Supporting Information

Comparative studies of oxaliplatin-based platinum(IV) complexes in different in vitro and in vivo tumor models

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1. ^{1}H NMR kinetics

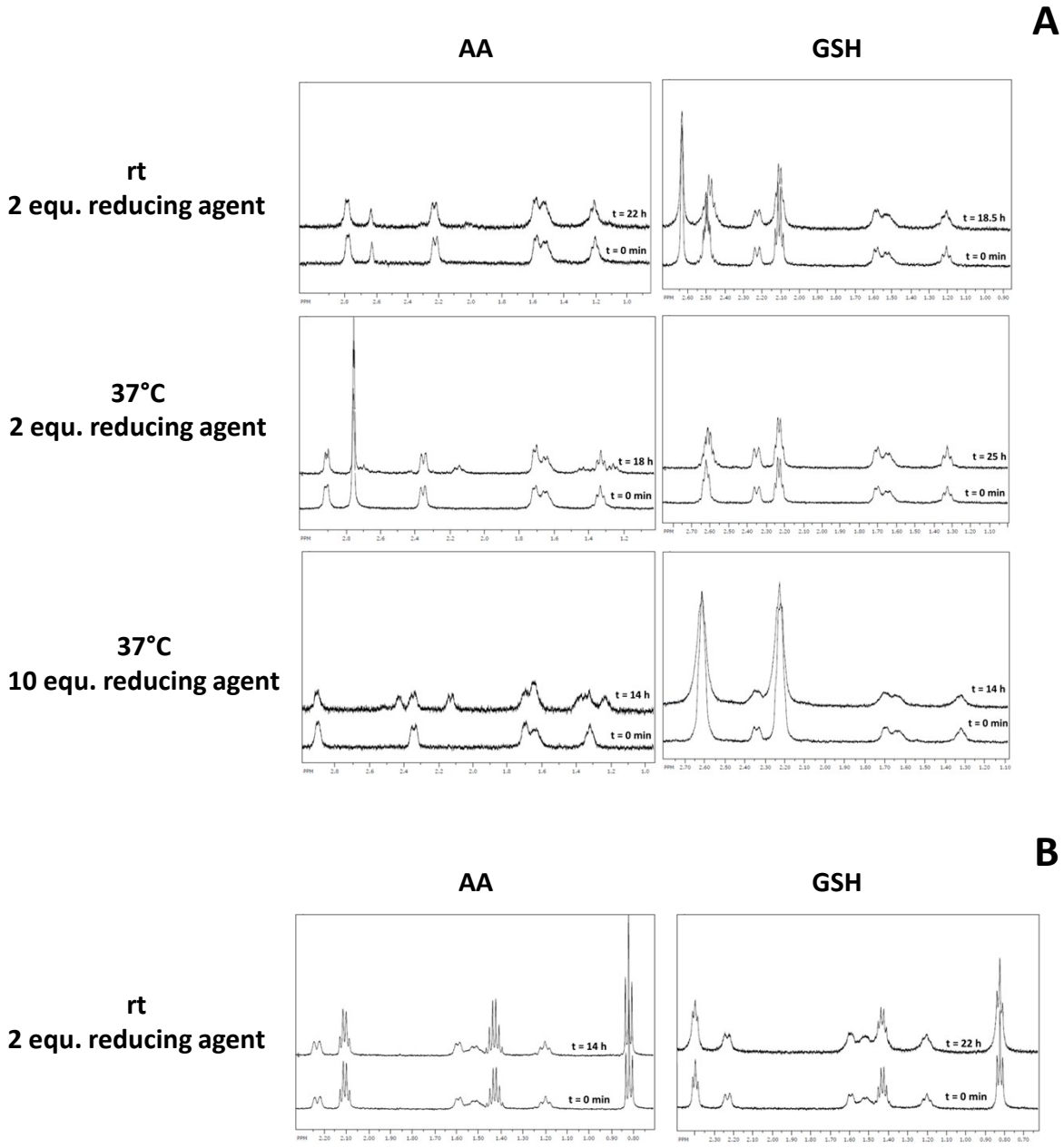


Figure S1 Reduction of **1** (A) or **2b** (B) by ascorbic acid (AA) or glutathione (GSH) at different molar ratios and different temperatures, measured by ^{1}H NMR over a time period of at least 14 h.

2. Cytotoxicity

2.1 MTT assay

Cytotoxicity was determined by the colorimetric MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide). Briefly, cells were harvested by trypsinization and seeded in medium into 96-well plates in volumes of 100 µL/well. Depending on the cell line, different cell densities were used to ensure exponential growth of the untreated controls during the experiment: 2.5×10^3 (HCT15), 1.5×10^3 (HCT116), 6.0×10^3 (HCT116oxR) cells per well. In the first 24 h, the cells were allowed to settle and resume exponential growth. Then, the test compounds were dissolved and serially diluted, both in RPMI 1640 medium, and added to the plates in volumes of 100 µL/well. After continuous exposure for 96 h (in the incubator at 37 °C and under 5% CO_2), the medium was replaced with 100 µL/well RPMI 1640 medium and MTT solution (MTT reagent in phosphate-buffered saline, 5 mg/mL) in a ratio of 6:1, and plates were incubated for further 4 h. Subsequently, the medium/MTT mixture was removed and the formed formazan was dissolved in DMSO (150 µL/well). Optical densities at 550 nm were measured (reference wavelength: 690 nm) with a microplate reader (ELX880, BioTek). The quantity of viable cells was expressed as the percentage of untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from the concentration-effect curves by interpolation. Every test was repeated in at least three independent experiments, each consisting of three replicates per concentration level.

Table 1 Cytotoxic activity in three different human colon carcinoma cell lines. IC_{50} (fifty percent inhibitory concentrations) given as means ± standard deviations from at least three independent experiments using exposure times of 96 h; RF … resistance factor.

	IC_{50} [µM], MTT			***RF***	
	HCT 15	**HCT 116**	**HCT116oxR**	***HCT15/ HCT116***	***HCT116oxR/ HCT116***
1	132 ± 10	40 ± 9	187 ± 45	*3.3*	*4.7*
2	76 ± 14	43 ± 7	793 ± 109	*1.8*	*18*
2a	21 ± 5	11 ± 2	264 ± 66	*1.9*	*24*
2b	37 ± 1	22 ± 3	198 ± 22	*1.6*	*8.9*
OxaliPt	1.0 ± 0.3	0.49 ± 0.07	32 ± 9	*2.1*	*66*
SatraPt	3.1 ± 0.5	1.9 ± 0.3	2.2 ± 0.1	*1.6*	*1.2*

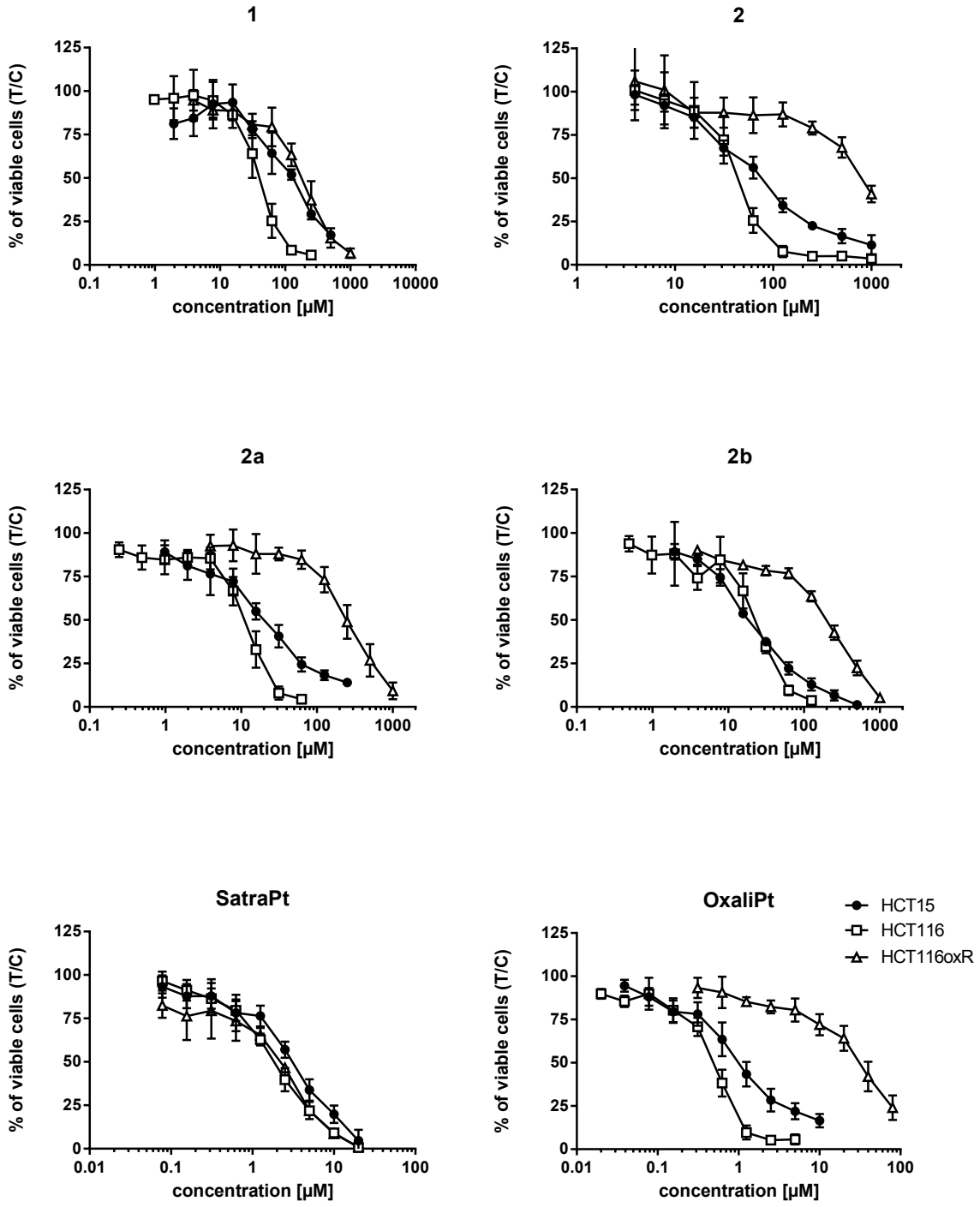


Figure S2 Cytotoxicity (concentration-effect curves) of all investigated complexes in HCT15, HCT116 and HCT116oxR cells, obtained by the colorimetric MTT after continuous exposure for 96 h.

