Supplementary Information

Probing Drug Delivery and Mechanisms of Action in 3D Spheroid Cells by Quantitative Analysis

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EXPERIMENTAL SECTION

Materials

Cisplatin and sodium acetate were purchased from Tokyo Chemical Industry (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Capricorn Scientific. Trypsin (0.25%), phosphate buffered saline (PBS), and antibiotics (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin B, 0.2 μm filtered) were purchased from Hyclone. Dimethyl sulfoxide (DMSO) and HPLC water were purchased from Junsei and J. T. Bakers, respectively. HNO_3 (65%), H_2O_2 (30%), phenol/chloroform/isoamyl alcohol (25:24:1, v/v; PCA solution) solution, and chloroform/isoamyl alcohol (24:1, v/v; CA solution) solution were purchased from Sigma-Aldrich. The former three agents were of ultra-trace metal grade quality suitable for inductively coupled plasma-mass spectrometry (ICP-MS) analysis, and the PCA and CA solutions were of biological grade quality. Ethyl alcohol (99.9%, AR grade) purchased from J.T. Bakers was used in all procedures. Tris-HCl buffer (1 M, pH 7.4) used in the biochemical applications was purchased from Biosesang. Lysis buffer [TE (10 mM Tris, 25 mM EDTA, pH 8) buffer, 1% SDS, 100 mM NaCl solution] was freshly prepared prior to the DNA extraction experiment.

Cell Culture

SK-N-SH, a neuroblastoma cell line, was purchased from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Welgene; LM002-08) containing l-glutamine supplemented with 10% FBS and antibiotics and filtered using a 0.2 μm syringe filter (Hyclone; Chicago, U.S.A). Cells were grown and incubated

at 37 ℃ in a humidified 5% CO_2 atmosphere. Cells were split every two days to maintain their confluence at 80–90%.

Cell viability assay in different solvents

Cells were cultured at a density of 15 000/100 μL in each well of a 96-well plate and were incubated for 24 h at 37 ℃ to allow complete attachment. Drug solutions were added to cell culture medium for final concentrations of 5, 10, 20, and 50 μM for cisplatin in media. For the presence of DMSO condition, the final concentration of DMSO was adjusted to 0.5% in the cell culture media to minimize the cytotoxic effects of DMSO. Cell viability was measured using a CCK-8 (Cell Counting Kit-8, Dojindo) assay according to manufacturer instructions at 24, 48, and 72 h after treatment with drug solutions. Briefly, 10 μL of CCK-8 solution was added to each well and incubated for 3 h at 37 ℃, and the change in absorbance for the CCK-8 reagent was detected at a wavelength of 450 nm. The absorbance of each well was corrected by subtraction of background signal, and was divided by those of control. Three repetitions of this cell viability assay were performed to demonstrate reproducibility.

Fluorescent staining of the 3D spheroid cells for confocal microscopy analysis

Two fluorescent moieties were used to stain the 3D spheroid cells to distinguish living and dead cells: calcein AM (3 mM in DMSO) (calcein AM, cell-permeant dye, Thermo Fisher Scientific) and propidium iodide (PI) solution (FxCycle™ PI/RNase Staining Solution, Thermo Fisher Scientific). The calcein AM was prepared to a 2× staining solution (6 μM) in PBS. Before fluorescent treatment, 60 μL of the media was removed and replaced with 50 μL of the 2× calcein

AM solution for a final well concentration of 3 μM. The plates were subsequently incubated at 37 °C and 5% CO_2 for 30 min. Afterwards, 10 μL of the PI solution was added to each well, and the plate was incubated for additional 30 min under the same conditions. Before confocal analysis, the 3D spheroid cells were carefully moved into a confocal dish (#100350, SPL life science) using a 1000 μL micropipette. When weak stimulation is applied to spheroid, the cells that are weakly attached to spheroids (e.g. dead cells and diseased cells) can be fully detached from the spheroid surface.

Treatment of 2D monolayer cells for quantitation of cellular uptake of Pt and DNA-Pt adduct

Cells (2.0×10^6 cells/7 mL) were seeded in a 100 mm culture dish and incubated for 24 h. Drugs were applied to each culture dish (5, 10, 20, and 50 μM for cisplatin in the presence or absence of 0.5% DMSO). After drug application, cells were harvested, and cell pellets were washed twice with 1X PBS. Cell pellets were dispersed with 1000 μL of PBS, and 100 μL was used for cellular uptake and the remaining 900 μL was used for DNA extraction and precipitation. The solutions for cellular uptake were centrifuged, and cell pellets were stored at -80 ℃ until ICP-MS analysis.

