Mechanism of action of *Viscum album* L. extracts in Ewing sarcoma

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by

Monika Twardziok (nee Dejewska)

from Graudenz (Poland)

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Abbreviations

BA	Betulinic acid
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
David	Database for Annotation, Visualization and Integrated Discovery
dH_2O	Demineralized water
DMSO	Dimethylsulfoxide
FDR	False discovery rate
GSEA	Gene set enrichment analysis
GO	Gene ontology
IAP	Inhibitor of apoptosis protein
IC50	half minimal inhibitory concentration of a substance
LC-MS/MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
ML	Mistletoe lectin
NAC	N-acetylcysteine
OA	Oleanolic acid
PBS	Phosphate buffered saline
PEP	posterior error probability
SDS	sodium dodecyl sulfate
TBST	Tris buffered saline with Tween-20
UA	Ursolic acid

For my sister, Agnieszka Dejewska (1984-2010).

Table of contents

1 Introduction

1.1 *Viscum album* L.

Viscum album L. (Loranthaceae), the European white berry mistletoe (Figure 1), is a species of mistletoe in the order Santalales. It is an evergreen hemiparasitic plant growing on several species of trees such as apple (Malus), oak (Quercus) and pine (Pinus) extracting their water and nutrients. The mistletoe plant contains a broad range of biologically active hydrophilic and lipophilic substances including mistletoe lectins, viscotoxins, triterpene acids, flavonoids and alkaloids (Franz *et al.*, 1981, Jung *et al.*, 1990, Orhan *et al.*, 2006, Nhiem *et al.*, 2013, Nazaruk *et al.*, 2015). Rudolf Steiner, who was the founder of anthroposophic medicine, established mistletoe therapy for cancer treatment in 1922. Nowadays, *Viscum album* L. extracts are the most widely used and studied plant extracts in alternative medicine and complementary cancer treatment, especially in Germany (Bar-Sela *et al.*, 2006, Bar-Sela, 2011).

Figure 1: *Viscum album* L. Photography of infested host tree (left) and white berries (right).

Standardized commercial *Viscum album* L. extracts are aqueous and contain mainly the hydrophilic mistletoe lectins and viscotoxins representing the best studied compounds of the mistletoe (Jung *et al.*, 1990, Maletzki *et al.*, 2013) (Figure 2). *Viscum album* L. extracts are usually given by injection under the skin or, less often, into a vein, into the pleural cavity or directly into the tumor. The host tree, harvest period and the manufacturing process have a big impact on the proportion of the constituents and

thus, on the effects of the *Viscum album* L. extracts (Büssing *et al.*, 1999a, Eggenschwiler *et al.*, 2006, Nazaruk *et al.*, 2015). The following paragraphs take a closer look on the main *Viscum album* L. components.

1.1.1 Mistletoe lectins

The mistletoe contains mistletoe lectin (ML) I, II and III which occur mostly in the berries of the plant. In mistletoe plants from deciduous trees predominates ML I, whereas mistletoe from fir and pine trees contains mainly ML III (Nazaruk *et al.*, 2015). Mistletoe lectins are a conjugate of a toxic A chain with enzymatic properties and a carbonhydrate-binding B chain. Since chain A inhibits ribosomal protein synthesis, mistletoe lectins I-III belong to type II ribosome-inactivating proteins (Franz *et al.*, 1982, Olsnes *et al.*, 1982, Dietrich *et al.*, 1992). Chain B binds specifically to D-galactose (ML I), D-galactose/*N*-acetyl-D-galactosamine (ML II) and *N*-cetyl-D-galactosamine (ML III) (Franz *et al.*, 1981) and mediates internalization by endocytosis (Więdłocha *et al.*, 1991, Jonas *et al.*, 1991). Aqueous European and Korean mistletoe (*Viscum coloratum*) extracts contain mainly the hydrophilic mistletoe lectins and are able to stimulate the immune system *in vitro* and *in vivo* (Hajto *et al.*, 1989, Ribereau-Gayon *et al.*, 1996, Elluru *et al.*, 2006, Lee *et al.*, 2007b, Lyu *et al.*, 2007, Gardin, 2009, Lee *et al.*, 2009). Further, they inhibit proliferation and induce apoptotic cell death in diverse cancer cell lines, for instance in cell lines derived from head and neck squamous cell carcinomas (Klingbeil *et al.*, 2013), rat glioma (Ucar *et al.*, 2012), acute lymphoblastic leukemia and its mouse model (Delebinski *et al.*, 2012), breast carcinoma (Beuth *et al.*, 2006) and pancreatic cancer (Rostock *et al.*, 2005). On mechanistic level *in vitro*, aqueous extracts of European and Korean mistletoe have been shown to activate PI3K/AKT and JNK/p38/MAPK signaling and the caspase-8 and/or -9 cascades in leukemia (Park *et al.*, 2000, Park *et al.*, 2012), head and neck squamous cell carcinoma (Klingbeil *et al.*, 2013) and hepatocellular carcinoma cell lines (Yang *et al.*, 2012b) as well as TLR4 signaling in bone marrow-derived dendritic cells (Kim *et al.*, 2014). Further, they were also shown to have an anti-angiogenetic effect *in vitro* and in a mouse model *in vivo* (Elluru *et al.*, 2009). On clinical level, there exist positive case reports of alternatively with mistletoe treated patients suffering from cutaneous squamous cell carcinoma (Werthmann *et al.*, 2013) and with a stage IIIC colon carcinoma (von Schoen-Angerer

et al., 2014). Data from clinical trials also exists, but most of the clinical trials in the past have been criticized due to weak study design (Bar-Sela *et al.*, 2006, Bar-Sela, 2011). A more recent randomized clinical trial with patients suffering from locally advanced or metastatic pancreatic cancer revealed a prolonged overall survival when patients were treated additionally to chemotherapy with *Viscum album* L. extracts (Tröger *et al.*, 2013). Similar benefits on the survival time show clinical trials with patients suffering from breast and gynecological cancer (Kienle *et al.*, 2009, Marvibaigi *et al.*, 2014). Other positive effects of aqueous *Viscum album* L. extracts, which were validated in clinical studies with cancer patients, include the reduction of chemotherapy side effects, improvement of the quality of life and the modulation of immune system (Horneber *et al.*, 2008, Kienle *et al.*, 2010, Kim *et al.*, 2012).

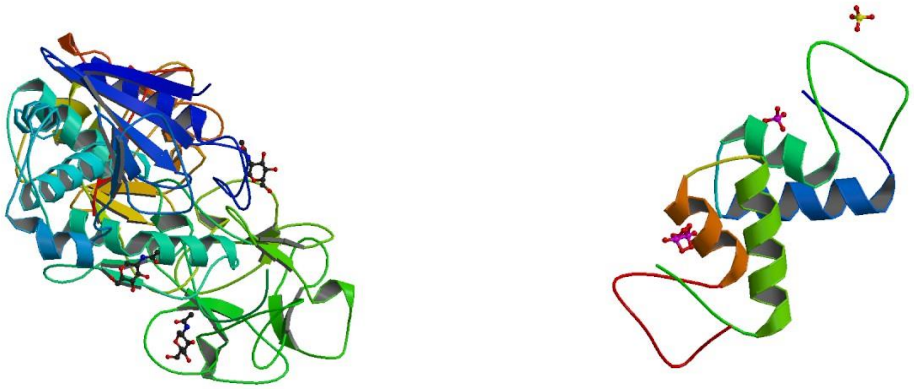

Figure 2: Crystal structures of *Viscum album* L. best studied hydrophilic components. Mistletoe lectin I (left) (Krauspenhaar *et al.*, 1999) and viscotoxin A3 (right) (Debreczeni *et al.*, 2003). Protein chains are colored from the N-terminal to the C-terminal using a rainbow color gradient (Protein Data Bank).

1.1.2 Viscotoxins

Viscotoxins belong to the thionin family type III and are characterized by the presence of three disulfide bridges (Orru *et al.*, 1997). They are small cysteine-rich, cationic proteins consisting of 46 amino acid residues and they exhibit six isomers (Nazaruk *et al.*, 2015), whereat the main three are viscotoxin A2, A3 and B (Pfüller, 2000). Viscotoxins occur in the leaves and stems of the mistletoe plant. As viscotoxins are hydrophilic, they occur in aqueous *Viscum album* L. extracts. Viscotoxins interact with biomembranes resulting in cytotoxicity by cell permeabilization (Coulon *et al.*, 2002, Coulon *et al.*, 2003, Giudici *et al.*, 2003). Viscotoxin A3 displays the most cytotoxicity while viscotoxin B is the less potent (Giudici *et al.*, 2003). The cytotoxic properties of viscotoxins have been demonstrated in HeLa cells (Konopa *et al.*, 1980), human lymphocytes and granulocytes (Büssing *et al.*, 1999b). A commercial standardized viscotoxin-rich *Viscum album* L. extract displayed cytostatic properties on myeloma and lymphoma cell lines (Kovacs *et al.*, 2006, Kovacs, 2010). Viscotoxins are also able to act immunomodulatory on granulocytes (Stein *et al.*, 1999) and to increase natural killer cell-mediated cytotoxicity (Tabiasco *et al.*, 2002).

1.1.3 Triterpene acids

The triterpene acids oleanolic acid (OA) and betulinic acid (BA) represent another potent group of mistletoe-derived substances. OA and BA belong to pentacyclic triterpenes (Figure 3). Triterpenes are terpenes consisting of six isoprene units resulting in a C-30 backbone. Tetracyclic and pentacyclic triterpenes are the most abundant triterpenes (Sheng *et al.*, 2011). OA and BA were firstly isolated from *Viscum album* L. in 1987 together with the triterpene β-amyrin acetate and a mixture of phytosterols (stigmasterol, β-sitosterol) and their glucosides (Fukunaga *et al.*, 1987). Triterpene acids are lipophilic and thus, their low solubility exclude them from commercially available aqueous *Viscum album* L. extracts (Jäger *et al.*, 2007). To overcome their solubility problem, an extraction method was established using sodium phosphate at pH 7.3. By using this extraction method, additionally lupeol and lupeol acetate were detected (Jäger *et al.*, 2007). Nowadays, triterpenes can be isolated from the mistletoe and solubilized by using 2-hydroxypropyl-β-cyclodextrins (Strüh *et al.*, 2012).

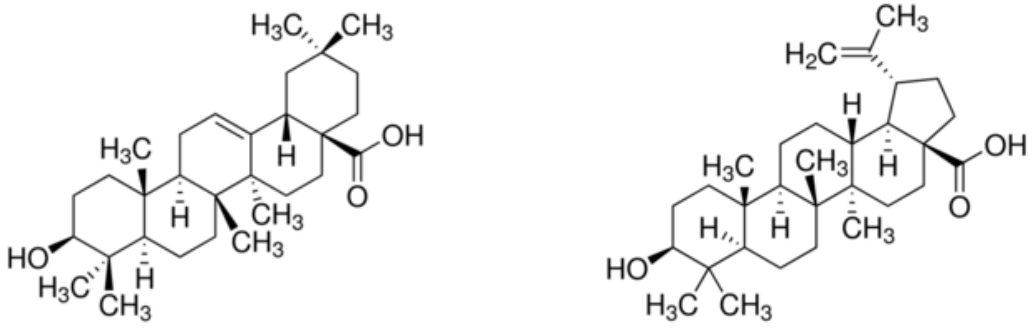

Figure 3: Structure of oleanolic and betulinic acid (from Sigma-Aldrich).

OA, its synthetic derivatives and BA have shown immunomodulating properties *in vitro* and *in vivo*, for example in cell lines and mouse models of colon and lung carcinoma, mouse models of cervical carcinoma, S180 sarcoma and hepatoma (Yun *et al.*, 2003, Deng *et al.*, 2009, Laszczyk, 2009, Nagaraj *et al.*, 2010, Rios, 2010, Wang *et al.*, 2012). Furthermore, OA, its derivatives and BA were able to inhibit cell growth and induce apoptosis in cell lines derived from neuroectodermal tumors (Fulda *et al.*, 1999, Laszczyk, 2009), leukemia (Urech *et al.*, 2005), ovarian cancer (Petronelli *et al.*, 2009b), non-small cell lung cancers (Lucio *et al.*, 2011) and breast cancer (Akl *et al.*, 2014), as well as neuroectodermal tumors *ex vivo* (Fulda *et al.*, 1999) and mouse models of melanoma (Lucio *et al.*, 2011, Strüh *et al.*, 2013). Recently, lipophilic *Viscum album* L. extracts, which contain mainly OA, revealed immunomodulatory effects on tumor cell co-cultured macrophages and modulated monocyte chemotactic transmigration *in vitro* exhibiting a multimodal concept of anti-cancer therapy in future (Estko *et al.*, 2015). The anti-tumoral effects of the triterpenes ursolic acid (UA) and BA are comparable to OA (Gheorgheosu *et al.*, 2014, Soica *et al.*, 2014a). Combination of OA and UA has been reported to act synergistically against melanoma cells *in vitro* and *in vivo* (Soica *et al.*, 2014b). On mechanistic level, triterpene acids also demonstrated the activation of PI3K/AKT and/or p38/JNK/MAPK signaling pathways in various cancer cell lines including osteosarcoma, pancreatic cancer, lung carcinoma (Liu *et al.*, 2014a, Liu *et al.*, 2014c) and melanoma (Tan *et al.*, 2003, Jin *et al.*, 2014).

1.2 Ewing sarcoma

Ewing sarcoma was named after the pathologist James Ewing, who first described this tumor as an endothelioma of bone in 1920 (Ewing, 1972). It is a rare bone malignancy, which may also concern soft tissues. The most affected areas of the body are the pelvis, the femur, the humerus, the ribs and clavicle. Ewing sarcoma is, after osteosarcoma, the second most common form of bone sarcoma in children and adolescents. In Germany, bone tumors amount to 5.2 % of cancer diagnoses per year in children under 18 years. The standardized incidence rate of Ewing sarcoma is about 2.8 per million children in Germany (Kaatsch *et al.*, 2014) and 2.6 per million children in the US (Society, 2014). Sex-specific incidence rates are for boys with 3.1 per million children slightly higher than for girls affecting 2.5 girls per million children. Ewing sarcoma peaks in the second life decade with a median age at diagnosis of 10 years 11 months and an age-specific incidence rate of 5.0 per million in children aged 10 to 14 years, while the incidence rate of children under 1 year is 0.4 per million (Kaatsch *et al.*, 2014).

1.2.1 Pathogenesis

The pathogenesis of Ewing sarcoma results from a balanced translocation involving the *EWSR1* gene on chromosome 22q12 and fusion partners from the *ETS* transcription factor family, mainly *FLI1* on chromosome 11q24 (85 %) or *ERG* on chromosome 21q22 (10 %) (Kelleher *et al.*, 2012). *EWSR1* encodes a multifunctional protein which is involved in many cellular processes as gene expression, cell signaling, and RNA processing and transport. The protein includes an N-terminal transcriptional activation domain and a C-terminal RNA-binding domain. The RNA binding domain of the *EWSR1* gene is exchanged with a DNA binding domain of an *ETS* gene family member generating fusions proteins, which code for chimeric transcription factors (Paronetto, 2013, Mackintosh *et al.*, 2013). Figure 4 shows a schematic of EWS-FLI1 t(11;22)(q24;q12) translocation.

