ATR Is a Therapeutic Target in Synovial Sarcoma

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Abstract

Synovial sarcoma (SS) is an aggressive soft-tissue malignancy characterized by expression of SS18–SSX fusions, where treatment options are limited. To identify therapeutically actionable genetic dependencies in SS, we performed a series of parallel, high-throughput small interfering RNA (siRNA) screens and compared genetic dependencies in SS tumor cells with those in >130 non–SS tumor cell lines. This approach revealed a reliance of SS tumor cells upon the DNA damage response serine/threonine protein kinase ATR. Clinical ATR inhibitors (ATRi) elicited a synthetic lethal effect in SS tumor cells and impaired growth of SS patient-derived xenografts. Oncogenic SS18–SSX family fusion genes are known to alter the composition of the BAF chromatin–remodeling complex, causing ejection and degradation of wild-type SS18 and the tumor suppressor SMARCB1. Expression of oncogenic SS18–SSX fusion proteins caused profound ATRi sensitivity and a reduction in SS18 and SMARCB1 protein levels, but an SSX1–SSX1 Δ71–78 fusion containing a C-terminal deletion did not. ATRi sensitivity in SS was characterized by an increase in biomarkers of replication fork stress (increased γH2AX, decreased replication fork speed, and increased R-loops), an apoptotic response, and a dependence upon cyclin E expression. Combinations of cisplatin or PARP inhibitors enhanced the antitumor cell effect of ATRi, suggesting that either single-agent ATRi or combination therapy involving ATRi might be further assessed as candidate approaches for SS treatment. Cancer Res; 77(24); 7014–26. ©2017 AACR.

Introduction

Synovial sarcoma (SS) is a rare, yet aggressive and difficult-to-treat type of soft-tissue sarcoma (STS) that has a variable age of onset but predominantly affects young adults. Although for patients with localized disease, wide surgical excision combined with radiotherapy can be curative, recurrent disease is common (1). In the metastatic setting, SS patients are treated with cytotoxic chemotherapies, including the topoisomerase inhibitor doxorubicin and/or the alkylating agent ifosfamide. Recently, the multikinase inhibitor pazopanib became the first targeted agent to be approved for the treatment of advanced SS after failure of anthracycline containing chemotherapy (2). Despite these multimodal therapy approaches, the outcome of metastatic SS patients remains poor; those with distant metastasis have a 10-year survival rate of only 8.9% compared with 69% for patients with localized tumors (3). These factors highlight that additional, more specific therapeutic approaches with greater efficacy are required to effectively manage this disease.

The main pathological driver event in SS is known, suggesting that in principle at least, mechanism-based targeted approaches to treating SS could be developed. The majority of SS are characterized by a t(X;18) reciprocal chromosomal translocation, often used as a diagnostic biomarker for the disease (4). These t(X;18) translocations fuse the first 10 exons of the SS18 (synovial sarcoma translocation, chromosome 18) gene to the last three exons of one of the SSX (synovial sarcoma, X breakpoint) family of genes, SSX1, SSX2, or SSX4 (4, 5), encoding either SS18–SSX1, SS18–SSX2, or SS18–SSX4 fusion proteins. SS display few other recurrent mutations (6).

A number of studies have aimed to identify the cellular functions of these oncogenic fusions as well as of their wild-type SS18 and SSX counterparts (7, 8). SS18–SSX oncproteins contribute to the dysregulation of gene expression through association with SWI/SNF (BAF) and polycomb chromatin remodeling complexes (9–11). BAF complexes mediate nucleosome remodeling via an ATP-dependent process and in doing so modulate transcription (12, 13), DNA repair, and the
maintenance of genomic integrity (13, 14). SS18–SSX1 fusion proteins displace wild-type SS18 and an additional BAF component, the tumor suppressor SMARCB1, from BAF complexes (7). The displacement of SMARCB1 from BAF leads to its proteasomal degradation, with reduced levels of BAF-associated SMARCB1 being a characteristic of SS tumor cell lines and tumors (7, 15).

