

High-throughput screening identifies proteasome inhibition as promising strategy for treatment of ALK-mutant neuroblastoma

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Abstract

Neuroblastoma (NB) is the most common extracranial malignant solid tumor in young children. NB has a high survival rate (> 80%) in localized stages, however, high-risk patients with metastatic disease have a much worse prognosis. Tumor-promoting alterations in NB include the amplification of *MYCN* or mutations in anaplastic lymphoma kinase (*ALK*), which are also well described as molecular features of high-risk neuroblastoma. To determine potential novel therapeutics, a high throughput screen of 198 compounds was undertaken using an in-house semi-automated pipeline across 10 NB cell lines. Supervised analysis identified proteasome inhibitors, particularly ixazomib citrate, as exhibiting selective toxicity in *ALK*-mutant cell lines. Viability and cell death assays revealed an *ALK*-mutation specific vulnerability at low nM ixazomib citrate concentrations. Furthermore, ixazomib citrate treatment of mice bearing *ALK*-mutant xenograft tumors significantly inhibited tumor growth and increased cleaved-caspase 3 expression, indicating enhanced tumor apoptosis. Taken together, our findings provide preclinical evidence suggesting that ixazomib citrate shows potential as a therapeutic agent for *ALK*-mutant NB.

Introduction

Neuroblastoma (NB) is a malignant embryonal tumor that arises from immature cells of the sympathetic nervous system. It is the most common extracranial malignant solid tumor in childhood, accounting for approximately 7.4% of all cancers in childhood and adolescence. Many NB patients are less than five years of age at diagnosis with a mean age of two years (1, 2). Moreover, NB is the most common cancer diagnosed in the first year of life (1, 3). NB tumors are known for spontaneously regressing in infants, however, NB in older patients are often resistant against standard of care therapy (4, 5). Although the average survival rate of NB patients is 80%, high-risk patients have a dismal prognosis, especially in case of disease relapse (2, 6).

To accurately determine the patient risk and to correlate clinical outcome, genetic factors such as amplification of the *MYCN* proto-oncogene, ploidy status, chromosomal deletions and gene mutations are considered (7, 8). For more than two decades, *MYCN* amplification is known as the most important prognostic factor associated with rapid tumor progression and poor prognosis (1, 9, 10). There also is evidence that telomere maintenance mechanisms are key determinants of patient outcome (11). Additional genes whose mutation has been commonly associated with NB include anaplastic lymphoma kinase (*ALK*), protein tyrosine phosphatase non-receptor type 11 (*PTPN11*), alpha thalassemia/mental retardation syndrome X-linked (*ATRX*), as well as the *MYCN* and *NRAS* proto-oncogenes. Among these driver genes *ALK* is the most altered gene besides *MYCN* amplification (12). Mutations in *ALK* can be either somatic or present in the germline of NB patients and have been suggested to drive particularly aggressive disease course with subsequent relapse (1, 13–15).

ALK is a receptor tyrosine kinase that is mainly expressed in the neural system and is predicted to be involved in the development of the brain and nervous system (16, 17). In NB, the oncogenic effect of *ALK*

is driven by gene amplification and activating mutations. While gene amplification results in increased protein expression, single-base pair missense mutations in the tyrosine kinase domain lead to ligand-independent signaling (16, 18, 19). Three key mutations are responsible for 85% of ALK mutations in NB, namely F1174L, F1245L and R1275Q (20). Interestingly, these mutations have been reported in the development of resistance to *ALK*-targeting therapy in *EML-ALK* rearranged lung cancer (21). Mutations in the *ALK* gene were identified to be the major cause of hereditary NB and somatic *ALK* mutations are detectable in 8–10% of sporadic NBs (14, 15, 22, 23). Importantly, although the frequency of *ALK* mutations is not very different between high- and low-risk neuroblastoma patients, *ALK* mutations or amplification are correlated with reduced event-free and overall survival (20, 24, 25). However, *ALK* mutations are mainly detected in NBs at an advanced diseases stage with an unfavorable outcome and often occur in *MYCN*-amplified tumors (20, 26). In NB relapses, there is a high incidence of new *ALK* mutations (25). *ALK* may also serve as a prognostic marker of disease status and represents an attractive target for therapy of NB in a subset of patients (20, 22, 23, 27, 28). In *ALK*-positive non-small cell lung cancer, targeting *ALK* has now moved into first-line therapy and led to impressive improvements of overall survival. Clinical trials failed to prove durable responses for *ALK*-directed monotherapy (20, 22, 23), but this might change with the advent of next-generation *ALK* inhibitors such as lorlatinib, especially in combination with chemotherapy (29). Thus, targeting *ALK* remains a promising strategy for *ALK*-mutant NBs but further investigation of parallel approaches is desirable.

In hematolymphoid cancers, proteasome inhibitors have evolved as an effective treatment strategy in various disease entities during the last decades (30). Their effect relies on inhibiting the cellular protein-degrading machinery and its downstream events. Some proteasome inhibitors, like bortezomib, carfilzomib and ixazomib citrate/MLN9708, have already been approved by the US Food and Drug Administration for the treatment of multiple myeloma (31–33). Ixazomib citrate, an orally available prodrug, is a reversible proteasome inhibitor (31, 33). The main mechanism of cytotoxicity for ixazomib citrate is based on the suppression of cell survival pathways, activation of apoptotic pathways and enhancement of endoplasmic reticulum stress (32, 33). Interestingly, the frequency of neuropathies after ixazomib citrate treatment is much lower than after treatment with bortezomib, where neuropathy is one of the main adverse effects (33, 34).

Herein, high-throughput drug screening was applied to NB. By including additional previously published brain tumors screening data (35), this study aimed to identify novel therapeutics in the high-risk NB. Specifically, supervised analyses were conducted between NB and the individual brain tumors, *MYCN* amplified vs non-amplified, and *ALK*-mutant vs *ALK*-wildtype NB. Interestingly, proteasome inhibitors were observed to be the top candidate between *ALK*-mutant vs *ALK*-wildtype NB and Ixazomib citrate was selected to be further characterized.

