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 performed a focussed viability screen with 200 small molecule kinase inhibitors in two 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. In order to identify the relevant targets underlying the specific vulnerability towards 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. Even though many patients initially respond well to chemotherapy, 40% of these usually develop lethal recurrent disease and 75%-80% of patients with metastatic Ewing’s sarcoma die within 5 years in spite of aggressive combinations of chemotherapy, radiation and surgery. In fact, metastasis is one of the most critical problems associated with Ewing’s sarcoma as approximately 15-20% of patients have overt metastasis upon diagnosis and a yet undefined percentage of patients is believed to carry micrometastasis.

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 about 85% of all cases and which results in the translation of the aberrant gene-product EWS-FLI1. The causal role of EWS-FLI1 in the pathogenesis of Ewing’s sarcoma results from the cooperativity of both fusion partners. While EWS contributes a strong transcriptional activation domain, FLI1 features an ETS-type DNA binding domain. Functionally, EWS-FLI1 acts as an aberrant transcription factor capable of deregulating more than 1000 direct and indirect target genes. EWS-FLI1 has been also described to act as transcriptional repressor. This specific oncogenic lesion is responsible for the highly malignant phenotype and poor prognosis typical for 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. However, so far none of these approaches has received clinical approval.
Protein kinases have received significant attention over the last decade since many of these play important roles e.g. in cancer and can be readily engaged by small molecules. Being safe and effective against many molecularly defined malignancies, such as chronic myeloid leukemia (CML)\textsuperscript{14}, ErbB2-driven breast\textsuperscript{15} or EGFR-dependent lung cancer\textsuperscript{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 like Philadelphia chromosome-positive acute lymphoblastic leukemia.\textsuperscript{17} Therefore, combination of targeted- and chemotherapy could be a viable therapeutic option in Ewing’s sarcoma.

Since Ewing’s sarcoma features the defined molecular lesion EWS-FLI1, an attractive option is the investigation of possible synthetic-lethal relationships.\textsuperscript{18} Globally, this concept has been exploited through genetic screens in yeast and RNAi screens in human cells.\textsuperscript{19, 20} Considering proven druggability, particularly kinome-wide RNAi screens are widely pursued.\textsuperscript{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. While 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,\textsuperscript{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.\textsuperscript{23} Chemical proteomics is a post-genomic 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.\textsuperscript{24-26}
Here, we apply such a multi-layered approach that combines a chemical biology viability screen with a focussed 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 Invesatigaciones Biomédicas, Madrid). Nilotinib, dasatinib, bosutinib and tozasertib were purchased from LC Labs (Woburn, MA, USA), lapatinib, etoposide, doxorubicin, erlotinib, sorafenib and sunitinib were purchased from Selleck Chemicals (Houston, TX, USA), Bafetinib was synthesized by WuXi AppTec (Shanghai, China). The customized kinase inhibitor library consisted furthermore of sub-libraries derived from Tocris, Calbiochem and Merck. All compounds were dissolved in DMSO as 10mM stock solutions.

**Western blotting**

Western blotting was performed as described in manufacturer's manual for antibodies against Aurora kinase 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 performed for 2 hours in the presence of 20µM MG-132.
Viability screen

Cells were plated at 40000 cells/ml (RPMI1640,10% FCS). Drugs were added after 24 hours and incubated for 72 hours. Viability was measured using the cell titer glo assay (Promega). EC₅₀ values were calculated using Spotfire (TIBCO) (duplicate analysis). All other viability measurements have been performed with cell titer glo in triplicate.

Chemical proteomics

Chemical proteomics experiments have been performed as described previously⁴⁸.

Apoptosis and cell cycle measurements

Cleaved caspase-3 was quantified by intracellular protein analysis using flow cytometry. 1x10⁶ cells were fixed 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 performed by staining DNA with propidium-iodide after 36h exposure to tozasertib.

Synergy determination

36-point dose-response matrices have been established as described elsewhere.⁴⁹

Knockdown assays

Knockdowns of Aurora kinases have been performed using ON-TARGETplus Dharmacon smartpools in triplicates (10 nM, 24 well plate). Sequences of EWS-FLI1 RT-primers used for evaluation of knockdown efficiency in inducible ASP14 cell line upon request.
In-vivo studies

