Systematic Screening Identifies Dual PI3K and mTOR Inhibition as a Conserved Therapeutic Vulnerability in Osteosarcoma

Ankita Gupte1,2, Emma K. Baker1,2, Soo-San Wan3,4, Elizabeth Stewart5, Amos Loh5, Anang A. Shelat6, Cathryn M. Gould7, Alistair M. Chalk1,2, Scott Taylor1,2, Kurt Lackovic3,4, Åsa Karlström5, Anthony J. Mutsaers1,2,8, Jayesh Desai9,10, Piyush B. Madhamshettiwar5, Andrew C.W. Zannettino11,12, Chris Burns3,4, David C.S. Huang3,4, Michael A. Dyer5,13, Kaylene J. Simpson7,14, and Carl R. Walkley1,2,15

Abstract

Purpose: Osteosarcoma is the most common cancer of bone occurring mostly in teenagers. Despite rapid advances in our knowledge of the genetics and cell biology of osteosarcoma, significant improvements in patient survival have not been observed. The identification of effective therapeutics has been largely empirically based. The identification of new therapies and therapeutic targets are urgently needed to enable improved outcomes for osteosarcoma patients.

Experimental Design: We have used genetically engineered murine models of human osteosarcoma in a systematic, genome-wide screen to identify new candidate therapeutic targets. We performed a genome-wide siRNA screen, with or without doxorubicin. In parallel, a screen of therapeutically relevant small molecules was conducted on primary murine– and primary human osteosarcoma–derived cell cultures. All results were validated across independent cell cultures and across human and mouse osteosarcoma.

Results: The results from the genetic and chemical screens significantly overlapped, with a profound enrichment of pathways regulated by PI3K and mTOR pathways. Drugs that concurrently target both PI3K and mTOR were effective at inducing apoptosis in primary osteosarcoma cell cultures in vitro in both human and mouse osteosarcoma, whereas specific PI3K or mTOR inhibitors were not effective. The results were confirmed with siRNA and small molecule approaches. Rationale combinations of specific PI3K and mTOR inhibitors could recapitulate the effect on osteosarcoma cell cultures.

Conclusions: The approaches described here have identified dual inhibition of the PI3K–mTOR pathway as a sensitive, druggable target in osteosarcoma, and provide rationale for translational studies with these agents. Clin Cancer Res; 21(14): 3216–29. ©2015 AACR.

Introduction

Osteosarcoma is the most common primary tumor of bone. It occurs mostly in the second decade of life and is the fifth most prevalent cancer in children. Patients with localized disease have 5-year survival rates of approximately 70%. However, at presentation, approximately 20% of patients have metastases and almost all patients with recurrent osteosarcoma have metastatic disease (1, 2). The 5-year survival for patients with metastatic disease is 20% (3, 4). Treatment relies on the use of chemotherapy and surgery, which cause considerable morbidity (5). Although substantial advances in our understanding of osteosarcoma biology and genetics have occurred, there have been no significant advances in therapy for the past 30 years.
Translational Relevance

Current therapy for osteosarcoma relies on surgery and conventional chemotherapy. In recent decades, survival rates have plateaued. Improvements in patient outcome have largely been derived from improved delivery of existing agents, rather than the introduction of novel agents. To probe for new susceptibilities, we have undertaken independent genome-wide siRNA and chemical screens in murine models of human osteosarcoma, identifying PI3K and mTOR pathways as therapeutic vulnerabilities. Our studies demonstrate that dual inhibition of the PI3K and mTOR pathways, but not either pathway independently, led to apoptosis of osteosarcoma cells. These findings were recapitulated in genetically diverse primary human osteosarcoma samples in separate facilities. These data demonstrate that dual PI3K–mTOR inhibitors warrant prioritization for development as a strategy to treat osteosarcoma.

(6, 7). The first whole-genome sequence of mutations in human osteosarcoma was recently generated, revealing TP53 pathway mutations in all samples assessed and recurrent somatic changes in RB1, ATRX, and DLEC2 in 29% to 53% of the tumors (8). This analysis highlighted the complexity of the osteosarcoma genome and provides invaluable information for improving preclinical modeling of osteosarcoma; however, it does not immediately reveal actionable strategies for improving therapy for patients.

To date, research has led to only a limited number of clinically relevant biologic insights (9, 10). Empirical evaluation of novel agents in human xenografts has not to date yielded any major translational advances (10). The only agent to show promise from these studies has been mTOR inhibitors such as rapamycin (11). Improvements in the delivery and application of existing treatments, rather than the introduction of new therapies, have seen some improvement in the management of osteosarcoma. Novel approaches to drug target identification are needed alongside robust preclinical testing platforms. The development of genetically engineered mouse models (GEMM), reflective of the human osteosarcoma, represents a critical component to improving patient outcomes and preclinical target validation (12). We previously developed a GEMM of the fibroblastic osteosarcoma subtype, through deletion of p53 and p16 from the osteoblast lineage that has been independently validated (13–15). We recently described the first murine model of osteoblastic osteosarcoma, the most common subtype of human osteosarcoma (16).

