ATR Is a Therapeutic Target in Synovial Sarcoma

Samuel E. Jones1,2,3, Emmy D.G. Fleuren1,2,4, Jessica Frankum1,2, Asha Konde1,2, Chris T. Williamson1,2, Dragomir B. Krastev1,2, Helen N. Pemberton1,2, James Campbell1,2, Aditi Gulati1,2, Richard Elliott1,2, Malini Menon1,2, Joanna L. Selfe5, Rachel Brough5,2, Stephen J. Pettitt1,2, Wojciech Niedzwiedz5, Winette T.A. van der Graaf6, Janet Shipley5, Alan Ashworth1,2, and Christopher J. Lord1,2

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 SSX18–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.

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 family fusion 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 oncoproteins 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 on 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 Aska-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-mutant isogenic cell lines have been described previously (19); all other cell lines were 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-mutant isogenic cell lines have been described previously (19); all other cell lines were

Cell culture
Yamato-SS and Aska-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-mutant isogenic cell lines have been described previously (19); all other cell lines were 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.

Materials and Methods
Cell culture
Yamato-SS and Aska-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-mutant isogenic cell lines have been described previously (19); all other cell lines were 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.
Edird 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 mmol/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.

Immunofluorescent imaging
Cells were plated onto poly-L-lysine Cellware 12-mm round microscope cover slips (Corning) in 6-well plates and allowed to adhere overnight. After drug exposure, cells were fixed in 4% (v/v) PFA (Sigma) and permeabilized with 0.2% (v/v) Triton X-100 (Sigma). Cells were then immunostained with antibodies targeting: γH2AX (Millipore, 05-636); 53BP1 (Novus, NB100-304); RAD51 (Abcam, AB13534); RNA/DNA hybrids (S9.6 ENH001, Kerafast); Nucleolin (Abcam, ab50279); or stained with DAPI. Cells were then fluorescent labeled with secondary antibodies: goat anti-mouse IgG (ThermoFisher Scientific, Alexa Fluor 555, A-21424) or goat anti-rabbit IgG (ThermoFisher Scientific Alexa Fluor Plus 488, A32731) for 60 minutes at room temperature before mounting on slides. Slides were imaged at 63 × on a Leica TCS SP2 confocal microscope. Cells were scored positive for γH2AX if greater than five foci could be counted within the DAPI-stained nucleus. Cells with pan-nuclear γH2AX staining were scored separately from those with γH2AX foci. Approximately 120 cells were scored for γH2AX for each condition. For quantification of nuclear S9.6 intensity, 30 individual cells were scored per condition, and ImageJ was used to generate nuclear masks based on DAPI staining. Nuclear S9.6 fluorescence intensity was then determined by subtracting the nucleolin signal and analyzing the intensity of the remaining S9.6 signal.

DNA fiber analysis
DNA fiber assays were carried out as described previously (24).

In vivo studies
In vivo efficacy studies were performed using SA13412 patient–derived SS xenografts at Crown Biosciences or using HS-SY-II tumor cells grown subcutaneously in the flank of female NOD SCID mice or Balb/C nude mice at the ICR, London, respectively. When tumors were established (~80 mm³), mice were randomized into treatment and vehicle groups. Animals bearing SA13412 PDXs were treated with either vehicle alone or VX970 (60 mg/kg; oral 4 consecutive days/week; n = 10 per group). Animals bearing HS-SY-II tumors were treated with vehicle, VX970 monotherapy (60 mg/kg; oral 4 consecutive days/week), cisplatin monotherapy (3 mg/kg; intraperitoneally once weekly), or the combination (n = 6–7 per group). Mice were treated until study end (6–12 weeks) or when the maximum tumor size was reached (defined as tumor size >15 mm in any direction for HS-SY-II experiments; or tumor volume >2000 mm³ for SA13412 PDX experiments). The SA13412 PDX study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH and the HS-SY-II experiment in accordance with ARRIVE guidelines, regulations set out in the UK Animals (Scientific Procedures) Act 1986, and in line with a UK Home Office–approved project license held by CIL and approved by the ICR ethics board.

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 (SS16-SSX1 or SS18-SSX2), and were amenable to high-efficiency siRNA transfection in a 384-well plate format (Fig. 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 druggable 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_{0,0} (concentration required to produce 50% reduction in cell survival) values of ≈0.1 μmol/L (Fig. 2B). SS tumor cell lines were also more sensitive to ATRi compared with HFF1 fibroblasts and nonmalignant 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 ATRi inhibitors, including the clinical ATRi AZD6738 (AstraZeneca; ref. 35) and the toolbox inhibitor 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 SSX18 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 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 ATRi 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).