2.2 Resazurin assay

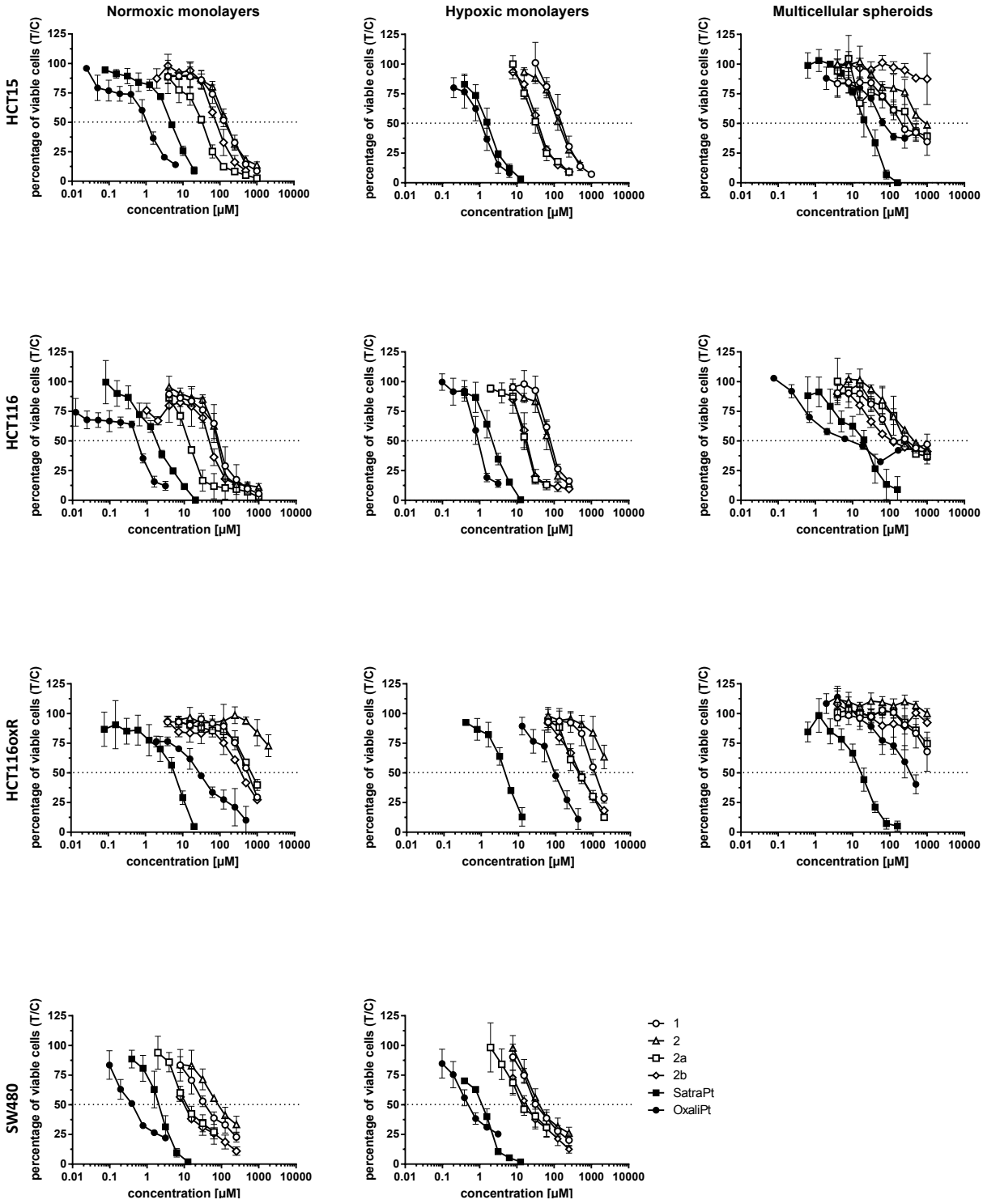


Figure S3 Cytotoxicity of all investigated complexes in HCT15, HCT116, HCT116oxR and SW480 cells; concentration-effect curves obtained by the fluorimetric reazurin assay after continuous exposure for 96 h. Left: normoxic monolayer cultures, middle: hypoxic monolayer cultures, right: multicellular spheroids.

3 Cell cycle studies

Method

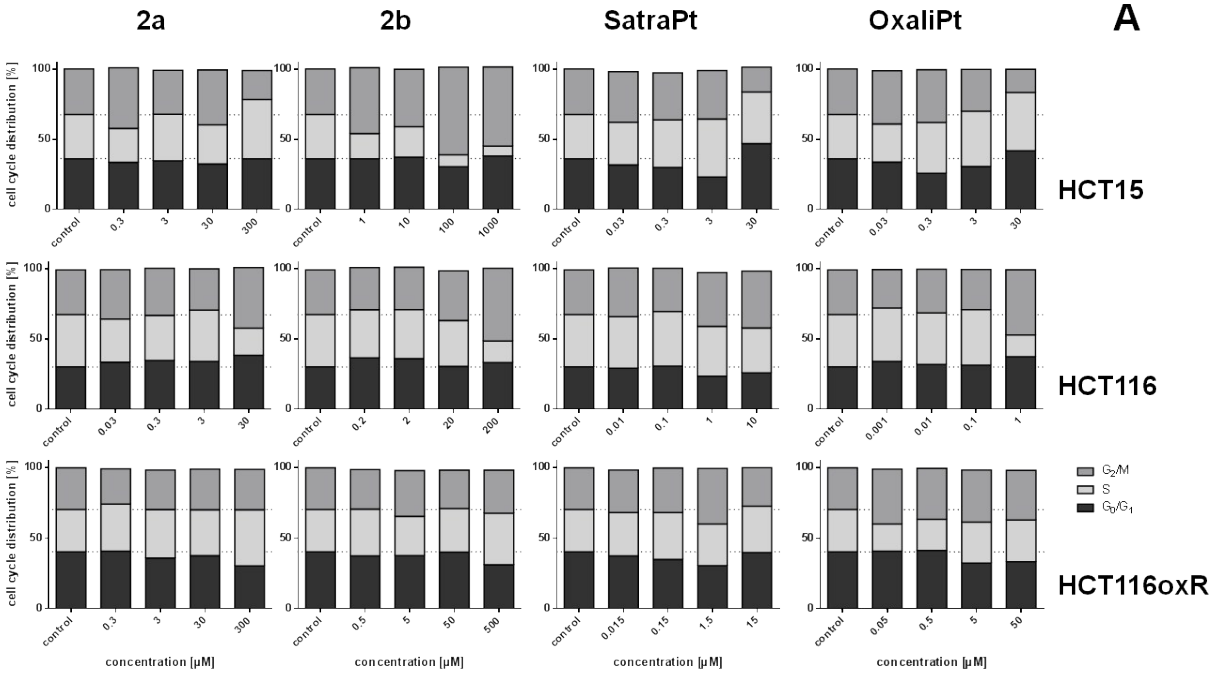
For cell cycle studies cells were harvested by trypsinization, and 6×10^4 cells/well (for 24 h incubation) and 4×10^4 cells/well (for 48 h incubation) were seeded into 48-well plates (250 µL/well). In the first 24 h, the cells were allowed to settle and resume exponential growth. Thereafter, the medium was removed, and 200 µL/well of test compounds in RPMI 1640 medium were added. After continuous exposure for 24 h or 48 h (in the incubator at 37 °C and under 5% CO_2), cells were washed with PBS and trypsinized. Trypsinization was stopped by addition of RPMI 1640 medium, cells were washed with PBS and resuspended in 200 µL PI/HSF buffer (0.1% v/v Triton X-100, 0.1% w/v sodium citrate, in PBS) containing 50 µg/mL propidium iodide (PI). After incubation overnight at 4 °C in the dark, 5×10^3 cells were measured by flow cytometry with a Millipore guava easy cyte 8HT instrument. Data were evaluated by FlowJo software (Tree Star) using Dean Jett Fox algorithms. Tests were repeated in at least three independent experiments.

Results

It is widely accepted that platinum drugs interact with DNA, forming cross-links that lead to distortion of the double helix.[5] Such interferences usually result in cell cycle perturbations. To analyze these alterations by flow cytometry, HCT15, HCT116 and HCT116oxR cells were stained with propidium iodide (PI) after 24 h and 48 h treatment. The highest concentration applied did not exceed IC50 values after 48 h. In all cell lines, effects on cell cycle distribution were found after both incubation times, whereby HCT116oxR is the least affected. At the highest concentration, 1 induces a pronounced G2/M phase arrest in all tested cell lines and incubation times (Fig. S5–S10). After 24 h, HCT15 cells get arrested in the S phase when treated with the highest concentration of 2a but show an even stronger increase of the G2/M phase at all tested concentrations of **2b** (Fig. S5A). When the incubation period is prolonged to 48 h, both compounds induce an S phase arrest (in the case of **2b** already at 1 µM). In the

same cell line, satraplatin and oxaliplatin increase the S phase already at lower concentrations after 24 h. A similar impact of oxaliplatin is detected after 48 h, whereas satraplatin induces a strong G_2/M phase (3 µM) or a G_1 phase arrest (30 µM) (Fig. 5B). After 24 h in HCT116 cells, **2a**, **2b** and oxaliplatin induce a decrease of the S phase fraction with a concurrent increase of G_1 and G_2/M phases, whereas satraplatin shows only a minor influence on cell cycle phases. After 48 h, the G1 phase rises during treatment with **2a** and oxaliplatin, while **2b** and satraplatin cause a strong G_2/M phase arrest at the highest concentration. As expected, only minor effects were observed in HCT116oxR cells after 24 h for **2a**, **2b**, oxaliplatin and satraplatin, but after 48 h oxaliplatin and satraplatin raise the G_2/M phase.

These experiments confirm that the cell cycle of colon carcinoma cell lines is affected in a time- and concentration-dependent manner by the treatment with platinum(IV) compounds featuring the oxaliplatin core. However, which phase of the cell cycle is affected, is highly dependent on the cell line, the compound or even the concentration. Overall, the cell cycle effects are consistent with a DNA-damaging mode of action. Any DNA damage exceeding the capacity of DNA repair will result in induction of apoptosis, which was addressed in the next part of the study.



