Tumor xenograft and immunohistochemistry

Eight-week-old female CD1 nu/nu mice (Charles River, Germany) were housed in Air Flow Cabinets (Ehret GmbH, Germany) and supplied with laboratory chow and water *ad libitum*. All experimental procedures were performed according to the German law for the protection of animals. For tumor induction, Colo 205 cells were harvested during logarithmic growth and adjusted to $5x10^7$ cells/ml in PBS. 100µl of cell suspension was subcutaneously injected into the flank region. EMD534085 was dissolved in 20% Cremophor RH and 0.066M phosphate buffer, pH 7.4 at concentrations of 10, 20 and 30 mg/kg. Treatment was initiated by interperitoneal administration when tumors were approximately 300mm^3. Animals were sacrificed at different time points after treatment and tumors were fixed in formalin and sectioned.

Two, 3µm serial sections of the xenografts were mounted on positively charged SuperFrost®Plus slides. Sections were deparaffinated using a Discovery™ staining instrument (Ventana Medical Systems, Inc., Tucson, USA). After deparaffinization, sections were heated for epitope retrieval in Tris-EDTA buffer pH 8. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Sections were incubated with the primary polyclonal rabbit anti-phospho histone H3 antibody (Cell Signaling Technology) at a dilution of 1:20 for 30 min at 37^{o}C, followed by biotin conjugated secondary goat anti-rabbit antibody (Vector) for 16 min at 37^{o}C. Positive labeling was visualized by incubating the slides with horseradish peroxidase conjugated streptavidin (DAB Map™, Ventana Medical Systems, Inc.), resulting in an insoluble dark brown precipitate that can be visualized. Sections were counterstained with haematoxylin.

The level of phospho-histone H3 in the xenografts was measured using the automated microscope/image analysis system Ariol SL-50 (Applied Imaging International Ltd., Newcastle upon Tyne, U.K.) scanning sections at 10X (scale x/y: 1 pixel = 0.73 x 0.73 μm^2). Twelve circular regions (input region area) of 0.03 mm^2 were set in areas of highest density of labeled cells and the mean signal was calculated. The brown color of the positive immunhistochemical labeling and the blue color of hematoxylin stained nuclei and cytoplasm was detected with the help of the image analysis software of the Ariol SL-50 by setting thresholds for "color", "hue", and "saturation". The percentage of positive nuclear area was calculated according to 100*[brown area/(brown area + blue area)]. All slides of one experiment were processed and analyzed in parallel.

Flow cytometry

500nM EMD534085 treated cells were trypsinized, resuspended in PBS, centrifuged for 5 min at 300 x g, resuspended and fixed in 10 ml ice-cold 80% ethanol/20% PBS for ≥2h at -20°C. Fixed cells were centrifuged for 5 min at 300 x g, washed in 10 ml PBS, centrifuged and resuspended in 1 ml of PBS, 0.2 mg/ml RNAse A, 0.1% triton X-100 and 25µg/ml propidium iodide for 30 min at 37°C. A Bectin Dickinson FACS Caliber, FACS Comp and Cell Quest Pro were used. Doublet discrimination excluded false 4N cells.