untreated cells, producing 50% cell death, respectively.

Protein carbonylation assay. Cells treated with **biFcT** (20 μM), **monoFcT** (20 μM) or **cisPt** (50 μM) for 24 or 48 hours were homogenized in distilled water (H_2O) using mechanical disruption. Samples were centrifuged, and the protein concentration was adjusted to 10 mg/mL using H_2O. A total of 100 μL of the sample containing 0.5 mg of protein was used per assay, with a reagent background control using 100 μL of H_2O. A volume of 100 μL of 2,4-Dinitrophenylhydrazine (DNPH) reagent was added to each sample, vortexed, and incubated for 10 minutes at room temperature. Next, 30 μL of Trichloroacetic Acid (TCA) solution was added, vortexed, and placed on ice for 5 minutes. The samples were centrifuged (2 minutes, maximum speed), and the supernatant was discarded. The pellet was washed with 500 μL of cold acetone, sonicated briefly, and incubated at -20°C for 5 minutes. After centrifugation, acetone was removed, and 200 μL of Guanidine solution was added. Samples were sonicated and incubated at 60°C for 15–30 minutes. After centrifugation, 100 μL of each supernatant was transferred to a 96-well plate. Absorbance was measured at 375 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek) to quantify protein carbonyl content. Results were expressed as ng of carbonyl/mg cell protein using previously prepared standard curve.

Evaluation of ferroptosis induction. To evaluate ferroptosis, cells were seeded in a 96-well plate at a density of 5.000 cells/well and allowed to adhere overnight. They were incubated with **biFcT** (20 μM) alone or together with ferrostatin-1 (50 μM) for 24 or 48 hours. After treatment, the medium was replaced with fresh medium containing a 1:10 dilution of WST-1 reagent, and the plate was incubated for 1 hour at 37°C. Absorbance was measured at 440 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek), with a reference wavelength of 636 nm. The relative absorbance of untreated control cells was set to 100% viability. Cell viability was expressed as a percentage relative to untreated control.

Lipid peroxidation. The lipid peroxidation was measured using MDA Assay Kit (ab118970) that quantifies malondialdehyde (MDA), a key marker of lipid peroxidation. Cells were seeded in 6-well plates and incubated in either fresh medium or medium containing **biFcT** (20 μM), **monoFcT** (20 μM) or **cisPt** (50 μM) for 24 or 48 hours. Following treatment, cell lysates were

prepared according to the manufacturer's instructions. The MDA assay was then performed by mixing the lysates with the thiobarbituric acid (TBA) and heating the samples at 95°C for 60 min, cool in ice bath for 10 min to form the MDA-TBA adduct. Fluorescence ($\lambda_{abs/em}$ = 532/553 nm) was measured to determine MDA levels using Synergy HT Multi-Mode Microplate Reader (BioTek). Results were expressed as nmol of MDA/mg cell protein using previously prepared standard curve.