Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

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Chapter 4

Inhibition of Bcl-2 family members sensitizes soft tissue leiomyosarcomas to chemotherapy

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British Journal of Cancer. 2016; 114(11): 1219-1226.

Abstract

BACKGROUND: Leiomyosarcoma is an aggressive soft tissue sarcoma with a 5-year survival rate of 15 to 60%. Treatment options for inoperable or metastatic patients are limited due to frequent resistance of tumours to chemotherapy and radiation. In this study, we hypothesized that anti-apoptotic Bcl-2 family proteins might contribute to leiomyosarcoma chemoresistance and therefore inhibition of Bcl-2 family proteins might sensitize leiomyosarcomas to conventional chemotherapy.

METHODS: Expression of the Bcl-2 family proteins Bcl-xL, Bcl-w and Bcl-2 was investigated using immunohistochemistry on a tissue microarray containing 43 leiomyosarcomas. Furthermore, we investigated whether ABT-737, a potent BH3 mimetic, sensitises leiomyosarcoma cells to doxorubicin treatment *in vitro*.

RESULTS: Seventy-seven percent, 84% and 42% of leiomyosarcomas demonstrated high expression of Bcl-2, Bcl-xL and Bcl-w, respectively. Single-agent treatment with ABT-737 resulted in a minor reduction of cell viability. However, combination treatment of ABT-737 and doxorubicin revealed synergism in all four cell lines, by inducing apoptosis.

CONCLUSION: In conclusion, Bcl-2 family proteins contribute to soft tissue leiomyosarcoma chemoresistance. Anti-apoptotic proteins are highly expressed in leiomyosarcoma of soft tissue, and inhibition of these proteins using a BH3 mimetic increases leiomyosarcoma sensitivity to doxorubicin.

Key words:
sarcoma, leiomyosarcoma, soft tissue tumour, apoptosis, Bcl-2, Bcl-xL, Bcl-w, ABT-737, chemoresistance.

Introduction

Leiomyosarcomas are malignant mesenchymal neoplasms displaying smooth muscle differentiation (1). They occur in patients over a wide age range with the highest incidence in the 5th and 6th decade of life. The 5-year survival rates range from 15 to 60% depending on the location, size and stage of disease at time of diagnosis (1). These low survival rates are in part attributable to low response rates to chemo- and radiotherapy (2).

Overexpression of multidrug resistance (MDR) proteins, like P-glycoprotein, only partly explains leiomyosarcoma chemoresistance. In addition, overexpression of Bcl-2 family proteins could contribute to chemo- and radiotherapy resistance, since chemo- and radiotherapy are DNA damaging therapies that provoke tumour regression by the activation of the intrinsic apoptotic pathway (3-5). Interestingly, immunohistochemistry shows high expression of the anti-apoptotic protein Bcl-2 in soft tissue sarcomas, including leiomyosarcoma (6, 7). Bcl family members, like Bcl-2, Bcl-xL (BCL2L1) and Bcl-w (BCL2L2) inhibit activation of the intrinsic apoptotic pathway by regulating the mitochondrial outer membrane permeabilization. Specifically, mitochondrial outer membrane pores are formed by dimerization of Bax and Bak proteins. These pores release cytochrome c from mitochondria which leads to activation of the intrinsic apoptotic cascade (5). By binding of the anti-apoptotic proteins Bcl-2/Bcl-xL to the pro-apoptotic family members Bak and Bax, the dimerisation of Bak and Bax is prevented and activation of the apoptotic pathway is inhibited.

In this study we hypothesized that the Bcl-2 family members play an important role in leiomyosarcoma chemoresistance and their inhibition might therefore render leiomyosarcoma cells sensitive to treatment with conventional chemotherapeutic agents. To explore the role of Bcl-2 family members in leiomyosarcoma chemoresistance we first evaluated the protein expression of the anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w in a large series of soft tissue leiomyosarcomas by immunohistochemistry, using clinically-annotated tissue microarrays. Subsequently, we determined whether the Bcl-2 pathway inhibitor ABT-737 sensitizes leiomyosarcoma cells to chemotherapy. ABT-737 is a potent BH3 mimetic that inhibits Bcl-2, Bcl-xL and Bcl-w at the mitochondrial membrane by binding to their BH3 domains (8). As chemotherapeutic agent we used doxorubicin which is widely used in the treatment of leiomyosarcoma (9, 10).

Material and methods

Tissue samples

Tissue microarrays were previously constructed containing leiomyosarcomas (soft tissue n=40, uterine n=3), leiomyomas of the uterus (n=7), myxofibrosarcomas (n=16), and undifferentiated pleomorphic/spindle cell sarcomas (n=28). These sarcomas all belong to the genetic subcategory of soft tissue sarcomas with complex karyotypes. Tissue microarrays (TMAs) were constructed using a semi-automated TMA apparatus (TMA Master; 3D Histech, Budapest, Hungary) (11). For each case, 1.767 mm^2 per core was present in triplicate. All samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch society of pathology (Federa). The samples were handled in a coded (pseudo-anonymised) fashion according to the procedures as accorded by the LUMC ethical board. In total, 40 soft tissue leiomyosarcomas were included from 39 patients (23 females and 16 males); of one patient both the primary tumor and a metastatic lesion were analyzed. In addition, three uterine leiomyosarcomas were included. The average age at diagnosis was 60 years. Clinicopathological information is shown in Suppl table 1. Patients were diagnosed between 1989 and 2011 in the LUMC (Leiden, the Netherlands). None of the patients received pre-operative chemotherapy or radiation therapy.