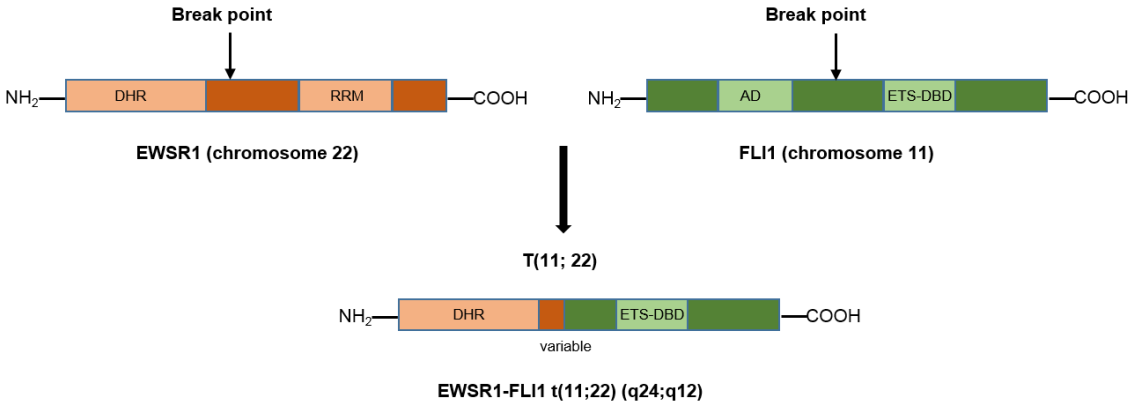

Figure 4: Scheme of EWS-FLI1 t(11;22)(q24;q12) translocation, inspired by Anderson *et al.*, 2012 (Anderson *et al.*, 2012). The EWSR1-FLI1 fusion protein consists of the N-terminal activation domain of EWS with multiple degenerate hexapeptide repeats (DHR) and the C-terminal ETS DNA-binding domain (ETS-DBD) of FLI1. The RNA recognition motif (RRM) of EWS and the activation domain (AD) of FLI1 are omitted in the fusion protein. Variation in the chromosomal break point sites result in multiple fusion types.

The consequences of a chimeric *EWSR1-ETS* transcription factor regarding tumorigenesis are manifold and lead to cell growth. EWS-FLI1, for instance, promotes cell survival and proliferation by activating genes as *NKX2.2* (Smith *et al.*, 2006), *GLI1* (Zwerner *et al.*, 2007), *DAX1* (Garcia-Aragoncillo *et al.*, 2008), *IGF1* and *TOPK* (Herrero-Martín *et al.*, 2009) and *EZH2* (Richter *et al.*, 2009). Furthermore, EWS-FLI1 represses genes that induce cell cycle arrest and apoptosis as *TGFB2* (Hahm, 1999) and *CDKN1A* (Nakatani *et al.*, 2003). Tumor development is a complex process that results from an interplay between mutations in oncogenes and/or tumor suppressors in a certain cellular context (Toomey *et al.*, 2010). Alterations in the RB and p53 pathways, for instance, may be cooperating mutations in Ewing sarcoma and also the IGF-1R pathway has been shown to be important, but there is still no complete understanding of the exact molecular combination (Toomey *et al.*, 2010). An important question also addresses the origin of Ewing sarcoma.

1.2.2 Origin

The origin of Ewing sarcoma has not been fully resolved yet. The main aspects of the controversial origin of Ewing sarcoma have been discussed in reviews (Lin *et al.*, 2011, Monument *et al.*, 2013). Histologically, it is an undifferentiated small round blue cell tumor with CD99 expression (Figure 5), which is a molecular marker for Ewing sarcoma (Ambros *et al.*, 1991). Ewing sarcoma cells display both, mesenchymal and neuroectodermal histological and immunohistochemical features (Desai *et al.*, 2010), but contrary to many other cancer models, a direct lineage-specific oncogenesis has not been demonstrated. Its morphology resembles primitive neuroectodermal cells. Therefore, Ewing sarcoma was once classified as a peripheral primitive neuroectodermal tumor (Cavazzana *et al.*, 1987). Some Ewing sarcomas express neuronal markers as the neuron-specific enolase and S-100 protein and Ewing sarcoma cells are able to differentiate into neuronal lineage *in vitro* by specific stimulation (Noguera *et al.*, 1992, Franchi *et al.*, 2001, Rorie *et al.*, 2004). Neural crest stem cells have been also implicated as the origin of Ewing sarcoma (Staege *et al.*, 2004, von Levetzow *et al.*, 2011). But since the fusion gene *EWS-FLI1* also plays a crucial role for the differentiation towards neuronal cells (Teitell *et al.*, 1999, Hu-Lieskovan *et al.*, 2005), the neuroectodermal features of Ewing sarcoma may be rather the result of the fusion gene than the origin of Ewing sarcoma.

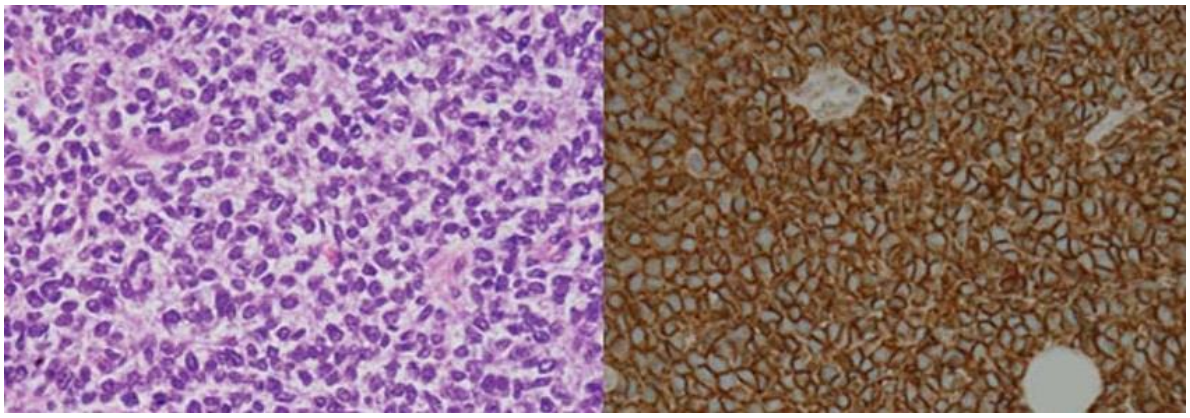

Figure 5: Microscopic images of typical Ewing sarcoma histopathology. Magnification x400, modified (Chuthapisith *et al.*, 2012). H&E staining (left) and CD99 immunostaining (right).

The other possible origin of Ewing sarcoma is the mesenchymal stem cell. Unlike primitive neuroectodermal cells, mesenchymal stem cells normally exist in bone and are the origin of bone development. They can show neuroectodermal features and express neural markers as S-100 (Torchia *et al.*, 2003, Takashima *et al.*, 2007). They are also able to differentiate into neural cells (Kopen *et al.*, 1999, Woodbury *et al.*, 2000) and appear as rounded cells expressing neuroectodermal markers when *EWS-FLI1* is introduced into the cells *in vitro* (Riggi *et al.*, 2008, Miyagawa *et al.*, 2008). Mesenchymal stem cells also express CD99, and CD99 expression can be enhanced by *EWS-FLI1* expression (Riggi *et al.*, 2008). In Ewing sarcoma cell lines, knockdown of *EWS-FLI1* has been shown to result in a differentiation towards osteogenic and adipogenic cells (Tirode *et al.*, 2007). Furthermore, *EWS-FLI1* expression in murine mesenchymal stem cells lead to sarcoma growth in mice (Castillero-Trejo *et al.*, 2005, Riggi *et al.*, 2005), while human mesenchymal stem cells transfected with *EWS-FLI1* were not able to induce tumor growth in mice (Riggi *et al.*, 2008). Moreover, the transcriptional profile of Ewing sarcoma cell lines with a *EWS-FLI1* knockdown is similar to the transcriptional profile of mesenchymal stem cells (Tirode *et al.*, 2007) and human fetal fibroblasts (Potikyan *et al.*, 2008). However, it is also conceivable that Ewing sarcoma derives from a neural-derived mesenchymal stem cell or an neural crest stem cell with mesenchymal potential (Toomey *et al.*, 2010) since it was demonstrated that neural-derived mesenchymal stem cells exist in the bone marrow of embryonic mice (Takashima *et al.*, 2007) and neural crest stem cells can differentiate toward mesenchymal lineages (Lee *et al.*, 2007a). Therefore, neural crest and mesenchymal stem cells do not exclude each other as putative origin of Ewing sarcoma (Riggi *et al.*, 2009, Toomey *et al.*, 2010).

1.2.3 Therapy and prognosis

Ewing sarcoma is an aggressive tumor that requires aggressive treatment. Therapy options have improved over the last years leading to a five-year survival rate of 70 %. The current first-line standard therapy consists of neoadjuvant therapy combining 4 to 6 chemotherapeutics including vincristine, doxorubicin, etoposide, cyclophosphamide, ifosfamide, and/or actinomycin-D, followed by surgery and/or radiotherapy (Ladenstein *et al.*, 2010, Amaral *et al.*, 2014). The most widely used combination in Europe is vincristine, ifosfamide, doxorubicin and etoposide or vincristine, actinomycin-D and ifosfamide, whereas in the United States it is vincristine, doxorubicin and cyclophosphamide or ifosfamide and etoposide (Hogendoorn *et al.*, 2010). There is no specific therapy that targets the pathogenetic mechanisms of Ewing sarcoma cells and drug resistance often occurs (Kelleher *et al.*, 2012, Amaral *et al.*, 2014). Additionally, the outcome for patients with relapse is very poor, only 10-15 % of the patients are being cured (Barker *et al.*, 2005, Stahl *et al.*, 2011). Patients with metastases have a survival rate of 25 % (Kelleher *et al.*, 2012). Metastases often occur in lungs (50 %), bones (25 %) and bone marrow (20 %) (Balamuth *et al.*, 2010). Several studies were performed with patients who harbor metastases outside the lungs using megatherapy (myeloablative high-dose chemotherapy with or without total-body irradiation, then transplantation of marrow or peripheral hematopoietic stem cells), but megatherapy was not able to improve survival (Burdach *et al.*, 2000, Meyers *et al.*, 2001, Balamuth *et al.*, 2010).

The stage, anatomic location and the size of the tumor also influence the prognosis and therapeutic outcome (Amaral *et al.*, 2014). Furthermore, several molecular features were reported to have an impact on prognosis, including alterations of *TP53* and *CDKN2A*, copy number alterations (Shukla *et al.*, 2013) and additional chromosomal aberrations, for instance gains of chromosome 1q (Mackintosh *et al.*, 2012). Moreover, the inhibitor of apoptosis protein (IAP) family member BIRC5 (survivin) has been identified as a poor prognostic factor in Ewing sarcoma (Greve *et al.*, 2012, Hingorani *et al.*, 2013). On the other hand, it was shown that the level of neural differentiation has no prognostic value (Parham *et al.*, 1999) and there is also no correlation with fusion type and prognosis (Ladenstein *et al.*, 2010, van Doorninck *et al.*, 2010). In order to improve prognosis and therapeutic outcome, innovative targeted therapies for Ewing sarcoma are under current investigation. One approach is to inhibit the fusion gene but

gene silencing by antisense cDNA and siRNA lacks bioavailability and is difficult to administer therapeutically. Therefore, the focus lies on targeting EWSR1-FLI1 protein interactions, for instance by inhibiting its interaction with RNA helicase A by small molecules. Other promising approaches include the inhibition of pathways related to IGF1 and mTOR, the inhibition of tyrosine kinases as c-KIT and PDGFR, interference with angiogenesis, modulation of epigenetics by demethylating agents or histone deacetylase inhibitors and NK cell-based immunotherapy (Balamuth *et al.*, 2010, Kelleher *et al.*, 2012, Amaral *et al.*, 2014). Despite the new targeted therapy approaches, only future will tell whether they succeed with all phases of clinical trials representing a new and better therapy for Ewing sarcoma patients.

1.3 Aim of the work

Drug discovery of new anti-tumor agents is crucial to improve survival of Ewing sarcoma patients. Combining active substances may offer benefits since some drug combinations are able to amplify each other creating synergistic effects. Natural substances in plant extracts contain diverse cytotoxic compounds and provide potential active ingredients for tumor therapy. The European mistletoe (*Viscum album* L.) contains triterpene acids, mistletoe lectins and viscotoxins, and all of them possess anticancer properties. *Viscum album* L preparations have been used for years in complementary cancer medicine additionally to conventional chemotherapy. However, standardized commercial *Viscum album* L. extracts are aqueous and contain only the hydrophilic mistletoe lectins and viscotoxins excluding the insoluble hydrophobic triterpenes. Triterpene acids can be solubilized by cyclodextrins allowing the combination of hydrophobic and hydrophilic active substances creating a total mistletoe effect. The aim of this work was to analyze the mechanism of action of a defined aqueous ML-containing extract (viscum) and a triterpene acids containing extract (TT) from *Viscum album* L. individually and in combination (viscumTT) in Ewing sarcoma cells. The focus hereby lied on synergistic effects of the viscumTT extract. In a first step, the strategy included the cytotoxic characterization of the extracts. Therefore, apoptosis and effects on proliferation were analyzed in Ewing sarcoma cell lines and patient-derived primary cells by using cell biology methods. Furthermore, the efficacy of the extracts was investigated in a Ewing sarcoma mouse model *in vivo*. In a second step, the molecular mechanism of viscum, TT and viscumTT was analyzed *in vitro* using diverse methods from molecular biology.

