An Integrated Chemical Biology Approach Identifies Specific Vulnerability of Ewing's Sarcoma to Combined Inhibition of Aurora Kinases A and B

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Abstract

Ewing's sarcoma is a pediatric cancer of the bone that is characterized by the expression of the chimeric transcription factor EWS-FLI1 that confers a highly malignant phenotype and results from the chromosomal translocation t(11;22)(q24;q12). Poor overall survival and pronounced long-term side effects associated with traditional chemotherapy necessitate the development of novel, targeted, therapeutic strategies. We therefore conducted a focused viability screen with 200 small molecule kinase inhibitors in 2 different Ewing’s sarcoma cell lines. This resulted in the identification of several potential molecular intervention points. Most notably, tozasertib (VX-680, MK-0457) displayed unique nanomolar efficacy, which extended to other cell lines, but was specific for Ewing's sarcoma. Furthermore, tozasertib showed strong synergies with the chemotherapeutic drugs etoposide and doxorubicin, the current standard agents for Ewing’s sarcoma. To identify the relevant targets underlying the specific vulnerability toward tozasertib, we determined its cellular target profile by chemical proteomics. We identified 20 known and unknown serine/threonine and tyrosine protein kinase targets. Additional target deconvolution and functional validation by RNAi showed simultaneous inhibition of Aurora kinases A and B to be responsible for the observed tozasertib sensitivity, thereby revealing a new mechanism for targeting Ewing’s sarcoma. We further corroborated our cellular observations with xenograft mouse models. In summary, the multilayered chemical biology approach presented here identified a specific vulnerability of Ewing’s sarcoma to concomitant inhibition of Aurora kinases A and B by tozasertib and danusertib, which has the potential to become a new therapeutic option.

Introduction

Ewing’s sarcoma is the second most frequent bone cancer occurring in children and young adults (1). Even though many patients initially respond well to chemotherapy, 40% of these usually develop lethal recurrent disease, and 75% to 80% of patients with metastatic Ewing's sarcoma die within 5 years despite aggressive combinations of chemotherapy, radiation, and surgery (2, 3). In fact, metastasis is one of the most critical problems associated with Ewing’s sarcoma, as approximately 15% to 20% of patients have overt metastasis upon diagnosis, and a yet undefined percentage of patients is believed to carry micrometastasis (4).

Thus, there is a profound need for novel therapies. Particularly desirable would be targeted therapies, which lack many of the acute and long-term side effects associated with classical chemotherapy, such as developmental impairment and elevated risk of cancer. At the molecular level, Ewing’s sarcoma is defined by a chromosomal translocation, such as t(11;22)(q24;q12), which accounts for approximately 85% of all cases and which results in the translation of the aberrant gene product EWS-FLI1 (5). The causal role of EWS-FLI1 in the pathogenesis of Ewing’s sarcoma results from the cooperativity of both fusion partners. Whereas EWS contributes a strong transcriptional activation domain, FLI1 features an ETS-type DNA-binding domain (5–7). Functionally, EWS-FLI1 acts as an aberrant transcription factor capable of deregulating more than 1,000 direct and indirect target genes (8). EWS-FLI1 has been also described to act as a...
transcriptional repressor (9). This specific oncogenic lesion is responsible for the highly malignant phenotype and poor prognosis typical of Ewing’s sarcoma. Despite the molecular understanding of the underlying pathology of the disease, development of targeted therapies has proven difficult. Promising steps have been made either by targeting the protein–protein interaction interface of EWS-FLI1 with its complex partner RNA helicase A or by inhibition of IGFR, CD99, or MGST1 (10–13). However, so far none of these approaches has received clinical approval.

Protein kinases have received significant attention over the past decade because many of these play important roles, for example, in cancer and can be readily engaged by small molecules. Being safe and effective against many molecularly defined malignancies, such as chronic myelogenous leukemia (CML; ref. 14), ErbB2-driven breast (15), or epidermal growth factor receptor–dependent lung cancer (16), kinase inhibitors are currently the most successful class of targeted drugs. Notably, some kinase inhibitors have also been combined with traditional chemotherapeutic agents for the treatment of particularly aggressive cancers such as Philadelphia chromosome–positive acute lymphoblastic leukemia (17). Therefore, combination of targeted therapy and chemotherapy could be a viable therapeutic option in Ewing’s sarcoma.