Despite an enhanced understanding of the SS18–SSX function, therapeutic targeting of these oncogenic proteins has not yet been achieved. One of the more recently used approaches to identifying therapeutic targets in cancer has been to identify and exploit genetic dependencies, such as synthetic lethal and gene addiction effects, that are associated with particular cancer driver gene defects. The potential of such an approach is best exemplified by the use of small-molecule PARP inhibitors in BRCA1/2-mutant cancers (16, 17). Because the key driver genotype of SS is well established, we sought to apply a similar approach to identify synthetic lethal interactions in SS. This identified an unexpected dependency in SS tumor cells upon the kinase ATR (Ataxia Telangiectasia mutated and Rad3-related), a key mediator of the DNA-damage response (DDR; ref. 18) that can be exploited with clinical ATR inhibitors.

Materials and Methods

Cell culture

Yamato-SS and Ask-SS cell lines were kindly provided by Kazuyuki Itoh and Norifumi Naka (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan); Akira Kawai (National Cancer Center Hospital, Tokyo, Japan) provided SYO-1 cells and Cinzia Lanzi (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) provided CME-1 cells. HS-SY-II cells were obtained from the RIKEN BioResource Center. HCT116 WT and ARID1A-mutated and Rad3-related, a key mediator of the DNA-damage response (DDR; ref. 18) that can be exploited with clinical ATR inhibitors.

Lentiviral SS18–mutant isogenic cell lines have been described previously (19); all other cell lines were supplied by ATCC. Cells were grown in 5% CO2 at 37°C in media described below, supplemented with 15% (HFF1) or 10% (all other cell lines) fetal calf serum (FCS, Gibco) and 1% antibiotics in GIPZ-infected cells using an EVOS FL imaging system. Cell viability was estimated 5 days after transfection using CellTiter-Glo assay (Promega). Data processing and quality controls were performed using the cellHTS2 R package as described previously (20, 21). In the case of the VX970 resistance screen, 24 hours after transfection, VX970 (final concentration 0.75 μmol/L) or vehicle (DMSO) was added to cells. Cells were then exposed to VX970 for 4 days and viability estimated with CellTiter-Glo assay as above. Drug effect (DE) Z-scores were calculated as described previously (22).

Lentiviral SS18–SSX expression

SS18–SSX1, SS18–SSX2, and the SS18–SSX1 A71–78 mutant cDNAs were cloned into the pLX301 lentiviral transfer vector (7). For viral infection, cells were plated in 6-well plates and exposed to 0.5 μL lentiviral filtrate. After 3 days, cells were transferred to a T25 flask containing 1 μg/mL puromycin (Gibco) to remove nontransduced cells. After 3 days selection in puromycin, cells were harvested and used for further analysis. GFP expression was confirmed in GFP+infected cells using an EVOS FL fluorescence microscope (Life Technologies).

SiRNA screens

Cells lines were transfected with a Dharmacon SMARTpool 384-well plate-arrayed siRNA library designed to target 714 kinases and kinase-related genes, 320 Wnt pathway–associated genes, 80 tumor suppressor genes, and 480 genes recurrently altered in human cancers (Supplementary Table S1) as described in ref. 20. Positive control (siPLK1) and multiple negative controls (siCON1 and siCON2; Dharmacon, catalog numbers D-001210-01-20 and D-001206-14-20, and AllStar; QIAGEN, catalog number 1027281) were included on every plate. Transfection reagents were as follows: SYO-1; Ask-SS; Yamato-SS and CME-1; RNAIMAX (Invitrogen); HS-SY-II, Lipofectamine 2000 (Invitrogen). Screens were performed in triplicate. Cell viability was estimated 5 days after transfection using CellTiter-Glo assay (Promega). Data processing and quality controls were performed using the cellHTS2 R package as described previously (20, 21). In the case of the VX970 resistance screen, 24 hours after transfection, VX970 (final concentration 0.75 μmol/L) or vehicle (DMSO) was added to cells. Cells were then exposed to VX970 for 4 days and viability estimated with CellTiter-Glo assay as above. Drug effect (DE) Z-scores were calculated as described previously (22).