2 Material and methods

2.1 Material

2.1.1 Equipment

Biological Safety Cabinet Maxisafe	Thermo Scientific, Dreieich, Germany
CASY Cell Counter	Schärfe System, Reutlingen, Germany
ChemiDoc Molecular Imager	Bio-Rad, München, Germany
Centrifuge MEGAFUGE 8	Thermo Scientific, Dreieich, Germany
Centrifuge MIKRO 22 R	Hettich, Tuttlingen, Germany
Centrifuge Rotanta 460R	Hettich, Tuttlingen, Germany
Cytoperm 2 CO_2 Incubator	Thermo Scientific, Dreieich, Germany
FACS Calibur	BD Biosciences, Heidelberg, Germany
Freezing Container Mr. Frosty	Thermo Scientific, Dreieich, Germany
Light Microscope Nikon TMS	Nikon, Tokio, Japan
Mastercycler	Eppendorf, Hamburg, Germany
Microplate Reader Multiskan Ascent	Thermo Scientific, Dreieich, Germany
NanoDrop 2000 spectrophotometer	Thermo Scientific, Dreieich, Germany
Pipettes Eppendorf Research Plus	Eppendorf, Hamburg, Germany
StepOnePlus Real-Time PCR System	Applied Biosystems, Darmstadt, Germany
Trans-Blot Turbo Transfer System	Bio-Rad, München, Germany

2.1.2 Consumables

CryoPure tubes, 1.6 mL	Sarstedt, Nümbrecht, Germany
FACS tubes	BD Biosciences, Heidelberg, Germany
Falcon tubes (5 mL; 10 mL)	BD Biosciences, Heidelberg, Germany
Cell Culture Flasks (T25 and T75)	BD Biosciences, Heidelberg, Germany
MicroAmp Fast Optical 96-Well Reaction Plate	Applied Biosystems, Darmstadt, Germany
MicroAmp Optical Adhesive Film	Applied Biosystems, Darmstadt, Germany

Microtiter Plates (6-, 12-, 96-well), flat bottom, for adherent cells	BD Biosciences, Heidelberg, Germany
Millex Syringe Filter Units, 0.22 µm	Merck Millipore, Darmstadt, Germany
PCR Single Cap 8er SoftStrips	Biozym Scientific, Hessisch Oldendorf, Germany
Pipette Tips (10/100/1000 µL)	Sarstedt, Nümbrecht, Germany
Scalpel, disponsable	Heinz Herenz, Hamburg, Germany
Serological Pipettes (5/10/25 mL)	BD Biosciences, Heidelberg, Germany
SafeSeal Reaction Tubes (1.5/2.0 mL)	Sarstedt, Nümbrecht, Germany
SafeSeal SurPhob Pipette Filter Tips (10/100/1000 µL)	Biozym Scientific, Hessisch Oldendorf, Germany
Syringe, 5 mL	BD Biosciences, Heidelberg, Germany
Trans-Blot Turbo transfer packs, nitro-cellulose	Bio-Rad, München, Germany

2.1.3 Chemicals and reagents

All chemicals were used in analytical quality.

Annexin V APC	BD Biosciences, Heidelberg, Germany
Ammoniumperoxidsulfate	Carl Roth, Karlsruhe, Germany
Bovine Serum Albumin (BSA)	Sigma Aldrich, Seelze, Germany
Bradford Protein Assay	Bio-Rad, München, Germany
Calciumchloride	Sigma Aldrich, Seelze, Germany
Carbonyl cyanide m-chlorophenyl hydrazine (CCCP)	Sigma Aldrich, Seelze, Germany
cOmplete Protease Inhibitor Cocktail	Roche Diagnostics, Mannheim, Germany
Dimethylsulfoxide (DMSO)	Sigma Aldrich, Seelze, Germany
Dithiothreitol	Carl Roth, Karlsruhe, Germany
Dulbecco´s PBS	Gibco, Darmstadt, Germany
Ethanol, absolute	Carl Roth, Karlsruhe, Germany
FACS-Flow	BD Biosciences, Heidelberg, Germany
Fetal Calf Serum	Biochrom, Berlin, Germany

Glycine	AppliChem, Darmstadt, Germany
HEPES	Sigma Aldrich, Seelze, Germany
IMDM with L-glutamine	Lonza, Köln, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Isoton II Diluent	Beckman Coulter, Krefeld, Germany
JC-1	AAT Bioquest, Sunnyvale, CA, USA
Laemmli sample buffer 4x	Bio-Rad, München, Germany
LPS-RS	InvivoGen, San Diego, CA, USA
Lysis Buffer 17	R&D systems, Minneapolis, MN, USA
N-acetylcysteine (NAC)	Sigma Aldrich, Seelze, Germany
Natriumchloride	Carl Roth, Karlsruhe, Germany
Pierce ECL Western Blotting Substrate	Life Technologies, Darmstadt, Germany
Penicillin-Streptomycin	Biochrom, Berlin, Germany
Pepstatin A	Sigma Aldrich, Seelze, Germany
Power SYBR Green Master Mix	Applied Biosystems, Darmstadt, Germany
Powdered Milk	Carl Roth, Karlsruhe, Germany
Precision Plus Protein Dual Color	Bio-Rad, München, Germany
Propidiumiodide	Sigma Aldrich, Seelze, Germany
Rotiphorese Gel-40 (Acrylamide)	Carl Roth, Karlsruhe, Germany
RPMI 1640	PAA Labortories, Cölbe, Germany
SB203580	Cell Signaling Technology, Danvers, USA
sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe, Germany
SP600125	Sigma Aldrich, Seelze, Germany
Tetramethylethylenediamine	Bio-Rad, München, Germany
Tris base	Carl Roth, Karlsruhe, Germany
Trypsin/EDTA, 0.05 %	Gibco, Darmstadt, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
Water, nuclease free	Thermo Scientific, Dreieich, Germany
Z-VAD-FMK	R&D systems, Minneapolis, MN, USA

2.1.4 Viscum album L. extracts

Viscum and TT extracts (lot #157) were prepared from *Viscum album* L. harvested from apple trees (malus) as previously described (Delebinski *et al.*, 2012, Strüh *et al.*, 2012) by the Birken AG (Niefern-Oeschelbronn, Germany), who kindly provided lyophilized viscum and TT extracts. Intact ML I (A+B chain) was quantified by ELISA in viscum extract (Jaggy *et al.*, 1995). OA and BA quantification was performed by GC-FID (Strüh *et al.*, 2012). Lyophilized viscum extract was reconstituted in phosphate-buffered saline (PBS) to a final concentration of 2 µg/mL intact ML I and < 1 µg/mL viscotoxins. Lyophilized TT extract (containing 2-hydroxypropyl-β-cyclodextrins) was reconstituted in PBS to a final concentration of 4000 µg/mL OA, 350 µg/mL BA and 317 mg/mL 2-hydroxypropyl-β-cyclodextrins. ViscumTT was obtained by adding both extracts to the cells. Before use in cell culture, extracts were sterile filtrated (0.22 µm).

2.1.5 Triterpene standards

OA (lot #153.1) and BA (lot #156) standards were kindly provided by the Birken AG (Niefern-Oeschelbronn, Germany), who bought OA and BA as pure substances from Extrasynthese (Lyon, France) and then solubilized them in 2-hydroxypropyl-β-cyclodextrins like the triterpene extract.

2.1.6 Buffers

Annexin binding buffer, 10x	0.1 M HEPES, 1.4 M NaCl, 25 mM $CaCl*2H_2O$, pH 7.4
Separating gel buffer	1.5 M Tris base, 0.4 % SDS, pH 8.8
Stacking gel buffer	0.5 M Tris base, 0.4 % SDS, pH 6.8
SDS-PAGE running buffer, 10x	250 mM Tris base, 1 % SDS (v/v), 2 M Glycine (w/v)
Blocking buffer	1x TBST, 5 % nonfat milk (w/v), pH 7.5
Tris-buffered saline with Tween, 10x	0.5 M Tris base, 1.5 M NaCl, 1 % Tween 20 (v/v), pH 7.6

10x buffers were diluted to 1x with demineralized water (dH_2O).

2.1.7 SDS-PAGE gels

Separating gel 12,5%		3.75 mL Rotiphorese Gel
	+	3.00 mL Separating Gel Buffer
	+	5.20 mL dH_2O
	+	96 µL 12.5 % Ammoniumperoxidsulfate (w/v)
	+	10 µL Tetramethylethylenediamine
Stacking gel 4.5 %		0.83 mL Rotiphorese Gel
	+	1.85 mL Stacking Gel Buffer
	+	4.80 mL dH_2O
	+	60 µL 12.5 % Ammoniumperoxidsulfate (w/v)
	+	7.5 µL Tetramethylethylenediamine

2.1.8 Kits

Cytotoxicity Detection Kit PLUS	Roche Diagnostics, Mannheim, Germany
Green Caspase Staining Kit	PromoKine, Heidelberg, Germany
High Capacity RNA-to-cDNA Kit	Applied Biosystems, Darmstadt, Germany
NucleoSpin RNA Kit	Macherey-Nagel, Düren, Germany
Proteome Profiler Human Apoptosis Array	R&D systems, Minneapolis, MN, USA
Venor®GeM Classic	Minerva Biolabs, Berlin, Germany

2.1.9 Antibodies

All used primary antibodies were derived from mouse or rabbit with reactivity against human proteins. The secondary antibodies (Goat Anti-Rabbit IgG-HRP #1706515 and Goat Anti-Mouse IgG-HRP #1721011) were supplied by Bio-Rad, München, Germany.

ß-Actin #A3854	Sigma Aldrich, Hamburg, Germany
BIRC5 #2803	Cell Signaling Technology, Danvers, MN, USA
CASP3 #9662	Cell Signaling Technology, Danvers, MN, USA
CD99 FITC #561986	BD Biosciences, Heidelberg, Germany
CLSPN #2800	Cell Signaling Technology, Danvers, MN, USA
EIF2AK3 #3192	Cell Signaling Technology, Danvers, MN, USA
HSPA5 #G8918	Sigma Aldrich, Hamburg, Germany
Isotype control #555573	BD Biosciences, Heidelberg, Germany
LC3B #2775	Cell Signaling Technology, Danvers, MN, USA
MCL1 sc-819	Santa Cruz Biotechnology, Heidelberg, Germany
PARP1 #9542	Cell Signaling Technology, Danvers, MN, USA
p-MAPK8 sc-6254	Santa Cruz Biotechnology, Heidelberg, Germany
p-MAPK14 #9211	Cell Signaling Technology, Danvers, MN, USA
PMAIP1 sc-56169	Santa Cruz Biotechnology, Heidelberg, Germany
TP53 sc-73566	Santa Cruz Biotechnology, Heidelberg, Germany
XIAP #610716	BD Biosciences, Heidelberg, Germany

2.1.10 Primers

Primers were predesigned and purchased from Integrated DNA Technologies (IDT, Leuven, Belgium) detecting all mRNA variants:

Hs.PT.58.14610020	DDIT3
Hs.PT.39a.22214836	GAPDH
Hs.PT.58.25094714.g	JUN
Hs.PT.58.3312889	MAP2K6

2.1.11 Cell lines

The human Ewing sarcoma cell lines TC-71 and MHH-ES-1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

2.1.12 Primary cells

A tumor sample was obtained as treatment residue from a 15-year-old girl with Ewing sarcoma during routine surgical resection at the Charité Universitätsmedizin Berlin Department of Pediatrics, Division of Oncology and Hematology. The sample was not explicitly collected for this research. Diagnosis was confirmed by histopathology. Written informed consent was obtained from the patient in accordance with the Declaration of Helsinki, approved by the local ethics committee of Charité - Universitätsmedizin Berlin.

2.2 Methods

2.2.1 Cell culture

All cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2. For subcultures, cells were washed with PBS, treated with 0.05 % trypsin/EDTA and incubated for 1 - 3 min at 37 °C. Afterwards, media was added and cells were counted by CASY Cell Counter, defined cell numbers were added to subcultures.

2.2.1.1 Cell lines

TC-71 cells were maintained in IMDM base medium with L-glutamine, while MHH-ES-1 were maintained in RPMI 1640 base medium with L-glutamine. Base medium was supplemented with 10 % heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin (1 % penicillin/streptomycin). The cell lines were cultured to 70 - 80 % confluence and to a maximum of 12 passages. Mycoplasma contamination

of cells was excluded by PCR using VenorGeM mycoplasma detection kit according to manufacturer´s instructions. For assays, $1x10^5$/mL TC-71 cells and $2x10^5$/mL MHH-ES-1 cells were seeded onto 6-well plates (2 mL), 12-well plates (1 mL), 96-well plates (100 µL), T25 cell culture flasks (5 mL) or T75 cell culture flasks (15 mL) depending on experimental set-up and were cultured 24 h to allow cell attachment. Next day, media was changed and cells were treated 24 h with viscum, TT or viscumTT added to fresh culture media. ML and OA concentrations were used as a measure of viscum and TT active agent concentration. Untreated control cells and with cyclodextrins treated control cells (further summed up and referred to as untreated control, for results see supplementary table S1) were used as reference.

2.2.1.2 Ewing sarcoma primary cells

The obtained tumor sample was dissected into smaller pieces immediately after surgical excision, then cultured as a primary explant in RPMI 1640 base medium with L-glutamine supplemented with 20 % heat-inactivated fetal calf serum and 1 % penicillin/streptomycin solution to obtain dissociated monolayer culture outgrowth from the explant. Confluent *ex vivo* cell cultures were treated within 4 trypsinized passages. CD99 expression confirmed *ex vivo* cultures as Ewing sarcoma. FISH analysis, performed by Dr. rer. nat. Lenze (Molecular pathology, Charité Berlin), confirmed the EWS-ETS translocation in the *ex vivo* culture. For assays, cells were seeded onto 12-well microtiter plates at $1.3x10^5$/well for 24 h to allow cell attachment. Next day, media was changed and cells were treated 24 h with viscum, TT or viscumTT added to fresh culture media. ML and OA concentrations were used as a measure of viscum and TT active agent concentration. Untreated control cells and with cyclodextrins treated control cells (further summed up and referred to as untreated control) were used as reference.

2.2.1.3 Cryopreservation of cells

Cryopreservation of cell lines and primary cells was performed to ensure cell maintenance and reproducible results. Therefore, cells were counted, then centrifuged at 220 *g*, 21 °C and resuspended in 90 % FCS and 10 % DMSO. Afterwards, cells were incubated for 90 min at -80 °C in freezing container following long-term storage in liquid nitrogen.

2.2.2 Ewing sarcoma xenografts

The Ewing sarcoma mouse experiment was performed by Epo Berlin-Buch GmbH, while statistical analysis and interpretation of the data was performed independently. Eight-week-old female NOD-scid IL2rγ null mice were obtained from Charles River Laboratories (Sulzfeld, Germany), housed in a pathogen-free facility under pathogen-free conditions and fed autoclaved standard diet (Sniff, Soest, Germany) with acidified drinking water *ad libitum*. TC-71 cells ($1x10^6$) were subcutaneously injected in saline into the left flank of 8 mice per treatment or control group. Intratumoral treatment with viscum, TT, viscumTT or cyclodextrins alone (control group) began on day 12 when tumors were palpable. Intravenous treatment with viscumTT or cyclodextrins began on day 3 after tumor cell injection. Mice were treated with increasing extract concentrations: 40/50/60 mg/kg OA (TT), 0.75/1.25/1.75 µg/kg ML (viscum) or a combination thereof (viscumTT). Doses were administered every 2 to 3 days (Monday/Tuesday/Wednesday/Thursday/Friday) and each dose was reapplied within 2 to 3 days. One positive control group received 4 mg doxorubicin/kg once intravenously on day 12. Body weight was measured before each treatment, and mice were carefully monitored for health and symptoms of toxicity. Animals were sacrificed by cervical dislocation at the end of the experiment or if mice were moribund (tumor volume > 1.2 cm^3 or > 10 % body weight lost). Animal experiments were performed in accordance with German legislation on the care and use of laboratory animals and in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia to minimize suffering. Approval for the study was obtained from the Regional Office for Health and Social Affairs (LaGeSo, approval G-0030/15).

