Cancer Therapy: Preclinical

Survivin Is a Viable Target for the Treatment of Malignant Peripheral Nerve Sheath Tumors

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Abstract

Purpose: To examine the role of survivin as a therapeutic target in preclinical models of human malignant peripheral nerve sheath tumors (MPNST)

Experimental Design: Survivin protein expression levels and subcellular localization were examined immunohistochemically in an MPNST tissue microarray. Human MPNST cells were studied in vitro and in vivo: real-time PCR, Western blotting, and immunocytochemical analyses were used to evaluate survivin expression and localization activation. Cell culture assays were used to evaluate the impact of anti-survivin–specific siRNA inhibition on cell growth and cell-cycle progression and survival. The effect of the small-molecule survivin inhibitor YM155 on local and metastatic MPNST growth was examined in vivo.

Results: Survivin was found to be highly expressed in human MPNSTs; enhanced cytoplasmic subcellular localization differentiated MPNSTs from their plexiform neurofibroma premalignant counterparts. Human MPNST cell lines exhibited survivin mRNA and protein overexpression; expression in both nuclear and cytoplasmic compartments was noted. Survivin knockdown abrogated MPNST cell growth, inducing G2 cell-cycle arrest and marked apoptosis. YM155 inhibited human MPNST xenograft growth and metastasis in severe combined immunodeficient (SCID) mice. Antitumor effects were more pronounced in fast-growing xenografts.

Conclusions: Our studies show an important role for survivin in human MPNST biology. Patients with MPNSTs should be considered for ongoing or future clinical trials that evaluate anti-survivin therapeutic strategies. Most importantly, future investigations should evaluate additional pathways that can be targeted in combination with survivin for maximal synergistic anti-MPNST effects. Clin Cancer Res; 18(9); 1–13. ©2012 AACR.

Introduction

Characterized by aggressive local growth, propensity for systemic spread, and marked resistance to conventional chemo- and radiotherapy, malignant peripheral nerve sheath tumors (MPNST) cause remarkable morbidity and mortality in afflicted individuals (1, 2). Development of more efficacious therapeutic strategies is critically needed and requires more comprehensive knowledge of molecular constituents driving MPNSTs. The tight association between MPNSTs and neurofibromatosis type 1 (NF1) is suggested by the reality that more than 50% of MPNSTs develop against the backdrop of this common genetic disorder (2), and that 8% to 13% of patients with NF1 will develop MPNSTs (2), suggesting a fundamental role for neurofibromin loss of function underlying MPNST inception (3). Deactivating mutations in the NF1 tumor suppressor gene, which encodes for the Ras-GTPase neurofibromin protein, are the hallmark of NF1; plexiform neurofibromas developing in patients with NF1 and MPNSTs arising within these typically deep seated lesions exhibit biallelic NF1 inactivation and consequential enhanced Ras pathway signaling. NF1 mutations also are observed in a portion of sporadic, non-NF1–associated MPNSTs (3). While neurofibromin loss and/or Ras pathway activation are early molecular events driving the development of MPNST premalignant stages, additional genetic and epigenetic alterations are most likely necessary for malignant transformation, disease progression, and metastasis (4). The disappointing results of MPNST clinical trials, singularly targeting the Ras pathway or

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-11-2592
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molecules within this signaling cascade (5), highlight the need to further identify additional MPNST-associated molecular aberrations, preferentially those that would be easily amenable to therapeutic targeting.

Originally identified in 1997 as a member of the inhibitor of apoptosis (IAP) family (6), survivin, encoded by the BIRC5 gene, has since been found to contribute to a multitude of critical biologic functions including cellular division, survival, and adaptation to stress (6, 7). While highly expressed during embryogenesis, survivin is largely undetectable in normal adult tissues and is restricted to the thymus, placenta, stem cell compartment, and basal epithelium of the colon (6, 8). However, survivin reexpression is commonly observed in transformed cells, and increased survivin levels have been found in multiple cancer types (9). Moreover, increased survivin expression levels have been found to correlate with adverse patient outcomes and resistance to therapy (10–13). Importantly, survivin has been shown to play a critical role in cancer, functioning as a convergence point for multiple signaling pathways controlling tumor maintenance and growth promotion (8). These attributes have rendered survivin a focus of intense investigation as a potentially worthy target for personalized molecular therapeutics (8). Anti-survivin treatment regimens have been easily amenable to therapeutic targeting.