Material and Methods

Cell lines and cell culture All NB cell lines were incubated at 37°C in a 5% CO₂ atmosphere and maintained as monolayer cultures. Cells were obtained from ATCC or DSMZ and regularly checked for

mycoplasma contamination. Authentication via STR genotyping was performed twice a year. Cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) except for NBL-S which were grown IMDM supplemented with 20% FBS and CLBGA cells which were maintained as described in Combaret *et al* (36). All cell lines were passaged with Trypsin-EDTA and re-seeded once or twice a week in ratios from 1:2–1:10. *MYCN* and *ALK* status is shown in **Table Supplemental 1**.

High-throughput drug screening Cells were processed using the high-throughput drug screening pipeline as reported in Pauck *et al* (35). High throughput screening results for medulloblastomas, atypical teratoid rhabdoid tumors and glioblastomas were generated in Pauck *et al*. Survival of cells was evaluated and presented as fitted area under the curve (fAUC, **Table Supplemental 1**).

In vivo evaluation All animal experiments were carried out in accordance with animal research legislation of the European Union (Directive 2010/63/EU). All study protocols were approved by the State Office for Nature, Environment and Consumer Protection of North-Rhine-Westphalia and the local ethics committee. Mice were housed in individually ventilated cages with *ad libitum* food and water and kept in a pathogen-free animal facility with a regular 14 hours light/10 hours dark cycle at constant room temperature of $22 \pm 2^\circ\text{C}$. SY5Y or Kelly cells (2×10^7 cells) were mixed with Matrigel® Matrix (BD Bioscience; 1:1 ratio) and injected into the flank of NMRI nude mice. Length (a), width (b), and height (c) of tumors were measured with digital calipers two times per week and tumor size was calculated as half the volume of an ellipsoid ($2/3 \pi abc$). When average tumor size of tumor-bearing mice was 100 mm^3 , mice were randomized into three groups and dosed orally twice a week with either a vehicle control (5% DMSO in 5% glucose), ceritinib (25 mg/kg; Axon Medchem), or ixazomib citrate (14 mg/kg; Selleckchem). Mice were sacrificed at the end of the experiment in accordance with institutional animal welfare guidelines. In a second cohort, mice were randomized as described above and treated for twice weekly until maximum average tumor size of the vehicle control group reached 1700 mm^3 whereby all mice were sacrificed for validation of cleaved-caspase 3 and *ALK* expression.

Immunohistochemistry Tissue sections (3 μm thickness) were prepared from formalin-fixed paraffin-embedded (FFPE) tumor tissue samples. Slides were stained using a Leica ST5010 Autostainer XL (Leica Biosystems) for *ALK* (Cell Signaling #3633, 1:100) or cleaved-caspase 3 (Cell Signaling #9664, 1:1000), then counter-stained with hematoxylin. Stained slides were scanned using an Axio Scan.Z1 (Zeiss).

Cell viability Cells were seeded into 96-well plates and, after 24 h of incubation, cells were treated with ixazomib citrate or DMSO as a negative control. After 72 h, the CellTiter-Glo® Luminescent Cell Viability Assay was performed according to the manufacturer's protocol. The CellTiter-Glo® Reagent was diluted (1:2 v/v) with PBS. Luminescence was measured using Spark 10M Microplate Reader.

Fluorescence-activated cell sorting (FACS) Cells were incubated in the presence of 25–50 nM ixazomib citrate, DMSO (negative control) or staurosporine (positive control) for 72 h and subsequently stained

with FITC Annexin V and PI according to the manufacturer's protocol. Flow cytometry analyses were performed using a CytoFLEX cytometer (Beckman Coulter).

Caspase 3/7 assay Cells were seeded into 96-well plates for Caspase 3/7 activity detection. After 24 h of incubation, cells were treated with ixazomib citrate or DMSO as a negative control as described above. After 24 h, the Caspase-Glo® 3/7 Assay was performed according to the manufacturer's protocol. Luminescence was measured using Spark 10M Microplate Reader.

DNA damage quantification Cells were processed in triplicate for γ H2A.X DNA damage detection as reported in Bartl *et al* (37).

Crystal violet staining Cells were seeded and treated with ixazomib citrate or DMSO as a negative control as described above. After 72 h, cells were washed with PBS, fixed with 10% formalin (30 min at RT) and stained with 0.1% crystal violet (1 h at RT). Afterwards, cells were washed with double distilled water and images were acquired.

Statistics All data are presented as mean $\pm$ SEM of at least three independent experiments, unless mentioned otherwise. Comparisons between groups were made employing t-test as appropriate. p-values ≤ 0.05 were considered significant.

Data Analysis NB datasets with *ALK* mutational information, GSE85047 and GSE16254, were downloaded from the R2 Genomics Analysis and Visualization Platform (<https://hgserver1.amc.nl/>) as pre-normalized data using MAS5.0 normalization. Next, Gene Set Enrichment Analysis (GSEA) was performed using the t values from the unpaired t tests of an *ALK*-mutant vs *ALK*-wildtype analysis. Gene sets were comprised of a manually curated list (**Table Supplemental 2**).

Results

High-throughput screening identifies potential therapeutic vulnerabilities in neuroblastoma.

To evaluate small molecule inhibitors as therapeutics for NB, we performed a high-throughput screen (HTS) using our in-house semi-automated platform in 10 NB cell lines. The library of compounds contained 198 inhibitors that are evaluated in late-stage clinical trials and included common approved chemotherapeutics serving as a reference. To distinguish NB-selective drug responses from more broadly conserved sensitivities across aggressive pediatric tumors, we used brain tumor cell lines as a biological distinct comparison cohort. We therefore included previously reported drug screening data from 13 atypical teratoid/rhabdoid tumors (ATRT), 8 glioblastoma (GB) and 11 medulloblastoma (MB) cell lines (32 in total) to evaluate the tumor-type specificity of all drugs applied (35). In total, using an average fAUC greater than 0.3 as a cutoff, 41 out of 198 (20.7%) compounds showed activity in our HTS (**Figure 1A**). Interestingly, NB cell lines and two MB cell lines clustered together in an unsupervised analysis, while the other entities were not separated by their drug sensitivity profiles. Next, we performed supervised analyses to identify potential therapeutic vulnerabilities in NB that were not targeting other

entities (**Figures 1 B-D**). In direct comparisons using only the 41 compounds showing activity, NB cell lines were significantly more sensitive to the PLK-inhibitor volasertib and the HDAC inhibitor belinostat than ATRT and GB cell lines, respectively (38, 39). As implied by the clustering, a single compound had different sensitivity profile when NB and MB cells were compared. Here, vinblastine sulfate, which inhibits microtubule formation, was the top-ranked hit (40). Combined analyses revealed that only the compound vinblastine sulfate had differential sensitivity in NB cell lines (**Figure 1E**). Dose-response curves show that vinblastine sulfate was significantly more sensitive in NB compared to other cell lines (**Figure 1F**).