2.2.3 Cell biological analyses

2.2.3.1 Measurement of cell proliferation

After incubating cells with viscum, TT or viscumTT versus untreated control cells for 24 h in 6-well plates, cells were harvested, washed with PBS (220 *g*, 5 min, 21 °C), then resuspended in 2 mL PBS and counted using a CASY Cell Counter. Cell proliferation was estimated from total cell numbers in treated cultures (started from defined cell numbers when cells were seeded out) compared to control cultures, which were set to 100 % of proliferation.

2.2.3.2 LDH assay

Early cytotoxicity was assessed after 2 h of incubation with viscum, TT or viscumTT by photometrically monitoring lactate dehydrogenase (LDH) release into culture medium using the Cytotoxicity Detection Kit according to manufacturer´s instructions. Therefore, 1x10^4 TC-71 cells and 2x10^4 MHH-ES-1 cells were seeded onto 96-well microtiter plates (100 µL) in technical triplicates (including background control, substance control, low control and high control) and cultured 24 h to allow cell attachment. Next day, culture media was replaced by media without fetal calf serum and cells were treated 2 h with viscum, TT or viscumTT added to culture media. After addition of Lysis solution, Reaction mixture and Stop solution, the microtiter plate was photometrically monitored at 490 nm. To determine the percentage of cytotoxicity, the average absorbance values of the triplicate samples and controls were calculated. Then the absorbance values obtained in the background control and substance control were subtracted and the resulting values were calculated in the following equation:

$$Cytotoxicity\ [\%] = \frac{experimental\ value - low\ control}{(high\ control - low\ control)} x\ 100$$

2.2.3.3 Measurement of apoptotic cell death

Apoptotic cell death was measured by annexin V/propidium iodide staining and flow cytometry. After incubating the cells with viscum, TT or viscumTT versus untreated control cells for 24 h in 6-well plates, cells were harvested and washed with PBS (220 *g*, 5 min, 21 °C), resuspended in 100 µL annexin V binding buffer and stained with 3 µL APC-conjugated annexin V for 20 min, 8 °C, in the dark. After annexin V staining, 1 µL of propidium iodide (1 mg/mL in PBS) was added and cells were immediately analyzed by flow cytometry using FL2 for propidium iodide fluorescence and FL4 for APC fluorescence. The results were evaluated with FlowJo Software (FlowJo, Ashland, OR, USA).

2.2.3.4 Measurement of mitochondria membrane potential

The mitochondrial membrane potential was measured by JC-1 staining and flow cytometry. After incubating cells with viscum, TT or viscumTT (versus untreated control cells) for 24 h in 6-well plates, cells were harvested, washed with PBS (220 *g*, 5 min, 21 °C), resuspended in 750 µL PBS and stained with 10 µL JC-1 (0.1 mg/mL in DMSO) for 30 min, 37 °C, in the dark. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 50 mM in DMSO) was added to untreated cells (1 µL) serving as positive control (mitochondria membrane disrupter). After staining, cells were washed twice with PBS (220 *g*, 5 min, 21 °C) and immediately analyzed by flow cytometry displaying intact mitochondrial membrane potential in red (FL2) and disrupted mitochondrial membrane potential in green fluorescence (FL1). The results were evaluated with FlowJo Software (FlowJo, Ashland, OR, USA).

2.2.3.5 Measurement of active caspases

CASP3, CASP8 and CASP9 activity was measured using the Green Caspase Staining Kit with the nontoxic, irreversibly capase-binding cell-permeables FITC-LEHD-FMK, FITC-IETD-FMK or FITC-DEVD-FMK according to manufacturer's directions. Briefly, after incubating cells with viscum, TT or viscumTT (versus untreated control cells) for 24 h in 6-well plates, cells were harvested, washed with PBS (220 *g*, 5 min, 4 °C), resuspended in 300 µL PBS and stained with 1 µL of specific capase-binding cell-permeable for 30 min, 37 °C, in the dark. After staining, cells were washed twice with the

kit´s washing buffer and immediately analyzed by flow cytometry detecting FL1 fluorescence. The results were evaluated with FlowJo Software (FlowJo, Ashland, OR, USA).

2.2.3.6 Inhibitor assays

TC-71 cells were pre-incubated for 1 h with following inhibitors before treatment with viscum, TT or viscumTT at the half minimal inhibitory concentrations (IC50) versus untreated control cells in 12-well microtiter plates: pan-caspase inhibitor Z-VAD-FMK (in DMSO), MAPK14 inhibitor SB203580 (in DMSO), MAPK8 inhibitor SP600125 (in DMSO), oxidative stress inhibitor N-acetylcysteine (NAC, in PBS), TLR4 inhibitor LPS-RS (in endotoxin-free water). DMSO, PBS or water were added to cells as solvent controls. Apoptotic cell death was measured flow cytometrically after annexin V/propidium iodide staining. The results were evaluated with FlowJo Software (FlowJo, Ashland, OR, USA).

2.2.3.7 CD99 immunostaining

CD99 expression was measured in *ex vivo* Ewing sarcoma cultures by flow cytometry with FITC-labeled anti-CD99 antibody versus the isotype control antibody. $1x10^6$ cells were washed with PBS + 0.5 % BSA (220 *g*, 5 min, 4 °C), then resuspended in 100 µL PBS + 0.5 % BSA and stained with 20 µL of the antibody for 30 min, 4 °C in the dark. After staining, cells were washed twice with PBS + 0.5 % BSA and immediately analyzed by flow cytometry detecting FL1 fluorescence. The results were evaluated with FlowJo Software (FlowJo, Ashland, OR, USA).

2.2.4 Molecular biological analyses

2.2.4.1 Antibody array

The Proteome Profiler Human Apoptosis Array detecting 35 apoptosis-related proteins was performed with TC-71 cells according to the manufacturer´s protocol. Briefly, after incubating the cells with ~IC50 concentrations (viscum 2 ng/mL ML I, TT 50 µg/mL OA, viscumTT 1 ng/mL ML I +10 µg/mL OA) of the extracts versus untreated control cells for 24 h in T75 cell culture flasks, TC-71 cells were harvested and washed twice with cold PBS, lysed in Lysis Buffer 17 containing cOmplete Protease Inhibitor Cocktail and Pepstatin A. Protein concentration was determined by Bradford assay. Lysates containing 300 µg protein were incubated with the array and visualized on a Molecular Imager ChemiDoc (including Image Lab software) to detect relative expression of apoptosis-related proteins. Untreated control cells were used as reference. Protein lysates were stored in -80 °C freezer.

2.2.4.2 Western blotting

Protein expression was validated using western blotting. TC-71 and MHH-ES-1 cells were incubated with increasing concentrations of the extracts (versus untreated control cells) for 24 h in T75 cell culture flasks. Then, cells were harvested and washed twice with cold PBS, lysed in Lysis Buffer 17 containing cOmplete Protease Inhibitor Cocktail and Pepstatin A according to the R&D systems instructions. Protein concentration was determined by Bradford assay. Cell lysates (30 µg protein/lane) were separated by SDS-PAGE using 4.5 % stacking gel and 12.5 % separating gel in 1x SDS running buffer for 20 min at 80 V, then 80 min at 110 V. Afterwards, proteins were transferred to nitrocellulose membranes by Trans-Blot Turbo Transfer System and Trans-Blot Turbo nitrocellulose transfer packs and incubated in blocking buffer for 1 h at room temperature. Then, blots were incubated overnight at 4 °C in TBST containing 5 % BSA and primary antibody. Next day, blots were washed 3x 10 min in TBST and incubated 1 h with HRP-conjugated secondary antibodies, then visualized by ECL solution on a Molecular Imager ChemiDoc (including Image Lab software). ß-Actin was used as loading control. Protein lysates were stored in -80 °C freezer.

2.2.4.3 Proteome profiling, pathway and protein network analysis

To analyze global protein expression after treatment with viscum, TT or viscumTT, a proteome profiling of TC-71 cells was performed by combined liquid chromatography/ mass spectrometry (LC-MS/MS). Therefore, biological triplicates of TC-71 cells were grown in T25 cell culture flasks and treated for 24 h with viscum, TT or viscumTT at ~ IC50 concentrations (viscum 2 ng/mL ML I, TT 50 µg/mL OA, viscumTT 1 ng/mL ML I and 10 µg/mL OA) versus untreated control cells. After incubation, cells were harvested and lysed in a denaturing buffer (6 M guanidinium chloride, 10 mM tris(2-carboxyethyl)phosphine, 40 mM chloroauric acid, 100 mM Tris pH 8.5). Then, lysates were sent to David Meierhofer´s lab at the Max Planck Institute for Molecular Genetics for a label-free LC-MS/MS analysis and data processing. There, the label-free software MaxLFQ (Cox *et al.*, 2014) was used for quantification (Cox *et al.*, 2008) and peptides were searched against the human proteome database UniProtKB with 88.717 entries, released in 11/2014. A false discovery rate (FDR) of 0.01 for proteins and peptides and a minimum peptide length of 7 amino acids were required. A maximum of two missed cleavages was allowed for the tryptic digest. Cysteine carbamidomethylation was set as fixed modification, while N-terminal acetylation and methionine oxidation were set as variable modifications. Further, principal component analysis and heat map of the replicates was carried out using the Perseus software v1.5.1.6.

Pathway analysis and interpretation of the data was performed independently by using Gene Set Enrichment Analysis (GSEA) v2.1.0 software tool (Subramanian *et al.*, 2005) in order to see if defined sets of proteins belonging to a certain pathway show statistically significant differences between treatments and control. GSEA standard settings were used, except the minimum size exclusion was set to 5 and KEGG v2.1.0 as well as reactome v5.0 were used as gene set database. The cutoff for significantly regulated pathways was set to be ≤ 0.05 p-value and ≤ 0.25 false discovery rate (FDR) (Benjamini *et al.*, 2001). For protein-protein interaction network analyses, String v10 bioinformatics database (Franceschini *et al.*, 2013) was applied to visualize networks of at least 2-fold up- or downregulated proteins with a confidence level of 0.7. Protein nodes, which were not integrated into the protein-protein interaction network, were removed.

2.2.4.4 RNA isolation

RNA of TC-71 cells was isolated by spin columns using NucleoSpin RNA Kit. Therefore, TC-71 cells were incubated with increasing concentrations of the extracts (versus untreated control cells) for 24 h in T75 cell culture flasks. Afterwards, cells were harvested and RNA was isolated according to the manufacturer´s protocol. Purity and concentration was determined by OD260/280 using NanoDrop 2000 spectrophotometer. RNA integrity was checked on a denaturing agarose gel. RNA was stored in -80 °C freezer.

2.2.4.5 Two-step real-time PCR

In the first step, cDNA was synthesized by using High Capacity RNA-to-cDNA Kit and 2 µg RNA from TC-71 cells (treated versus control) according to the manufacturer´s protocol. In the second step, quantification of gene expression was performed by real-time PCR (qPCR) on a StepOnePlus System in 96-well fast plates under standard conditions (10 min, 95 °C; 15 sec, 95 °C and 60 sec, 60 °C, 40x) and automatic threshold setting using Power SYBR Green Master Mix including ROX as passive reference. A “no template control” and a “no reverse transcriptase control” were included. The quantitative PCR reaction was set up in total volume of 20 µl containing 5 ng cDNA and 500 nM primers. Primer efficiency was tested ranging between 90-100 %. GAPDH levels were used for normalization (reference). Melt curves were analyzed and validated. The relative expression of genes was calculated by ΔΔCT method (Livak *et al.*, 2001) using the following equation:

$$\Delta\Delta CT = \left(CT_{target,\ untreated} - CT_{reference,\ untreated}\right) - \left(CT_{target,\ treated} - CT_{reference,\ treated}\right)$$

The fold-change of gene expression relative to untreated control was derived from the following formula:

$$fold - change = 2^{-\Delta\Delta CT}$$

2.2.4.6 Transcriptome profiling and pathway analysis

To analyze global gene expression after treatment with viscum, TT or viscumTT, a transcriptome profiling of TC-71 cells was performed by mRNA sequencing. Therefore, TC-71 cells were treated in T75 cell culture flasks with the extracts for 24 h in ~ IC50 concentrations (viscum 2 ng/mL ML I, TT 50 µg/mL OA, viscumTT 1 ng/mL ML I and 10 µg/mL OA) versus untreated control cells in one single experiment. After total RNA isolation of treated and control cells using NucleoSpin RNA Kit, samples were sent to mRNA sequencing and data processing at Bernd Timmermann´s lab at the Max Planck Institute for Molecular Genetics, Berlin. Before sequencing, Illumina TruSeq RNA sample preparation including a polyA-selection step via oligo-dT-beads was used to gain mRNA and to generate cDNA libraries. Samples were sequenced by paired-end mRNA sequencing using Illumina HiSeq 2500 (50 bp reads). For data processing, reads were mapped uniquely to human genome hg19 using CLC genomics software, while normalization and identification of differentially expressed genes was performed using the Bioconductor open source software DESeq v3.1 (Anders *et al.*, 2010) using the negative binomial distribution and calculating fold-change relative to untreated control cells, FDR (Benjamini *et al.*, 2001) and its p-value. Heat map illustrating differentially expressed genes ($p \leq 0.05$) as log2 fold change relative to untreated control cells was performed by using the R free software (Team, 2008).

Pathway analysis and interpretation of the data was performed independently with top differentially expressed genes ($p \leq 0.01$) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7(Huang *et al.*, 2008) with standard settings in order to identify differentially regulated pathways between treatments and control. The cutoff for significantly regulated pathways was set to be $p \leq 0.05$ and FDR ≤ 0.1.

2.2.5 Statistical analyses

In vitro experiments were performed in at least three independent experiments (except the Proteome Profiler Human Apoptosis Array and transcriptome profiling, which were carried out once). In detail, flow cytometric analyses, measurement of cell proliferation and qPCR (including RNA isolation) were performed in four, while LDH assay and inhibitor assays were carried out in three independent experiments. Mean results ±

standard deviation are plotted in bar or line graphs. Western blots and proteome profiling were performed in three independent experiments. *Ex vivo* experiments were performed in four independent experiments to asses induction of apoptosis (n = 4) and two independent experiments to asses proliferation inhibition, mitochondrial membrane potential and activation of CASP8 and CASP9 (n = 2) due to limited cell number and culture period. *In vivo* mouse experiment was performed once and mean tumor volumes of 8 mice per group were used to asses tumor growth.

Webb´s fractional product (Webb, 1963) (Fp) was used to determine whether the effect of viscumTT *in vitro* and *ex vivo* was additive (Fp = 1), antagonistic (Fp < 1) or synergistic (Fp > 1) and was calculated in the following two equations with $E1;2_{calculated}$ = calculated effect of viscumTT; E1 = observed effect of TT; E2 = observed effect of viscum; $E1;2_{observed}$ = observed effect of viscumTT:

$$E1;2_{calculated} = (E1 + E2) - (E1\ x\ E2)$$

$$Fp = \frac{E1;2_{observed}}{E1;2_{calculated}}$$

Since most of the obtained data of *in vitro* and *ex vivo* cell biological measurements as well as the *in vivo* data was not normally distributed, a non-parametric test method was needed for multi-sample analysis (treatment with viscum, TT or viscumTT in different concentrations). Therefore, the Kruskal-Wallis one-way analysis of variance was chosen to test whether there is a significant treatment effect ($p \leq 0.05$). Dunn´s test was applied as post hoc test comparing all groups with all groups and using FDR correction. A FDR ≤ 0.1 was considered as significant. Dunn´s test results of treatment groups (viscum, TT or viscumTT) compared to untreated control group are displayed in the supplementary data (Table S2-S7).