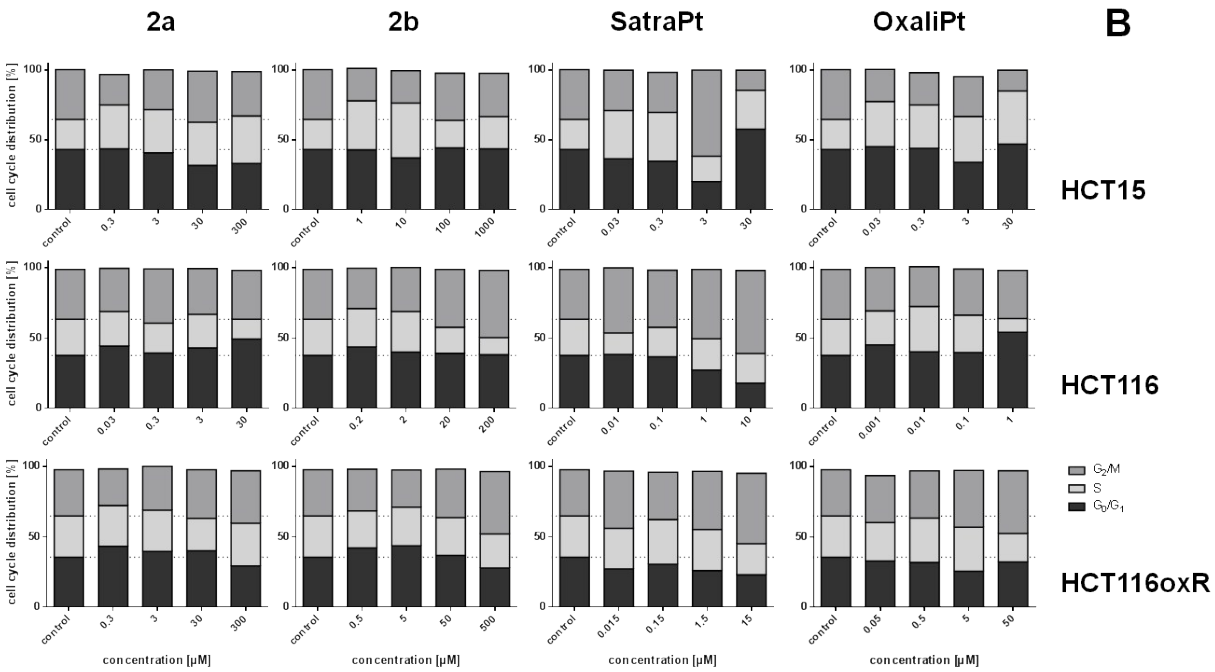


Figure S4 Impact of compounds **2a**, **2b**, satraplatin and oxaliplatin on the cell cycle distribution of HCT15, HCT116 and HCT116oxR cells after 24 h (A) and 48 h (B) continuous exposure with the indicated concentrations. Cells were stained with PI, measured by flow cytometry, and distribution was evaluated by FlowJo software using Dean Jett Fox algorithms. Results represent means of at least three independent experiments.

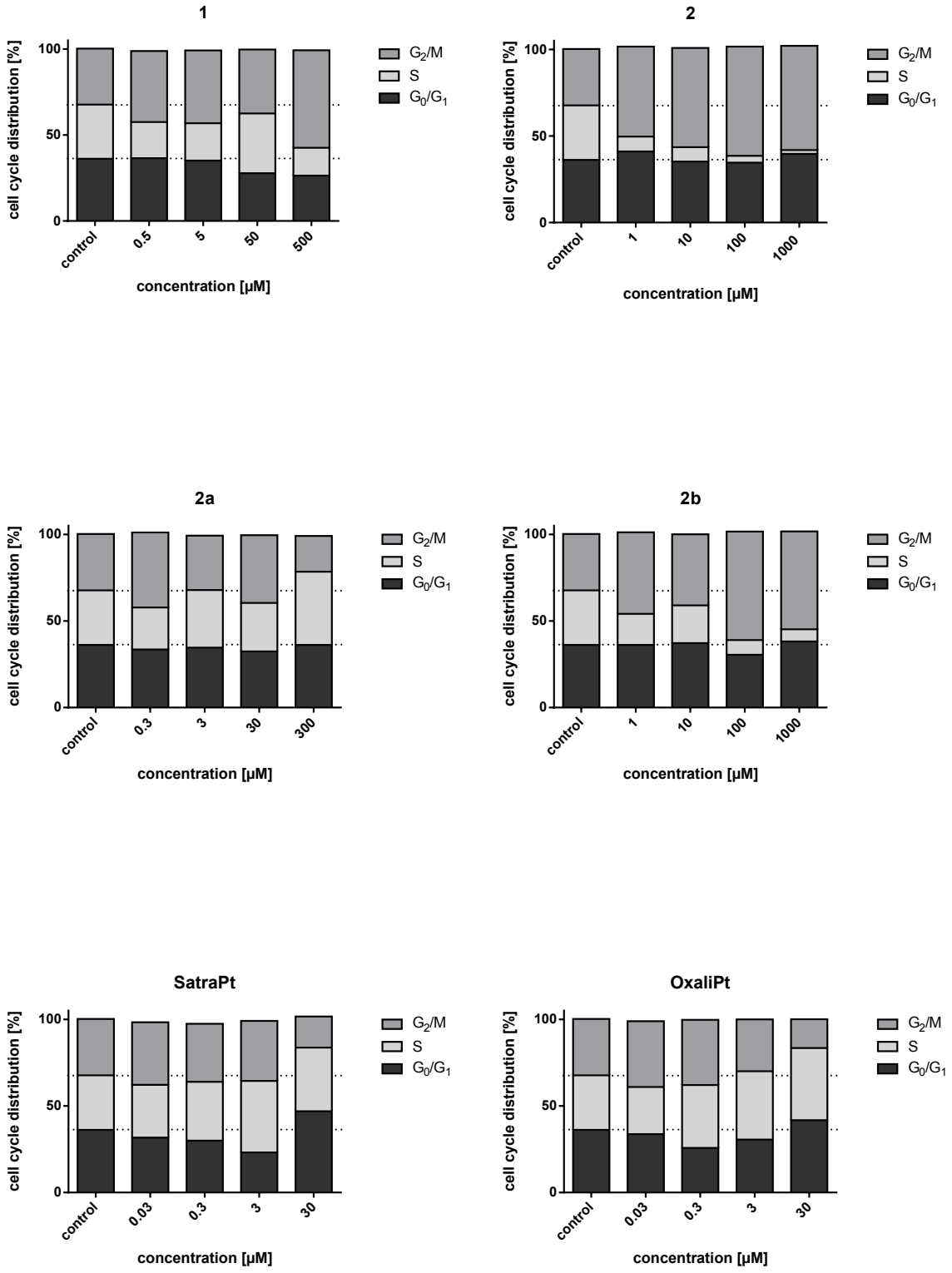


Figure S5 Impact of platinum compounds on the cell cycle distribution in HCT15 cells. PI staining and flow cytometric analyses were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

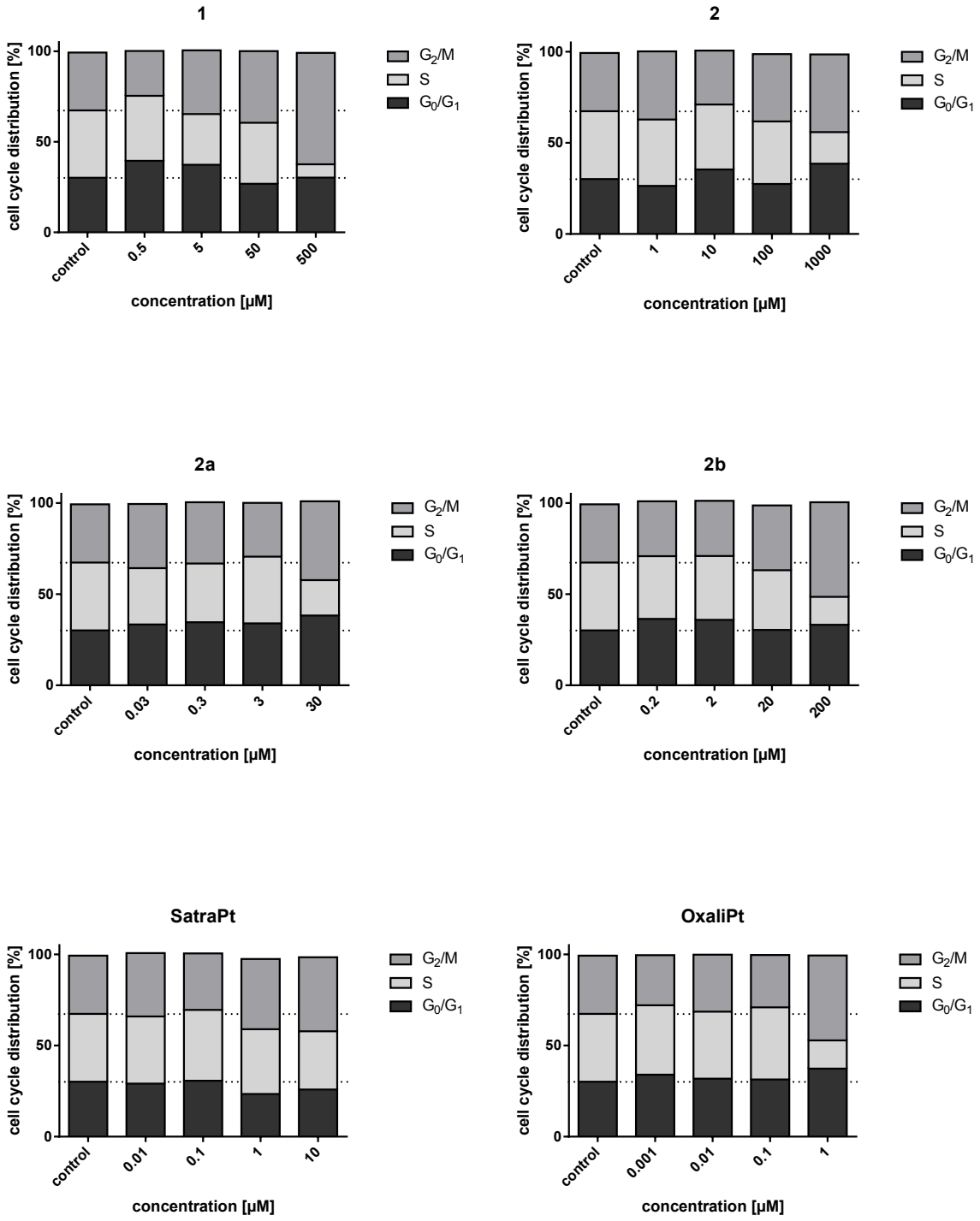


Figure S6 Impact of platinum compounds on the cell cycle distribution in HCT116 cells. PI staining and flow cytometric analyses were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

