Inhibition of SAPK2/p38 Enhances Sensitivity to mTORC1 Inhibition by Blocking IRES-Mediated Translation Initiation in Glioblastoma

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Abstract
A variety of mechanisms confer hypersensitivity of tumor cells to the macrolide rapamycin, the prototypic mTORC1 inhibitor. Several studies have shown that the status of the AKT kinase plays a critical role in determining hypersensitivity. Cancer cells in which AKT activity is elevated are exquisitely sensitive to mTORC1 levels, maintaining the translation of crucial mRNAs involved in cell-cycle progression in the face of global eIF-4E–mediated translation inhibition. The activation of this salvage pathway is dependent on SAPK2/p38-mediated activation of IRES-dependent initiation of the cyclin D1 and c-MYC mRNAs, resulting in the maintenance of their protein expression levels. Here, we show that both genetic and pharmacologic inhibition of SAPK2/p38 in glioblastoma multiforme cells significantly reduces rapamycin-induced IRES-mediated translation initiation of cyclin D1 and c-MYC, resulting in increased G1 arrest in vitro and inhibition of tumor growth in xenografts. Moreover, we observed that the AKT-dependent signaling alterations seen in vitro are also displayed in engrafted tumors cells and were able to show that combined inhibitor treatments markedly reduced the mRNA translational state of cyclin D1 and c-MYC transcripts in tumors isolated from mice. These data support the combined use of SAPK2/p38 and mTORC1 inhibitors to achieve a synergistic antitumor therapeutic response, particularly in rapamycin-resistant quiescent AKT-containing cells. Mol Cancer Ther; 10(12); 2244–56. ©2011 AACR.

Introduction
Glioblastoma is the most common malignant brain tumor in adults and is generally highly refractive to current chemotherapies (1). In addition, these tumors are highly invasive, rendering surgical resection difficult under the best circumstances, with median survival times of only 12 to 15 months following diagnosis (2). The mTOR kinase has emerged as an attractive target for current therapeutic approaches, however, monotherapies using first generation rapalogs have been ineffective (3). Thus, combinatorial therapeutic approaches targeting additional relevant molecules would be of interest.

The signaling state of a cell can have profound effects on responses to individual chemotherapeutic agents. Several mechanisms have been described that confer hypersensitivity of tumor cells to the prototypic mTORC1 inhibitor rapamycin (4, 5). Many tumor types have displayed pronounced hypersensitivity to rapamycin, which is dependent on the relative level of AKT kinase activity. Tumor cells in which AKT is hyperactive due to gene amplification, loss of the tumor suppressor PTEN, or hyperactive PI-3 kinase activity are markedly more sensitive to mTORC1 inhibitor induced G1 arrest as compared with cells harboring relatively quiescent AKT (6–8). Our previous studies have shown that this differential sensitivity can be, in part, due to the continued IRES-initiated mRNA translation of the cyclin D1 and c-MYC transcripts following the global inhibition of eIF-4E–mediated protein synthesis (9, 10). This IRES-mediated protein synthesis of cyclin D1 and c-MYC is sufficient to promote cell-cycle progression, rapamycin resistance, and requires the ITAF hnRNP A1 (11).

The SAPK2/p38 kinase is activated by many compounds and environmental stimuli, including rapamycin, which results in dual phosphorylation on threonine/
tyrosine residues found in its T-loop (12). Activated SAPK2/p38 subsequently phosphorylates downstream effectors such as ATF2 and PRAK (13, 14). In this fashion, SAPK2/p38 is thought to play an important regulatory role coordinating cellular processes including growth, differentiation, cell death, and responses to chemotherapeutics.

During the course of our studies, we noted that rapamycin treatment activated SAPK2/p38 kinase activity in an AKT-dependent fashion and was required for cyclin D1 and c-MYC IRES activity following rapamycin exposure (10). Because c-MYC IRES function has also been shown to require SAPK2/p38 activity following genotoxic stress (15), we investigated whether SAPK2/p38 inhibition would augment rapamycin-mediated cytostasis and confer AKT-dependent hypersensitivity in tumors with relatively quiescent AKT activity. Here, we show that inhibitors of SAPK2/p38 selectively inhibit both cyclin D1- and c-MYC rapamycin-induced IRES activity and induces G1 arrest in rapamycin-resistant quiescent AKT-containing glioblastoma lines. Furthermore, we observed a synergistic antiproliferative effect of these compounds when used in combination against xenografted cells. Finally, we show that the mRNA translational state of the cyclin D1 and c-MYC mRNAs is markedly reduced in quiescent AKT-containing tumors following combination therapy.

Materials and Methods

Reagents and plasmids

The dicistronic plasmid pRF and the IRES containing plasmids pRCND1F, pRmycF, and pRP27F have been described previously (9) and contain the minimal IRES sequences for each of the 3 full-length mRNA leaders for their respective transcripts. Rapamycin and SB203580 were obtained from LC Laboratories. LY2228820 was from Selleck Chemicals and 4-hydroxy-tamoxifen (4OHT) was from Sigma.