While extensively studied in a wide range of human malignancies, a role for survivin deregulation in MPNST biology has yet to be determined. Several previously published reports suggest that survivin is aberrantly expressed in MPNSTs (16, 17). Specifically, Storlazzi and colleagues identified the BIRC5 genetic region on chromosome 17 as commonly amplified in human MPNST samples; high-level amplification correlated with poor prognosis (17). Overexpression of survivin mRNA in MPNSTs compared with neurofibroma has been observed by 3 independent groups (16, 18, 19). Finally, a recent immunohistochemistry-based study showed survivin protein expression in 52 human MPNST samples (20). Building on these initial observations, the current study sought to further determine the potential role of survivin as a MPNST biomarker, to elucidate the functional consequences of survivin overexpression in these tumors, and, most importantly, to assess the efficacy of survivin blockade as an anti-MPNST therapeutic strategy.

Materials and Methods

Cell lines and reagents

MPNST cell lines used 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), the MPNST642 isolated in our laboratory (21), 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 (22). Primary human adult Schwann cell cultures established from human cauda equina nerves were provided by Dr. Patrick Wood (Miami Project, University of Miami, Miami, FL) and maintained as previously described (23). DNA fingerprinting (short tandem repeat) was conducted as previously described (21) for all MPNST cell lines, confirming that no cross-contamination has occurred. The small-molecule survivin inhibitor YM155 was purchased from ChemieTek. For in vitro studies, the drug was dissolved in dimethyl sulfoxide (DMSO) and stored in −20°C. For in vivo experiments, YM155 (dosed at 6 mg/kg/d) was dissolved and diluted in saline immediately before administration. Commercially available antibodies were used for immunoblotting, immunohistochemical (IHC), or immunocytochemical detection of the full-length wild-type (WT) survivin (polyclonal; Abcam), XIAP (polyclonal; Abcam), cleaved caspase-3 (polyclonal; Abcam), the 85-kDa fragment of cleaved PARP (clone Y34; Abcam), total PARP (clone 46D11; Cell Signaling Technology), cleaved caspase-3 (polyclonal; BioCare Medical), α-tubulin (Santa Cruz Biotechnology), Ki67 (Thermo/Lab Vision), Lamin A/C (Santa Cruz Biotechnology), and β-actin (Santa Cruz Biotechnology). Hoechst (Invitrogen) was used as a nuclear stain for immunocytochemical analysis.
In vitro assay

Specific experiments.

medium. Cells were harvested at indicated time points for added for 24 hours, followed by incubation in regular mixture of siRNA (20 nmol/L) and X-tremeGENE (6 mm).

Immunohistochemistry and immunocytochemistry

As previously described (24–26). For TMA analysis, survivin expression was scored by 2 independent observers (A.J. Lazar and M.P. Ghadimi). Intensity was graded as none (- 0), weak/low (= 1), moderate (= 2), and high (= 3); percentage of positively staining cells was also determined. Scoring was conducted separately for the nuclear and cytoplasmic intracellular compartments.

siRNA transfection procedures

siRNAs (20 nmol/L, pools targeting survivin and control nontargeting constructs; Thermo Scientific) were introduced into cells by X-tremeGENE as per manufacturer’s instructions (Roche). Briefly, 2 x 10^5 cells were plated in each well of a 6-well plate and incubated overnight. A mixture of siRNA (20 nmol/L) and X-tremeGENE (6 μL) diluted in 100 μL Dulbecco’s Modified Eagle’s Medium was added for 24 hours, followed by incubation in regular medium. Cells were harvested at indicated time points for specific experiments.

In vitro growth assays

MTS assays were conducted with CellTiter96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega Corp) as per the manufacturer’s instructions. Absorbance was measured at a wavelength of 490 nm, and the absorbance values of treated (or of survivin knocked down) cells are presented as a percentage of the absorbance of untreated cells. Colony formation assay was conducted for pretreatment analysis; MPNST cells were treated in culture dishes for 24 hours with DMSO (control) or YM155. One hundred viable cells per well were replated and allowed to grow in normal medium (no drug) for 10 days and then stained for 30 minutes at room temperature with a 6% glutaraldehyde and 0.5% crystal violet solution. For continuous treatment studies, 100 viable cells per well were plated and treated with YM155 or DMSO alone for 10 consecutive days. Pictures were captured digitally and surface area covered by cells (as surrogate to colony number) was determined with ImageJ software. Anchorage-independent growth: MPNST cells were treated with DMSO (control) or YM155 for 24 hours in a 6-well plate. A total of 1 x 10^3 viable cells were plated in a 24-well plate in culture medium containing 0.33% agarose overlying a 0.7% agarose layer. Cells were incubated for 3 weeks at 37°C. Cells were stained with p-iodonitrotetrazolium violet (1 mg/mL) for 24 hours at 37°C.