2x10^6 TC-71 cells were injected into the gastrocnemious muscle of 5- to 7-week-old SCID/bg mice (Charles River, Sulzfeld, Germany). Single primary tumors developed in >90% of mice over 2 weeks. Mice were randomized into three groups with 11 animals each and intraperitoneally injected with Tozasertib (50 mg/kg 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 mM phosphate buffer. Two diameters of the tumor sphere were measured every 2 days. Tumor volumes were approximated using the formula \((D \times d 2/6) \times \pi\) (where \(D\) is the longer, \(d\) the shorter diameter). When tumors reached 2000 mm^3, 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 <0.05 were regarded as significant. For synergy determination, Bliss additivity was used to predict the combined response C for two single agents with their effects A and B \((C = A + B - A*B)\) 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 performed a focussed screen by probing two 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 five different concentrations ranging from 16 nM to 10 µM. While 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 EC_{50} values below 1 µM (Figure 1, Supplementary Table 1). Among these were staurosporine and several of its derivates like midostaurin (N-benzoyl-staurosporine) or UCN-01 (7-hydroxystaurosporine). Cumulatively, the known cognate targets of the identified screening hits, such as cyclin dependent kinases (CDKs), casein-kinases (CKs) and Aurora kinases (AURKs), 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 EC_{50} of 20 nM in SK-N-MC and the second most efficacious compound with an EC_{50} of 30 nM in SK-ES-1. Tozasertib and also danusertib (PHA-739358), another potent screening hit with EC50 values of 47.6nM (SK-M-MC) and 25.5 nM (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.\textsuperscript{22, 27, 28} 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.\textsuperscript{29, 30}

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 EC_{50} values for these cell lines are consistent with previous observations, displaying a window of activity between 8.8 nM and 55 nM for tozasertib and 22 nM and 37 nM for danusertib (Figure 2A, Supplementary Table 2). 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 EC_{50} values that are considerably lower than in the other cell lines. (Figure 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 FDA-approved or in later stages of clinical trials, but, most importantly, have well characterized target spectra across near kinome-wide kinase panels which were established previously^{22, 24, 25, 31, 32}. In fact, these studies show that collectively these drugs affect about 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 (Figure 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
In order to elucidate the mechanism of action of tozasertib in Ewing’s sarcoma and furthermore compare its target-ID with the drug panel mentioned above, we pursued a chemical proteomics
approach as an initial step of target deconvolution. Based on available co-crystal structure information, we designed an analogue of tozasertib (c-tozasertib), which features an \( n \)-aminobutyl linker (Figure 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 (Figure 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 like AURKA, ABL, SRC and FGFR1, we also identified new potential targets, such as FAK, MAP2K5, MAP3K2 and TBK1 (Figure 3C, Supplementary Table 3).

Comparison of the tozasertib target spectrum with the deconvolution panel drugs highlighted Aurora kinases A and B as unique targets, since they were about 50-fold more potently inhibited by tozasertib (and danusertib) than by any other drug (Supplementary Table 4). Any remaining target was at least as strongly affected by one 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 performed competitive pulldown experiments with SK-ES-1 cell lysates by co-treatment with original tozasertib or DMSO. Interaction of AURKA and B with c-tozasertib was successfully competed for with tozasertib. Taken together, we therefore focussed on the effects of Aurora kinase inhibition in Ewing’s sarcoma cells (Figure 3D).

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

We asked the question, if 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 Figure 1). RNAi knockdown experiments were performed in SK-ES-1 (and SK-N-MC, Supplementary Figure 2) 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 to RD upon knockdown of Aurora kinase B (Figure 4A). While the effect of AURKB knockdown on viability of SK-ES-1 appears stronger than for AURKA, 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 AURKA, which is known to correlate with kinase activity, as well as phosphorylation of the validated AURKB downstream target histone 3 S10 in the mid-nanomolar range. These results validate Aurora kinase A and B as functional targets of tozasertib in Ewing's sarcoma cells and corroborate our results from the chemical proteomics binding assay (Figure 4B).

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

We performed apoptosis measurements via intracellular FACS 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 (Figure 4C). SK-ES-1 displays higher sensitivity towards tozasertib treatment as compared to 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 Figure 3).
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\textsuperscript{33} and that transient transfection of EWS-FLI1 results in mislocalization of Aurora kinase B followed by mitotic defects\textsuperscript{34}. 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\textsuperscript{35}. As expected, we observed down-regulation 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 five days of EWS-FLI1 knockdown, which was sufficient to discern differences elicited by tozasertib. We next tested whether the absence of EWS-FLI1 can confer resistance to pan-Aurora kinase inhibitors within this time window as opposed to an un-induced state. 48 hours after induced 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 towards tozasertib as compared to the non-induced state (Figure 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 Figure 4).