Compounds

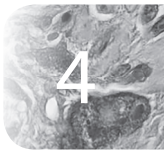
Doxorubicin was obtained from the in-house hospital pharmacy in a 0.9% NaCl solution. ABT-737 (Selleckchem, Houston, USA) was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

In this study we included four leiomyosarcoma cell lines (LMS04, LMS05, IB133 and IB140). These cell lines were established from primary tumours and for the experiments described herein were used between passages 20-50. LMS04 was derived from a high grade retroperitoneal metastasis from the uterus and LMS05 was derived from a grade 2 soft tissue leiomyosarcoma (Suppl table 2). IB140 was derived from a FNCLCC grade 2 leiomyosarcoma from the upper leg and IB133 was derived from a FNCLCC grade 3 retroperitoneal leiomyosarcoma. *TP53* mutation analysis was determined by Sanger sequencing. In addition, the

cell lines were screened for hotspot mutations using the Ion AmpliSeq™ Cancer Hotspot panel v2 (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Fifty reads were considered enough to draw reliable conclusions. The coverage of identified SNPs was ranging from 50 up to 1995 reads.

The leiomyosarcoma cell lines LMS04 and LMS05 were cultured in RPMI1640 medium with 15% FBS and 1% penicillin/streptomycin and the cell lines IB140 and IB133 were cultured in RPMI1640 with 10% FBS, 1% glutamax and 1% pen/strep. The HeLa cell line was included as a reference and cultured in RPMI1640 with 10% FBS and 1% pen/strep. All cell lines were repeatedly checked for mycoplasm and authentication of the cell lines was determined by short tandem repeat (STR) typing (GenePrint 10 system, Promega, Leiden, The Netherlands).



Immunohistochemistry

To detect the expression of anti-apoptotic proteins, immunohistochemistry on TMA was performed according to standard laboratory procedures (11). Further details of the Bcl-2, Bcl-xL and Bcl-w antibodies are shown in Suppl table 3. Scoring was performed by two observers (MAdG and JVMGB) independently and discrepant cases were discussed to reach consensus. Both intensity (0=absent, 1=weak, 2=moderate and 3=strong expression) and percentage of positive tumour cells (0=0%, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) were assessed and scores were added up (12).

Cell viability assays

To analyse the effect of treatment with doxorubicin and ABT-737, cell viability assays were performed with Alamar blue (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Absorbance was measured with a light spectrometer (Victor V, 1420 Multilabel counter; Perkin Elmer, Groningen, The Netherlands).

The dose-response curves of single treatments with ABT-737 and doxorubicin were determined for each cell line in a range from 0.001 to 12.8μM. For doxorubicin, it is accepted that an *in vivo* range of 5 to 100 μM corresponds to an *in vitro* range from 1 to 10 μM (13). All cell lines were treated with ABT-737 for 24 hours and subsequently doxorubicin for 48 hours. Because in only one of the four cell lines (LMS05) large effects could be observed, the other three cell lines were treated with doxorubicin and ABT-737 combined for 72

hours. The concentrations of doxorubicin used for the combination treatments were selected per cell line, based on the individual dose-response curves after 48 hour (not shown) and 72 hour doxorubicin treatment. Cell viability was calculated relative to untreated cells, all experiments were repeated three times in triplicate.

To evaluate whether the combination treatments of doxorubicin and ABT-737 were synergistic drug interactions, a simplified version of the Bliss independence model was applied (14). When two drugs exert their effects independent to one another, the resulting relative viability after drug treatment is expected to equal the product of the relative viability after treatment with the individual drugs. When the observed viability after combination treatment is lower than the expected viability, this is an indication of a synergistic drug interaction. When the observed viability is higher than the expected based on the individual drug effects, the compounds inhibit each other's effects (antagonism).

Western blot

To evaluate the expression of Bcl-2, Bcl-xL, Bcl-w, Mcl-1, PARP and α-tubulin before and after treatment we performed western blots according to the Cell Signaling Technology protocol enhanced chemiluminescence. Cell lysates were made from cultured cell lines LMS04, LMS05, IB133 and IB140 treated with doxorubicin (respectively 0.2, 0.6, 0.7 and 0.5μM) and with ABT-737 (5μM) for 24 hours. In order to obtain cell lysates, hot SDS was added to the cells and the solution was heated up to 100°C. Gels were loaded with 20μg lysate. Antibodies, described in detail in Suppl table 4, were dissolved in TPS Tween 0.05% in which the blots were also washed. As a positive control for all antibodies except for PARP, the HeLa cell line was included. For PARP, Jurkat apoptosis cell lysate (Cell Signaling Technology) was included as a positive control.

