



Universiteit
Leiden
The Netherlands

Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

Graaff, M.A. de

Citation

Graaff, M. A. de. (2017, February 7). *Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue*. Retrieved from <https://hdl.handle.net/1887/45811>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/45811>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/45811> holds various files of this Leiden University dissertation

Author: Graaff, M.A. de

Title: Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

Issue Date: 2017-02-07

Chapter 7

High-throughput screening of myxoid liposarcoma cell lines reveals survivin as a potential novel druggable target

Marieke A. de Graaff, Shruti Malu, Irma Guardiola, Alwine B. Kruisselbrink,
Yvonne de Jong, Willem E. Corver, H. Gelderblom, Patrick Hwu, Torsten O.
Nielsen, Alexander J. Lazar, Neeta Somaiah, Judith V.M.G. Bovée

Submitted

Abstract

Myxoid liposarcoma is a soft tissue sarcoma characterized by a recurrent t(12;16) translocation. Although tumours are initially radio- and chemosensitive, the management of inoperable or metastatic MLS can be challenging. Therefore, our aim was to identify novel targets for systemic therapy. We performed an *in vitro* high-throughput drug screen using three myxoid liposarcoma cell lines (402-91, 1765-92, DL-221), which were treated with 273 different drugs, including various targeted inhibitors, FDA approved drugs, natural products and chemotherapeutic agents, at four different concentrations. Cell lines and tissue microarrays were used for validation. As expected, all cell lines revealed a strong growth inhibition to conventional chemotherapeutic agents, such as anthracyclines and taxanes. A good response was observed to compounds interfering with Src and the mTOR pathway, which are known to be affected in these tumours. Moreover, we identified survivin (BIRC5) as a potential novel target, since a strong inhibitory effect was seen at low concentration using the survivin inhibitor YM155. Sensitivity was confirmed in 402-91 and 1765-92 cells in dose response curves and siRNA for BIRC5 decreased cell viability. Survivin is multifunctional protein involved in the correct segregation of sister chromatids during mitosis. Secondly, it has an anti-apoptotic function. Interestingly, immunohistochemistry revealed abundant expression of survivin restricted to the nucleus in all 32 tested primary tumor specimens. Inhibition of survivin in 402-91 and 1765-92 by YM155 increased the percentage S-phase but did not induce apoptosis. In conclusion, using a 273 compound drug screen we confirmed previously identified targets (mTOR, Src) in myxoid liposarcoma. In addition, we identified survivin as a potential novel target for therapy. However, although a strong decrease in cell viability is observed using the survivin inhibitor YM155, the increased percentage S-phase combined with the lack of apoptosis warrant further investigation before application in the treatment of metastatic myxoid liposarcoma.

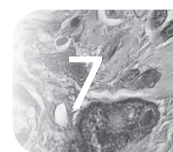
Keywords: myxoid liposarcoma, soft tissue sarcoma, drug screen, survivin, YM155, mTOR, targeted therapy

Introduction

Myxoid liposarcoma (MLS) is a malignant soft tissue tumour accounting for 20-30% of the liposarcomas and roughly 5% of all soft tissue sarcomas (1). These tumours are histopathologically characterized by a proliferation of stellate spindle cells with monomorphic ovoid nuclei, embedded in a myxoid matrix with a plexiform vasculature (1). High grade tumours are defined by having more than 5% of closely packed small blue round cells with high nuclear/cytoplasm ratio and scant stroma. Myxoid liposarcoma is genetically characterized by a reciprocal translocation $t(12;16)(q13;p11)$, generating a fusion product of FUS and DDIT3. The chimeric fusion oncoprotein acts as an aberrant transcription factor, and is known to influence the expression of several genes, including inhibition of adipogenic transcription factors C/EBP α and PPAR γ (2, 3).

MLS tumours are initially sensitive to conventional chemo- and radiation therapy, but despite adequate local treatment, up to 40% can progress to local or distant relapse (4-7). Myxoid liposarcoma exhibits a unique metastatic pattern, as tumour cells tend to spread to other soft tissue sites, before metastasizing to the lungs. The disease can become quite extensive and management of metastatic or otherwise inoperable tumours often is challenging. This is reflected by the variable 5-year survival rates reported in several studies, which range from 8% for advanced disease to around 83% to 93% for cases with purely myxoid and localized tumours (5-9).

In addition to doxorubicin and ifosfamide, recently eribulin, a microtubule-dynamics inhibitor, was shown to offer a survival benefit when compared with dacarbazine in the third-line setting in liposarcomas and is now FDA approved (10). Moreover, myxoid liposarcoma was shown to be sensitive to trabectedin (ET-743, Ecteinascidin), a natural alkylating agent derived from a marine tunicate (11). The drug has a complex mechanism of action that is not entirely elucidated, but involves binding to the DNA-minor groove, interaction with DNA repair complexes and additional effects on the tumour microenvironment (12). Unfortunately, similar to other systemic therapies, resistance develops and the antitumour effect of trabectedin has been shown to diminish after some time on treatment (13). Therefore new therapeutic approaches are warranted to improve the outcome of advanced or metastatic myxoid liposarcoma.



Over the past decades, therapeutic progress has been hampered by the sparse availability of representative preclinical models. For many years only two published cell lines (403-91 and 1765-92) were widely available, both of which were SV40 immortalized (14, 15). Recently, we reported on the generation of a novel myxoid liposarcoma cell line (DL-221) and ancillary mouse xenograft model (16). This newly established cell line is so far the only known myxoid liposarcoma cell line that underwent spontaneous immortalization.

Here we used all three available myxoid liposarcoma cell lines in an *in vitro* high-throughput drug screen to search for novel therapeutic agents that have the potential to enter future clinical trials. Drug screens are regularly used and contribute to the discovery of new candidate targets in cancer therapies (17, 18); furthermore, the pathways targeted by effective drugs can yield insights into tumor biology. In addition to the conventional chemotherapeutic agents used in daily practice, such as anthracyclines and taxanes, we found that YM155, a survivin inhibitor, also strongly decreased tumour growth. Strong nuclear accumulation of survivin was observed in 100% of myxoid liposarcomas, and confirmed to be essential for tumour growth. Inhibitors of survivin may therefore represent novel and promising therapeutic options in myxoid liposarcoma.

