# Chapter 5

# Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma *in vitro*: BCL-2 family members cause chemoresistance

This chapter is based on the manuscript: van Oosterwijk JG, Herpers B, Meijer D, Briaire de Bruijn IH, Cleton-Jansen AM, Gelderblom H, van de Water B, Bovée JVMG. *Ann Onc. 2012; 23(6): 1617-26*

**Abstract**

Chondrosarcomas are malignant cartilage forming tumors notorious for their resistance to conventional chemo- and radiotherapy. Postulated explanations describe the inaccessibility due to abundant hyaline cartilaginous matrix, presence of multi-drug resistance (MDR) pumps, and expression of anti-apoptotic BCL-2 family members.
We studied the sensitivity of chondrosarcoma cell lines (SW1353, CH2879, JJ012, OUMS27) and 2 primary cultures for doxorubicin and cisplatin. We examined the role of extracellular matrix using 3D pellet models and MDR pump activity using FACS analysis. The role of BCL2 family members was investigated using the BH3 mimetic ABT-737.
Chondrosarcoma cells showed highest resistance to cisplatin. 3D cell pellets, morphologically strongly resembling chondrosarcoma *in vivo*, confirmed nuclear incorporation of doxorubicin. MDR pump activity was heterogeneous among cultures. Chondrosarcoma cells responded to ABT-737 and combination with doxorubicin led to complete loss of cell viability and apoptosis with cytochrome C release.
Despite MDR pump activity and abundance of hyaline cartilaginous matrix, doxorubicin is able to accumulate in the cell nuclei. By repairing the apoptotic machinery we were able to sensitize chondrosarcoma cells to doxorubicin and cisplatin, indicating an important role for BCL-2 family members in chemoresistance and a promising new treatment strategy for inoperable chondrosarcoma.

**Introduction**

Chondrosarcoma is a malignant hyaline cartilaginous tumor of the bone, and is the second most common primary bone malignancy in humans. Several subtypes of chondrosarcoma exist, with conventional chondrosarcoma being the most common (~80%) (1). Conventional chondrosarcomas (CS) can occur either in the medulla of the bone (central chondrosarcoma) or at the surface (peripheral chondrosarcoma). As chondrosarcomas have clinically proven to be resistant to conventional chemo- and radiotherapy, no (systemic) treatment can be offered for high grade, irresectable, or metastatic tumors (2;3). Recent literature focuses on investigating activated pathways and assessing their validity as novel targeted treatment strategies for these inoperable tumors (4).

Several hypotheses explaining primary resistance in conventional chondrosarcoma have been postulated. It is suggested that chemoresistance is caused by a possible impediment of chemotherapeutic agents to penetrate the extracellular matrix (5). In grade I chondrosarcomas, there is a vast amount of hyaline extracellular matrix (3;6-8). Low grade chondrosarcomas are composed of slowly dividing cells while conventional chemo- and radiotherapy target rapidly dividing cells (9). In higher grade chondrosarcomas, the extracellular matrix appears more myxoid and cells divide more rapidly but especially in these tumors

chemoresistance confers an important clinical problem. Alternatively, the activity of multi-drug resistance (MDR) pumps may cause chemoresistance in chondrosarcoma, as has been described in various cancer types. The role of P-glycoprotein in resistance to doxorubicin has been shown in two chondrosarcoma cell lines (10) and retrospective studies (11). Finally, the parathyroid hormone related peptide (PTHrP) pathway was found to be activated in chondrosarcoma with high BCL-2 expression, correlating with increasing histological grade (6;8). Since BCL-2 is an anti-apoptotic protein, its aberrant expression may contribute to chondrosarcoma resistance (12).

In order to determine the cause of chemoresistance in conventional central chondrosarcoma, we tested the different hypotheses described above: 3D pellet models were used to investigate the role of matrix surrounding the tumor cells. We assessed the activity of the multidrug resistance pumps and established the role of BCL-2 family members. Our data indicate that tumor matrix and MDR pumps are not critical to chemoresistance of chondrosarcoma whereas the activity of anti-apoptotic BCL-2 family members controls the onset of cell killing caused by classical anticancer drugs.

## Methods

*Compounds*

Doxorubicin and cisplatin were obtained from the in-house hospital pharmacy in a 0.9% NaCl solution. Therapeutic concentrations of doxorubicin in patients are 5-100μM with an *in vitro* range of 1-10 μM, for cisplatin these are 3-13μM with an *in vitro* range of 1-50μM (13). ABT-737 (Abbott Laboratories Inc, IL, USA) and R-roscovitine (R7772, Sigma Aldrich, Zwijndrecht, The Netherlands) were dissolved in DMSO.

*Cell culture*

Acute Lymphatic Leukemia cell line HL-60, chondrocyte cell line LBPVA (14), osteosarcoma cell line MNNG, as well as chondrosarcoma cell lines and primary cultures (table 5.1), were cultured in RPMI1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 1% L-glutamax, 1% penicillin/streptomycin (100U/mL), and 10% (cell lines) or 20% (primary cultures), heat-inactivated Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Normal human mesenchymal stem cells (MSC) L2069 (15) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 1 mg/ml glucose, 2% penicillin/streptomycin (P/S), and 10% Fetal Bovine Serum acetyl salicyclic acid (ASA) (FBS ASA; Hyclone, Logan, UT). Cells were grown at 37°C in a humidified incubator with 95% air and 5% $CO_2$. All primary chondrosarcoma cultures were generated from chondrosarcomas surgically resected in our institute. All samples were coded 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). Cells were cultured until stably multiplying and chondrogenic phenotype was confirmed using RT-PCR for collagen I, IIB, III, and X, aggrecan, and SOX9 (16). DNA was isolated from the cells for subsequent TP53 mutation analysis (17). Identity of cell lines was confirmed using the PowerPlex® 1.2 system after completion of experiments (Promega Benelux BV, Leiden, The Netherlands).