FACS analysis

Cells were cultured in 6-well plates and exposed to either 0.5 μmol/L VX970 or DMSO. Cells were pulse-labeled with EdU for 1 hour prior to fixation in ice-cold 100% (v/v) ethanol and stored at −20°C until use. EdUrd was fluorescently labeled by conjugation to the Alexa488-488 fluorophore using the Click-IT EdU FITC Imaging Kit (Invitrogen).
EdUrd Flow Cytometry Kit (Life Technologies) according to the manufacturer's protocol. Total DNA content was assessed by incubation of cells for 15 minutes in 1.43 μmol/L DAPI (Sigma) in PBS. Cell-cycle profiles were generated using a BD LSR-II flow cytometer (BD Biosciences) and analysis was performed using BD FACSDIVA software V8.01. Flow cytometry was performed according to the manufacturer's guidelines.

**Results**

**RNA interference profiling of SS cell lines identifies ATR as a candidate genetic dependency**

To identify candidate therapeutic targets in SS, we carried out large-scale small interfering RNA (siRNA) screens in five commonly used SS tumor cell lines; HS-SY-II, Aska-SS, Yamato-SS, CME-1, and SYO-1 (Fig. 1A). These cell lines were selected as they harbor either one of the two most common SS-associated fusions (SS18-SSX1 or SS18-SSX2), and were amenable to high-efficiency siRNA transfection in a 384-well plate format (1B–D). After optimizing high-throughput transfection conditions, each cell line was reverse-transfected with a 384-well plate-arrayed siRNA library designed to target 1,600 genes (Supplementary Table S1 and Methods), including pharmacologically tractable kinase-coding and kinase-related genes, Wnt-pathway–associated genes given their role in SS (11, 25, 26) and genes recurrently altered in human cancers (27). Cell viability was measured after 5 days, and these data were then used to identify those siRNAs that caused significant tumor cell growth inhibition (i.e., genetic dependencies). Robust Z-scores were calculated from three replica screens (Methods and Supplementary Table S4) to estimate the effect of siRNAs on tumor cell inhibition.

To identify genetic dependencies associated with SS, we compared the siRNA Z-scores from the SS tumor cell line screens to siRNA Z-score profiles of >130 tumor cell lines from a diverse set of cancer histotypes (20). We noted a series of profound SS-specific genetic dependencies, including the proto-oncogene Mouse Double Minute 2 homologue (MDM2), the gene encoding the calcium-binding protein Calmodulin (CALM1; Fig. 1E and F), genes associated with Wnt signaling (e.g., those encoding the Wnt ligand WNT7B and the β-catenin interacting protein, BCL9L; ref. 28; Fig. 1G and H) and the drugable Never in Mitosis-A (NIMA) family members (29) NEK1, 2, and 4 (Fig. 1I–K).

The substantial variation in siRNA Z-scores within the SS tumor cell lines for these genes, however, suggested that these were not highly penetrant effects (i.e., profound effects in the vast majority of SS tumor cell lines) and might therefore have limited utility as targets in SS. More penetrant genetic dependencies associated with SS included the DREAM complex kinase, DYRK1A (Fig. 1L) and the DDR kinase ATR (Fig. 1M, described below). DYRK1A inhibition is synthetic lethal with RB1 (pRb) tumor suppressor defects in osteosarcoma (OS; ref. 20). We found the sensitivity of SS tumor cell lines to DYRK1A siRNA to be of a scale equivalent to that in RB1-null OS tumor cell lines (Fig. 1L), suggesting that DYRK1A, which is amenable to small molecule inhibition (20, 30), might be worthy of further assessment as a candidate therapeutic target in SS.

We also found a series of genes involved in the DDR network (31) to be candidate genetic dependencies in SS, including ATR, the ATR-activating proteins RAD9A and RAD18, and two tumor suppressor genes involved in double-strand break repair by homologous recombination (HR), BRCA1 and BRCA2 (Fig. 1M–Q), suggesting a reliance upon processes that are associated with the stability and repair of replication forks.