Materials and Methods

Cell culture

The myxoid liposarcoma cell lines 402-91 and 1765-92 (both kindly provided by Pierre Åman, Sahlgrenska Cancer Centre, Department of Pathology, Institute of Biomedicine, University of Gothenburg, Sweden) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Landsmeer, The Netherlands) and 1% penicillin-streptomycin (100U/ml). DL-221 was cultured with DMEM supplemented with 10% FBS and 1% pen/strep. All included cell lines have been well characterized for possible alterations in myxoid liposarcomas. All three cell lines are *PIK3CA* wild type, 402-91 and 1765-92 are *TP53* wild type and only DL-221 has two *TP53* mutations (T125R and N239D)(16). Cells were maintained in a humidified 5% CO₂ incubator at 37°C. Cell lines were tested on a regular basis for mycoplasma infections. STR (short tandem repeat) typing was performed before and after the experiments

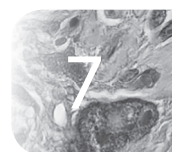
to confirm cell line identity using the Cell ID GenePrint 10 system (Promega, Leiden, The Netherlands).

Drug screen

A high throughput drug screen was performed in which a selection of 273 drugs (Suppl. Table S1) out of 2100 of the Bioactive Compound Library L1700 (Selleckchem, Houston, TX, USA) were tested on the three myxoid liposarcoma cell lines. Selection was based on potential clinical relevance of drugs. Drug stocks were dissolved in DMSO and stored in aliquots at -80°C. Cells were seeded in 96-well plates 24 hour before treatment. Addition of the drugs was performed with the Freedom EVO® 200 liquid handling platform (Tecan, Männedorf, Switzerland) and final treatment concentrations were 1, 10, 100 and 1000 nM, each in triplicate. Cells were treated for 72 hours and thereafter CellTiter-Blue Cell Viability Assay (#G8081, Promega, Madison, WI) was added and incubated for 3 or 4 hours. Plates were read at room temperature using Fluoroskan Ascent™ FL fluorometer (Thermo Fisher Scientific Inc., Pittsburgh, PA), measuring the fluorescence at 530 Ex/604 Em. Data was analyzed by Tableau (Tableau, Seattle, WA, USA). A compound was considered effective if able to reduce the cell viability >50% at a drug concentration of 100 nM in at least two cell lines.

Compounds and cell viability assays

For single agent validation studies, the survivin suppressant YM155, sepantronium bromide (S1130, Selleckchem), everolimus (S1120, Selleckchem), panobinostat (LBH-589, 13280, Cayman Chemical, Ann Arbor, MI, USA) and trabectedin (ET-743, PharmaMar, Madrid, Spain) were dissolved in DMSO according to the manufacturers' instructions. Z-vad-FMK (550377, BD bioscience) was used as a general caspase inhibitor and also dissolved in DMSO. Myxoid liposarcoma cell lines were plated in 96-well plates 24 hr before treatment. Cells were treated with YM155 using concentrations from 0.01 till 5000 nM, in 11 steps, for 72 hours. Cell viability experiments were performed three times, in triplicate. As myxoid liposarcomas are known to respond well to trabectedin, combination treatments with YM155 were performed to investigate possible synergism or antagonism of both drugs. Concentrations used for combination treatment were for both drugs selected around the IC_{25} , IC_{50} and IC_{75} values of single drug treatment for each cell line.



This resulted in nine different dose combinations per cell line, and drugs were added simultaneously. After 72 hours incubation a Presto blue assay (Life-Technologies, Scotland, UK) was performed. Fluorescence was measured at 590nm on a fluorometer (Victor³V 1420 multilabel reader, Perkin Elmer, The Netherlands). Combination experiments were performed twice, in triplicate. To evaluate whether the combination treatments were synergistic, a simplified version of the Bliss independence model was applied (19).

siRNA knockdown

For siRNA knockdown experiments, validated smartpool siRNAs targeting BIRC5 and control siRNAs (siGAPDH and siPLK1) were purchased from Dharmacon (GE Life Sciences, Landsmeer, the Netherlands). Reversed siRNA (50nM) transfection was carried out using DharmaFECT3 transfection reagent (Thermo Fisher Scientific Inc.) in triplo using 5000-10000 cells/well, the experiments were performed twice. After 24 hours evaluation of gene knockdown qRT-PCR were performed and expression levels were normalized towards housekeeping genes CPSF6, GPR108 and CYP11B1 (20). After 72 hours Presto Blue assay and protein analysis by Western blot analysis were performed.

Immunohistochemistry

Survivin expression was investigated in myxoid liposarcoma tissue samples using a previously constructed tissue micro-array with tumour samples from 32 patients diagnosed in the Leiden University Medical Center (21). The tumours of 15 patients consisted of a purely myxoid (M), intermediate cell density (I) or round cell (RC) morphology. The other 17 patients had tumours containing a combination of two histological areas (M+I, M+RC or I+RC); in these cases both areas were included, resulting in a total of 49 tumour samples. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies). Cell pellets of the three untreated myxoid liposarcoma cell lines were fixed in formalin and embedded in paraffin by using Shandon[™] Cytoblock[™] (Thermo Fisher Scientific Inc.). Sections were incubated with rabbit anti-survivin monoclonal antibody (71G4B7, #2808, Cell Signaling Technology, Leiden, The Netherlands) in a 1:100 dilution. Staining was visualized with DAB+ substrate Chromogen System (DAKO, Heverlee, Belgium). Placenta tissue was included as a control.

Both nuclear and cytoplasmic expression was scored separately by two independent observers (JVMGB, MAG). A semi-quantitative scoring system was used, combining the staining intensity (0=negative, 1=weak, 2=moderate, 3=strong) and the percentage of positive tumour cells (0=0%, 1=1-25%, 2=25-50%, 3=51-75% and 4=76-100%) as described previously (22).

RNA isolation and quantitative real-time PCR

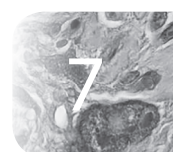
RNA was isolated from myxoid liposarcoma cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by a purification procedure using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. Q-PCR was performed for the isoforms wild-type (WT) survivin, survivin 2b and survivin Δ ex3 with primers described previously (23). Expression levels were normalized towards housekeeping genes HPRT, GAPDH and TBP. Results are depicted relative to survivin WT expression for each cell line.

Apoptosis

Induction of apoptosis was assessed using the Caspase-Glo® 3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, MLS cells were plated into white walled 96 well plates (Corning, Fisher Scientific, Landsmeer, The Netherlands) and incubated with YM155 at the IC₇₅ concentrations, as determined by cell viability assays. After 24 hr treatment the substrate was added in a 1:1 dilution and incubated for 30 minutes at room temperature. Luminescence was measured with a luminometer (Victor³V 1420 multilabel reader). Experiments were performed twice.

