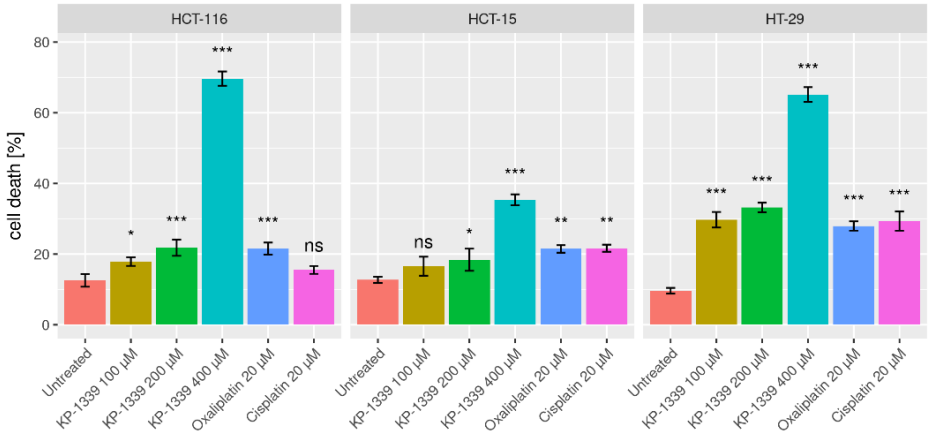
Electronic Supplementary Material (ESI) for Metallomics.

First-in-class Ruthenium Anticancer Drug (KP1339/IT-139) Induces an Immunogenic Cell Death Signature in Colorectal Spheroids *in vitro*

Debora Wernitznig, Konstantinos Kiakos, Giorgia Del Favero, Nathalie Harrer, Herwig Machat, Annika Osswald, Michael A. Jakupec, Andreas Wernitznig, Wolfgang Sommergruber and Bernhard K. Keppler.*

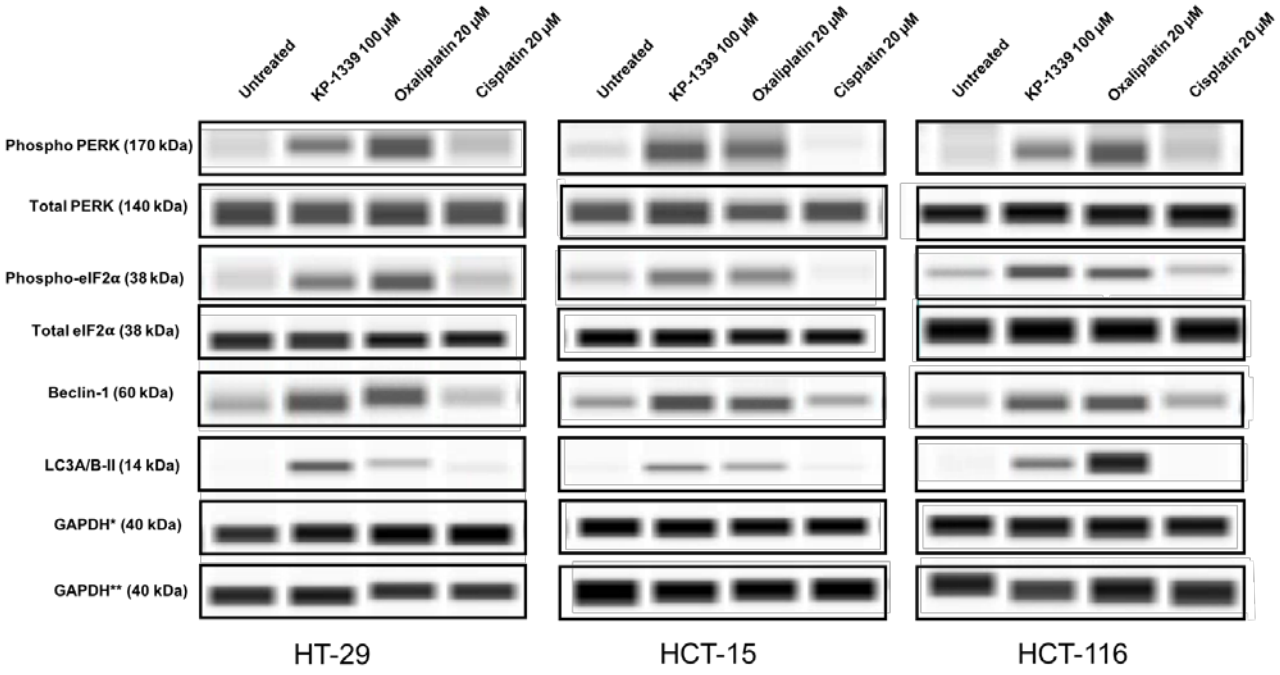
Supporting Information (Supplementary)

Supplementary Figure 1 (SF1)