Caspase assay

To confirm that the decrease in cell viability was caused by apoptosis, caspase assays were performed according to the caspase-Glo 3/7 assay protocol of Promega. LMS04, LMS05, IB133 and IB140 cell lines were treated for 24 hours with ABT-737 (5μM), doxorubicin (0.2, 0.6, 0.7 and 0.5μM respectively) or a combination. A 1:1 ratio of Caspase-Glo ® 3/7 reagent volume per sample was used. As negative controls, untreated cells were included. For each condition,

Z-VAD-FMK, an irreversible pan-caspase inhibitor (25μM, Promega) was used. The luciferase units of each sample were measured by the VICTOR3 Label counter with emission filter D572 after 1 hour.

Statistics

GraphPad Prism for Windows version 6.02 (GraphPad Software, La Jolla California, USA) was used to analyze the results. The statistical difference between 2 groups was assessed by an unpaired t-test, *P-values* were considered statistically different if $P<0.05$). Statistical differences in overall survival were calculated with the log-rank (Mantel-Cox) test. Also GraphPad was used for dose-response curves and calculation of IC_{50} values.

Results

High expression of Bcl family proteins in leiomyosarcomas

Immunohistochemistry for anti-apoptotic proteins using tissue microarrays revealed that Bcl-xL and Bcl-w were higher expressed in leiomyosarcomas compared to uterine leiomyomas (Figure 1). Thirty three out of 43 (77%) leiomyosarcomas showed high Bcl-2 expression (IHC score >3). For Bcl-xL and Bcl-w this was 36 out of 43 (84%) and 18 out of 43 (42%), respectively (Table 1). Interestingly, Bcl-xL and Bcl-w were expressed at significantly lower levels in uterine leiomyomas ($p=<0.001$), while Bcl-2 expression was significantly higher in leiomyomas, compared to leiomyosarcomas ($p=0.042$). The three uterine leiomyosarcomas showed a slightly higher Bcl-2 expression compared to the leiomyomas, although this is not statistically significant ($p=0.224$) (Suppl figure 1A). The anti-apoptotic proteins were also expressed in other high grade sarcomas (myxofibrosarcoma and undifferentiated pleomorphic sarcoma) (Table 1).

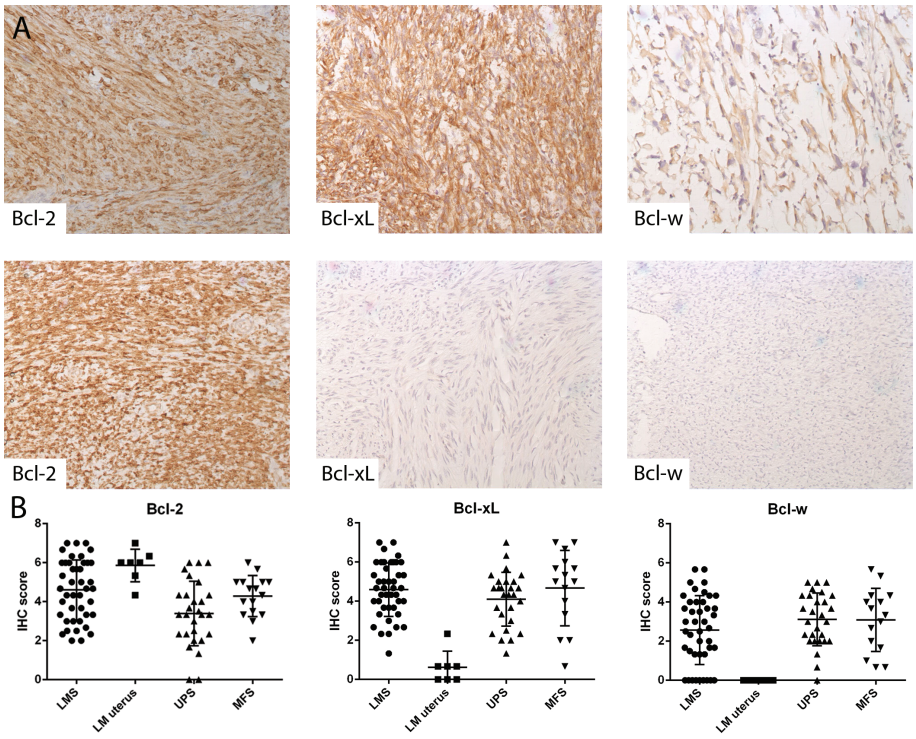


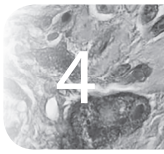
Figure 1. Protein expression of Bcl-2, Bcl-xL and Bcl-w determined by immunohistochemistry. (**A**) In the top panel Bcl-2, Bcl-xL and Bcl-w expression of a representative leiomyosarcoma is displayed were the bottom panel shows expression of a representative uterine leiomyoma (20x magnification). Leiomyosarcomas showed overall higher expression of Bcl-xL and Bcl-w, whereas both leiomyosarcomas and uterine leiomyomas revealed a high expression of Bcl-2. (**B**) The difference in Bcl-2, Bcl-xL and Bcl-w protein expression of leiomyosarcoma (LMS), uterine leiomyoma (LM uterus), myxofibrosarcoma (MFS) and undifferentiated pleomorphic sarcoma (UPS) illustrated by dot plots confirmed an overall higher expression of Bcl-xL and Bcl-w in leiomyosarcomas as compared to uterine leiomyomas.