Because Ewing’s sarcoma features the defined molecular lesion EWS-FLI1, an attractive option is the investigation of possible synthetic lethal relationships (18). Globally, this concept has been exploited through genetic screens in yeast and RNAi screens in human cells (19, 20). Considering proven druggability, particularly kinome-wide RNAi screens are widely pursued (21). Alternatively, the kinome can be perturbed with small molecules. Both approaches display distinct advantages and a certain level of complementarity. However, there is a general difference in removing a protein by RNAi or inhibiting it by a small molecule. Although genomic approaches are not limited by the druggable chemical space, dose dependencies are easier to investigate using small molecules. Furthermore, kinase inhibitors are notoriously promiscuous (22), which offers the advantage of modulating multiple nodes at the same time and therefore provides a higher chance of uncovering complex mechanistic relationships. However, dissecting these relationships requires suitable downstream target identification and deconvolution approaches, such as chemical proteomics (23). Chemical proteomics is a postgenomic version of classical drug affinity chromatography enabled by high-resolution tandem mass spectrometry and downstream bioinformatics analysis, which can identify the cellular target spectra of screening hits (24–26).

Here, we apply such a multilayered approach that combines a chemical biology viability screen with a focused kinase inhibitor library and chemical proteomics–based target identification. Subsequent target deconvolution by RNAi identified parallel inhibition of Aurora kinases as a specific vulnerability of Ewing’s sarcoma.

Materials and Methods

Cell culture and reagents

SK-ES-1, SK-N-MC, TC-71, A673, STA-7.2, RD, Rh30, U2OS, K562, and KU812 were obtained from the American Type Culture Collection; ASP14 was a gift of Javier Alonso (Instituto de Investigaciones Biomédicas). Nilotinib, dasatinib, bosutinib, and tozasertib were purchased from LC Laboratories; lapatinib, etoposide, doxorubicin, erlotinib, sorafenib, and sunitinib were purchased from Selleck Chemicals, and bafetinib was synthesized by WuXi AppTec. Furthermore, the customized kinase inhibitor library consisted of sublibraries derived from Tocris, Calbiochem, and Merck. All compounds were dissolved in dimethyl sulfoxide (DMSO) as 10 mmol/L stock solutions.

Western blotting

Western blotting was carried out as described in the manufacturer’s manual for antibodies against Aurora kinases A (#3092; Cell Signaling) and B (611038; BD Transduction Laboratories) and actin (#AAN01; Cytoskeleton). Aurora A p-T288 (#3079; Cell Signaling) and pH3S10 (#05-806; Millipore) were detected after 12 hours nocodazole arrest. Drug treatment was carried out for 2 hours in the presence of 20 μmol/L MG-132.

Viability screen

Cells were plated at 40,000 cells/mL (RPMI 1640; 10% fetal calf serum). Drugs were added after 24 hours and incubated for 72 hours. Viability was measured by using the CellTiter-Glo Assay (Promega). Half-maximal effective concentration (EC50) values were calculated by using Spotfire (TIBCO; duplicate analysis). All other viability measurements have been conducted with CellTiter-Glo in triplicate.

Chemical proteomics

Chemical proteomics experiments have been carried out as described previously (27).

Apoptosis and cell-cycle measurements

Cleaved caspase-3 was quantified by intracellular protein analysis, using flow cytometry. A total of 1 × 10⁶ cells were fixed by using paraformaldehyde, washed with PBS, and stored overnight at −20°C in methanol. Antibodies against cleaved caspase-3 (Cell Signaling; #9661) and PE-conjugated goat anti-mouse (Southern Biotech; #4030_09) have been used. Cell-cycle analysis was carried out by staining DNA with propidium iodide after 36 hours exposure to tozasertib.

Synergy determination

Thirty-six-point dose–response matrices have been established as described elsewhere (28).

Knockdown assays

Knockdowns of Aurora kinases have been carried out by ON-TARGETplus Dharmacon SMARTpools in
triplicates (10 nmol/L, 24-well plate). Sequences of EWS-FLI1 RT primers were used for evaluation of knockdown efficiency in inducible ASP14 cell line upon request.