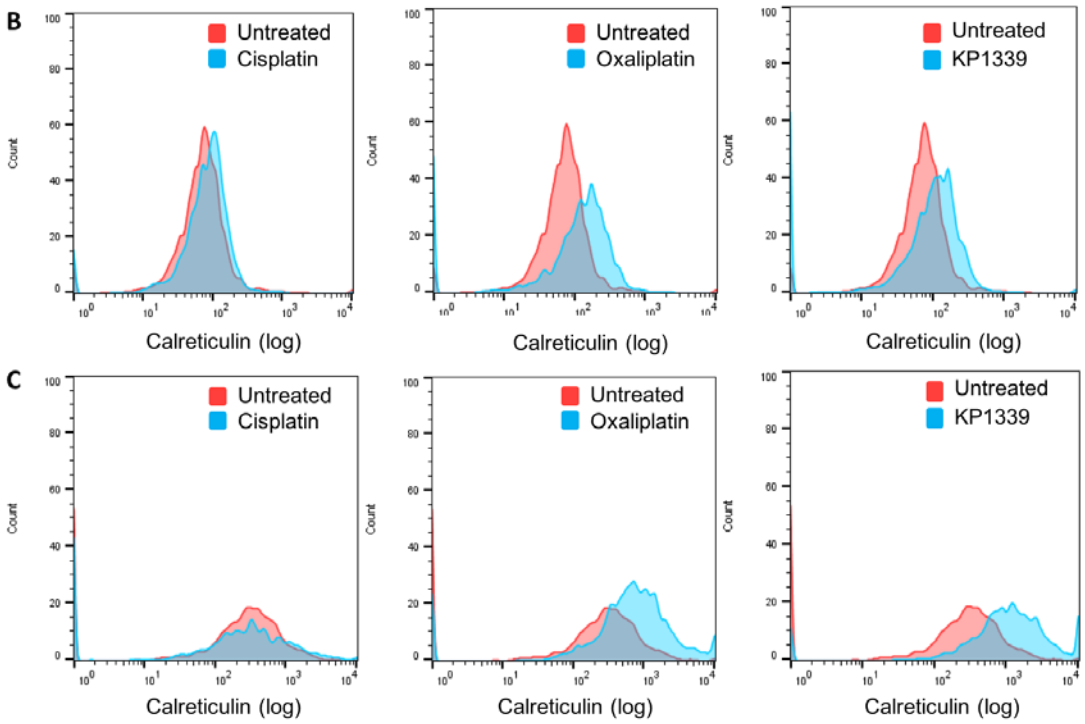
Supplementary Figure 1: Tumor spheroids were treated for 24 h, stained with annexin-V and propidium iodide and analyzed by flow cytometry. Mean + STD (*p≤0.05, **p≤0.01, ***p≤0.001, Tukey's range test), not significant (ns).

Supplementary Figure 2 (SF2)



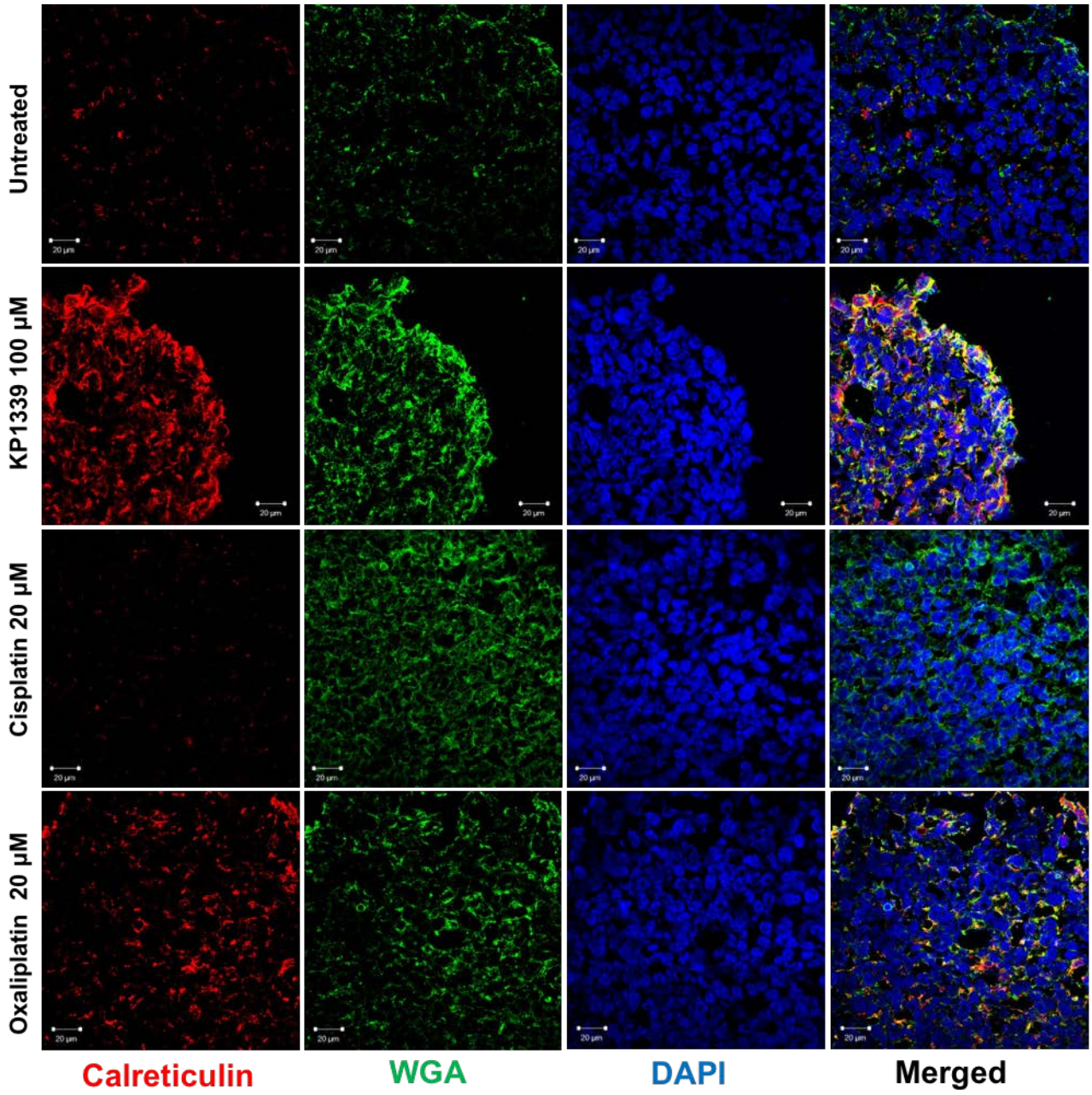
Supplementary Figure 2: Representative immunoblot images from a capillary Western blot system. The total load of PERK, eIF2α, Beclin-1, LC3A/B and GAPDH, respectively, of HT-29, HCT-15 andHCT-116 cells from spheroids is shown. For PERK and eIF2α the phosphorylation status is also analyzed, whereby phosphorylation is only observed upon treatment with KP1339 and oxaliplatin for 24 h. Beclin-1 and LC3A/B-II are upregulated in KP1339 and oxaliplatin treated spheroids only. *GAPDH: Loading control for pPERK, tPERK, p-eIF-2α, Beclin-1 and LC3A/B-II. **GAPDH: Loading control for t-eIF2α