Western blot

After 24 hours YM155 treated and untreated MLS cells or 72 hours after siRNA transfection MLS cells were collected in hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP) for protein analysis as described previously (24). Briefly, 5ug or 10 µg protein of each sample was size-fractionated on 12% (TGX Stain-Fast Cast Acrylamide kit, BIO-RAD Laboratories) separating gels. Jurkat cell lysate treated with 25 µM etoposide (Cell Signaling (#2043)) was used as positive control for PARP and cleaved PARP detection. Proteins were transferred to a PVDF membrane and incubated overnight at 4°C with primary antibodies (all from Cell Signalling); PARP antibody (clone 46D11), survivin antibody (clone 71G4B7) or GAPDH antibody (clone D16H11). As a



loading control, α -tubulin staining (clone DM1A, Sigma-Aldrich, Zwijndrecht, The Netherlands) was included. After incubation with the secondary HRP-conjugated antibody for 30 minutes at room temperature, membranes were treated with ECL2 substrate (Pierce™, Thermo Scientific) as per manufacturer's instructions, and chemiluminescence was captured using ECL hyperfilm (Amersham, GE Healthcare Life Sciences, Eindhoven, The Netherlands).

Cell cycle analysis

Cell lines were cultured in T25 flasks in amounts ensuring 70%-90% confluency when harvested and treated with the YM155 IC_{50} concentrations. After 48hr treatment cells were prepared for flow cytometric analysis. In short, cells were harvested, counted and fixed in ice-cold methanol. Next, cells were washed, treated with RNase and stained for DNA using propidium iodide (PI) and stored one over night at 4°C. Next day, cells were analysed using an LSRII flowcytometer (BD biosciences). A blue 488 nm, 20 mW laser was used for excitation. PI fluorescence was collected using a 610/20 band pass filter. Data analysis was performed by using WinList 8 remotely connected to ModFit LT 4 (Verity Software House, Topsham, ME) (25). Each data file contained at least 10,000 single cell events. A one-compartment polynomial model was used for calculating the percentage G_1 , S and G_2M phase of the cell cycle. This statistical model showed the best fit.

Statistical analysis

Dose response curves and IC_{50} values were determined using GraphPad Prism (version 6.05, GraphPad Software, La Jolla, CA). Mann-Whitney testing was performed to investigate differences in survivin expression (GraphPad Prism).

Results

Identification of 27 compounds effective against myxoid liposarcoma tumour cells

Out of 273 tested compounds, 27 drugs were shown to be effective in myxoid liposarcoma tumour cell lines *in vitro*, which was defined as a loss of >50% cell viability in two or all three cell lines at a drug concentration of 100 nM (Figure 1, Supplementary Table 1). All three myxoid liposarcoma cell lines

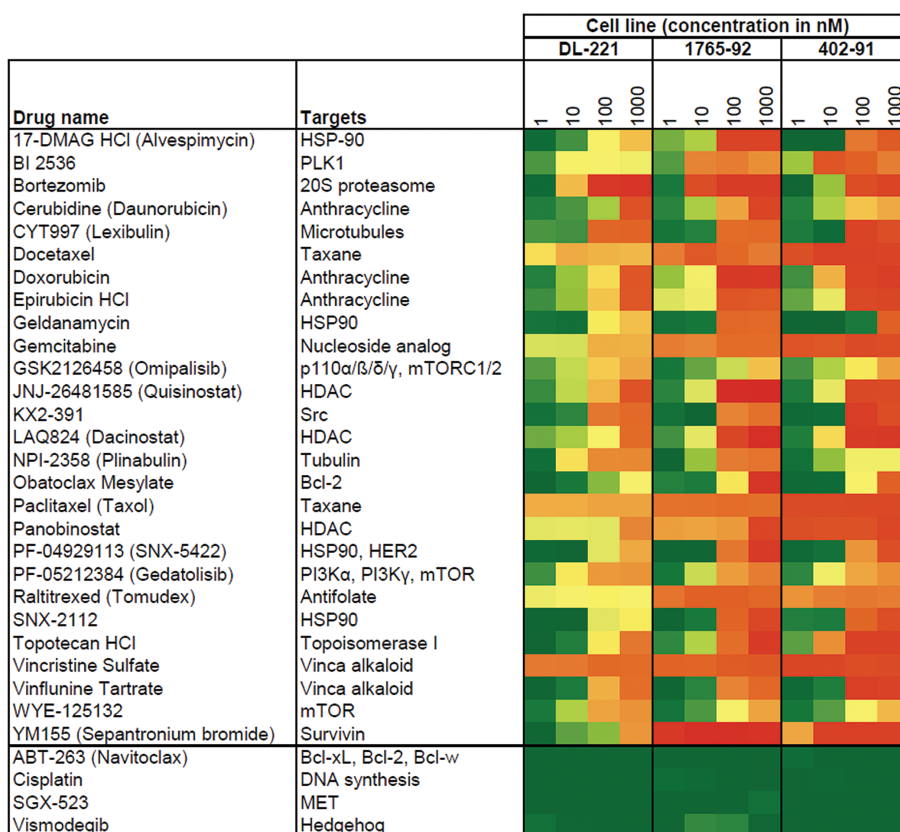
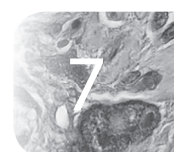


Figure 1. Hits of high throughput *in vitro* drug screen of three MLS cell lines.

List of 27 drugs with a reduction in cell viability of >50% in two or all three cell lines at a drug concentration of 100 nM. Strong inhibitory effect of the survivin inhibitor YM155 is observed in two out of three MLS cell lines. Also a good response is observed to several conventional chemotherapeutics, like doxorubicin, gemcitabine and paclitaxel. Per drug four concentrations (1, 10, 100 and 1000 nM) are tested, green boxes correspond to a high cell viability (~100%) and red boxes to a loss of cell viability (~0%). For comparison, at the bottom, four compounds are randomly shown that did not meet the criteria.

showed a strong response (>80% loss of cell viability) to treatment with the anthracyclines doxorubicin and epirubicin at dose concentrations of both 100 nM and 1000 nM (402-91 and 1765-92) and 1000 nM (DL-221) (Figure 1). The tested taxanes, docetaxel and paclitaxel, as well as a member of another class of mitotic inhibitors, vincristine, revealed a strong decrease in cell viability at the lowest concentration of 1 nM (average cell viabilities of the three cell lines respectively ~22%, ~24% and ~19% after 72 hours of treatment). In the drug screen YM155, a survivin suppressant, showed in the 402-91 and 1765-92 cell



line a very strong decrease in cell viability (average ~13% cell viability) at all four drug concentrations. In the DL-221 cell line there was a moderate reduction of the cell viability after treatment with YM155 at concentrations of 10, 100 and 1000 nM (ranging from 81% to 30% cell viability). Other interesting agents in the list are 17-DMAG HCl, geldanamycin, SNX-5422 and SNX-2112, all targeting HSP90. The list also contains several drugs affecting the PI3K/AKT/mTOR pathway, including omipalisib, gedatolisib and WYE-132. The HDAC inhibitors quisinostat, dacinostat and panobinostat showed a strong inhibitory effect in all three cell lines. KX2-391, an agent interfering with the proto-oncogene Src, also was effective in all three cell lines.