**In vivo studies**
A total of 2 × 10⁶ TC-71 cells were injected into the gastrocnemius muscle of 5- to 7-week-old SCID/bg mice (Charles River). Single primary tumors developed in more than 90% of mice over 2 weeks. Mice were randomized into 3 groups with 11 animals each and intraperitoneally injected with tozasertib (50 or 100 mg/kg) or vehicle twice daily for 6 days. Tozasertib was first dissolved in DMSO at 100 mg/mL and then further diluted in 50% PEG 400 in 50 mmol/L phosphate buffer. Two diameters of the tumor sphere were measured every 2 days. Tumor volumes were approximated by using the formula: \( V = (D \times d)^2 \times \pi \), where \( D \) is the longer and \( d \) is the shorter diameter. When tumors reached 2,000 mm³, experiments were terminated. Mice studies were approved by the state regulatory board.

**Statistical considerations**
Two-way ANOVA test for the log of tumor growth ratio was used for statistical analysis of the pairwise differences between in vivo treatment groups. Tumor growth ratio was the volume of the tumor observed at a given day versus day 1. \( p \) values less than 0.05 were regarded as significant. For synergy determination, Bliss additivity was used to predict the combined response \( C \) for 2 single agents with their effects \( A \) and \( B \) (\( C = A + B - A \times B \)), where each effect is expressed as fractional inhibition between 0 and 1.

**Results**

**Chemical biology screen reveals sensitivity of Ewing’s sarcoma cell lines to several small molecule protein kinase inhibitors**
To elucidate vulnerabilities of Ewing’s sarcoma, we carried out a focused screen by probing 2 different Ewing’s sarcoma cell lines (SK-ES-1 and SK-N-MC) with a library of 200 small molecule protein kinase inhibitors. Each compound was tested at 5 different concentrations ranging from 16 nmol/L to 10 μmol/L. Although the majority of compounds showed no or at best moderate effects, a subset of 16 (SK-ES-1) and 20 compounds (SK-N-MC), respectively, showed significant impairment of cellular viability, as indicated by EC₅₀ values less than 1 μmol/L (Fig. 1; Supplementary Table S1). Among these were staurosporine and several of its derivatives such as midostaurin (N-benzoyl-staurosporine) or UCN-01 (7-hydroxyxystaurosporine). Cumulatively, the known cognate targets of the identified screening hits, such as cyclin-dependent kinases (CDK), casein kinases (CK), and Aurora kinases, are predominantly implicated in cell cycle control. Tozasertib (VX-680, MK-0457) was prominently represented in both cell lines, as it was the most efficacious compound with an EC₅₀ of 20 nmol/L in SK-N-MC and the second most efficacious compound with an EC₅₀ of 30 nmol/L in SK-ES-1. Tozasertib and also danusertib (PHA-739358), another potent screening hit with EC₅₀ values of 47.6 nmol/L (SK-M-MC) and 25.5 nmol/L (SK-ES-1), have initially been developed as Aurora kinase inhibitors but are known to target many other serine/threonine and tyrosine kinases as well (22, 29, 30). For instance, both compounds have been shown to potently inhibit ABL, its CML-relevant counterpart BCR-ABL, and some of the clinically relevant mutants, such as the gatekeeper mutant T315I, and are or have been in clinical trials for this indication (31, 32).

**Effects of tozasertib and danusertib are specific for Ewing’s sarcoma cells**
To extend the initial observation made in SK-ES-1 and SK-N-MC, we determined individual drug effects of tozasertib and danusertib on cellular viability across a broader range of concentrations and Ewing’s sarcoma cell lines (A673, TC-71, STA-ET-7.2). The derived EC₅₀ values for these cell lines are consistent with previous observations, displaying a window of activity between 8.8 and 55 nmol/L for tozasertib and 22 and 37 nmol/L for danusertib (Fig. 2A; Supplementary Table S2). To further investigate the specificity and relative potency of these drugs for Ewing’s sarcoma, we determined their effects on cell lines derived from the pediatric tumors rhabdomyosarcoma (RD, Rh30) and osteosarcoma (U2OS), as well as CML (K562, KU812). This comparison revealed specificity for Ewing’s sarcoma cells, as they displayed EC₅₀ values that are considerably lower than those in the other cell lines (Fig. 2A).