There was no association between expression and histological grade (Suppl figure 1A). The Kaplan-Meier survival curve showed a trend for high expression of Bcl-2, Bcl-xL and Bcl-w to be associated with shorter overall survival time (Suppl figure 1B). However, the differences in overall survival between the groups of patients with an immunohistochemical score ≤3 or >3 were not significant (p=0.5347 (Bcl-2), p=0.5490 (Bcl-xL) and p=0.2495 (Bcl-w)). Sixteen patients were diagnosed with a metastasis after a period of treatment. In the Kaplan-Meier survival curves no correction was added for metastasis or treatment.

Table 1. Expression of Bcl-2, Bcl-xL and Bcl-w in leiomyosarcomas, leiomyomas and sarcomas.

Expression	LM	LMS	P-value LM vs LMS	MFS	UPS/SCS
Bcl-2					
Low	0/7 (0%)	10/43 (23%)	0.0419	2/16 (13%)	11/28 (39%)
High	7/7 (100%)	33/43 (77%)		14/16 (87%)	17/28 (61%)
Bcl-xL					
Low	7/7 (100%)	7/43 (16%)	<0.0001	3/15 (20%)	7/28 (25%)
high	0/7 (0%)	36/43 (84%)		12/15 (80%)	21/28 (75%)
Bcl-w					
Low	7/7 (100%)	25/43 (58%)	0.0004	7/15 (47%)	14/28 (50%)
High	0/7 (0%)	18/43 (42%)		8/15 (53%)	14/28 (50%)

Leiomyosarcoma (LMS), uterine leiomyoma (LM uterus), myxofibrosarcoma (MFS) and undifferentiated pleomorphic/spindle cell sarcoma (UPS/SCS). Low = IHC score ≤ 3; high = IHC score > 3.



Doxorubicin and ABT-737 act synergistically in leiomyosarcoma cell lines

Since Bcl-2, Bcl-xL and Bcl-w were highly expressed in the majority of leiomyosarcomas, four leiomyosarcomas cell lines (LMS04, LMS05, IB133 and IB140) were selected for further functional evaluation. Clinicopathological as well as genetic characteristics of these cell lines are shown in Suppl table 2. *TP53* mutations were found in LMS04 and IB133, while IB140 demonstrated a *CTNNB1* variation (Suppl table 2 and suppl figure 2).

The response to ABT-737 and doxorubicin were determined separately after 24, 48 and 72 hours of treatment. All cell lines showed maximal response at 72 hours of ABT-737 treatment. IB140 and IB133 demonstrated a slight decrease in cell viability compared to LMS04 and LMS05, however due to the limited sensitivity of single treatment with this drug, no reliable absolute IC_{50} could be calculated (Figure 2A). The cell lines showed a maximal reduction in cell viability of 60-95% after single doxorubicin treatment (Figure 2B). The calculated absolute IC_{50} values of doxorubicin treatment for the four cell lines LMS04, LMS05, IB140 and IB133 were respectively: 0.198; 0.265; 1.314 and 0.580 μM.

To determine possible synergy of combination treatment, ABT-737 (1, 5 and 10μM) was administered for the first 24 hours, followed by the addition

of doxorubicin for 48 hours. A synergistic effect of ABT-737 was found, most pronounced in LMS05 (Figure 2C) and only a moderate effect was observed in IB140, IB133 and LMS04 (not shown). Therefore, these last three cell lines were treated simultaneously with ABT-737 and doxorubicin for 72 hours. The expected cell viability of combination treatment was calculated and compared with the observed viability. For five out of six tested sequential drug combinations in LMS05 a statistical significant difference ($P<0.005$) was found between the expected and observed cell viability, indicating synergism (Figure 2C). IB133 and IB140 both showed synergistic effects with pronounced reduction of cell viability in the three drug combinations with the higher doxorubicin concentration ($p<0.005$) after synchronous treatment for 72 hour (Figure 2E and 2F). In LMS04, on the other hand, the difference between the observed and expected effect of the synchronous combination treatment was most clear in the two combinations with the highest (10μM) ABT-737 concentration ($p=0.07$ and $p<0.005$) (Figure 2D).