DNA was extracted using the phenol-chloroform extraction method and purified using ethanol precipitation. Briefly, cell pellets were suspended in 1.2 mL of lysis buffer combined with 6 μL of freshly added proteinase K solution (20 mg/mL). After incubation at 60 ℃ for 15 h with shaking, 1.2 mL of PCA solution was added to the cell lysates, and the mixture was vortexed thoroughly until emulsion formation. The solution was then centrifuged for 5 min at 1700 × *g* to separate the

liquid layer, and the aqueous (top) layer was transferred to new tubes. Subsequently, 1 vol. of CA solution was added, vortexed, and centrifuged. This step was repeated twice for complete elimination of phenol and protein. For precipitation of extracted DNA, 2 vol. of 100% ethanol and 0.1 vol. of 3 M sodium acetate were added. The solutions were mixed until white precipitation (DNA precipitant) was observed. After centrifugation for 3 min at 1700 × *g*, the supernatant was gently removed, and the pellet was rinsed twice with 70% ethanol. DNA pellets were stored at -80 ℃ until ICP-MS analysis.

Preparation of ICP-MS samples by wet digestion

Before quantification of cellular uptake and DNA-Pt adduct formation of cisplatin with ICP-MS, wet digestion was performed to ensure the solid-phase samples (cell pellets and DNA) were suitable for analysis. The cell pellets and DNA previously obtained were suspended in 30.0 μL of 100% HNO_3 and 20.0 μL of 36.5% HCl heated to 70 ℃ for 1 h. HCl was added to stabilize Pt atom. H_2O_2 (42.8 μL; an oxidant) was then added and the mixture was heated for an additional 3 h at 70 ℃. The digested samples were diluted to 1.5 mL with HPLC water to adjust the final concentration of nitric acid to 2%.

Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS was performed using Bruker Compact Q-TOF mass spectrometer (Bruker Daltronik GmbH, Bremen, Germany). The sample was sprayed into the ESI source at a flow rate of 10 μL/min. The source temperature of 80 °C, capillary voltage of 3.5 kV were used. Cisplatin was

prepared with 500 μM in presence of 5 % DMSO and incubated at 25 °C. Before ESI-MS analysis, cisplatin was diluted to 10 μM. The final DMSO concentration was set to 0.1 %. ESI-MS spectra of cisplatin was obtained after incubation for 0, 5 min, 30 min, 1 h, 3 h, 24 h.