Identification of NB vulnerabilities using HTS

When analyzing NB samples, an unsupervised hierarchical analysis of HTS results did not reveal distinct subgroups within NB (**Figure 2A**). As a proof-of-concept, we subsequently compared *MYCN*-amplified (n= 4) versus non-amplified cell lines (n= 6) in a supervised analysis. Using the 41 active compounds, only a single compound was found to be enriched in the *MYCN*-amplified models compared to the non-amplified cell lines (**Figure 2B, C**). Interestingly, we identified elesclomol, which promotes cuproptosis through increase of reactive oxygen species (41), as the target of *MYCN*-amplified cells (**Figure 2D**). This compound has not been associated with NB to date, but it has been studied in the context of high *MYC* (41, 42). We next used the *ALK* mutation status as a variable to uncover new potential *ALK*-directed therapeutics for NB. Surprisingly, we found that ceritinib, which has been evaluated in clinical trials for *ALK*-mutant NB, only showed a modest trend toward *ALK*-mutant cells being more sensitive (**Supplemental Figure 1**). However, with an fAUC less than 0.3, ceritinib was not part of the 41 active compounds. Interestingly, supervised analysis revealed higher sensitivity to bortezomib and ixazomib citrate, two proteasome inhibitors (31, 33, 43) in *ALK*-mutant versus *ALK*-wildtype cell lines (**Figure 3A, B**). Both, bortezomib and ixazomib citrate were significantly more potent in reducing cell viability of *ALK*-mutant cells (**Figure 3C**). Ixazomib citrate was prioritized for further experiments since it is an orally available compound formulated as a citrate ester prodrug (MLN9708) that is rapidly hydrolyzed to the active form (MLN2238) (43).

ALK-mutant neuroblastoma are sensitive to proteasome inhibitors

Further validation experiments were performed using two NB cell lines with an *ALK* F1174L mutation (Kelly and SY5Y) as well as the *ALK* R1275Q mutation (CLBGA). IMR32, NBL-S and SK-N-FI cells were chosen as *ALK*-wildtype NB cell lines. Concentrations between 25 nM and 50 nM were sufficient to abrogate clonogenic growth of *ALK*-mutant NB models, while *ALK*-wildtype NB cells were more resistant (**Supplemental Figure 2**). Cell viability assays confirmed that cell viability relative to DMSO control for ixazomib citrate was much lower in *ALK*-mutant cell lines (52% viability) compared to *ALK*-wildtype models (85% viability) suggesting that *ALK*-mutant NB cells are more sensitive to ixazomib citrate (**Figure 4A**). Treatment with ixazomib citrate also significantly increased apoptosis in *ALK*-mutant but not in *ALK*-wildtype cells as deduced from Annexin V staining and caspase 3/7 activation assays (**Figure 4B, C, Supplemental Figure 3A**). An increase from 3.81% to 52.2% apoptosis after ixazomib citrate

treatment was observed in the *ALK*-mutant cell lines, whereas the apoptosis fraction was observed to be 5.5% and 6.4% for the control and ixazomib citrate treated *ALK*-wildtype lines, respectively. Additionally, there was a 2-fold increase in caspase 3/7 activity in the *ALK*-mutant lines compared to the *ALK*-wildtype cells after treatment (**Figure 4C**). Finally, immunofluorescence staining for γ H2A.X revealed DNA double-strand breaks, with *ALK*-mutant cells showing higher DNA damage than *ALK*-wildtype cells after ixazomib citrate treatment (**Figure 4D, Supplemental Figure 3B**). Statistically significantly higher DNA damage was observed in *ALK* mutated NB cell lines (2.5 times increased DNA damage in the *ALK*-mutant vs 1.3 in the wildtype), indicating that DNA damage contributes to ixazomib citrate's mechanism of action.

Proteasome inhibition exhibits in vivo efficacy in *ALK*-mutant NB xenografts

Upon subcutaneous injection of SY5Y and Kelly *ALK*-mutant cells into the flanks of immunocompromised mice, tumors were allowed to reach a volume of 100 mm³, when treatment was started with either ceritinib or ixazomib citrate (**Figure 5 A-B**). Both treatments significantly reduced tumor growth compared to vehicle controls. Immunohistochemical analyses of tumors at prespecified endpoints (i.e. when tumors in controls reached the stop criterion) revealed that total *ALK* levels were not reduced by treatments. Instead, both treatments induced apoptosis in SY5Y-induced tumors, while only ixazomib citrate caused elevated expression of cleaved-caspase 3 in Kelly-induced tumors (**Figure 5 C-D**). Together, these data indicate that proteasome inhibition is as effective as targeted treatments in controlling growth of *ALK*-dependent NB in *in vitro* and *in vivo*.

Proteasome processing leads to ixazomib sensitivity

Finally, we performed GeneSet Enrichment Analysis (GSEA) on two NB datasets with *ALK* mutational information. The patient datasets GSE85047 (n=283) and GSE16254 (n=122) showed PROTEASOME_26S_COMPLETE (q value = 0.04 and q value = 0, respectively) in common during a focused proteasome specific analysis (**Table Supplemental 3**). The enrichment of the 26S proteasome gene signature in *ALK*-mutant patient tumors suggest that these tumors may have increased proteasomal protein-processing demands and, therefore, are more susceptible to proteasome inhibitors.