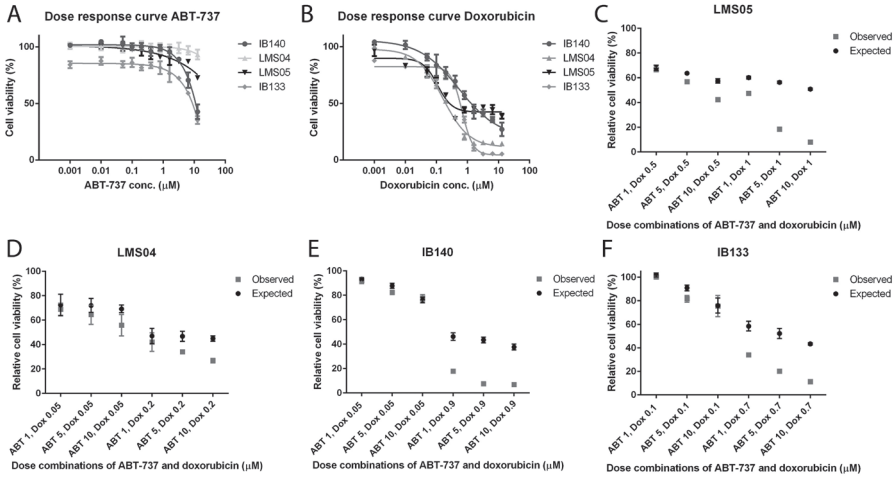


Figure 2. Dose response curves of the four cell lines treated with ABT-737 and doxorubicin. (**A**) Dose response curves of ABT-737 ranging from 0.001-12.8μM after 72 hours of treatment showed limited effect of this single drug treatment. (**B**) Dose response curves of doxorubicin ranging from 0.001-12.8μM after 72 hours of treatment showed reductions till 5% cell viability. (**C**) Comparison of observed and expected relative cell viability of LMS05 after sequential treatment with ABT-737 for 24 hours followed by 48 hours treatment with doxorubicin. Strong synergism was seen in LMS05. (**D-F**) Comparison of observed and expected relative cell viability of the cell lines LMS04, IB140 and IB133 after synchronous combination treatment with ABT-737 and doxorubicin for 72 hours. A moderate synergistic effect was observed in IB140 and IB133, and LMS04 revealed a mild synergistic effect at ABT-737 10μM.

Western blot performed on untreated cells confirmed expression of all three anti-apoptotic proteins in the four cell lines. Among these, Bcl-xL was most abundantly expressed. Expression of Bcl-2 was highest in LMS05, followed by LMS04, while expression was almost absent in IB133 and IB140 (Figure 3). Mcl-1 was expressed in all cell lines, with a comparable intensity as the HeLa control. LMS04 even showed a stronger Mcl-1 expression. Treatment with ABT-737 and/or doxorubicin did not significantly alter the expression levels of Mcl-1, Bcl-w, Bcl-xL or Bcl-2.

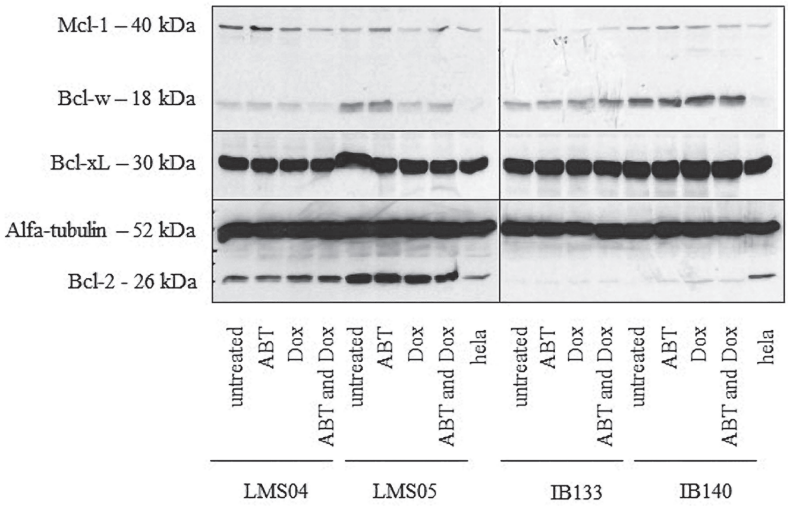


Figure 3. Western blots performed on untreated, single agent treated or combination-agent treated cells. The expression of Mcl-1, Bcl-w, Bcl-xL, α-tubulin and Bcl-2 in four leiomyosarcoma cell lines showed that Bcl-2 was expressed in LMS05 and LMS04, while it was almost absent in the IB133 and IB140 cell lines. Bcl-xL was most abundantly expressed in all four cell lines. Mcl-1 was expressed in all cell lines.

Combination treatment induces apoptosis

Using caspase Glo3/7 assays we demonstrated that reductions in cell viability were caused by apoptosis. All four cell lines showed a dramatic increase in caspase activity after combination treatment compared to single treatment (Figure 4A). Addition of the pan-caspase inhibitor Z-VAD-FMK blocked the increase in luciferase units, which indicates that the luciferase units correspond to apoptosis. Furthermore, induction of cleaved PARP, an indicator for apoptosis was greater in all 24 hour combination treated lysates, compared to the single-agent treated or untreated samples (Figure 4B) (13).