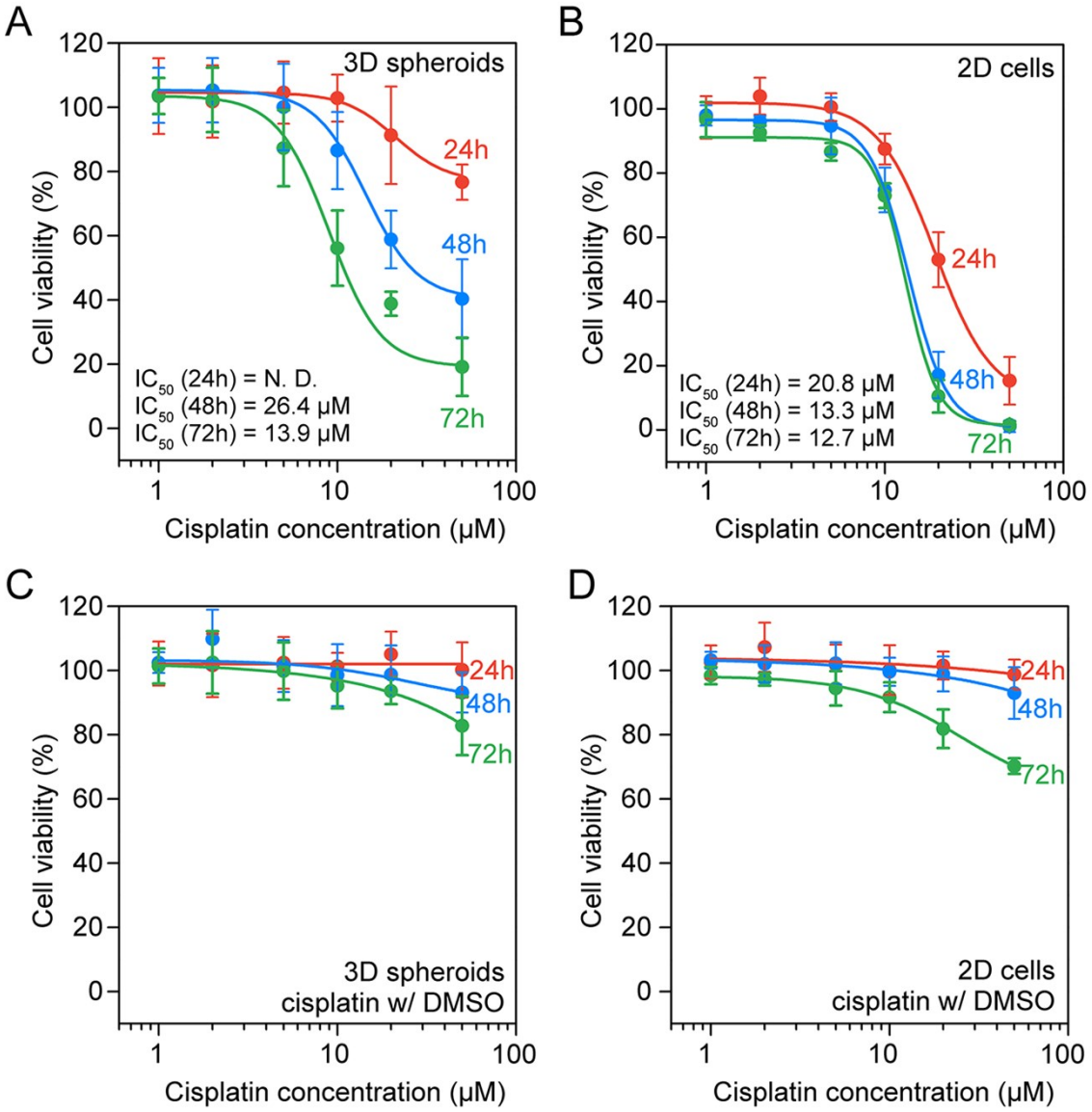

Fig. S1. Cell viability of 3D spheroids (A, C) and 2D monolayer cells (B, D) treated with cisplatin (A, B) in the absence of and (C, D) in the presence of DMSO for 24 (red), 48 (blue), and 72 h (green). IC_{50} concentrations determined from the plotted curve are indicated in the figure (detailed values are indicated in Table S2). N. D. indicates not detected within the investigated concentration range.