Advances in technology and the capacity for high-throughput phenotypic and chemical screens offer considerable promise for identifying new therapeutic agents. Screening approaches afford an opportunity for an unbiased, saturation coverage of the genome. Genome-wide siRNA screens offer an unmatched probing of the genetics of osteosarcoma, but the immediate clinical utility of many identified candidates, such as transcription factors, may be limited as they are not modifiable using current therapeutic approaches. Chemical screens with drugs that are in clinical or preclinical use interrogate a limited spectrum of targets but, if validated, offer the prospect of a more rapid clinical application if validated (17). Given the limited advances in translating basic knowledge of osteosarcoma biology to patient benefit an approach using systematic screening of drugs or focusing on defining genetic susceptibilities of osteosarcoma could offer a new means to identify new potential candidates for either preclinical testing or further development.

Here, we report results from parallel screens using primary cell cultures derived from murine osteosarcoma models that faithfully replicate the human disease (13, 16). First, a whole-genome siRNA screen for enhancers of cell death was performed. The screen was conducted with either siRNA alone or as siRNA with doxorubicin, a standard-of-care chemotherapy for osteosarcoma (5). Second, a curated drug/chemical library predominantly targeting kinases or known targets was screened against three independent primary cell cultures derived from paired primary and metastatic osteosarcoma. Validation across mouse- and human biopsy–derived primary osteosarcoma cell cultures established the robustness of our analyses. These complementary chemical and genetic strategies have converged to provide independent evidence that concurrent targeting of protein translation and growth control pathways, in particular the PI3KCA and mTOR pathways, represent tractable targets in osteosarcoma.

Materials and Methods

Osteosarcoma cell cultures and mouse models

Primary mouse osteosarcoma cell cultures were derived from tumors generated in either Osx-Cre p53<sup>fl/fl</sup>pRb<sup>fl/fl</sup> mice (fibroblastic osteosarcoma model; ref. 13) or Osx-Cre TRE-p53.1224 pRb<sup>fl/fl</sup> model (osteoblastic osteosarcoma model; all obtained directly from mouse models of osteosarcoma; ref. 16). Experiments using mouse osteosarcoma models were approved by the St. Vincent’s Hospital AEC. Primary human osteosarcoma (OS17) was derived from a primary human osteosarcoma xenograft generously provided by Dr. Peter Houghton ( Nationwide Children’s Hospital, Ohio). Excess, deidentified tumor material was collected from patients with osteosarcoma at St. Jude Children’s Research Hospital (SICRH) in agreement with local institutional ethical regulations and Institutional Review Board (IRB) approval. Primary human osteosarcomas (SIOs001112_X1, SIOs010929_X1, SJOs001107_X1, and SJOs001107_X2) were derived from primary human xenografts collected from patients with osteosarcoma at SICRH. Human osteosarcoma cell lines MG-63, Saos-2, U2OS, G-292, 143B, and SISA-1 were purchased from the ATCC (MG63 from ECACC cell line remainder from the ATCC; no authentication performed by the authors). Normal human osteoblasts were derived from bone marrow aspirates from the posterior iliac crest of healthy human adult donors (17–35 years of age), with informed consent (ImVS/SA Pathology normal bone marrow donor program RAH#940911A). The cells were outgrown from the bony spicules that were collected following filtration of the BM through a 70-μm filter. Cell lines were grown in αMEM with 10% FCS (non-hep inactivated), 1% penicillin/streptomycin and 1% Glutamax supplement.

High-throughput siRNA whole-genome screen

Osteosarcoma cell line 494H (Crel:lox model derived cell line representative of fibroblastic osteosarcoma passage 8 from derivation from primary tumor) were grown and transfected with SMARTpool siRNAs using DharmaFECT lipid 3
transfection reagents (Dharmacon GE). The mouse siRNA library (Dharmacon GE) consists of 16,874 individual protein coding genes consisting of the following gene families all designed to RefSeq52. Cells were reverse transfected (18) at 400 cells per well with 40 nmol/L of the siGEMONE SMARTpool library complexed for 20 minutes with DharmaFECT 3 using the Sciclane ALH3000 (Caliper Life Sciences) and BioTek 406 (BioTek) liquid handling robotics. Cell lines were transfected in quadruplicate (day 0) and one set of duplicates treated as control (siRNA only), and a second set of duplicates treated after 48 hours (day 2) with 100 nmol/L doxorubicin. At 120 hours posttransfection (day 5), the cell viability for each well was quantified on the basis of the direct measurement of intracellular ATP using the CellTiter-Glo luminescent assay (Promega) at a 1:2 dilution. Full analysis methods are provided in the Supplementary Information. RNAi screen data are available from PubChem with PubChem accession ID #1053208 (primary screen) and PubChem AID# 1053209 (validation screen) and in Supplementary Dataset S1. RNA-seq data from mouse osteosarcoma models are deposited in GEO (GSE58916).