### ATRi 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 tumor cells were also reliant upon additional proteins involved in transcriptional machineries, often associated with R-loops, DNA–RNA hybrid structures that activate ATR-mediated DDRs (38). We reasoned that an enhanced transcriptional program caused by SS fusion expression might cause R-loops. R-loop frequency can be estimated by the immunohistochemical detection of DNA–RNA hybrid structures (38). We found that the exposure of SYO-1 cells to VX970 enhanced the R-loop IHC signal (P < 0.001, Student’s t test; Fig. 3H). The expression of the SS18–SSX1 fusion in HCT116 cells also caused a modest but significant increase in R-loop IHC signal (P < 0.001, Student t test), which was enhanced by the addition of VX970 (P < 0.001, Student t test, Fig. 3I).

As previous studies have demonstrated that ATRi prevents normal replication of DNA under oncogene-induced replication fork stress (39), we performed FACS cell-cycle analysis to determine whether a similar effect operated in SS cells. SYO-1 cells were pulse-labeled with EdUrd, which is incorporated into DNA during active DNA synthesis. Cells exposed to VX970 exhibited a profound reduction in the fraction of cells in active S-phase (from 30% to 8% after 48 hours VX970 exposure; Fig. 3J and Supplementary Fig. S4D), suggesting that ATRi exposure in SS tumor cells impaired DNA replication, consistent with our previous observations.

### ATRi 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/pan nuclear staining in SYO-1 cells following VX970 exposure (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. F, Percentage of SYO-1 cells with elevated 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. At least 100 tracks were measured for each condition. Average fork speed SYO-1 exposed to DMSO = 0.51 kb/minute; average fork speed SYO-1 exposed to VX970 = 0.3 kb/minute. Error bars, SD. P value represents paired t test. G, Replication fork rates as measured from DNA fiber monitoring in HCT116 cells expressing ectopic SS18–SSX1 cDNA (fusion) exposed to DMSO or 500 nmol/L VX970 for 2 hours. At least 100 tracks were measured for each condition. Error bars, SD. P values represent paired t test. H, Box and whisker plot of R-loop (S9.6 nuclear staining) intensity in SYO-1 cells expressing ectopic SS18–SSX1 cDNA (fusion) exposed to DMSO or 500 nmol/L VX970. h, hours of exposure. One hundred images were captured and scored for >5 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.
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.
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. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. E and F, MacSynergy plots for SYO-1 (E) and HS-SY-II (F) 5S 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. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. 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.
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 chemosensitivity 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 and/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 values 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 xenographs (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–FLI1 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).
Writing, review, and/or revision of the manuscript: S.E. Jones, E.D.G. Fleuren, C.T. Williamson, J.L. Selle, S.J. Petitt, W.T.A. van der Graaf, J. Shipley, A. Ashworth, C.J. Lord

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.D.G. Fleuren, A. Konde, C.J. Lord

Study supervision: D.B. Krastev, J.L. Selle, J. Shipley, A. Ashworth, C.J. Lord

Acknowledgments
We thank John Polland and Philip Reaper (Vertex) for providing ATR inhibitors; Akira Kawai (National Cancer Center Hospital, Tokyo, Japan; SYO-1), Kazuyuki Itoh, and Noritumi Naka (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan; Yamato-SS and Aika-SS), and Cinzia Lanzi (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; CME-1) for kindly providing the SS cell lines. We also thank Cigal Kadoch (Dana Farber/Harvard Cancer Center, Boston, MA) for useful discussions.

References

Grant Support
This work was funded by a Cancer Research UK Programme Grant (grant number C347/A8363) to C.J. Lord. S.E. Jones was supported by a Wellcome Trust Studentship. E.D.G. Fleuren is supported by a Rubicon Fellowship (NWO 019.153LW.035). We acknowledge NIHR funding to the Royal Marsden Bio-medical Research Centre.

The costs of publication of this article were delayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 11, 2017; revised August 21, 2017; accepted October 10, 2017; published OnlineFirst October 16, 2017.
ATR Is a Therapeutic Target in Synovial Sarcoma

Samuel E. Jones, Emmy D.G. Fleuren, Jessica Frankum, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-17-2056

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2017/10/14/0008-5472.CAN-17-2056.DC1

Cited articles
This article cites 46 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/77/24/7014.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/77/24/7014.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/77/24/7014.
Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.