Validation of selected hits using cell viability assays

We selected the survivin inhibitor YM155, the mTOR inhibitor everolimus, and the broad-spectrum HDAC inhibitor panobinostat (LBH-589) for further validation using cell viability assays. YM155 was of particular interest because this was the drug with the best antitumor effect at the lowest treatment dose in 2 of the 3 cell lines. The 402-91 and 1765-92 cell lines were confirmed to be highly sensitive to YM155 and showed IC_{50} values of 6.3 nM and 5.1 nM respectively (Figure 2A). The IC_{50} value of DL-221 was 194 nM, which is consistent with the results observed in the drug screen. In all three cell lines an inhibition of cell viability (~50%) was observed at a low dose concentration (~5 nM) of everolimus (Supplementary Figure 1A). However, with increasing dose no further decrease of cell viability was observed. The tested cell lines 402-91 and DL-221 demonstrated an IC_{50} value of 28 nM and 49 nM respectively after treatment with panobinostat (Supplementary Figure 1B).

To confirm the importance of BIRC5 in myxoid liposarcoma cell survival, siRNA experiments were performed. Knockdown of the survivin gene by smartpool siBIRC5 resulted in a partial decrease of viability in all three cell lines (402-91 69%, 1765-92 55% and DL-221 69%). The transfection was successful as all three myxoid liposarcoma cell lines and the control siRNA GAPDH showed a minor reduction of cell viability, whereas transfection with the positive control siPLK1 resulted in all three cell lines in a pronounced decrease in cell viability. Moreover, western blot analysis after transfection with siBIRC5 confirmed a decrease in survivin protein in all three cell lines (Supplementary Figure 2).

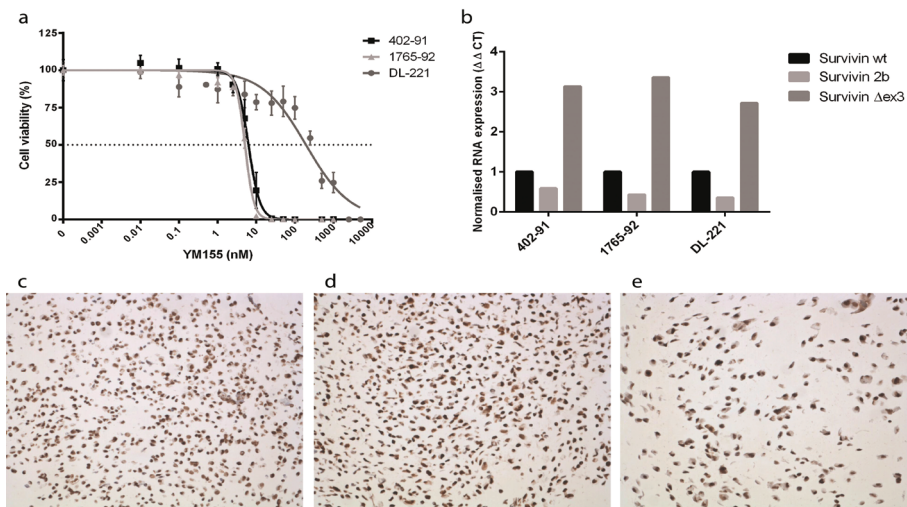


Figure 2. Role of survivin in myxoid liposarcoma cell lines.

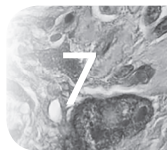
Dose response curves for YM155 (72 hours) in myxoid liposarcoma cell lines. Error bars represents three experiments performed in triplicates (a). Normalised RNA expression of three survivin isoforms in cell lines showing relative abundance of the $\Delta ex3$ isoform (b). Survivin immunohistochemistry of FFPE cell pellets revealed a strong nuclear survivin expression in the cell lines 402-91 (c), 1765-92 (d) and DL-221 (e) (20x magnification).

No synergistic effect of everolimus or YM155 and trabectedin

As trabectedin (ET-743) is effective and therefore often used in the treatment of myxoid liposarcoma, we investigated possible synergy between trabectedin and two of the validated screen hits, YM155 or Panobinostat. Combination treatment of trabectedin with YM155 showed no statistically significant difference between the expected and the observed cell viability in all three tested cell lines, indicating absence of synergism or antagonism for this drug combination (Supplementary Figure 3A, C, E). The combination of trabectedin and panobinostat was evaluated in the 402-91 and DL-221 cell lines, but again no statistical significant synergism or antagonism was observed (Supplementary Figure 3B, D).

Strong nuclear survivin expression in all myxoid liposarcoma cell lines and primary tumours

Since YM155 was most efficient in decreasing myxoid liposarcoma cell viability, we evaluated the protein expression of survivin in the myxoid liposarcoma cell lines. The FFPE cell pellets of the three cell lines showed strong nuclear survivin



expression and a weak cytoplasmic expression in all three cell lines (Figure 2C-E). No difference in the expression levels was observed.

Moreover, we examined the relative distribution of the three most common survivin isoforms: survivin WT, survivin 2b and survivin Δ ex3 by qRT-PCR. All cell lines revealed a similar mRNA expression pattern, with survivin Δ ex3 being most highly expressed, followed by survivin WT, while the lowest expression was found for survivin 2b (Figure 2B).

Next we studied a cohort of myxoid liposarcoma primary tumour samples. The tumour samples of all 32 patients (100%) on the tissue microarrays demonstrated strong nuclear expression of survivin protein (Figure 3A). No statistically significant differences were observed between the myxoid, intermediate and round cell components ($p > 0.05$) (Figure 3B,C). Cytoplasmic staining was absent, with the exception of a single round cell liposarcoma with moderate cytoplasmic staining.



Figure 3. High nuclear expression of survivin in myxoid liposarcomas.

Immunohistochemical analysis of nuclear survivin expression in 32 MLS patients showing high expression in tumour components with myxoid, intermediate and round cell morphology (a). High nuclear survivin expression in tumour with myxoid morphology (b). High nuclear survivin expression in tumour with round cell morphology (c)(20x magnification).

YM155 does not induce apoptosis but increases the S-phase in two out of three MLS cell lines

To further evaluate the mechanism of action of YM155 in myxoid liposarcoma cell lines, we evaluated PARP expression in all three myxoid liposarcoma cell lines using Western blot analysis, but no cleaved PARP was found in the cell lines (Figure 4A) after 24 hours of YM155 treatment. Caspase 3/7 activity after treatment with the survivin inhibitor YM155 at IC_{75} concentration also revealed no significant increase in caspase 3/7 activity after 24 hours (data not shown).