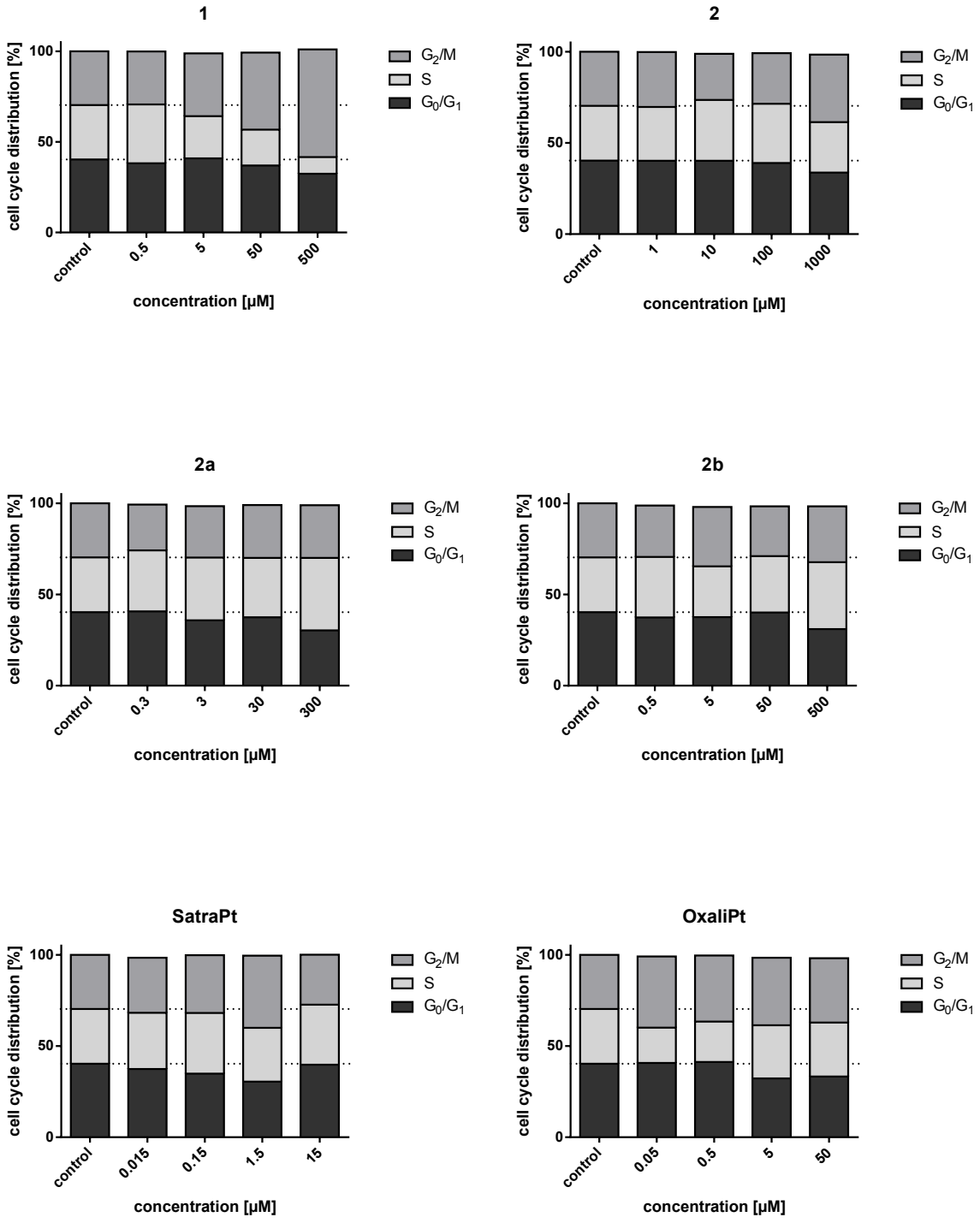


Figure S7 Impact of platinum compounds on the cell cycle distribution in HCT116oxR cells. PI staining and flow cytometric analyses were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

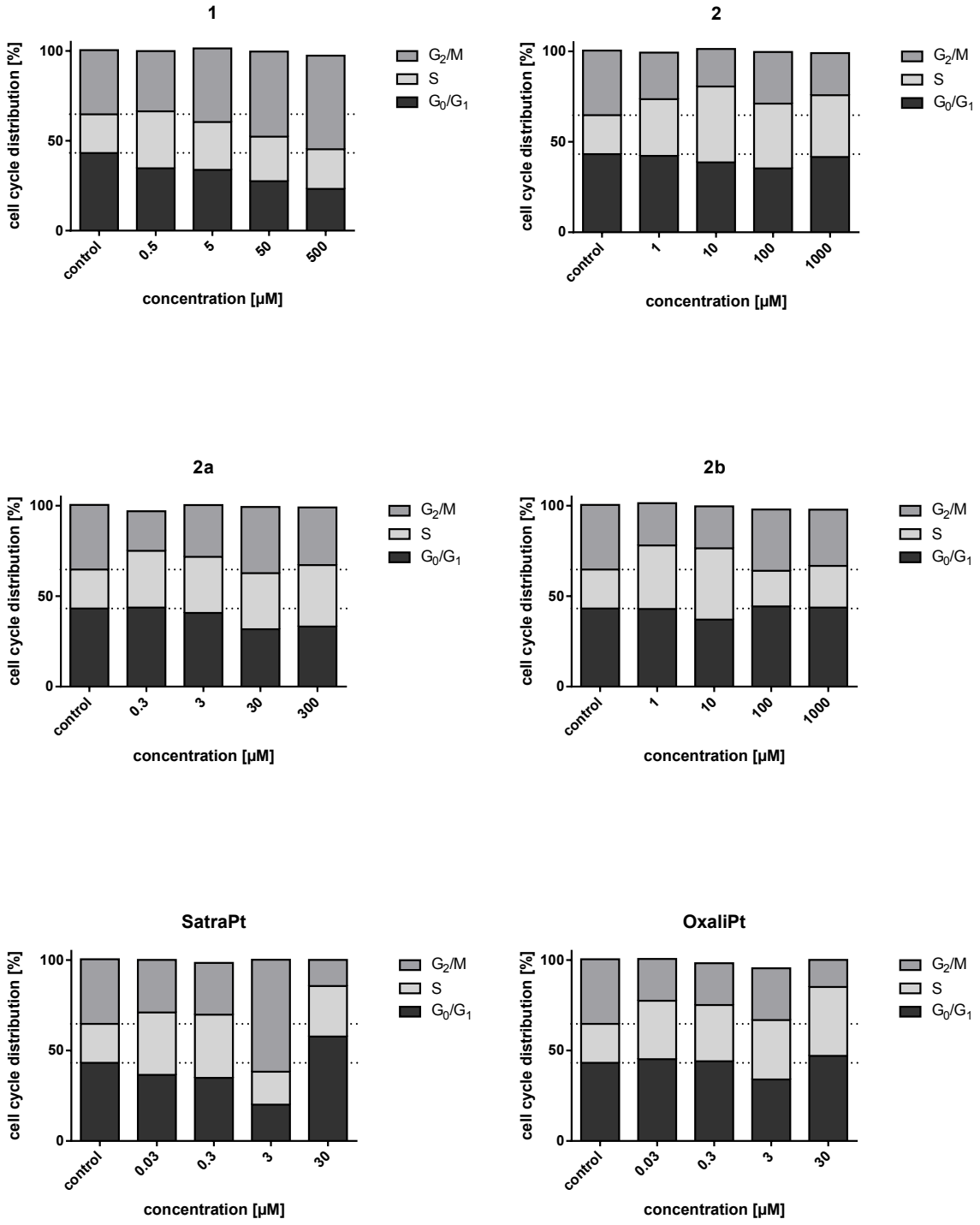


Figure S8 Impact of platinum compounds on the cell cycle distribution in HCT15 cells. PI staining and flow cytometric analyses were performed after 48 h continuous exposure at the indicated concentrations. Results are mean percentages of at least three independent experiments.