Cellular assays

Western blot analyses were conducted by standard methods (27). Subcellular (nuclear and cytoplasmic) protein fractionation and isolation was conducted with the Nuclear and Cytoplasmic Extraction Reagents Kit (NE-PER Thermo Scientific) as per manufacturer’s instructions. Cell-cycle progression was measured via propidium iodide (PI) staining/fluorescence-activated cell-sorting (FACS) analysis (27), and apoptosis was measured via Annexin-V/PI staining FACS analysis with the Apoptosis Detection Kit I (BD Biosciences) as per manufacturers’ recommendations. Real-time PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) were carried out as we have previously described (27); primers (survivin forward: 5’-GGACCACCGCATCTCTCATCAT-3’ and reverse: 5’-GTTCCCTCTATGGGTTGTCGTCA-3’) were obtained from Sigma.

In vivo animal models

All animal procedures and care were approved by the MD Anderson Cancer Center (Houston, TX) Institutional Animal Care and Use 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 the effect of treatment on local tumor growth, trypan blue staining confirmed viable MPNST cells [STS26T and MPNST724; 1 x 10^6 to 2 x 10^6] 0.1 mL Hank’s balanced salt solution (HBSS)/mouse] was used. Cell suspensions were injected s.c. into the flank of 6-week-old female hairless severe combined immunodeficient (SCID) mice (n = 7–8/treatment group) and growth was measured twice weekly; after establishment of palpable lesions (average diameter ~4–5 mm), mice were assigned to treatment groups as described below. An experimental lung metastasis MPNST model was used to evaluate the growth of metastases. STS26T cells (1 x 10^6) 0.1 mL HBSS/mouse) were injected into the tail vein of female SCID mice. Two weeks after injection, mice were allocated to treatment groups as described below. Therapeutic regimen and dose followed previous reports (28, 29). YM155 [6 mg/kg/d; several preclinical studies (29, 30) have shown antitumor efficacy with doses of 1 to 10 mg/kg/d, with the latter being the maximal tolerated dose] or saline was delivered via a micro-osmotic pump (Alzet model 1003D, DURECT Corporation) that was implanted s.c. on day 1 of treatment into each mouse; these pumps enable continuous drug delivery for 3 consecutive days. Previous studies have shown that this regimen results in steady-state levels of YM155 in plasma and enhanced tumor bioavailability (30). YM155 continuous infusion was found superior to intravenous daily bolus or intermittent schedules (30). The pumps were replaced on day 8 for a total of 6 treatment days. Mice were followed for tumor size, well-being, and body weight and sacrificed when control group tumors reached an average of 1.5 cm in their largest dimension. Tumors were resected, weighed,
and fixed in formalin and paraffin-embedded for immunohistochemical studies. For lung metastatic studies, mice were followed for body weight and well-being and sacrificed 3 weeks after start of treatment. Lungs were resected, evaluated macroscopically for tumor load, and weighed and fixed in formalin and paraffin-embedded for hematoxylin and eosin (H&E) staining.

Statistics
Several alternative statistical tests were used to determine the correlation between survivin expression and clinical factors such as histology, NF1 status, and disease status including Spearman’s correlation coefficient, \( \chi^2 \), and Fisher’s exact test. Correlation between survivin and other molecular biomarkers was evaluated by Spearman’s correlation coefficient analyses. Kaplan–Meier analyses were used to determine the potential impact of survivin expression levels on MPNST disease-specific survival. All computations were conducted with SAS for Windows (release 9.2; SAS Institute). Cell culture–based quantitative assays were repeated at least 3 times and mean \( \pm \) SD was calculated. Cell lines were examined separately. For outcomes that were measured at a single time point, two-sample \( t \) tests were used to assess the differences. Differences in xenograft growth \textit{in vivo} were assessed by a 2-tailed Student \( t \) test. Significance was set at \( P \leq 0.05 \).

