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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 demonstrated 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 inhibitors while cells in which the kinase is quiescent are relatively resistant. Our previous work has demonstrated that a transcript specific protein synthesis salvage pathway is operative in cells with quiescent AKT 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 demonstrate that both genetic and pharmacological inhibition of SAPK2/p38 in glioblastoma multiforme (GBM) 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 demonstrate 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 anti-tumor therapeutic response, particularly in rapamycin resistant quiescent AKT-containing cells.

Keywords

mTOR; p38 MAPK; AKT; IRES; glioblastoma multiforme

Introduction

Glioblastoma is the most common malignant brain tumor in adults and is generally highly refractive to current chemotherapies (1). Additionally, these tumors are highly invasive rendering surgical resection difficult under best circumstances with median survival times of only 12–15 months following diagnosis (2). The mTOR kinase has emerged as an attractive target for current therapeutic approaches, however monotherapies utilizing 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 which 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 to 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). Since 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 anti-proliferative effect of these compounds when used in combination against xenografted cells. Finally, we demonstrate 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 as well as the IRES containing plasmids pRCND1F, pRmycF and pRp27F have been described previously (9) and contain the minimal IRES sequences for each of the three 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 ATCC. The U87 line was stably transduced with a retroviral construct containing a wild-type *PTEN* gene or empty vector (EV) to generate U87^{PTEN} and U87^{EV} (gift from I. Mellinghoff and C. Sawyers) (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 performed using X-treme GENE Q² transfection reagent (Roche Applied Science) according to the manufacturer's recommendations. siRNA transfections targeting human SAPK2/p38 were performed 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 nM, SB203580 at 4 μ M and LY2228820 at 200 nM for 24 h treatments unless otherwise indicated.

IRES Reporter Assays

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

Protein analysis

Immunoblots were performed using standard procedures. Briefly, cells were lysed in 50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na₃VO₄, 10 mM NaF, 2 mM PMSF, 0.5 mM EDTA, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. Samples were resolved by 10% or 12% SDS-PAGE, transferred to PVDF (0.2 μ M) 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-AKT-(S473), phospho-S6K1-(T389) and S6K1 were from Cell Signaling. Phospho-PRAK (T182) and phospho-ATF-2-(T69/71) were from Abcam. Blots were then incubated in appropriate horseradish peroxidase-conjugated secondary antibody and then detected using ECL plus (Amersham Biosciences). For the *in vitro* kinase assays, cleared supernatants were incubated with anti-SAPK2/p38 antibody and protein A-Sepharose overnight. Pellets were washed three times in lysis buffer and once in kinase buffer containing 25 mM HEPES, pH 7.4, 25 mM β -glycerolphosphate, 25 mM MgCl₂, 0.5 mM EDTA and 0.5 mM dithiothreitol. Kinase reactions were performed in the presence of 100 mM ATP and 2 μ g of ATF-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 2×10^4 /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-sulphophenyl]-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 FITC-conjugated anti-annexin V antibody (Abcam). For TUNEL staining of sections, slides were stained using 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

SCID mice were injected subcutaneously with single cell suspensions of the U87_{EV}, U87_{PTEN}, LN229_{EV} and LN229_{MER-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 (eight 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 histological sectioning. Tumor growth delay values were calculated as previously defined (19).

Polysome Analysis

Extraction and display of polysomes was performed as previously described (10, 20). Briefly, fresh tumors were minced and homogenized in buffer containing 1% Triton X-100, 1% deoxycholate, 400 mM KOAc, 25 mM HEPES, 15 mM MgOAc, 1 mM DTT, 200 μ M cycloheximide and 80 U/ml RNase Out at 4 °C. Nuclei and mitochondria were removed by centrifugation and supernatants were layered onto 15–50% sucrose gradients and spun at 38,000 rpm for 2 h 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/monosomal and polysomal fractions. These RNAs (100 ng) were used in real time quantitative RT-PCR analysis for the indicated mRNAs using amplicons located within the coding regions. Real time amplifications were carried out on a Eppendorf Mastercycler equipped with a Realplex² optical module (Eppendorf AG, Germany) and normalized to actin 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 analysis of variance models using Systat 13 (Systat Software, Chicago, IL). P values of less than 0.05 were considered significant.