Discussion

Development of resistance of *ALK*-mutant neuroblastomas to *ALK* inhibitors

Development of small-molecule inhibitors of *ALK* for the treatment of *ALK*-mutant NB has been evaluated for over a decade. Crizotinib, the first-generation *ALK* inhibitor, was approved by the FDA in 2011 for *ALK*-mutant non-small cell lung cancer (NSCLC) (44) and later, in 2018, for pediatric use in anaplastic large-cell lymphoma (45). In 2021, it was tested in patients with relapsed or refractory *ALK*-mutant NB, but the results showed limited efficacy (46). Currently, lorlatinib, a third-generation *ALK* inhibitor, is under evaluation for relapsed NB due to its ability to target the prevalent F1174L mutation, which causes resistance to crizotinib treatment (13). Lorlatinib is also specifically promising as it is

effective in a broader range of mutations (29). Resistance to the second-generation inhibitor ceritinib has also been observed (46–49). This resistance is not restricted to F1174L, but to other mutations such as F1245L and R1275Q which differ in oncogenic activity and inhibitor sensitivity (20). Since crizotinib fails to address most *ALK* mutations present in NB, there is a clear need to develop newer *ALK* inhibitors (50). Considering resistance mechanisms to these inhibitors, investigating alternative therapeutic options is crucial. This is even more true as in our screening, *ALK* inhibitors were not enriched as significantly more effective against *ALK*-mutant NB cell lines.

Importance of novel therapeutic strategies for neuroblastoma

Unbiased screening for tumor cell sensitivity towards treatment using approved drugs might serve as a shortcut to accelerate clinical developments. In our screen, we have identified Ixazomib citrate, which is approved for the treatment of multiple myeloma (51, 52) as a candidate for targeting *ALK*-mutant NB. Ixazomib citrate is promising due to its oral formulation and a relatively mild side effect profile (34) making it a noteworthy lead compound for children with NB. Unlike direct *ALK* inhibitors, ixazomib citrate does not target *ALK* signaling but instead interferes with proteasome function, thereby inducing proteostasis/proteotoxic-stress and apoptosis. The mechanism in non-small cell lung cancer (NSCLC) is suggested to involve NOXA and MCL1 (53), these were not significantly different when comparing *ALK*-mutant and *ALK*-wildtype NB (data not shown), but our data suggests that proteasome-processing leads to protease induced stress and then cell death in NB, especially with activity at low nanomolar concentrations leading to significant induction of apoptosis and DNA damage. More broadly, several alternative therapeutic strategies are being explored for high-risk or relapsed NB, including anti-GD2 immunotherapy (54, 55), radiolabeled MIBG therapy (56, 57), epigenetic inhibitors (58, 59), cell-cycle inhibitors (60), and cellular immunotherapies such as CAR-T cells (61). These approaches reflect the increasing need to move beyond conventional chemotherapy and single-agent therapeutics toward strategies that exploit distinct biological dependencies of aggressive NB. Within this landscape, proteasome inhibition may represent a complementary therapeutic approach, particularly in settings where resistance to direct *ALK* inhibition limits treatment efficacy. Our findings therefore support further investigation of ixazomib citrate, alone or in rational combinations with *ALK* inhibitors or established neuroblastoma treatment backbones, as a potential strategy for high-risk *ALK*-mutant NB.

Limitations of this study

As *ALK*-mutant NB is genetically heterogeneous, we did not cover the mutational spectrum of *ALK* mutations in NB that are associated with different resistance patterns. Moreover, the *ALK*-mutant models used here may differ not only in *ALK* status but also in additional genomic alterations, including *MYCN* status and other lineage- or cell-state-specific features. Therefore, *ALK* mutation may serve as a marker of ixazomib citrate sensitivity, but the causal relationship between *ALK* activation and proteasome inhibitor vulnerability remains to be fully established. Modulation of *ALK* signaling, including

ALK knockdown, overexpression of mutant *ALK*, or comparison of isogenic models would help to determine how *ALK* mutations contribute to ixazomib citrate sensitivity. In addition, the *in vivo* studies were performed in subcutaneous immunocompromised xenograft models, which do not fully recapitulate the features of high-risk neuroblastoma. Future studies using orthotopic, metastatic, patient-derived, or minimal residual disease models would further clarify the translational potential of ixazomib citrate.

As an additional limitation, our study focused primarily on ixazomib citrate monotherapy. Given the current clinical development of next-generation *ALK* inhibitors such as lorlatinib, an important next step will be to test whether ixazomib citrate synergizes with *ALK* inhibition, chemotherapy, or other therapeutic modalities. Combination approaches may be particularly relevant because *ALK* inhibitor monotherapy has historically shown limited durability in neuroblastoma, whereas combined treatment could target both oncogenic signaling and proteotoxic stress adaptation. Dose–response matrix experiments, assessment of synergy, and evaluation of toxicity will be necessary to define rational combinations. It will also be important to identify biomarkers beyond *ALK* mutation that predict response to proteasome inhibition, such as proteasome activity, ER-stress markers, apoptotic priming or DNA-damage response defects.

Summary

Using a high-throughput drug screening pipeline, we demonstrate that ixazomib citrate is one of the top-ranked inhibitors differentially affecting *ALK*-mutant and *ALK*-wildtype NB cell lines. In validation experiments, *ALK*-mutant NB cell lines were confirmed to have decreased cell viability, increased apoptosis and increased DNA damage compared to *ALK*-wildtype NB models upon treatment with ixazomib citrate. In a subcutaneous tumor model in immunocompromised mice, ixazomib citrate controlled the growth of *ALK*-mutant NB with comparable efficacy as *ALK* inhibition.