Results
Human MPNSTs exhibit increased survivin levels; cytoplasmic expression is significantly enhanced in MPNSTs as compared with plexiform neurofibroma
Survivin overexpression has been previously shown for many cancer types (9). Supported by previous publications suggesting \textit{BIRC5} gene amplification and enhanced survivin mRNA expression in MPNSTs (16–18), we examined survivin protein expression levels and intracellular localization in a cohort of human samples. A previously constructed, clinically annotated TMA was used, containing human MPNST and plexiform neurofibroma samples (24). Survivin IHC analysis was conducted (Fig. 1A). Both normal peripheral nerves were negative for survivin expression. All MPNSTs showed survivin expression; both nuclear and cytoplasmic distributions were noted (Fig. 1A). Weak nuclear expression levels were found in 31 (49%), moderate in 28 (45%), and high in 4 (6%); an average of 73% (\( \pm \)18%) of tumor cells per sample exhibited positive nuclear staining. Cytoplasmic expression levels were low in 3 (5%), moderate in 13 (21%), and high in 47 (74%); an average of 83% (\( \pm \)11%) of tumor cells per sample exhibited positive cytoplasmic staining. Similarly, all plexiform neurofibroma expressed survivin. Low nuclear levels were found in 9 (43%), moderate in 8 (38%), and high in 4 (19%); an average of 52% (\( \pm \)23%) of tumor cells per sample exhibited...
positive nuclear staining. Cytoplasmic expression levels were low in 8 (38%), moderate in 11 (52%), and high in 2 (10%); an average of 80% (±9%) of tumor cells per sample exhibited positive cytoplasmic staining. Notably, whereas no difference in nuclear survivin expression was found between MPNST and plexiform neurofibroma–derived specimens, a statistically significant enhanced cytoplasmic survivin expression was identified in MPNSTs (P < 0.0001; Spearman’s rank test; Fig. 1B). No correlation between survivin expression levels and MPNST disease status (i.e., primary, recurrent, or metastatic) or NF1 versus sporadic lesions was identified. Kaplan–Meier analysis was further conducted to determine whether survivin expression levels correlate with MPNST disease outcome; only scoring results of evaluable localized MPNST samples (n = 48) were included. No correlation was found between either cytoplasmic or nuclear survivin expression levels or MPNST patient disease-specific survival. In summary, MPNSTs and their precursor lesions, plexiform neurofibroma, both commonly express survivin; cytoplasmic survivin expression is markedly more pronounced in malignant lesions. In contrast to findings in other malignancies, we did not identify survivin expression to be a molecular prognosticator of MPNST disease outcome.

Next, we assessed whether there was a correlation (Spearman rank correlation analyses) between survivin expression levels (cytoplasmic and nuclear) and other biomarkers, with our MPNST TMA, including Ki67 (as a marker of proliferation), p53 (commonly deregulated in MPNSTs; ref. 31), VEGF (as a marker of angiogenesis), and Ras pathway signaling effectors including pMEK, pAKT, and the mTOR downstream target pS6RP. No association between Ki67 or p53 intensity and survivin was identified. However, enhanced cytoplasmic survivin intensity was found to directly and statistically significantly correlate with increased VEGF (r = 0.28, P = 0.013), pMEK (r = 0.48, P < 0.0001), pAKT (r = 0.29, P = 0.012), and pS6RP (r = 0.33, P = 0.007) expression levels. Interestingly, increased nuclear survivin expression was found to inversely correlate with VEGF (r = −0.33, P = 0.004) and pAKT (r = −0.32, P = 0.008) intensities.

Survivin is highly expressed in human MPNST cell lines

Next, we determined survivin expression levels in a panel of human MPNST cell lines to confirm that our experimental model recapitulated the findings in human samples and thus could be used to further evaluate the potential function of this protein in MPNSTs (Fig. 2A). WT survivin mRNA levels were found to be markedly increased in all MPNST cell lines as compared with normal human Schwann cell and did not correlate with NF1 (S462, ST88, MPNST642) versus sporadic (STS26T and MPNST724) disease background, cell growth (growth rates, STS26T > S462 > MPNST724 > ST88 > MPNST642), or p53 mutational status (WT = ST88 and MPNST642; mutated/null = MPNST724, S462, and STS26T; 29). Similarly, WT survivin protein expression was enhanced in MPNST cell lines, and no other bands were identified to suggest expression of survivin splice variants. Of note, survivin protein expression and survivin mRNA levels did not entirely match, possibly
suggesting that additional posttranscriptional factors may contribute to survivin protein levels, whereas a closer association with MPNST cell growth rate was noted. Finally, as observed in human MPNST specimens, survivin expression was noted in both nuclear and cytoplasmic cellular compartments of MPNST cells (Fig. 2B).