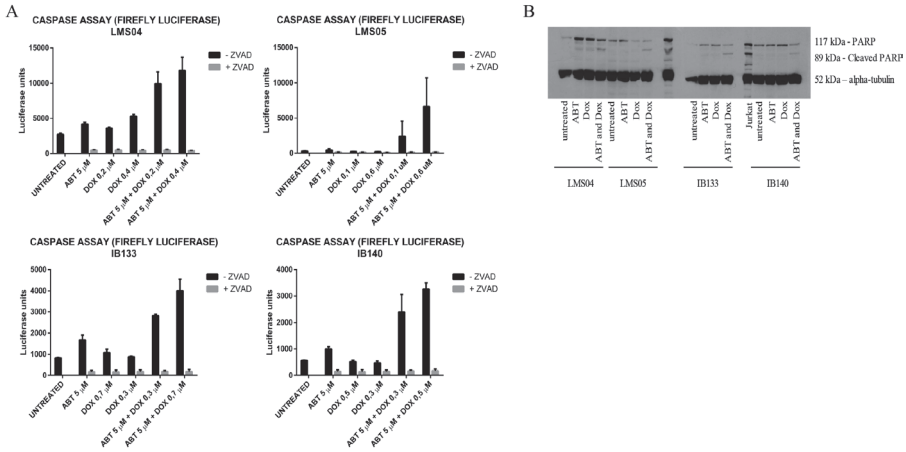


Figure 4. Caspase Glo3/7 assay and PARP western blots to evaluate apoptosis after single-agent and combination treatments. (**A**) Amount of luciferase units measured in combined treated cells was significantly higher compared to single and untreated cells. For each condition also the pan-caspase inhibitor (Z-VAD-FMK) was included which show low or no expression of luciferase units. (**B**) Cleaved PARP expression was confirmed in all combined treated cells by western blot.

Discussion

In this study we found high expression of Bcl-2 family members in leiomyosarcomas and demonstrate a role for these proteins in the response of leiomyosarcomas to conventional chemotherapy. More specifically, inhibition of Bcl-2 family members using the ABT-737 BH3 mimetic rendered leiomyosarcoma cell lines more sensitive to doxorubicin, suggesting new therapeutic options for patients with metastatic or inoperable disease.

We demonstrated a high protein expression of Bcl-xL and Bcl-w in leiomyosarcomas compared to benign uterine smooth muscle tumours. Bcl-2 expression was higher in leiomyomas compared to leiomyosarcomas, which was also reported by others (15, 16). Some studies showed a correlation between high Bcl-2 expression and longer disease-specific survival in uterine leiomyosarcomas (15-18). In contrast, we observed a trend for high Bcl-2 expression with shorter survival. The difference might be due to differences in biological behavior between uterine and soft tissue leiomyosarcomas as our series contains only three uterine leiomyosarcomas.

These results are consistent with observations in other tumour types, including sarcomas, in which *in vitro* studies have also demonstrated an important role for the Bcl-2 family members in chemoresistance, and that

tumour cells could be sensitized to doxorubicin by inhibition of these proteins, using ABT-737 (19). The addition of doxorubicin after ABT-737 treatment resulted in a complete loss of cell viability in chondrosarcoma. In other tumour cell lines, including castration-resistant prostate cancer (CRPC), malignant peripheral nerve sheath tumour (MPNST), acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML), ABT-737 also showed synergy in combination with chemotherapeutic agents (20-23).

Treatment with ABT-737 alone resulted only at a high concentration in a small reduction of the cell viability in the IB133 and IB140 cell lines. Therefore, we concluded that *in vitro* treatment with ABT-737 as single agent has almost no influence on the cell viability. Other studies in chondrosarcomas and small cell lung carcinomas also revealed limited activity of ABT-737 monotherapy (24). To explore a possible role of the Bcl-2 family members in response to conventional chemotherapy, we combined ABT-737 with doxorubicin, which is commonly used as single agent in the treatment of high grade soft tissue sarcomas (9, 10). Indeed, a synergistic effect was seen as cell viability decreased and apoptosis could be shown. Interestingly, single treatment with doxorubicin decreased cell viability, but had no impact on apoptosis. This suggests that even though cells stopped proliferating and lost their viability, they failed to activate their apoptotic machinery at the doses tested, most probably due to expression of Bcl-2 family members. This is in accordance with the low response rates and the poor impact of chemotherapeutic agents on overall survival of patients.

The four leiomyosarcoma cell lines were analyzed using the AmpliSeq™ Cancer Hotspot Panel v2. We detected a *CTNNB1* S45C variation in IB140, with a coverage of 61 reads. Revision of the corresponding primary tumour confirmed the diagnosis of leiomyosarcoma as well as the presence of the same mutation (Suppl fig 2). Nuclear staining for β-catenin was absent, so the significance of the S45C *CTNNB1* alteration in this tumour remains unclear. Two cell lines showed alterations in *TP53*. The leiomyosarcoma cell lines which showed the highest synergy (LMS05 and IB140) have a wild type *TP53* gene, whereas the cell line with the lowest synergy (LMS04) did not. TP53 is a major protein in the apoptotic signaling pathway and when *TP53* is mutated the apoptotic response is impaired (25). Furthermore, wild type *TP53* increases the responsiveness to chemotherapy by down-regulating multidrug resistance-1 expression (26). Even though the numbers are small, a mutation in this key protein might explain the difference in treatment response for LMS04 and IB133.