3 Results

3.1 Viscum album L. extracts inhibit proliferation *in vitro*

In order to examine the anti-proliferative potential of viscum, TT and viscumTT, the cell proliferation of TC-71 and MHH-ES-1 cells was measured after incubation for 24 h with increasing concentrations of the *Viscum album* L. extracts. Viscum and viscumTT displayed a significant and concentration-dependent inhibition of proliferation in both cell lines with up to 80 % inhibition in TC-71 cells and 50-55 % in MHH-ES-1 cells, whereby viscumTT displayed a stronger effect than viscum alone (Figure 6). TT had no effect on MHH-ES-1 cells, but displayed a moderate (not significant) inhibitory effect up to 60 % at the highest TT concentration (50 µg/mL) in TC-71 cells (Figure 6).

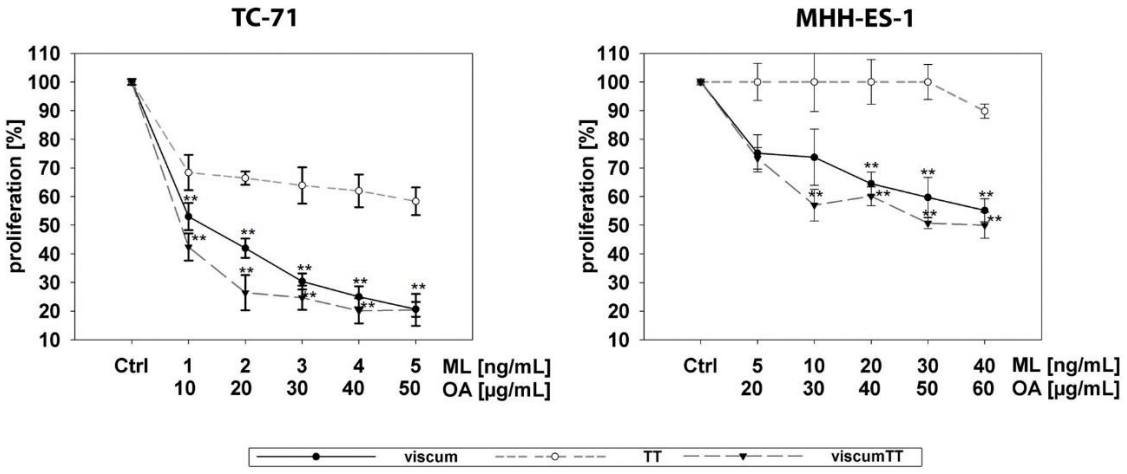

Figure 6: *Viscum album* L. extracts inhibit proliferation in Ewing sarcoma cell lines. TC-71 and MHH-ES-1 cells were treated with increasing concentrations of viscum, TT or viscumTT for 24 h and proliferation was calculated from total cell numbers in cultures started from defined cell numbers compared to control cultures. Cells were counted using a CASY Cell Counter. The results are presented as the percentage of untreated control (Ctrl) cultures ± SD, and are the mean of 4 independent experiments. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum and viscumTT).

In order to detect synergism of the combinatory viscumTT extract, Web´s fractional product was calculated. ViscumTT displayed no synergistic anti-proliferative effect (Figure 6). The results indicate that the anti-proliferative effect of the extracts is concentration- and cell type-dependent.

3.2 *Viscum album* L. extracts show no early cytotoxicity via necrosis *in vitro*

Necrosis is a rapid type of cell death which leads to the loss of membrane integrity and therefore results in inflammation (Elmore, 2007). In order to exclude an early cytotoxicity of viscum, TT or viscumTT via necrotic cell death, LDH release from cells to culture medium was measured after 2 h incubation with the extracts. Neither cell line released more than 15 % LDH in response to treatment with any extract when compared to untreated control cells (Figure 7).

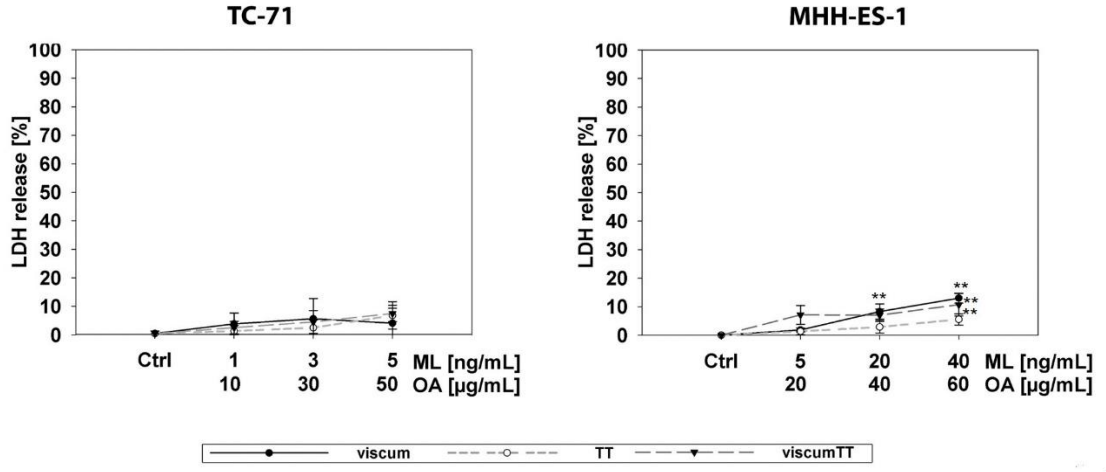

Figure 7: *Viscum album* L. extracts show no early cytotoxicity via necrosis *in vitro*. TC-71 and MHH-ES-1 cells were incubated with increasing concentrations of viscum, TT or viscumTT for 2 h before assessing early cytotoxicity via LDH release into the culture medium. The results are presented as the percentage of untreated control (Ctrl) cultures ± SD, and are the mean of 3 independent experiments. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) for MHH-ES-1 cells and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups.

The results reveal that the extracts do not exhibit early cytotoxicity via necrotic cell death when treated with the extracts for a short time period. However, the results suggest that necrotic cell death caused by the extracts is concentration-dependent.

3.3 *Viscum album* L. extracts induce apoptosis *in vitro*

To further analyze the cytotoxic potential of the extracts, TC-71 and MHH-ES-1 cells were incubated for 24 h with viscum, TT or viscumTT. Afterwards, cells were stained with annexin V/propidium iodide and the fraction of apoptotic cells was flow cytometrically assessed. Viscum and viscumTT induced apoptosis in both cell lines in a significant and dose-dependent manner (Figure 8). Up to 80 % of TC-71 cells and up to 50 % of MHH-ES-1 cells underwent apoptosis after treatment with the highest applied viscum concentration.

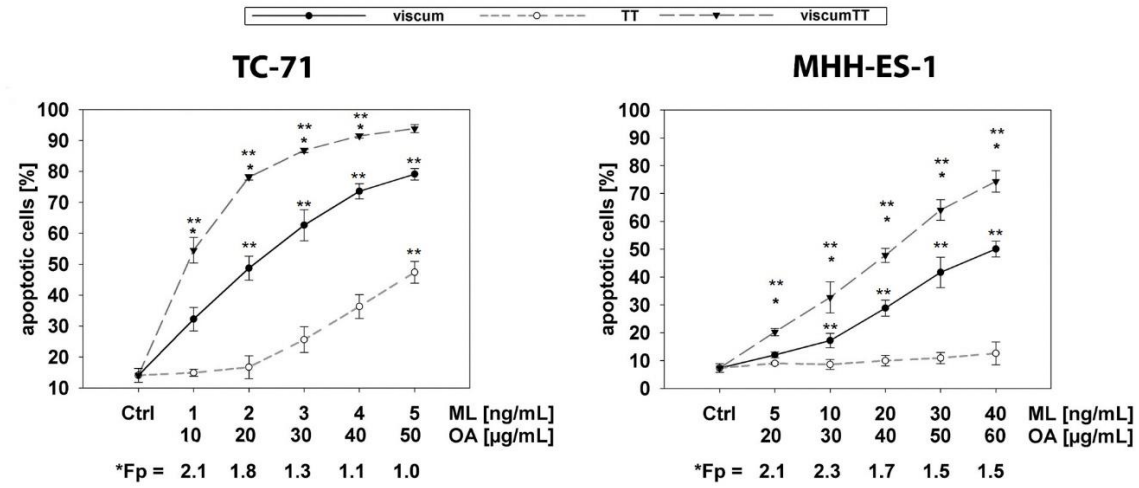

Figure 8: *Viscum album* L. extracts induce apoptosis in Ewing sarcoma cell lines. TC-71 and MHH-ES-1 cells were incubated for 24 h with increasing concentrations of viscum, TT or viscumTT, then cultures were stained with annexin V and propidium iodide and examined flow cytometrically. The percentage of dead cells ± SD in each treatment group are shown from 4 independent experiments compared to untreated control cells (Ctrl). Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. A synergistic effect of the combined viscumTT extract above single extracts was calculated by Webb´s fractional product (*Fp > 1). Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum, TT and viscumTT).

When treated with the combinatory viscumTT extract, apoptosis induction was even higher. The TT extract induced a significant and dose-dependent apoptosis only in TC-71 cells (Figure 8). IC50 was approximately reached in TC-71 cells for viscum at 2 ng/mL ML I, for TT at 50 µg/mL OA and for viscumTT at 1 ng/mL ML I and 10 µg/mL OA. In MHH-ES-1 cells, IC50 was approximately reached for viscum at 40 ng/mL ML I and for viscumTT at 20 ng/mL ML I and 40 µg/mL OA. In order to investigate synergism of the combinatory viscumTT extract, Web´s fractional product was calculated pointing out a synergistic effect of viscumTT on apoptosis in both cell lines when compared to the single extracts (Figure 8).

Western blot analyses detecting PARP1 and CASP3 cleavage validated the concentration-dependent apoptosis induction by viscum and viscumTT in both cell lines indicating the involvement of caspases in apoptosis induction (Figure 9). Furthermore, the TT extract displayed only significant PARP1 and CASP3 cleavage in TC-71 cells and not in MHH-ES-1 cells (Figure 9) confirming the flow cytometry results.

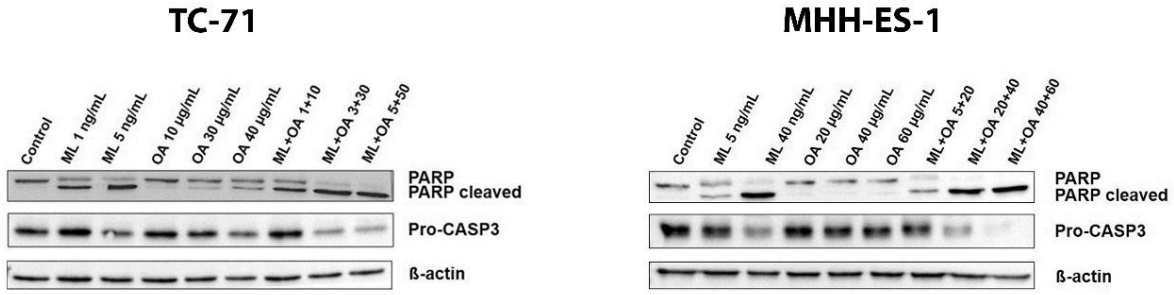

Figure 9: Western blots confirm apoptosis in Ewing sarcoma cell lines. PARP1 cleavage and CASP3 activation was assessed in whole-cell extracts using western blotting from cells treated for 24 h with increasing concentrations of viscum, TT or viscumTT. β-actin was used as loading control, and images shown are representative for the results in 3 independent experiments. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration.

3.4 Viscum and viscumTT induce depolarization of mitochondria membrane in Ewing sarcoma cell lines

To more closely assess the mechanism behind the cytotoxicity of *Viscum album* L. extracts, mitochondrial involvement during apoptotic induction was analyzed in TC-71 and MHH-ES-1 cells. Therefore, cell cultures were treated for 24 h with viscum, TT or viscumTT and were flow cytometrically assessed after JC-1 staining detecting mitochondrial membrane potentials relative to untreated control cultures. Treatment with viscum or viscumTT resulted in a significant dose-dependent loss of mitochondrial membrane potential in TC-71 and MHH-ES-1 cells. Moreover, viscumTT displayed a synergistic effect on the mitochondrial membrane depolarization peaking at 80 % suppression of membrane potential in both Ewing sarcoma cell lines (Figure 10) validating the synergistic apoptosis induction.

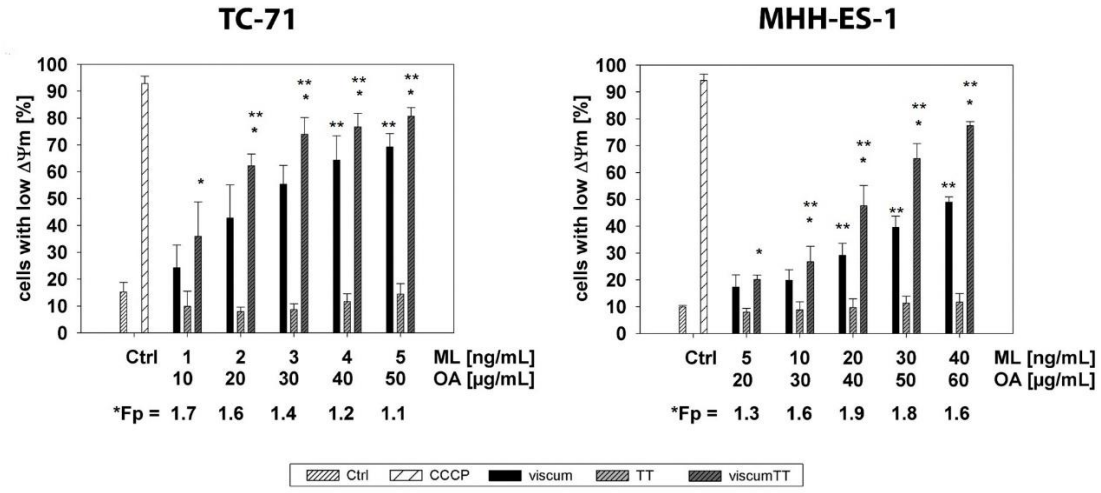

Figure 10: Viscum and viscumTT induce apoptosis via mitochondrial depolarization. TC-71 and MHH-ES-1 cells treated for 24 h with increasing concentrations of viscum, TT or viscumTT and were assessed for mitochondrial involvement in apoptosis using JC-1 staining and flow cytometry. Bars depict the percentage of cells with low mitochondrial membrane potential ($\Delta\Psi_{m,}$ ± SD, error bars) in each treatment group compared to untreated control cells (Ctrl) averaged from 4 independent experiments. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was used as positive control. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. A synergistic effect of combined viscumTT extract was calculated by Webb´s fractional product (*Fp > 1). Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum and viscumTT).