*Cell counting and viability assay*

Cell counting for proliferation assay was performed with a Casey ® cell counter (Roche Applied Sciences, Almere, the Netherlands). Chondrosarcoma cell lines and primary cultures were plated in 96 well plates for viability assessment ($2\text{-}5x10^4$ cells/well for cell lines and $2x10^5$ for primary cultures) and allowed to adhere overnight. Cells were incubated with the drugs for 24, 48, or 72 hours, after which a WST-1 assay was performed according to manufacturer's instruction (Cat. No. 11 644 807 001; Roche Diagnostics GmbH, Penzberg, Germany) and analyzed with a light spectrometer (Victor$^3$V, 1420 Multilabel counter, Perkin Elmer, NL). All experiments were performed in triplicate at least three times. Graphs show data from one representative experiment. Error bars indicate standard deviation. Synergy was calculated using the Chou & Talalay method for Combination Index calculations (18).

*3D Pellet model*

3D cell pellets were generated from cultures CH2879, OUMS27, L835, and MSCs L2069 as described (15). Cell pellets were allowed to differentiate for 4 weeks after which they were treated with either 1μM or 10μM doxorubicin 2x week. After a total of 6 weeks, pellets were harvested, washed in PBS 3x, fixed in formalin, embedded in paraffin. Pellet slides were mounted using 4'-6-diamidino-2phenylindole (DAPI) -containing VECTASHIELD (Vector Laboratories, Burlingame, CA) and examined under a Leica DM500B fluorescent microscope. Doxorubicin incorporation was recorded at 480nm. Pellet morphology and matrix formation were examined using H&E staining and 1% toluidine blue staining (Brocacef Holding, Maarssen, The Netherlands). Immunohistochemistry for Ki-67 (DAKO, Heverlee, Belgium) and cleaved caspase 3 (Cell Signaling, Leiden, The Netherlands) were performed as described (19). Percentage of positive cells was calculated using NuclearQuant software (3DHISTECH, Budapest, Hungary).

*Multidrug resistance pump assay*

Activities of three multidrug resistance pumps Multidrug Resistance Protein 1 (MDR1), Multidrug Resistance-Associated Protein 1 (MRP1), and Breast Cancer Resistance Protein (BCRP) were measured using the MDQ assay (SOLVO Biotechnology, Budapest, Hungary) according to manufacturer's instructions in all cell lines. Ligand incorporation was analyzed using FACS

Table 5.1. Description of chondrosarcoma cell lines and primary cultures

| Cell Line | Bone of origin | Grade | Gender | Age | Passage | Expression cartilage markers (Real-Time PCR) | P53 | MDR pump activity | Reference |
|---|---|---|---|---|---|---|---|---|---|
| SW1353 | Humerus | II | F | 72 | 21 | Col 1, 2B, 3,10,aggr, sox9[a] | V203L | MDR1 | ATCC |
| OUMS27 | Humerus | III | M | 65 | 27 | Col 1,2B,3,10,aggr [a] | 12434-35 del CC | MDR1/ BCRP | (44) |
| CH2879 | Chest wall | III | F | 35 | >80 | Col 1, 2A [a] | wildtype | MRP1/ MDR1/ BCRP | (45) |
| JJ012 | Femur | II | M | 39 | 9 | Aggr, sox9 [a] | G199V | MRP1/ MDR1/ BCRP | (46) |
| L835[b] | Humerus | III | M | 55 | 15 | Col 1, 2A, 3, 10, aggr [a] | wildtype | MDR1/ BCRP | |
| L869[b] | Tibia | II | M | 52 | 27 | Col 1,2B,3,10,aggr [a] | wildtype | MRP1/ MDR1/ BCRP | |

analysis on a BD™ LSR II flow cytometer. Calcein incorporation was recorded at 488nm, and mitoxantrone at 633nm. Dead cells were labeled with Propidium Iodide (Sigma Aldrich, Zwijndrecht, The Netherlands) (20) and gating was set at 10.000 live cells. Means of Geometric counts were calculated from all events. Experiments were performed in triplicate on one day using the same instrument settings.

*BCL-2 and MCL1 expression analysis*

A standard quantitative reverse transcriptase PCR (qRT-PCR) with SYBR green (16) was performed for MCL1 and BCL-2. Primers were designed using primer3 software (http://frodo.wi.mit.edu/primer3/) (Table 5.2) and ordered from ISOGEN Bioscience BV (Maarssen, The Netherlands). Relative gene expression levels were normalized for the amount of cDNA input using the genes *CYPa, CPSF, and GPR108, based on their constant expression in chondrosarcoma (21). For immunoblotting, 20µg of each sample was run on SDS-PAGE and proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, UK) using electrophoresis. Membranes were preincubated with skinned milk in phophate buffered saline-Tween 0.05%. Rabbit monoclonal antibodies against BCLXL (54H6), BCL-2 (50E3), and MCL1 (4572) were obtained from Cell Signaling Technology (Leiden, the Netherlands). Mouse monoclonal antibody against alpha-tubulin (DM1A) was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).*

**Table 5.2: Primers used in qRT-PCR**

| **Primer** | | **Sequence** |
|---|---|---|
| **MCL1** | forward | 5’ GGT GGC ATC AGG AAT GT 3’ |
| | reverse | 5’ ATC AAT GGG GAG CAC T 3’ |
| **BCL-2** | forward | 5’ ACA TCG CCC TGT GGA TGA CT 3’ |
| | reverse | 5’ GGG CCG TAC AGT TCC ACA AA 3’ |

