Supplementary Materials and Methods

Cell-lines and reagents

MPNST cell lines utilized for our studies included the NF1-associated: S462 (provided by Dr Lan Kluwe, University Hospital Eppendorf, Hamburg, Germany), ST88-14 (provided by Dr Jonathan Fletcher, Brigham and Women's Hospital, Boston, MA), MPNST642 isolated in our laboratory (30), and the sporadic MPNST cell lines STS26T (provided by Dr Steven Porcelli, Albert Einstein College of Medicine, Bronx, NY) and MPNST724 (provided by Dr Jonathan Fletcher); these were propagated and maintained as previously described (31). We acquired these cell lines between 2008-2011; all were authenticated using DNA fingerprinting (short tandem repeat [STR]) as previously described (30), confirming that no cross contamination has occurred. Cell lines utilized were re-fingerprinted, as per above, during their use for the current study. STS26T, MPNST724, and ST88 cell lines, stably transfected to express GFP-LC3, were utilized for autophagy assessment; over-expressing cells were FACS-sorted on the basis of GFP expression. Compounds utilized in our studies included the small molecule dual PI3K/mTOR inhibitor, XL765 (provided by Exelixis, South San Francisco, CA and Sanofi-Aventis, Vitry/Seine, France), the dual PI3K/mTOR inhibitor, PI103 (Tocris, Ellisville, MO), and the mTORC1 inhibitor rapamycin (Calbiochem, Darmstadt, Germany). The autophagy modifiers bafilomycin and chloroquine were obtained from Sigma (St Louis, MO). Commercially available antibodies were used for immunoblot or immunohistochemical detection of: AKT, pAKT (S473), 4EBP1, p4EBP1, S6-kinase (S6K), pS6K (T389), S6 Ribosomal Protein (S6RP), pS6RP (S235/236), ULK1, and LC3B (Cell Signaling, Danvers, MA); GFP, beclin, actin (Santa Cruz, Santa Cruz, CA); cleaved PARP (Abcam, Cambridge, MA); Ki-67 (MIB-1; Dako, Carpenteria, CA); cleaved caspase- 3 (BioCare medical, Concord, CA), and CD31 (PharMingen, San Diego, CA).

Cellular assays

MTS and clonogenicity assays were performed as previously described (Lahat G et al, PLoS ONE 2010;5:e10105.). MTS assays were repeated trice (500-3000 cells were cultured); mean ± SD was calculated; cell lines were examined separately. XL765 IC_{50} concentrations were calculated from MTS results using GraphPad Prism® software. Cell cycle progression was measured via **PI staining/FACS analysis** (Zhu QS et al. Cancer Res 2008;68:2895–903); apoptosis was measured via **Annexin V/PI staining FACS analysis** using the Apoptosis Detection kit I (BD Biosciences) per manufacturers' recommendations. **Western blot analysis** WB was performed by standard methods. Briefly, 25-50 μg of proteins extracted from cultured cells were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked and blotted with relevant antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected by ECL chemiluminesence (Amersham Biosciences, Plc., UK). RTPCR was conducted as we have previously described (Jin Z et al. Clin Cancer Res 2008;14:5033–42); ATG7 primers (forward: 5'-ACCCAGAAGAAGCTGAACGA-3' and reverse: 5'-CTCATTTGCTGCTTGTTCCA-3') and GADPH primers (forward: 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse: 5'-GAAGATGGTGATGGGATTTC-3') were obtained from Sigma.

Autophagy related assays

Transmission Electron Microscopy (TEM; enabling the identification of autophagic structures in different stages of autophagosome maturation) and quantification of acidic vesicular organelles (using acridine orange and determined via FACS analysis). Conversion of soluble LC3-I to lipid bound LC3-II is associated with the formation of autophagosomes. WB analysis was used to evaluate expression of LC3-II (normalized to actin); enhanced expression is an indicator of increased number of autophagosomes. The inhibitors chloroquine (CQ) and bafilomycin (BFA) block the last stages of productive autophagy thus result in autophagosome accumulation; WB

for LC3-II in the presence of these inhibitors (expected to show increased LC3B-II levels) was conducted. Combining CQ/BFA with compounds that enhance productive autophagy is expected to show further increase in LC3B-II expression. Here we tested the effects of XL765 or PI103 in combination CQ or BFA on the expression of LC3B-II. In addition, MPNST cells stably transduced with LC3-GFP were used to evaluate the impact of XL675/PI103 on autophagosome accumulation (via GFP immunofluorescence, assessing GFP puncta formation) and productive autophagy. Regarding the later, when GFP-LC3 is delivered to a lysosome the LC3 part of the chimera is sensitive to degradation, whereas the GFP protein is relatively resistant to hydrolysis. Therefore, the appearance of free GFP on WB (blotted for GFP) can be used to monitor lysis of the inner autophagosome membrane and breakdown of the cargo. Finally, the impact of autophagy blockade (achieved by knockdown of the ATG genes beclin or ATG7 or pharmacologically using CQ or BFA) on apoptotic response to PI3K/mTOR inhibitors was assessed (apoptosis was evaluated as per above)

Transfection procedures

siRNAs (20nM pools targeting beclin, ATG7, and ULK1control non-targeting constructs; Thermo Scientific, Waltham, MA) were introduced into cells using X-treme Gene per manufacturer's instructions (Roche, Mannheim, Germany). Briefly, $2x10^5$ cells were plated in each well of a six-well plate and incubated overnight. A mixture of siRNA (20nM) and X-treme Gene (6μl) diluted in 100μl Dulbecco's modified Eagle medium (DMEM) was added for 24 hr, followed by incubation in regular medium. Cells were harvested at indicated time points for specific experiments.

In vivo xenograft therapeutic experiments

All animal procedures and care were approved by the MD Anderson Cancer Center Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals". For experiments evaluating effect of XL765 monotherapy on local tumor growth trypan blue staining confirmed viable MPNST cells (STS26T and MPNST724; 1 and 2 x 10^6/0.1 mL HBSS/mouse, respectively) were used. Cell suspensions were injected subcutaneously into the flank of 6-7 week old female hairless SCID or SCID mice and growth was measured twice weekly. After establishment of palpable lesions (average diameter ~4-5mm), mice were randomly assigned to receive either vehicle control or XL765 (30mg/kg). XL765 was solubilized in water/10mM HCl and was administered by oral gavage twice daily, five days/week. Treatment continued until mice in control group mandated euthanasia. Tumors were resected, weighed, and fixed in formalin and paraffin-embedded for immunohistochemical studies. Immunohistochemistry on paraffinized, xenograft-derived specimen was conducted as previously described (35). An experimental lung metastasis MPNST model was used to evaluate growth of metastases. STS26T cells (1 x 10^6/0.1 mL HBSS/mouse) were injected into the tail vein of female SCID mice. Ten days after injection mice were allocated to treatment groups as per above; treatment was continued for three weeks. For lung metastasis studies, mice were followed for body weight and well being and sacrificed after three weeks after start of treatment. Lungs were weighed and evaluated macroscopically for tumor load.

To test the effect of XL765 in combination with autophagy blockade a four armed study was designed: 1) control vehicle; 2) XL765 (at doses and regimen as per above); 3) chloroquine (50mg/kg/d for five days a week); and, 4) combination of XL765 and chloroquine (doses and schedule as above). Of note, all mice were treated with XL765 alone (started when tumors were ~4-5mm) and were randomly assigned to the treatment groups above only after tumors reached

~6mm in largest dimension. Differences in xenograft growth (tumor/metastases) *in vivo* were assessed using a two-tailed Student's t-test. Significance was set at $P \leq 0.05$.