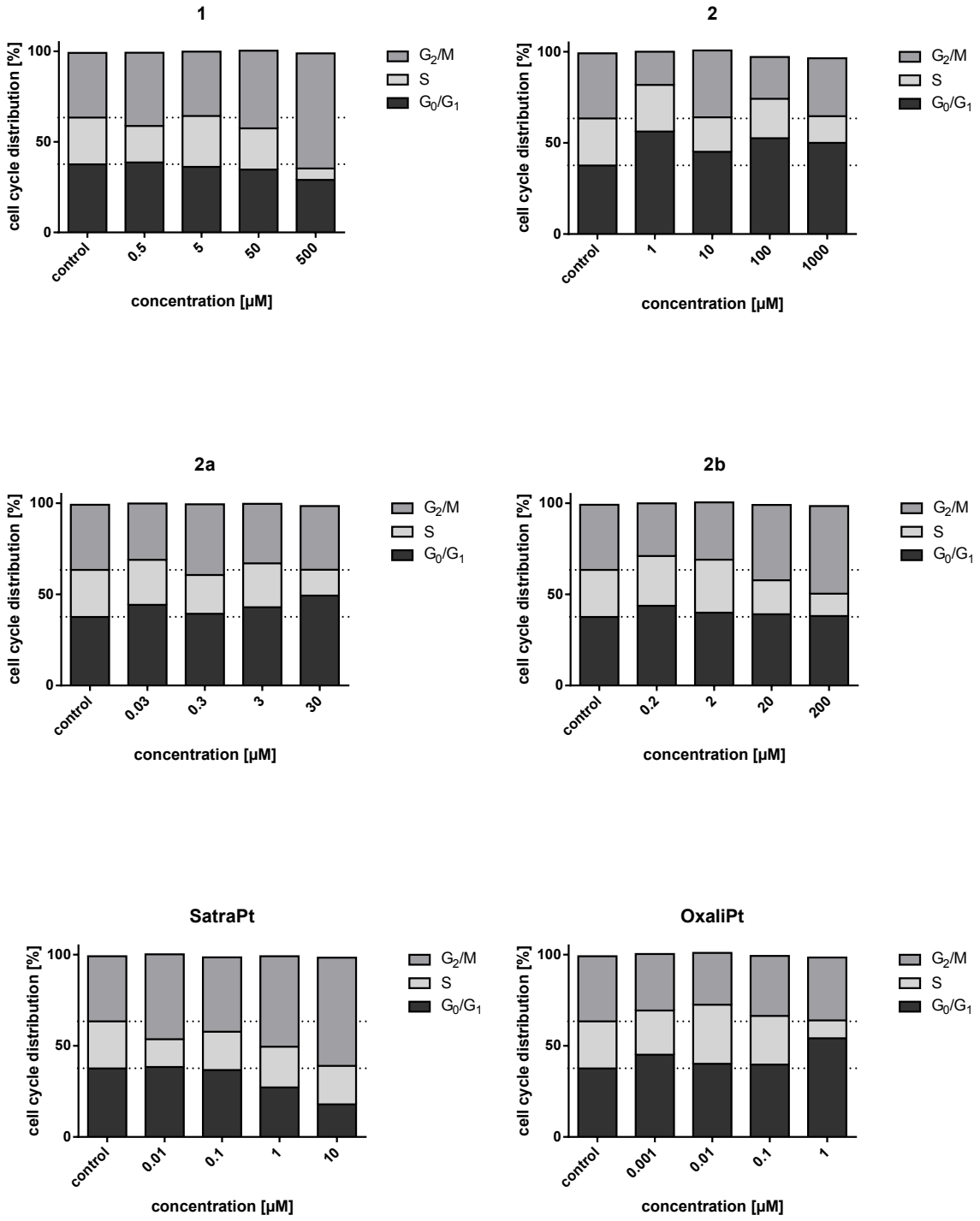


Figure S9 Impact of platinum compounds on the cell cycle distribution in HCT116 cells. PI staining and flow cytometric analyses were performed after 48 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

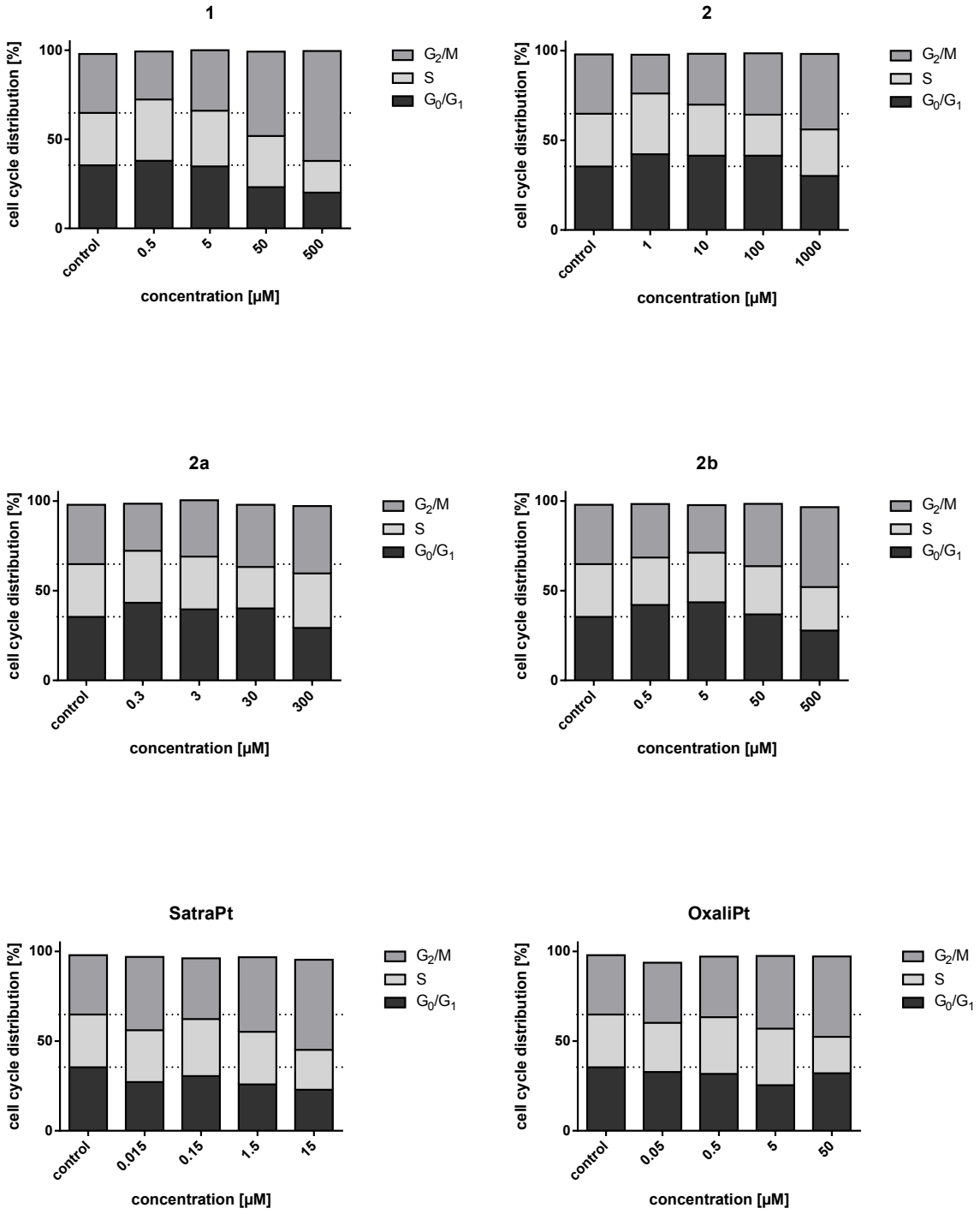


Figure S10 Impact of platinum compounds on the cell cycle distribution in HCT116oxR cells. PI staining and flow cytometric analyses were performed after 48 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

4. Apoptosis induction

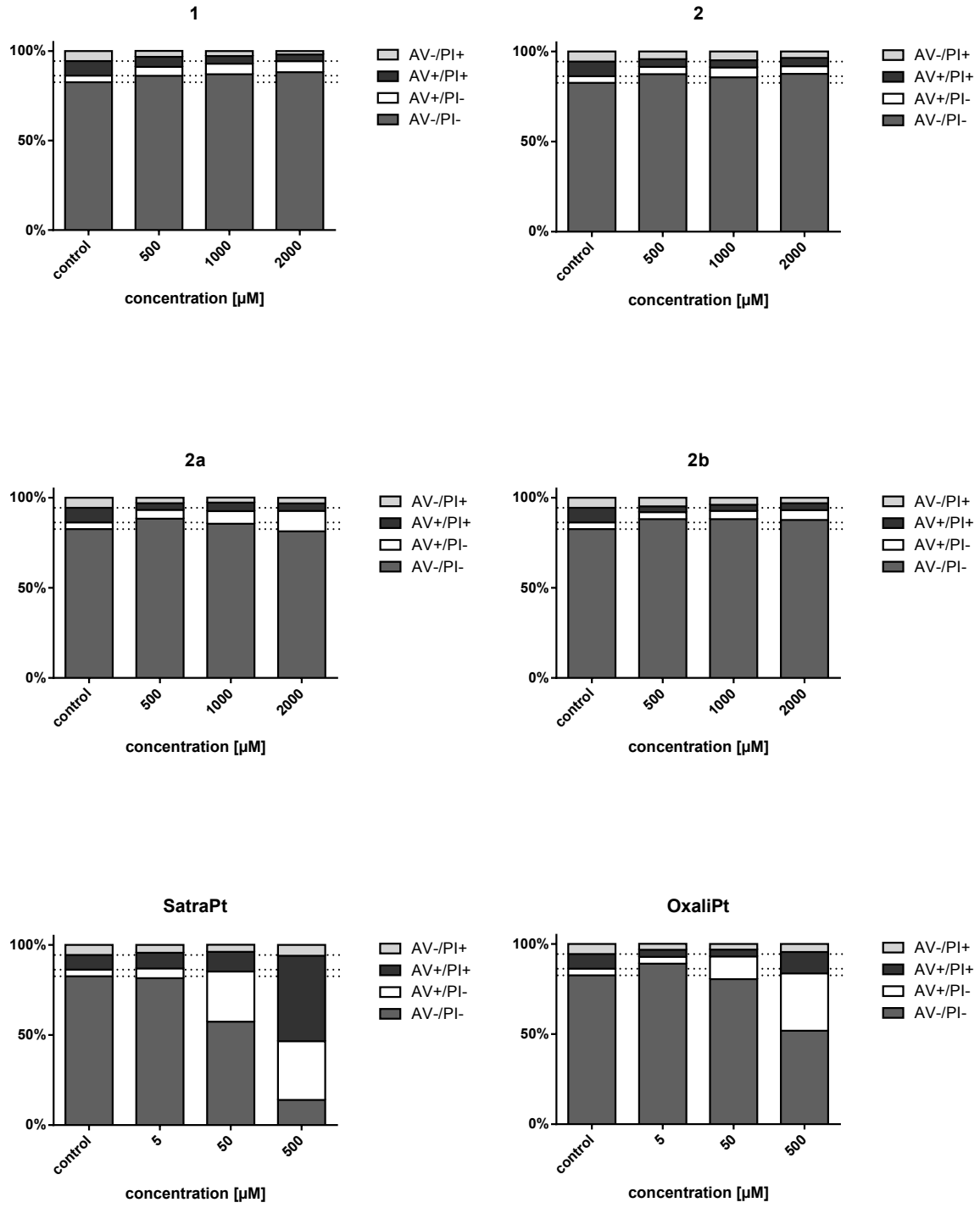


Figure S11 Induction of apoptosis by platinum complexes in HCT15 cells. Annexin V-FITC/PI staining and flow cytometry were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