Supplementary Figure 3 (SF3)



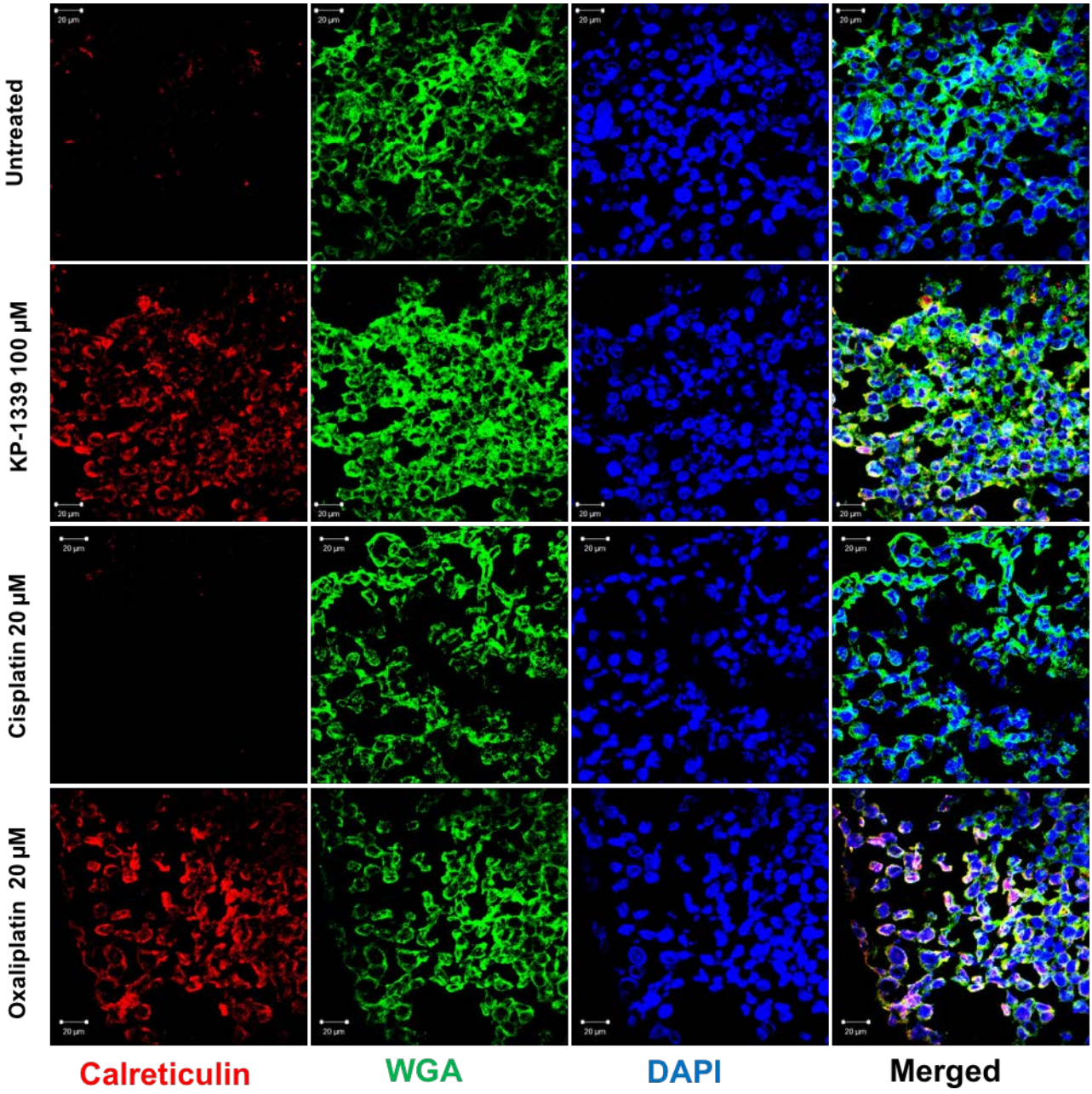
Supplementary Figure 3: Flow cytometry histograms showing increased CRT expression on the surface of HT-29 (left) and HCT-15 (right) spheroids treated with KP1339 and oxaliplatin for 24 h, over untreated (control) and cisplatin treated spheroids.