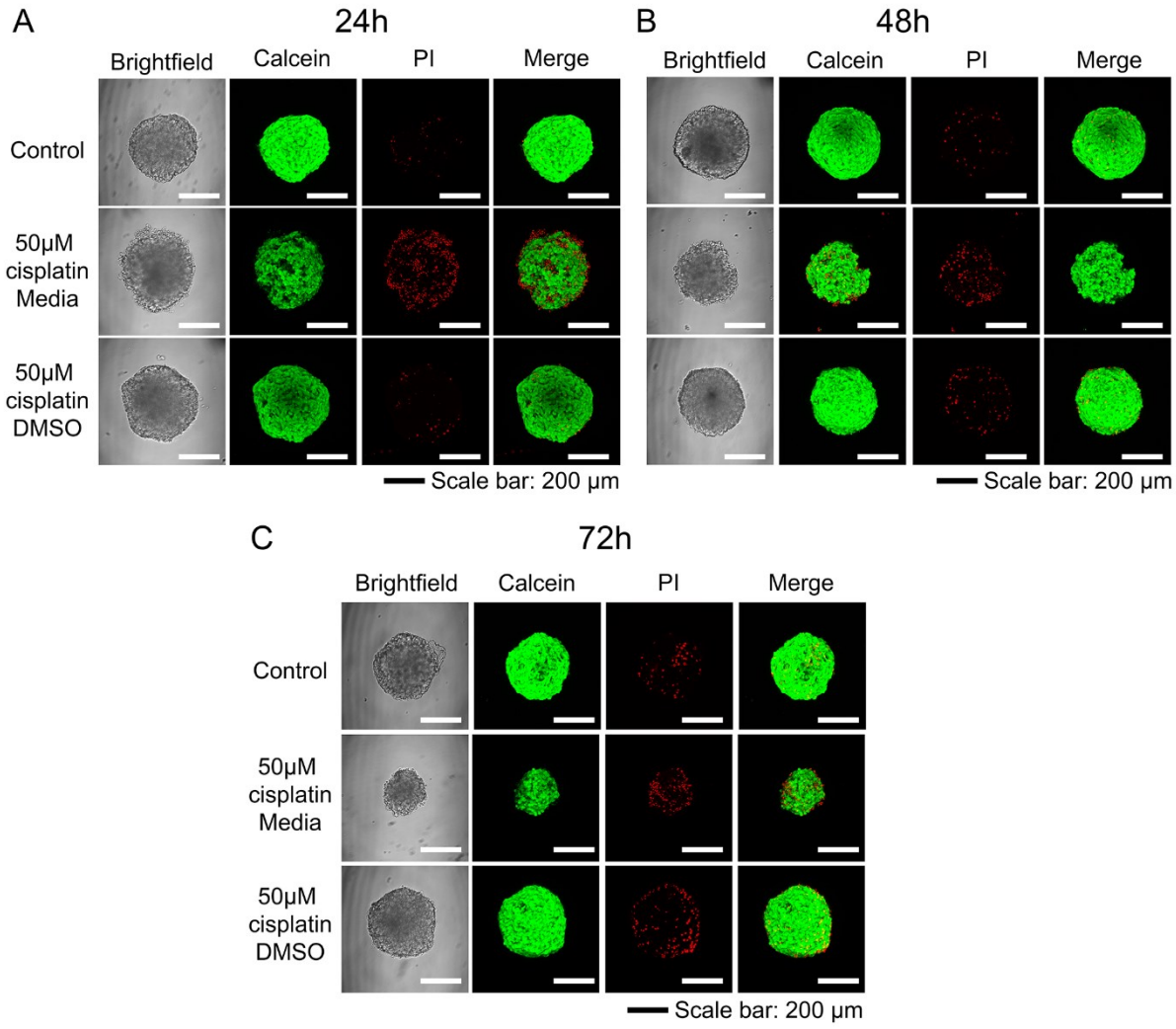

Fig. S2. Confocal images of 3D spheroids treated with 50 μM cisplatin for (A) 24, (B) 48, and (C) 72 h in the presence and the absence of DMSO. Spheroid cells were stained with calcein AM (green) and PI (red) for living and dead cells, respectively. Brightfield images of spheroids are also shown.

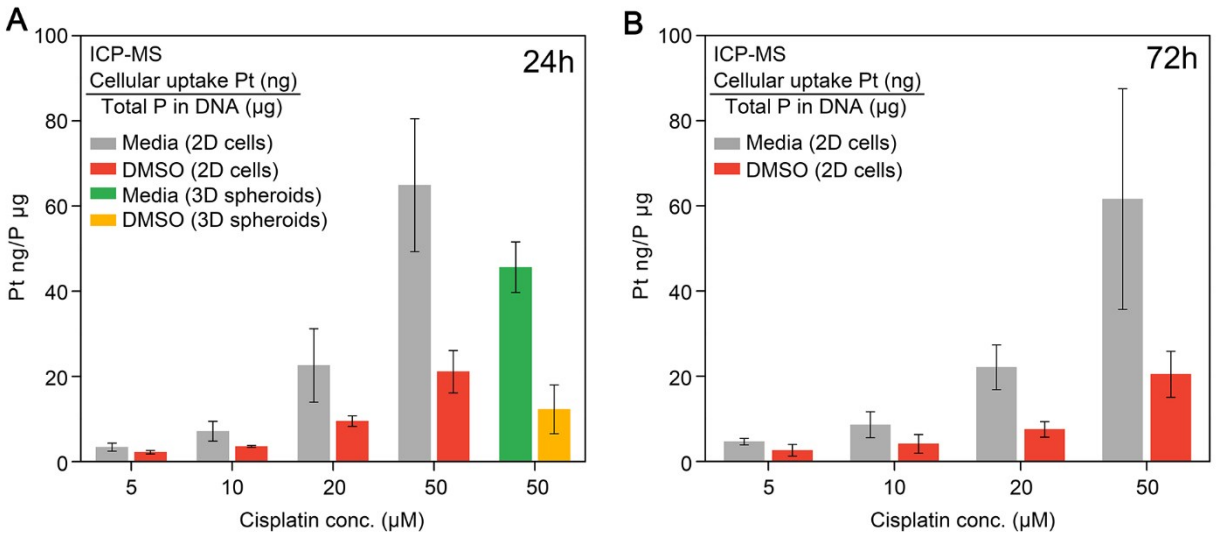

Fig. S3. Quantitative analysis of cellular uptake of cisplatin using ICP-MS. SK-N-SH cells in 2D monolayers and 3D spheroids were treated with cisplatin dissolved in media or DMSO for (A) 24h and (B) 72 h. After harvesting, digested cells were injected into ICP-MS to determine ^{195}Pt intensity. The amount of DNA in each sample was measured by quantifying ^{31}P, which is a representative element comprising the phosphodiester backbone of DNA. Values represent the mean ± SD of three independent experiments.