Treatment with TT did not affect the mitochondrial membrane potential significantly in both cell lines (Figure 10). These results reveal that viscum and viscumTT induce apoptosis via the involvement of the intrinsic apoptosis pathway.

3.5 TT-mediated apoptosis induction is driven by the combination of oleanolic and betulinic acid

To further analyze the apoptosis induction in TC-71 cells by the triterpene extract, cells were incubated for 24 h with increasing concentrations of either TT or with an OA and BA standard separately and in combination. Afterwards, cells were stained with annexin V/propidium iodide and JC-1 followed by flow cytometry. Treatment with the standards displayed, like TT, a significant dose-dependent induction of apoptosis. However, treatment with OA standard alone displayed a stronger effect than the TT extract with ~ 80 % apoptotic cells and even ~ 70 % loss of mitochondria membrane potential. By contrast, BA standard alone showed a weaker effect than TT with ~ 35 % apoptotic cells and a similar effect as TT on the mitochondria membrane potential (Figure 11). The combination of OA and BA, mimicking the TT extract, showed a slightly higher effect on apoptosis induction than TT and a similar low effect on mitochondria membrane depolarization as the TT extract (Figure 11). The results indicate, that the combination of OA and BA is responsible for the cytotoxic effect of the TT extract. However, the combination of OA and BA is less potent than OA alone, but more effective than BA alone. Furthermore, high OA concentrations alone, which effectively induce apoptosis (OA 50 µg/mL), result in the depolarization of mitochondria membrane.

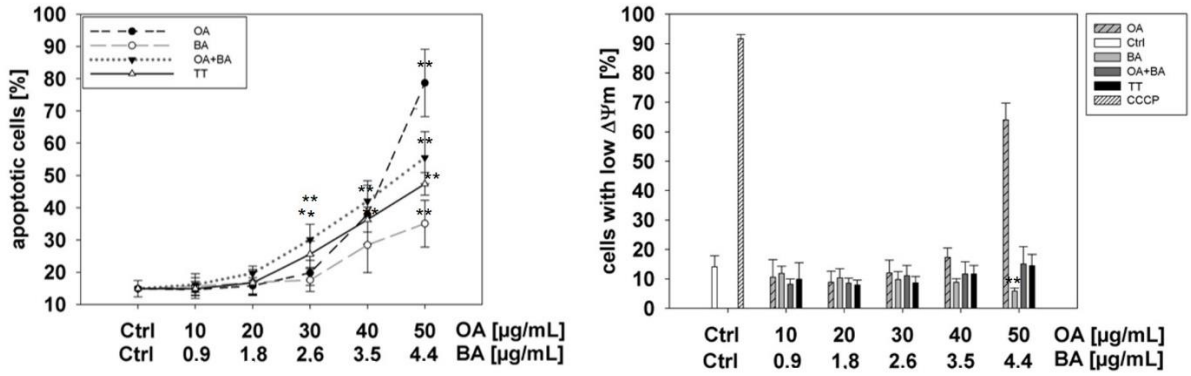

Figure 11: Oleanolic acid is more potent and betulinic acid is less potent than the TT extract. TC-71 cells were treated for 24 h with increasing concentrations of TT or an oleanolic acid (OA) and betulinic acid (BA) standard separately and in combination followed by flow cytometric analysis of apoptotic cells (annexin V/propidium iodide staining, left) and cells with low mitochondria membrane potential (JC-1 staining, right). Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was used as positive control for JC-1 staining. The percentage of apoptotic cells and cells with low mitochondrial membrane potential ($\Delta\Psi_m$, ± SD, error bars) is shown in each treatment group compared to untreated control cells (Ctrl) averaged from 3 independent experiments. OA and BA concentrations were used as a measure of TT active agent concentration. A synergistic effect of combined OA+BA standard was calculated by Webb´s fractional product (*Fp > 1). Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups displayed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (TT, OA, BA, OA+BA).

3.6 Viscum and viscumTT activate caspases *in vitro*

To further investigate the mechanisms of apoptosis, the activation of CASP8, CASP9 and CASP3 was measured in TC-71 and MHH-ES-1 cells after 24 h treatment with viscum, TT or viscumTT. Both cell lines showed a similarly potent and significant concentration-dependent activation of caspases in TC-71 and MHH-ES-1 cells after viscum or viscumTT treatment. Thereby, caspase activity reached 50-60 % upon viscum treatment and up to 80-90 % upon viscumTT treatment (Figure 12). Calculation of Webb´s fractional product revealed that viscumTT synergistically increased caspase activation (Figure 12). In line with the previous results, TT treatment increased caspase activation by only ~15 % (not significant) in TC-71 cells and did not affect caspase activation in MHH-ES-1 cells (Figure 12) when compared to the basal caspase activity of untreated control cells indicating that other mechanisms are responsible for the moderate TT-mediated apoptosis in TC-71 cells. The results show that in addition to the

intrinsic (mitochondrial) apoptosis pathway also the extrinsic apoptosis pathway plays a role in viscum- and viscumTT-mediated apoptosis.

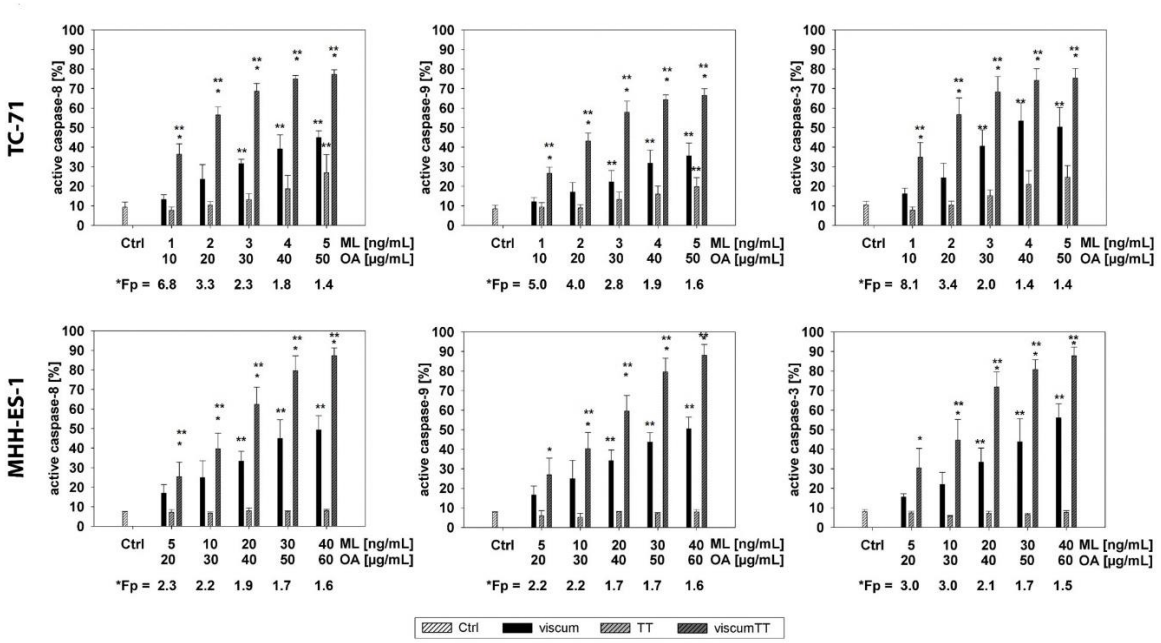

Figure 12: Viscum and viscumTT activate caspases. TC-71 and MHH-ES-1 cells were treated 24 h with either viscum, TT or viscumTT. CASP9, CASP8, CASP3 activation was measured using FITC-LEHD-FMK, FITC-IETD-FMK and FITC-DEVD-FMK staining followed by flow cytometry. Bars represent mean activation (± SD, error bars) in treatment groups from 4 independent experiments compared to untreated control cultures. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. A synergist effect of the combined viscumTT extract was calculated by Webb´s fractional product (*Fp > 1). Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum and viscumTT).

In order to further illuminate the role of caspases in mistletoe-mediated apoptosis, TC-71 cells were treated for 24 h with viscum, TT or viscumTT in the presence or absence of the Z-VAD-FMK pan-caspase inhibitor. Z-VAD-FMK significantly decreased apoptotic induction by up to 70 % in cells treated with viscum or viscumTT and up to 30 % in cells treated with TT (Figure 13), validating the essential role of caspases in viscum- and viscumTT-induced apoptosis.

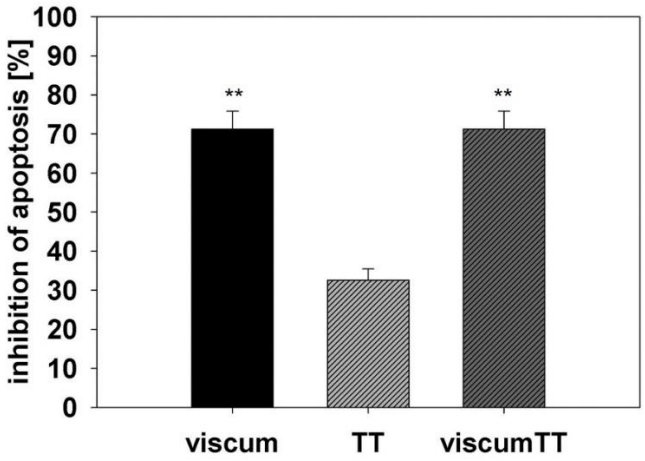

Figure 13: Pan-caspase inhibitor reduces apoptosis induction in TC-71 cells by *Viscum album* L. extracts. TC-71 cells were treated with viscum, TT or viscumTT for 24 h with or without 40 µM Z-VAD-FMK (pan-caspase inhibitor), then apoptotic cells were detected using annexin V/propidium iodide staining and flow cytometry. Bars represent the mean (± SD, error bars) of 3 independent experiments. Results are expressed as percentages of the untreated control cultures. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum and viscumTT).

3.7 Viscum and viscumTT induce apoptosis and inhibit proliferation *ex vivo*

To verify the *in vitro* results *ex vivo*, cultures of primary Ewing sarcoma cells were treated with increasing concentrations of viscum, TT or viscumTT for 24 h followed by flow cytometric analysis of apoptotic cells, mitochondria membrane potential and caspase activation. TT alone had no effect on primary Ewing sarcoma cells *ex vivo* (Figure 14), similarly to MHH-ES-1 cells *in vitro*. Treatment with viscum or viscumTT significantly induced apoptosis in a concentration-dependent manner in primary Ewing sarcoma cells *ex vivo* accompanied by a loss of mitochondrial membrane potential and CASP8 and CASP9 activation (Figure 14). Importantly, the synergistic effect of viscumTT was validated in primary Ewing sarcoma cells *ex vivo* (Figure 14).

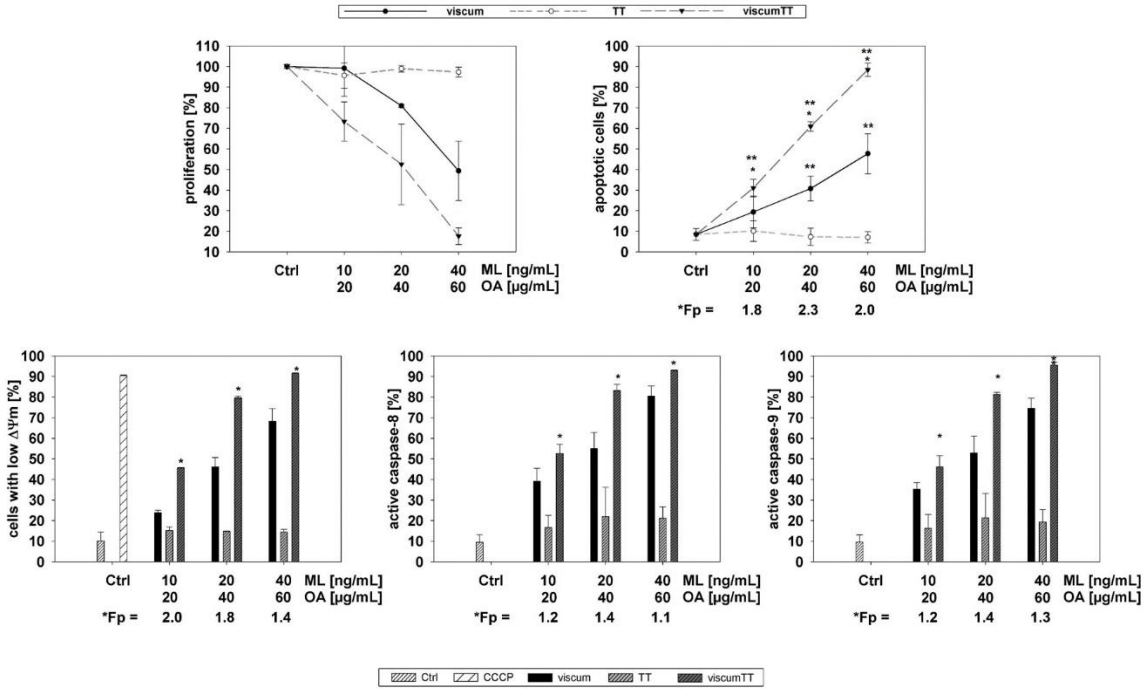

Figure 14: Viscum and viscumTT induce apoptosis and inhibit proliferation *ex vivo*. Primary Ewing sarcoma cells from a 15-year-old girl were grown as short-term cultures *ex vivo*, and treated with increasing concentrations of viscum, TT or viscumTT for 24 h. Induction of apoptosis (n = 4), proliferation inhibition (n = 2), mitochondrial membrane potential (n = 2) and activation of CASP8 and CASP9 (n = 2) were measured flow cytometrically with the same assays described for the cell lines. Values are the means of n experiments ± SD (error bars). Controls (Ctrl) were untreated cultures grown in parallel. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration. A synergistic effect of the combined viscumTT extract was calculated by Webb´s fractional product (*Fp > 1). Kruskal-Wallis one-way analysis revealed a significant treatment effect in apoptosis induction ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum and viscumTT).

3.8 *Viscum album* L. extracts reduce tumor volume in a Ewing sarcoma mouse model

In order to test the cytotoxicity of viscum, TT and viscumTT *in vivo*, established human Ewing sarcoma xenografts, with tumors grown subcutaneously in female NOD-scid IL2rγ null/TC-71 mice, were treated with the extracts. Viscum, TT or viscumTT were injected intratumorally every 2 to 3 days with 40/50/60 mg/kg OA (TT), 0.75/1.25/1.75 µg/kg ML (viscum) or a combination thereof (viscumTT). The mice received a total of 5 treatments of viscum, TT or viscumTT and each dose was administered twice within 2 to 3 days. Additionally, viscumTT was also intravenously given in a total of 5 applications (with each dose being administered twice within 2 to 3 days) in one treatment group to assess systemic effects on tumor growth. A positive control group was included receiving a single intravenous dose of the classical cytostatic agent, doxorubicin 4 mg/kg. The negative control group received cyclodextrins either intratumorally or intravenously.