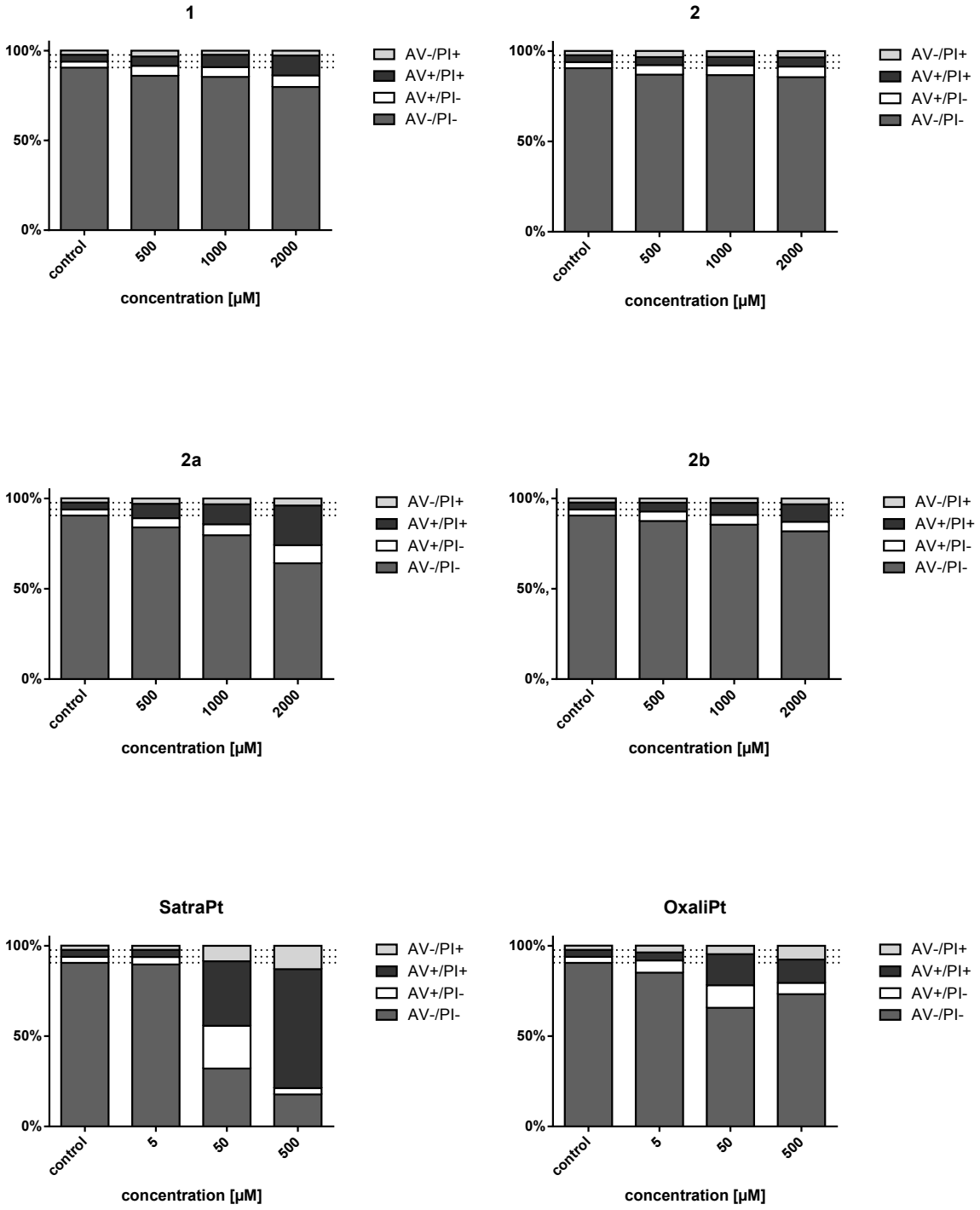


Figure S12 Induction of apoptosis by platinum complexes in HCT116 cells. Annexin V-FITC/PI staining and flow cytometry were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

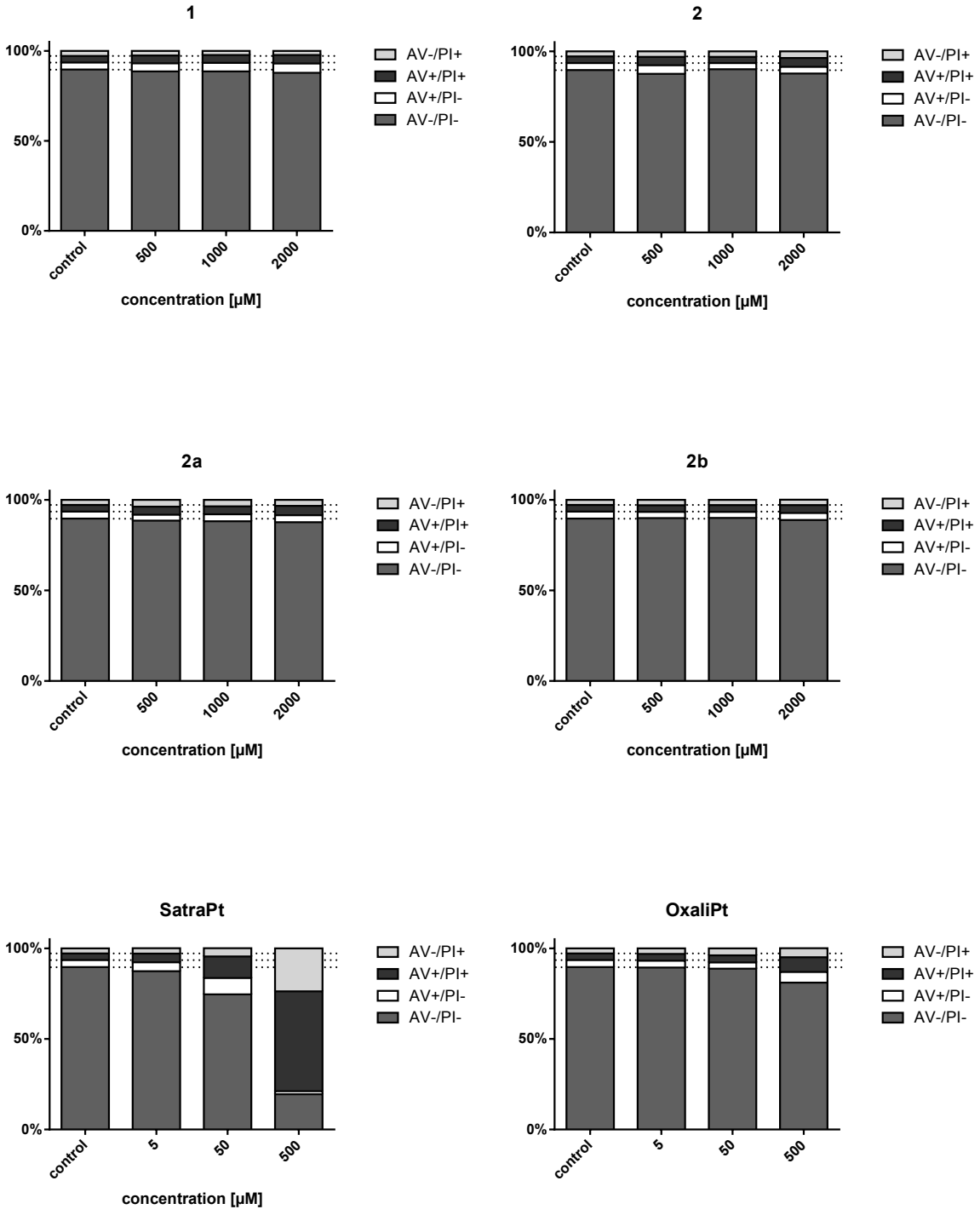


Figure S13 Induction of apoptosis by platinum complexes in HCT116oxR cells. Annexin V-FITC/PI staining and flow cytometry were performed after 24 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

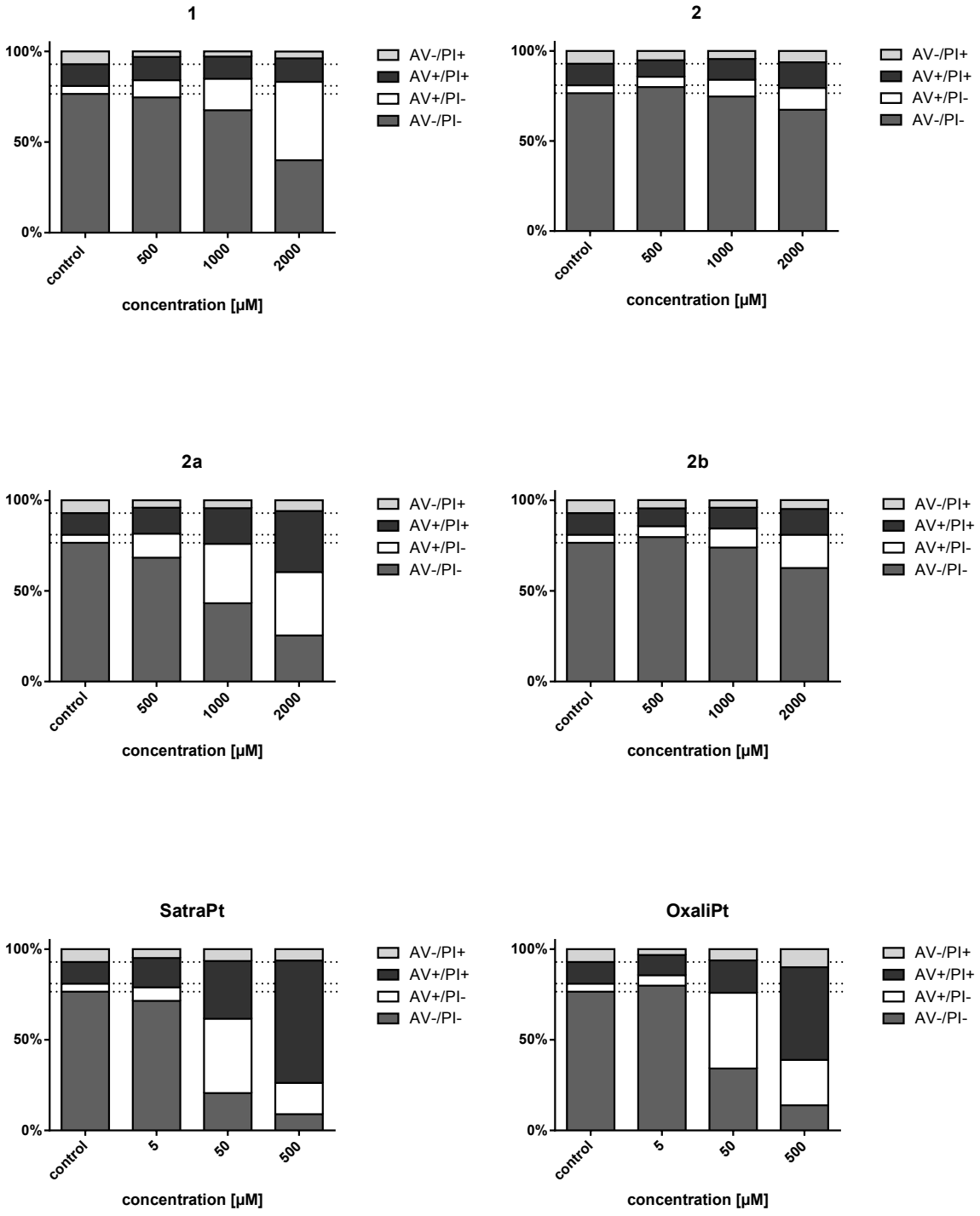


Figure S14 Induction of apoptosis by platinum complexes in HCT15 cells. Annexin V-FITC/PI staining and flow cytometry were performed after 48 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