Small-molecule screen against primary mouse osteosarcoma paired samples
Three independent sets of paired primary and metastatic osteosarcoma cell lines [from fibroblastic osteosarcoma model, less than or equal to passage 8 from derivation; ref. 13] were plated at 400 cells per well in 384-well plates. After 24 hours, the cells were treated with the 131 different compounds in an 11-point dose response ranging from 0.01 to 10 nmol/L. All assays were performed in duplicate. At 48 hours after treatment, the cell viability for each well was quantified on the basis of the direct measurement of intracellular ATP using the CellTiter-Glo luminescent assay (Promega) at a 1:2 dilution. All luminescent measurements were taken on the EnSpire plate reader (PerkinElmer). Data were plotted and the IC_{50} value calculated using Prism 6 software.

Small-molecule screen against primary human xenograft-derived osteosarcoma
Excess, deidentified tumor material was collected from patients with osteosarcoma at SICRH in agreement with local institutional ethical regulations and IRB approval. Small-molecule/drug screening was performed as previously described (19, 20).

Cell death assays
Cells were treated with the indicated drug or vehicle control for 48 hours before harvesting. Cells were washed with PBS and 100,000 cells were stained in 1 Annexin Binding buffer (BD Biosciences) diluted 1:20 with Annexin V-APC (1 mg/mL; BD Pharmingen) and 7-Aminoactinomycin D (7AAD; 100 mg/mL; Life Technologies) for 15 minutes protected from light. Following the addition of 4 volumes of 1 Annexin Binding buffer, apoptotic cells were quantified using FACS (LSRFortessa; BD Biosciences). Live cells (Annexin V negative, 7AAD low) and cells in early and late stages of apoptosis (Annexin V positive, 7AAD low/high) were analyzed using FlowJo software (v8.8.7). Each cell line was assessed in duplicate in three separate biologic experiments.

Statistical analysis (excluding siRNA screen, which is described separately)
Each experiment is represented as the mean ± SEM calculated from biologic replicates unless otherwise stated. The unpaired parametric Student t test was used to compare results unless otherwise stated. Where indicated in figure legends, the unpaired nonparametric Mann–Whitney ranks test or two-way ANOVA were used to compare groups. Data were log-transformed to normalize variances before two-way ANOVA. In all analyses, statistical significance is reported: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. Prism 6.0e software was used for statistical analyses.

Results
Whole osteosarcoma genome siRNA screen to identify osteosarcoma susceptibilities
We sought to identify, in a genome-wide fashion, inducers of osteosarcoma cell death, and in parallel sensitizers to doxorubicin-induced cell death. Doxorubicin is a standard-of-care agent in the management of osteosarcoma (5). The screen was performed in early passage cell cultures established from the murine Osx-Cre p53^{fl/fl}pRb^{fl/fl} osteosarcoma model (fibroblastic osteosarcoma; ref. 13). A genome-wide screen was performed using a library containing >16,000 siRNAs targeting murine protein-coding genes (each siRNA pool has four individual siRNA against the same target). Mouse osteosarcoma cells were treated with either siRNA alone or, on a duplicate plate, siRNA in combination with 100 nmol/L doxorubicin (siRNA + doxorubicin; Fig. 1A). The dose of doxorubicin was titrated to achieve approximately 45% cell death as a single agent. All plates were screened in duplicate under both conditions. A robust Z-score sample–based normalization strategy was performed across the screen. To be classified as a hit, a candidate had to have a robust Z-score ≤–2 and a viability of equal to or less than 70% (fold change of ≤0.7) when compared with the respective nontargeting siRNA control for each treatment arm (NT1, Fig. 1B and C). A total of approximately 270 hits were identified from the primary screen that passed statistical analysis and robust Z-score stratification. A difference of at least 25% between siRNA alone and siRNA + doxorubicin was categorized as an interaction after filtering based on robust Z-scores (Fig. 1D). We further triaged candidates based on their expression in RNA-seq/microarray datasets from GEMM osteosarcoma models. The top 299 candidates that enhanced cell death were then validated by deconvolution of the SMARTpool into its constituent four individual siRNAs and rescreening using the same assay. Of the primary screen hits, 113 validated with three or more of the individual siRNAs and were classified as medium confidence hits. In total, approximately 50% of the primary screen hits validated (Fig. 2A–C). A range of candidates that reduced cell viability independently of whether doxorubicin was present in addition to those that specifically enhanced the toxicity of doxorubicin were identified (Fig. 2A–D). Although not strongly powered to detect loss of function alleles involved in resistance to doxorubicin, several candidates were identified (Fig. 2A), including siRNA, against topoisomerase 2a (Top2a), the cellular target of doxorubicin (21, 22), and a number of proapoptotic genes such as Bid (Fig. 1B, Supplementary Fig. S1A and S1B). A significantly higher...
false-positive rate on the candidates that prevented cell death was apparent, with only Top2a and Bid robustly validating of the 60 candidates tested. From the enhancers of doxorubicin induced death, we identified 266 siRNA candidate genes (1.576%) that fulfilled the classification criteria (subset visualized in Fig. 2B). Analysis of the hits interacting with doxorubicin revealed a marked enrichment of candidates on chromosome 7 \( P = 4.77 \times 10^{-11}, \) FDR = 3.68 \times 10^{-8}, statistics derived from DAVID analysis), relevant as the loss of one to two copies of chromosome 7 from a tetraploid karyotype was recurrently noted in the murine osteosarcoma models (16). Using Ingenuity Pathway Analysis software, we tested whether the identified hits functioned in overlapping pathways/gene-expression signatures (Fig. 2A and B). Pathways enriched included those associated with RNA transport (Ran) and RNA splicing, in particular the U2 splicing complex (Fig. 2A and B, Supplementary Fig. S1C). Eleven of the 13 identified U2 snRNP complex members in mammals were identified in the screen. In contrast, we did not observe enrichment for any other snRNP complexes, suggesting a specific requirement for the U2 snRNP complex in osteosarcoma pathogenesis (Fig. 2B). The most significantly enriched pathways were associated with protein translation and mTOR signaling (Fig. 2A–D).