Declarations

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figures

Figure 1

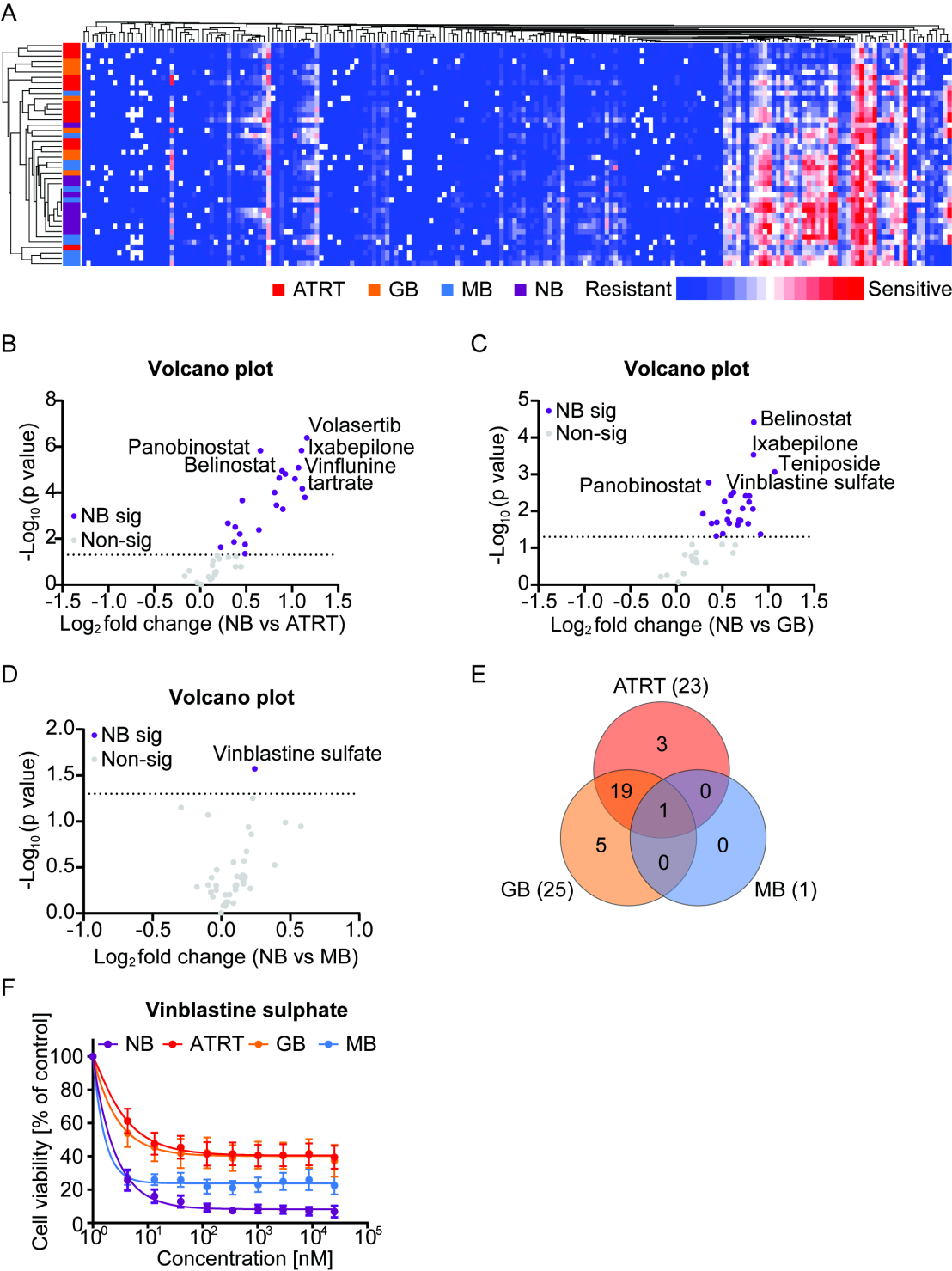


Figure 1

High-throughput screening shows potential vulnerabilities in neuroblastoma cell lines compared to brain tumors cell lines. **A** Unsupervised hierarchical clustering of the fitted area under the curve (fAUC) values of neuroblastoma cell lines (purple, NB=10), atypical teratoid/rhabdoid tumor cell lines (red,

ATRT=13), glioblastoma cell lines (orange, GB=8) and medulloblastoma cell lines (blue, MB=11). Red coloring represents samples which are sensitive to a specific compound, whereas blue indicates resistance. **B-D** Supervised analysis of ATRT (**B**), GB (**C**) and MB (**D**) cell lines using volcano plots. Each dot represents a drug, while color-coding indicates disease-specific activity. NB specific compounds are in purple, colored based on specific disease (see above) or light grey for non-significant drugs. Additionally, the top five compounds for NB are highlighted. ("sig" is short for significant.) **E** Venn diagram highlighting nine compounds that are in common between the three previous analyses (**B-D**). **F** Heatmap of fAUCs for NB, ATRT, GB and MB showing the nine common compounds seen in the Venn diagram (**E**).