**Survivin promotes MPNST cell growth, cell-cycle progression, and survival**

We next conducted loss of survivin function experiments to determine its contribution to MPNST cell growth. Anti-survivin siRNA–specific constructs were used to achieve knockdown; a nontargeting construct was used as control. Marked survivin knockdown was noted 48 hours after transfection (Fig. 3A). No decrease in the expression of the IAP proteins XIAP (Fig. 3A), cIAP1, or cIAP2 (Supplementary Fig. S1) was noted in response to anti-survivin siRNA transfection. MTS assays showed significant ($P < 0.05$) decreases in tumor cell growth at 24 and 48 hours (experiments were initiated 24 hours after transfection). PI staining/FACS analyses showed significant ($P < 0.05$) $G_2$–M cell-cycle arrest 48 hours after transfection (Fig. 3B). These assays also showed increased sub-$G_0$ fractions in MPNST cells, secondary to survivin knockdown. Annexin-V/PI staining FACS analyses confirmed a statistically significant ($P < 0.05$) increase in apoptosis 72 hours after siRNA transfection and Western blot analyses showed increased cleaved PARP (85-kDa fragment) expression in survivin knocked down cells (Fig. 3C and Supplementary Fig. S2). Taken together, these studies showed a role for survivin in MPNST cell growth, cell-cycle progression, and survival, thereby supporting further investigation of anti-survivin therapeutic strategies in our MPNST experimental models.

**YM155, a small-molecule survivin inhibitor, exerts marked anti-MPNST effects in vitro and in vivo**

The anti-MPNST effects of YM155, a small-molecule inhibitor of survivin currently under clinical investigation, were next considered. YM155 was identified by a cell-based chemical library screen to specifically inhibit survivin promoter activity (30). It is suggested to exert its antitumor effects through inhibition of survivin mRNA transcription, resulting in decreased protein expression, whereas not impacting the expression of other IAP proteins such as cIAP1, cIAP2, or XIAP (30). Western blot analyses confirmed that YM155 induced a dose- (in the nanomolar range) and treatment time–dependent decrease in survivin expression in MPNST cell lines (Fig. 4A). No decrease in the expression of the IAP proteins, XIAP and cIAP1, was observed in response to YM155 (Fig. 4A). MTS assays likewise showed a marked YM155 dose-dependent decrease in MPNST cell growth after 48 and 96 hours of treatment (Fig. 4B). YM155 effects were found to correlate with cell line growth rates. A less pronounced effect was shown in MPNST642 cells; among the human MPNST cell lines tested, this specific cell line exhibited the slowest growth rate and lowest survivin expression level. On the basis of these initial results, YM155 doses used for the remaining cell culture–based assays ranged between 1 and 10 nmol/L; these doses are lower than the YM155 plasma levels achievable in humans (32, 33), hence clinically relevant. YM155 administration significantly ($P < 0.05$) abrogated the colony-forming capacity and anchorage-independent growth of MPNST cells (Fig. 4C and D). Compatible with the effects observed above in response to survivin knockdown, YM155 treatment resulted in abrogated $G_2$–$M$ cell-cycle progression ($P < 0.05$) and increased sub-$G_0$ fraction in MPNST cells (Fig. 5A). Finally, a statistically significant ($P < 0.05$) increase in tumor cell apoptosis was noted in response to YM155 (evaluated after 48 hours of treatment; Fig. 5B) and an increase in the expression of cleaved PARP (85-kDa fraction) was observed (Fig. 5B and Supplementary Fig. S2).