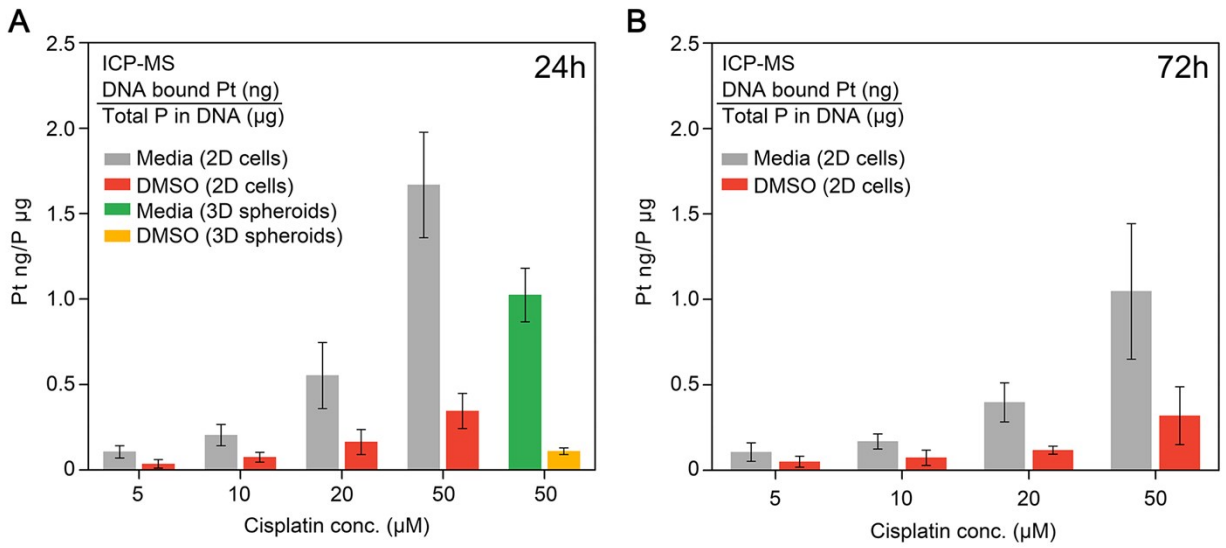

Fig. S4. Quantitative analysis of DNA-Pt adducts of cisplatin using ICP-MS. SK-N-SH cells in 2D monolayers and 3D spheroids were treated with cisplatin dissolved in media or DMSO for (A) 24h and (B) 72 h. After harvesting, digested cells were injected into ICP-MS to determine ^{195}Pt intensity. The extracted, precipitated, and digested DNA was injected into ICP-MS to quantify ^{195}Pt and ^{31}P. The amount of DNA in each sample was measured by quantifying ^{31}P, which is a representative element comprising the phosphodiester backbone of DNA. Values represent the mean ± SD of three independent experiments.