Results

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

In our experiments we utilized two pairs of isogenic 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}) (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), as well as its involvement in the support of c-MYC IRES activity in response to various genotoxic stresses (15). As shown in figure 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 demonstrated 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 U87^{PTEN} cells demonstrated a marked increase in phospho-ATF2 as compared to its counterpart elevated AKT-containing U87^{EV} cells following rapamycin exposure. Similarly, LN229^{MER-AKT} in the absence of the inducer 4OHT, containing quiescent AKT levels, displayed markedly elevated SAPK2/p38 activity, as did the LN229^{EV} 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 performed IRES assays utilizing the reporter constructs shown in figure 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 U87^{EV} and U87^{PTEN} cells in which SAPK2/p38 was knocked down via RNAi. siRNAs targeting SAPK2/p38 effectively reduced levels to below detection via immunoblot while a non-targeting scrambled control siRNA had no appreciable effect on expression (supplementary Fig. 1A). U87^{EV} or U87^{PTEN} 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 U87^{PTEN} quiescent AKT-containing cells relative to elevated AKT-containing U87^{EV} cells in control and scrambled siRNA transfected 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 LN229^{EV} and LN229^{AKT-MER} cell line pair. As shown in figure 1E, in the absence of 4OHT, quiescent AKT-containing LN229^{EV} and LN229^{AKT-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. 1B). 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 pharmacological approach to inhibiting SAPK2/p38 via the specific inhibitor SB203580 or, the potentially more clinically relevant inhibitor LY2228820, would have effects on rapamycin induced IRES activity. As shown in figure 1F, U87^{EV} 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 p27^{Kip1} IRES displayed increased IRES activity following rapamycin exposure in several cell lines which was not AKT-dependent (9). Activity from this IRES was also not sensitive to either of the SAPK2/p38 inhibitors. U87^{PTEN} 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 LN229^{EV} and LN229^{MER-AKT} cell line pair. LN229^{MER-AKT} in the presence of 4OHT did not demonstrate cyclin D1 or c-MYC IRES activity while LN229^{EV} cells displayed significant IRES activity which 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 LN229_{EV} cells in the absence or presence of the inhibitors (data not shown). These data demonstrate 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 U87_{EV} cells to rapamycin was associated with decreased cyclin D1 and c-MYC protein levels while in relatively quiescent AKT containing U87_{PTEN} cells, cyclin D1 and c-MYC expression increased (Fig. 2A). Treatment with SB203580 alone did not significantly affect cyclin D1 or c-MYC expression in either cell line (U87_{EV} vs. U87_{PTEN}), however cotreatment with rapamycin and SB203580 markedly reduced the rapamycin-stimulated cyclin D1 and c-MYC protein levels observed in the quiescent AKT-containing U87_{PTEN} 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. Additionally, this suggests that the AKT-dependent alterations observed in cyclin 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 SAPK2/p38, PRAK and ATF2. Both phospho-threonine 182 PRAK and phospho-threonine 69/71 ATF2 levels were inhibited by SB203580 alone and in combination with rapamycin in elevated and quiescent AKT-containing lines. Induced LN229_{AKT-MER} cells also displayed markedly reduced cyclin D1 and c-MYC expression levels following rapamycin treatment, however in quiescent AKT containing LN229_{EV} cells cyclin D1 and c-MYC levels increased as observed previously (Fig. 2B) (11). Cotreatment with both rapamycin and SB203580 inhibited cyclin D1 and c-MYC expression in both lines irrespective of AKT activity. Treatment with the inhibitors alone or in combination also had similar effects on substrate phosphorylations for S6K, mTORC1/2 and SAPK2/p38 as observed in the paired U87 lines. Rapamycin alone blunted mTORC1 activity while not significantly affecting mTORC2 and SB203580 effectively inhibited SAPK2/p38 activity. These data support the specific and effective inhibition of SAPK2/p38 and mTORC1 activities under these conditions.