Cell culture and transfections

The U87 and LN229 parental lines were originally obtained from American Type Culture Collection. The U87 line was stably transduced with a retroviral construct containing a wild-type PTEN gene or empty vector (EV) to generate U87PTEN and U87EV (gift from I. Mellinghoff and C. Sawyers; ref. 9). The LN229 (PTEN; wt) was transfected with a myristoylated AKT-estrogen receptor ligand-binding domain fusion (AKT-MER) cloned into pTracer-SV40 and stably expressing clones isolated and previously characterized (11). The parental and transfected lines were authenticated by genotyping. Transfections were done using X-treme GENE Q2 transfection reagent (Roche Applied Science) according to the manufacturer’s recommendations. Short interfering RNA (siRNA) transfections targeting human SAPK2/p38 were done using double-stranded RNAs directed at sequences within the coding region and 3’ UTR (ON-TARGETplus SMARTpool, Thermo Scientific). An siRNA with a scrambled sequence was used as a negative targeting control. Rapamycin was used at 100 nmol/L, SB203580 at 4 μmol/L and LY2228820 at 200 nmol/L for 24 hours treatments unless otherwise indicated.

IRES reporter assays

The indicated dicistronic mRNA reporters were cotransfected into cells with pSVβ-galactosidase to normalize for transfection efficiency as described previously (16). Cells were harvested 18 hour after transfection and Renilla, firefly and β-galactosidase activities determined (Dual-Glo luciferase and β-galactosidase assay systems; Promega).

Protein analysis

Immunoblots were done using standard procedures. Briefly, cells were lysed in 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L Na3VO4, 10 mmol/L NaF, 2 mmol/L PMSF, 0.5 mmol/L EDTA, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Samples were resolved by 10% or 12% SDS-PAGE, transferred to polyvinylidene difluoride (0.2 μm/L) membranes and probed with antibodies to the following proteins: cyclin D1 (BD Biosciences), c-MYC (clone 9E11, Upstate Biotechnology), actin (Sigma), SAPK2/p38, phospho-SAPK2/p38 (T180/Y182), phospho-β-glycerol phosphate, 25 mmol/L HEPES, pH 7.4, 25 mmol/L β-glycerol phosphate, 25 mmol/L MgCl2, 0.5 mmol/L EDTA, and 0.5 mmol/L dithiothreitol. Kinase reactions were done in the presence of 100 mmol/L ATP and 2 μg of ATP-2 as a substrate. Phosphorylation of ATF-2 was assessed by immunoblotting using phospho-ATF-2 (T69/71) antibody.

Cell proliferation/cell-cycle distribution/apoptosis assays

Cells were plated into 96-well plates at 2 × 104/well. After culturing with the indicated inhibitors for the time points described, cell numbers were measured by 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Roche) as described by the manufacturer. Cell-cycle analysis was done by propidium iodide staining of cells and flow cytometry as previously described (17). Cells were stained for Annexin V using a fluorescein isothiocyanate-conjugated anti-Annexin V antibody (Abcam). For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining of sections, slides were stained using...
Figure 1. AKT-dependent SAPK2/p38 activation and regulation of IRES activity following mTORC1 inhibition. A, U87EV, U87PTEN, LN229MER-AKT, and LN229EV were treated with rapamycin (100 nmol/L) for 24 hour and immunoblot assays were done for the indicated proteins. LN229MER-AKT or LN229EV cells were pretreated with 4OHT (1 μmol/L, 24 hours). B, SAPK2/p38 in vitro kinase assays of immunoprecipitated SAPK2/p38 from U87EV, U87PTEN, LN229MER-AKT, and LN229EV cells treated with rapamycin or 4OHT as in A. C, schematic diagrams of the dicistronic IRES containing mRNA reporters. D, cyclin D1
the TACSXL DAB In Situ Apoptosis Detection kit ( Trevigen) according to the manufacturer’s instructions and counterstained with hematoxylin. The combination index (18) was determined by using CALCUSYN software (Biosoft).

Xenograft models

Severe combined immunodeficient (SCID) mice were injected subcutaneously with single cell suspensions of the U87EV, U87PTEN, LN229EV, and LN229MER-AKT as described previously (11). For the LN229 xenografts, AKT activity was induced by intraperitoneal maintenance injections of 4OHT in peanut oil. Tumor growth was measured and mice were randomized into treatment cohorts when tumors reached 200 mm³. Treatment (8 mice/experimental group) was given via intraperitoneal injection for 10 consecutive days. Tumor growth was assessed by direct measurement on the indicated days following the start of the drug treatments. Tumor volume was determined using the formula $L \times W^2 \times 0.5$, where $L$ is the longest length, and $W$ is the shortest length. Tumors were harvested and extracts prepared for immunoblot analyses or fixed in 10% neutral buffered formalin and embedded in paraffin for histologic sectioning. Tumor growth delay values were calculated as previously defined (19).