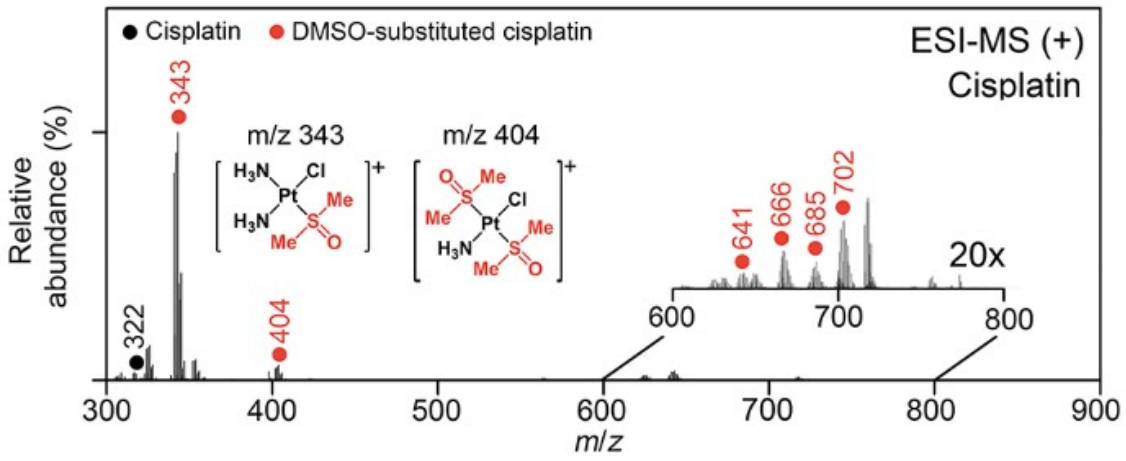

Fig. S5. ESI-MS spectrum of 10 μM cisplatin dissolved in 0.1% DMSO after incubation for 24 h at 25 ℃. DMSO-cisplatin peaks were observed at m/z = 343 $[Pt(NH_3)_2Cl(DMSO)]^+$ and m/z = 404 $[Pt(NH_3)Cl(DMSO)_2]^+$. Amine mediated cisplatin dimer peak with a DMSO ligand ($[Pt(NH_3)Cl_2$-NH_2-$Pt(Cl_2)DMSO]^+$), amine mediated cisplatin dimer peak with two DMSO ligands ($[Pt(NH_3)Cl(DMSO)$-NH_2-$Pt(NH_3)Cl(DMSO)]^+$), and chloride mediated cisplatin dimer peak with two DMSO ligands ($[Pt(NH_3)Cl(DMSO)$-Cl-$Pt(NH_3)Cl(DMSO)]^+$) were observed at m/z 641, 666, and 685, respectively (More details of detected ions are summarized in Table S6).

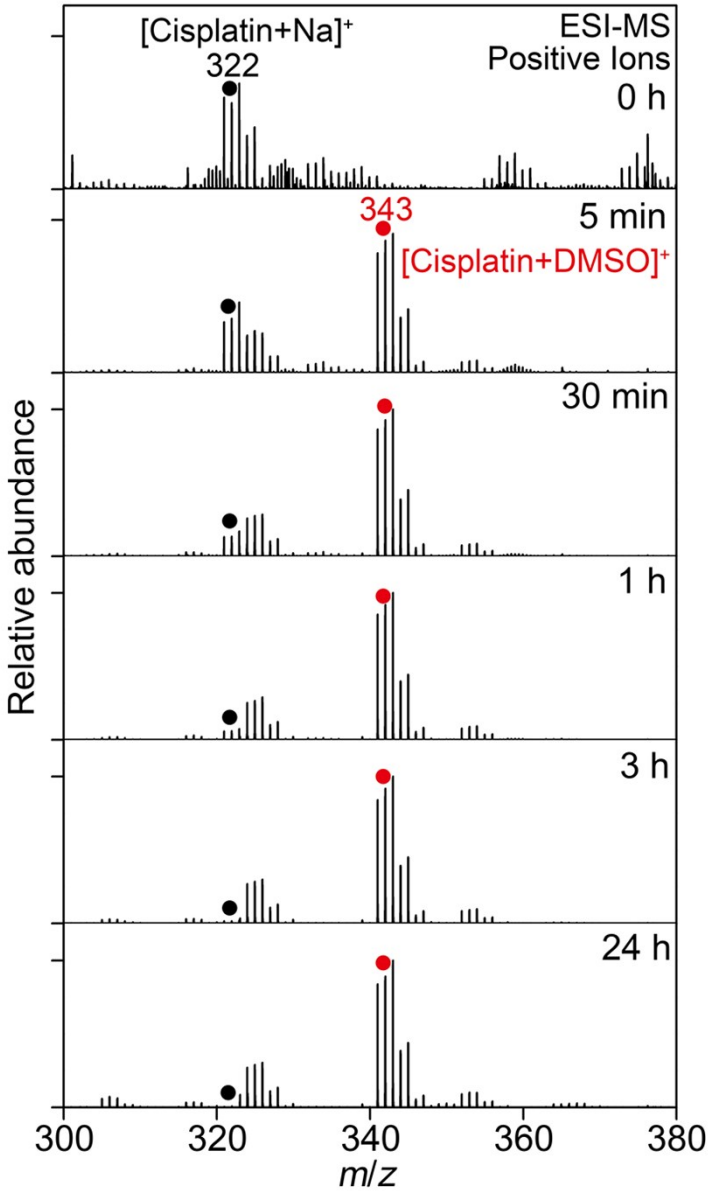

Fig. S6. The time resolved ESI-MS spectrum of cisplatin dissolved in DMSO and incubated for 0 to 24 h at 25 ℃, observed in the range of *m*/*z* 300–380. The sodiated adduct of cisplatin and DMSO-cisplatin complex was observed at *m*/*z* 322 $[Pt(NH_3)_2Cl_2+Na]^+$ and *m*/*z* = 343 $[Pt(NH_3)_2Cl(DMSO)]^+$. The calculated and observed *m*/*z* of DMSO-cisplatin complex are indicated in Table S6.