These results indicate that YM155 does not induce apoptosis in myxoid liposarcoma.

We subsequently performed cell cycle analysis to evaluate a possible effect of YM155 on cell cycle regulation. After 48 hours, YM155 treated cells revealed an increase in the S-phase fraction and a decrease in the G1-fraction as compared to untreated 402-91 and 1765-92 cells. In DL-221 no clear changes were observed in cell cycle distribution after YM155 treatment (Figure 4B).

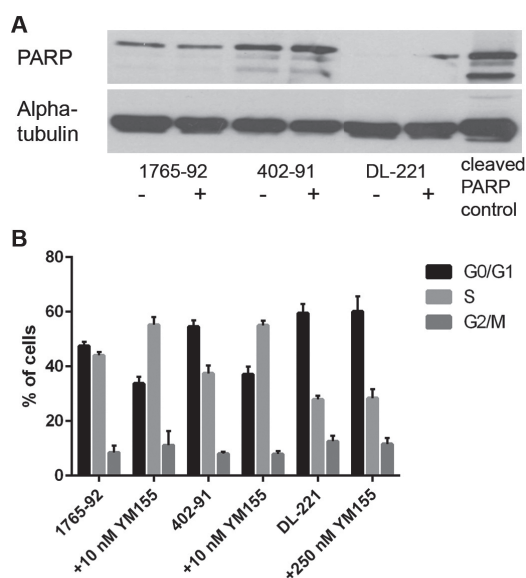
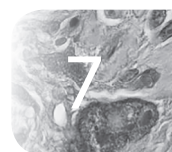


Figure 4. Effect of YM155 on apoptosis and cell cycle.

YM155 does not cause PARP dependent apoptosis, but increases S-phase in 2 of the myxoid liposarcoma cell lines. Western blot analysis for PARP and cleaved PARP expression in myxoid liposarcoma cell lines (a). FACS cell cycle analysis of myxoid liposarcoma cell lines treated with YM155 for 48 hours, measured in two independent experiments. Two cell lines, 402-91 and 1765-92, show a decrease in G₁ and an increase in S-phase after treatment. DL-221 does not show a difference in cell cycle distribution (b).

Discussion

Myxoid liposarcoma initially often responds well to treatment with radiotherapy, conventional chemotherapeutics, or trabectedin, however after several treatment cycles, the effect of treatment is observed to decrease. Therefore, for patients with locally advanced or metastatic disease new therapeutic options are highly warranted in order to improve survival. In this study our aim



was to identify novel candidate targets for treatment by performing a high throughput drug screen with multiple targeted (tyrosine kinase) inhibitors as well as substances of other classes.

The 27 most effective compounds (Figure 1) include the conventional chemotherapeutic agents used in daily practice, such as anthracyclines and taxanes, as anticipated. In addition, the results included three potent inhibitors of the PI3K/AKT/mTOR pathway (omipalisib, gedatolisib and WYE-125132). A subset of myxoid liposarcomas demonstrate dysregulation of this pathway by either *PIK3CA* mutation, loss of PTEN expression or Akt activation (26-28). No large series on mTOR inhibition in myxoid liposarcoma patients are available, although in two cases a minor response has been observed (29) and a clinical trial is ongoing (COSYMO www.clinicaltrials.gov NCT02821507). In the current study we found a decrease of cell viability down to 50% at low dose treatment with everolimus. As a plateau phase was reached, this drug is only able to kill a part of the cell population, suggesting that in clinical studies the drug will need to be combined with another (chemotherapeutic) drug.

The Src inhibitor KX2-391 is another interesting hit confirming previous preclinical results, as two previous studies reported that the Src pathway is highly active in myxoid liposarcoma (30, 31). KX2-391 is the first clinical Src inhibitor targeting the peptide substrate-binding site, with higher *in vitro* potency than dasatinib (32). In a small phase I study with several solid malignancies favourable pharmacokinetics and antitumour activity were observed (33).

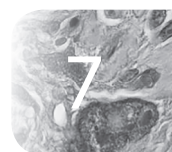
We demonstrate that inhibition of survivin by YM155 results in a significant decrease of cell viability in two out of three cell lines (402-91 and 1765-92). YM155 blocks BIRC5 at the promoter region. In addition to this inhibition at the transcriptional level, also at the post-transcriptional level survivin can be downregulated by the addition of CDK or HSP90 inhibitors which are known to interact with the expression of BIRC5 in the cells (34). Interestingly, four of the 27 hits emerging from the compound screen target HSP90 (Figure 1), and demonstrate a strong inhibitory effect on cell viability. A recent study also demonstrates an important role for HSP90 inhibitor 17-DMAG resulting in decreased phosphorylation of several receptor tyrosine kinases and demonstrating massive tumour cell death in a xenograft model (35).

Survivin (BIRC5) is a member of the inhibitor of apoptosis family and is a multifunctional protein that is involved in several important cellular processes

in both the nucleus and the cytoplasm. Survivin interacts with aurora B kinase and both are part of the chromosomal passenger complex, which forms at the kinetochore in the nucleus and regulates microtubule-kinetochore attachment ensuring proper segregation of the sister chromatids during mitosis (36, 37). Cytoplasmic survivin is involved in two processes, on one hand the binding of XIAP, which inhibits caspase-9 and prevents activation of the apoptotic pathway. In addition, survivin is able to activate AKT and to upregulate $\alpha 5$ integrin, resulting in stimulation of cell motility. Normally, survivin is expressed during fetal development and also in certain differentiated tissues. High expression of survivin has been found in several malignancies, including sarcomas such as malignant peripheral nerve sheath tumor, pleomorphic liposarcoma, uterine leiomyosarcoma, chondrosarcoma and Ewing sarcoma (38-43).

All primary myxoid liposarcoma tumor samples, as well as the three cell lines, showed high nuclear expression of survivin. There are multiple survivin isoforms described. Several studies show a particular overexpression of survivin $\Delta ex3$ in several malignancies, including for example breast and colon carcinoma and soft tissue sarcomas (44, 45). The expression of this isoform is also associated with a worse prognosis. We here show that also in the myxoid liposarcoma cell lines the survivin $\Delta ex3$ isoform is relatively abundant. Successful knockdown of BIRC5 by siRNA resulted in a decreased cell viability which confirms that survivin is indeed important for the survival of myxoid liposarcoma tumor cells.