Polysome analysis

Extraction and display of polysomes was done as previously described (10, 20). Briefly, fresh tumors were minced and homogenized in buffer containing 1% Triton X-100, 1% deoxycholate, 400 mmol/L KOAc, 25 mmol/L HEPES, 15 mmol/L MgOAc, 1 mmol/L DTT, 200 μmol/L cycloheximide, and 80 U/mL RNase A at 4°C. Nuclei and mitochondria were removed by centrifugation and supernatants were layered onto 15% to 40% sucrose gradients and spun at 38,000 rpm for 2 hours at 4°C in a SW40 rotor (Beckman Instruments). Centrifuged gradients were fractionated using a gradient fractionator system (Brandel Instruments) at a flow rate of 3 mL/min. The polysome profile of the gradient was monitored via UV absorbance at 260 nm. RNA was precipitated and subsequently pooled into nonribosomal/monomosomal and polysomal fractions. These RNAs (100 ng) were used in real-time quantitative reverse transcriptase-PCR (qRT-PCR) analysis for the indicated mRNAs using amplicons located within the coding regions. Real-time amplifications were carried out on the amplicons are available upon request.

mRNA levels as previously described (16). Primers for the amplicons are available upon request.

Statistical analysis

Significance between groups for all experiments was done with Student’s t test and ANOVA models using Systat 13 (Systat Software). $P$ values of less then 0.05 were considered significant.

Results

AKT-dependent SAPK2/p38 activation and IRES activity following mTORC1 inhibition

In our experiments, we used 2 pairs of isogenic glioblastoma multiforme (GBM) lines, which differ dramatically in their degree of AKT kinase activation and have been described previously (11). U87 cells harbor a mutant nonfunctional PTEN and as a result display elevated AKT activity (9). These cells were stably transduced with an adenoviral vector expressing native PTEN (U87empty vector, $U87_{EV}$ and $U87_{PTEN}$). The LN229 GBM line contains a functional PTEN tumor suppressor and was stably transfected with a myr-AKT-MER fusion, which is a fusion protein consisting of the active form of AKT fused to the ligand-binding domain of the estrogen receptor (MER; LN229empty vector, LN229$_{EV}$ and LN229$_{MER-AKT}$; ref. 11). This fusion is conditionally regulatable via the addition of the MER ligand, 4-hydroxy-tamoxifen (4OHT) exhibiting elevated AKT activity in its presence and is inactive in its absence (21). The relative expression of the transgenes and the marked differential mTORC1 inhibitor sensitivities of these paired lines have been previously shown (9, 11). Our previous data implicated the differential activation of SAPK2/p38 kinase following mTORC1 inhibition in an AKT-dependent manner (10). Data from other laboratories also supported the activation of SAPK2/p38 by rapamycin (22, 23), and its involvement in the support of c-MYC IRES activity in response to various genotoxic stresses (15). As shown in Fig. 1A, rapamycin exposure resulted in a marked activation of SAPK2/p38 activity in quiescent AKT-containing lines ($U87_{PTEN}$ and LN229$_{MER-AKT}$ (in the absence of 4OHT) and LN229$_{EV}$ (irrespective of ligand)). However, in lines containing elevated AKT activity rapamycin exposure markedly inhibited SAPK2/p38 activity ($U87_{EV}$, LN229$_{MER-AKT}$ (in the presence of 4OHT)), consistent with the known negative regulation of AKT on SAPK2/p38 activity (24, 25). The downstream effector of SAPK2/p38, ATF2 also showed increased phosphorylation in an in vitro kinase assay conducted from extracts taken from...
either quiescent or active AKT-containing cells following rapamycin treatment (Fig. 1B). As shown, SAPK2/p38 immunoprecipitated from quiescent AKT-containing U87PTEN cells showed a marked increase in phospho-ATF2 as compared with its counterpart elevated AKT-containing U87EV cells following rapamycin exposure. Similarly, LN229MER-AKT in the absence of the inducer 4OHT, containing quiescent AKT levels, displayed markedly elevated SAPK2/p38 activity, as did the LN229EV line irrespective of 4OHT following rapamycin treatment. To then determine whether rapamycin-induced cyclin D1 and c-MYC IRES activity required SAPK2/p38 kinase, we conducted IRES assays using the reporter constructs shown in Fig. 1C. These dicistronic mRNA reporters contain the indicated IRES sequences within the intercistronic region between Renilla and firefly open reading frames, thus Renilla luciferase activity is a readout of cap-dependent translation while firefly translation is directed by the respective IRES. These plasmids were then transiently transfected into U87EV and U87PTEN cells in which SAPK2/p38 was knocked down via RNAi. SiRNAs targeting SAPK2/p38 effectively reduced levels to below detection via immunoblot while a nontargeting scrambled control siRNA had no appreciable effect on expression (Supplementary Fig. S1A). U87EV or U87PTEN cells were subsequently transiently transfected with the indicated siRNAs and IRES reporter plasmids (Fig. 1D) and Renilla and firefly luciferase activities determined in the absence or presence of rapamycin. As observed previously (10, 11), cyclin D1 and c-MYC IRES activity was markedly induced by rapamycin specifically in U87PTEN quiescent AKT-containing cells relative to elevated AKT-containing U87EV cells in control and scrambled siRNA transected groups. However, in cells treated with siRNAs targeting SAPK2/p38 both cyclin D1 and c-MYC IRES activity was curtailed following rapamycin exposure. Similar data were observed with the conditionally inducible AKT cell line LN229EV and LN229AKT-MER cell line pair. As shown in Fig. 1E, in the absence of 4OHT, quiescent AKT-containing LN229EV and LN229AKT-MER displayed high levels of both cyclin D1 and c-MYC IRES activity in control and nontargeting scrambled siRNA-treated groups, which was inhibited by SAPK2/p38 knockdown (see also Supplementary Fig. S1B). In the presence of 4OHT, differential AKT-dependent cyclin D1 and c-MYC IRES activity was observed as before, which was also abrogated by knockdown of SAPK2/p38. We also determined whether a pharmacologic approach to inhibiting SAPK2/p38 via the specific inhibitor SB203580 or, the potentially more clinically relevant inhibitor LY2228840 would have effects on rapamycin induced IRES activity. As shown in Fig. 1F, U87EV elevated AKT-containing cells did not exhibit significant rapamycin inducible cyclin D1 or c-MYC IRES activity as previously observed (10) and was unaffected by SB203580. We had previously determined that the p27Kip1 IRES displayed increased IRES activity following rapamycin exposure in several cell lines that was not AKT-dependent (9). Activity from this IRES was also not sensitive to either of the SAPK2/p38 inhibitors. U87pten cells, containing relatively quiescent AKT displayed a marked induction of rapamycin stimulated cyclin D1 and c-MYC IRES activity as before, which was inhibited by either SB203580 or LY2228840 by comparable amounts. Again similar data was obtained for the LN229EV and LN229AKT-MER cell line pair. LN229AKT-MER in the presence of 4OHT did not show cyclin D1 or c-MYC IRES activity while LN229EV cells displayed significant IRES activity that was sensitive to both SAPK2/p38 inhibitors. Cyclin D1 and c-MYC IRES activities of these lines in the absence of 4OHT did not differ from those values obtained for the control LN229EV cells in the absence or presence of the inhibitors (data not shown). These data show that AKT-dependent rapamycin stimulated cyclin D1 and c-MYC IRES activity requires SAPK2/p38 function.