Supplementary Figure 4 (SF4)



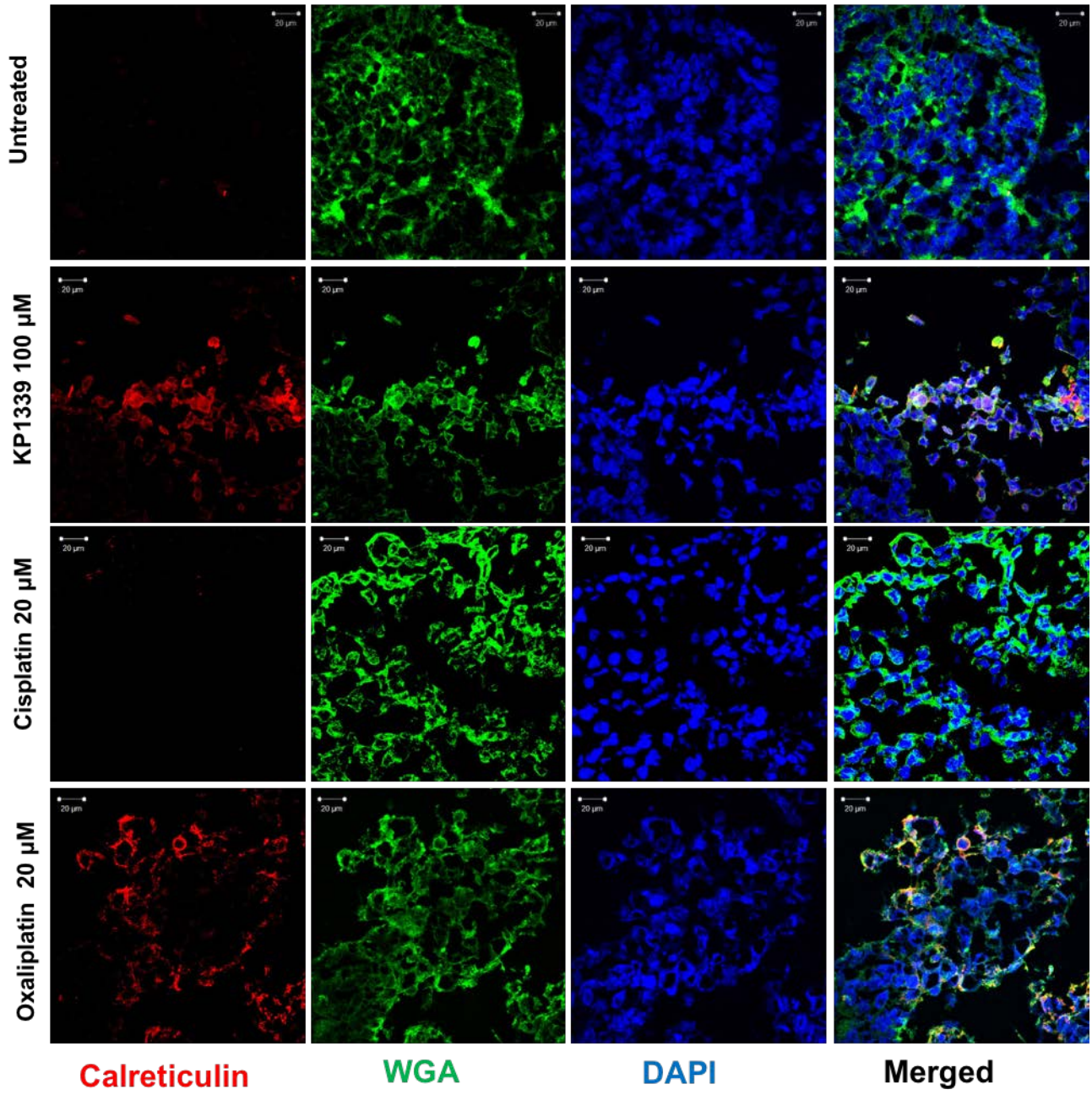
Supplementary Figure 4: Representative immunofluorescence analysis (confocal microscopy) of paraformaldehyde-fixed (PFA) HCT-116 spheroids. Exposure of calreticulin on the cell membrane is observed after 24h treatment with KP1339 (100 μM) and oxaliplatin (20 μM), as indicated by co-localization of calreticulin and membrane associated wheat germ agglutinin (WGA).

