Electronic Supplementary Information (ESI)

Plecstatin-1 induces an immunogenic cell death signature in colorectal tumor spheroids

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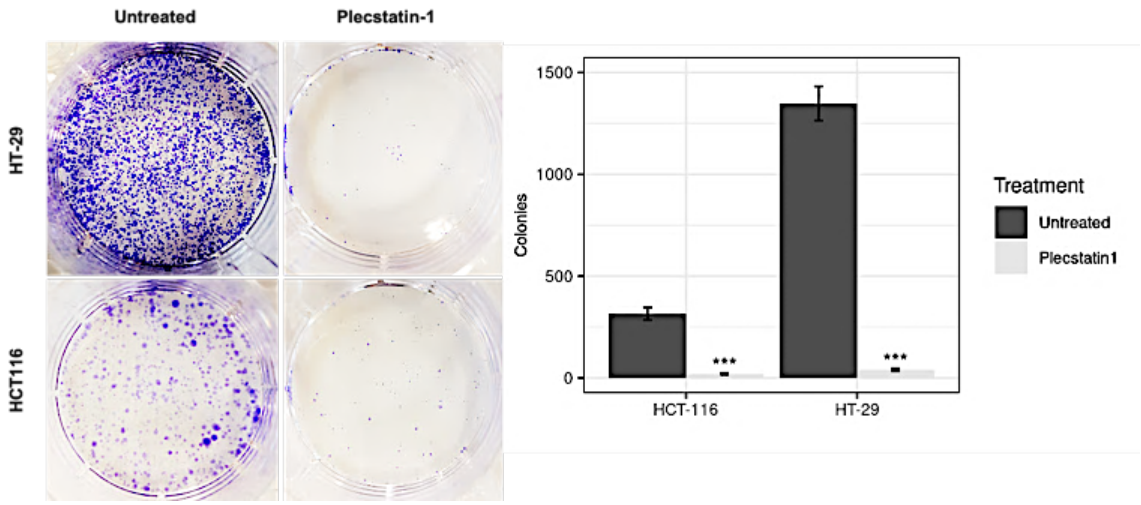
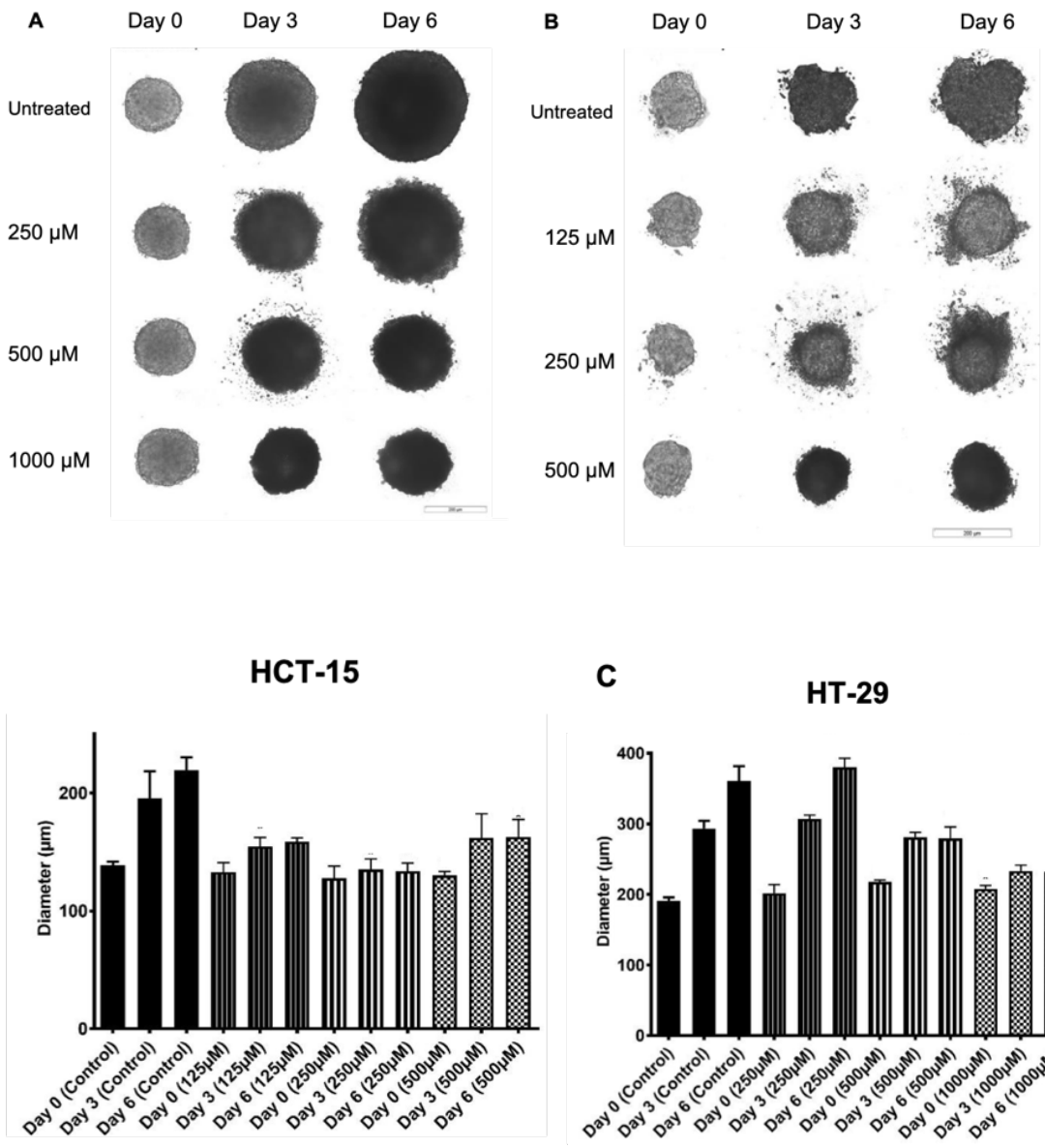
	IC_{50} [µM]	
Cell lines	**2D cell culture**	**3D tumour spheroids**
HCT-15	32 ± 4	>200
HCT-116	18 ± 5	>200
HT-29	24 ± 2	>200

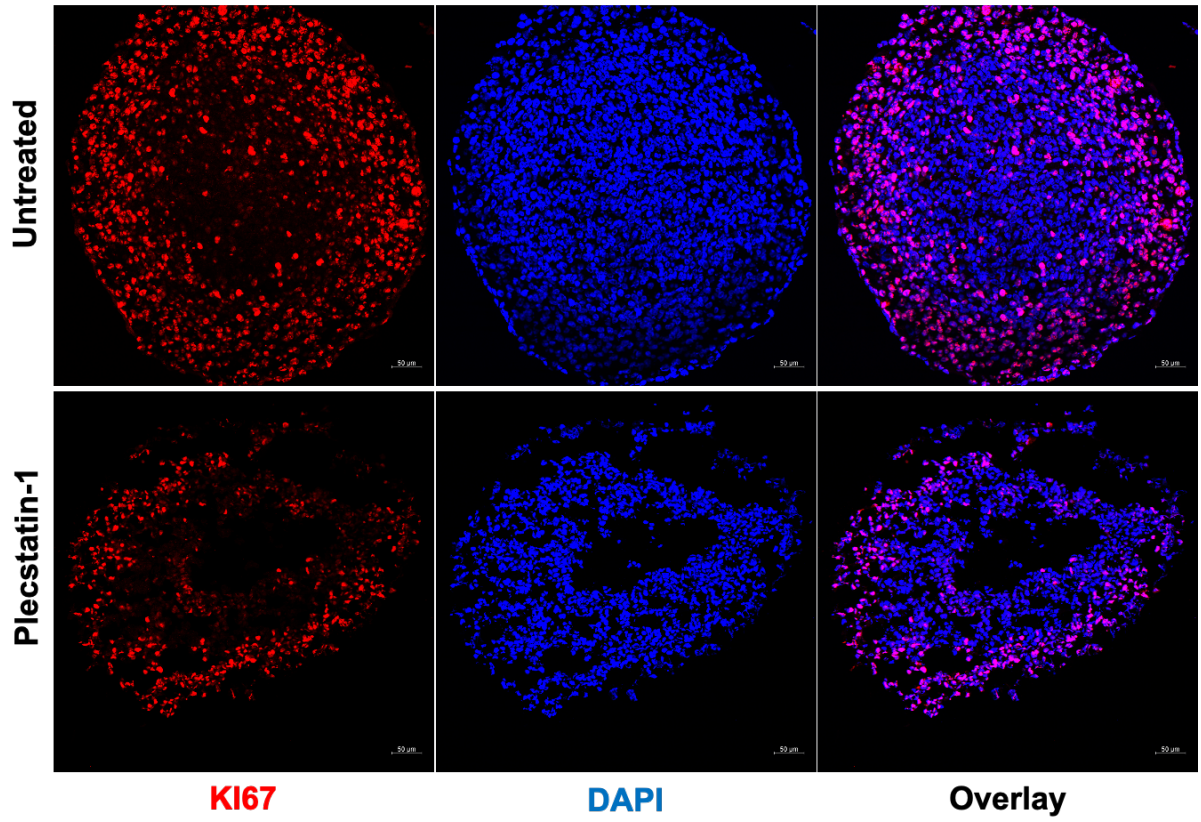
Supplementary Table ST1: Concentrations for inhibiting 50% of cell growth (IC_{50}-values, expressed in µM) for three colon adenocarcinoma cell lines grown as monolayers (2D) and as multicellular spheroids (3D) treated with plecstatin-1. The compound was more potent in 2D compared to 3D.

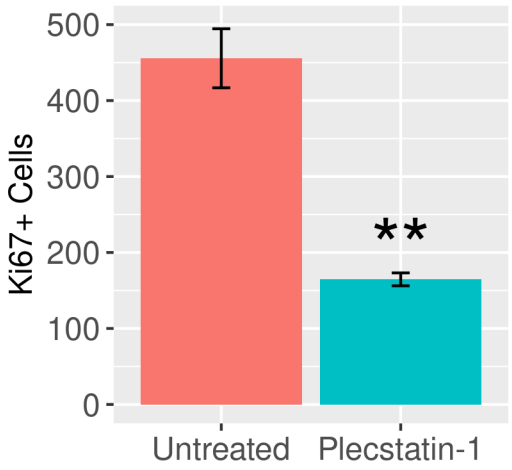


Supplementary Figure SF1: Representative pictures of the colony formation assay with plecstatin-1-treated HT-29 and HCT-116 cancer cells in monolayers. Plecstatin-1 treatment reduced proliferation of HT-29 and HCT-116 cells *in vitro.* Treatment with plecstatin-1 significantly decreased the colony formation potential in HT-29 and HCT-116 cells (Mean $\pm$ STD, ***$p \leq 0.001$).