**Signaling effects of SAPK2/p38 and mTORC1 inhibition**

We then examined the effects of combining rapamycin and SB203580 on relevant signaling pathways targeted by these inhibitors. Exposure of active AKT-containing U87EV cells to rapamycin was associated with decreased...
cycdin D1 and c-MYC protein levels while in relatively quiescent AKT containing U87PTEN cells, cycdin D1 and c-MYC expression increased (Fig. 2A). Treatment with SB203580 alone did not significantly affect cycdin D1 or c-MYC expression in either cell line (U87EV vs. U87PTEN), however, cotreatment with rapamycin and SB203580 markedly reduced the rapamycin-stimulated cycdin D1 and c-MYC protein levels observed in the quiescent AKT-containing U87PTEN line. Treatment with rapamycin was associated with reduced phospho-S6K levels and had no significant effect on serine 473 phosphorylation of AKT suggesting mTORC1 was effectively inhibited and that mTORC2 activity was not altered by exposure. In addition, this suggests that the AKT-dependent alterations observed in cycdin D1 and c-MYC levels are not a consequence of changes in mTORC2 activity. We also assessed the phosphorylation state of the downstream effectors of mTORC2 activity. We also assessed the phosphorylation state of the downstream effectors of mTORC2 activity. Treatment of U87EV or U87PTEN cells with rapamycin induced G1 arrest and apoptosis of quiescent AKT-containing cells. The hallmark outcome of rapamycin treatment in most cell types is G1 arrest (26), although in some cells apoptotic death can be induced (27). Our previous studies have shown that relative AKT activity has a profound effect on the ability of rapamycin to induce G1 arrest (9). To test whether rapamycin induced AKT-dependent G1 arrest could be potentiated, or apoptosis induced by additional SAPK2/p38 inhibition, we determined the cell-cycle distributions of the U87 and LN229 paired cell lines following cotreatments with the inhibitors. As shown in Fig. 3A, high AKT-containing U87EV cells were sensitive to rapamycin as compared with control (increased from 34% G1/G0 to 52% G1/G0) while quiescent AKT-containing U87PTEN cells were relatively resistant (28% G1/G0 in control to 30% G1/G0 rapamycin treated). Treatment of U87EV or U87PTEN cells with SB203580 alone did not have a significant effect on the