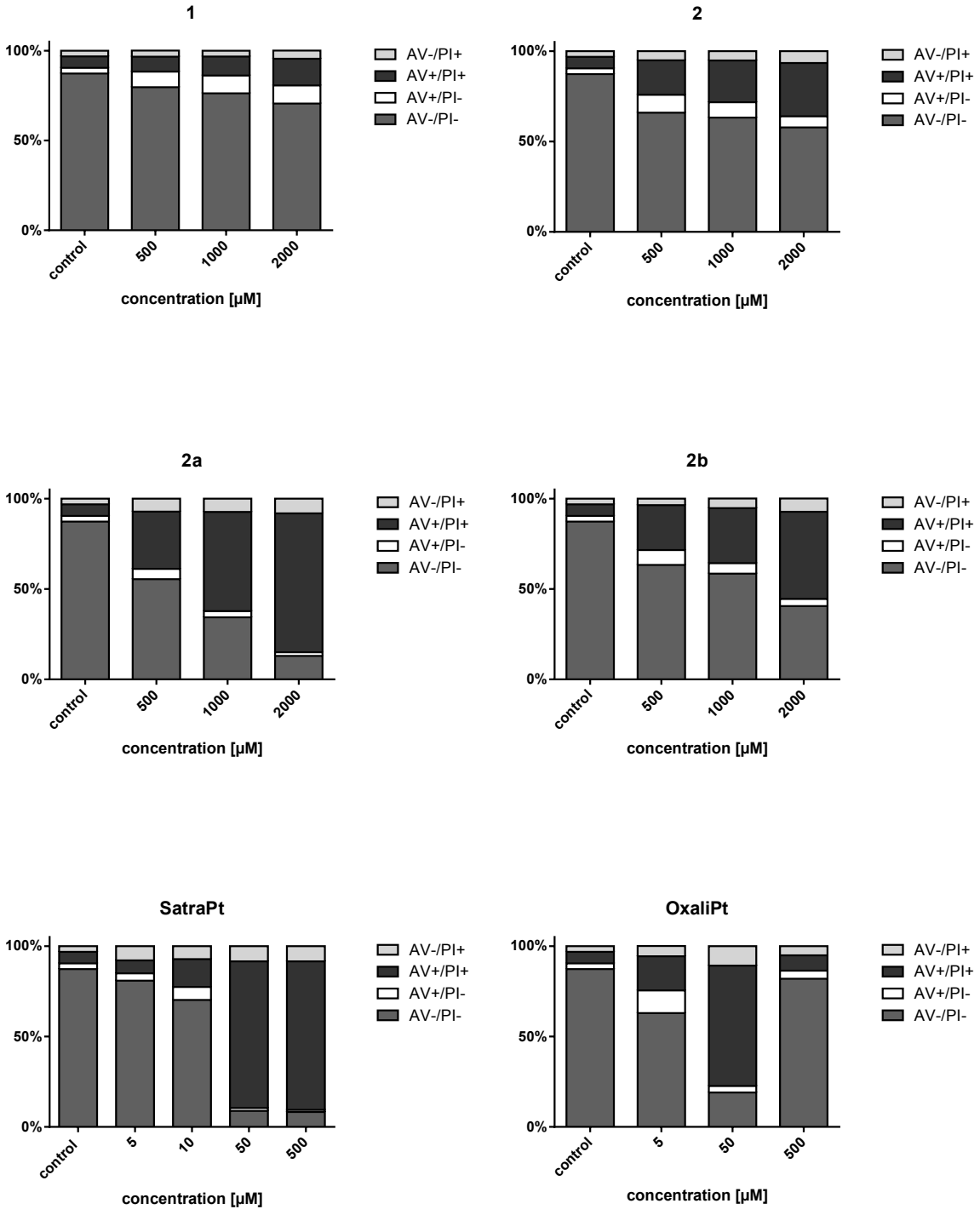


Figure S15 Induction of apoptosis by platinum complexes in HCT116 cells. Annexin V-FITC/PI staining and flow cytometry were performed after 48 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

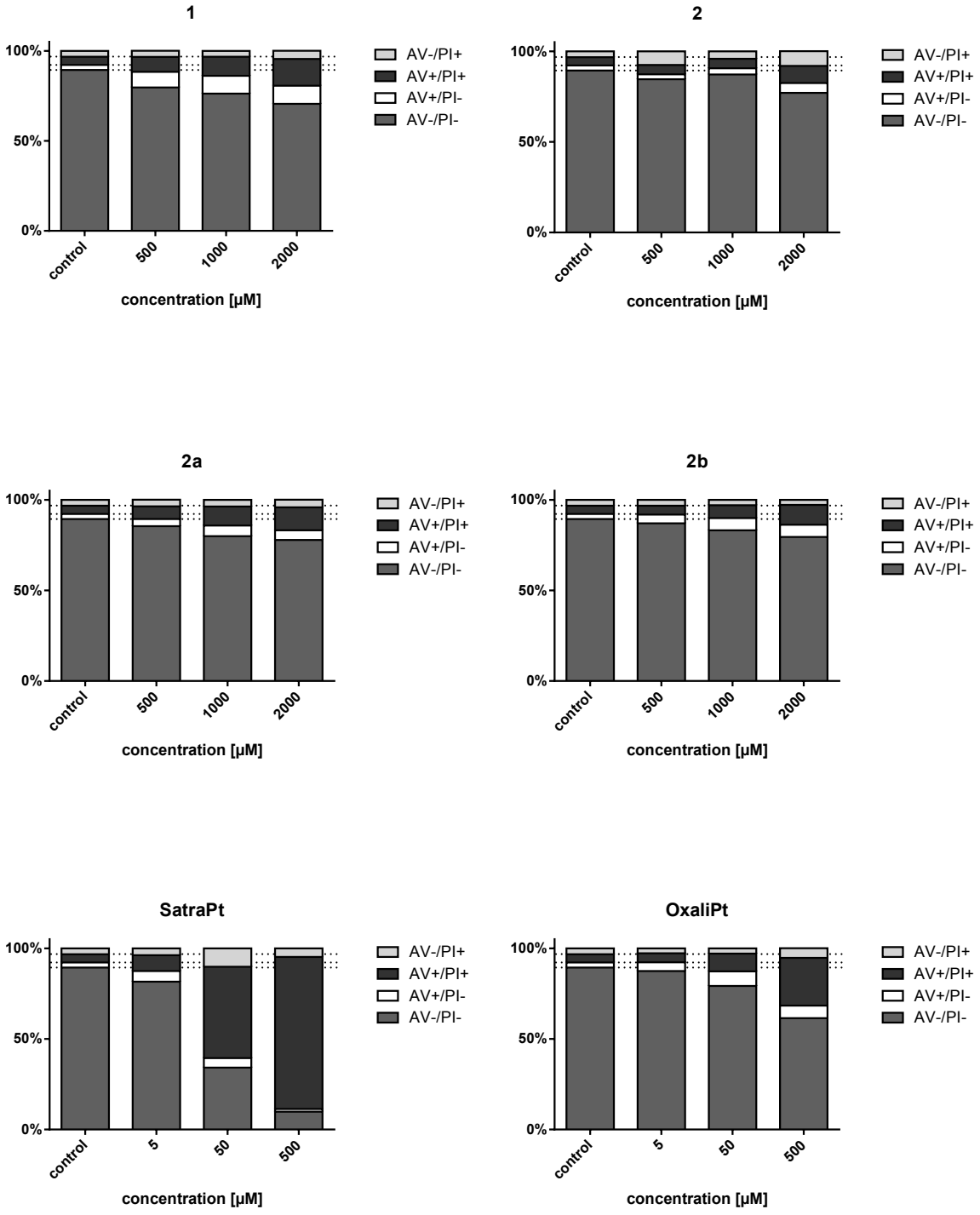


Figure S16 Induction of apoptosis by platinum complexes in HCT116oxR cells. Annexin V-FITC/PI staining and flow cytometry were performed after 48 h continuous exposure to the indicated concentrations. Results are mean percentages of at least three independent experiments.