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 cell lines,
SK-ES-1 and A673, tozasertib proved to be the most effective agent of the three (Figure 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 performed pair-wise drug combination viability assays and constructed three-dimensional dose-response surfaces delimited by the respective single dose-response curves. These experimentally derived dose-response surfaces were subsequently correlated to predicted values that were, based on the course of the single dose-response curves, generated employing the Bliss additivity model\textsuperscript{36}. 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 two chemotherapeutic agents in both cell lines, but 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 (Figure 5B, Supplementary Figure 5, Supplementary Table 5).

**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 performed. $2 \times 10^6$ TC-71 Ewing’s sarcoma cells were orthotopically injected in the gastrocnemius muscle of 5-7 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 2000 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 to vehicle control (Figure 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. This strategy allows for the discovery of novel agents with disease-relevant activity and the dissection of their 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. Due to the promiscuous nature of drugs in general and kinase inhibitors in particular, this panel covers a wide range of the druggable kinome.

Our screen identified a number of kinase inhibitors potently killing Ewing’s sarcoma cell lines. In light of that many EWS-FLI1-upregulated target genes have been linked to cell cycle control and that knockdown of EWS-FLI1 results in growth arrest, it is notable that the cognate targets, such as cyclin dependent kinases (CDKs), casein kinases (CKs) and aurora kinases (AURKs), 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. 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. However, targeting multiple gene-products simultaneously can also be of significant benefit if several of these targets show disease relevance. 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. 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 like 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.

Torasertib 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. Of the approximately 20 tozasertib targets identified in these cells, aurora kinases were highlighted by our deconvolution approach as potentially relevant targets. AURKA and AURKB are serine/threonine kinases that play critical roles in mitosis. Among other functions, they are implicated in spindle assembly (AURKA), regulation of the mitotic checkpoint and cytokinesis (AURKB). AURKA 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 AURKB (17p13.1), is not commonly amplified in human cancers. Nevertheless, increased mRNA- and protein levels of AURKB have been reported in colorectal cancers. 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. In the context of pediatric cancers, aurora kinase A has been implicated in chemosensitivity of medulloblastoma cells. Furthermore, the AURKA-specific inhibitor MLN8237 has recently
been demonstrated to have efficacy in pediatric acute lymphoblastic leukemia and neuroblastoma, but interestingly to somewhat lesser extent also in Ewing’s sarcoma.\textsuperscript{47} This report is consistent with our observations described here. Thus, knockdown of AURKA by RNAi reduces viability of SK-ES-1 cells to a minor extent, which is more pronounced over a longer period of time (Supplementary Figure 2). Furthermore, tozasertib is approximately twice as potent as MLN8237 on a cellular level, which could be attributed to the additional inhibitory effect on AURKB (Figure 4A, B). However, reducing the levels of a protein by RNAi might not always be comparable with its pharmacological inhibition. Therefore, an entirely unambiguous evaluation of individual contributions of AURKA and AURKB 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.\textsuperscript{33} Our results furthermore show concomitant knockdown of aurora kinases A and B in Ewing’s sarcoma cells to display a potentiating effect as compared to single knockdown of either kinase. This effect appears 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,\textsuperscript{27, 28} this suggests a compound-intrinsic synergy, which might explain the specific vulnerability of Ewing’s sarcoma cells towards 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 towards tozasertib treatment as compared to 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, this 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\textit{ 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.\textsuperscript{42} 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 towards 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.

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References


**Figure 1** Focussed kinase Inhibitor screen reveals sensitivity of Ewing's sarcoma cell lines to small molecule protein kinase inhibitors. (A) EC$_{50}$ values for inhibition of cell viability of all 200 protein kinase inhibitors in SK-N-MC cells. Compounds with EC$_{50}$ values <1 µM are displayed in the corresponding bargraph. Viability measurements have been conducted using the cell titer glo viability assay as described in materials and methods. (B) Same as for (A) but using SK-ES-1 cells. Full dataset see Supplementary Table 1. Toza: tozasertib, Danu: danusertib, Stau: staurosporine.

**Figure 2** Tozasertib and danusertib selectively and potently impair viability of Ewing’s sarcoma cell lines. (A) Tozasertib and danusertib show selectivity towards Ewing’s sarcoma cell lines over other pediatric sarcoma (RD, Rh30, U2OS) - and leukemic (K562, KU812) cell lines. (B) EC$_{50}$ values of danusertib and tozasertib compared to the drug deconvolution panel in the different Ewing’s sarcoma cell lines. EC$_{50}$ determination based on measurements using the viability assay as described in materials and methods. Only tozasertib and danusertib
treatments result in EC50 values in the low nanomolar range. EC50 values that exceeded 5 µM are displayed as 5 µM.