In a previous study, p53 overexpressing chondrosarcomas displayed higher levels of nuclear survivin expression and *TP53* mutant chondrosarcoma cell lines were more sensitive to YM155 compared to *TP53* wild-type chondrosarcoma cell lines (40). The myxoid liposarcoma cell line DL-221 is the only one carrying a *TP53* mutation while 402-91 and 1765-92 are *TP53* wild-type (16). Also, we did not observe a difference in survivin expression among the three myxoid liposarcoma cell lines, and as such the mechanism in myxoid liposarcoma might be different as compared to chondrosarcoma. Inhibition of survivin by p53 is regulated via several signal transduction pathways and alterations in one of these pathways might underlie a possible difference (46). A possible explanation for the observed difference might be the interaction between HDAC proteins and p53 at the promoter region of survivin. Also the presence of methyl residues on the survivin promoter block binding of p53, resulting in induced survivin expression. Next, survivin expression is also



influenced by other transcription factors, of which NF- κ B is an important one as NF- κ B is deregulated in myxoid liposarcoma as it is influenced by the FUS-DDIT3 fusion protein. However, as there is only one TP53 mutant myxoid liposarcoma cell line available, this number is too small to draw any conclusions.

Previously we and others showed that inhibition of survivin by YM155 is effective *in vitro* in several other malignancies, including squamous cell carcinoma, gastrointestinal stromal tumor and chondrosarcoma (40, 47, 48). Evaluation of the effect of YM155 in *in vivo* models for gastric carcinoma and osteosarcoma demonstrated a suppression of tumor growth in mice (49, 50). Interestingly, dual treatment with YM155 and temsirolimus, an inhibitor of mTOR, demonstrated an improved antitumor effect in a renal cancer model (51). However, in most of these carcinoma models survivin inhibition induces apoptosis, while for myxoid liposarcoma as well as for chondrosarcoma (40) survivin expression was predominantly nuclear. This can be contributed to its role in maintaining proper sister chromatid segregation during mitosis. Strikingly, after treatment with YM155 we observed an increase in percentage of cells in S-phase in 402-91 and 1765-92 (wild-type *TP53*). In DL-221 (*TP53*^{mut}) phases of the cell cycle were left unchanged while the caspase 3/7 pathway seems not to be activated, possibly associated with the lack of cytoplasmic survivin staining. This should be further explored since a high S-phase is significantly associated with a poor prognosis (52, 53). Furthermore, survivin knock-downs by shRNA increased chromosomal instability, irrespective of p53 (54). As together with a high S-phase this might lead to clonal selection and outgrowth of resistant clones, this warrants further investigation.

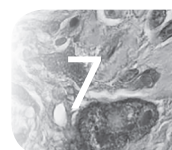
In conclusion, using a large compound screen we identified 27 compounds that are effective in decreasing cell viability of myxoid liposarcoma cells. We identified survivin as a potential novel target in myxoid liposarcoma. The survivin protein is expressed in 100% of tumour samples and two of three tumour cell lines are highly sensitive for treatment with YM155. However, despite a strong decrease in cell viability, the S-phase increased, which warrants further investigation before treating advanced myxoid liposarcoma patients with YM155 targeting survivin.

Disclosure/conflict of interest

The authors have no disclosure or conflict of interest to declare.

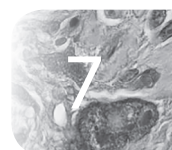
References

1. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
2. Perez-Mancera PA, Bermejo-Rodriguez C, Sanchez-Martin M, et al. FUS-DDIT3 prevents the development of adipocytic precursors in liposarcoma by repressing PPARGgamma and C/EBPalpha and activating eIF4E. *PLoS One*. 2008;3(7):e2569.
3. Aman P, Dolatabadi S, Svec D, et al. Regulatory mechanisms, expression levels and proliferation effects of the FUS-DDIT3 fusion oncogene in liposarcoma. *The Journal of pathology*. 2016;238(5):689-99.
4. ten Heuvel SE, Hoekstra HJ, van Ginkel RJ, et al. Clinicopathologic prognostic factors in myxoid liposarcoma: a retrospective study of 49 patients with long-term follow-up. *Ann Surg Oncol*. 2007;14(1):222-9.
5. Fuglo HM, Maretty-Nielsen K, Hovgaard D, et al. Metastatic pattern, local relapse, and survival of patients with myxoid liposarcoma: a retrospective study of 45 patients. *Sarcoma*. 2013;2013:548628.
6. Moreau LC, Turcotte R, Ferguson P, et al. Myxoid/round cell liposarcoma (MRCLS) revisited: an analysis of 418 primarily managed cases. *Ann Surg Oncol*. 2012;19(4):1081-8.
7. Nishida Y, Tsukushi S, Nakashima H, et al. Clinicopathologic prognostic factors of pure myxoid liposarcoma of the extremities and trunk wall. *Clin Orthop Relat Res*. 2010;468(11):3041-6.
8. Hoffman A, Ghadimi MP, Demicco EG, et al. Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. *Cancer*. 2013;119(10):1868-77.
9. Baxter KJ, Govsyeyev N, Namm JP, et al. Is multimodality therapy necessary for the management of pure myxoid liposarcomas? A multi-institutional series of pure myxoid liposarcomas of the extremities and torso. *Journal of surgical oncology*. 2015;111(2):146-51.
10. Schoffski P, Chawla S, Maki RG, et al. Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. *Lancet*. 2016;387(10028):1629-37.
11. Gordon EM, Sankhala KK, Chawla N, et al. Trabectedin for Soft Tissue Sarcoma: Current Status and Future Perspectives. *Advances in therapy*. 2016;33(7):1055-71.
12. D'Incalci M, Galmarini CM. A review of trabectedin (ET-743): a unique mechanism of action. *Mol Cancer Ther*. 2010;9(8):2157-63.
13. Grosso F, Sanfilippo R, Viridis E, et al. Trabectedin in myxoid liposarcomas (MLS): a long-term analysis of a single-institution series. *Ann Oncol*. 2009;20(8):1439-44.
14. Aman P, Ron D, Mandahl N, et al. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). *Genes Chromosomes Cancer*. 1992;5(4):278-85.
15. Thelin-Jarnum S, Lassen C, Panagopoulos I, et al. Identification of genes differentially expressed in TLS-CHOP carrying myxoid liposarcomas. *Int J Cancer*. 1999;83(1):30-3.
16. de Graaff MA, Yu JS, Beird HC, et al. Establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the FUS-DDIT3 translocation. *Lab Invest*. 2016;96(8):885-94.
17. Halvorson KG, Barton KL, Schroeder K, et al. A high-throughput in vitro drug screen in a genetically engineered mouse model of diffuse intrinsic pontine glioma identifies BMS-754807 as a promising therapeutic agent. *PloS one*. 2015;10(3):e0118926.
18. Eriksson A, Osterroos A, Hassan S, et al. Drug screen in patient cells suggests quinacrine to be repositioned for treatment of acute myeloid leukemia. *Blood cancer journal*. 2015;5:e307.
19. Wong M, Tan N, Zha J, et al. Navitoclax (ABT-263) reduces Bcl-x(L)-mediated chemoresistance in ovarian cancer models. *Mol Cancer Ther*. 2012;11(4):1026-35.
20. Hameetman L, Rozeman LB, Lombaerts M, et al. Peripheral chondrosarcoma progression is accompanied by decreased Indian Hedgehog signalling. *The Journal of pathology*. 2006;209(4):501-11.
21. Endo M, de Graaff MA, Ingram DR, et al. NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. *Mod Pathol*. 2015;28(4):587-95.