**ATR genetic dependency in SS can be elicited with clinical ATR inhibitors**

The identification of ATR as a candidate genetic dependency was particularly interesting for a number of reasons: (i) compared
Figure 1.
Genetic dependency profiling in synovial sarcoma cells identifies ATR as a candidate genetic dependency. A, Overview of the five SS cell lines and their characteristic SS18-SSX driver fusions. B, Schematic of siRNA screening procedure and data processing. C, Scatter plot illustrating distribution of siRNA Z-scores for negative (siCON1, siCON2, and AllSTAR) and positive (siPLK1) controls, as well as Z-scores for the library siRNAs in the SYO-1 siRNA screen. Dots represent siRNA Z-scores from individual wells in the library. Each well contained a siRNA SMARTPool designed to target one gene. Error bars, SD. D, Scatter plot illustrating the correlation of siRNA Z-scores between replica 1 and 2 in the SYO-1 screen. E–Q, siRNA Z-score plots illustrating genetic dependencies associated with SS. Error bars, SD from median effects. P value represents Mann–Whitney test.
with other cancer histologies, we found the five SS tumor cell lines to be amongst the most sensitive tumor cell lines to ATR siRNA and to respond in a relatively consistent fashion (Fig. 2A), suggesting a relatively penetrant effect; (ii) relatively little is understood about the sensitivity of SS tumors to small-molecule DDR inhibitors, such as ATR inhibitors; and (iii) ATR inhibitors such as VX970 and AZD6738 have recently entered clinical trials for cancer treatment (e.g., clinicaltrials.gov NCT02157792, NCT02223923), suggesting that this genetic dependency could be clinically actionable.

Having confirmed the sensitivity of SS tumor cell lines to ATR siRNA in post-screen validation experiments (Supplementary Fig. S1A–S1C), we assessed the sensitivity of the five SS tumor cell lines to the clinical ATR inhibitor (ATRi), VX970 (Vertex Pharmaceuticals/Merck KGaA). Compared with previously validated ATRi-resistant HCT116 colorectal cancer cells (32), all five SS cell lines were profoundly sensitive to VX970 (ANOVA \( P < 0.0001 \)), each exhibiting \( SF_{0.50} \) (concentration required to cause 50% reduction in cell survival) values of \( \approx 0.1 \) \( \mu \)mol/L (Fig. 2B). SS tumor cell lines were also more sensitive to ATRi compared with HFF1 fibroblasts and nontumoral epithelial MCF10A cells (Supplementary Fig. S1D). Defects in the tumor suppressor gene ARID1A cause ATRi sensitivity, both in vitro and in vivo (32). We found SS tumor cell lines to be as sensitive to VX970 as ARID1A-defective HCT116 cells (HCT116/ARID1A/−; Fig. 2C, ref. 19). Furthermore, when comparing the VX970 sensitivity of SS tumor cell lines to those with other molecular defects associated with ATRi sensitivity, namely, ATM gene defects (33), ARID1A mutations (32) and Ewing sarcoma (EWS) associated EWS–FLI fusions (34), the SS tumor cell lines showed a similar extent of VX970 sensitivity to EWS tumor cells and ARID1A-defective tumor cells (Fig. 2D; Supplementary Fig. S1E). This consistent in vitro sensitivity of SS tumor cell lines to ATRi was also observed with other ATR inhibitors, including the clinical ATRi AZD6738 (AstraZeneca; ref. 35) and the toolbox inhibitors AZ20 (ref. 35; AstraZeneca) and VE821 (Vertex Pharmaceuticals; Supplementary Fig. S1F–S1H; ref. 36), suggesting that these effects were not specific to VX970 and represented a drug class effect. We next assessed whether the clinical ATRi VX970 could inhibit SS tumor growth in vivo. Treatment of mice bearing established patient-derived SS xenografts (PDX SA13412; SS18–SSX1 translocation-positive) with VX970 caused a significant inhibition of tumor growth (ANOVA \( P < 0.0001 \), Fig. 2E) and extended the survival of tumor-bearing mice (log-rank test \( P = 0.0451 \), Fig. 2F).