Combination of SAPK2/p38 and mTORC1 inhibition induces 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 demonstrated 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 figure 3A, high AKT-containing U87_{EV} cells were sensitive to rapamycin as compared to control (increased from 34% G₁/G₀ to 52% G₁/G₀) while quiescent AKT-containing U87_{PTEN} cells were relatively resistant (28% G₁/G₀ in control to 30% G₁/G₀ rapamycin treated). Treatment of U87_{EV} or U87_{PTEN} cells with SB203580 alone did not have a significant effect on the 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 U87_{PTEN} 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. Additionally, the percent of apoptotic cells, determined by annexin V staining, indicated a modest but significant

increase in the percentage of apoptotic cells in U87^{PTEN} line treated with both inhibitors (3.1% apoptotic cells rapamycin treatment alone to 13.6% apoptotic cells combination treatment). We also performed these analyses using the LY2228820 SAPK2/p38 inhibitor as shown in figure 3B. Treatment of either U87^{EV} or U87^{PTEN} cells with LY2228820 alone had little effect on cell cycle distribution as compared to control, however cotreatment with both rapamycin and LY2228820 resulted in a substantial increase in the percentage of cells arrested in G₁ in quiescent AKT-containing U98^{PTEN} cells (32% G₁/G₀ LY2228820 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 & D). LN229^{MER-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 LN229^{EV} were resistant (33% G₁/G₀ control to 31% G₁/G₀ rapamycin treated) (Fig. 3C). Neither of the SAPK2/p38 inhibitors alone had significant effects on the cell cycle distributions of LN229^{AKT-MER} nor LN229^{EV} cells (Fig 3C & D). Combining rapamycin and SB203580 treatments again resulted in a significant increase in the number of LN229^{EV} cells in G₁/G₀ (LN229^{EV}; 34% G₁/G₀ rapamycin alone to 50% G₁/G₀ combination treatment), as was the case when rapamycin and LY2228820 were combined (LN229^{EV}; 29% G₁/G₀ rapamycin alone to 58% G₁/G₀ combination treatment). Treatment of LN229^{MER-AKT} or LN229^{EV} cells with the inducer 4OHT had no significant effects on cell cycle distribution as compared to values obtained in its absence (data not shown). A significant increase in the number of apoptotic cells was also detected in LN229^{EV} cells following cotreatment with both SAPK2/p38 and mTORC1 inhibitors. These data demonstrate that SB203580 or LY2228820 significantly enhanced rapamycin induced G₁ 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 figure 4A, no significant inhibition of cell growth was observed at any of the concentrations tested up to 10 μ M 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 figure 4B, U87^{EV} elevated AKT-containing cell growth was inhibited by rapamycin (IC₅₀ = 0.9 nM) in a dose-dependent manner which was not potentiated by the addition of either SB203580 or LY2228820, however in U87^{PTEN} quiescent AKT-containing cells treatment with SB203580 at 4 μ M and LY2228820 at both 200 and 400 nM concentrations resulted in synergistic inhibition of cell growth over a wide range of rapamycin concentrations tested (SB203580; CI = 0.3 at ED₅₀ ratio of 1:1000, LY2228820; CI = 0.2 at ED₅₀ ratio of 1:400) (28, 29). In the LN229 cell line pair (Fig. 4C), LN229^{MER-AKT} cells containing elevated AKT activity were highly susceptible to growth inhibition induced by rapamycin (IC₅₀ = 1 nM) as compared to their relatively resistant counterpart line LN229^{EV} (IC₅₀ = 2 μ M). Again, inhibition of SAPK2/p38 via SB203580 or LY2228820 treatment did not significantly potentiate the effects on cell proliferation in rapamycin treated elevated AKT-containing line LN229^{MER-AKT}, however cotreatment with either of the SAPK2/p38 inhibitors resulted in a marked synergistic effects on the antiproliferative capacity of rapamycin in resistant LN229^{EV} cells (SB203580; CI = 0.4 at ED₅₀ ratio of 1:1000, LY2228820; 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 demonstrate 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 performed 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 mm³, 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 figure 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 U87_{EV} cells receiving monotherapy 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 U87_{PTEN} 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 U87_{EV} or U87_{PTEN} 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 U87_{EV} and U87_{PTEN} 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 to double vehicle. Similar AKT-dependent growth effects were observed for LN229_{MER-AKT} and LN229_{EV} tumors following mono- or combination therapies with SB203580 and rapamycin (Fig. 5C & D). Tumor growth rates of elevated AKT activity containing LN229_{MER-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 LN229_{EV} 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 LN229_{MER-AKT} or LN229_{EV} 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 LN229_{EV} tumors (88% inhibition at end of dosing period, tumor growth delay, 25 days) relative to double vehicle. Tumor growth rates of the LN229_{MER-AKT} and LN229_{EV} 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 demonstrate 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 figure 6A, elevated AKT-containing tumors (U87_{EV}, LN229_{MER-AKT}) rapamycin monotherapy resulted in decreased cyclin D1/c-MYC levels while in relatively quiescent AKT-containing tumors (U87_{PTEN}, LN229_{EV}) 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 (U87_{EV} vs. U87_{PTEN} and LN229_{MER-AKT} vs. LN229_{EV}) 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 figure 6C, significant staining was evident in quiescent AKT-containing tumors (U87^{PTEN}, LN229^{EV}) from mice which 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 employed 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 demonstrated that cyclin D1 and c-MYC IRES activity nearly exclusively directs protein synthesis of these two 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 performed polysome analysis of freshly harvested U87^{EV} and U87^{PTEN} tumors following the last day of inhibitor dosing. Polysomes were isolated from homogenized extracts of U87^{EV} and U87^{PTEN} 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 non-ribosomal/monosomal fractions. Transcripts sedimenting within the heavy polysomal fractions are undergoing active translation while those present within the non-ribosomal/monosomal fractions are relatively poorly translated. Spectrophoretic monitoring of fractions at 260 nm was used to identify polysome and non-ribosomal containing fractions and monitor polysome integrity (see supplementary Fig. 2). As shown in figure 6D, U87^{EV} or U87^{PTEN} tumors from mice which received double vehicle treatments, cyclin D1 and c-MYC transcripts are present at approximately 40 – 45% of total cyclin D1 and c-MYC mRNA. Mice that received rapamycin monotherapy exhibited markedly different cyclin D1 and c-MYC mRNA translational states. In U87^{EV} tumors cyclin D1 and c-MYC mRNA were mostly associated with non-ribosomal/monosomal material, while in U87^{PTEN} tumors 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 non-ribosomal fractions demonstrating 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 demonstrate differential AKT-dependent cyclin D1 and c-MYC mRNA translational efficiency in actual tumors. Mice which received SB203580 monotherapy displayed similar cyclin D1 and c-MYC mRNA translational states from U87^{EV} or U87^{PTEN} 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 non-ribosomal 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 demonstrated 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 utilized two isogenic cell line pairs that differ in their relative degrees of AKT activity to demonstrate 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 demonstrated 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 demonstrate that SAPK2/p38 activity is strongly induced following rapamycin exposure in the absence of AKT activity (Fig. 1A & B). Following appropriate stimulation, SAPK2/p38 MAPKs are activated by dual phosphorylation in their activation loop sequence Thr-Gly-Tyr by three 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 figure 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 anti-tumor effects of rapamycin in combination with other chemotherapeutic agents *in vitro*, as well as decreases in 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 U87^{EV} and U87^{PTEN} xenografts following monotherapy with rapamycin and combination therapy (not shown). Rapamycin is known to exhibit anti-angiogenic effects in xenografts (35, 37) and this activity could contribute to the anti-tumor responses following combination therapy.