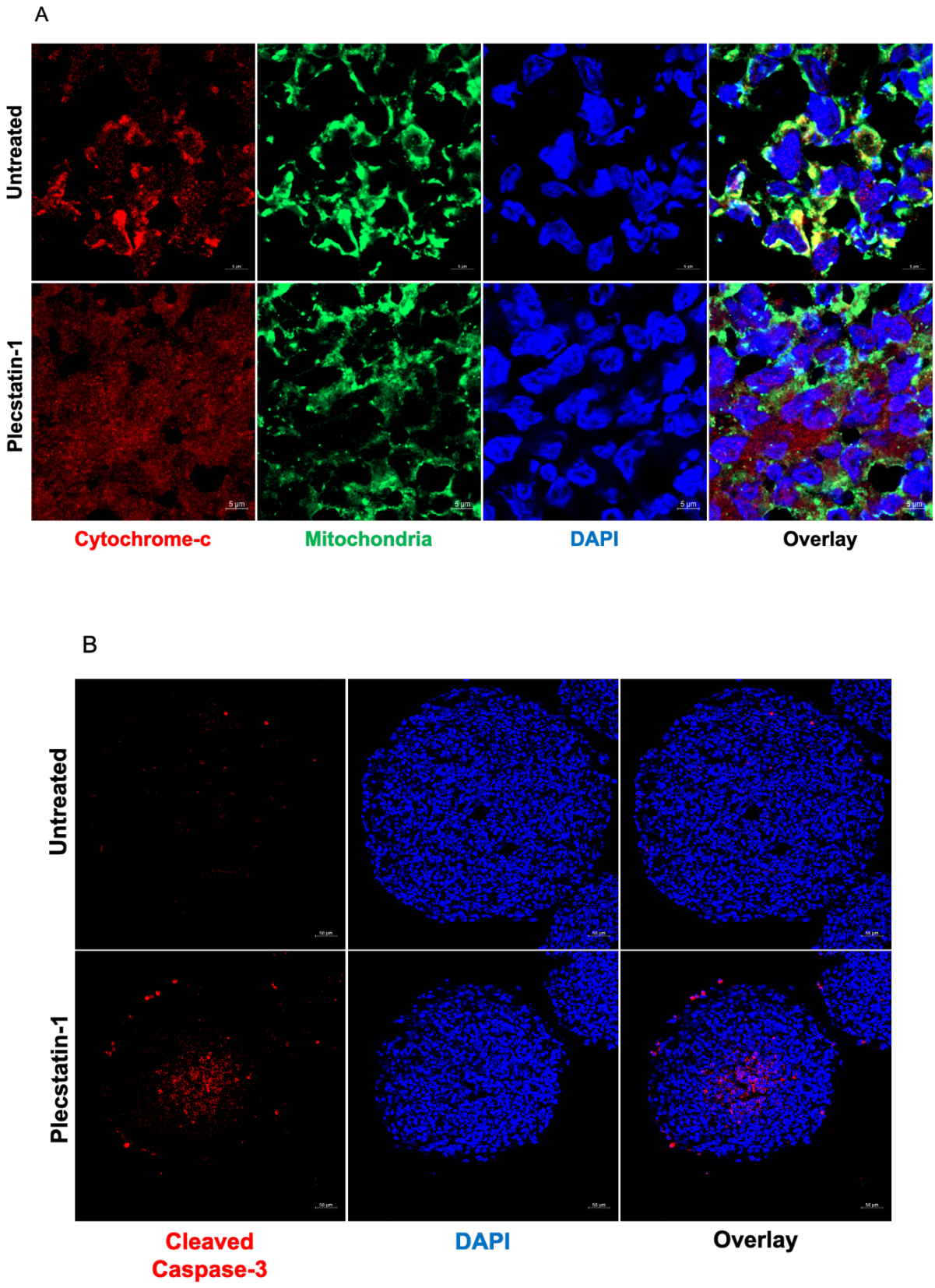


Supplementary Figure SF2: Representative pictures of HT-29 (**A**) and HCT-15 (**B**) multicellular spheroids treated with plecstatin-1 for 6 days. Changes in the morphology can be observed in both cell lines upon treatment with plecstatin-1. (**C**) Plecstatin-1 treatment inhibited growth in both cell lines, and even at sub-IC_{50} concentrations in HCT-15 spheroids.



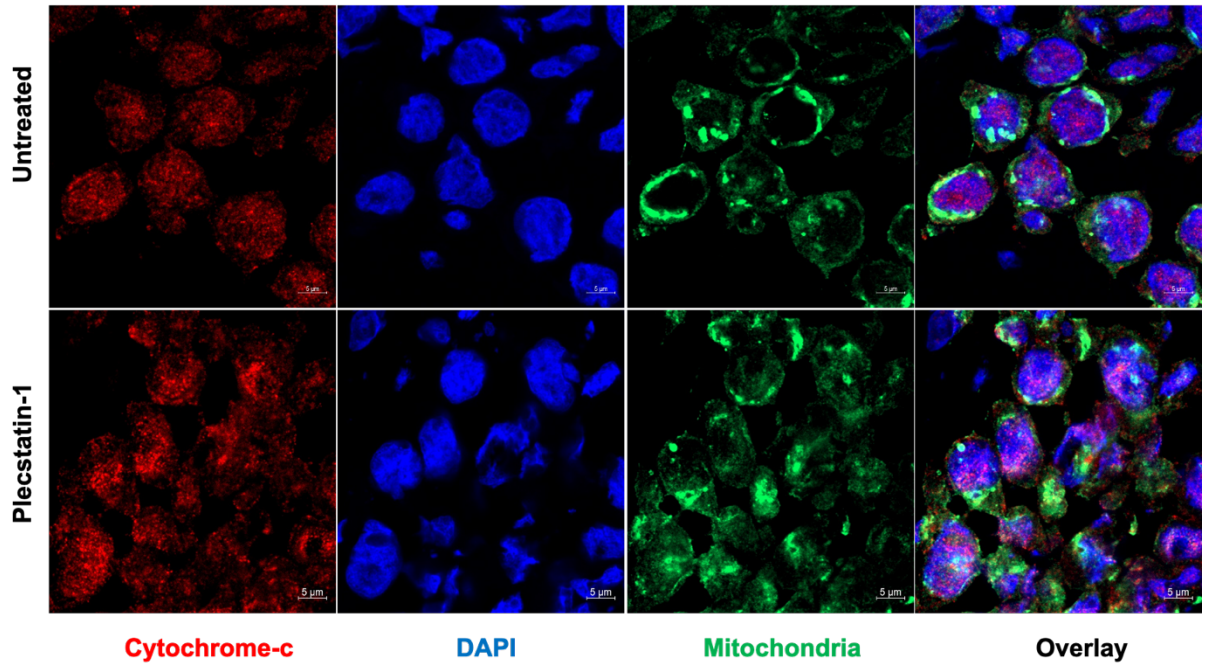


Supplementary Figure SF3: Representative immunofluorescence analysis (confocal microscopy) of paraformaldehyde (PFA) -fixed HCT-116 spheroids. A 3-fold reduction in the number of KI67^{+} cells is observed after 72 h treatment with plecstatin-1 (200 µM) in comparison to the untreated control. Scale Bar 50 µm. (t-test, p-value = 0.004).

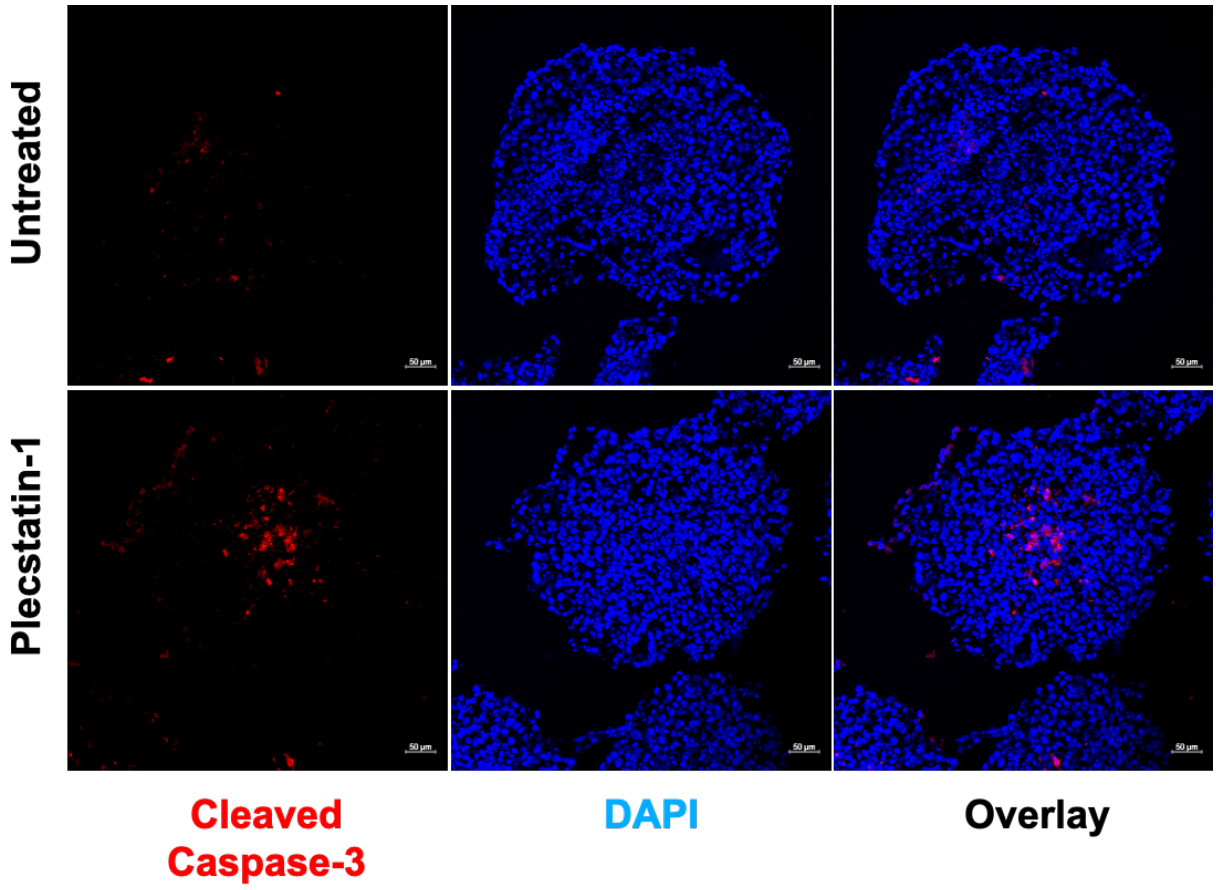


Supplementary Figure SF4: (A) Representative immunofluorescence analysis (confocal microscopy) of HCT-116 spheroids. Mitochondria integrity is shown in untreated spheroids,

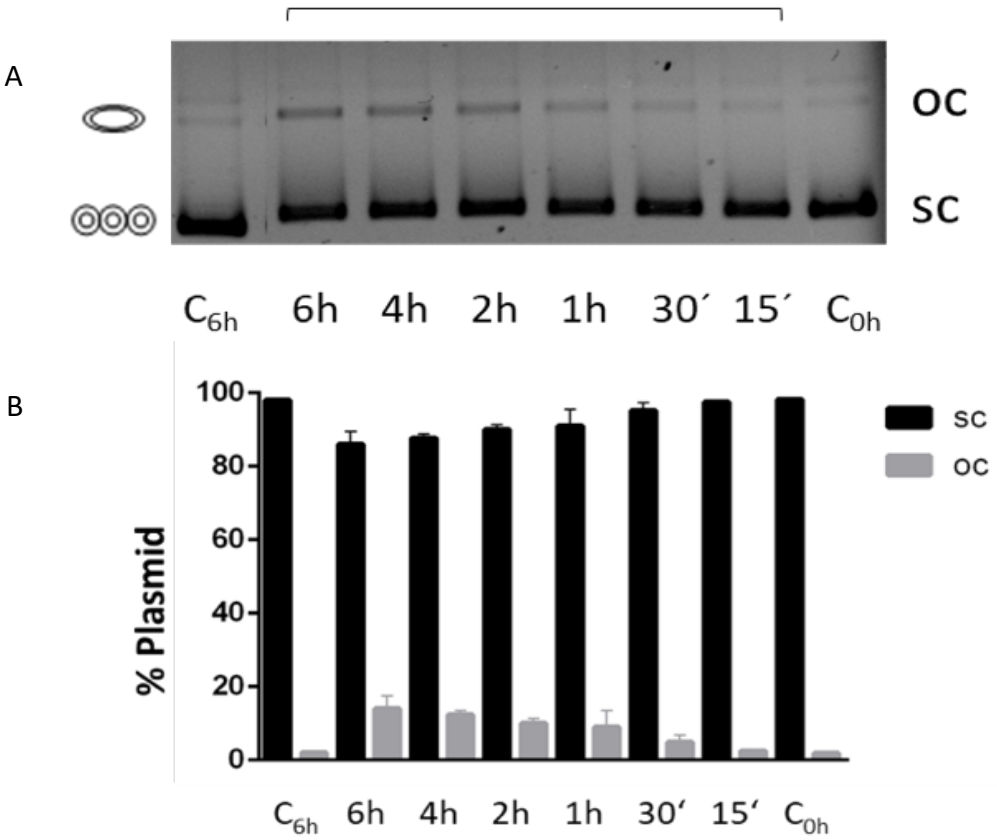
while cytochrome c release from mitochondria is observed upon treatment with plecstatin-1 (200 µM) for 72 h. Note that the cytochrome-c immunostaining overlapped as indicated by yellow regions with the mitochondrial marker in the cytoplasm of control cells. However, in plecstatin-1 treated cells cytochrome-c leaked from mitochondria and was distributed distinctly from the mitochondrial marker. Scale bar 5 µm. (B) Representative immunofluorescence analysis by confocal microscopy of PFA fixed HCT-116 spheroids. Plecstatin-1 (200 µM) induced the activation of caspase-3 after 72 h treatment. Scale bar 50 µm.