Combination of SAPK2/p38 and mTORC1 inhibition induces G1 arrest and apoptosis of quiescent AKT-containing cells

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Figure 3. Inhibition of SAPK2/p38 induces G1 arrest in rapamycin-resistant quiescent AKT-containing GBM lines. Cell-cycle phase distributions were determined on U87EV and U87PTEN cell treated with + rapamycin and + SB203580 (A) or with + rapamycin and + LY228820 (B) as shown. Percent apoptotic cells as determined via Annexin V staining is also shown below each graph. C and D were identical to A and B, respectively, except LN229MER-AKT and LN229EV cells induced with 4OHT were used. One of 3 experiments with similar results are shown.
relative cell-cycle distributions, however, combining rapamycin and SB203580 markedly increased the percentage of cells arrested in G1. Most notably, rapamycin-resistant quiescent AKT-containing U87PTEN cells shifted significantly to the G1/G0 phase (30% G1/G0 rapamycin treatment alone to 58% G1/G0 combination treatment). SB203580 and rapamycin cotreatment did not enhance the rapamycin induced G1 arrest displayed in elevated AKT containing U87 EV cells. In addition, the percent of apoptotic cells, determined by Annexin V staining, indicated a modest but significant increase in the percentage of apoptotic cells in U87PTEN line treated with both inhibitors (3.1% apoptotic cells rapamycin treatment alone to 13.6% apoptotic cells combination treatment). We also conducted these analyses using the LY2228820 SAPK2/p38 inhibitor as shown in Fig. 3B. Treatment of either U87EV or U87PTEN cells with rapamycin and SB203580 markedly increased the percentage of cells arrested in G1. Most notably, rapamycin-LY2228820 combination treatment (30% G1/G0 rapamycin alone to 54% G1/G0 cotreatment). The percentage of apoptotic cells also increased from 2.9% following treatment with rapamycin alone to 11.7% after cotreatment. We also determined the cell-cycle distributions of the LN229 cell line pair following SAPK2/p38 and mTORC1 inhibitor cotreatments (Fig. 3C and D). LN229MER-AKT cells containing elevated AKT activity responded to rapamycin exposure by arresting in G1 (32% G1/G0 control to 58% G1/G0 rapamycin treated), however, LN229EV were resistant (33% G1/G0 control to 31% G1/G0 rapamycin treated; Fig. 3C). Neither of the SAPK2/p38 inhibitors alone had significant affects on the cell-cycle distributions of LN229AKT-MER nor LN229EV cells (Fig. 3C and D). Combining rapamycin and SB203580 treatments again resulted in a significant increase in the number of LN229EV cells in G1/G0 (LN229EV; 34% G1/G0 rapamycin alone to 50% G1/G0 combination treatment), as was the case when rapamycin and LY2228820 were combined (LN229EV; 29% G1/G0 rapamycin alone to 58% G1/G0 combination treatment).
treatment). Treatment of LN229\textsubscript{MER-AKT} or LN229\textsubscript{EV} cells with the inducer 4OHT had no significant effects on cell-cycle distribution as compared with values obtained in its absence (data not shown). A significant increase in the number of apoptotic cells was also detected in LN229\textsubscript{EV} cells following cotreatment with both SAPK2/p38 and mTORC1 inhibitors. These data show that SB203580 or LY2228820 significantly enhanced rapamycin induced G\textsubscript{1} arrest and increased apoptosis in quiescent AKT-containing GBM cells.