Supplementary Figure 5 (SF5)



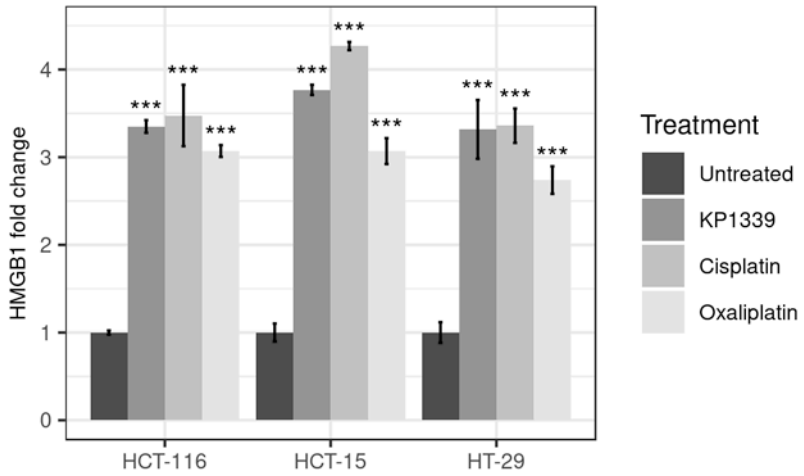
Supplementary Figure 5: Immunofluorescence analysis (confocal microscopy) of paraformaldehyde-fixed (PFA) spheroids. Exposure of calreticulin on the cell membrane is observed after treatment with KP1339 and oxaliplatin for 24 h, as indicated by the co-localization of calreticulin and membrane associated wheat germ agglutinin (WGA) in HT-29 spheroids.

Supplementary Figure 6 (SF6):



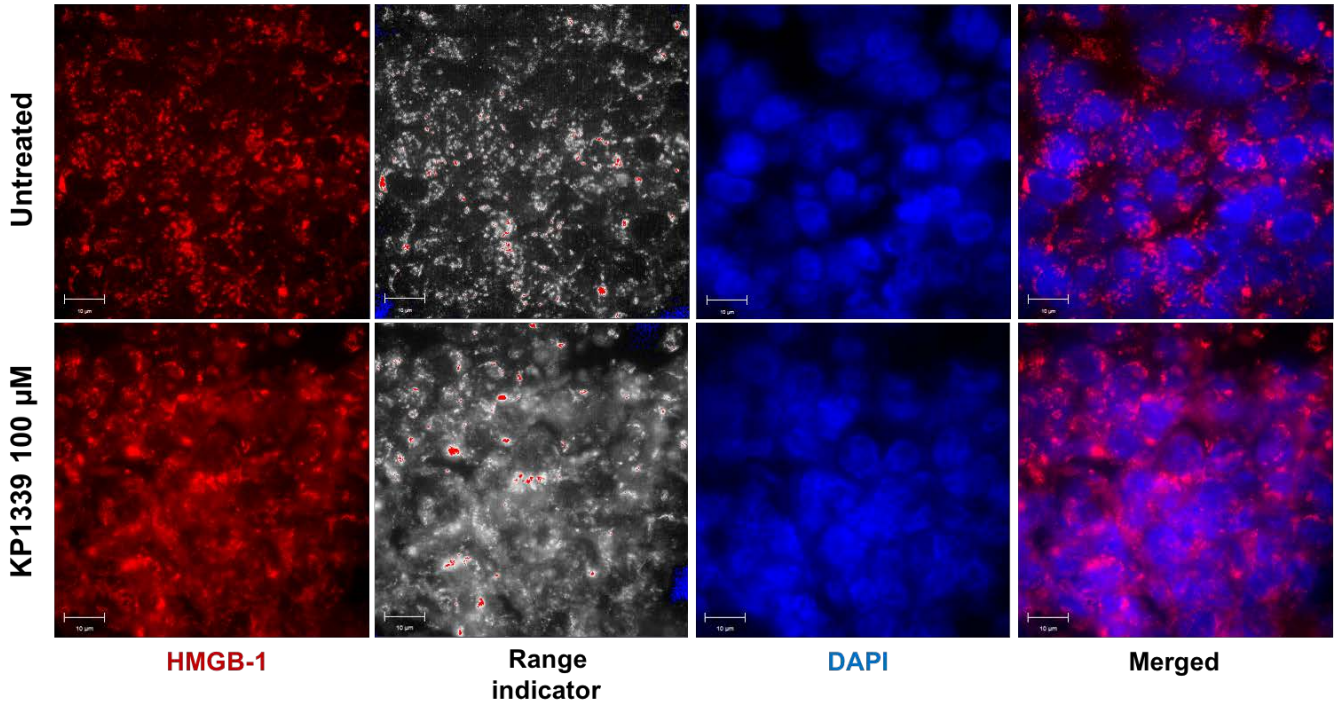
Supplementary Figure 6: Immunofluorescence analysis (confocal microscopy) of paraformaldehyde-fixed (PFA) spheroids. Exposure of calreticulin on the cell membrane is observed after treatment with KP1339 and oxaliplatin for 24 h, as indicated by the co-localization of calreticulin and membrane associated wheat germ agglutinin (WGA) in HCT-15 spheroids.