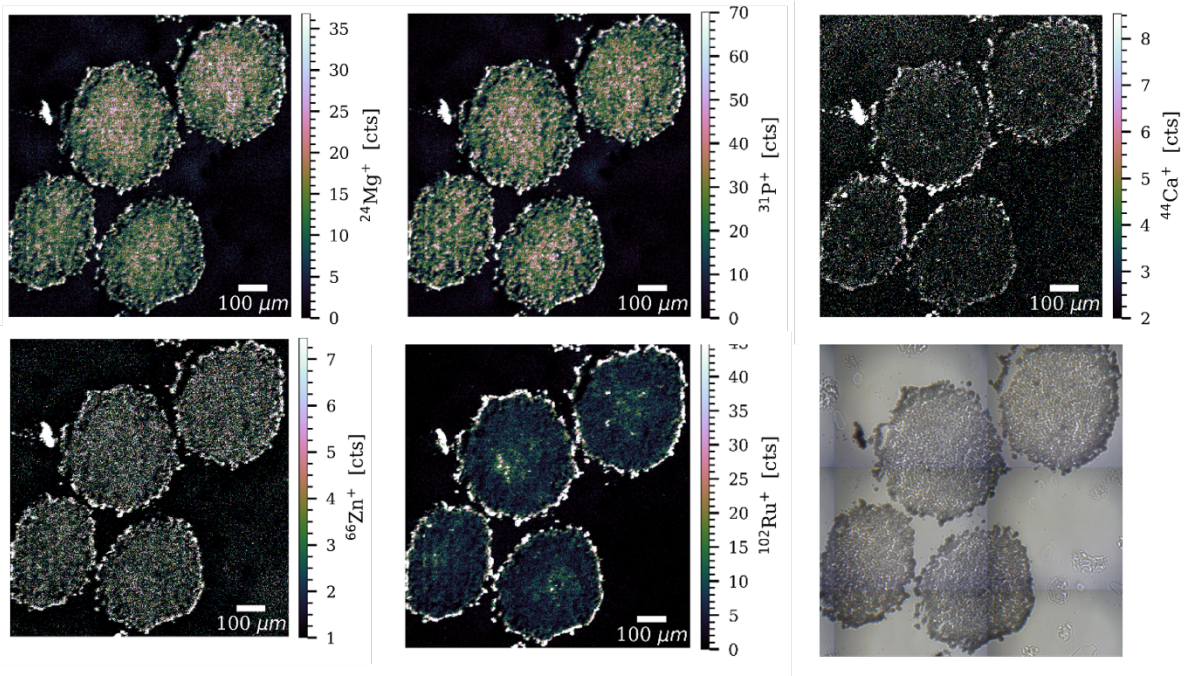
Supplementary Figure SF5: Representative immunofluorescence analysis (confocal microscopy) of HT-29 spheroids. Mitochondria integrity is shown in untreated spheroids, while a mild cytochrome c release from mitochondria is observed upon treatment with plecstatin-1 for 72 h. Scale bar 5 µm.



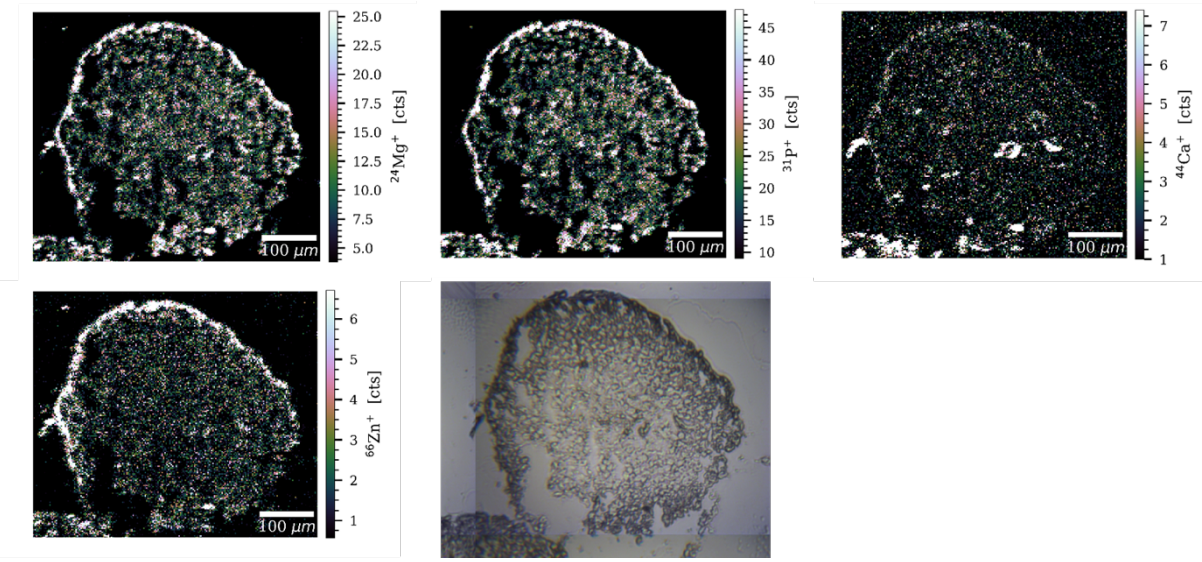
Supplementary Figure SF6: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Plecstatin-1 induces cleavage of caspase-3 after 72 h treatment, however the increased signal is not so pronounced as observed in HCT-116 spheroids. Scale bar 50 µm.



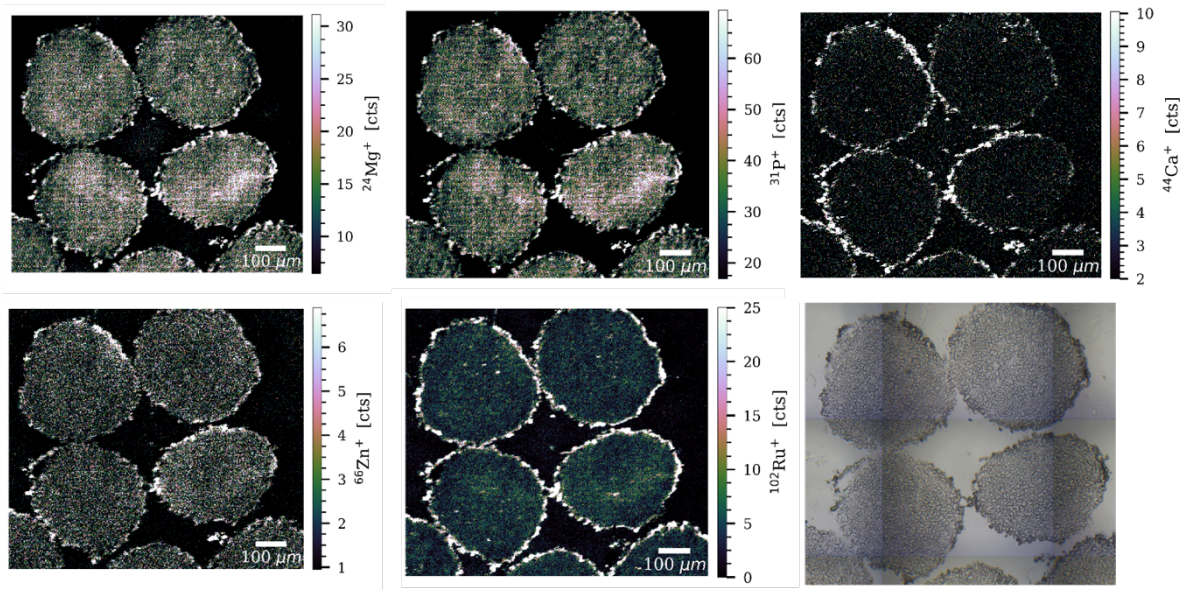
Supplementary Figure SF7: (**A**) Results of the supercoiled pUC19 DNA cleavage assay of the ruthenium complex by using agarose gel electrophoresis. The pUC19 plasmid was incubated with plecstatin-1 (50 µM) for 15 min, 30 min, 1 h, 2 h, 4 h and 6 h at 37 °C in 1x TE buffer. On the presented image, the 'C_{6h}' lane corresponds to the untreated control sample for the total duration of the experiment (6 h incubation), while 'C_{0h}' corresponds to the equivalent, non-modified DNA sample, at the end of the incubation (0 h). (**B**) The electropherogram A was analysed by the ImageJ 1.51j8 software. The intensity of bands corresponding to the supercoiled (sc) and open circular (oc) species were quantified and presented as percentage of the whole plasmid DNA content.



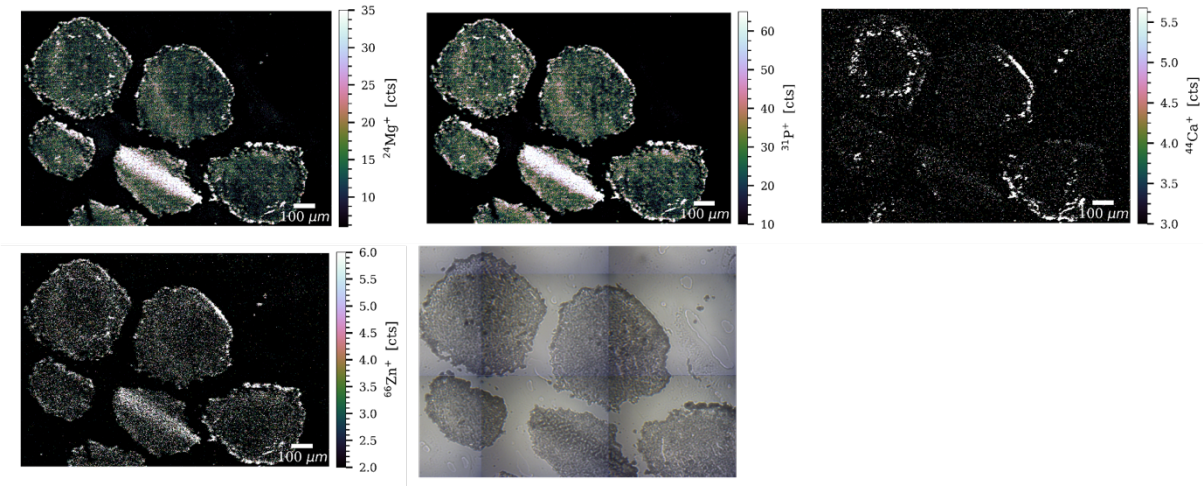
Supplementary Figure SF8: Signal intensity maps of $^{24}Mg^+$, $^{31}P^+$, $^{44}Ca^+$, Zn, and $^{102}Ru^+$ obtained by LA-ICP-TOF-MS of HT-29 tumor spheroids after treatment with 400 µM of plecstatin-1 for 5 days. High-resolution laser ablation images were obtained with a pixel size of 2.5 µm and a repetition rate of 200 Hz**.**