Furthermore, the cell lines showed Mcl-1 expression at the same level or stronger as compared to the HeLa control, and it has been reported that Mcl-1 in HeLa cells plays an important role in the sensitization to ABT-737 (27). High Mcl-1 levels are associated with resistance of cell lines to ABT-737 and downregulation of Mcl-1 sensitizes carcinoma cells to doxorubicin (27-29). This suggests that Mcl-1 expression might be important in the anti-apoptotic mechanisms in the LMS cell lines, and modulation of this protein level might be a target to increase the effect of ABT-737 and doxorubicin treatment. Treatment with ABT-737, doxorubicin or a combination did not change the expression levels of Bcl-xL, Bcl-w or Bcl-2. This is comparable to other studies in which there were only slight differences in the expression of Bcl-2 and Bcl-xL after treatment (21, 30). Thus, ABT-737 and doxorubicin do not down regulate the expression of the Bcl-2 family members at the protein level, and may instead block their inhibitory effect on Bak/Bax proteins in the mitochondrial membrane (31).

The effect of ABT-263 (navitoclax®, Abbott), a structural analogue of ABT-737, has been analyzed in toxicity phase I and II studies in small cell lung carcinomas. Thrombocytopenia was the most frequently observed adverse effect. However, marrow compensation platelet counts showed partial recovery when dosing was sustained, and in the week without dosing, recovery to baseline was seen. Diarrhea, vomiting, nausea and fatigue were other common adverse effects (24, 32). These adverse events are also frequently seen after chemotherapeutic treatment but are most of the time not life-threatening, suggesting that the preclinical evidence generated in this report may be realistically translated to clinical application.

In conclusion, our results show that inhibition of Bcl-2 family members using ABT-737 renders leiomyosarcoma cell lines more sensitive to doxorubicin, especially in the presence of wildtype *TP53*, suggesting new potential therapeutic options for leiomyosarcoma patients with metastatic or inoperable disease.

Acknowledgements

The authors thank Inge Briaire-de Bruijn and Yvonne de Jong for expert technical assistance.

Conflict of interest

The authors declare no conflict of interest.

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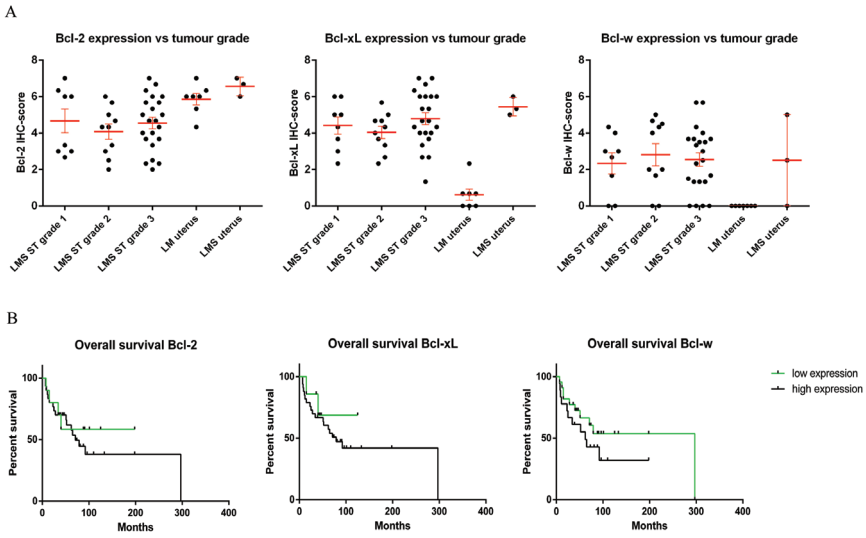
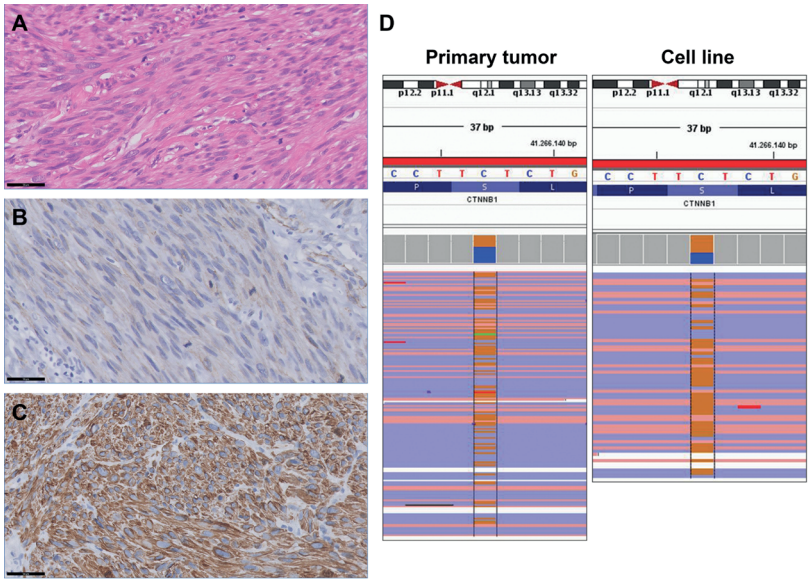
Supplementary Information



Supplementary figure 1. Immunohistochemistry score in relation with histological grade and overall survival. (**A**) The overall score was not correlated with histological grade. (**B**) Leiomyosarcoma patients were divided into two groups (high >3 and low ≤3) based on the immunohistochemical score of Bcl-2, Bcl-xL and Bcl-w. A shorter overall survival was seen for patients with a high expression of Bcl-2, Bcl-xL and Bcl-w, although the difference was not significant.