Finally, we compared the efficacy of tozasertib and danusertib in killing Ewing’s sarcoma cell lines with a panel of kinase inhibitors (nilotinib, dasatinib, bosutinib, bafetinib, danusertib, tozasertib, lapatinib, erlotinib, sorafenib, and sunitinib) that are for the largest part already approved by the Food and Drug Administration or in later stages of clinical trials, but, most importantly, have well characterized target spectra across near kinome-wide kinase panels that were established previously (22, 24, 25, 33, 34). In fact, these studies show that collectively these drugs affect approximately 300 kinases, more than 200 of which are significantly inhibited at concentrations that are achievable in patients. We hypothesized that a combined comparison of the target spectra and the cellular efficacy of tozasertib with those well-characterized agents would facilitate subsequent target deconvolution. Tozasertib and danusertib were 60-fold more potent than the next best drug (bosutinib; SKI-606) and more than 100-fold more potent than the remaining drug panel (Fig. 2B). Therefore, we conjectured that the target spectrum of tozasertib must show significant differences to the less effective compounds and that differentially inhibited kinases would be prioritized for validation experiments.
Tozasertib interacts with 20 protein kinases in Ewing’s sarcoma cells

To elucidate the mechanism of action of tozasertib in Ewing’s sarcoma and furthermore compare its target ID with the drug panel mentioned earlier, we pursued a chemical proteomics approach as an initial step of target deconvolution (26). On the basis of the available cocrystal structure information, we designed an analogue of tozasertib (c-tozasertib) which features an N-aminobutyl linker (Fig. 3A). This modification allowed for immobilization on NHS-activated sepharose beads and subsequent affinity purification of interacting proteins without affecting kinase binding properties as confirmed by comparison of tozasertib and c-tozasertib for inhibition of ABL kinase activity (Fig. 3B). We identified a total of 20 and 16 kinases binding to c-tozasertib in lysates of the Ewing’s sarcoma cell line SK-ES-1 and A673, respectively. Besides already known tozasertib targets such as Aurora kinase A, ABL, SRC, and FGFR1 (22), we also identified new potential targets, such as FAK, MAP2K5, MAP3K2, and TBK1 (Fig. 3C; Supplementary Table S3).

Comparison of the tozasertib target spectrum with the deconvolution panel drugs highlighted Aurora kinases A and B as unique targets, because they were approximately 50-fold more potently inhibited by tozasertib (and danusertib) than by any other drug (Supplementary Table S4). Any remaining target was at least as strongly affected by 1 or more other drugs within the panel and therefore was unlikely to account for the observed selectivity. To further validate the interaction between c-tozasertib and the endogenous Aurora kinases expressed in Ewing’s sarcoma cells, we carried out competitive pull-down experiments with SK-ES-1 cell lysates by cotreatment with original tozasertib or DMSO. Interaction of Aurora kinases A and B with c-tozasertib was successfully competed for with tozasertib (Fig. 3D). Taken together, we...
therefore focused on the effects of Aurora kinase inhibition in Ewing’s sarcoma cells.

**Combined inhibition of Aurora kinases A and B underlies tozasertib effect on Ewing’s sarcoma cells**

We asked the question, whether inhibition of Aurora kinases A and/or B represents the relevant mechanism of action of tozasertib in the killing of Ewing’s sarcoma cells. First, we confirmed expression of Aurora kinases A and B for all Ewing’s sarcoma cell lines used in this study via immunoblotting (Supplementary Fig. S1). RNAi knockdown experiments were carried out in SK-ES-1 (and SK-N-MC; Supplementary Fig. S2), as well as in the tozasertib-insensitive rhabdomyosarcoma control cell line RD, using siRNAs against Aurora kinases A and B. We observed similar impairment of viability for both cell lines when knocking down Aurora kinase A, but significantly reduced viability of SK-ES-1 compared with RD upon knockdown of Aurora kinase B (Fig. 4A). Whereas the effect of Aurora kinase B knockdown on viability of SK-ES-1 seems stronger than for Aurora kinase A, simultaneous knockdown of both Aurora kinases resulted in a dramatic reduction of viability in the Ewing’s sarcoma cells, but only a mild effect in the control cell line, which cannot be attributed to knockdown of either kinase alone. This suggests a combined mode of action that may underlie the potent effect of the pan-Aurora kinase inhibitors tozasertib and danusertib.

Furthermore, in SK-ES-1 cells, tozasertib completely abrogated phosphorylation of T288 on Aurora kinase A, which is known to correlate with kinase activity, as well as phosphorylation of the validated Aurora kinase B downstream target histone 3 S10 in the mid-nanomolar range. These results validate Aurora kinases A and B as functional targets of tozasertib in Ewing’s sarcoma cells and corroborate our results from the chemical proteomics binding assay (Fig. 4B).