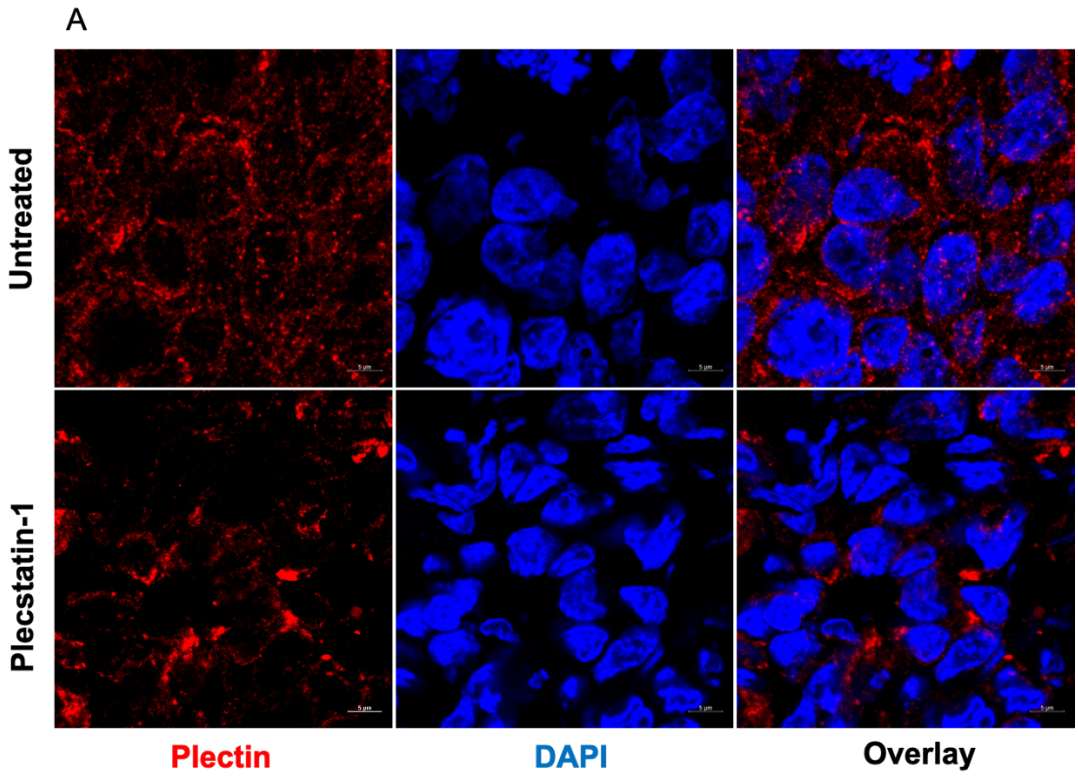
Supplementary Figure SF9: Signal intensity maps of $^{24}Mg^+$, $^{31}P^+$, $^{44}Ca^+$, Zn, and $^{102}Ru^+$ obtained by LA-ICP-TOF-MS of untreated HT-29 tumor spheroids. High-resolution laser ablation images were obtained with a pixel size of 2.5 µm and a repetition rate of 200 Hz**.**

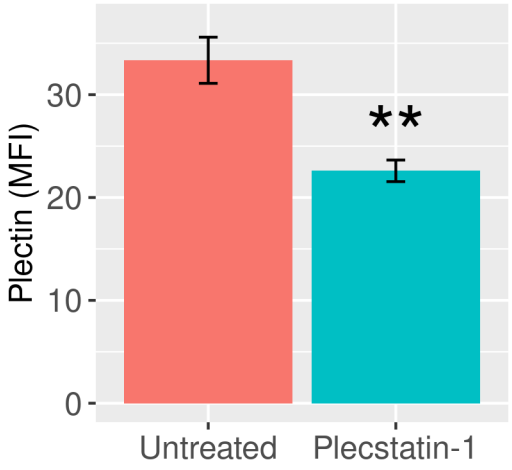


Supplementary Figure SF10: Signal intensity maps of $^{24}Mg^+$, $^{31}P^+$, $^{44}Ca^+$, Zn, and $^{102}Ru^+$ obtained by LA-ICP-TOF-MS of HCT-116 tumor spheroids after treatment with 400 µM of plecstatin-1 for 5 days. High-resolution laser ablation images were obtained with a pixel size of 2.5 µm and a repetition rate of 200 Hz.

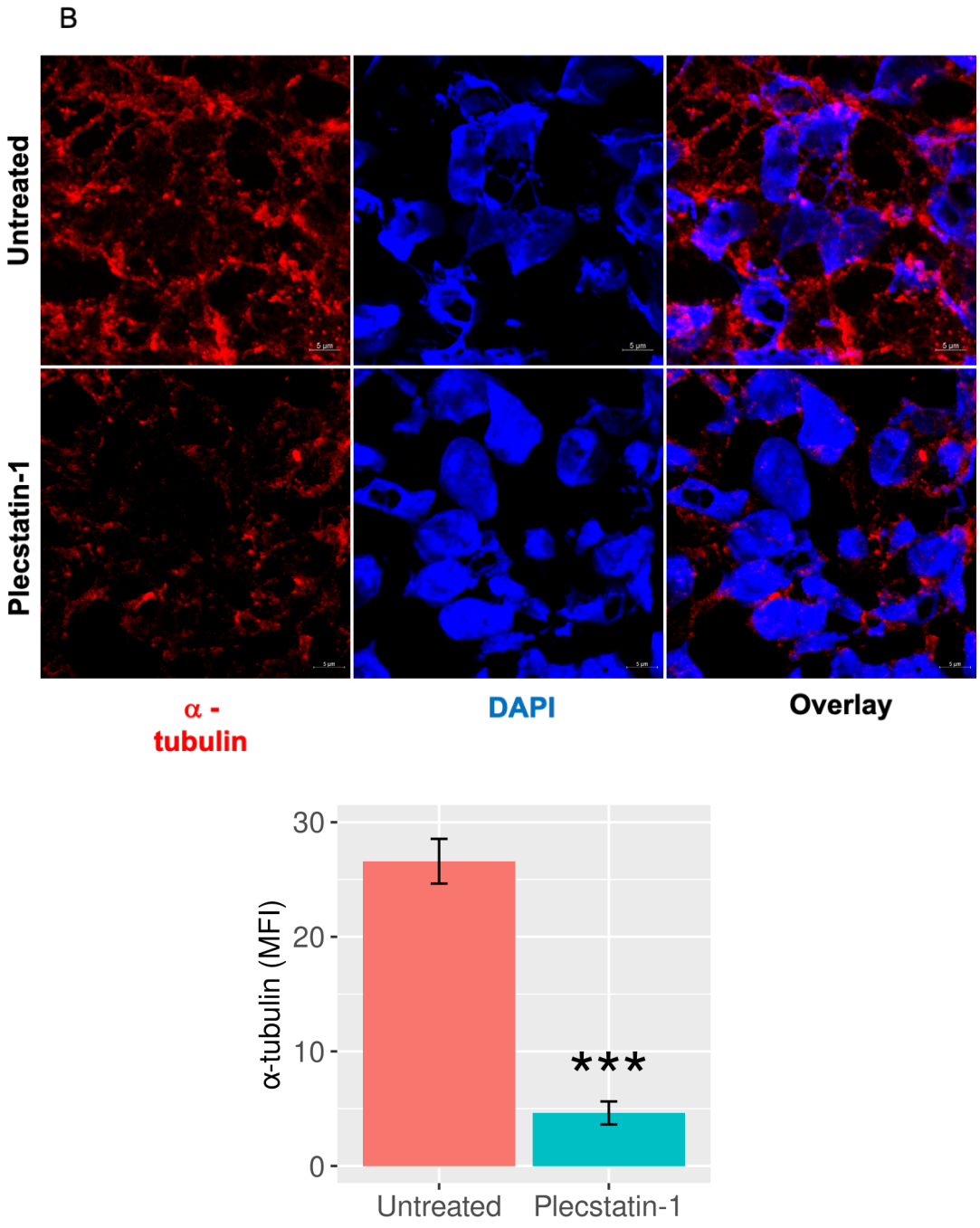


Supplementary Figure SF11: Signal intensity maps of $^{24}Mg^+$, $^{31}P^+$, $^{44}Ca^+$, Zn, and $^{102}Ru^+$ obtained by LA-ICP-TOF-MS of untreated HCT-116 tumor spheroids. High-resolution laser ablation images were obtained with a pixel size of 2.5 µm and a repetition rate of 200 Hz.



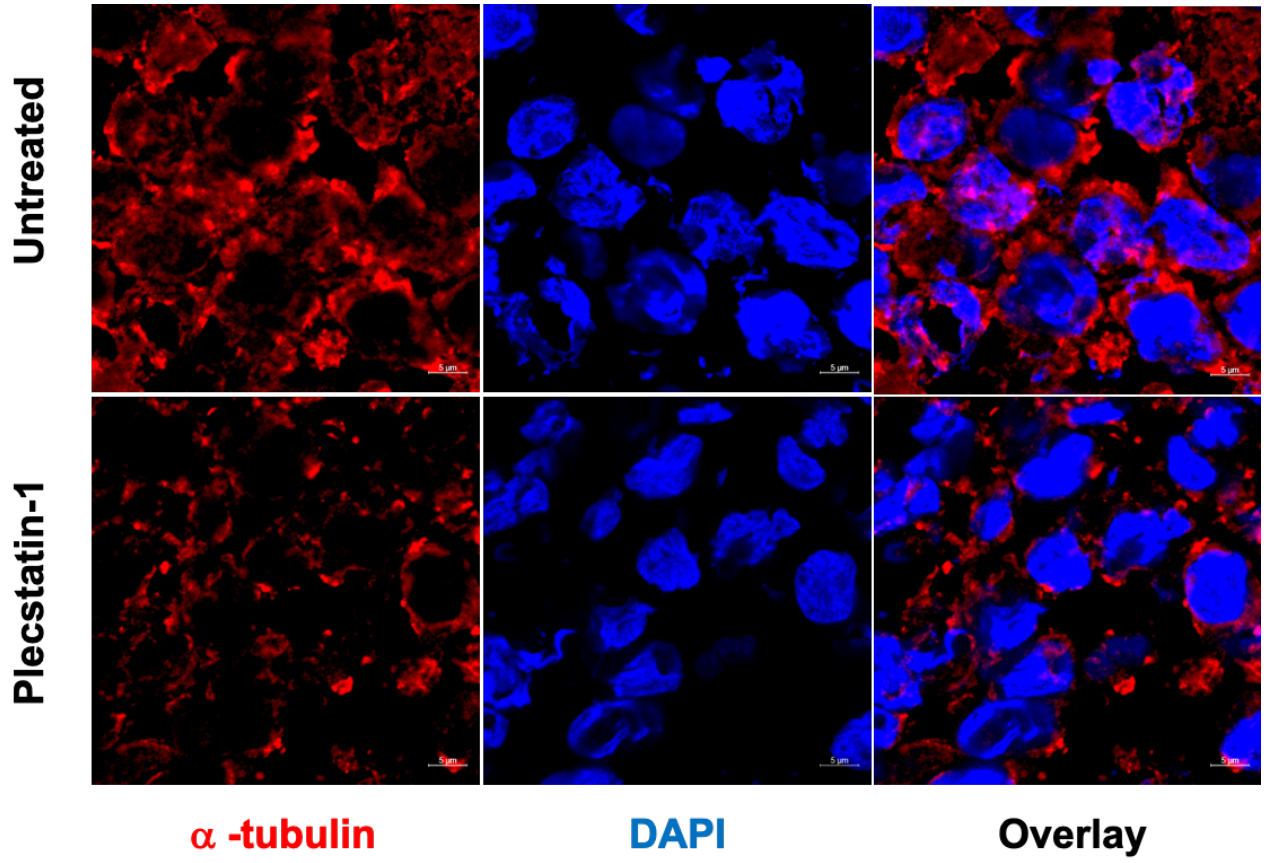


Supplementary Figure SF12: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labelled against a plectin antibody. Plecstatin-1 treatment (200 µM) led to a collapse of the plectin-network. The mean fluorescence intensity (MFI x10^3) of plectin was measured and compared between untreated and treated spheroids. A reduction in the MFI could be observed in plecstatin-1 treated spheroids in comparison to untreated controls. Scale bar 5 µm. (t-test, p-value = 0.006).