Next, we tested whether small molecules targeting the identified pathways demonstrated toxicity against osteosarcoma cells.
Protein translation and RNA-splicing enhance osteosarcoma cell death. A, analysis of pathways enriched in the primary screen from both arms (siRNA alone; top), those enriched within hits that enhanced doxorubicin-induced cell death (middle) and those that increased cell survival in the presence of doxorubicin (bottom). Data plotted as log-fold enrichment (P value) based on Ingenuity Pathway Analysis. B, visualization of the individual candidate hits based on subcellular localization (from Ingenuity Pathway Analysis software) with specific pathways that are enriched indicated within each colored box based on the validated primary screen results described in part A. C, secondary screen deconvolution analysis of the candidates identified in the primary screen. Primary screen candidates were secondarily screened as individual siRNAs and those with 3 or 4 of 4 validating the primary screen were considered high-confidence candidates; 2 of 4 medium confidence and 1 or less not considered further. D, examples of individual target effects in the primary and secondary screen. Top, mean viability of duplicate samples for each treatment arm from the primary siRNA screen; bottom, viability following transfection of the individual siRNA validation for each target in the secondary screen. In the bottom, each point represents an individual siRNA from within the pooled complex used for the primary screen with mean ± SEM for each gene.
The agents were tested against a primary human osteosarcoma xenograft–derived cell culture (OS17) and murine primary tumor–derived cultures from both fibroblastic (494H) and osteoblastic (148I) osteosarcoma models. We tested a Ran inhibitor, an inhibitor of the U12 splicing complex member Sf3b spliceostatin A (SSA; ref. 23), and the proteasome inhibitor bortezomib (Fig. 3A–C). All agents showed activity against the osteosarcoma cells, although the specificity of the bortezomib response was indeterminate, given its reported in vitro activity against a large panel of solid tumors. Given the enrichment of the U2 snRNP specifically in the siRNA screen and the potent activity of SSA, we sought to further understand the effects of impaired U2 snRNP activity in osteosarcoma. We treated fibroblastic and osteoblastic mouse osteosarcoma cells with nontargeting or siRNA against Sf3b1 and U2af1, both components of the U2 snRNP (Fig. 3B). In both cases, cell death as measured by cleaved caspase-3 levels was observed. Treatment of human or mouse osteosarcoma cells with SSA resulted in impaired splicing of p27 and detection of an aberrant protein isoform (p27*), a known consequence of Sf3b1 inhibition (Fig. 3C, Supplementary Fig. S2). Consistent with the siRNA results, SSA treatment resulted in massive induction of apoptosis in fibroblastic and osteoblastic murine osteosarcoma, consistent with the low nmol/L IC50 in these cells. These studies identify a range of susceptibilities in osteosarcoma cells that had not been previously observed. Furthermore, these targets were validated by independent means using chemical or drugs that target these processes.

Kinase inhibitor screen: dual PI3K–mTOR inhibitors show strong activity against osteosarcoma

The activity of a chemical library of 131 primarily kinase inhibitors, either currently in clinical trials and those that either failed to work or discontinued trials due to lack of efficacy or toxicity, were characterized by IC50 in both fibroblastic and osteoblastic murine osteosarcoma, (Fig. 3A). Figure 3A demonstrates the presence of an abnormally spliced p27 isoform (indicated as p27*) accompanied by cleaved caspase-3 in SSA-treated cells.