To determine whether these in vitro observations might be recapitated in vivo, we carried out a series of therapeutic experiments with human MPNST xenograft mouse models. First, we examined the effect of YM155 on STS26T growth (Fig. 6A). YM155 (6 mg/kg/d) therapy was initiated after establishment of tumor (5 mm in largest dimension). Therapy was administered with subcutaneously implanted micro-osmotic pumps, delivering a 3-day continuous infusion; pumps were implanted on days 1 and 8 of treatment as per previously published reports (28, 29). Control mice were treated with saline (carrier) equivalently delivered. YM155 was well-tolerated and no significant weight loss was observed. YM155 treatment markedly abrogated tumor growth; the average size of control-treated tumors at study termination was 1,109 mm$^3$ ($\pm 167$) versus 251 mm$^3$ ($\pm 134$) of YM155-treated tumors ($P < 0.0001$; Fig. 6A). Moreover, treatment with YM155 significantly reduced tumor weight compared with control ($P = 0.005$). Average tumor weights at study termination were $0.82 \pm 0.18$ and $0.36 \pm 0.19$ in control and YM155 groups, respectively (Fig. 6A). Tumor sections from each experimental arm containing viable cells were selected for IHC studies. A marked decrease in survivin expression was observed in YM155-treated tumors (Fig. 6A). Furthermore, a pronounced decrease in MPNST cell proliferation (evaluated via Ki67 staining) and a demonstrable increase in tumor cell apoptosis (evaluated with cleaved caspase-3 immunohistochemistry) were also noted.

We next evaluated the effects of YM155 in a second MPNST xenograft model derived from s.c. injection of MPNST724 cells; YM155 doses and regimens were as described above. YM155-treated mice exhibited smaller tumors at study termination. The average size and weight of YM155-treated tumors were 702 mm$^3$ ($\pm 362$) and 1.53 g ($\pm 0.58$) for the control group (Fig. 6B). While statistically significant ($P < 0.05$), the effects of YM155 on MPNST724 xenografts were less profound than its in vivo anti-STS26T effects and are probably more consistent with cytostasis than cytolysis per se. This difference might be related to the inherently slower growth of MPNST724 xenografts than STS26T, which was also reflected in the lower baseline Ki67 expression in these tumors. Similar to the IHC results
obtained from the STS26T therapeutic experiments, a decrease in survivin and Ki67 and an increase in cleaved caspase-3 expression was found in MPNST724 YM155–treated xenografts (Fig. 6B).

Finally, we used the STS26T experimental lung metastasis model to evaluate whether YM155 can affect the growth of MPNST pulmonary metastases. Pumps were implanted 2 weeks after tumor cell tail vein injection and then replaced.

Figure 3. Survivin knockdown abrogates MPNST cell growth, cell-cycle progression, and survival. A, anti-survivin siRNA (20 nmol/L pool) resulted in pronounced survivin knockdown (KD) in MPNST cell lines 48 hours posttransfection [non targeting (NT-siRNA) constructs were used as control; Western blot analyses, top]. No decrease in the expression of the IAP protein XIAP was noted. Survivin KD significantly inhibited MPNST cell growth. MTS assays were conducted for 24 and 48 hours (48 and 72 hours posttransfection, respectively); B, survivin KD resulted in a statistically significant G2 cell-cycle arrest in MPNST cells (48 hours posttransfection; graphs represent at least 3 independent experiments). Increased sub-G1 fraction (sG1) was also noted. C, survivin KD induced marked apoptosis in MPNST cells (assays were conducted 72 hours after siRNA transfection) as is shown via Annexin-V/PI staining FACS analyses (graphs represent at least 3 independent experiments) and increased cleaved PARP expression (cl. PARP; with an antibody directed against the 85-kDa fraction of PARP; Western blot analyses further show marked survivin KD at this time point). *, statistically significant effects (P < 0.05). FITC, fluorescein isothiocyanate.
one time 7 days later. Mice were sacrificed 3 weeks after treatment initiation. A significant difference in average lung weight was found between control (0.47 ± 0.13 g) and treated mice (0.24 ± 0.07 g; P = 0.0004; Fig. 6C). Macroscopic lung metastasis was observed in all (n = 8) control mice but not in YM155-treated mice. H&E staining identified large lung tumor deposits in all control mice, whereas only 2 of the YM155-treated mouse lungs exhibited microscopic lesions (Fig. 6C).

Discussion

Driven by the critical need to identify MPNST molecular deregulations amenable to therapeutic targeting, studies here focused on the multifunctional protein survivin. More than a decade of intensive research has illuminated the fundamental role survivin plays across a broad range of cancer histologies (9). Multiple molecular mechanisms have been identified that drive aberrant survivin expression in cancer, including amplification of the BIRC5 genetic locus (on
chromosome 17q25), BIRC5 demethylation, enhanced transcription, and deregulated cellular signaling pathways for which survivin acts as a convergence point (34–36). While not previously extensively studied in MPNSTs, published data suggest that BIRC5 amplification is a common molecular event in these devastating malignancies (17).