Supplementary figure 2. Histology, immunohistochemistry and NGS analysis of the IB140 cell line and the corresponding primary tumour. (**A**) H&E staining of the primary tumour showed high mitotic activity, (**B**) no nuclear β-catenin expression, and (**C**) a strong and diffuse staining for caldesmon. The

morphology and immunohistochemistry are highly characteristic of leiomyosarcoma. (**D**) NGS analysis of the primary tumour and the cell line showed *CTNNB1 c.134C>G* (p.S45C) variation in the cell line (total reads 61; 24 C (39%), 37 G (61%)) and primary tumour (total reads 2787; 1642 C (59%), 1135 G 41%)).

Supplementary table 1. Clinicopathological data of leiomyosarcoma patients.

Sample	Age	Gender	P/R/M	Tumour localisation	Grade
1	39	M	P	Jejunum	1
2	60	F	P	Rectum	1
3	55	F	P	Retroperitoneum	1
4	76	F	R	Retroperitoneum	1
5	41	M	P	Soft tissue jaw	1
6	73	M	R	Duodenum	1
7	48	F	P	Rectus abdominis	1
8	32	M	P	Musculus psoas	1
9	23	M	R	Lower extremity	2
10	74	M	P	Upper extremity	2
11	56	F	P	Lower extremity	2
12	79	M	P	Subcutis back	2
13	43	M	P	Upper extremity	2
14	80	F	P	Lower extremity	2
15	30	M	P	Colon	2
16	76	F	P	Lower extremity	2
17	65	F	P	Lower extremity	2
18	55	M	M	Liver	2
19	80	F	P	Lower extremity	3
20	86	M	P	Lower extremity	3
21	43	F	P	Pelvis	3
22	67	F	P	Upper extremity	3
23	68	F	P	Lower extremity	3
24	70	M	P	Lower extremity	3
25	65	F	P	Lower extremity	3
26	70	M	P	Lower extremity	3
27	56	F	P	Lower extremity	3
28	75	M	P	Lower extremity	3
29	67	F	P	Upper extremity	3
30	41	F	P	Lower extremity	3
31	70	F	P	Retroperitoneum	3
32	84	F	P	Upper extremity	3
33	74	F	M	Pleura	3
34	50	F	P	Arteria femoralis	3
35	58	M	P	Lower extremity	3
36	52	F	P	Lower extremity	3
37	48	M	P	Stomach	3
38	76	F	P	Lower extremity	3
39*	50	F	P	Retroperitoneum	3
40*	50	F	M	Abdomen	3
41	39	F	P	Uterus	NA

Supplementary table 1. Continued

Sample	Age	Gender	P/R/M	Tumour localisation	Grade
42	61	F	P	Uterus	NA
43	52	F	P	Uterus	NA

P=primary tumor; R=recurrence; M=metastasis; NA=non applicable; *primary and metastatic tumour sample from the same patient. None of the included samples were pretreated with chemotherapy or radiation.

Supplementary table 2. The characteristics of the four leiomyosarcoma cell lines.

Cell lines	Passage number	Diagnosis and grade	Patient	*TP53*[$]	Cancer Hotspot panel
LMS04	P45	LMS III	F/54	Homozygous deletion	KDR
LMS05	P34	LMS II ST	M/76	Wild type: exon 4-9	No mutations
IB133	P21	LMS III RP	F/56	Deletion: exon 2 and 3	No mutations
IB140	P22	LMS II ST	M/51	Wild type: exon 1-11	CTNNB1 S45C[#]

[#] The mutation was also found in the primary tumour, which upon revision demonstrated abundant mitotic activity, strong expression of heavy caldesmon, and lack of nuclear staining for beta-catenin, consistent with the diagnosis of leiomyosarcoma (Suppl fig 2).

$ determined by Sanger sequencing.

LMS: leiomyosarcoma, RP: retroperitoneum, ST: soft tissue.

Supplementary table 3. Details of antibodies used for immunohistochemistry.

Antibody	Manufacturer	Dilution	Antigen retrieval
Bcl-2	Abcam	1/6	Citrate, pre-incubation 5% Elk milk
Bcl-xL	Cell signaling	1/3	Citrate, DAB+
Bcl-w	Abcam	1/3	Citrate, DAB+

Supplementary table 4. Details of antibodies used for western blot.

Antibody/clone	Species	Manufacturer	Dilution
Bcl-2/50E3	Rabbit	Cell signaling technology, Leiden, The Netherlands	1:1000
Bcl-xL/54H6	Rabbit	Cell signaling technology	1:1000
Bcl-w/31H4	Rabbit	Cell signaling technology	1:1000
Mcl-1	Rabbit	Cell signaling technology	1:1000
PARP/46D11	Rabbit	Cell signaling technology	1:1000
Alpha-tubulin/DM1A	Mouse	Abcam, Cambridge, UK	1:1000