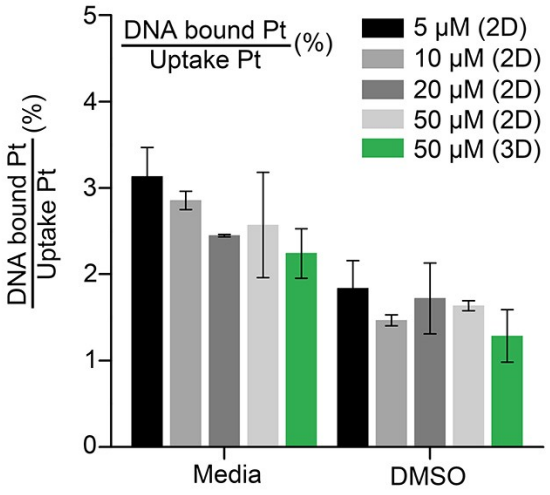

Fig. S7. Relative amount of DNA-Pt adducts normalized by cellular uptake Pt for cisplatin 24 h from drug application to cells in 2D monolayers and 3D spheroids. Values represent the mean ± SD of three independent experiments.

Table S1. The instrumental settings used during the ICP-MS measurements

Parameter	Pt	P
RF power(W)	1550	
Carrier gas flow rate (L/min)	0.88	
Integration time(sec)	0.0999	
Points/isotope	3	
Replicate	30	
Sweeps/Replicate	100	
Acquisition mode	Single quad	MS/MS (O_2)
Measured ion	$^{195}Pt^{+}$	$^{31}P^{16}O^{+}$
Mass pair	Q2 = 195	Q1 = 31, Q2 = 47
Reaction gas flow rate(%)	-	30
Cell entrance (V)	-50	-50
Cell exit (V)	-60	-58
Energy discrimination	-	-7 V
Stabilization time(sec)	-	15

Table S2. Summary of IC_{50} values of cisplatin with and without DMSO against SK-N-SH neuroblastoma cells cultured in 3D spheroids and 2D monolayers. N. D. indicates not detected within the investigated concentration range.

		IC50		
Condition	Culture dimension	24 h	48h	72 h
Media	2D	20.80 ± 2.3	13.34 ± 0.42	12.67 ± 0.98
	3D	N. D.	26.40 ± 1.14	13.94 ± 4.30
DMSO	2D	N. D.	N. D.	N. D.
	3D	N. D.	N. D.	N. D.

Table S3. Cell viability of neuroblastoma cell SK-N-SH in 2D culture dish and 3D spheroids exposed to 5, 10, 20, and 50 μM of cisplatin for 24, 48, and 72 h. Values represent the mean ± SD of three independent experiments.

			Cell viability (%)		
Condition	Culture dimension	Cisplatin (μM)	24 h	48h	72 h
Media	2D	5	100.6 ± 4.3	94.7 ± 8.8	86.7 ± 2.7
		10	87.5 ± 4.8	74.7 ± 7.0	73.0 ± 3.9
		20	53.0 ± 8.6	17.1 ± 7.3	10.5 ± 5.1
		50	15.3 ± 7.4	1.2 ± 1.8	1.6 ± 1.0
	3D	5	104.6 ± 9.7	100.1 ± 12.0	87.3 ± 11.8
		10	102.8 ± 7.3	86.5 ± 7.0	56.1 ± 11.7
		20	91.3 ± 15.2	58.8 ± 9.0	39.8 ± 3.7
		50	76.7 ± 5.5	40.3 ± 12.3	19.2 ± 9.1
DMSO	2D	5	101.9 ± 6.2	102.2 ± 6.5	94.4 ± 5.4
		10	100.1 ± 7.7	99.6 ± 4.4	87.8 ± 7.0
		20	101.5 ± 4.4	98.9 ± 5.4	77.4 ± 6.8
		50	98.8 ± 4.6	93.0 ± 8.1	70.2 ± 2.4
	3D	5	102.4 ± 8.0	101.4 ±8.1	99.8 ± 8.9
		10	101.2 ± 4.3	98.5 ± 9.6	95.2 ± 7.0
		20	105.0 ± 7.0	98.7 ± 9.1	93.6 ± 4.1
		50	100.2 ± 8.6	93.1 ± 6.2	82.8 ± 9.1

Table S4. Quantitative analysis of cellular uptake of cisplatin in the presence of DMSO using ICP-MS. Values represent the mean ± SD of three independent experiments.

Cellular uptake Pt / P amount of DNA (ng/μg)					
Culture dimension	Cisplatin (μM)	24 h		72 h	
		Media	DMSO	Media	DMSO
2D	5	3.64 ± 0.95	1.95 ± 0.39	4.66 ± 0.75	2.62 ± 1.39
	10	7.99 ± 2.31	5.11 ± 0.21	8.64 ± 3.05	4.17 ± 2.18
	20	22.59 ± 8.61	9.52 ± 1.24	22.13 ± 5.25	7.54 ± 1.83
	50	64.89 ± 15.6	21.13 ± 4.97	61.60 ± 25.9	20.48 ± 5.38
3D	50	45.65 ± 5.93	9.02 ± 1.79	-	-

Table S5. Quantitative analysis of DNA-Pt adducts of cisplatin in the presence of DMSO using ICP-MS. Values represent the mean ± SD of three independent experiments.