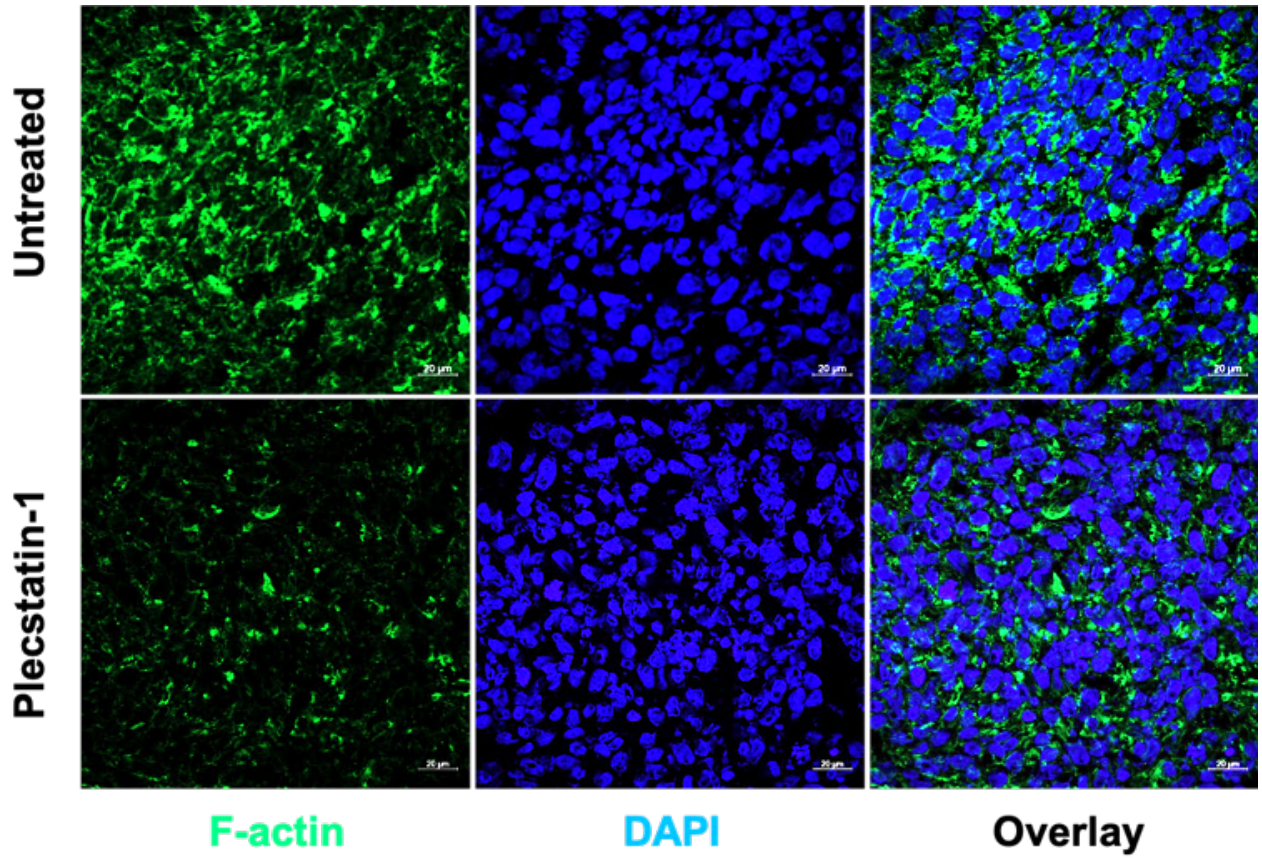


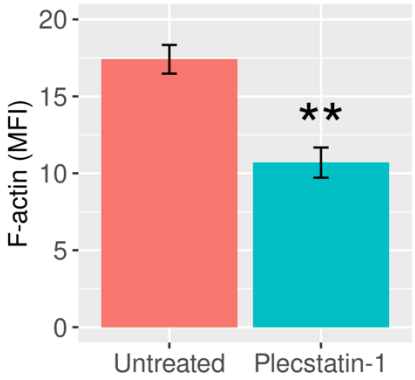
Supplementary Figure SF13: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labelled against an α-tubulin antibody. Treatment with plecstatin-1 (200 µM) led to a major collapse of the α-tubulin network**.** The mean fluorescence intensity (MFI x10^3) of a-tubulin was measured and compared between untreated and treated spheroids. A 5-fold reduction in the MFI could be

observed in plecstatin-1 treated spheroids in comparison to untreated controls. (t-test, p-value = 4.2×10^{-4})

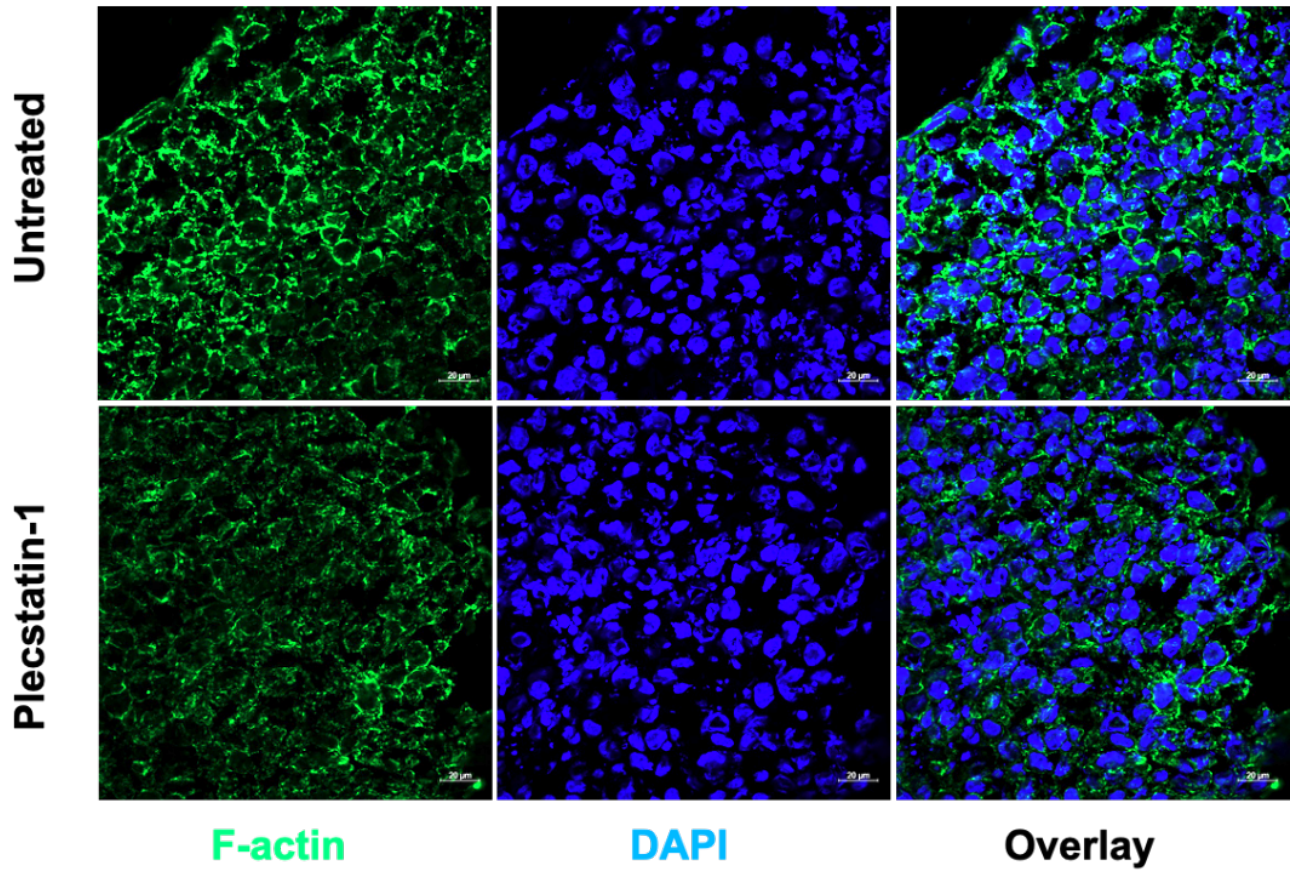


Supplementary Figure SF14: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Spheroids were labeled against an α-tubulin antibody. Treatment with plecstatin-1 induced a significant loss of α-tubulin, an important component of the cytoskeleton.