*Apoptosis assay*

20.000 cells were grown in black 96-well microclear plates (Greiner®, Sigma-Aldrich, Zwijndrecht, The Netherlands) to perform a live cell apoptosis assay based on the binding of AnnexinV-Alexa633 conjugate (prepared as in (22)) to phosphatidyl-serine on the outer membrane of apoptotic cells. Increase of fluorescent apoptotic cells after drug exposure was followed by time-lapse imaging with 30 minute intervals using the BD Pathway® 855 (Becton Dickinson, Breda, The Netherlands) for 24 hours. Time series were quantified using in house developed macros for Image-Pro Plus (Media Cybernetics, Bethesda, USA). Apoptotic cells were expressed as the total pixel area of fluorescent objects in each frame. Drugs were added 0, 0 and 24, or 0, 24 and 48 hours before imaging; last 24 hours of treatment of each culture was measured using AnnexinV labeling

immediately prior to imaging. To establish apoptosis specificity of the assay, all caspase activity was blocked using the pan-caspase inhibitor, z-VAD-fmk (Bachem-Holding AG, Weil am Rhein, Germany) at 50µM, 30 minutes before and continued during treatment and imaging. All experiments were performed in triplicate and at least three times. Error bars show standard deviation from one representative experiment.

*Immunofluorescence*
Cells were fixed in 4% buffered formaldehyde (Added Pharma, Oss, The Netherlands). Washing steps were performed with 1x PBS and blocking with 0.5% BSA + 0.1% Triton X100 in 1x PBS (TBP) at room temperature. Immunofluorescent staining was performed for cytochrome C (556432, BD PharMingen, CA, USA) and cleaved caspase 3 (9664, Cell Signaling, Leiden, The Netherlands) overnight after which washing steps were performed with TBP. Cells were post-fixed in 4% formaldehyde for 5 minutes. Imaging was performed at a Nikon TiE2000 confocal laser scanning microscope (Nikon, Amstelveen, The Netherlands) at 20x magnification.

## Results

*Chondrosarcoma cultures are sensitive to doxorubicin while resistant to cisplatin*
For doxorubicin an $IC_{50}$ of 6µM was observed in the most responsive cell line (OUMS27) and an $IC_{50}$ of >100µM in the least responsive cell line (SW1353) (figure 5.1A, table 5.3). Cell cultures showed a poor response to cisplatin treatment; total resistance even at 80µM was observed for L869 and SW1353 (figure 5.1B, table 5.3). Live cell imaging with AnnexinV labeling, performed with 10µM doxorubicin and 50µM cisplatin, showed a significant induction of apoptosis ($p<0.05$ one-way ANOVA) for cell lines responsive to doxorubicin, such as OUMS27, but not after cisplatin treatment (figure 5.1C). For non responsive cultures, such as L835, no significant induction of apoptosis could be achieved after either treatment (figure 5.1D). All apoptosis could be inhibited using a general caspase inhibitor zVAD-fmk ($p<0.05$ one-way ANOVA) (figure 5.1C, D). p53 analysis showed three cultures to be negative for p53 mutations with no correlation of mutation status with drug response (table 5.1).

*3D pellets show chondrosarcoma morphology and doxorubicin incorporation*
3D pellet cultures were created from OUMS27, CH2879, and L835 and 1 MSC culture. Cell pellets had a maximal diameter of about 1mm. 3D pellets made from the MSC culture showed ample matrix surrounding the cells, resembling the cellular make-up of a low grade chondrosarcoma (figure 5.2A, Ai). 3D cell pellets from high grade chondrosarcoma cell lines (grade II, III) morphologically resembled high grade chondrosarcoma (figure 5.2B, Bi) showing high cellularity.

Doxorubicin incorporation showed nuclear accumulation at both 1µM and 10µM, even in L835 which shows resistance to doxorubicin at 10µM (figure 5.2D-Dii), and in MSC cell pellets with ample matrix formation (results not shown). Ki-67 staining showed decreased proliferation in all pellets after treatment although this was not significant (figure 5.2Ci, E-Eii). Cleaved caspase 3 staining on pellets showed 10% of cells had undergone apoptosis in non-treated pellets, whereas pellets treated with doxorubicin showed caspase activation in almost all cells ($p<0.05$, one-way ANOVA, figure 5.2C, F-Fii). Activation of caspase in cell pellets as opposed to the resistance we showed in figure 5.1 in the 2D cultures is due to differences in exposure to doxorubicin: 3D pellets were treated 4x and 2D cultures only once.