Figure 2

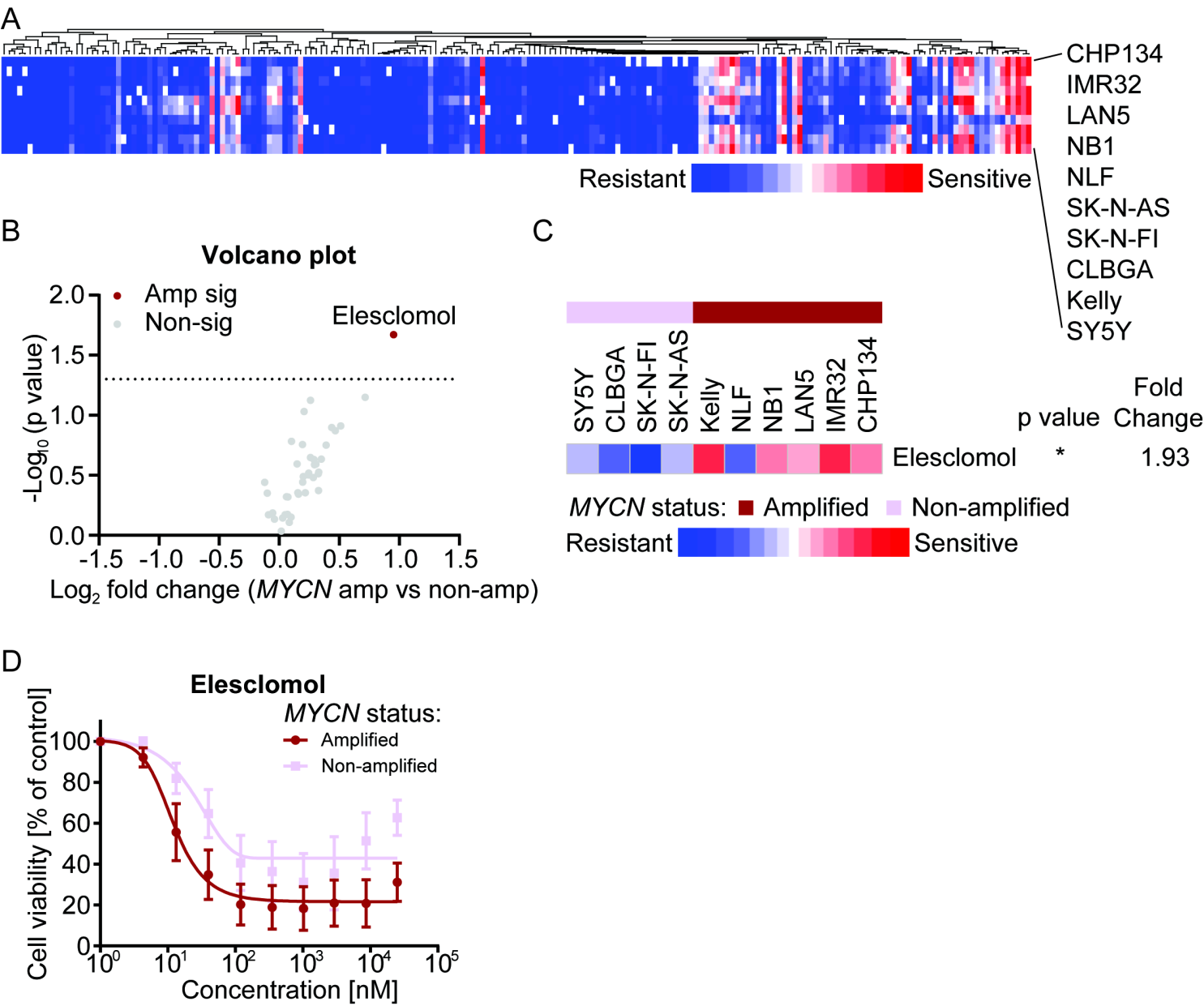


Figure 2

Supervised analysis of high-throughput screening based on MYCN amplification status. **A** Heatmap of fAUCs for NB samples and all compounds in the HTS screen (n= 198). Red coloring represents samples which are sensitive to a specific compound, whereas blue shows resistance. **B-C**Supervised analysis of MYCN-amplified (4 cell lines) versus non-amplified samples (6 cell lines). **B** Volcano plot shows significant compounds for MYCN-amplified in red. Top compound is highlighted. **C**Heatmap showing significant compound based on a t test. p value and fold change are shown in a MYCN-amplified vs non-amplified supervised analysis (* <0.05). **D** Dose-response curve of elesclomol showing cell viability compared to control on the y-axis and dose in nM along the x-axis. MYCN-amplified samples are shown in red and non-amplified samples are shown in pink.