SS18–SSX1 or SS18–SSX2 fusion proteins induce ATRi sensitivity and reduce SMARCB1 and SS18 protein levels

To establish a causative link between the expression of oncogenic SS18–SSX fusions and ATRi sensitivity, we ectopically expressed SS18–SSX1 or SS18–SSX2 cDNAs in cells and assessed ATRi sensitivity. Many cell lines, including HFF1 fibroblasts, were unable to maintain long-term survival in the face of either SS18–SSX1 or SS18–SSX2 cDNA expression, precluding the use of these models in drug-sensitivity assays where prolonged cell culture is required. However, we found that ATRi-resistant HCT116 cells were able to tolerate lentiviral expression of SS18–SSX1 or SS18–SSX2 cDNA (Supplementary Fig. S2A–S2C). Furthermore, expression of SS18–SSX1 or SS18–SSX2 in HCT116 cells recapitulated three features previously associated with SS fusion gene expression, namely: (i) a modest reduction in endogenous SS18 expression (7); (ii) a reduction in levels of the SWI/SNF component SMARCB1 (7); and (iii) upregulation of the canonical Wnt pathway target gene AXIN2 (Fig. 2G and H), effects replicated in short-term U2OS (osteosarcoma) and HFF1 (fibroblast) cell cultures (Supplementary Fig. S2C–S2E). Amino acid residues at the C-terminus of the SS18–SSX1 fusion protein are critical for the displacement of SMARCB1 from SWI/SNF complexes (7). In comparison with the expression of full-length SS18–SSX1 or SS18–SSX2 fusion proteins, expression of an SS18–SSX1 variant with the final eight residues of SSX1 deleted (Δ71–78) did not cause a reduction in SMARCB1 levels, nor an increase in AXIN2 mRNA (Fig. 2G and H). Having established that SS18–SSX fusion expression could recapitulate some of the molecular features associated with SS fusion gene expression, we assessed the effects of these fusions upon ATRi sensitivity. We found both SS18–SSX1 and SS18–SSX2 cDNAs caused a significant (ANOVA \( P < 0.0001 \)) enhancement in sensitivity to a series of distinct ATRi inhibitors, whereas the expression of the SS18–SSX1 variant with the final eight residues of SSX1 deleted (Δ71–78) mutant isoform did not (Fig. 2I–K), establishing a causative link between the expression of oncogenic fusion genes and ATRi sensitivity. Furthermore, siRNA-mediated gene silencing of SMARCB1 in HCT116 cells also caused VX970...
sensitivity (ANOVA, P < 0.001; Supplementary Fig. S2F and S2G), suggesting that the SWI/SNF defect caused by SS fusion genes could, in principle, be responsible for ATR inhibitor sensitivity.

As defects in DDR pathways, such as ATM and ATR activation, have been associated with increased ATRi sensitivity (33, 37), we assessed whether defects in ATM/ATR activation following DNA damage in SS tumor cell lines could explain the ATRi sensitivity. However, we did not detect a profound dysfunction in the ability of SS tumor cells to elicit ATR signaling in response to either cisplatin or hydroxyurea, or ATM signaling in response to ionizing radiation (IR; Supplementary Fig. S3A–S3C). Furthermore, we found that SS tumor cells generated nuclear RAD51 foci in response to ATRi, hydroxyurea, or IR (Supplementary Fig. S3D), suggesting that a defect in RAD51-mediated DNA repair processes might not explain ATRi sensitivity. ATRi sensitivity has also been associated with a reduction in chromatin bound topoisomerase levels (32). Again, we did not observe a clear defect in chromatin-TOP2A levels that could explain the profound ATRi sensitivity (Supplementary Fig. S3E–S3J).