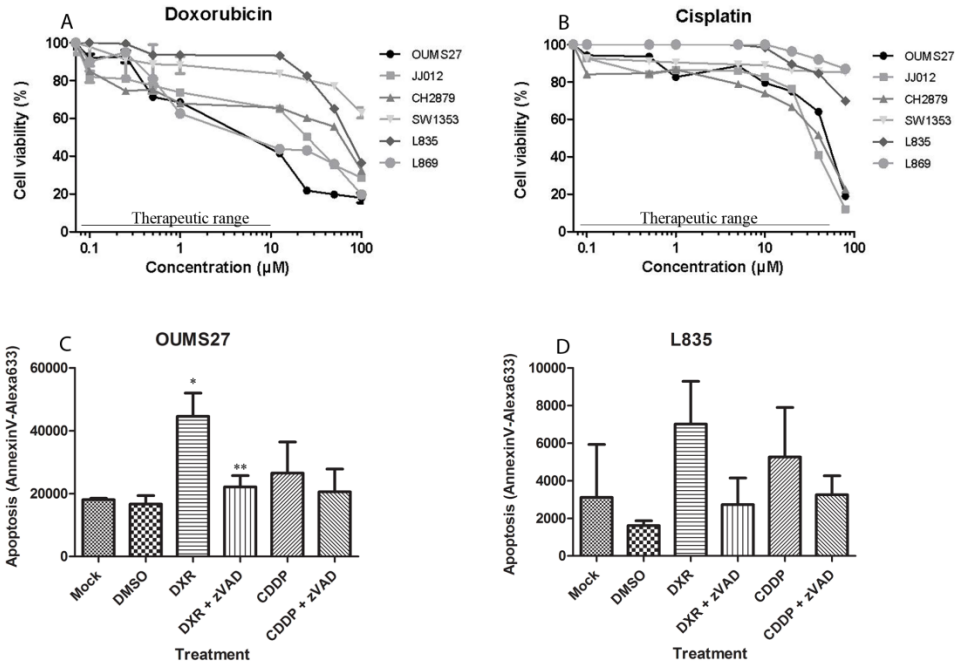


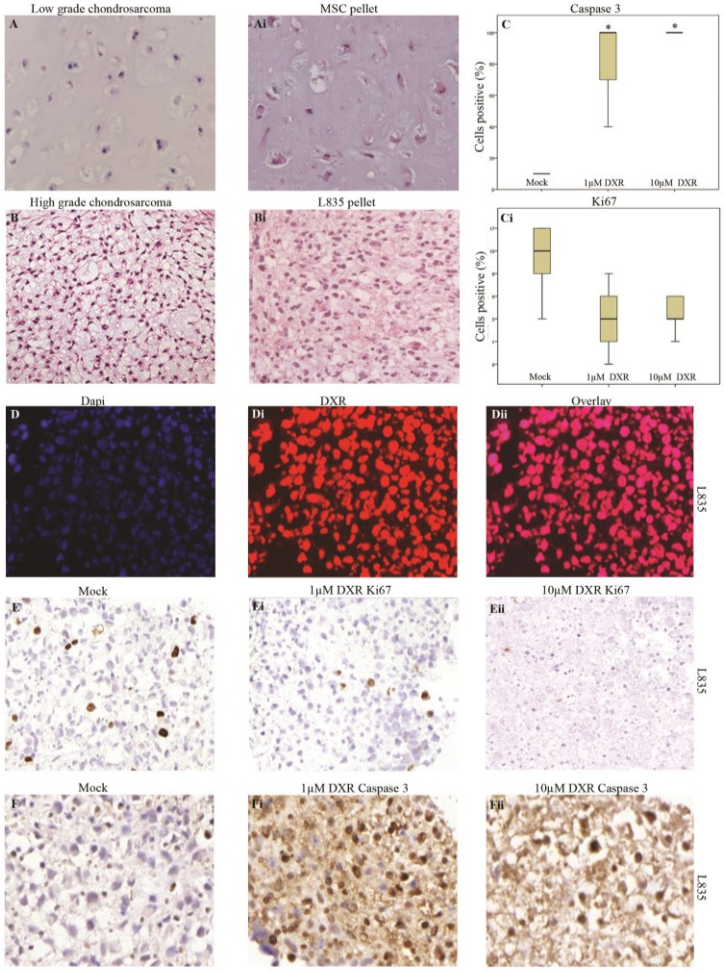

**Figure 5.1. Cell viability of chondrosarcoma cells is inhibited and apoptosis induced by high dose doxorubicin but not cisplatin**. Dose response curves for chondrosarcoma cell lines show sensitivity to doxorubicin at high concentrations (A) and resistance to cisplatin (B) after 72 hours incubation. OUMS27 cells show a significant increase of apoptosis (AnnexinV) after addition of doxorubicin 1µM (DXR), but not after addition of cisplatin 5µM (CDDP) (C). Addition of zVAD-fmk (zVAD) before doxorubicin allowed for significant inhibition of apoptosis (fluorescence intensity shown by AnnexinV binding, measured over 24hrs). L835 shows no significant induction of apoptosis after either treatment (D). Error bars in graphs show standard deviations from 3 measurements.
*significant increase of apoptosis compared to mock (medium only treatment) and DMSO treatment (one-way ANOVA, $p<0.05$) ** significant decrease of apoptosis compared to treatment without zVAD (one-way ANOVA, $p<0.05$)

**Table 5.3. IC50 concentrations and Combination Indices per cell line**

| | DXR (µM)[a] | CDDP (µM) [a] | ABT-737 (µM) [a] | Combination DXR (µM) [b] | Combination ABT-737 (µM) [b] | Combination index (CI) [d e] | Combination CDDP (µM) [c] | Combination ABT-737 (µM) [c] | Combination index (CI) [d f] |
|---|---|---|---|---|---|---|---|---|---|
| **OUMS27** | 6 | 50 | 25 | 0,1 | 5 | 0,22 | 0,1 | 5 | 0,20 |
| **CH2879** | 60 | 40 | 20 | 0,1 | 0,25 | 0,01 | 1 | 0,1 | 0,03 |
| **JJ012** | 25 | 35 | 100 [g] | 0,1 | 0,5 | 0,01 | 0,1 | 5 | 0,05 |
| **SW1353** | 150[g] | 400 [g] | 135 [g] | 1 | 1 | 0,01 | 0,5 | 5 | 0,04 |
| **L835** | 70 | 200 [g] | 60 [g] | 0,25 | 0,5 | 0,01 | 5 | 5 | 0,11 |