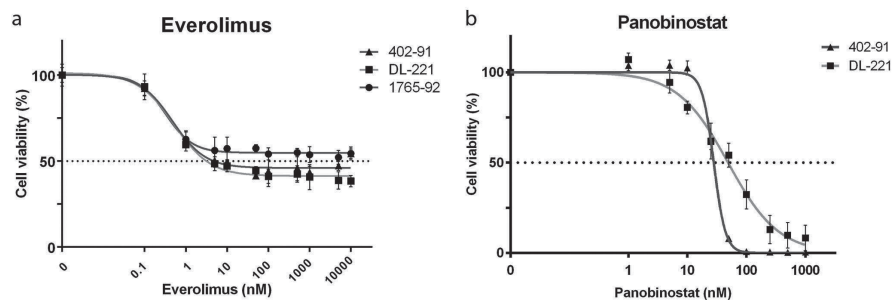


22. Bovee JV, Cleton-Jansen AM, Kuipers-Dijkshoorn NJ, et al. Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in the origin of peripheral and central chondrosarcoma. *Genes Chromosomes Cancer*. 1999;26(3):237-46.
23. Pavlidou A, Dalamaga M, Kroupis C, et al. Survivin isoforms and clinicopathological characteristics in colorectal adenocarcinomas using real-time qPCR. *World journal of gastroenterology*. 2011;17(12):1614-21.
24. Willems SM, Schrage YM, Baelde JJ, et al. Myxoid tumours of soft tissue: the so-called myxoid extracellular matrix is heterogeneous in composition. *Histopathology*. 2008;52(4):465-74.
25. van Haaften C, Boot A, Corver WE, et al. Synergistic effects of the sesquiterpene lactone, EPD, with cisplatin and paclitaxel in ovarian cancer cells. *J Exp Clin Cancer Res*. 2015;34:38.
26. Demicco EG, Torres KE, Ghadimi MP, et al. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. *Mod Pathol*. 2012;25(2):212-21.
27. Negri T, Virdis E, Brich S, et al. Functional mapping of receptor tyrosine kinases in myxoid liposarcoma. *Clin Cancer Res*. 2010;16(14):3581-93.
28. Barretina J, Taylor BS, Banerji S, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet*. 2010;42(8):715-21.
29. Sanfilippo R, Dei Tos AP, Casali PG. Myxoid liposarcoma and the mammalian target of rapamycin pathway. *Curr Opin Oncol*. 2013;25(4):379-83.
30. Willems SM, Schrage YM, Bruijn IH, et al. Kinome profiling of myxoid liposarcoma reveals NF-kappaB-pathway kinase activity and casein kinase II inhibition as a potential treatment option. *Mol Cancer*. 2010;9:257.
31. Sievers E, Trautmann M, Kindler D, et al. SRC inhibition represents a potential therapeutic strategy in liposarcoma. *Int J Cancer*. 2015;137(11):2578-88.
32. Lau GM, Lau GM, Yu GL, et al. Expression of Src and FAK in hepatocellular carcinoma and the effect of Src inhibitors on hepatocellular carcinoma in vitro. *Digestive diseases and sciences*. 2009;54(7):1465-74.
33. Naing A, Cohen R, Dy GK, et al. A phase I trial of KX2-391, a novel non-ATP competitive substrate-pocket- directed SRC inhibitor, in patients with advanced malignancies. *Invest New Drugs*. 2013;31(4):967-73.
34. Chen X, Duan N, Zhang C, et al. Survivin and Tumorigenesis: Molecular Mechanisms and Therapeutic Strategies. *Journal of Cancer*. 2016;7(3):314-23.
35. Safavi S, Jarnum S, Vannas C, 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(1):433-45.
36. McKenzie JA, Grossman D. Role of the apoptotic and mitotic regulator survivin in melanoma. *Anticancer research*. 2012;32(2):397-404.
37. van der Waal MS, Hengeveld RC, van der Horst A, et al. Cell division control by the Chromosomal Passenger Complex. *Experimental cell research*. 2012;318(12):1407-20.
38. Wurl P, Kappler M, Meye A, et al. Co-expression of survivin and TERT and risk of tumour-related death in patients with soft-tissue sarcoma. *Lancet*. 2002;359(9310):943-5.
39. Hingorani P, Dickman P, Garcia-Filion P, et al. BIRC5 expression is a poor prognostic marker in Ewing sarcoma. *Pediatric blood & cancer*. 2013;60(1):35-40.
40. de Jong Y, van Oosterwijk JG, Kruisselbrink AB, et al. Targeting survivin as a potential new treatment for chondrosarcoma of bone. *Oncogenesis*. 2016;5:e222.
41. Ghadimi MP, Liu P, Peng T, et al. Pleomorphic liposarcoma: clinical observations and molecular variables. *Cancer*. 2011;117(23):5359-69.
42. Ghadimi MP, Young ED, Belousov R, et al. Survivin is a viable target for the treatment of malignant peripheral nerve sheath tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18(9):2545-57.
43. Lusby K, Savannah KB, Demicco EG, et al. Uterine leiomyosarcoma management, outcome, and associated molecular biomarkers: a single institution's experience. *Annals of surgical oncology*. 2013;20(7):2364-72.