DNA bound Pt / P amount of DNA (ng/μg)					
Culture dimension	Cisplatin (μM)	24 h		72 h	
		Media	DMSO	Media	DMSO
2D	5	0.106 ±0.036	0.036 ±0.025	0.106 ±0.054	0.050 ±0.032
	10	0.204 ±0.062	0.075 ±0.028	0.180 ±0.044	0.074 ±0.045
	20	0.553 ±0.193	0.164 ±0.073	0.436 ±0.114	0.118 ±0.024
	50	1.668 ±0.309	0.346 ±0.104	1.240 ±0.396	0.320 ±0.169
3D	50	1.024 ±0.157	0.159 ±0.019	-	-

Table S6. Peaks of cisplatin-DMSO complex ions observed in ESI-MS spectra.

Complex	*m*/*z* (calculated)	*m*/*z* (observed)
$[Pt(NH_3)_2(Cl)(DMSO)]^+$	342.0007	342.0072
$[Pt(NH_3)(Cl)(DMSO)_2]^+$	402.9881	402.9957
$[(NH_3)(Cl)Pt(\mu Cl)_2\ Pt(NH_3)(DMSO)]^+$	606.9032	606.9108
μCl-$[Pt(NH_3)(Cl)(DMSO)]^+$	623.9297	623.9358
$\{[Pt(NH_3)_2(Cl)_2][Pt(NH_3)_2(Cl)(DMSO)]\}^+$	640.9563	640.9616
μNH_2-$[Pt(NH_3)(Cl)(DMSO)]_2^+$	665.9670	665.9705
μCl-$[Pt(NH_3)(Cl)(DMSO)]_2^+$	684.9171	684.9294
$\{[Pt(NH_3)_2(Cl)(DMSO)][Pt(NH_3)(Cl)_2(DMSO)]\}^+$	701.9436	701.9517

Table S7. Cell viability, drug uptake, and DNA-Pt adduct formation after 24 and 72 h of drug application in absence and presence of DMSO in 2D monolayer cells. Values represent the mean ± SD of three independent experiments.

Condition	Applied Cisplatin (μM)	24h		
		Cell viability	Uptake Pt	DNA Bound Pt
Media	5	100.6 ± 4.3	3.64 ± 0.95	0.11± 0.04
	10	87.5 ± 4.8	7.99 ± 2.31	0.20± 0.06
	20	53.0 ± 8.6	22.6 ± 8.61	0.55± 0.19
	50	15.3 ± 7.4	64.9 ± 15.6	1.67± 0.39
DMSO	5	101.9 ± 6.2	1.95 ± 0.39	0.04± 0.02
	10	100.1 ± 7.7	5.11± 0.21	0.08± 0.03
	20	101.5 ± 4.4	9.52± 1.24	0.16± 0.07
	50	98.8 ± 4.6	21.1± 4.97	0.35± 0.10
Condition	Applied Cisplatin (μM)	72h		
		Cell viability	Uptake Pt	DNA Bound Pt
Media	5	86.7 ± 2.7	4.66 ± 0.76	0.11 ± 0.05
	10	73.0 ± 3.9	8.64 ± 3.04	0.18 ± 0.04
	20	10.5 ± 5.1	22.1 ± 5.25	0.44 ± 0.11
	50	1.6 ± 1.0	61.6 ± 25.9	1.24 ± 0.40
DMSO	5	94.4 ± 5.4	2.62 ± 1.38	0.05 ± 0.03
	10	91.7 ± 4.6	4.17 ± 2.19	0.07 ± 0.04
	20	81.8 ± 6.0	7.54 ± 1.83	0.12 ± 0.02
	50	70.2 ± 2.4	20.5 ± 5.38	0.32 ± 0.17

Table S8. Cell viability, drug uptake, and DNA-Pt adduct formation after 24 h of drug application in absence and presence of DMSO in 3D spheroid cells. Values represent the mean ± SD of three independent experiments.

Condition	Applied Cisplatin (μM)	24h		
		Cell viability	Uptake Pt	DNA Bound Pt
Media	50	76.7 ± 5.5	45.6 ± 5.9	1.02 ± 0.16
DMSO	50	100.2 ± 8.6	9.02 ± 1.79	0.11 ± 0.02