[a] Concentrations needed to achieve 50% reduction in cell viability
[b] Concentrations needed to achieve 50% reduction in cell viability during combination assay with Doxorubicin and ABT-737
[c] Concentrations needed to achieve 50% reduction in cell viability during combination assay with Cisplatin and ABT-737
[d] Combination index as calculated according to the Chou & Talalay method for synergistic relationships. A combination index <1 indicates synergy between drugs.
[e] Combination index for the combination Doxorubicin and ABT-737
[f] Combination index for the combination Cisplatin and ABT-737
[g] Values are approximations as IC50 values were not achieved in experiments

**Figure 5.2. 3D pellet models show similar cellular organization as chondrosarcoma tissues and doxorubicin incorporation.** Low grade chondrosarcoma (A) strongly resembles 3D pellet created from MSC cell culture (L2069) (A'). B: high grade chondrosarcoma strongly resembles 3D pellet created from L835, a grade III chondrosarcoma cell culture (B'). D-D": Fluorescence microscopy of L835 pellet at 120x: dapi staining (D) shows nuclear organization, doxorubicin incorporation is strong at 10µM (D'), overlay (D") shows doxorubicin has successfully entered the nuclei. Pooled data of pellets (L2069, OUMS27, CH2879, and L835) with error bars indicating lowest and highest counts, shows that cleaved caspase 3 staining is present in 100% of cells after 10µM doxorubicin (C) and Ki67 staining in about 5% of cells (C'). Staining for Ki67 (E-E") and cleaved caspase 3 (F-F") in L835 pellets treated with 0, 1, and 10 µM doxorubicin show decrease in number of cells proliferating and an increase in apoptosis. After treatment with 10µM, cellularity decreases, pictures were obtained at 40x magnification




* Significant difference between treated and untreated (one-way ANOVA $p < 0.05$)

*MDR pump activity is found in chondrosarcoma cultures*

We determined MDR pump activity in chondrosarcoma cell lines and primary cultures, and in a chondrocyte cell line LBPVA (14). Pump activity was compared to HL-60, described to have high MDR1 activity, and to osteosarcoma cell line MNNG which has been described to express MDR1 and MRP1, with higher MDR1 activity (23). This was confirmed using our MDQ assay. Considerable heterogeneity was observed in the activity of MDR pumps throughout the cell lines, with MDR1 (p-glycoprotein) demonstrating activity in all cell lines except OUMS27 and LBPVA. MRP1 activity was observed in LBPVA, 2 cell lines (JJ012, CH2879) and primary cultures. L869, JJ012, and CH2879 showed activity of all three pumps, with highest activity observed in L869 (figure 5.3, table 5.1).

*Inhibition of BCL-2 family members but not MCL-1 reduces cell viability and increases apoptosis*

RT-PCR confirmed mRNA expression of BCL-2 and BCL-$X_L$, with expression levels similar to that of HL-60 (figure 5.4A) in which these expression levels have been linked to chemoresistance (24). Western blot analysis showed high expression of BCL-$X_L$ in all cell lines (figure 5.4B) while BCL-2 expression was less uniform. Using ABT-737, HL-60 showed complete loss in cell viability at 0.5μM, as reported (25). In chondrosarcoma cell lines, CH2879 and OUMS27 showed the best response, with $IC_{50}$s of 20μM and 25μM, respectively. Cell counting showed cell proliferation at 10μM to correspond with cell viability (figure 5.5). L835 showed intermediate response to ABT-737 ($IC_{50}$ 60μM), for other cell lines, the $IC_{50}$ values exceeded 100μM (figure 5.4C, table 5.3). Live cell imaging during 24 hours of ABT-737 (25μM) treatment showed a significant increase of apoptotic cells compared to DMSO treatment, all of which could be inhibited using the caspase inhibitor zVAD-fmk (figure 5.4E,F). It has been described that in tumors resistant to BCL-2 inhibition, MCL1 upregulation might play a role (26). Indeed RT-PCR demonstrated 2-fold increases of MCL1 mRNA expression in chondrosarcoma cell lines compared to household genes (fig 5.4A). Also, MCL1 expression was confirmed at the protein level using western blot (fig 5.4B) although lower than BCL-2 or BCL-$X_L$. We used R-roscovitine, a non-specific CDK inhibitor described to downregulate MCL-1 (27) to assess the role of MCL-1 in the response to ABT-737. High concentrations of R-roscovitine (5μM and 10μM) failed to induce reduction in cell viability (figure 5.4D) or apoptosis on its own or in combination with ABT-737 (results not shown).