44. Taubert H, Kappler M, Bache M, et al. Elevated expression of survivin-splice variants predicts a poor outcome for soft-tissue sarcomas patients. *Oncogene*. 2005;24(33):5258-61.
45. Necoechea-Campion R, Chen CS, Mirshahidi S, et al. Clinico-pathologic relevance of Survivin splice variant expression in cancer. *Cancer Lett*. 2013;339(2):167-74.
46. Sah NK, Seniya C. Survivin splice variants and their diagnostic significance. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2015;36(9):6623-31.
47. Zhang W, Liu Y, Li YF, et al. Targeting of Survivin Pathways by YM155 Inhibits Cell Death and Invasion in Oral Squamous Cell Carcinoma Cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2016;38(6):2426-37.
48. Falkenhorst J, Grunewald S, Muhlenberg T, et al. Inhibitor of Apoptosis Proteins (IAPs) are commonly dysregulated in GIST and can be pharmacologically targeted to enhance the pro-apoptotic activity of imatinib. *Oncotarget*. 2016.
49. Cheng XJ, Lin JC, Ding YF, et al. Survivin inhibitor YM155 suppresses gastric cancer xenograft growth in mice without affecting normal tissues. *Oncotarget*. 2016;7(6):7096-109.
50. Zhang Z, Ma L, Wang J. YM155 exerts a growth inhibitory effect on human osteosarcoma in vitro and in vivo. *Oncology reports*. 2015;34(2):1074-80.
51. Carew JS, Espitia CM, Zhao W, et al. Targeting Survivin Inhibits Renal Cell Carcinoma Progression and Enhances the Activity of Temozolomide. *Mol Cancer Ther*. 2015;14(6):1404-13.
52. Bagwell CB, Clark GM, Spyrtos F, et al. DNA and cell cycle analysis as prognostic indicators in breast tumors revisited. *Clinics in laboratory medicine*. 2001;21(4):875-95.
53. Dayal JH, Sales MJ, Corver WE, et al. Multiparameter DNA content analysis identifies distinct groups in primary breast cancer. *British journal of cancer*. 2013;108(4):873-80.
54. Wiedemuth R, Klink B, Topfer K, et al. Survivin safeguards chromosome numbers and protects from aneuploidy independently from p53. *Molecular cancer*. 2014;13:107.

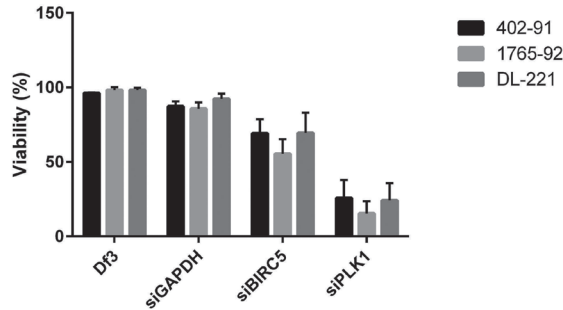


Supplementary Information

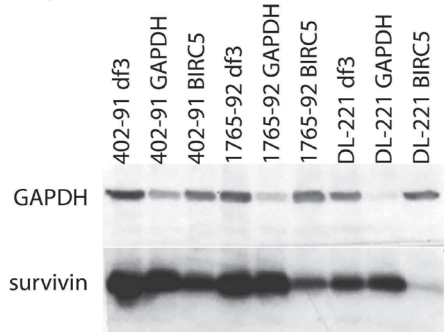


Supplementary Figure 1.
Dose response curves of myxoid liposarcoma cell lines after monotherapy with everolimus (a) and panobinostat (b) after 72 hours of treatment.

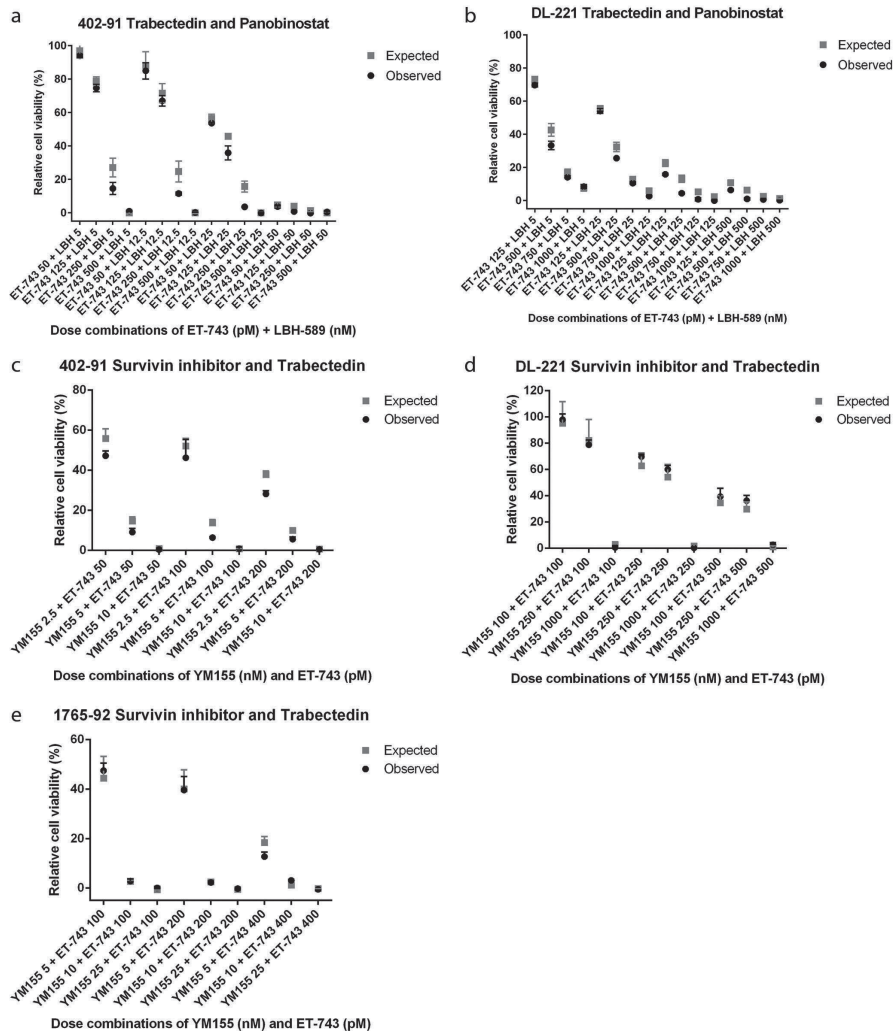
a. Viability after 72 hours siRNA in MLS cell lines



b. Western blot with reduced GAPDH and survivin protein after siRNA



Supplementary Figure 2. Knockdown BIRC5.
BIRC5 and PLK1 siRNA show a decrease in cell viability, whereas the transfection agent alone or control siGAPDH hardly affect cell viability (a). A reduction in GAPDH and survivin protein expression was found after transfection with respectively siGAPDH and siBIRC5 in all three cell lines (b).



Supplementary Figure 3. Combination treatment of cell line 402-91 and DL-221 with panobinostat and trabectedin (a and b) showed no synergism or antagonism. Combination therapy of three MLS cell lines with trabectedin and YM155 also demonstrated no synergism or antagonism (c-e).