**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 to tozasertib (squares, solid line) as shown by in vitro kinase inhibition assays, which have been performed as described previously31 (C) Target profile of tozasertib in SK-ES-1 and A673 cells. Kinase targets identified in both cell lines are displayed in blue, 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. Full data provided in the Supplementary Table 3. (D) Competitive binding of tozasertib at 10 µM with immobilized c-tozasertib for Aurora kinases A and B in SK-ES-1 cell lysates as indicated by immunoblots.

**Figure 4** Combined inhibition of Aurora kinases A and B underlies tozasertib effect on Ewing’s sarcoma cells. (A) Comparable knockdown levels in SK-ES-1 and RD result in similar impairment of viability regarding aurora kinase A, but substantially reduced viability regarding aurora kinase B that is even more pronounced concerning the parallel knockdown of aurora kinases A and B. (B) Phosphorylation events indicative of activity of Aurora kinase A (pAurkA Thr288) and B (pH3S10) are reduced in nocodazole-arrested cells upon increasing concentrations of tozasertib. (C) Induction of apoptosis of Ewing’s sarcoma cells increases with escalating concentrations of tozasertib as specified by elevated levels of cleaved caspase-3
positive cells. (D) Doxycycline-inducible knockdown of EWS-FLI1 in ASP14 cells results in downregulation of aurora kinases and causes resistance to tozasertib.

**Figure 5** Tozasertib is more effective in comparison to- and shows synergistic potential with etoposide and doxorubicin. (A) Dose-response curves of tozasertib, doxorubicin and etoposide in SK-ES-1 and A673. (B) Combined effect of tozasertib with etoposide or doxorubicin exceeds Bliss prediction indicating synergy. Needle-graphs indicate deviation from Bliss-predicted additivity. Dose-response surfaces are centered on the EC$_{50}$ of each compound in the respective cell lines. Analysis was performed in triplicates. Values depicted represent absolute deviations. Observed values were divided through standard deviations plus 15$^{th}$ percentile (Supplementary Table 5).

**Figure 6** Tozasertib causes reduction of tumor growth in vivo. After orthotopical injection of $2\times10^6$ Ewing’s sarcoma cells (TC-71) in the gastrocnemous muscle, mice were treated with vehicle control (n=11), 50 mg/kg (n=11) or 100 mg/kg (n=11) tozasertib for 6 days. Further tumor growth was monitored until day 11. Two-way ANOVA test for the log of tumor growth ratio reveals 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.
Winter et al., Figure 2
Winter et al., Figure 3

A

\[
\text{Tozaserib (VX-880, MK-0457)}
\]

\[
\text{c-Tozaserib}
\]

B

\[
\begin{align*}
\text{% ABL Kinase Activity} & \quad c \text{ [nM]} \\
\hline
& 0 \quad 25 \quad 50 \quad 75 \quad 100 \quad 0.1 \quad 1 \quad 10 \quad 100 \quad 1000 \quad 10000 \\
\hline
\text{Tozaserib} & \downarrow \\
\text{c-Tozaserib} & \downarrow \\
\end{align*}
\]

C

\[
\begin{align*}
\text{FGFR1} & \quad \text{SRC} \\
\text{LYN} & \quad \text{FGFR1} \\
\text{FAK} & \quad \text{ZAK} \\
\text{ACVR1} & \quad \text{MAP3K2} \\
\text{MAP4K5} & \quad \text{MAP2K5} \\
\text{PAK4} & \quad \text{TC1} \\
\text{CMGC} & \quad \text{AGC} \\
\text{AURKA} & \quad \text{AURKB} \\
\text{AURKB} & \quad \text{AURKA} \\
\text{ULK1} & \quad \text{BIKE} \\
\text{TBK1} & \quad \text{AAK1} \\
\text{SK-ES-1} & \quad \text{Sk-ES-1+A673} \\
\text{AMPK1} & \quad \text{CAMK} \\
\text{STE} & \quad \text{TKL} \\
\text{TK} & \quad \text{TK}
\end{align*}
\]

D

\[
\begin{align*}
\text{TCL} & \quad \text{c-Tozaserib} & \quad \text{Competition} \\
+ & - & \\
\text{a-AURKA} & & \\
\text{a-AURKB} & & \\
\end{align*}
\]
Winter et al. Figure 6
Molecular Cancer Therapeutics

An integrated chemical biology approach identifies specific vulnerability of Ewing's sarcoma to combined inhibition of Aurora kinases A and B

Georg E. Winter, Uwe Rix, Andrej Lissat, et al.

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