ATR causes apoptosis and replication fork stress in SS tumor cells
In addition to ATR, our siRNA screens suggested that SS tumor cells were also reliant upon additional proteins involved in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O). As well as in the replication fork stress response, including RAD9A and RAD18 (Fig. 1N and O).

ATR sensitivity in SS tumor cells is cyclin E dependent
To gain more insight into the processes involved in ATRi sensitivity, we took a relatively unbiased approach using an siRNA ‘resistance’ screen to identify genes that could reverse the ATRi-sensitivity phenotype in an SS tumor cell line. SYO-1 cells were reverse-transfected in a 384-well plate format with the siRNA library described earlier, and 24 hours later exposed to either a relatively high concentration of VX970 (0.75 μmol/L) or vehicle (DMSO) for four continuous days (Fig. 4A). Results of three highly correlated replica screens were combined in the final analysis. By comparing siRNA effects in VX970-exposed versus

Figure 3.
ATR inhibition causes apoptosis and replication fork stress in SS tumor cells. A, Bar chart illustrating a dose-dependent increase in Caspase Glo activity in SYO-1 cells exposed to VX970 for 48 hours. Caspase Glo luminescence was normalized to cell viability as determined by CellTiter-Glo and calculated as fold change compared with DMSO-exposed cells. P values represent Student’s t test. Error bars, SD. B, Western blot illustrating accumulation of cleaved PARP1 and γH2AX upon VX970 exposure in SYO-1 SS cells. C, Western blot illustrating accumulation of γH2AX after exposure of HS-SY-II cells to 500 nmol/L VX970. Two independently derived lysates were generated at each time point as shown. D, Representative confocal microscopy images illustrating elevated 53BP1 (green) and γH2AX (red) foci/pam nuclear staining in SYO-1 cells following exposure to 500 nmol/L VX970 (8 hours) or 2 mmol/L hydroxyurea (HU; positive control), compared with DMSO-exposed controls. E, Bar chart showing percentage of SYO-1 cells with elevated γH2AX levels following exposure to 500 nmol/L VX970. h, hours of exposure. One hundred images were captured and scored for γH2AX foci or pan-nuclear γH2AX staining. Hydroxyurea exposure was used as a positive control. All P values were calculated using Student’s t tests. Error bars, SD from triplicate experiments. F, Replication fork rates as measured from DNA fiber monitoring in SYO-1 cells exposed to VX970 for 48 hours. Error bars, SD from triplicate experiments. G, Cell-cycle profiles for EdU incorporation in SYO-1 cells exposed to DMSO or 500 nmol/L VX970 for 2 hours. At least 100 tracks were measured for each condition. Error bars, SD. H, Box and whisker plot of R-loop (S9.6 nuclear staining) intensity in HCT116 cells expressing ectopic SSX1 fusion protein in IHC116 cells. Numbers indicate fraction of cells present in the different cell-cycle phases after 16, 24, and 48 hours VX970 exposure.
DMSO-exposed cells we identified those siRNAs that caused VX970 resistance (see Materials and Methods), quantifying resistance-causing effects as drug effect (DE) Z-scores (Supplementary Table S5; Fig. 4B). The most profound ATRi resistance-causing effect identified in this screen was caused by siRNA designed to target the cyclin E encoding gene CCNE1 (Fig. 4B; DE Z-score 7.7). Multiple independent CCNE1 siRNAs derived from the SMARTpool reduced cyclin E protein expression (Fig. 4C) and caused a statistically significant reduction in ATRi sensitivity in subsequent dose–response survival assays in both SYO-1 and HS-SY-II SS cells (Fig. 4D and E; ANOVA P < 0.0001). We found that the CCNE1 siRNA SMARTpool used in the siRNA screen also reduced the extent of VX970-induced apoptosis in SYO-1 cells (Fig. 4F). As cyclin E has been implicated in enhancing replication fork stress (40, 41), we next tested the ability of CCNE1 siRNA to reverse the γH2AX response to VX970 in SYO-1 and HS-SY-II SS cells.
Figure 5.
Identification of candidate drug combinations with ATR inhibitors in SS. A and B, Heat maps summarizing the results from high-throughput chemosensitization screens using VX970 in SYO-1 (A) and HS-SY-II (B) cells. Drug sensitization effects are shown as drug effect (DE) Z-scores for each concentration of the top 10 chemosensitizing molecules out of 79 library small molecules used in combination with VX970. Concentration (nmol/L) denotes the concentration of the library small molecules used in combination with VX970. Library small molecules are rank ordered top to bottom according to average VX970 drug effect Z-scores; library small molecules causing VX970 chemosensitization are ranked at the top of the heat map.
C and D, Dose–response curves in Yamato-SS cells exposed to escalating concentrations of VX970 combined with the PARP inhibitors olaparib (C) or talazoparib (BMN673; D) for 5 days. Error bars, SD. P values were calculated with a two-way ANOVA compared with cells exposed to VX970 alone.
E and F, MacSynergy plots for SYO-1 (E) and HS-SY-II (F) SS cells exposed to escalating concentrations of VX970 combined with cisplatin. The 3D synergy plots represent synergy volumes in μmol/L²; volumes > 120 μmol/L² were considered as synergistic (details in Materials and Methods) and are shown in bold. G and H, Dose–response curves for SYO-1 (G) and HS-SY-II (H) cells exposed to escalating concentrations of VX970 combined with cisplatin. Error bars, SD. P values were calculated with a two-way ANOVA compared with cells exposed to VX970 alone.
I, Kaplan–Meier survival curves for tumor-size-related survival (defined by a tumor reaching the tumor size limit of 15 mm in any one direction) demonstrating a significant delay in time to reach the tumor size limit in the combined treatment group as compared with controls. P value calculated by log-rank test.