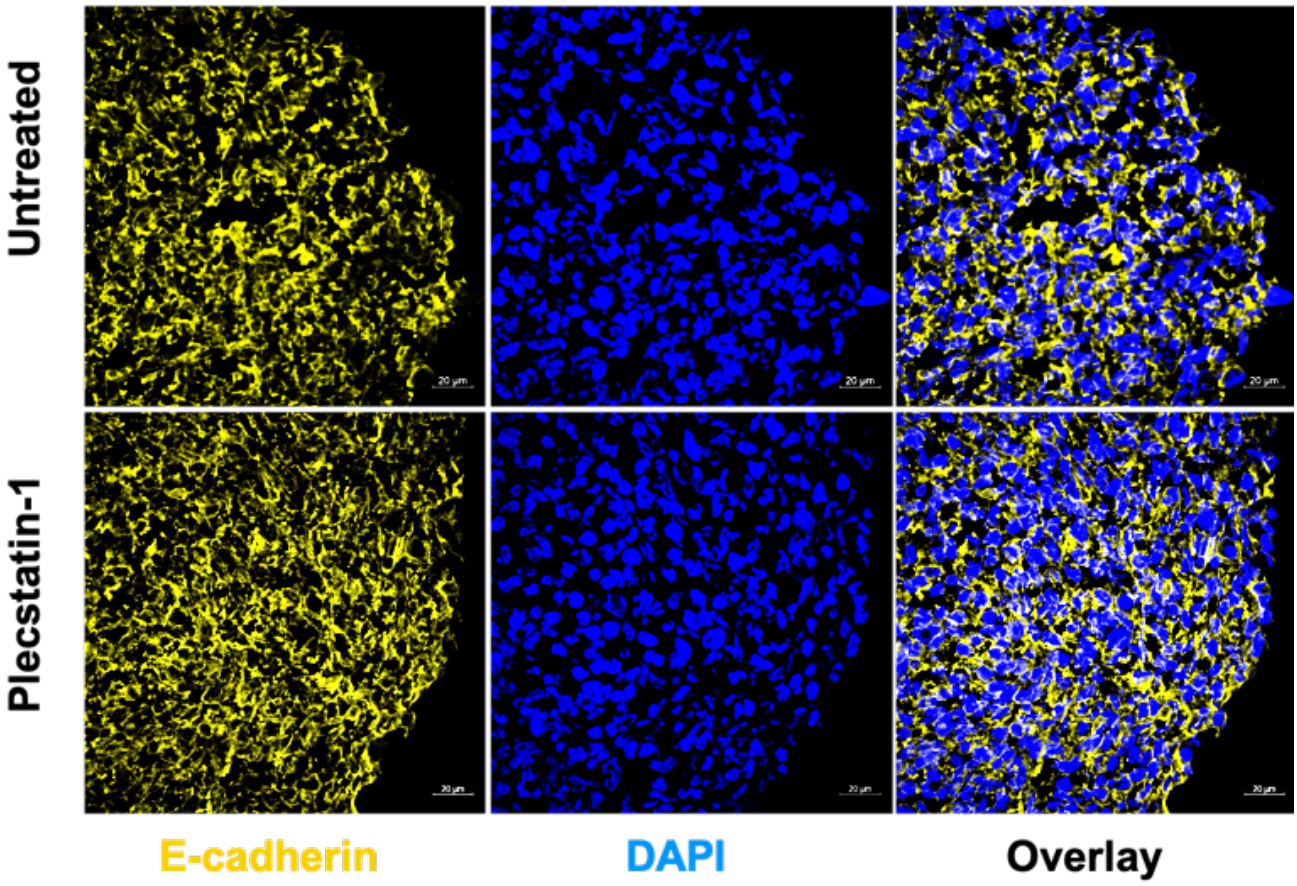




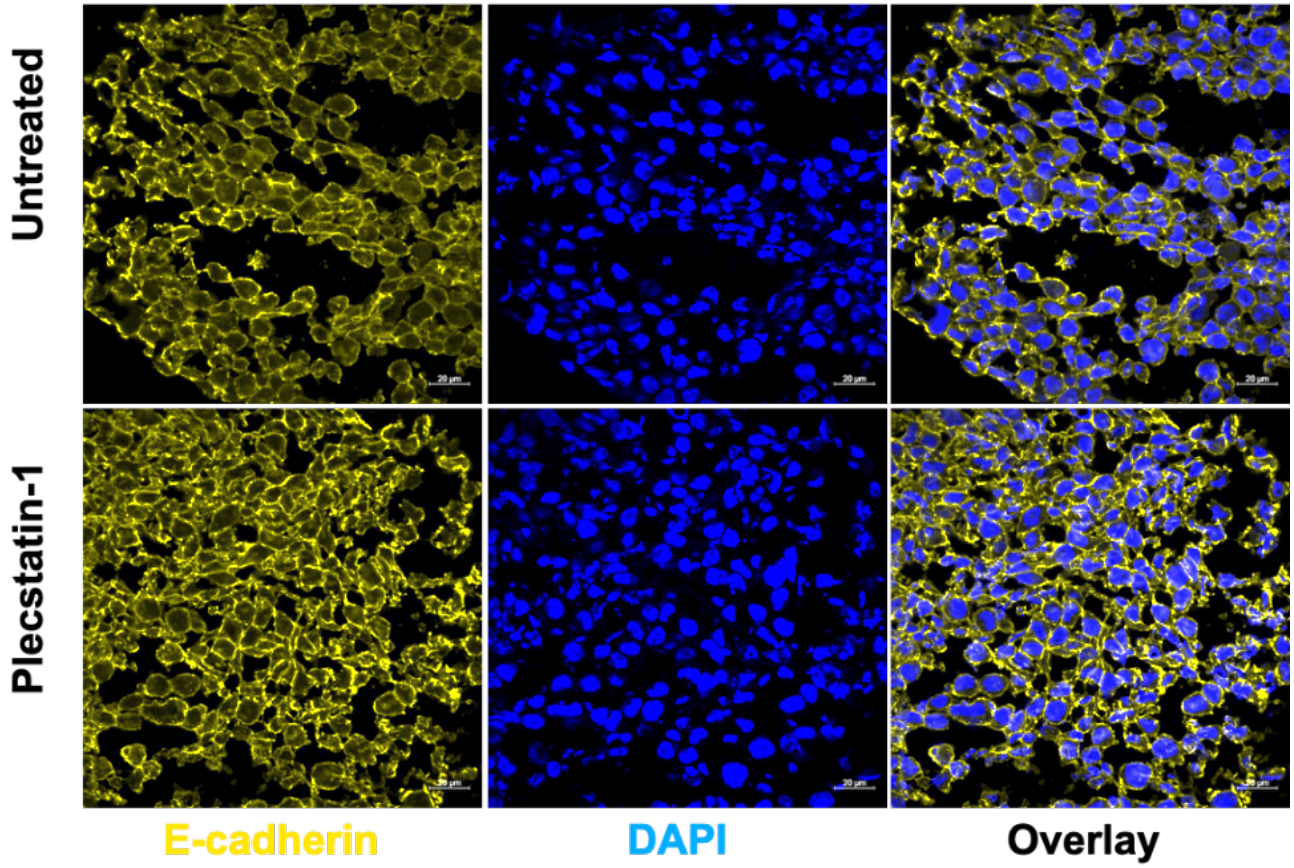
Supplementary Figure SF15: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labelled with phalloidin-Alexa Fluor 488 to detect F-actin. Disruption of the F-actin network can be observed in plecstatin-1 treated spheroids. The mean fluorescence intensity ($x10^3$) of F-actin was measured and compared between untreated and treated spheroids. A ca. 1.5-fold reduction in the MFI could be observed in plecstatin-1 treated spheroids in comparison to untreated controls. (t-test, p-value = 0.001).



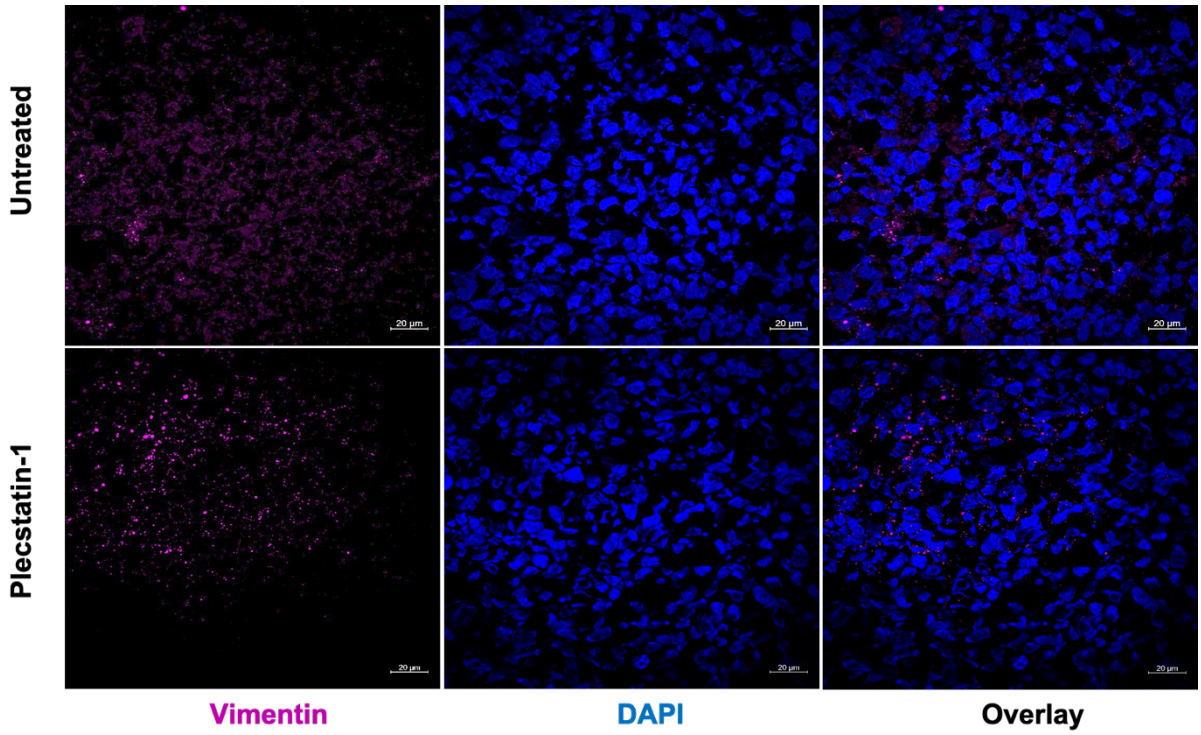
Supplementary Figure SF16: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Spheroids were labeled with phalloidin-Alexa Fluor 488 to detect F-actin. Disruption of the F-actin network can be observed in plecstatin-1 treated spheroids, although to a lesser extent compared to HCT-116 spheroids.



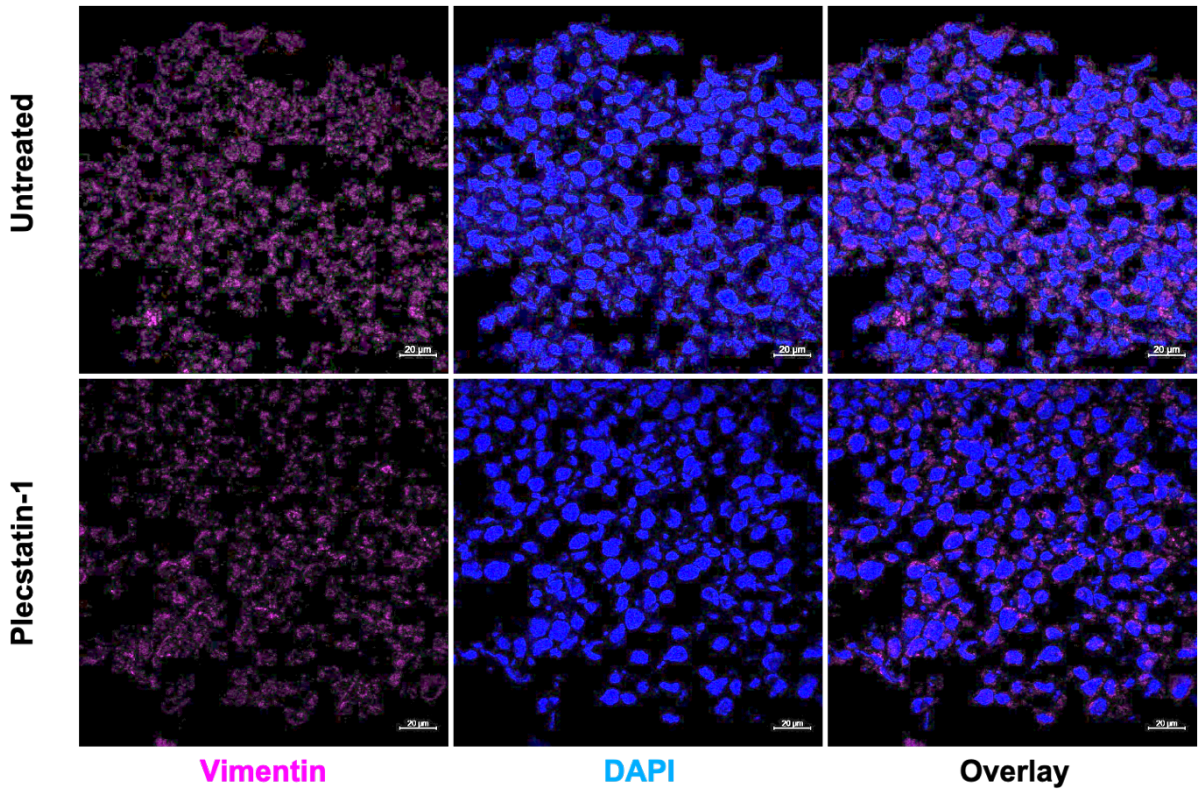
Supplementary Figure SF17: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labelled against an E-cadherin antibody. No qualitative loss could be observed in the E-cadherin fluorescence signal between untreated and plecstatin-1 spheroids.



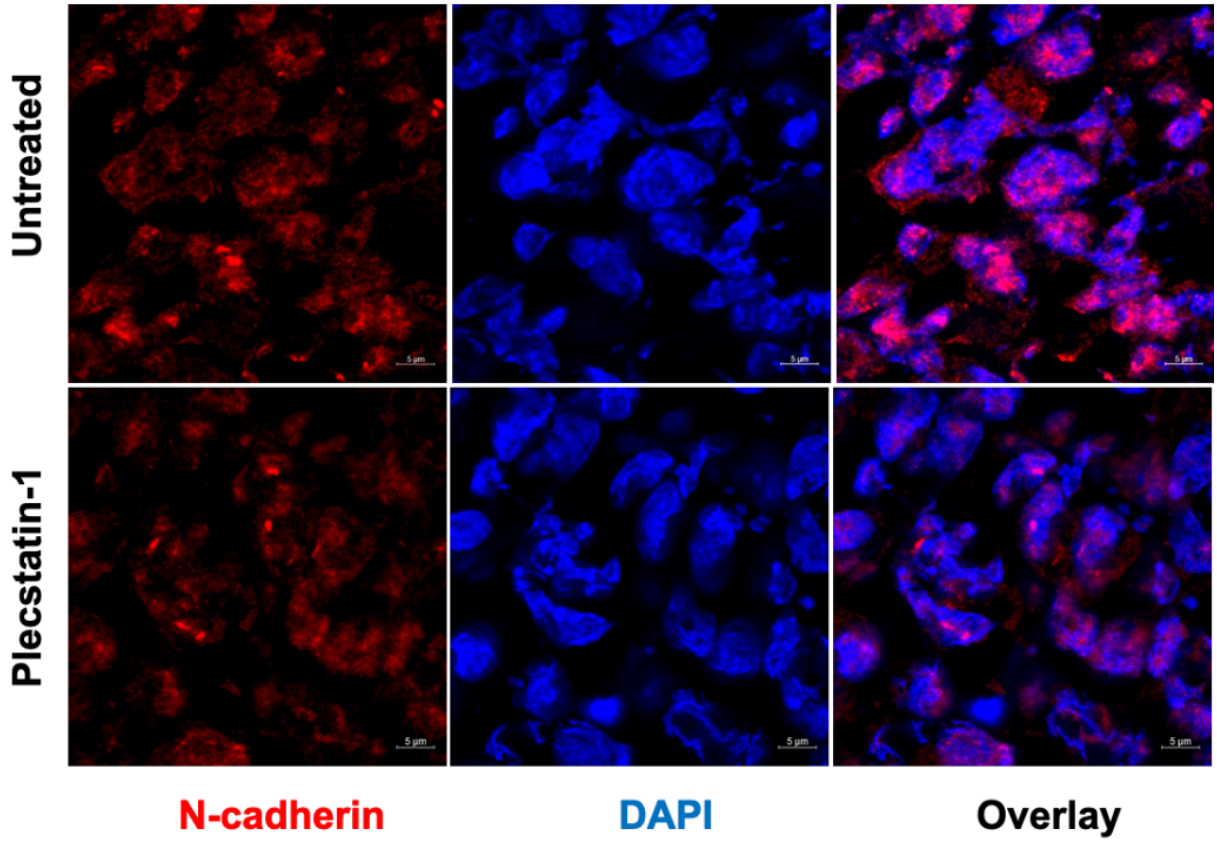
Supplementary Figure SF18: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Spheroids were labeled against an E-cadherin antibody. No qualitative loss could be observed in the E-cadherin fluorescence signal between untreated and plecstatin-1 spheroids.