Figure 3

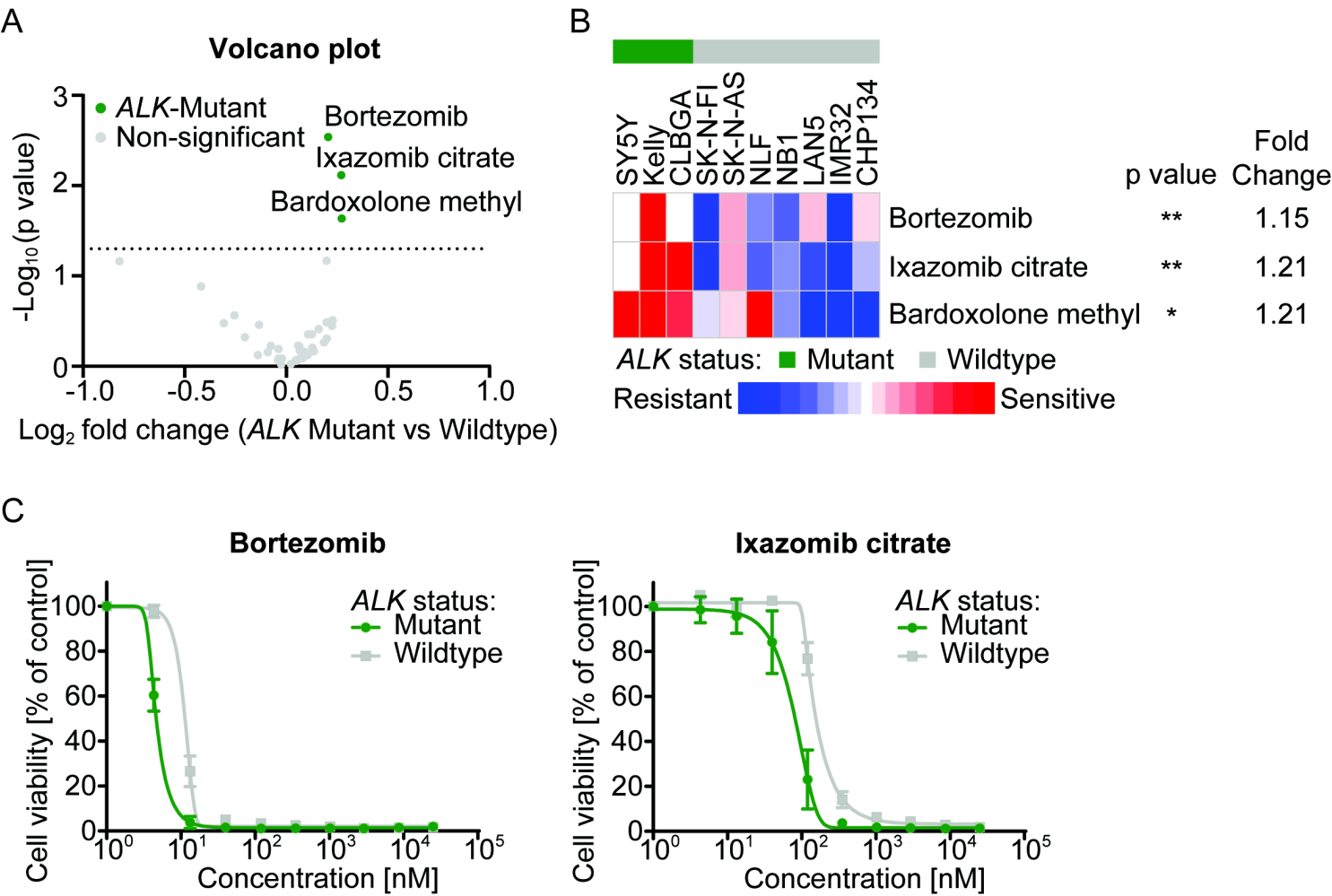


Figure 3

Proteasome inhibitors show differential sensitivity in ALK-mutant versus wildtype NB cell lines. **A-B** Supervised analysis of ALK-mutant (3 samples) versus ALK-wildtype samples (7 samples). **A** Volcano plot highlights compounds that are specifically active against ALK-mutant samples (green) when compared to wildtype samples. **B** Heatmap showing significant differences in sensitivity towards ALK-mutant and ALK-wildtype highlighted in green and grey, respectively. p value and fold change rely on the

comparison of *ALK*-mutant vs *ALK*-wildtype cell lines (*<0.05 and **<0.001, t test). **C** Dose response curves of the top two hits, bortezomib and ixazomib citrate, indicate higher sensitivity in *ALK*-mutant cells (green) compared to *ALK*-wildtype cells (grey).

Figure 4

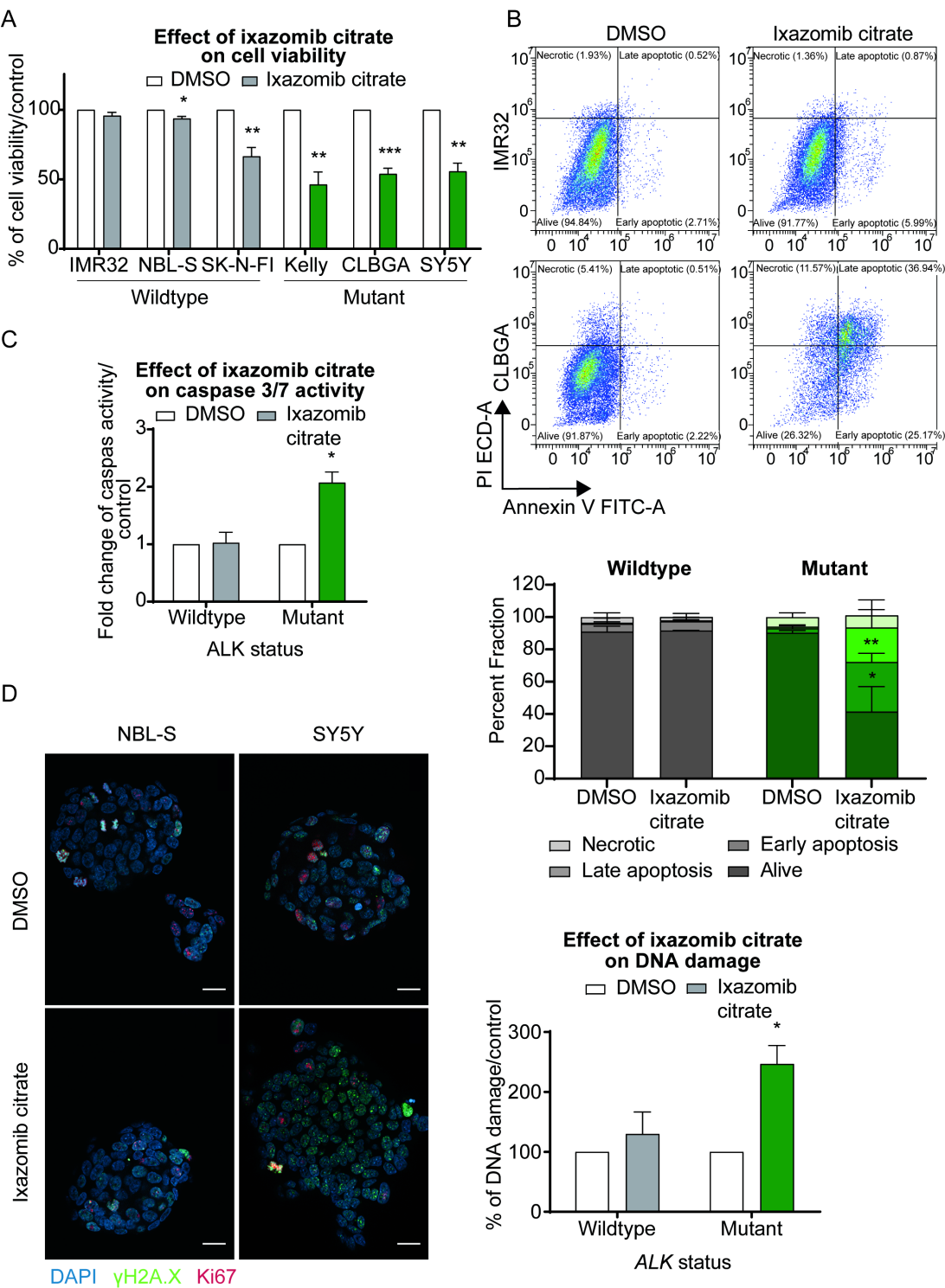


Figure 4

Ixazomib citrate induces cell death and DNA damage in *ALK*-mutant but not *ALK*-wildtype NB cell lines.