**Tozasertib treatment induces apoptosis and cell-cycle arrest**

We carried out apoptosis measurements via intracellular fluorescence-activated cell sorting analysis of cleavage of caspase-3 as well as flow cytometric analysis of cell cycle with increasing concentrations of tozasertib. In both cell lines, SK-ES-1 and TC-71, tozasertib induced apoptosis as indicated by increasing levels of cells positive for cleaved caspase-3 (Fig. 4C). SK-ES-1 displays higher sensitivity toward tozasertib treatment than TC-71, thus recapitulating our findings of the viability assay. Moreover, flow cytometric cell-cycle analysis revealed tozasertib-induced arrest in G2–M phase (Supplementary Fig. S3).

**Induced knockdown of EWS-FLI1 causes downregulation of Aurora kinases A and B and confers resistance to tozasertib**

It has been shown that EWS-FLI1 directly upregulates both Aurora kinases (35) and that transient transfection of EWS-FLI1 results in mislocalization of Aurora kinase B followed by mitotic defects (36). We investigated whether
the specific vulnerability of Ewing’s sarcoma for tozasertib and danusertib is a consequence of the functional relationship between EWS-FLI1 and the Aurora kinases. Therefore, we used the A673-derived cell line ASP14 that allows for a doxycycline-inducible knockdown of EWS-FLI1 (37). As expected, we observed downregulation of Aurora kinases A and B at 24 and 48 hours after doxycycline-induced depletion of EWS-FLI1.

Knockdown of EWS-FLI1 has been shown to reduce viability of Ewing’s sarcoma cells. Therefore, we examined whether the time window from doxycycline induction to reduction of both Aurora kinases on the protein level (48 hours) would allow for a subsequent 72-hour drug treatment. We observed a reduction of viability of 56% after 5 days of EWS-FLI1 knockdown, cells were treated either with tozasertib or with etoposide, a topoisomerase II inhibitor, as a control drug. Doxycycline induction severely reduced the relative sensitivity toward tozasertib as compared with the noninduced state (Fig. 4B). This shift was seen only to a much lesser extent with etoposide showing that tozasertib sensitivity of Ewing’s sarcoma cells is dependent on EWS-FLI1 expression (Supplementary Fig. S4).

**Tozasertib synergizes with current chemotherapeutic options**

To further investigate the therapeutic potential of pan-Aurora kinase inhibition in Ewing’s sarcoma, we compared tozasertib with the chemotherapeutic agents, etoposide and doxorubicin, both of which are among the standard treatment options for Ewing’s sarcoma. In both

**Figure 3.** Characterization of the tozasertib target spectrum in Ewing’s sarcoma cells (SK-ES-1, A673) by chemical proteomics. A, chemical structures of tozasertib and the coupleable analogue c-tozasertib. B, c-tozasertib (triangles, dashed line) retains c-ABL inhibitory potential compared with tozasertib (squares, solid line) as shown by in vitro kinase inhibition assays, which have been carried out as described previously (31). C, target profile of tozasertib in SK-ES-1 and A673 cells. Kinase targets identified in both cell lines are displayed in blue, and those only identified in SK-ES-1 in brown. Color intensities correlate with the observed amino acid sequence coverage of the respective targets thereby providing a semiquantitative surrogate parameter for interaction strength. All kinases that bound to c-tozasertib in A673 cells were also found in SK-ES-1 cells. Aurora kinase B is marked with an asterisk as it is being masked by Aurora kinase A peptides. Complete data are provided in the Supplementary Table S3. D, competitive binding of tozasertib at 10 μmol/L with immobilized c-tozasertib for Aurora kinases A and B in SK-ES-1 cell lysates as indicated by immunoblots.
cell lines, SK-ES-1 and A673, tozasertib proved to be the most effective of the 3 agents (Fig. 5A). We were also interested in investigating possible synergistic or antagonistic effects resulting from the combination of pan-Aurora kinase inhibitors with these agents. Therefore, we carried out pairwise drug combination viability assays and constructed 3-dimensional dose–response surfaces delimited by the respective single dose–response curves. These experimentally derived dose–response surfaces were subsequently correlated to predicted values, based on the course of the single dose–response curves, that were generated using the Bliss additivity model (38). Calculating the differential volumes between predicted and measured inhibition allowed estimation of synergy over a broad concentration range and different ratios. This analysis revealed strong synergistic interactions between tozasertib and the 2 chemotherapeutic agents in both cell lines, particularly between tozasertib and etoposide in A673 cells. The largest positive deviation from predicted values and therefore the highest synergy was detected at low nanomolar concentrations of tozasertib and mid-nanomolar concentrations of etoposide, both of which are readily achievable in cancer patients (Fig. 5B; Supplementary Fig. S5; Supplementary Table S5).