ATR in Synovial Sarcoma
to ATRi exposure. We found that CCNE1 siRNA reduced both γH2AX and RPA phosphorylation in response to ATRi exposure (Fig. 4G), an observation also reproduced by immunofluorescent detection of γH2AX (Fig. 4H). However, cyclin E protein expression itself was not regulated by ectopic expression of SS18–SSX1, SS18–SSX2, or SS18–SSX1 A71–78 fusions (Supplementary Fig. S4E). Several likely explanations might account for the role of cyclin E in these phenotypes; the presence of an SS fusion gene might create a genomic context (e.g., a chromatin remodeling event) that allows cyclin E to mediate replication fork stress. Alternatively, the reduction in cyclin E expression in SS tumor cells might independently reduce or delay entry into S phase, thus minimizing the effects of an ATR inhibitor that would otherwise mediate its cytotoxic effects by targeting cells undergoing DNA replication. Indeed, we found that CCNE1 siRNA reduced the fraction of SS tumor cells in S phase (Supplementary Fig. S4F and S4G), although such an observation might not completely discount the possibility that cyclin E in S phase might also play a role in ATRi sensitivity in SS cells.

Identification of candidate drug combinations with ATR inhibitors in SS

To improve the likelihood of a significant and sustained clinical response to ATR-targeted therapy, we also assessed the effects of combination therapy approaches with ATRi in SS tumor cells. To do this, we used high-throughput chemosensitization screens in SYO-1 and HS-SY-II cells to identify ATRi combinatorial effects. Here, we used a bespoke drug library encompassing 79 different small molecules, which are either already used in cancer treatment or are in late-stage development (see Materials and Methods). To maximize the possibility of identifying drug combination effects, each small molecule in the library was used in eight concentrations (see Materials and Methods). From this screen, we identified several drugs that sensitized both SYO-1 and HS-SY-II cells to VX970. These included the three clinical PARPi inhibitors rucaparib, olaparib, and talazoparib (BMN673), the WEE1 kinase inhibitor MK1775, the CHK1 kinase inhibitor SAR20106, the Topoisomerase inhibitor camptothecin, and the replication inhibitor sapacitabine (Fig. 5A and B), all agents that either cause or enhance replication fork stress. In validation experiments in a third SS tumor cell line, Yamato-SS, we found that the addition of either olaparib or talazoparib enhanced the effects of VX970 (Fig. 5C and D). Synergistic effects of combined ATR and PARP inhibition have been reported in other tumor types as well (42–44).