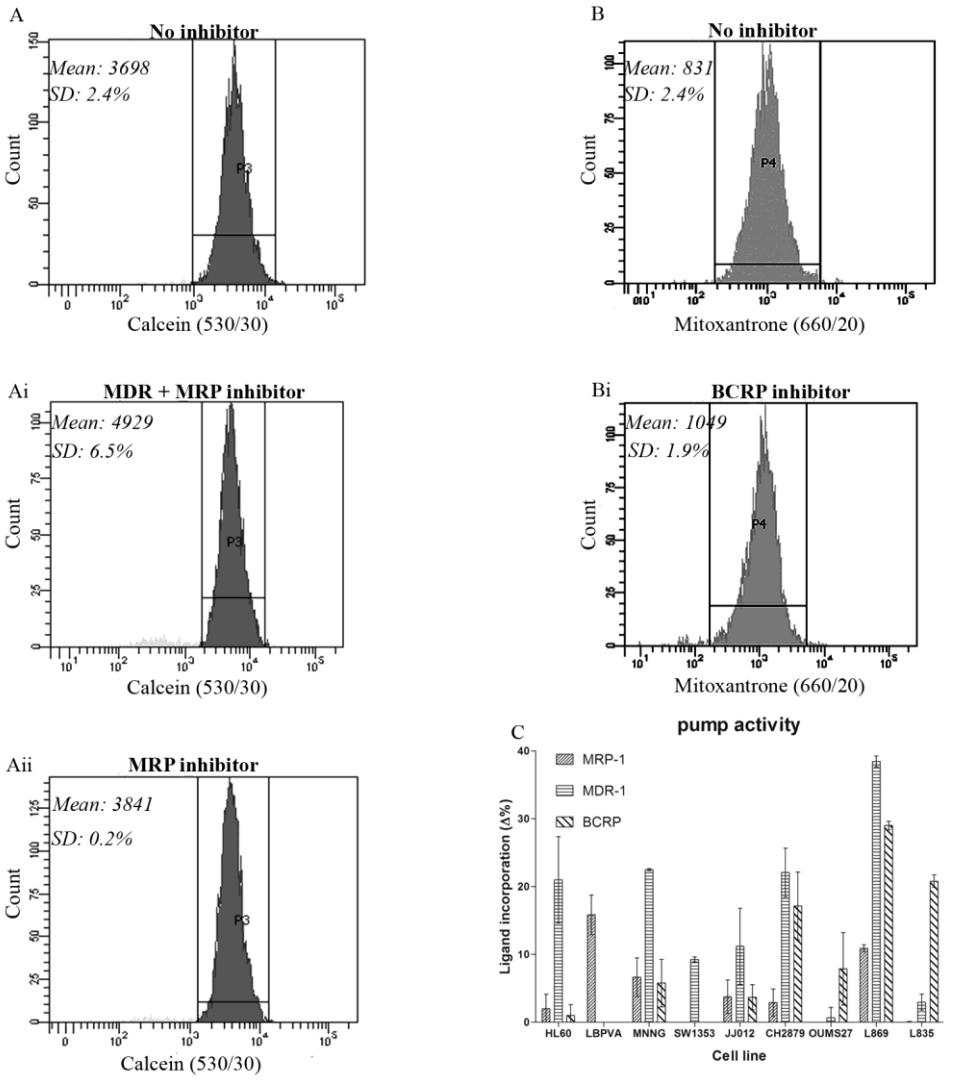



**Figure 5.3. Activity of Multi-Drug-Resistance pumps in chondrosarcoma cultures.**
Ligand incorporation with and without the presence of pump inhibitors was measured using FACS analysis. A, B:FACS analysis of L835; insets show mean GEO counts with the standard deviation. C: graphical representation of pump activity per cell line. Ligand incorporation (Δ%) represents the difference of total GEO counts with inhibitor to total GEO counts without inhibitor. Activity was compared to HL-60 cell line and to an osteosarcoma cell line (MNNG). MRP1 activity was higher than in HL-60 in the chondrocytic cell line and in L869, but equal in JJ012 and CH2879. Activity of the MDR1 pump (p-glycoprotein) was observed in nearly all cultures. BCRP activity varied highly among cultures.

*BCL-2 inhibition can reverse chemoresistance of chondrosarcoma*

Simultaneous addition of ABT-737 and doxorubicin or cisplatin to cell cultures did not show additive effects. Combination treatments proved most effective with respect to cell viability when at least 24 hours were left in between drug administration, with ABT-737 treatment before and after doxorubicin or cisplatin addition (results not shown). The combination of doxorubicin or cisplatin with ABT-737 allowed for the reduction of both treatments to sublethal concentrations (table 5.3). Combination Index (CI) calculations for synergy analysis revealed high synergy between ABT-737 and both doxorubicin and cisplatin; a mean CI of 0.05 for the combination with doxorubicin and a mean CI of 0.09 for the combination with Cisplatin (table 5.3). The combination of doxorubicin with ABT-737 was most effective also with respect to apoptosis (figure 5.6). Live cell imaging to evaluate apoptosis was performed using 5μM ABT-737 and 1μM doxorubicin or 5μM cisplatin. For most cell lines a clear advantage was observed when ABT-737 treatment was performed 24 hours before doxorubicin or cisplatin treatment; with doxorubicin being the most effective combination (figure 5.6A, C; OUMS27 and CH2879 shown as representatives for these four cell lines). For L835, ABT-737 treatment showed the largest increase in apoptosis when administered after doxorubicin or cisplatin (figure 5.6B). Immunofluorescence for cytochrome C combined with Hoechst staining 24hrs after addition of each drug showed increase in cytochrome C release after addition of each drug; after the third cycle of drug addition almost all cells had undergone apoptosis and few nuclei could be identified (figure 5.6D).