The administered extract concentrations were well-tolerated without significant weight loss or toxicity symptoms. Because three mice from control group treated with cyclodextrins intratumorally had to be taken of the study on day 21 due to tumor size, but all remaining mice were not sacrificed on that day for organizational reasons, tumor volume on day 21 was set as endpoint measurement. All intratumorally injected extracts inhibited tumor growth by 50-70 % compared to controls intratumorally treated with cyclodextrins. However, tumor volumes displayed a large variance within the groups and only intratumoral viscum treatment revealed significant results (Figure 22). Intravenously administered viscumTT also significantly inhibited tumor growth by 65 % compared to control mice receiving cyclodextrins intravenously, and achieved a comparable effect to doxorubicin, which reduced tumor growth by 62 % (Figure 22). Taken together, viscum injected intratumorally and intravenously administered viscumTT effectively suppressed Ewing sarcoma growth in a xenograft mouse model.

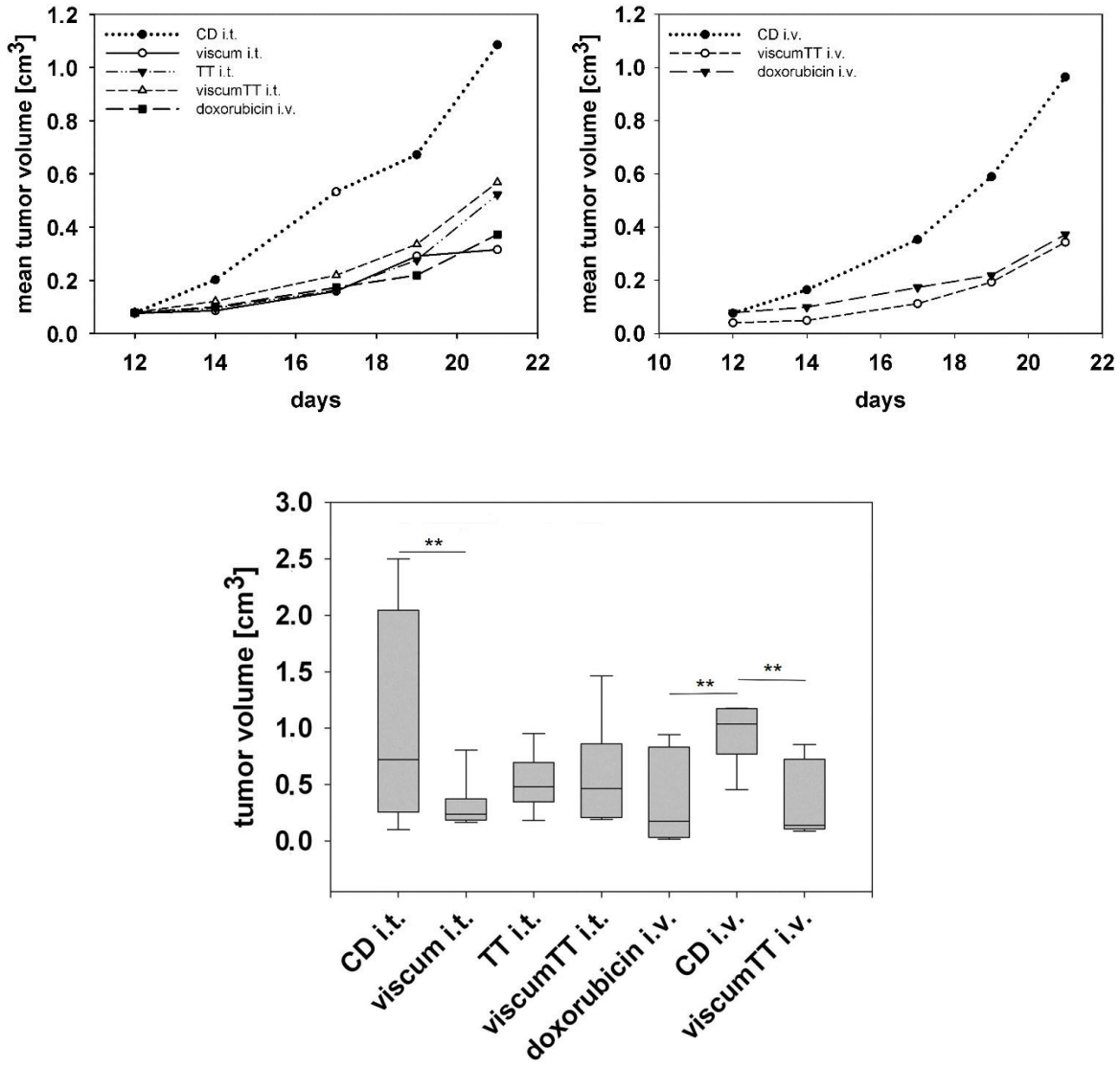

Figure 22: *Viscum album* L. extracts reduce the growth of human Ewing sarcoma xenograft tumors in mice. Established Ewing sarcoma xenograft tumors in NSG mice were intratumorally injected with increasing concentrations of 40/50/60 mg oleanolic acid/kg mouse weight (TT i.t.), 0.75/1.25/1.75 µg mistletoe lectin I/kg mouse weight (viscum i.t.) or a combination thereof (viscumTT i.t.) every 2 to 3 days with a total of 5 treatment cycles. One treatment group received viscumTT intravenously (viscumTT i.v.) every 2 to 3 days with a total of 5 treatment cycles, and one positive control group was intravenously treated once with 4 mg/kg doxorubicin (doxorubicin i.v.), a clinically used classical cytostatic agent. The negative control group received the solubilizing agents, cyclodextrins (CD), which was injected either 5 times intratumorally (i.t.) or intravenously (i.v.). Kruskal-Wallis one-way analysis revealed a significant treatment effect ($p < 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed a significant effect (**FDR ≤ 0.1) between the CD i.t. control group and viscum i.t. as well as between the CD i.v. control group and viscumTT i.v. or the positive control group, doxorubicin i.v., displayed in Box-and-Whisker plots (each represents one treatment group including 8 mice).

3.9 *Viscum album* L. extracts alter the expression of apoptosis related proteins in Ewing sarcoma cell lines

To further investigate the mistletoe-induced apoptosis, the R&D Systems Proteome Profiler Human Apoptosis Array was performed detecting the expression of proteins related to apoptosis. Therefore, TC-71 cells were treated with ~ IC50 concentrations of viscum, TT or viscumTT for 24 h and cell lysates were incubated with array membranes. The array detected a dose-dependent shift of many proteins associated with apoptosis in the protein extracts from the treatment groups. Viscum, TT and viscumTT reduced expression of the IAP family members, BIRC2 (cIAP1), BIRC3 (cIAP2), XIAP and BIRC5, and to a lesser extent, the BCL2 protein and tumor suppressor protein TP53, compared to untreated control cells (Figure 15A). BIRC5 downregulation was particularly prominent. Interestingly, the expression of claspin (CLSPN), a protein needed for efficient DNA replication, was strongly suppressed by viscum, TT and viscumTT (Figure 15A). TT also strongly upregulated expression of the heat shock 60kDa protein 1 (HSPD1) (Figure 15A).

Since the array could only be performed on a single treatment experiment, selected results were validated in TC71 and MHH-ES-1 lysates from three independent experiments using western blotting. Western blots confirmed the downregulation of BIRC5, XIAP, TP53 and CLSPN by viscum, TT and viscumTT in Ewing sarcoma cells *in vitro* (Figure 15B). Since the pro-apoptotic BCL2 family member PMAIP1 (formerly NOXA) was shown to play a role in polychemotherapy-induced apoptosis of BA and doxorubicin (Ehrhardt *et al.*, 2012), next the expression of PMAIP1 was analyzed in protein extracts from cells treated with viscum, TT or viscumTT. TT treatment resulted in an upregulation of PMAIP1 expression in both cell lines, whereas viscum and viscumTT did not affect PMAIP1 expression compared to the untreated control cell protein lysates (Figure 15B). Furthermore, the anti-apoptotic MCL1, an opponent of PMAIP1, was effectively downregulated by viscum and viscumTT in both cell lines, but its expression was not affected after TT treatment when compared to untreated control cells (Figure 15B). These data suggest that TT upregulates pro-apoptotic PMAIP1 but is not able to down-regulate MCL1, possibly leading to a less effective apoptosis induction as viscum or viscumTT. Taken together, the results indicate that the cytotoxic agents derived from mistletoe invoke several signaling pathways to induce apoptosis, which might contribute to the synergistic effect of the total mistletoe extract viscumTT.

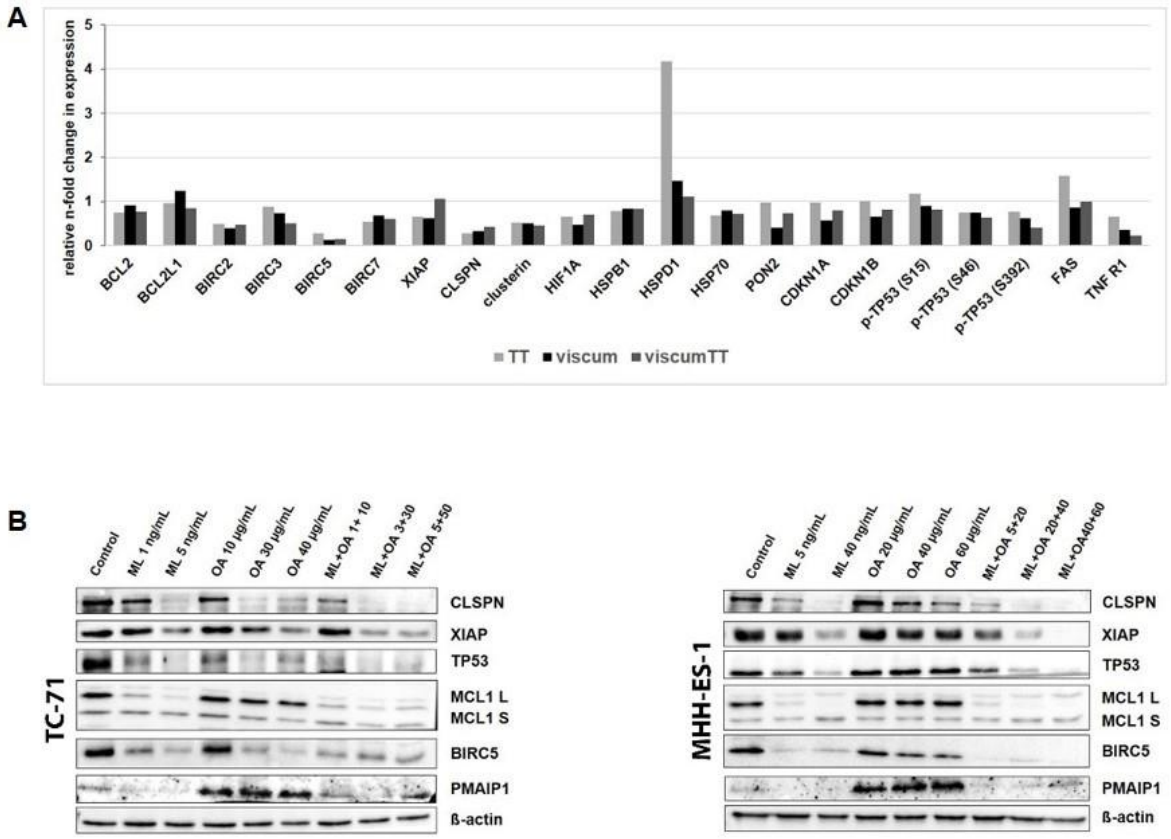

Figure 15: Viscum, TT and viscumTT alter apoptosis-related protein expression. A. Whole-cell protein lysates from TC-71 cells after 24 h of treatment with viscum, TT or viscumTT in ~ IC50 concentrations were analyzed using the R&D systems Proteome Profiler Human Apoptosis Array (n = 1). Bars represent the n-fold change in apoptosis-related protein expression relative to untreated control cell protein expression. **B.** TC-71 and MHH-ES-1 cells were treated with increasing concentrations of viscum, TT or viscumTT for 24 h, then expression of selected apoptosis-related proteins was examined in whole-cell lysates using western blotting. β-actin was used as loading control, and representative pictures are shown from 3 independent experiments. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration.

3.10 *Viscum album* L. extracts alter the transcriptomic profile of TC-71 cells

In order to gain global insights into the effects of the single extracts and the synergistic effect of viscumTT on the transcriptome of Ewing sarcoma cells, mRNA-sequencing of TC-71 cells was performed. Therefore, cells were treated for 24 h with viscum, TT or viscumTT in ~ IC50 concentrations in reference to untreated control cells. Sequencing analysis of total mRNA detected > 62,400 transcripts belonging to > 17,300 genes. Treatment with viscum or viscumTT displayed a strong alteration of the transcriptomic profile with a similar transcriptomic pattern of differentially expressed genes, and resulted in a differential expression of 1004 genes for viscum and 1069 genes for viscumTT treatment. Most of the differentially expressed genes were upregulated relative to untreated control cells (Figure 16). TT treatment had a lesser impact on the TC-71 transcriptome, causing the differential expression of 249 genes (Figure 16).

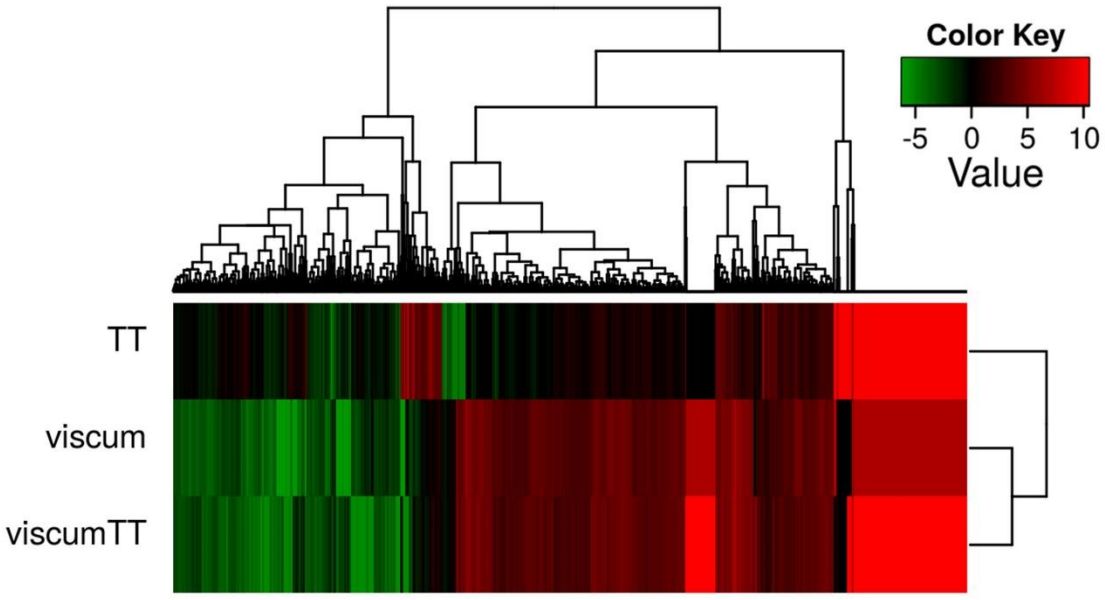

Figure 16: Viscum, TT and viscumTT alter the transcriptomic profile. TC-71 cells were incubated for 24 h with viscum, TT or viscumTT in ~ IC50 concentrations followed by mRNA sequencing. Gene expression was calculated as the relative log2 fold change relative to untreated control cells in one experiment (n = 1, $p \leq 0.05$). Heat map displays the upregulation of genes relative to untreated control cells in red and the downregulation in green.