**Figure 3.** Small-molecule validation of siRNA candidates. A, dose–response viability curves of human (OS17) and mouse fibroblastic (494H) or osteoblastic (148I) osteosarcoma cell cultures treated with the Ran inhibitor Importazole either alone (red line) or in combination with doxorubicin (blue line). Data, IC50 values (n = 2–4 independent replicates); sensitivity of human (+doxorubicin), murine fibroblastic, and osteoblastic osteosarcoma to the proteasome inhibitor bortezomib; sensitivity of human (+doxorubicin), murine fibroblastic, and osteoblastic osteosarcoma to the Sf3b1 inhibitor spliceostatin A (SSA). B, treatment of murine fibroblastic and osteoblastic osteosarcoma for 72 hours with mock, nontargeting (NT), Sf3b1, or U2af1 siRNA. Western blots were probed for the presence of cleaved caspase-3 and actin as a control. C, murine fibroblastic and osteoblastic osteosarcoma were treated for 48 hours with SSA. Western blot analysis demonstrates the presence of an abnormally spliced p27 isoform (indicated as p27*) accompanied by cleaved caspase-3 in SSA-treated cells.
trial or were not developed further, as single agents was also assessed. The screen was conducted in duplicate on three sets of paired primary and metastatic early passage osteosarcoma cell cultures derived from the murine fibroblastic osteosarcoma model (Fig. 4A). Osteosarcoma culture (494H) was also used for the siRNA screen. Cell viability was determined after 48 hours exposure to the agents and a dose–response curve established.

The chemical screen identified eight compounds with activity as single agents in the sub-1 μmol/L range (6.1%, Fig. 4A). Of the active agents, there were two multitarget inhibitors (ponatinib and dasatinib), a CHK inhibitor (AZD7762), flavopiridol (CDK inhibitor), and a PIK-1 inhibitor [BI-2536(R-)]. PIK-1 siRNA was used as a positive control for our siRNA screen, so it was known that inhibition of PIK-1 was toxic to osteosarcoma cells. Of particular interest were three compounds with an overlapping spectrum of targets. PIK-75, GSK2126458, and BEZ-235. All target PI3K together with mTOR and/or DNA-PK (Supplementary Fig. S3). The effect was specific to these dual inhibitors as we did not observe low IC50 values with an additional 14 agents with PI3K-restricted activity. In addition, the specific mTORC1/2 inhibitor (Ki11063794), the DNA-PK inhibitor (NU7441), and several specific Akt inhibitors (Akt-I-1; Akt-I-1/2) did not exhibit potent activity (sub-1 μmol/L, Fig. 4B). These findings suggested that concurrent inhibition of both the PI3K and another pathway (mTOR or DNA-PK) was most likely responsible for the observed activity. We did not observe any differential sensitivity between the primary and metastatic paired samples. The chemical overlap of the results from chemical and genetic screening revealed a profound enrichment for cell growth/protein translation regulation pathways, as a vulnerability in osteosarcoma. We further tested PIK-75, GSK2126458, and BEZ-235 across a panel of osteosarcoma cultures encompassing murine osteoblastic osteosarcoma, murine fibroblastic osteosarcoma, an established human osteosarcoma line (MG63), and a primary human osteosarcoma xenograft–derived cell culture (OS17). The activity was confirmed with independent stocks of drug and facilities and on all osteosarcoma cell cultures tested (Fig. 4C).

**Primary human osteosarcoma also display sensitivity to dual PI3K–mTOR inhibitors**

Our data indicated that dual targeting of the PI3K–mTOR pathway represents an effective strategy to induce osteosarcoma cell death. To extend our analysis and expand the genetic diversity of the primary human osteosarcoma that were assessed, we tested a targeted range of small-molecule inhibitors against a panel of acute short-term cultures of primary osteosarcoma material derived from orthotopic xenografts (8, 20). The established human osteosarcoma cell lines Saos-2 and U2OS were also included. Strikingly, the most active agents against primary human osteosarcoma cultures were dual PI3K and mTOR inhibitors (GSK2126458, PKI-587, BEZ-235, and BGT-226; Fig. 4D). Therefore, the sensitivity to concurrent inhibition of PI3K and mTOR pathways is conserved across species and subtypes. These data provide a strong rationale, obtained from systematic screening, for the testing of dual PI3K–mTOR pathway inhibitors in osteosarcoma.