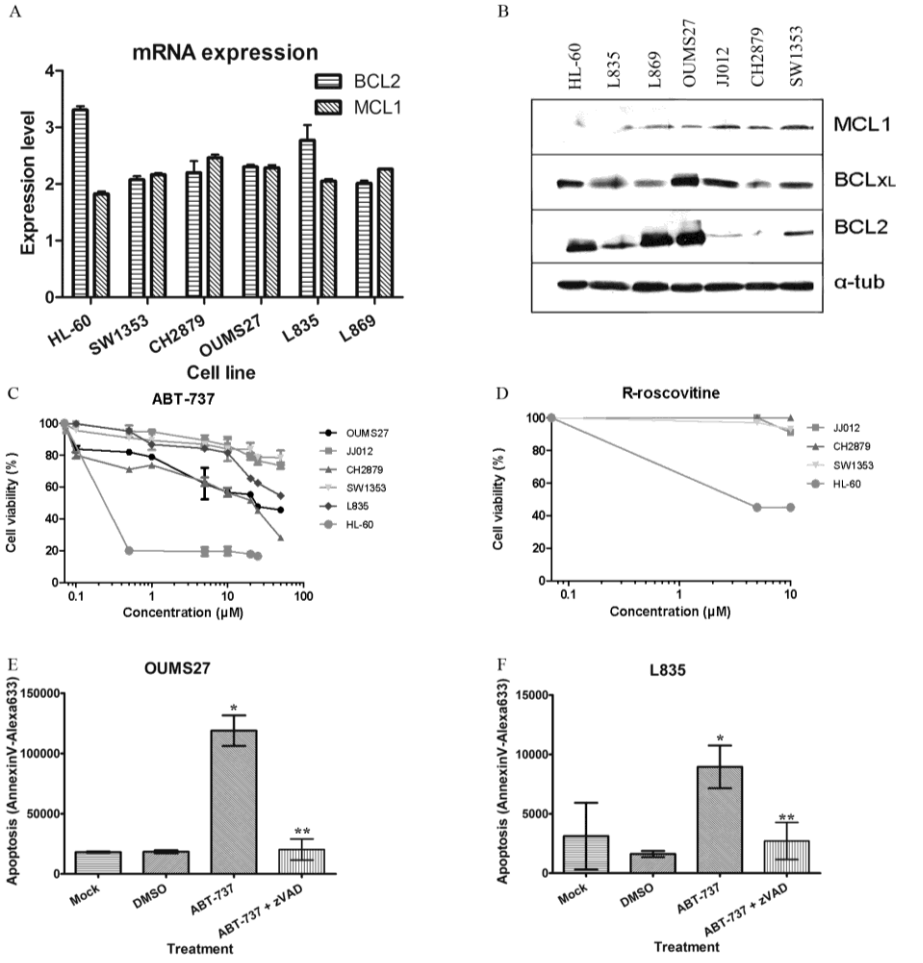



**Figure 5.4. BCL-2 but not MCL1 overexpression protects chondrosarcoma cell lines from apoptosis.** A: normalized mRNA expression levels of BCL-2 and MCL1 in chondrosarcoma cell lines showing constant expression in chondrosarcoma. B: Western Blot analysis of MCL1, BCL-XL, and BCL-2 expression in chondrosarcoma cell lines. BCL-2 and BCL-XL are present in all cell lines, and markedly higher than MCL1. C,D: Cell lines were treated with ABT-737 (C) or R-roscovitine (D) for 72 hrs. Dose response curves show sensitivity to ABT-737 at high concentrations and resistance to R-roscovitine for mitochondrial activity. E,F: OUMS27 and L835 cells show apoptosis (fluorescence intensity shown by AnnexinV binding) after 72hrs of 25μM ABT-737 treatment. Addition of zVAD before ABT-737 allowed for significant inhibition of apoptosis (AnnexinV binding). Error bars in graphs show standard deviations from 3 measurements.
*significant increase of apoptosis compared to mock (medium only treatment) and DMSO treatment (one-way ANOVA, $p<0.05$) ** significant decrease of apoptosis compared to treatment without zVAD (one-way ANOVA, $p<0.05$)

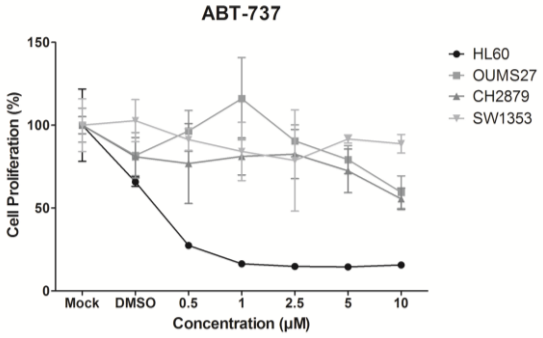



**Figure 5.5 ABT-737 successfully inhibits cell proliferation of chondrosarcoma cells.** Chondrosarcoma and HL-60 cells were treated with medium (mock), DMSO, or ABT-737 for 72hrs. Cell count for mock treatment was put at 100% proliferation. HL-60 cells showed some sensitivity to DMSO treatment. For all cell lines, sensitivities reduction in cell proliferation (cell number) were comparable to those observed in cell viability.