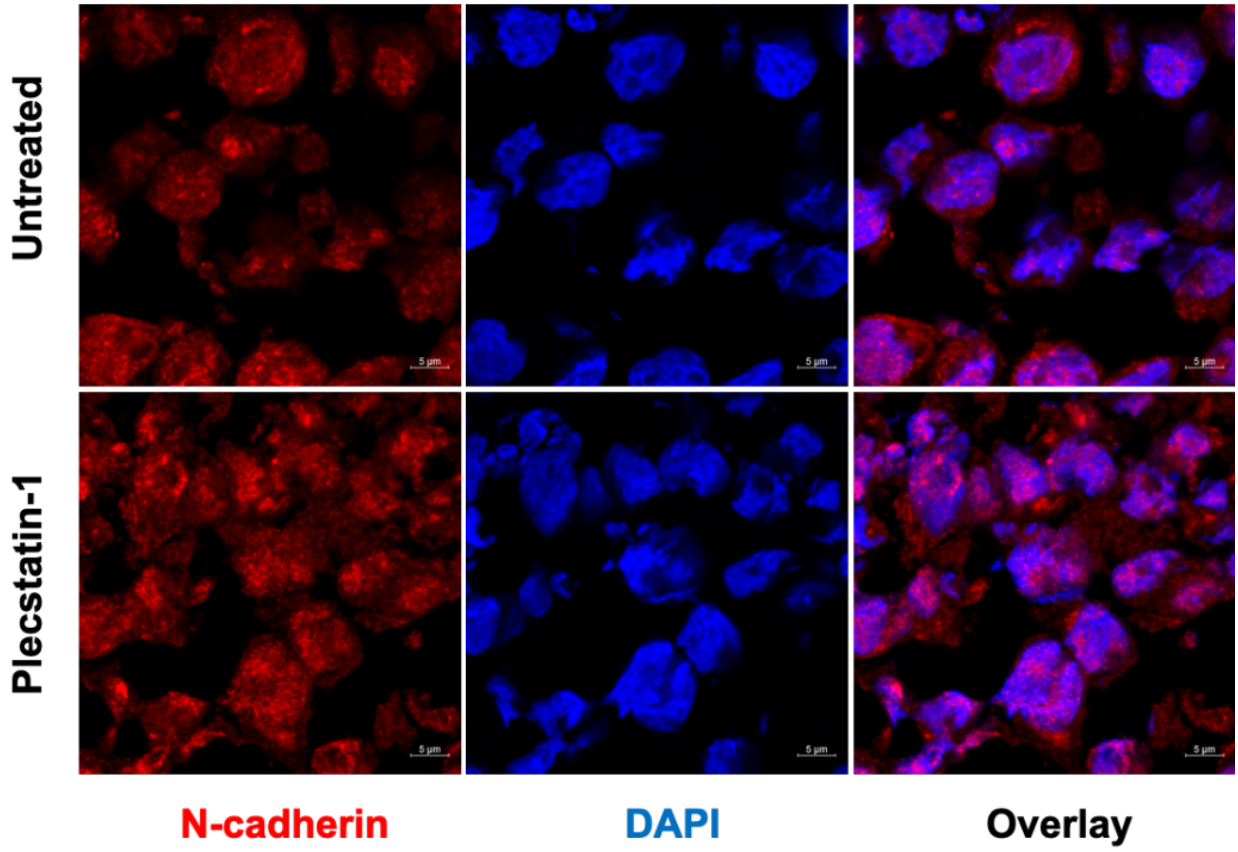
Supplementary Figure SF19: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labeled against a vimentin antibody. In plecstatin-1 treated spheroids a loss in the vimentin signal can be observed in comparison to untreated cells.



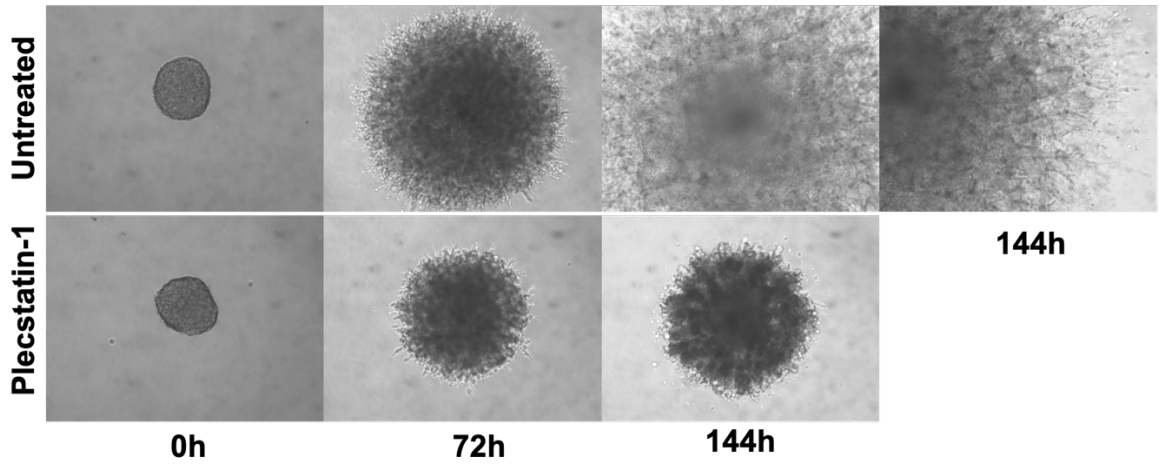
Supplementary Figure SF20: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Spheroids were labelled against a vimentin antibody. In plecstatin-1 treated spheroids a loss in the vimentin fluorescence signal can be observed in comparison to untreated cells.

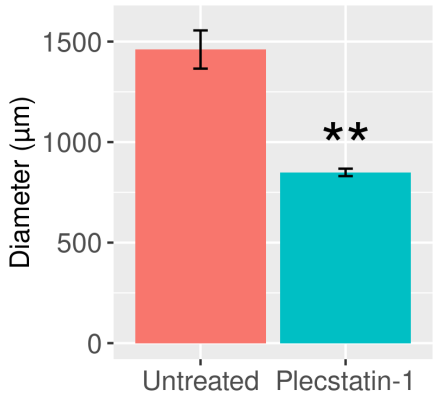


Supplementary Figure SF21: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HCT-116 spheroids. Spheroids were labeled against an N-cadherin antibody. No qualitative increase could be observed in the N-cadherin fluorescence signal between untreated and plecstatin-1 spheroids.

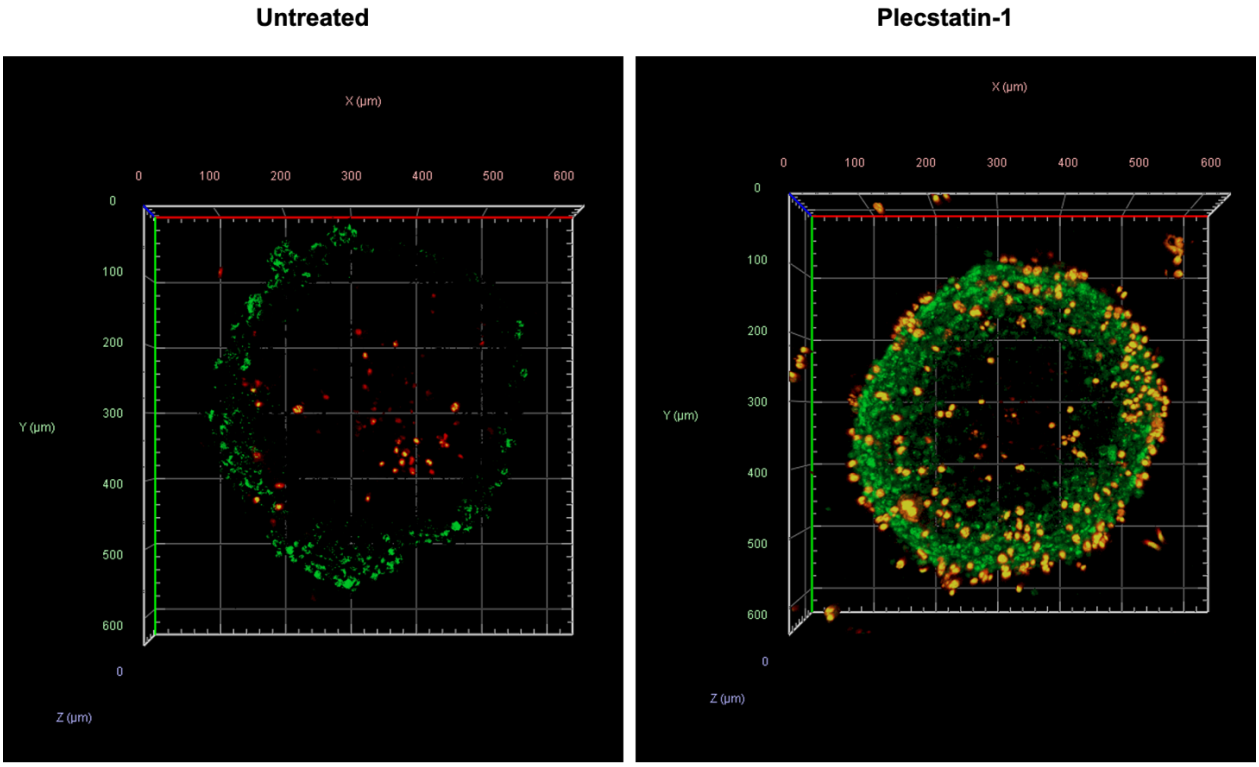


Supplementary Figure SF22: Representative immunofluorescence analysis (confocal microscopy) of PFA fixed HT-29 spheroids. Spheroids were labeled against an N-cadherin antibody. No qualitative increase could be observed in the N-cadherin fluorescence signal between untreated and plecstatin-1 spheroids.