Tozasertib causes reduction of tumor growth in a xenograft mouse model

To assess the potential of tozasertib to reduce Ewing’s sarcoma tumor growth in vivo, mouse xenograft experiments were conducted. A total of $2 \times 10^6$ TC-71 Ewing’s sarcoma cells were orthotopically injected in the gastrocnemius muscle of 5- to 7-week-old SCID/bg mice. Mice were examined on a daily basis for tumor formation.
After tumors were palpable, mice were treated twice daily intraperitoneally with vehicle \((n = 11)\), 50 mg/kg \((n = 11)\), or 100 mg/kg \((n = 11)\) tozasertib for 6 days. Subsequently, further tumor growth was monitored until day 11 when first tumors in the vehicle-treated group reached a volume exceeding 2,000 mm\(^3\) initially set as termination criterion. Although treatment was stopped after day 6, a dose-dependent reduction in tumor growth was observed comparing the 100 mg/kg group with the vehicle-treated control group or the 50 mg/kg group that did not show an effect compared with vehicle control (Fig. 6). No toxic deaths occurred.

**Discussion**

We describe an integrated chemical biology approach based on small molecule screening combined with proteomics-assisted drug target identification and validation (23). This strategy allows for the discovery of novel agents with disease-relevant activity and the dissection of their
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Figure 6. Tozasertib causes reduction of tumor growth in vivo. After orthotopic injection of $2 \times 10^6$ Ewing’s sarcoma cells (TC-71) in the gastrocnemius muscle, mice were treated with vehicle control (n = 11), 50 mg/kg (n = 11), or 100 mg/kg (n = 11) tozasertib for 6 days. Furthermore, tumor growth was monitored until day 11. Two-way ANOVA test for the log of tumor growth ratio revealed significant differences between the 100 mg/kg treated group versus the vehicle-treated control group ($P = 9.876e-06$) and versus the 50 mg/kg treated group ($P = 3.591e-07$), whereas no significant difference was found between the control group and the 50 mg/kg treated group.

molecular mechanism of action. In turn, this provides for a better understanding of the underlying disease biology. We have applied this approach to Ewing’s sarcoma, a pediatric bone cancer with high metastatic potential and unfavorable long-term prognosis in need for novel therapeutic options. Considering the significant side effects of high-dose chemotherapy it would be advantageous to implement not just improved but furthermore targeted therapies. Therefore, we have screened a library of 200 kinase inhibitors. Because of the promiscuous nature of drugs in general and kinase inhibitors in particular, this panel covers a wide range of the druggable kinaseome.

Our screen identified a number of kinase inhibitors potently killing Ewing’s sarcoma cell lines. In light of the fact that many EWS-FLI1–upregulated target genes have been linked to cell cycle control and that knockdown of EWS-FLI1 results in growth arrest (8), it is notable that the cognate targets, such as CDKs, CKs, and Aurora kinases, of the majority of our screening hits were implicated in cell cycle regulation. Consistently, the CDK inhibitor roscovitine has been previously described as an effective inducer of apoptosis in Ewing’s sarcoma cell lines (39). However, for CDKs and also for CKs we found a number of validated small molecule inhibitors among the ineffective compounds as well. A similar observation was made with inhibitors targeting phosphatidylinositol-3 kinases and AKT signaling, suggesting the possibility of off-target effects.

Kinase inhibitors are enriched for polypharmacologic features. Potentially, this can translate into initially unexpected side effects as predicted previously (40). However, targeting multiple gene products simultaneously can also be of significant benefit if several of these targets show disease relevance (41). In fact, as redundancy and buffering capacities are inherent features of many biological systems, several phenotypes of interest will only be revealed by such higher-order perturbations (42). These aspects are an advantage of multilayered chemical biology approaches and allowed the discovery that both Aurora kinases A and B contribute to the effect of tozasertib cooperatively. Moreover, additional tozasertib targets such as FAK and SRC were revealed by chemical proteomics and might indicate, due to their established role in promoting metastasis, a potential impact of tozasertib that extends beyond the scope of our initial screen (43).