**Induction of osteosarcoma cell apoptosis by dual inhibition PI3K and mTOR pathways**

Of the small-molecule inhibitors identified, PIK-75 cannot be directly clinically applied and BEZ-235 has ceased clinical development. Therefore, we tested compounds that could be clinically translated, including GSK2126458, and included a second PI3K–mTOR active agent, PKI-587 (Supplementary Fig. S3A–S3C; refs. 24, 25). GSK2126458 and PKI-587 could be combined with doxorubicin in vitro with no reduced effectiveness of either agent (Supplementary Fig. S3D–S3G). Both agents inhibited phosphorylation of known targets downstream of PI3K–mTOR in a dose-dependent manner in mouse osteosarcoma subtypes and human primary osteosarcoma cell cultures (Fig. 5A). Next, we assessed whether GSK2126458 and PKI-587 were inducing changes in cell-cycle distribution or cell death. There was a significant increase in cells in the G0–G1 phase of the cell cycle in both mouse and human osteosarcoma cells treated with GSK2126458, and to a lesser magnitude with PKI-587 (Fig. 5B). In all cells tested, there was a dose-dependent increase in the induction of apoptosis as determined by Annexin V/7AAD staining or the presence of cleaved caspase-3 (Fig. 5C and D). In all three osteosarcoma cell models tested, GSK2126458 was more potent at inducing apoptosis than PKI-587, despite similar effectiveness at inhibiting downstream signaling events (Fig. 5C and D).

**Combining PI3KCA and mTOR inhibitors as a therapeutic approach**

The activity of the dual PI3K–mTOR inhibitors corresponded most closely with activity against PI3KCA (Fig. 4B). We determined the expression of the different catalytic subunits of PI3K in murine osteosarcoma subtypes and in human osteosarcoma and normal human osteoblasts. The murine osteosarcoma tumors had the highest expression of Pik3ca and Pik3cb with low to undetectable levels of Pik3cd and Pik3cg (Fig. 6A). This pattern of expression was mirrored in normal human osteoblasts and osteosarcoma samples (Fig. 6A). Given the relative isoform specificity of PIK-75, GSK2126458, and BEZ-235, this indicated that the activity was most likely primarily mediated by inhibition of PI3KCA together with mTOR. To directly test the requirement for...
**Figure 5.**
Dual PI3K-mTOR inhibitors induce apoptosis in osteosarcoma cells. A, analysis of target inhibition downstream of PI3K-mTOR activity was assessed by Western blot analysis of the indicated proteins against murine fibroblastic, murine osteoblastic, and primary human osteosarcoma. Each cell type was treated with the calculated IC50 and Emax, respectively, for each drug. B, cell-cycle distribution of murine fibroblastic osteosarcoma or primary human osteosarcoma cells treated with the indicated concentrations of GSK2126458 and PKI587 for 24 hours, statistical comparison performed comparing the proportions of G0-G1 phase cells between vehicle and each treatment. C, GSK2126458 and PKI587 induce apoptosis as assessed by annexin V/7AAD staining (D) or cleaved caspase-3 Western blot analysis in a dose-dependent manner. Where indicated data presented as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (n = 3 technical replicates/cell line, three independent cell lines tested as indicated).
inhibition of PIK3CA, we used siRNA to knockdown the PIK3CA and PIK3CB. Knockdown of PIK3CA in mouse osteosarcoma and PIK3CA in human osteosarcoma cells resulted in significantly reduced viability (Fig. 6B). When the siRNA was combined with the specific mTOR inhibitor everolimus, an increased cell death compared with that achieved with either agent alone was observed. The increased death from concurrent inhibition was more pronounced with PIK3CA compared with PIK3CB knockdown, although the reduced viability with PIK3CB siRNA would suggest some contribution from this isomor to the overall effect (Fig. 6B, Supplementary Fig. S4). We tested whether knockdown of mTOR, Raptor or Rictor could cooperate with inhibition of PIK3ca. The knockdown of mTor was the most effective and when combined with the PIK3CA-specific inhibitor BYL719 resulted in significantly reduced cell viability compared with the siRNA alone (Fig. 6C, Supplementary Fig. S4). These data demonstrate that specific targeting of either the PI3K or mTOR pathways in isolation is not as effective at inducing osteosarcoma cell death as dual inhibition.

Finally, we sought to determine whether combinations of specific inhibitors of the PI3K and mTOR pathway could be used to recapitulate the activity of the dual inhibitors. This may be an approach amenable to translation as several pathway–specific inhibitors have progressed in clinical trials or, in the case of the everolimus, been approved (26). We tested combinations of either the pan-PI3K inhibitor BKM120 or the PIK3CA-specific inhibitor BYL719 with everolimus. When tested individually, all agents failed to demonstrate significant activity (Fig. 6D). When combined, we observed a synergistic interaction between the BLY719 and everolimus based on BLISS synergy scores (Fig. 6D, Supplementary Fig. S5). Both agents were tolerated alone or in combination by normal osteoblasts in vitro, consistent with in vivo reports with these and similar agents (Supplementary Fig. S6; refs. 28, 29). Therefore, agents currently in clinical trials with activity against PIK3CA and mTOR could be combined and demonstrate efficacy against osteosarcoma in vitro. These data demonstrate the feasibility of dual targeting of the PI3K–mTOR pathway in osteosarcoma and propose a new therapeutic approach in this cancer.