The current study further expands these initial observations showing enhanced survivin protein expression in human MPNST specimens. Studies presented here did not identify survivin expression as a predictor of MPNST patient disease-specific survival. This is in contrast to observations made in many other solid malignancies including non–small cell lung cancer, gastric cancer, colorectal cancer, breast carcinomas, neuroblastoma, and osteosarcoma, where survivin expression levels were found to correlate with poor patient outcome (10–13). However, our negative results are potentially confounded by the relatively small number of evaluable localized MPNST samples and should perhaps be reexamined with a larger cohort of specimens.

In alignment with its diverse molecular functions, survivin has shown a dynamic intracellular expression pattern, localizing to both the cytoplasm and the nucleus of tumor cells (37). Interestingly, nuclear survivin has been shown to be a predictor of favorable outcome in non–small cell lung cancer and osteosarcoma (18, 38) while portending a poor prognosis in mantle cell lymphoma and squamous cell carcinomas of the esophagus (38). Enhanced cytoplasmic survivin expression has been shown to correlate with

Figure 5. YM155 inhibits MPNST cell-cycle progression and survival in vitro. A, YM155 induces a statistically significant G2 cell-cycle arrest and increases in sub-G1 (sG1) fraction in MPNST cells (graphs represent at least 3 independent experiments). B, moreover, YM155 (48 hours of treatment) exerts a statistically significant increase in tumor cell apoptosis (graphs represent at least 3 independent experiments). Increased cleaved PARP (85-kDa fraction) expression can also be seen. * , statistically significant effects ($P < 0.05$). FITC, fluorescein isothiocyanate.
Figure 6. YM155 exerts marked anti-MPNST effects in vivo. A, SCID mice bearing STS26T xenografts (~4–5 mm in average larger dimension) were implanted with a subcutaneous micro-osmotic pump delivering YM155 (6 mg/kg/d) or vehicle only in a continuous fashion for 3 consecutive days. Pumps were replaced once (on day 8; arrows denote days of pump implantation and bolded line days of treatment). YM155 markedly abrogated tumor growth ($P < 0.0001$; top graph). Moreover, treatment with YM155 significantly reduced tumor weight compared with control ($P = 0.005$; bottom graph). IHC analyses showed decreased survivin and Ki67 and increased cleaved caspase-3 (CC3) expression in YM155-treated xenografts (scale bars are included). B, experiment was repeated as above for MPNST724 xenografts. YM155 treatment was found to delay tumor growth, a statistically significant decrease in tumor size ($P < 0.01$; top graph) and tumor weight ($P < 0.05$; bottom graph) was observed at study termination. IHC analyses results (right) aligned with the STS26T findings above (scale bars are included). C, STS26T lung metastases–bearing mice were treated with YM155. A significant ($P < 0.001$; right) difference in average lung weight between control and YM155-treated mice was found at study termination. Pulmonary metastases were macroscopically observed in all control mice, but not in YM155-treated mice. H&E staining further showed large lung tumor deposits in control mice lungs, but no ($n = 6$) or only small microscopic lesions ($n = 2$) in the YM155-treated group (scale bars are included). *$, statistically significant effects ($P < 0.05$).
shorter disease-free survival in patients with oral squamous cell carcinoma (39). Possibly related to the above observations, it has been previously suggested that the antiapoptotic effects of survivin are greatest when the protein is localized in the cytoplasm, whereas nuclear survivin may play a more critical role in mitosis (40). Together, it is clear that the prognostic and functional significance of these distinct collections of subcellular survivin are yet to be fully appreciated and delineated (15). In our study, we noted survivin expression in both the nuclear and cytoplasmic cellular compartments of MPNSTs. Interestingly, whereas equivalent nuclear survivin expression levels could be observed when comparing MPNSTs with their premalignant plexiform neurofibroma precursors, cytoplasmic expression levels were markedly increased in the malignant lesions. It is well established that progression of neurofibromas to MPNSTs mandates accumulation of genetic and epigenetic alterations that drive transformation and subsequent tumor progression (4); our findings possibly justify evaluating the role of cytoplasmic survivin in this process. Of note, phosphoinositide 3-kinase (PI3K), AKT, mTOR, and MEK/ERK signaling have previously been identified to regulate survivin expression (41). Dereglulation of these pathways is commonly observed in MPNSTs (42); published data from our laboratory have identified enhanced AKT, mTOR, and MEK activation in human MPNSTs samples compared with plexiform neurofibroma tissues (24). Moreover, in the current study, we found a direct correlation between cytoplasmic survivin expression levels and those of the activated forms of MEK, AKT, and S6RP. These results offer at least one possible molecular mechanism underlying enhanced cytoplasmic survivin expression in MPNSTs.