Supplementary Figure 7 (SP7)



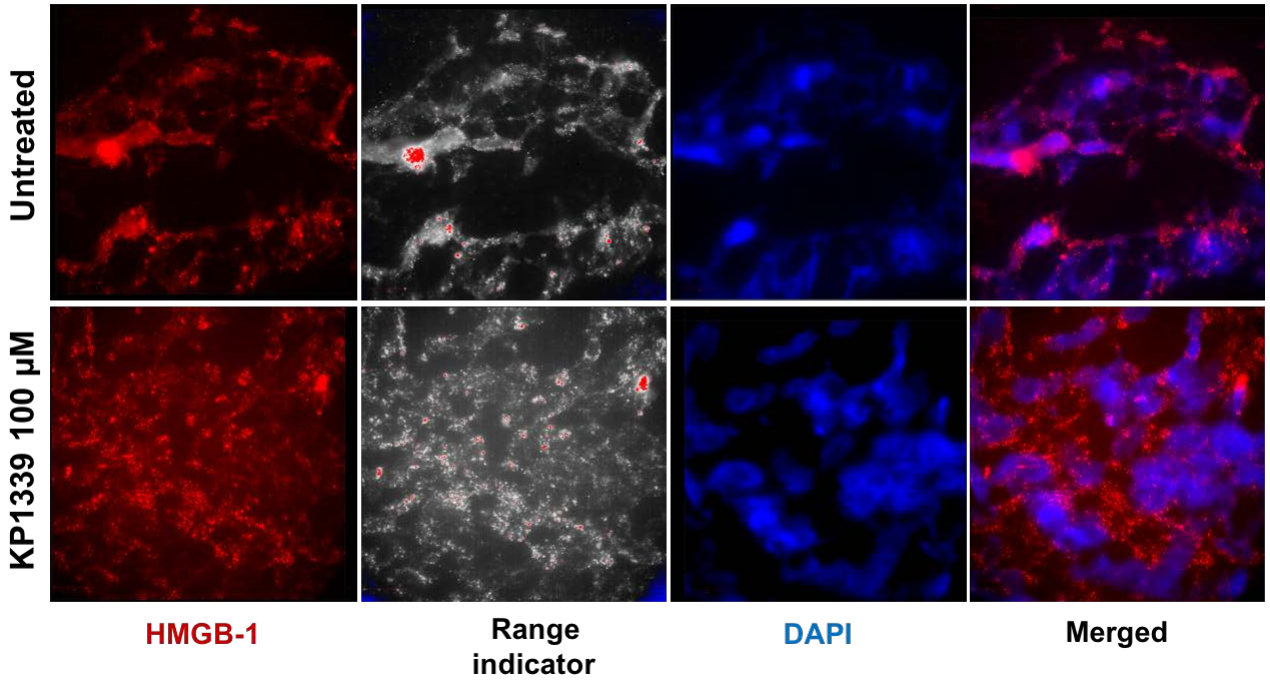
Supplementary Figure 7: HMGB-1 release upon treatment for 24h with KP1339 (100 μM), oxaliplatin (20μM) and cisplatin (20 μM). Mean + STD (*p≤0.05, **p≤0.01, ***p≤0.001; Tukey's range test).

Supplementary Figure 8 (SF8):



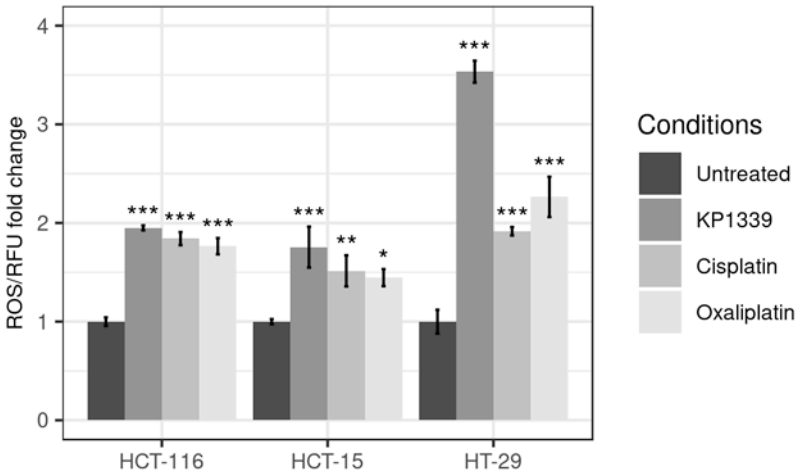
Supplementary Figure 8: Representative immunofluorescence analysis (Structure Illumination Microscopy) of paraformaldehyde-fixed (PFA) spheroids. Release of HMGB-1 (red and range indicator) into the cytoplasm of HT-29 spheroids is observed after 24h treatment with KP1339 (100 μM).