5. Reactive oxygen species

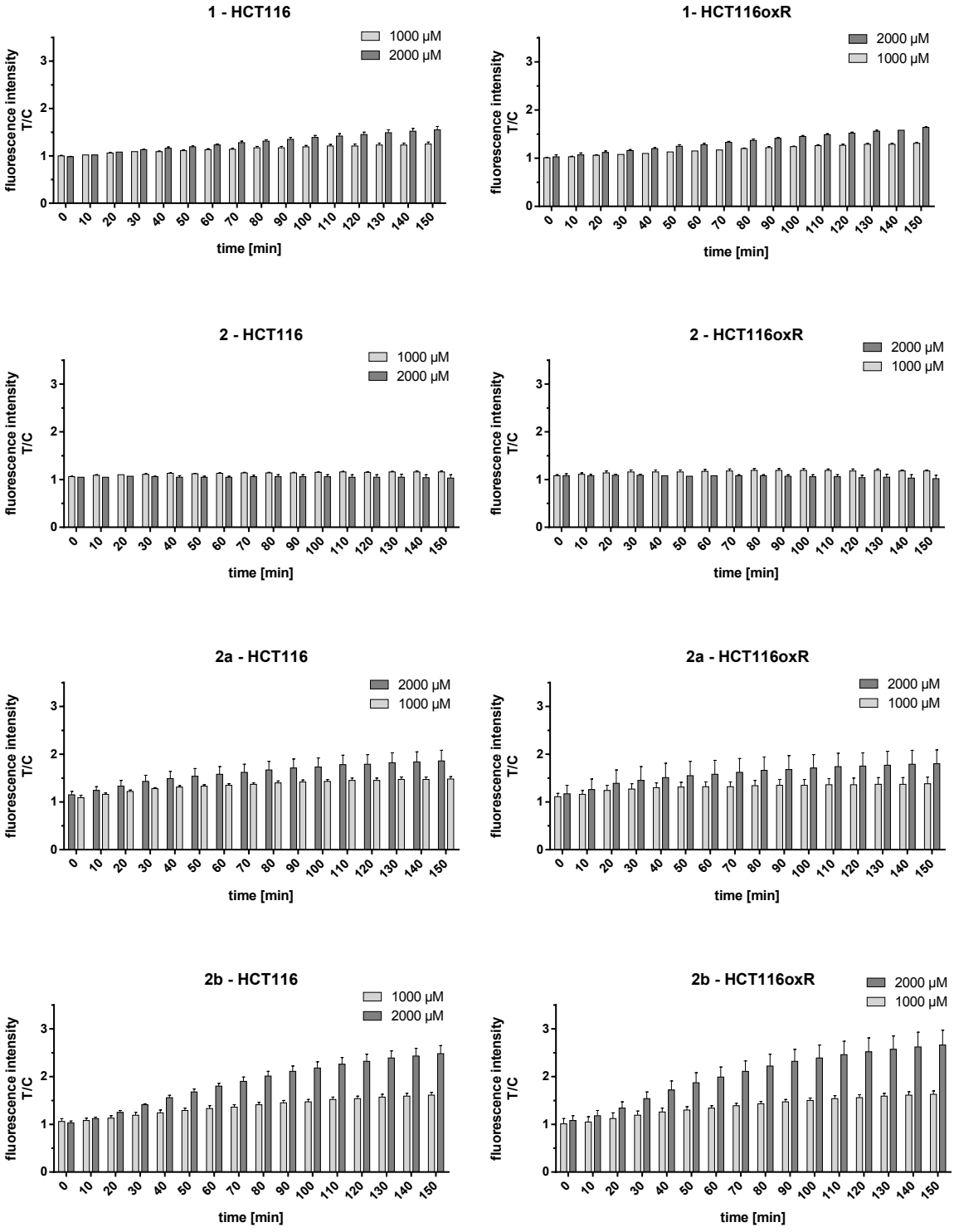


Figure S17 Fluorescence intensity of DCF, measured every 10 min during incubation with platinum complexes at the indicated concentrations. Results are means ± standard deviations of at least three independent experiments for **2a**, **2b** and satraplatin and two independent experiments for **1**, **2** and oxaliplatin where no or negligible effects were observable.

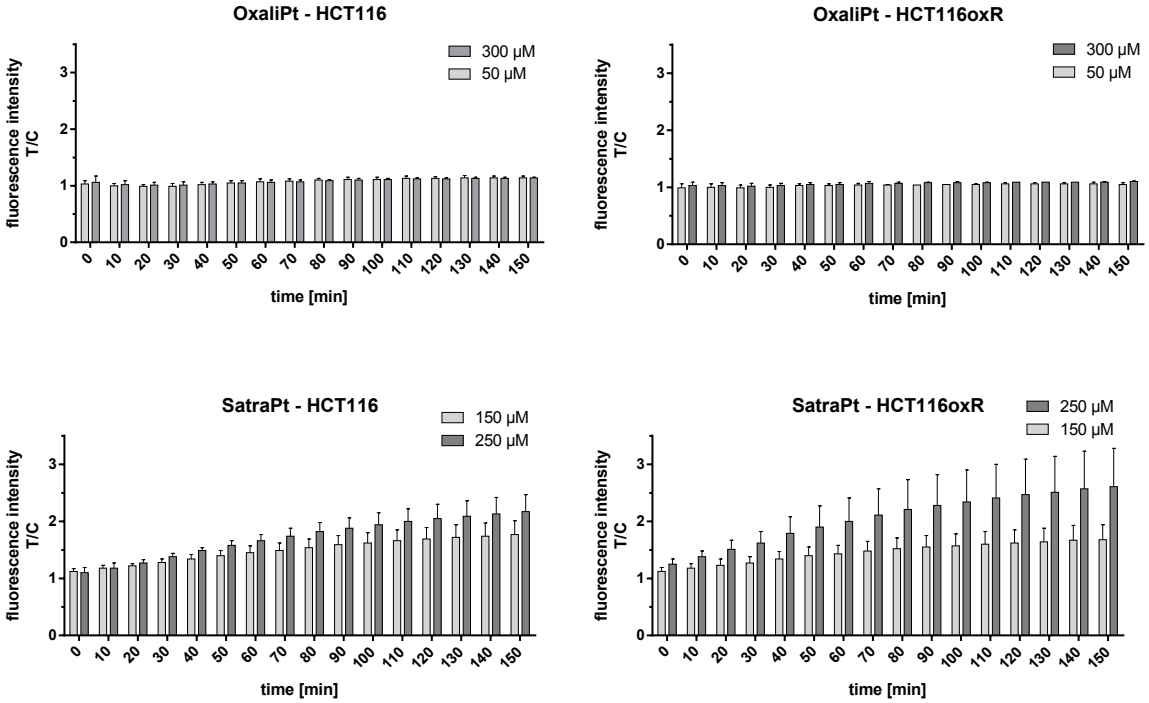


Figure S18 (continued) Fluorescence intensity of DCF, measured every 10 min during incubation with platinum complexes at the indicated concentrations. Results are means ± standard deviations of at least three independent experiments for **2a**, **2b** and satraplatin and two independent experiments for **1**, **2** and oxaliplatin where no or negligible effects were observable.

6. Apoptosis/mitosis in tumor tissue

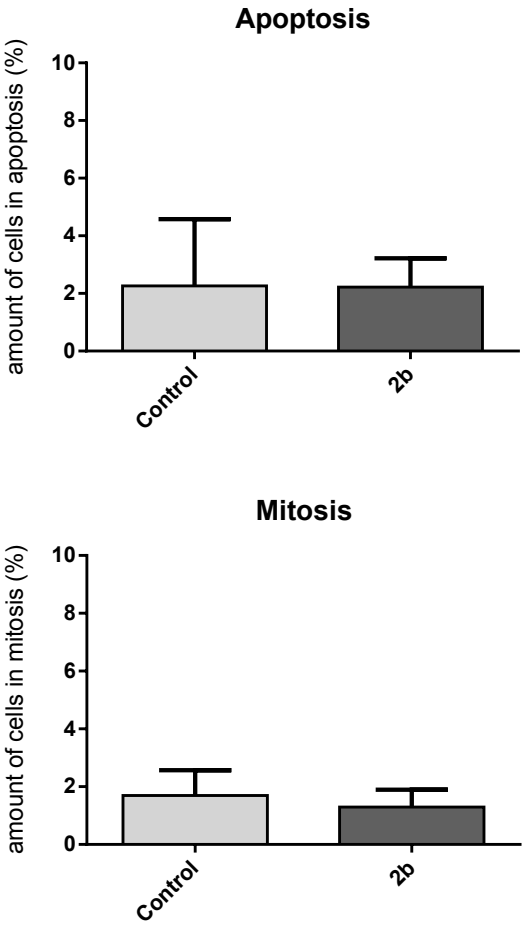


Figure S19 H&E staining of CT26 tumor tissue in BALB/c mice. Apoptotic and mitotic cells were counted in Image J and are given as percentages of total cell counts.

7. Platinum levels in tumor tissue

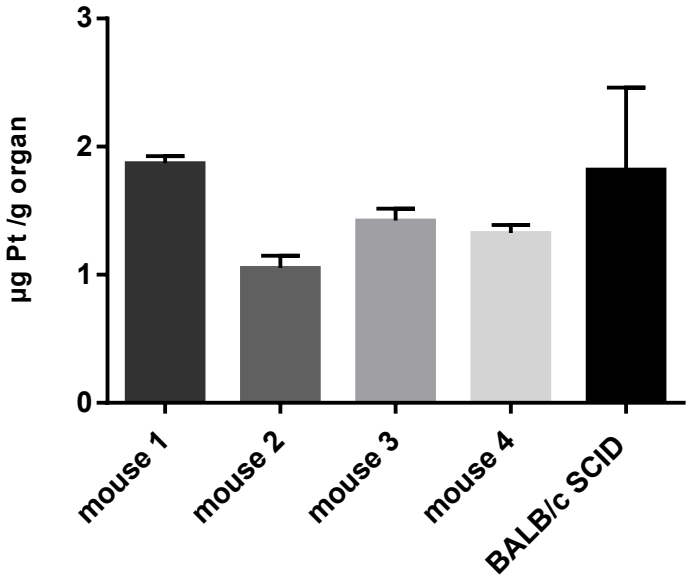


Figure S20 Platinum levels in CT26 tumor tissue from mice treated with **2b**, determined by ICP-MS.

8. CT26 – MTT tests

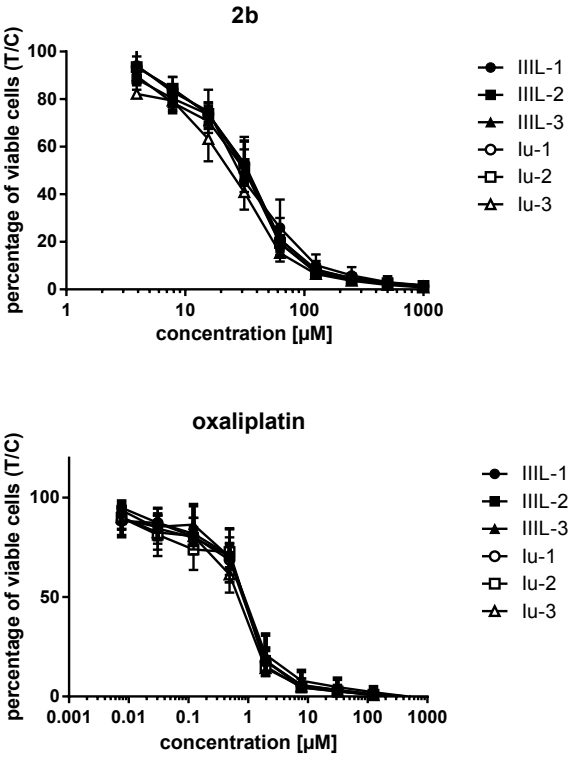


Figure S21 Cytotoxicity (concentration-effect curves) of **2b** and oxaliplatin in CT26 cells isolated from treated (IIIL-1-3) and control (Iu-1-3) animals, obtained by the colorimetric MTT assay after continuous exposure for 96 h.