Discussion

To identify new therapeutic targets for osteosarcoma, we have made use of murine osteosarcoma models combined with primary human osteosarcoma xenograft derived cell cultures (13, 16). These independent approaches converged to identify dual inhibition of the PI3K–mTOR pathways as a species conserved sensitivity in osteosarcoma. The use of the primary tumor–derived material from multiple species strengthens the confidence in our data and provides a new approach to integrating mouse models into preclinical target identification and validation. We observed in vitro activity against osteosarcoma of the dual PI3K–mTOR inhibitor GSK2126458 (Supplementary Fig. S7). Supporting our results, are previous data testing a range of compounds that target single arms of these pathways with evidence for some, albeit limited, in vivo activity against human osteosarcoma xenografts (11, 30–33). Dual PI3K–mTOR inhibitors were far more potent that PI3K-selective agents on established osteosarcoma cell lines, further validating our observations with primary osteosarcoma–derived cells (34). Most recently, exome capture of human osteosarcoma and shRNA screening independently confirmed that the PI3K–mTOR pathway is an attractive candidate in osteosarcoma, supported by the data from the screening and validation described here (35).

Of the pathways identified in our siRNA screen, the regulation of RNA processing and splicing in osteosarcoma is an intriguing finding. We recovered 11 of 13 members of the U2 snRNP complex without significant enrichment for other complexes of the RNA-splicing machinery in cells treated with siRNA and doxorubicin (36). The osteosarcoma cells were highly sensitive to inhibition of Sfas1 with SSA (23). Changes in RNA splicing are recognized as major contributors to cancer, particularly hematologic malignancy (37). Recently, other genetics screens have identified RNA-splicing machinery as preferentially required in cancer cells, with PRPF6 important in colon cancer (38) and PHF5a in brain cancer (39). These data suggest that aberrant RNA splicing in osteosarcoma are important in maintaining the tumor state and warrant further investigation. The identity of the aberrantly spliced RNAs causing osteosarcoma cell death osteosarcoma cells is currently unknown; however, one candidate group of transcripts encode pro-death proteins (40). We identified proapoptotic effectors as suppressors of doxorubicin-induced cell death in our siRNA screen, providing a plausible link to the sensitivity of osteosarcoma cells to U2 snRNP inhibition. Another intriguing candidate was Mesogenin (Msgn), a bHLH transcription factor that was the most potent inducer of osteosarcoma cell death irrespective of the presence of doxorubicin. Although there is limited information relating to Msgn, the Msgn mouse has defects in skeletal development and Msgn acts downstream of the Wnt and β-catenin pathways, both implicated in osteosarcoma (41, 42). It would be interesting to determine whether Msgn is required for normal osteoblast survival once the lineage is established, or analogous to the requirement for another bHLH transcription factor SCL/tal-1 in hematopoiesis, it is only required developmentally for lineage specification (43). If this were the case then developing strategies to target Msgn may be of value.

Given the overlap between the siRNA and chemical/drug screen results, we focused our attention on understanding the contribution of the PI3K–mTOR pathway in osteosarcoma. Recently, several groups have reported that agents working within this pathway have some activity in osteosarcoma (28, 29). Our preclinical screening has indicated the requirement for concurrent inhibition of PI3K, in particular PIK3CA, and mTOR pathways. The screens reported herein provide a rationale for combining PI3KCA and mTOR inhibitors, rather than the use of either inhibitor alone, in osteosarcoma. Recent osteosarcoma studies from other groups have drawn attention to this pathway (28, 29, 44–46), yet our study is distinct from these as we have identified this pathway as a result of independent chemical and genetic screens, and can demonstrate that dual pathway inhibition is the most potent. We further define that PIK3CA activity is the driver of this PI3K activity in both human osteosarcoma and mouse models of osteosarcoma. The sensitivity to PI3K–mTOR inhibitors is conserved across osteosarcoma subtypes and across species, greatly strengthening the case that this pathway should be tested clinically.

Our results using GSK2126458 and PKI-587 contrast with those described by Gobin and colleagues (28), who reported the effects of BEZ-235. We identified BEZ-235 as active in our chemical screen. We observe robust induction of apoptosis...
Figure 6. PIK3CA and mTOR inhibition mediates the sensitivity of osteosarcoma cells to agents targeting these pathways. A, transcript levels of Pik3c isoforms were quantified from RNA-seq of murine fibroblastic and osteoblastic osteosarcoma or by real-time PCR in human osteosarcoma cells compared with normal human osteoblast cells. (Continued on the following page.)
with both GSK2126458 and PKI-587 in murine and in primary human osteosarcoma–derived cells at significantly lower doses than that reported on established murine or human osteosarcoma cell lines (28, 29). Gobin and colleagues reported a cell-cycle arrest in the absence of apoptosis when treating with up to 25 μmol/L BEZ-235 or the PI3KCA-specific inhibitor BYL719. The discrepancy in effects is most likely a reflection of the cells used for the respective studies. Our study has used early passage primary osteosarcoma cell cultures derived from either GEMM (at least three independent cultures of each subtype) or xenografts. In contrast, Gobin and colleagues used a range of long established osteosarcoma derived cell lines. Therefore, the age and nature of the cells used may lead to divergent effects on cell survival. The ant osteosarcoma activity of the PI3K–mTOR inhibitors on primary osteosarcoma cells is mediated via induction of apoptosis and cell-cycle arrest.