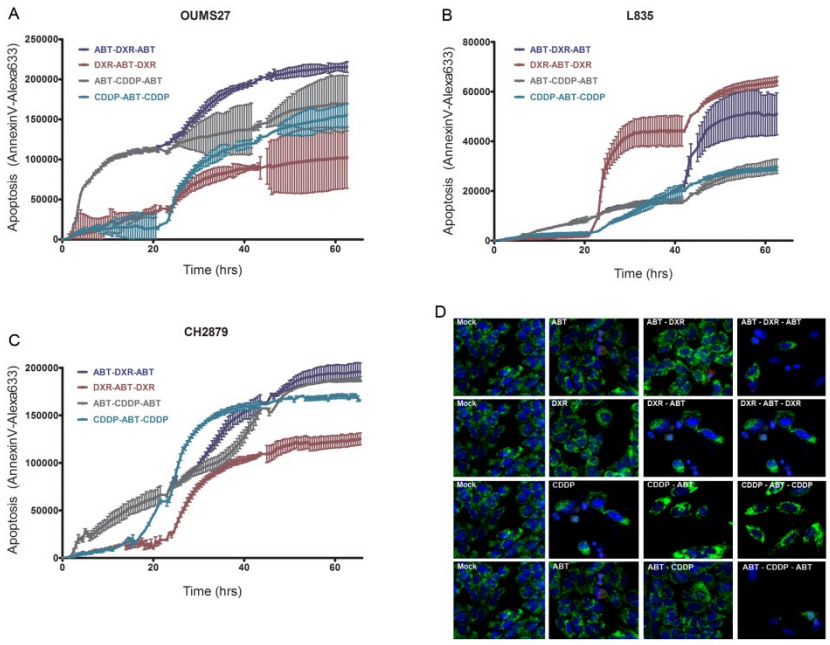



**Figure 5.6. Combination therapy of ABT-737 with doxorubicin and cisplatin induces apoptosis in chondrosarcoma cells**. Cells were treated with respective drugs for 24hrs time periods, apoptosis was monitored with AnnexinV staining. Y axes show total pixel area of AnnexinV positive cells. Combination therapy of ABT-737 with doxorubicin was most effective in inducing apoptosis in all cell lines. OUMS27 and CH2879 (A,C) were most responsive to treatment with ABT-737 before and after doxorubicin (DXR) treatment, whereas L835 (B) seemed slightly more sensitive to doxorubicin treatment before and after ABT-737. Error bars show standard deviation from 3 measurements. D: Increase in cytochrome C release (green) is observed during treatment, with loss of cells at the end of treatment (Hoechst, blue), caspase 3 release (red) could be observed after ABT-737 addition (20x magnification).

## Discussion

Chondrosarcomas are resistant to conventional chemotherapy and radiotherapy. In the ongoing pursuit to find new, targeted, treatment strategies we set out to define the reasons for chemoresistance. Postulated hypotheses regarding the mechanisms underlying chemoresistance include the high abundance of cartilaginous matrix, the expression of MDR pumps, and the expression of anti-apoptotic proteins (5).

We first assessed the sensitivity of our cell lines and primary cultures to single treatments of doxorubicin and cisplatin, the most commonly used chemotherapeutic agents in sarcomas, using 2D cell culture. All cultures responded poorly, especially to cisplatin. As 2D cultures lack matrix formation, we used 3D pellet cultures, shown to be good models for cartilage formation (28;29), to study the effect of the chondroid matrix on diffusion of therapeutic agents *in vitro*. We here demonstrate 3D pellets of MSC and chondrosarcoma cell lines to strongly resemble low grade and high grade chondrosarcoma, respectively. Previously, doxorubicin was reported to remain in chondrosarcoma cells during washout experiments in 2D cell cultures (30;30;31). Using 3D pellets we now show that the chondroid matrix does not hinder doxorubicin from entering the nuclei of the cells.

Numerous reports on the role of MDR pumps in chemoresistance exist. MRP1 (encoded by ABCC1) expression has been described to play a role in resistance to cisplatin (32). P-glycoprotein (ABCB1) activity (33) and BCRP (ABCG2) activity are described to play a role in doxorubicin efflux (34). P-glycoprotein expression has previously been found on the surface of the chondrosarcoma cell lines JJ012 and SW1353 (30;31;35) and endogenous P-glycoprotein expression has been described in human growth plate, suggesting a physiological role (10). Recently, ABT-737 has been shown to be a substrate for P-glycoprotein (36). We show strong variation in the activity of MDR pumps over a wide panel of cell cultures and pump activity could not be correlated with the *in vitro* response to doxorubicin, cisplatin or ABT-737.

Thus, we demonstrate that despite high matrix production (MSCs) and P-glycoprotein activity (CH2879, L835), doxorubicin is able to enter the nuclei of the cells. This suggests a different, more specific mechanism possibly including a defective apoptotic pathway. It has been described that mutations in p53 can influence the response to DNA damaging agents such as doxorubicin (37). However, we found no correlation between p53 status and response.

We previously demonstrated BCL-2 expression in 63-71% of high grade conventional chondrosarcoma (6;8). We investigated the role of anti-apoptotic BCL-2 family members using the BH3 mimetic ABT-737. ABT-737 is a small-molecule inhibitor of the BCL-2 family and has been reported to be effective in, among others, myeloma and ovarian cancer cell lines (38-40). The apoptotic machinery in chondrosarcoma cultures was activated through inhibition of BCL-2 family members with ABT-737 and cells were sensitized to doxorubicin and cisplatin. Using the BH3 mimetic ABT-737 proved more effective than the

previously reported use of small interfering RNA (siRNA) against BCL-2 which was unable to induce apoptosis on its own (30), supporting a role for other BCL-2 family members. Moreover, recently, a small IAP (inhibitor of apoptosis) member, survivin was shown to be highly expressed in chondrosarcoma, indicating that also other factors contribute to a defective apoptotic machinery in chondrosarcoma (41). We show high synergy between ABT-737 and doxorubicin or cisplatin with combination indices for both combinations far below 1. Synergistic effects were observed only when combination treatments were administered with a 24 hour time gap and not when administered simultaneously, this is in concordance with the findings published on ABT-263 which accelerates apoptosis during drug-induced mitotic arrest (42). These data support that aberrant expression of BCL-2 family members, but not MCL-1, in chondrosarcoma debilitates the apoptotic pathway as it should be activated in response to conventional chemotherapy, and that combination strategies are successful in overcoming this resistance mechanism.

In conclusion, using a 3D pellet model we have shown that doxorubicin is able to diffuse across at least 1mm chondroid matrix surrounding chondrosarcoma cells and that it is able to enter and accumulate in the nuclei despite MDR pump activity. We showed *in vitro* that complete apoptosis can be achieved in chondrosarcoma cells at low concentrations of doxorubicin and cisplatin if combined with ABT-737, pointing towards an important role for BCL-2 and BCL-$X_L$ in chemoresistance. The orally available homologue of ABT-737; ABT-263, is used in clinical trials with low side effects (43). Our results strongly support the combination treatment of chondrosarcoma patients with ABT-263, with our data supporting a stronger synergistic effect in combination with doxorubicin.

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