Supplementary Figure 9 (SF9):



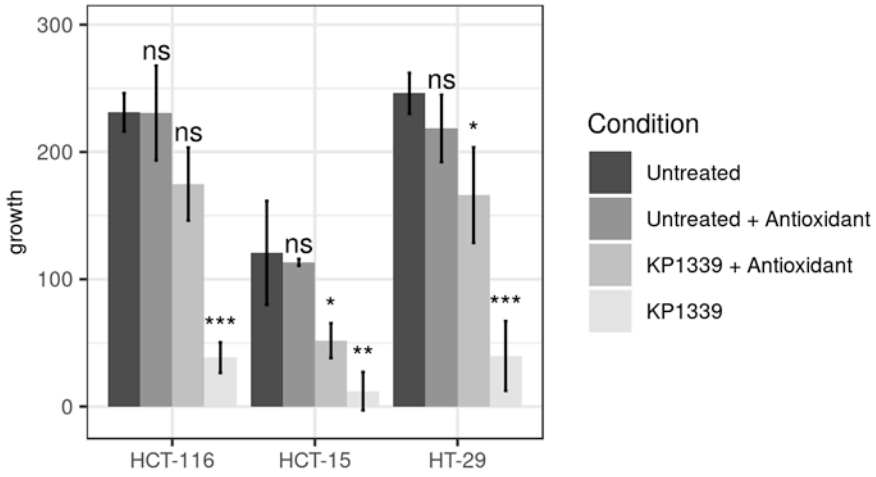
Supplementary Figure 9: Representative immunofluorescence analysis (Structure Illumination Microscopy) of paraformaldehyde-fixed (PFA) spheroids. Release of HMGB-1 (red and range indicator) into the cytoplasm of HCT-15 spheroids is observed after 24h treatment with KP1339 (100 µM).

Supplementary Figure 10 (SF10)



Supplementary Figure 10: ROS induction upon treatment for 24h with KP1339 (100 µM), oxaliplatin (20µM) and cisplatin (20 µM). Mean + STD (*p≤0.05, **p≤0.01, ***p≤0.001; Tukey's range test).

Supplementary Figure 11 (SPF11):



Supplementary Figure 11: Growth (in µm) of spheroids treated for 96h with KP1339 (100 µM), KP1339 + antioxidant (100 µM), untreated + antioxidant or and untreated only. Mean + STD (*p≤0.05, **p≤0.01, ***p≤0.001; Tukey's range test; not significant (ns).

Material and Methods:

Cell Culture. Human colon carcinoma cell lines HCT-116 and HT-29 were kindly provided by Dr. Brigitte Marian, Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, and HCT-15 was obtained from ATCC® (CCL-247™). HCT-116 and HT-29 cells were maintained in McCoy's 5a medium supplemented with 10% fetal calf serum (FCS) and L-glutamine. HCT-15 cells were grown in RPMI 1640 medium supplemented with 10% FCS and L-glutamine. All cells were cultured as adherent monolayers in 75 cm^2 flasks and kept in a humidified incubator at 37 °C with 5% CO_2. All cell culture media and reagents were obtained from Sigma-Aldrich (Vienna, Austria), and all plastic dishes, plates and flasks were from StarLab (Germany) unless stated otherwise.

Spheroid formation. All experiments were performed using spheroids. For spheroid generation, HCT-116, HT-29 and HCT-15 cells were harvested from culture flasks by trypsinization, resuspended in their respective supplemented medium and seeded in ultra-low attachment round-bottom 96-well plates (Nunclon Sphera™, Thermo Fisher Scientific) at a density of 10.000 viable cells per well. Plates were incubated at 37 °C with 5% CO_2 for 96 hours to allow spheroid formation and then used for the experiments.

Compounds. All compounds: KP1339, oxaliplatin and cisplatin were synthesized at the Institute of Inorganic Chemistry, University of Vienna, as described previously[26,46,47].

Alamar Blue Test. KP1339 was first dissolved in DMSO, and stock solutions were prepared in the appropriate medium according to the cell line and diluted stepwise to obtain a serial dilution. 100 µl of the respective dilutions were added to each well, and the plates were incubated for 96 hours at 37 °C with 5% CO_2. A 440 µM resazurin sodium salt (Sigma-Aldrich, Austria) in PBS solution was prepared and 20 µl were added to each well. The plates were incubated for 16 hours at 37 °C with 5% CO_2. Fluorescence was measured with a Synergy HT reader (BioTek).

Treatment. For testing the ability of KP1339 to induce ICD *in vitro,* 3D tumor spheroids were first treated with the compounds as follows: KP1339 (100 µM), oxaliplatin (20 µM) and cisplatin (20 µM) were used as positive and negative controls, respectively. Spheroids were incubated with the compounds for 24 hours at 37 °C with 5% CO_2 and then processed for further experiments. All the concentrations were carefully selected based on the IC50 values obtained by the Alamar Blue test. The IC50 values after 96 h of drug exposure were selected for performing the experiments.