A Cell viability as assessed using CellTitre Glo (CTG) 72 hours post exposure to DMSO or ixazomib citrate. Data was normalized to each cell line's DMSO. The white bar indicates DMSO control whereas dark bar shows ixazomib citrate treatment (grey for wildtype and green for *ALK*-mutant; n=3). **B** (Above) Representative dot plots of Annexin V/PI staining visualized 72 hours post DMSO or ixazomib citrate treatment. Annexin V was visualized using the FITC channel and PI using the EDC channel (additional visualizations in **figure supplemental 2A**; n=1). (Below) Quantification of Annexin V/PI stain showing viable, early- or late-stage apoptotic, and necrotic cell populations (biological triplicate). Grey bars indicate wildtype samples and green are *ALK*-mutant samples. **C** Caspase 3/7 ratio was assessed 24 hours post-treatment with DMSO or ixazomib citrate. Data was normalized to each cell line's DMSO. The white bar indicates DMSO control whereas dark bar shows ixazomib citrate treatment (grey for wildtype and green for *ALK*-mutant; n=3). **D** DNA damage was assessed 48 hours post treatment with DMSO or ixazomib citrate. Representative images show fixation and staining of slides with DAPI, γ H2A.X and Ki67 (additional images in **figure supplemental 2B**). Size bars indicate 20 μ m. (Right) Quantification of γ H2A.X foci in ixazomib citrate-treated cells (dark bar) compared to DMSO control (white bar). Grey and green colours indicate *ALK*-wildtype and *ALK*-mutant cells, respectively (n=3). (*<0.05, **<0.001 and ***<0.0001)

Figure 5

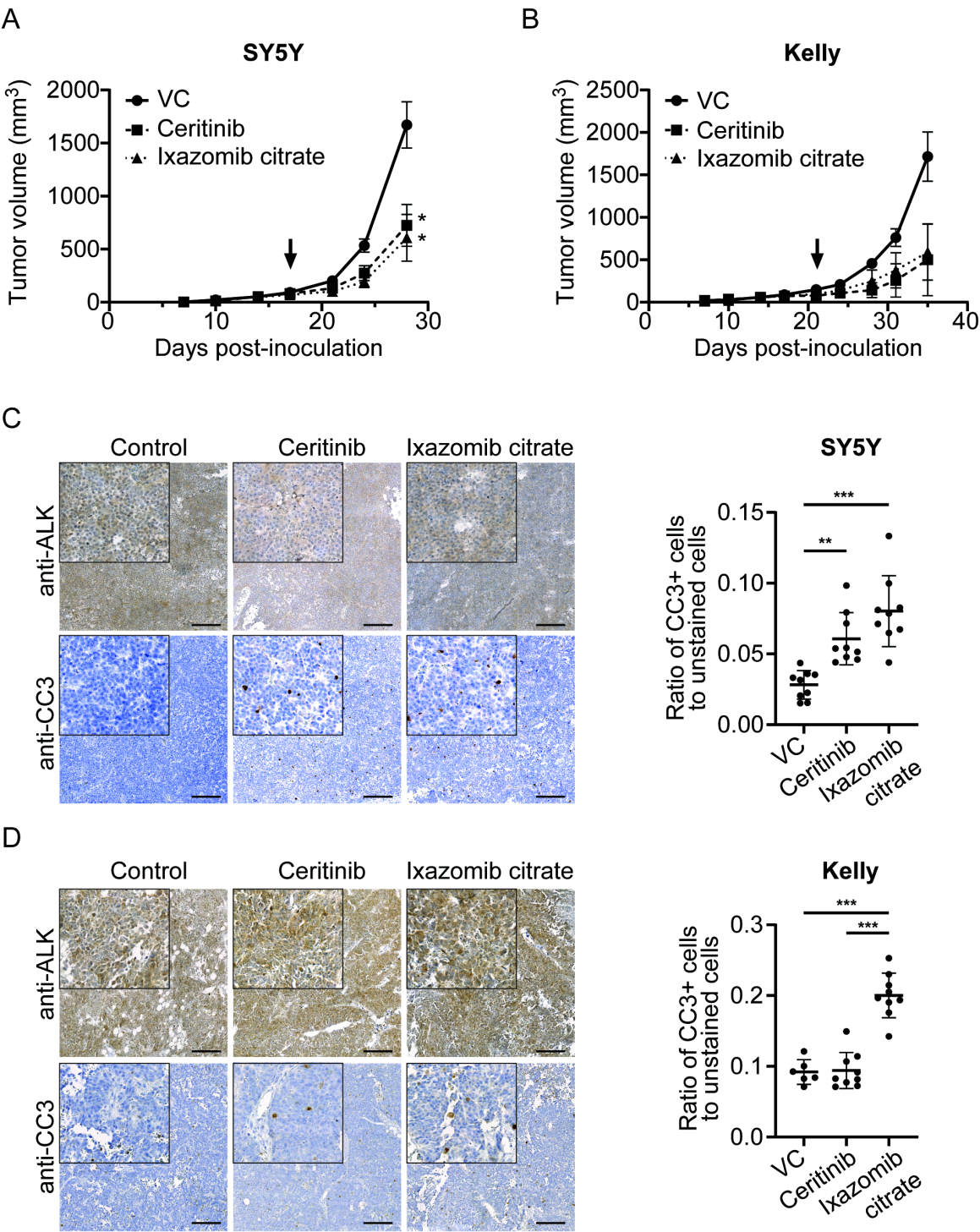


Figure 5

Ixazomib citrate treatment increases cleaved-caspase 3 in mice compared to control mice. A-B *In vivo* experiments with SY5Y (**A**) and Kelly (**B**) cells show a reduction in tumor size (left) when comparing vehicle control (VC) to either ceritinib or ixazomib citrate treated mice. Treatment began at day 17 for mice implanted with SY5Y cells and 21 for mice implanted with Kelly cells. **C-D** Immunohistochemistry shows ALK and cleaved-caspase 3 (CC3) expression in control, ceritinib or ixazomib citrate treated

mouse samples for SY5Y (**C**) and Kelly (**D**) cells. Inset shows three times higher magnification than original image. (Right) Quantification of immunohistochemistry of mice treated with vehicle control (VC), ceritinib or ixazomib citrate. Size bars indicate 200 μm . (* <0.05 , ** <0.001 and *** <0.0001).

Supplementary Files

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