We also assessed known synergistic combinations with ATRi, as well as assessing the effects of combining ATRi with standard-of-care agents used in SS. For example, in non-SS tumor cells, the combination of VX970 with platinum salts has been reported as synergistic (33); we found that the combination of VX970 and cisplatin generated a synergistic effect on cell inhibition in both SYO-1 and HS-SY-II cells, generating synergy volumes of 465 μmol/L² and 334 μmol/L², respectively (Fig. 5E–H). This combinatorial effect also elicited a survival benefit in mice bearing HS-SY-II SS tumor xenografts (P = 0.0424, log-rank test, Fig. 5I). Finally, we also assessed combinations of VX970 when used with cytotoxic agents used in the treatment of SS (doxorubicin and the alkylating chemotherapeutic cyclophosphamide) as well as the targeted drug used in SS treatment pazopanib (Supplementary Fig. S5A–S5F; ref. 2).

We observed largely nonsynergistic effects in each of these cases, although in HS-SY-II cells, the combination of VX970 plus the active derivate of cyclophosphamide (4-HC) caused a mild synergistic effect (Supplementary Fig. S5B and S5E).

Discussion

In this study, high-throughput siRNA screening of SS cell lines identified a selective and novel dependency upon the DDR-related kinase ATR. Validation studies confirmed a synthetic lethal interaction between SS18–SSX fusion genes and ATR and established that ATRi might be used to exploit this effect.

The sensitivity of SS to DDR-targeting drugs was rather unexpected. To date, much of the focus on identifying tumor subtypes that might respond to targeted DDR targets has centered upon tumor subtypes such as high-grade serous ovarian cancers, where homologous recombination (HR) defects with expected genomic rearrangements exist (31). Although genomic instability has been reported in SS, this is generally restricted to adult patients and those with advanced disease; the majority of patients with SS have tumors that do not exhibit mutations or chromosomal alterations other than a pathognomonic SS18–SSX translocation (6, 45, 46). Interestingly, ATRi sensitivity has also been recently reported in models of EWS, a sarcoma of adolescent and young adult (AYA) age that is generally characterized by a single oncogenic fusion driver event (EWS–FLI translocations ref. 34). In our in vitro assays, SS cells appeared to have comparable ATRi sensitivity to EWS–FLI1-positive EWS tumor cells. This might suggest that assessing ATRi inhibitor sensitivity in additional AYA and/or other translocation-associated sarcoma subtypes might also identify similar vulnerabilities.

At the mechanistic level, our data suggest that the expression of SS18–SSX fusion genes generates a relatively moderate form of replication fork stress that is enhanced by ATR inhibition (e.g., Fig. 3F and G). Our analysis of ATRi sensitivity in SS tumor cells suggests that this phenotype is dependent on a known mediator of replication fork stress, cyclin E (Fig. 4; refs. 40, 41) but is not necessarily caused by an overt increase in cyclin E expression, profound defects in either ATM, ATR, or chromatin bound topoisomerase levels or a defect in RAD51 localization to the site of DNA damage (Supplementary Fig. S3). Another possibility might be that the expression of SS fusion genes and the subsequent effects on SWI/SNF function cause subtle yet critical differences in the chromatin structure of the genome that induces an increased impairment of replication fork progression and thus an enhanced reliance upon ATR (and sensitivity to ATRi); this model would be a form of induced essentiality (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.E. Jones, E.D.G. Fleuren, J. Shipley, A. Ashworth, C.J. Lord

Development of methodology: S.E. Jones, D.B. Krastev, C.J. Lord

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Jones, E.D.G. Fleuren, J. Frankum, A. Konde, C.T. Williamson, H.N. Pemberton, R. Elliott, M. Menon, R. Brough, W. Niedzwiedz, C.J. Lord


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References


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