The analysis of cyclin D1 and c-MYC mRNA translational state in U87^{EV} and U87^{PTEN} 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). While 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 bearing 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 demonstrate 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 utility.

Alternatively, developing inhibitors of specific ITAF-IRES interactions may prove more fruitful in this regard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

AKT-dependent SAPK2/p38 activation and regulation of IRES activity following mTORC1 inhibition. **A**, U87_{EV}, U87_{PTEN}, LN229_{MER-AKT} and LN229_{EV} were treated +/- rapamycin (100 nM) for 24 h and immunoblot assays performed for the indicated proteins. For LN229_{MER-AKT} or LN229_{EV} cells were pretreated with 4OHT (1 μ M, 24 h). **B**, SAPK2/p38 *in vitro* kinase assays of immunoprecipitated SAPK2/p38 from U87_{EV}, U87_{PTEN}, LN229_{MER-AKT} and LN229_{EV} cells treated with rapamycin or 4OHT as in **A**. **C**, Schematic diagrams of the dicistronic IRES containing mRNA reporters. **D**, Cyclin D1 and c-MYC IRES activity in U87_{EV} and U87_{PTEN} cells following SAPK2/p38 knockdown (control, scr = scrambled non-targeting siRNAs, SAPK2/p38 targeting siRNAs). Relative fold change in firefly luciferase activity is shown as compared with activities obtained in the absence of rapamycin and normalized to values obtained for pRF in each cell line. The means +S.D. are shown (n = 3). **E**, As in **D** except the myrMER-AKT expressing LN229_{MER-AKT} and control LN229_{EV} cells were used. Left panel is without 4OHT and right panel is with 4OHT treatment (1 μ M, 24 h). **F**, AKT-dependent cyclin D1, c-MYC and p27^{Kip1} IRES activity in U87_{EV} and U87_{PTEN} (left top, left bottom, respectively) and LN229_{MER-AKT} and LN229_{EV} (right top, right bottom, respectively) transiently transfected with the indicated IRES reporters and treated with rapamycin alone, rapamycin plus SB203580 or rapamycin plus LY2228820. Firefly luciferase values expressed as in **D**. The means +S.D. are shown (n = 3).