A large phase II trial with single-agent mTOR inhibitor ridaforolimus in sarcomas noted some, albeit limited, activity in osteosarcoma. Two partial responses in 54 patients with bone sarcomas were treated (47). BEZ-235 has in vivo activity in preclinical osteosarcoma models, supporting our preliminary data (28). However, BEZ-235 has been discontinued from further development, emphasizing our focus on clinically usable agents. We demonstrate in vivo activity of the dual inhibitors GSK2126458 and PKI-587 and the combination of the PI3KCA-specific inhibitor BYL719 and the mTORC1 inhibitor everolimus. BYL719 and everolimus are currently being tested together clinically, a phase I/II trial in patients with in breast cancers, based on the observation that dual inhibition of PIK3CA and mTORC reversed resistance of PIK3CA-mutant breast cancer to BYL719 monotherapy (48). This will act as a good indicator of how readily combinable these two agents are in terms of clinical potency, manageable off-target effects, and possible pharmacologic interactions.

Through combining siRNA screening with targeted chemical library screens, we have identified a number of candidate targets in osteosarcoma. The most directly translatable pathway is PI3K–mTOR dual inhibition, with a number of agents already in clinical trials or approved. The screens described herein provide a rational basis for detailed in vivo orthotopic pharmacokinetic and pharmacodynamic studies, and the assessment of these agents in preclinical phase I, II, and III studies that will be required. Furthermore, using independent approaches and validation across species, osteosarcoma subtypes and facilities, we provide evidence that PI3K–mTOR dual inhibition is an attractive therapeutic candidate for osteosarcoma.

Disclosure of Potential Conflicts of Interest
J. Desai is a consultant/advisory board member for GlaxoSmithKline, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: A.J. Mutsaers, D.C.S. Huang, K.J. Simpson, C.R. Walkley
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gupke, E.K. Baker, S.S. Wan, E. Stewart, A. Loh, S. Taylor, Å. Karlström, C. Burns, D.C.S. Huang, M.A. Dyer, K.J. Simpson, C.R. Walkley
Writing, review, and/or revision of the manuscript: E.K. Baker, A.A. Shelat, A.M. Chalk, K. Lackovic, Å. Karlström, A.J. Mutsaers, J. Desai, A.C.W. Zannettino, C. Burns, D.C.S. Huang, K.J. Simpson, C.R. Walkley
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.K. Baker, S.S. Wan, E. Stewart, C.M. Gould, Å. Karlström, A.C.W. Zannettino, D.C.S. Huang, C.R. Walkley

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(Continued) Data are presented as TMH (trimmed mean of M values) normalized read counts (n = 3/subtype) for RNA-seq or mean relative expression to PGK1 ± SEM (n = 4–7) for QPCR. B, human and mouse osteosarcoma cells were transfected with 25 nm SMARTpool siRNAs targeting Pik3ca/b or Pik3ca/B, respectively, or a pool of nontargeting siRNAs (NT2) for 24 hours then treated with the indicated concentrations of the mTOR inhibitor everolimus for 48 hours. Cell viability was assessed relative to Non-T DMSO-treated cells. Data, mean ± SEM (n = 3). C, mouse fibroblastic osteosarcoma cells (494H) were transfected with siRNAs targeting mTor, Raptor or Rictor or a pool of nontargeting siRNAs (Non-T) for 24 hours then treated with the indicated concentrations of the mTOR inhibitor everolimus for 48 hours. Cell viability was assessed relative to Non-T DMSO-treated cells. Data, mean ± SEM (n = 3). *P < 0.05, **P < 0.05 calculated using Student t tests. D, treatment of murine osteosarcoma cells (494H) with the pan-PI3K inhibitor BKM120, PI3KCA-specific inhibitor BYL719 or the mTOR inhibitor Everolimus as single agents. Data, mean ± IC50 values ± SEM (n = 3/treatment); needle graphs show synergy effects in mouse fibroblastic osteosarcoma cells treated with combinations of Everolimus and BYL719 for 72 hours. Synergy is represented as a percentage of deviation from a predicted additive response (Bliss additivity). Deviations greater than 15% were considered synergistic (red baseline). Data, mean deviation (n = 3).
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