**Synergistic antiproliferative effects of SAPK2/p38 and mTORC1 inhibitors**

To support the previously observed effects on cell-cycle arrest, we determined whether the combination of SAPK2/p38 inhibitors and rapamycin would have effects on in vitro growth of the paired U87 and LN229 cell lines in XTT assays. We initially determined whether active or quiescent AKT-containing lines were sensitive to growth inhibition following exposure to SB203580 or LY2228820 alone. As shown in Fig. 4A, no significant inhibition of cell growth was observed at any of the concentrations tested up to 10 \textmu\text{mol/L} with either inhibitor. We subsequently determined the effects of SB203580 or LY2228820 on the AKT-dependent inhibition of growth of the paired U87 and LN229 lines following rapamycin exposure. As shown in Fig. 4B, U87\textsubscript{EV} elevated AKT-containing cell growth was inhibited by rapamycin (IC\textsubscript{50} = 0.9 nmol/L) in a dose-dependent manner, which was not potentiated by the addition of either SB203580 or LY2228820, however, in U87\textsubscript{PTEN} quiescent AKT-containing cells treatment with SB203580 at 4 \textmu\text{mol/L} and LY2228820 at both 200 and 400 nmol/L concentrations resulted in synergistic inhibition of cell growth over a wide range of rapamycin concentrations tested (SB203580; CI = 0.3 at ED\textsubscript{50} ratio of 1:1,000, LY2228820; CI = 0.2 at ED\textsubscript{50} ratio of 1:400; refs. 28, 29). In the LN229 cell line pair (Fig. 4C), LN229\textsubscript{MER-AKT} cells containing elevated AKT activity were highly susceptible to growth inhibition induced by rapamycin (IC\textsubscript{50} = 1 nmol/L) as compared with their relatively resistant counterpart line LN229\textsubscript{EV} (IC\textsubscript{50} = 2 \textmu\text{mol/L}). Again, inhibition of SAPK2/p38 via SB203580 or LY2228820 treatment did not significantly potentate the effects on cell proliferation in rapamycin treated elevated AKT-containing line LN229\textsubscript{MER-AKT}, however,

![Figure 5. In vivo inactivation of SAPK2/p38 and mTORC1 inhibits tumor growth of quiescent AKT-containing xenografts. SCID mice with the indicated xenografts of the paired U87 and LN229 cells, A–D, were treated with indicated schedules of double vehicle, rapamycin (black bar), SB203580 (white bar), or combination for 10 consecutive days and tumor growth was assessed every 2 days following initiation of treatment (start, day 0). Mice with LN229\textsubscript{MER-AKT} and LN229\textsubscript{EV} xenografts received 4OHT via daily maintenance injections.](image-url)
Figure A: Western blot analysis showing expression levels of cyclin D1, c-myc, P-AKT (S473), P-S6K (T389), P-p38 (T180/Y182), and p38 in U87, U87_Pten, LN229, and LN229_WT cell lines.

Figure B: Immunohistochemical staining of double vehicle and rapamycin (rapa) treated SB203580 with or without rapa.

Figure C: Quantification of TUNEL-positive cells in U87, U87_Pten, LN229, and LN229_WT cell lines under different treatments.

Figure D: Quantitative PCR analysis of actin, c-myc, and cyclin D1 expression in U87 and U87_Pten cell lines treated with double vehicle, rapa 4 mg/kg, SB203580 8 mg/kg, and rapa 4 mg/kg + SB203580 8 mg/kg.
cotreatment with either of the SAPK2/p38 inhibitors resulted in a marked synergistic effects on the antiproliferative capacity of rapamycin in resistant LN229Ev cells (SB203580; CI = 0.4 at ED$_{50}$ ratio of 1:1,000, LY2288820; CI = 0.3 ratio 1:400). A dose-dependent inhibition of SAPK2/p38 activity by the inhibitors was observed and suggested that near complete ablation of SAPK2/p38 function was required to elicit maximal antiproliferative effects (Supplementary Fig. 2). These data show the strong synergistic antiproliferative effects of combined mTORC1 and SAPK2/p38 treatment in quiescent AKT-containing cells.