Figure 2.

Cell signaling effects of SAPK2/p38 and mTORC1 inhibition. **A**, U87_{EV} and U87_{PTEN} cells were treated with rapamycin and SB203580 as indicated and immunoblotted for the proteins shown. **B**, as in **A** except the LN229_{MER-AKT} and LN229_{EV} lines were used and both pretreated with 4OHT. ** denotes MER-AKT fusion, * denotes endogenous AKT.

Figure 3.

Inhibition of SAPK2/p38 induces G₁-arrest in rapamycin resistant quiescent AKT-containing GBM lines. Cell cycle phase distributions were determined on U87_{EV} and U87_{PTEN} cell treated with **A**, +/- rapamycin and +/- SB203580 or with **B**, +/- rapamycin and +/- LY2228820 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 LN229_{MER-AKT} and LN229_{EV} cells induced with 4OHT were used. One of three experiments with similar results are shown.

Figure 4.

Antiproliferative effects of SAPK2/p38 and mTORC1 inhibition in GBM lines containing differential AKT activity. **A**, Growth inhibition of paired U87 and LN229 cell lines following 48 h culture in SB203580 (left panel) or LY2228820 (right panel). Data represent mean $\pm$ SD of three independent experiments. **B**, Combination analysis of rapamycin and SAPK2/p38 inhibitors in U87_{EV} (left graph) or U87_{PTEN} (right graph) cells were treated with the indicated doses of rapamycin alone or in combination for 48 h and percent growth relative to control cultures was assessed via XTT assay. Control cells were treated with DMSO vehicle. Data are means $\pm$ S.D., $n = 3$. **C**, As in **B**, except LN229_{MER-AKT} (left panel) and LN229_{EV} (right panel) were used and were cultured in the presence of 4OHT.

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 assessed every two days following initiation of treatment (start, day 0). Mice with LN229_{MER-AKT} and LN229_{EV} xenografts received 4OHT via daily maintenance injections.

Figure 6. Effects of combination therapy on signaling pathways, apoptosis and mRNA translational state in U87_{EV} and U87_{PTEN} xenografts. **A**, U87_{EV}, U87_{PTEN}, LN229_{MER-AKT} and LN229_{EV} xenografted tumors from mice receiving double vehicle, monotherapy with the indicated inhibitor or combination therapy were harvested (day 10) and immunoblot analyses performed for the proteins shown. ** denotes MER-AKT fusion, * denotes endogenous AKT. **B**, Representative TUNEL assay results from quiescent AKT containing U87_{PTEN} xenografted tumors receiving the indicated therapies. As shown only rare apoptotic (brown) signals in monotherapy treated animals and significantly greater numbers of apoptotic cells in combination therapy treated mice. **C**, Quantification of *in situ* TUNEL assay results. The data are expressed as the number of positive apoptotic bodies divided by high power field (hpf) (10–12 hpf/tumor). Values are means + S.D. * = $P < 0.05$. **D**, Polysome distributions of cyclin D1, c-MYC and actin mRNAs from xenografted U87_{EV} or U87_{PTEN} tumors harvested from mice receiving the indicated treatment schedules. Tumor extracts were subjected to sucrose density gradient centrifugation, fractionated and pooled into a nonribosomal, monosomal fraction (N, white bars) and a polysomal fraction (black bars). Purified RNAs were used in real time quantitative RT-PCR analysis to determine the distributions of cyclin D1, c-MYC and actin mRNAs across the gradients. RT-PCR measurements were done in quadruplicate and the means and + S.D. are shown.