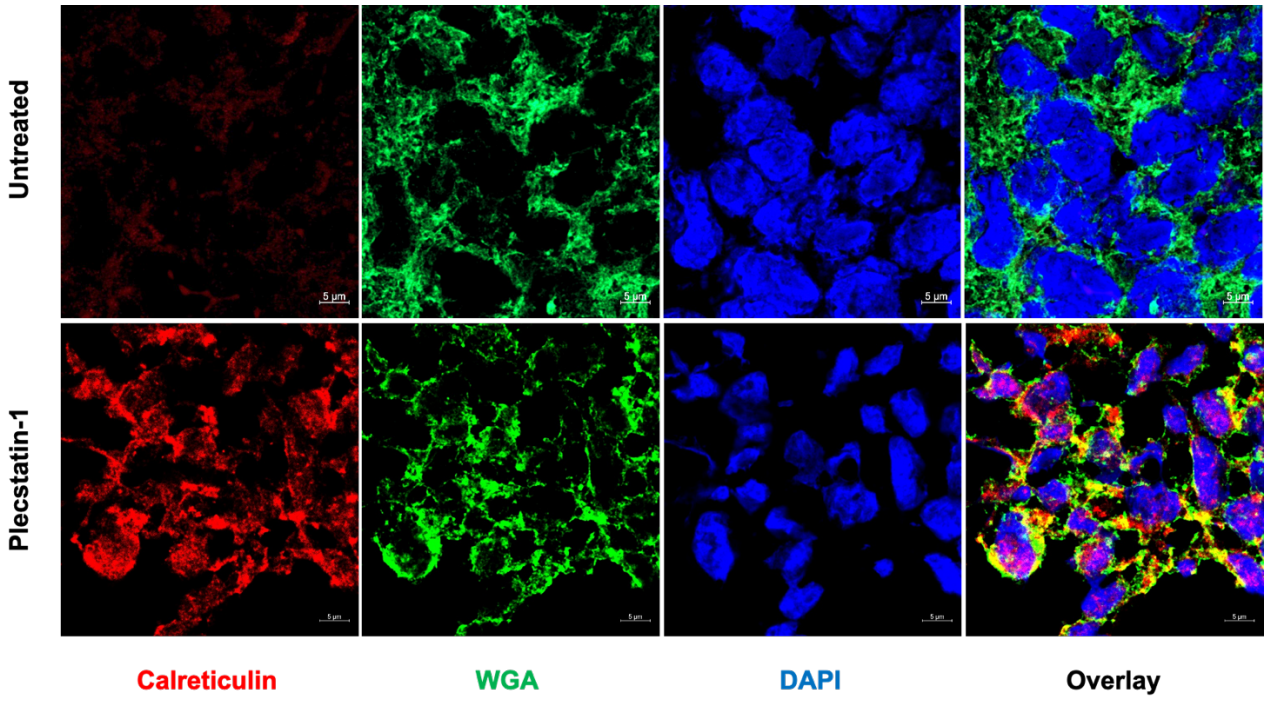




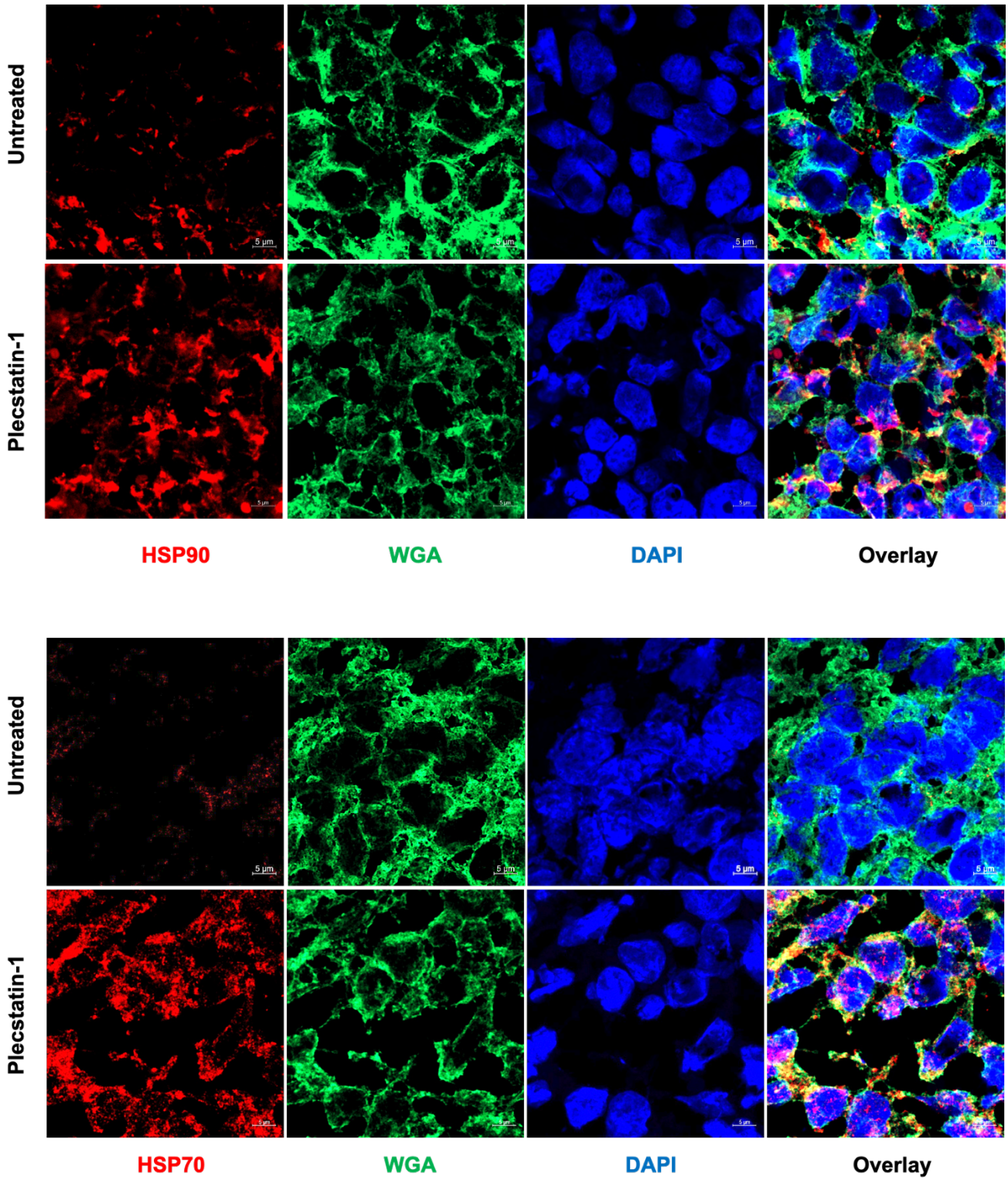
Supplementary Figure SF23: HT1080 spheroids were incubated for 144 h with plecstatin-1 or were left untreated. After 72 h of drug treatment, there is a ca. 2-fold difference in the sizes of the treated versus untreated spheroids. Furthermore, untreated cells display a very invasive morphology, characterized by long cellular protrusions and larger diameter sizes. The massive invasiveness observed in untreated spheroids after 144h of incubation didn't allow to entirely depict the cellular protrusions in one single picture using the smallest magnification (4x). (t-test, p-value = 0.006).



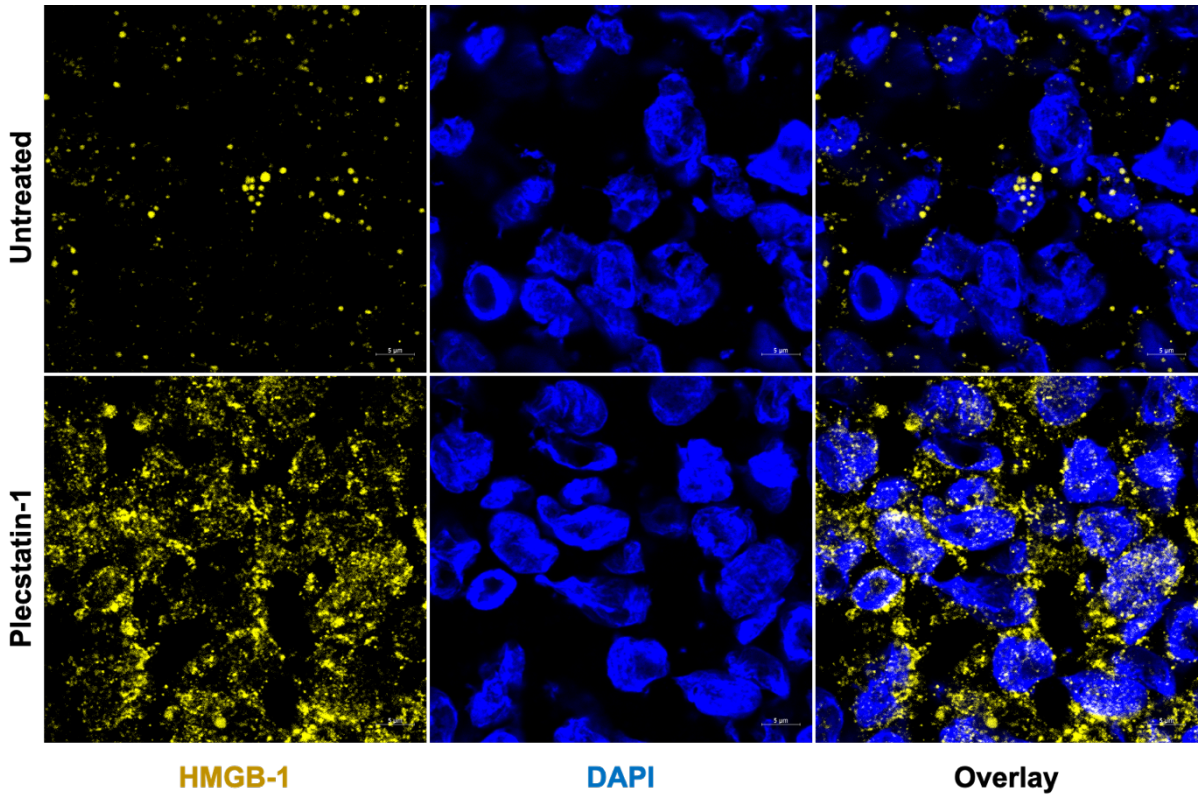
Supplementary Figure SF24: Representative three-dimensional reconstruction of HCT-116 spheroids double-labelled with Cellrox® and PI. Confocal microscope images were obtained from a stack of optical sections from the spheroids. Green puncta correspond to ROS^+ cells, red puncta to PI^+ and yellow puncta ROS^+/PI^+ (colocalization). The untreated sample (on the left) displays few ROS^+ cells. There is an increase in ROS^+ cells in plecstatin-1 samples (on the right) treated for 24 h.



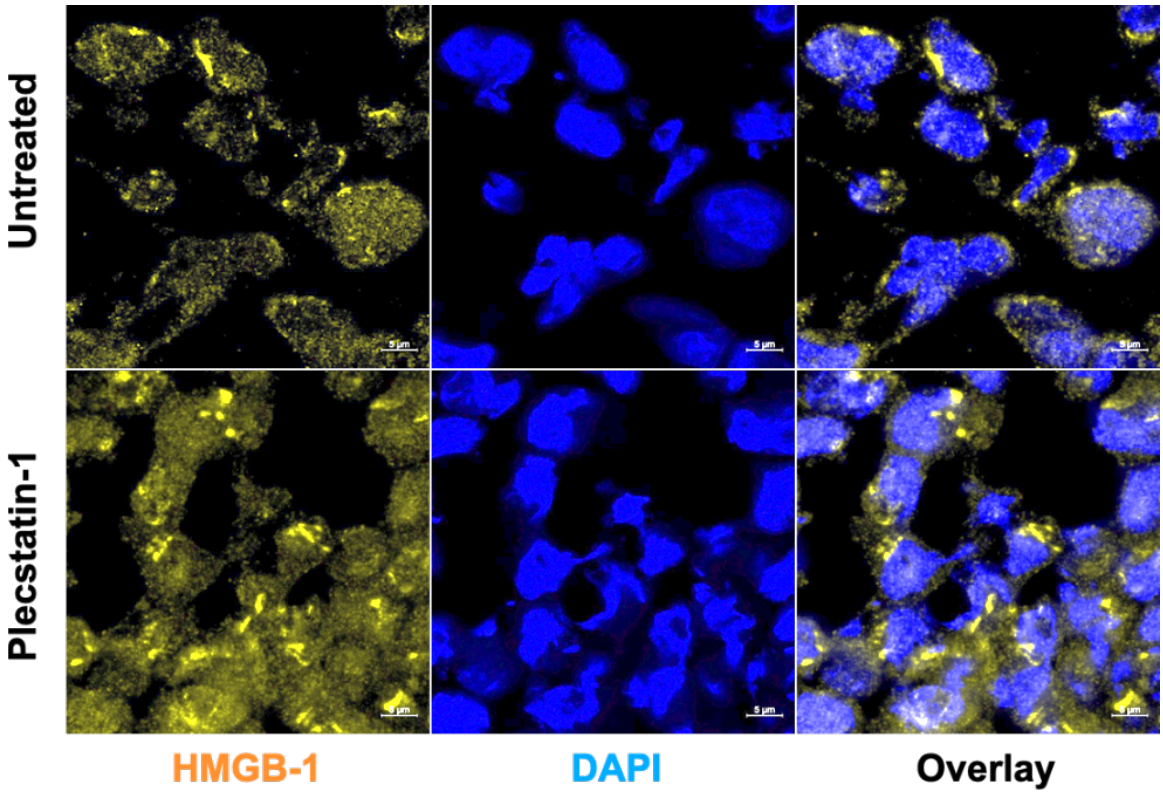
Supplementary Figure SF25: Representative pictures of HCT-116 spheroids. Spheroids were labelled against a calreticulin antibody, as well as WGA for membrane staining. CRT translocation can be observed upon treatment with plecstatin-1 after 24 h (colocalization yellow regions).



Supplementary Figure SF26: Representative immunofluorescence analysis (confocal microscopy) of HCT-116 spheroids. Spheroids were labelled against either against HSP90 or HSP70 antibodies, as well as wheat germ agglutinin (WGA) for membrane staining and DAPI for counterstaining. HSP90 and HSP70 induction and co-localization to membrane areas was observed upon treatment with plecstatin-1 after 24 h (colocalization yellow regions).



Supplementary Figure SF27: Representative immunofluorescence analysis (confocal microscopy) HCT-116 spheroids. Spheroids were labelled against a HMGB-1 antibody. HMGB-1 translocation to and enrichment in the cytoplasm can be observed upon treatment with plecstatin-1 after 72 h.



Supplementary Figure SF28: Representative immunofluorescence analysis (confocal microscopy) HT-29 spheroids. Spheroids were labeled against a HMGB-1 antibody. HMGB-1 translocation to and enrichment in the cytoplasm can be observed upon treatment with plecstatin-1 after 72 h.