**In vivo effects of SB203580 and rapamycin combination therapy in xenografts**

To determine whether the combination of SAPK2/p38 and mTORC1 inhibitor cotherapy would be efficacious in vivo, we conducted xenograft studies with the paired U87 and LN229 lines in mice. Mice were subcutaneously implanted with tumor cells and once tumors were palpable and reached ~200 mm$^3$, mice were randomized into treatment groups receiving double vehicle, rapamycin (1 mg/kg/d), SB203580 (1.5 mg/kg/d), and rapamycin (1 mg/kg/d) + SB203580 (1.5 mg/kg/d). As shown in Fig. 5, mice with the indicated xenografted tumors (panels A–D) received the various dosing schedules and tumor size assessed by direct measurements. Xenografts of elevated AKT-containing U87Ev cells receiving mono-therapy with rapamycin resulted in significant inhibition of tumor growth rate (Fig. 5A, 76% inhibition at end of dosing period, tumor growth delay 16.5 days) whereas U87PTEN quiescent AKT-containing tumors were relatively refractory to treatment (Fig. 5B, 23% inhibition at end of dosing period, tumor growth delay 5.5 days). Tumor growth following monotherapy with SB203580 of either U87Ev or U87PTEN xenografts also did not differ significantly and exhibited similar growth rates to double vehicle controls consistent with the lack of effects of this inhibitor alone in vitro. However, in both U87Ev and U87PTEN xenografts tumor growth rate was inhibited to a comparable extent following combination therapy (77% and 70% inhibition, respectively at end of dosing period; tumor growth delay 24 and 24.5 days, respectively) as compared with double vehicle. Similar AKT-dependent growth effects were observed for LN229MER-AKT and LN229Ev tumors following mono- or combination therapies with SB203580 and rapamycin (Fig. 5C and D). Tumor growth rates of elevated AKT activity containing LN229MER-AKT tumors were markedly inhibited by monotherapy with rapamycin (69% inhibition at end of dosing period, tumor growth delay 20.5 days) relative to double vehicle treatment control tumors, whereas the relatively quiescent AKT-containing LN229Ev tumors did not respond to treatment as effectively (29% inhibition at end of dosing period, tumor growth delay 5.5 days). Monotherapy with SB203580 did not inhibit tumor growth rates of LN229MER-AKT or LN229Ev tumors relative to double vehicle controls as previously observed with the U87 cell line pair. The combination of SB203580 and rapamycin was significantly more efficacious in the rapamycin monotherapy resistant LN229Ev tumors (88% inhibition at end of dosing period, tumor growth delay 25 days) relative to double vehicle. Tumor growth rates of the LN229MER-AKT and LN229Ev tumors were comparably inhibited by combination therapy (85% and 88% inhibition, respectively at end of dosing period; tumor growth delay 24 and 25 days, respectively). These data show a significant inhibition of quiescent AKT-containing xenograft tumor growth by combination SB203580 and rapamycin treatment. We also confirmed the signaling effects of these treatments on the harvested tumors following dosing. As shown in Fig. 6A, elevated AKT-containing tumors (U87Ev, LN229MER-AKT) rapamycin monotherapy resulted in decreased cyclin D1/c-MYC levels while in relatively quiescent AKT-containing tumors (U87PTEN, LN229Ev) rapamycin treatment increased cyclin D1/c-MYC expression correlating well with the signaling alterations observed in vitro. Differential AKT activity was sustained in both paired xenografted cell lines (U87Ev vs. U87PTEN and LN229MER-AKT vs. LN229Ev) irrespective of inhibitor treatments. Treatment of mice with either rapamycin monotherapy or SB203580 and rapamycin combination therapy comparably inhibited mTORC1 activity as determined by phosphorylated threonine 389 S6 kinase levels in all xenografts. Monotherapy with SB203580 had no appreciable effect on phospho-T389 S6 kinase levels in any of the engrafted cell lines and monotherapy with SB203580 alone or combination therapy with SB203580 and rapamycin comparably inhibited SAPK2/p38 activity in all the tumors, while monotherapy with rapamycin had no observable effect. Finally, we also monitored the induction of apoptosis via TUNEL staining of tumor sections (Fig. 6B). As can be seen in Fig. 6C, significant staining was evident in quiescent AKT-containing tumors (U87PTEN, LN229Ev) from mice that received combination therapy, corroborating the increase in apoptotic cell observed in...
our in vitro experiments treated with these inhibitors (see Fig. 3). No significant loss in body weight was noted in these models at the concentrations of inhibitors used, suggesting that they were well tolerated in vivo.

Cyclin D1 and c-MYC mRNA translational state in rapamycin-resistant quiescent AKT-containing tumors in response to SAPK2/p38 and mTORC1 inhibitors

We have previously shown that cyclin D1 and c-MYC IRES activity nearly exclusively directs protein synthesis of these 2 determinants in the face of rapamycin exposure in the quiescent AKT setting (16). To attempt to correlate the AKT-dependent alterations in cyclin D1/c-MYC expression mediated by the inhibitor therapies in xenografted tumors with actual changes in mRNA translational efficiency of these transcripts, we conducted polysome analysis of freshly harvested U87EV and U87PTEN tumors following the last day of inhibitor dosing. Polysomes were isolated from homogenized extracts of U87EV and U87PTEN tumor xenografts from mice treated with the mono- and combination therapies as before (see Fig. 5A–D and Fig. 6A). Polysomes were separated via sucrose density gradient sedimentation and fractionated into heavy polysomal and nonribosomal/monosomal fractions. Transcripts sedimenting within the heavy polysomal fractions are undergoing active translation while those present within the nonribosomal/monosomal fractions are relatively poorly translated. Spectrophotometric monitoring of fractions at 260 nm was used to identify polysome and nonribosomal containing fractions and monitor polysome integrity (see Supplementary Fig. S2). As shown in Fig. 6D, U87EV or U87PTEN tumors from mice that received double vehicle treatments, cyclin D1 and c-MYC transcripts were predominantly polysomal. We also monitored actin mRNA polysome distribution in these tumors and as shown this mRNA whose translation initiation is mediated by eIF-4E was redistributed to nonribosomal fractions showing effective inhibition of cap-dependent protein synthesis by rapamycin therapy. These data are consistent with our previous in vitro mRNA translational state experiments (10, 16) and show differential AKT-dependent cyclin D1 and c-MYC mRNA translational efficiency in actual tumors. Mice that received SB203580 monotherapy displayed similar cyclin D1 and c-MYC mRNA translational states from U87EV or U87PTEN explants as tumors from double vehicle-treated mice, however, cyclin D1 and c-MYC transcripts from tumors excised from mice receiving combination therapy displayed a significant redistribution of cyclin D1 and c-MYC mRNA to nonribosomal fractions. Importantly, this shift in polysome association occurred in quiescent AKT-containing tumors suggesting that cyclin D1 and c-MYC IRES-mediated protein synthesis was inhibited in these tumors.