In addition to aberrant survivin expression in human MPNSTs, our data further suggest that survivin plays an important role in the MPNST protumorigenic phenotype enhancing tumor cell growth, cell-cycle progression, and survival. These survivin-induced effects are not unique to MPNSTs and are similar to those previously shown in other solid and hematologic malignancies (6, 30, 35, 41). As such, our study expands previous preclinical observations to include the devastating malignancy MPNSTs as a cancer type in which survivin might play a role and further highlights the potential use of this protein as an attractive and possibly universal target for molecularly based anticancer therapies. A wide variety of anti-survivin therapeutic strategies, including antisense oligonucleotides, ribozymes, siRNA-based approaches, immunotherapy, and small molecular weight inhibitors, are currently in various stages of development (8). YM155 is one such extensively tested inhibitor and has already received clinical attention (15). An imidazolium-based compound originally identified by Nakahara and colleagues (30) through a high-throughput compound screen to specifically inhibit survivin transcription, the exact mechanisms of YM155 action are yet to be elucidated. This compound induces marked antitumor effects in multiple preclinical cancer models including prostate cancer, non–small cell lung cancer, melanoma, and non–Hodgkin lymphoma (30, 43, 44). These encouraging results have led to the development of initial phase I/II YM155 human clinical studies, which have shown favorable toxicity and tolerability profiles (32, 33, 45). Furthermore, signs of efficacy, albeit modest, have been noted including partial responses in non–Hodgkin lymphoma and in heavily pretreated patients with refractory non–small cell lung carcinoma. Our studies confirm that human MPNST cells are highly sensitive to survivin knockdown, resulting in marked cell-cycle arrest and apoptosis. Seeking to establish translational applicability, we examined the potential efficacy of YM155 in our preclinical model. These studies showed promising YM155-induced anti-MPNST effects in vitro, where a dose-dependent decrease in survivin expression, tumor cell growth, cell-cycle progression, and survival were observed at low nanomolar concentrations. Furthermore, YM155 inhibited the local and metastatic growth of human MPNST xenografts in SCID mice; an especially pronounced effect was noted in the STS26T rapid growth in vivo model. While these results are very encouraging (and possibly support the inclusion of patients with MPNSTs in anti-survivin–based clinical trials), it is of note that the YM155-induced complete regression reported in other tumor models using similar (or even lower) drug dose and regimen (43, 44) was not observed in our MPNST xenografts. In that, these latter observations may more closely resemble the effects observed in YM155 monotherapy clinical trials, a more efficacious therapeutic strategy will mandate combining anti-survivin strategies with other MPNST-relevant conventional and/or molecularly targeted agents. Clinical studies combining YM155 with conventional chemotherapies (e.g., taxanes and carboplatin) or biologic agents (e.g., rituximab) for the treatment of several cancer types are currently ongoing (ClinicalTrials.gov), buttressed by recent evidence for enhanced antitumor activity of such combinations in preclinical models (46, 47). Future preclinical studies combining YM155 with agents previously showing promising effects in MPNSTs such as tyrosine kinase inhibitors (e.g., MET; ref. 48) or inhibitors of cellular signaling (e.g., AKT, mTOR, and MEK; refs. 24, 49) and others are currently being planned.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Acknowledgments

The authors thank the expert assistance in figure preparation provided by Kim Vu.

Grant Support

The manuscript was supported in part by an NIH/NCI R01CA133845 (to D. Lev), an Amschwand Foundation Seed Grant (to D. Lev), a Deutsche Forschungsgemeinschaft Fellowship grant (supporting M.P. Gadhimi), and a NIH/NCI STS2CA09599-21 training grant (supporting K. Lusby). MDACC cell line characterization Core Facility was further supported by an NCI Cancer Center Support Grant (CA16672).

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Received October 7, 2011; revised February 7, 2012; accepted February 29, 2012; published OnlineFirst March 8, 2012.
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Clinical Cancer Research

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Markus P. Ghadimi, Eric D. Young, Roman Belousov, et al.

Clin Cancer Res  Published OnlineFirst March 8, 2012.

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