Flow Cytometry Analysis. After treatment, spheroids were collected and pooled. Samples were washed with PBS followed by dissociation with Tryple Express (Gibco, Austria) for 15 minutes at 37 °C. 500 µl medium containing 10% FCS were added to each tube to stop trypsinization. Samples were centrifuged at 2200 g for 3 minutes and resuspended in ice cold FACS buffer containing the primary antibody: Calreticulin (#ab2907 Abcam, United Kingdom) and incubated for 30 minutes at 4 °C. Samples were washed twice and resuspended in FACS buffer containing the secondary antibody Goat Anti Rabbit Alexa Fluor 488 (# ab150077Abcam, United Kingdom) and incubated at 4 °C for 20 minutes. Samples were washed twice, resuspended in FACS buffer containing propidium iodide and transferred into 96-well plates for measurements. Samples were measured immediately by using a

Guava Soft flow cytometer (Merck/Millipore, Germany). For the apoptosis assay, samples were incubated with anti-annexin-V FITC conjugated antibody (eBioscience) for 15 minutes followed by nuclei staining using propidium iodide (Sigma Aldrich) in annexin-V binding buffer solution. Samples were analyzed immediately by using a Guava EasyCyte flow cytometer (Merck/Millipore). The results were analyzed using FlowJo software.

Immunofluorescence staining. Spheroids were collected, pooled and embedded in TissueTek (Sakura, Japan). Samples were frozen at -80 °C until further processing. Samples were cut in 5 µm thick sections by using a Cryostat (Leica, Germany) and placed onto Superfrost slides (Thermo Fisher Scientific, Austria). For immunofluorescence staining, slides were brought to room temperature and fixed with a 0,25% PFA solution for 15 minutes followed by 3 washing steps with PBS. Samples were then permeabilized with a 0.1% Triton-X solution for 10 minutes for the following target/staining: HMGB-1(#3935, Cell Signaling Technology). In the case of Calreticulin (#ab2907 Abcam, United Kingdom), samples were not permeabilized. After additional washing steps, samples were incubated with the respective primary antibody at 4 °C overnight. Samples were washed with PBS and incubated with the secondary antibody: Goat anti-rabbit Alexa Fluor 594 (abcam #ab150080) for 45 minutes at room temperature. For Calreticulin, slides were incubated with a wheat germ agglutinin solution (Invitrogen) according to the manufacturer's instructions for 20 minutes and washed three times with PBS. Slides were mounted with ProlonGold Antifade Reagent with DAPI (Molecular Probes) and analyzed with a Confocal Microscope. For the SIM imaging a Laser Scanning Microscope Zeiss 710 with Elyra system PS.1 was used. Images were acquired with Plan Apochromat 63X/1.4 oil DIC M27 objective and using 3 grid rotations. Images were processed with the software Zen Zeiss applying deconvolution algorithm for the rendering of the HMGB1. Range indicator: gray (min.) to red (max.) scale.

Reactive Oxygen Species (ROS) generation. Spheroids were washed twice with warm HBSS supplemented with 1% FCS. Cells were stained for 2 hours with a 25 µM DCFH-DA solution (Sigma Aldrich) at 37 °C. Cells were washed twice with HBSS supplemented with 1% FCS. Test compound dilutes were prepared as described before, but in phenol-free media supplemented with 1% FCS and 100 µl were added to each well. Fluorescence was measured for 8 hours with a Synergy HT reader (BioTek) at 516 nm.

ATP Measurements. Spheroids were treated for 24 hours, and supernatants were collected and centrifuged at 5000 g for 5 minutes. Supernatants were transferred into a white opaque flat-bottom 96-well CulturPlate (PerkinElmer), and 100 µl of Cell titer Glo solution (Promega) were added to each well. Samples were incubated for 15 minutes at room temperature, and luminescence was recorded by using a Synergy HT reader (BioTek).

Western Blotting. Spheroids were collected, pooled and washed three times with cold PBS and placed on ice. Cells were then lysed for 3 hours with lysis buffer consisting of Cell Lytic M (Sigma Aldrich), Phosphatase inhibitor cocktail (Roche, Switzerland) and cOmplete (Roche, Switzerland). Lysates were centrifuged at 14000 g for 30 minutes at 4 °C, and supernatants were transferred to a fresh microfuge tube. For protein concentration measurements, 5 µl of sample were mixed with 1 mL Bradford solution (Biorad), and wave lengths were measured with a spectrophotometer at 590 nm. A WES Protein Simple System was used for protein detection according to manufacturer's instructions. The following antibodies were used: Calreticulin (#2891), PERK (#3192), phospho-PERK (#3179), phospho-eIF-2alpha (#9721), eIF-2alpha (#5324), LC3A/B (#12741) and Beclin-1 (#3495), all from Cell Signaling Technologies.

ELISA HMGB-1. Supernatants originated from the 3D cultures were collected after 24 hours of drug-treatment (KP1339, oxaliplatin, cisplatin or untreated), centrifuged and stored at -20°C until the measurements. Supernatants were brought to room temperature and were processed according to the manufacturer's instructions (Human HMGB1 ELISA Kit, FineTest). OD absorbance values were measured Synergy HT reader (BioTek) at 450 nm.

Statistics. All the results originate from at least three technical and biological replicates. ANOVA and Tukey's range test were applied to analyze the apoptosis, ROS, HMGB-1 and ATP data.

References.

[26] L. S. Flocke, R. Trondl, M. A. Jakupec, B. K. Keppler. *Ivest. New Drugs*, **2016**, *34*, 261–268
[46] Y. Kidani, K. Inagaki. *J. Medi. Chem.* **1978**, 21(12):1315-1318.
[47] W. Peti, T. Pieper, M. Sommer, B. K. Keppler, G. Giester.*Eur. J. Inorg. Chem.* **1999**, 1551-1555.