Discussion

The successes achieved with some targeted therapies have shown dramatic efficacy with relatively little toxicity. However, drug resistance has emerged as a significant obstacle impeding curative monotherapies with signal transduction inhibitors. Combining inhibitors of multiple signaling pathways with functional roles in resistance may prove efficacious. In this report, we used 2 isogenic cell line pairs that differ in their relative degrees of AKT activity to show that mTORC1 and SAPK2/p38 inhibition have strong synergistic antiproliferative properties in vitro and in vivo in the quiescent AKT setting. Previously, we have shown that continued IRES-dependent protein synthesis of the cyclin D1 and c-MYC mRNA was induced following mTORC1 inhibition leading to rapamycin resistance in cells harboring little AKT activity. This induction of IRES activity was shown to be dependent on SAPK2/p38 activity. These studies provided a biochemical rationale for investigating whether the combination of small molecule inhibitors targeting these pathways would generate antiproliferative responses.

Our data show that SAPK2/p38 activity is strongly induced following rapamycin exposure in the absence of AKT activity (Fig. 1A and B). Following appropriate stimulation, SAPK2/p38 MAPKs are activated by dual phosphorylation in their activation loop sequence Thr-Gly-Tyr by 3 dual specificity MAP2Ks (MKK3, 4 and 6) (30). The relative contribution of different MAP2Ks to SAPK2/p38 activation depends on the stimulus and cell type due to variations in MAP2K expression levels among cell types (31). Several MAP3Ks (MAP2K kinases) have also been shown to trigger SAPK2/p38 activation including ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual-leucine-zipper-bearing kinase 1) and MLK3 (mixed-lineage kinase 3). The ability of rapamycin to dissociate the PP52A-B regulatory subunit (PR72) from the PP5-ASK1 complex leading to increased ASK1 activity (32) provides a ready mechanistic explanation as to how rapamycin exposure may lead to SAPK2/p38 activation. However, as AKT is known to negatively regulate ASK1 (33, 34), clearly additional mechanisms may be operative in active AKT-containing cells in which we observed increased SAPK2/p38 activity following rapamycin exposure. It was also observed that neither cyclin D1 nor c-MYC rapamycin-induced IRES activity returned to basal levels following SAPK2/p38 inhibition (see Fig. 1) and is suggestive of additional SAPK2/p38-independent signaling contributions. We are currently addressing these mechanistic questions.

Previous reports have described additive and synergistic antitumor effects of rapamycin in combination with other chemotherapeutic agents in vitro, and decreases in
pHsensitive proliferation of tumor xenografts and tumor vasculature (29, 35, 36). We also observed significant decreases in tumor vasculature as determined by VEGF staining in both U87EV and U87PTEN xenografts following monotherapy with rapamycin and combination therapy (not shown). Rapamycin is known to exhibit antiangiogenic effects in xenografts (35, 37) and this activity could contribute to the antitumor responses following combination therapy.

The analysis of cyclin D1 and c-MYC mRNA translational state in U87EV and U87PTEN tumor xenografts support the notion that IRES-mediated translation of these determinants is reduced following combination therapy with the inhibitors (Fig. 6D). These data are consistent with the known requirement for SAPK2/p38 activity for c-MYC IRES activity, and more recently SAPK2/p38 activity has been implicated in the regulation of XIAP IRES activity (15, 38). Although long-term stable expression of IRES containing dicistronic reporters has been achieved in mice (39), our efforts thus far in generating stable long-term expression of cyclin D1 and c-MYC IRES baring dicistronic reporters in cell lines have been unsuccessful, hampering the ability to directly assess IRES activities in xenografts. Inducible expression of these reporters may permit analyses of IRES activities in tumors.

In conclusion, our studies show that the combination of SAPK2/p38 inhibition and rapamycin may provide an effective therapeutic strategy in those GBMs harboring normal PTEN status and quiescent AKT. Furthermore, it is possible that these effects may be generalizable to other tumor types, as our previous studies also suggest that AKT-dependent cyclin D1 and c-MYC IRES activity requires SAPK2/p38 activity in prostate cancer lines as well (10). With the development of potent and selective SAPK2 inhibitors, with possibly more favorable toxicity profiles (40, 41), this treatment option may find clinical use. Alternatively, developing inhibitors of specific ITAF-IRES interactions may prove more fruitful in this regard.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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