SUPPLEMENTARY MATERIALS AND METHODS

Cabenda Immunohistochemistry for CD31 and EGFR

Cryosections were fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature. Vasculature was stained using a 1:200 dilution of rat-anti-PECAM/CD31 (BD Biosciences) and 1:200 fluorescent Alexa 647 anti-rat secondary (Invitrogen). EGFR was detected using a 1:50 dilution of rabbit-anti-EGFR (Cell Signaling) and a 1:200 Alexa 546 anti-rabbit secondary. Following fluorescence imaging, slides were counterstained with haematoxylin, dehydrated and mounted using Permount (Fisher Scientific) was quantified by measuring the percentage of stain-positive cells within viable tumor areas using a robotic fluorescent microscope and customized ImageJ software by Cabenda Pharmaceuticals Research (Vancouver, BC, Canada).

Cabenda Immunohistochemistry for $DiOC_7(3)$, CD31, TUNEL, pimonidazole & BrdUrd

Prior to immunostaining, slides were imaged for $DiOC_7(3)$ tissue fluorescence to visualize blood flow. Cryosections were fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature. Vasculature was stained using a 1:2000 dilution of anti-PECAM/CD31 (clone 2H8) and 1:200 fluorescent Alexa 647 anti-hamster secondary (Invitrogen, Burlington, ON, CA). Hypoxia was detected via bound pimonidazole adducts using a 1:2000 polyclonal rabbit-anti-pimonidazole and a 1:200 Alexa 488 anti-rabbit secondary. Apoptosis was detected using a TUNEL kit (Roche Diagnostics) with a TMR red tagged dUTP. Slides were imaged for fluorescence and then transferred to distilled water for 10 min and then treated with 2 M HCl at room temperature for 1 hour followed by neutralization for 5 min in 0.1 M sodium borate. Slides were then washed in distilled water and transferred to a PBS (phosphate buffered saline) bath. BrdUrd incorporated into DNA was detected using a 1:500 dilution of monoclonal rat anti-BrdUrd (clone BU1/75) followed by 1:200 dilution of anti-mouse peroxidase conjugate antibody (Sigma) and 1:10 dilution of metal enhanced DAB substrate (Pierce, Rockford, IL). Slides were counterstained with haematoxylin, dehydrated and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Image acquisition

The imaging system consists of a robotic fluorescence microscope (Zeiss), a cooled, monochrome CCD camera (Retiga), a motorized slide loader and x-y stage (Ludl Electronic Products) and customized ImageJ software (NIH) running on a Macintosh computer (Apple). The system allows tiling of adjacent microscope fields of view. Using this system, images of entire tumor cryosections 1-2 cm^2 were captured at a resolution of 0.75 μm/pixel. Images were reduced to 1.5 μm/pixel prior to analysis to reduce file size.

Image analysis for EGFR, BrdUrd, TUNEL & pimonidazole mapping

Using NIH-Image and user supplied algorithms, images of CD31 fluorescence and EGFR, BrdUrd, TUNEL or pimonidazole staining from each tumor section were overlaid and areas of necrosis and staining artifacts manually removed. On the fluorescence image, CD31-positive regions were identified by selecting all pixels 15 standard deviations above the tissue background levels. CD31 positive regions that were less than 5 μm^2 in size were considered artifacts and automatically removed from the analysis. BrdUrd and positive staining was identified by selecting pixels that were 5 standard deviations above tissue background levels. Measuring the distance from each point in the tissue to the nearest CD31 positive object and noting if it is BrdUrd positive or negative was used to determine the relation between proliferation and distance to the nearest blood vessel. The data was tabulated so as to determine the fraction of BrdUrd positive pixels of the total number pixels found at each distance to a blood vessel. Analysis of EGFR, TUNEL and pimonidazole profiles were carried out in a similar fashion but using average signal intensity as a function of distance to the nearest vessel rather than the fraction of tissue above threshold. Overall BrdUrd positive staining and average EGFR, TUNEL or pimonidazole intensity was calculated from images of entire tumor sections following removal of necrotic regions and tissue artifacts (folds, tears, debris etc). Percent necrosis was calculated from the fraction of each tissue section that exhibited confluent necrosis.

Immunohistochemical staining for EGFR L858R, total S6, phospho-S6, Ki-67, HSP70 and HSP27

Briefly, slides were soaked in xylene, passed through graded alcohols and placed in distilled water. Slides were then pre-treated with Citrate buffer (pH 6, Invitrogen/ Life Technologies, for anti-S6, phospho S6, HSP27, HSP70), EDTA buffer (pH 8, Invitrogen, for anti-EGFR L858R), or DAKO retrieval solution (for anti-Ki67) in a steam pressure cooker (Decloaking Chamber, BioCare Medical) as per manufacturer's instructions followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO USA) for 5 minutes to quench endogenous peroxidase activity, washed and incubated with serum-free protein block (DAKO) for 20 minutes prior to applying antibody.