Tozasertib and danusertib were, apart from staurosporine, the most potent screening hits with low- to mid-nanomolar activity in Ewing’s sarcoma cell lines. Both compounds have been developed as pan-Aurora kinase inhibitors (29, 30). Of the approximately 20 tozasertib targets identified in these cells, Aurora kinases were highlighted by our deconvolution approach as potentially relevant targets. Aurora kinases A and B are serine/threonine kinases that play critical roles in mitosis (44). Among other functions, they are implicated in spindle assembly (Aurora kinase A), regulation of the mitotic checkpoint, and cytokinesis (Aurora kinase B; ref. 45). Aurora kinase A is located at the genomic locus 20q13.2, often found to be amplified in several tumors and is a known oncogene capable of transforming fibroblasts. The genomic locus of Aurora kinase B (17p13.1) is not commonly amplified in human cancers. Nevertheless, increased mRNA and protein levels of Aurora kinase B have been reported in colorectal cancers (46). Consequently, Aurora kinases have gained significant attention as candidate targets in drug discovery, resulting in the development of various small molecule inhibitors that are currently in different stages of clinical trials, such as danusertib (29, 30, 44, 47). In the context of pediatric cancers, Aurora kinase A has been implicated in chemosensitivity of medulloblastoma cells (48). Furthermore, the Aurora kinase A–specific inhibitor MLN8237 has recently been shown to have efficacy in pediatric acute lymphoblastic leukemia and neuroblastoma, but interestingly to somewhat lesser extent also in Ewing’s sarcoma (49). This report is consistent with our observations described here. Thus, knockdown of Aurora kinase A by RNAi reduces viability of SK-ES-1 cells to a minor extent, which is more pronounced over a longer period of time (Supplementary Fig. S2). Furthermore, tozasertib is approximately twice as potent as MLN8237 on a cellular level, which could be attributed to the additional...
inhibitory effect on Aurora kinase B (Fig. 4A and B). However, reducing the levels of a protein by RNAi might not always be comparable with its pharmacologic inhibition. Therefore, an entirely unambiguous evaluation of individual contributions of Aurora kinases A and B to the observed phenotype is not possible.

It is noteworthy that Aurora kinases A and B have been described to be upregulated in Ewing’s sarcoma by EWS-FLI1 (35). Our results furthermore show concomitant knockdown of Aurora kinases A and B in Ewing’s sarcoma cells displays a potentiating effect as compared with single knockdown of either kinase. This effect seems to be specific for Ewing’s sarcoma, as it is not observed in rhabdomyosarcoma cells. As tozasertib and danusertib are inhibiting Aurora kinases A and B with similar potencies (29, 30), this suggests a compound-intrinsic synergy, which might explain the specific vulnerability of Ewing’s sarcoma cells toward these drugs. This is in line with our observation that downregulation of Aurora kinases A and B as a consequence of conditional EWS-FLI1 knockdown confers relative resistance toward tozasertib treatment as compared with an uninduced state or an unrelated control drug. Considering that EWS-FLI1 is the molecular lesion defining Ewing’s sarcoma and would be, if not for lack of chemical tractability, a very attractive drug target, constitutes an interesting higher-order synthetic lethal relationship.

The mouse xenograft studies showed that this specific vulnerability of Ewing’s sarcoma cells translates also into reduced tumor growth rates in vivo. Furthermore, it has been suggested that Aurora kinase inhibition might cooperate with chemotherapeutic drugs that induce DNA damage and cause cell-cycle arrest (44). Consistently, we observed strong synergy of tozasertib with the current standard chemotherapeutic drugs in Ewing’s sarcoma, doxorubicin and etoposide, at low and therapeutically achievable dosages. Several Aurora kinase inhibitors, among them danusertib, are already in clinical trials for various cancers including some pediatric tumors, but not yet Ewing’s sarcoma. Therefore, we believe that the specific vulnerability of Ewing’s sarcoma cells toward pan-Aurora kinase inhibitors described here may represent an attractive and novel therapeutic option, the clinical evaluation of which could profit from ongoing similar trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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