Several components of immune and cellular stress response were within the significantly regulated genes by viscum, TT or viscumTT treatment. The 40 most significantly regulated genes by viscum, TT or viscumTT treatment of TC-71 cells are shown in the table 1a-1c. Notably, viscum and viscumTT treatment resulted in the upregulation of e.g. *JUN*, *DDIT3* and *CXCL8* (formerly *IL8*) and the downregulation of *MAP2K6* (Table 1a and 1c), while *KLHDC7B*, *NUPR1* and *CYTH3* (formerly *GPR1*) were upregulated by TT treatment (Table 1b). The upregulation of *JUN* and *DDIT3* and the downregulation of *MAP2K6*, which are involved in MAPK signaling, was confirmed by qPCR (Figure 17).

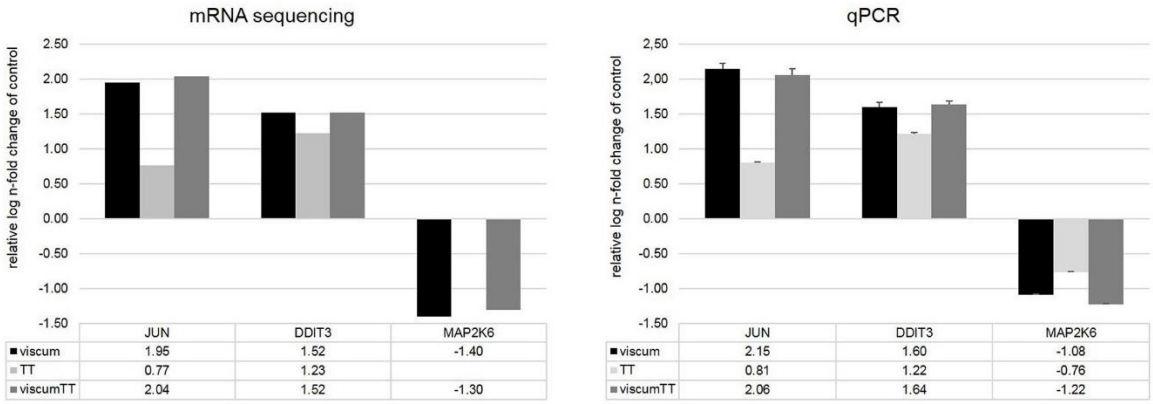

Figure 17: Viscum, TT and viscumTT alter the expression of MAPK signaling related genes. TC-71 cells were incubated for 24 h with viscum, TT or viscumTT in ~ IC50 concentrations. In addition to mRNA sequencing, qPCR was performed to detect the expression of *JUN*, *DDIT3* and *MAP2K6*. The relative gene expression compared to untreated control cells, which was detected by qPCR, was calculated by ΔΔCT method. Bars of qPCR results represent mean fold change (± SD, error bars) in treatment groups from 4 independent experiments relative to untreated control cells. Bars of mRNA sequencing results represent mean fold change in treatment groups from one experiment relative to untreated control.

Table 1a: The 40 most significantly regulated genes by viscum treatment in TC-71 cells as fold-change relative to untreated control cells.

ENSTID	Gene	p-Value	Control	Viscum-treated	Fold-change
ENST00000357727	CREB5	1.32E-07	0.00	1451.99	N/A
ENST00000482692	CREB5	7.45E-06	0.00	343.33	N/A
ENST00000249330	VGF	8.98E-06	0.00	320.98	N/A
ENST00000371222	JUN	5.63E-05	206.19	18,368.89	89.09
ENST00000409458	GPNMB	3.06E-04	2.11	194.91	92.17
ENST00000414584	LINC00152	3.07E-04	1.06	144.84	136.98
ENST00000223095	SERPINE1	4.02E-04	0.00	82.26	N/A
ENST00000239938	EGR1	4.95E-04	540.32	21,507.12	39.80
ENST00000215885	PLA2G3	5.36E-04	998.16	24.14	0.02
ENST00000550903	PPP1R12A	5.84E-04	0.00	72.42	N/A
ENST00000394684	SGMS2	6.46E-04	27.49	1,063.96	38.70
ENST00000592209	WASH5P	6.98E-04	0.00	67.95	N/A
ENST00000369165	HIST2H4A	7.56E-04	1.06	103.71	98.09
ENST00000289352	HIST1H4H	8.81E-04	27.49	947.73	34.47
ENST00000346473	DDIT3	9.51E-04	29.61	985.28	33.28
ENST00000296252	LIPH	9.61E-04	1.06	95.67	90.48
ENST00000533621	BCLAF1	9.68E-04	0.00	59.90	N/A
ENST00000429295	MRPL23	9.79E-04	9.52	357.63	37.58
ENST00000304218	HIST1H1E	1.00E-03	6.34	257.50	40.59
ENST00000474904	GPATCH4	1.04E-03	1.06	92.09	87.09
ENST00000403537	THUMPD2	1.07E-03	0.00	58.12	N/A
ENST00000464213	CD36	1.13E-03	1.06	91.20	86.25
ENST00000532854	BMS1P2	1.23E-03	2.11	118.02	55.81
ENST00000424148	KRTAP5-AS1	1.25E-03	0.00	53.64	N/A
ENST00000489615	RABGAP1L	1.29E-03	4.23	176.13	41.64
ENST00000506674	MRPL1	1.29E-03	6.34	234.25	36.92
ENST00000569313	CTD-2636A23.2	1.36E-03	96.22	1.79	0.02
ENST00000570279	CFDP1	1.47E-03	1.06	82.26	77.79
ENST00000377831	HIST1H3D	1.52E-03	1.06	79.57	75.26
ENST00000200453	PPP1R15A	1.54E-03	432.46	11,251.11	26.02
ENST00000490692	PCSK9	1.60E-03	66.61	0.89	0.01
ENST00000461675	MRPS31	1.61E-03	0.00	49.17	N/A
ENST00000556330	SRSF5	1.84E-03	7.40	232.46	31.41
ENST00000446547	RAB5A	1.86E-03	4.23	152.89	36.15
ENST00000481490	FSBP	1.89E-03	6.34	202.96	31.99
ENST00000554437	SCFD1	1.90E-03	0.00	47.39	N/A
ENST00000317216	EGR3	1.93E-03	10.57	303.99	28.75
ENST00000506974	NDUFS4	1.99E-03	10.57	298.62	28.24
ENST00000369317	TXNIP	2.08E-03	118.43	2783.27	23.50
ENST00000435030	KIF5C	2.10E-03	2.11	96.56	45.66

* $p \leq 0.05$ regarded as significant, N/A not available

Table 1b: The 40 most significantly regulated genes by TT treatment in TC-71 cells as fold-change relative to untreated control cells.

ENSTID	Gene	p-Value	Control	TT-treated	Fold-change
ENST00000395676	KLHDC7B	4.06E-07	2.11	2127.00	1005.80
ENST00000252809	GDF15	2.22E-06	1.06	853.00	806.72
ENST00000441316	RP4-756G23.5	3.65E-06	0.00	451.00	N/A
ENST00000324873	NUPR1	1.77E-05	2.11	556.00	262.92
ENST00000407325	GPR1	4.52E-05	1.06	295.00	278.99
ENST00000496593	RPLP0P2	1.04E-04	1.06	220.00	208.06
ENST00000266646	INHBE	1.11E-04	16.92	1306.00	77.20
ENST00000450895	AP000322.53	1.44E-04	0.00	125.00	N/A
ENST00000416826	RP11-125B21.2	1.98E-04	1.06	175.00	165.50
ENST00000218867	SGCG	2.23E-04	1.06	168.00	158.88
ENST00000257570	OASL	2.38E-04	1.06	158.00	149.43
ENST00000572856	DLGAP1-AS2	2.73E-04	0.00	97.00	N/A
ENST00000426475	AC007405.6	3.06E-04	0.00	96.00	N/A
ENST00000273353	MYH15	3.20E-04	16.92	888.00	52.49
ENST00000582155	NFE2L1	3.48E-04	0.00	92.00	N/A
ENST00000409652	APOL6	3.79E-04	0.00	89.00	N/A
ENST00000371930	ANKRD22	3.82E-04	0.00	89.00	N/A
ENST00000371826	IFIT2	3.95E-04	1.06	130.00	122.95
ENST00000445281	OSTN	4.15E-04	2.11	181.00	85.59
ENST00000265598	LAMP3	4.66E-04	3.17	217.00	68.41
ENST00000338333	FBLL1	7.12E-04	2.11	149.00	70.46
ENST00000512913	SUB1	9.31E-04	0.00	65.00	N/A
ENST00000405493	RAPGEF3	1.01E-03	0.00	63.00	N/A
ENST00000409458	GPNMB	1.21E-03	2.11	115.00	54.38
ENST00000415913	IDH1	1.26E-03	7.40	270.00	36.48
ENST00000288976	PTPDC1	1.65E-03	0.00	53.00	N/A
ENST00000409039	DNAH10	1.73E-03	6.34	213.00	33.57
ENST00000415282	IDH1	1.74E-03	11.63	344.00	29.58
ENST00000303965	ARAP2	1.82E-03	0.00	50.00	N/A
ENST00000580037	NFE2L1	1.91E-03	0.00	50.00	N/A
ENST00000519505	ESRP1	1.94E-03	0.00	50.00	N/A
ENST00000536222	NFE2L1	2.76E-03	1.06	68.00	64.31
ENST00000493793	EPS15	2.95E-03	6.34	175.00	27.58
ENST00000434245	RP11-495P10.8	3.18E-03	2.11	86.00	40.67
ENST00000547303	DDIT3	3.20E-03	4.23	122.00	28.85
ENST00000287713	NMNAT2	3.27E-03	0.00	41.00	N/A
ENST00000264474	CSTA	3.66E-03	6.34	161.00	25.38
ENST00000454170	ESRP1	4.10E-03	0.00	38.00	N/A
ENST00000361488	FAM110B	4.40E-03	4.23	113.00	26.72
ENST00000380659	TLR7	4.56E-03	3.17	89.00	28.06

* $p \leq 0.05$ regarded as significant, N/A not available

Table 1c: The 40 most significantly regulated genes by viscumTT treatment in TC-71 cells as fold-change relative to untreated control cells.

ENSTID	Gene	p-Value	Control	ViscumTT-treated	Fold-change
ENST00000307407	IL8	7.32E-08	4.23	5983.00	1414.59
ENST00000357727	CREB5	2.91E-07	0.00	1084.00	N/A
ENST00000377507	TNFRSF9	3.11E-07	0.00	1072.00	N/A
ENST00000482692	CREB5	3.73E-06	0.00	439.00	N/A
ENST00000249330	VGF	4.04E-06	0.00	427.00	N/A
ENST00000371222	JUN	3.12E-05	206.19	22,624.00	109.73
ENST00000371826	IFIT2	1.77E-04	1.06	176.00	166.45
ENST00000409458	GPNMB	1.83E-04	2.11	235.00	111.12
ENST00000445961	RPS9	3.21E-04	0.00	91.00	N/A
ENST00000414584	LINC00152	3.79E-04	1.06	133.00	125.78
ENST00000223095	SERPINE1	4.00E-04	0.00	82.00	N/A
ENST00000264930	SLC12A7	4.16E-04	22.20	1029.00	46.34
ENST00000257570	OASL	4.52E-04	1.06	124.00	117.27
ENST00000424148	KRTAP5-AS1	5.15E-04	0.00	76.00	N/A
ENST00000326577	TNFRSF12A	5.35E-04	2.11	163.00	77.08
ENST00000369165	HIST2H4A	5.71E-04	1.06	115.00	108.76
ENST00000304338	PPP4R4	5.83E-04	3.17	200.00	63.05
ENST00000394684	SGMS2	6.59E-04	27.49	1049.00	38.16
ENST00000474904	GPATCH4	6.65E-04	1.06	109.00	103.09
ENST00000239938	EGR1	7.35E-04	540.32	18,438.00	34.12
ENST00000200453	PPP1R15A	7.87E-04	432.46	14,397.00	33.29
ENST00000429295	MRPL23	8.81E-04	9.52	370.00	38.88
ENST00000550903	PPP1R12A	8.92E-04	0.00	61.00	N/A
ENST00000377831	HIST1H3D	9.32E-04	1.06	96.00	90.79
ENST00000304218	HIST1H1E	9.33E-04	6.34	263.00	41.46
ENST00000346473	DDIT3	9.43E-04	29.61	982.00	33.17
ENST00000533621	BCLAF1	1.04E-03	0.00	58.00	N/A
ENST00000295927	PTX3	1.06E-03	0.00	61.00	N/A
ENST00000259874	IER3	1.07E-03	2.11	126.00	59.58
ENST00000592209	WASH5P	1.07E-03	0.00	57.00	N/A
ENST00000461675	MRPS31	1.21E-03	0.00	55.00	N/A
ENST00000542735	DND1	1.23E-03	0.00	56.00	N/A
ENST00000506674	MRPL1	1.23E-03	6.34	237.00	37.36
ENST00000538682	SLC3A2	1.47E-03	7.40	250.00	33.78
ENST00000296252	LIPH	1.55E-03	1.06	79.00	74.71
ENST00000423282	STAT1	1.58E-03	1.06	79.00	74.71
ENST00000380659	TLR7	1.59E-03	3.17	135.00	42.56
ENST00000419736	MIR4435-1HG	1.63E-03	0.00	50.00	N/A
ENST00000564543	RP11-20I23.1	1.66E-03	99.39	2.00	0.02
ENST00000173785	KLF6	1.68E-03	0.00	50.00	N/A

* $p \leq 0.05$ regarded as significant, N/A not available

To look more deeply into functions affected at the transcript level by mistletoe extract treatment, gene enrichment and functional annotation analysis was performed with all genes displaying a p-value < 0.01 for differential expression changes from the control cells (p ≤ 0.01, viscum = 163 genes, TT = 63 genes and viscumTT = 190 genes). Viscum and viscumTT treatment affected gene ontology (GO) clusters for the “positive regulation of cell death”, “response to reactive oxygen species” or “oxidative stress” and “MAPK signaling” (Table 2). However, the GO “MAPK signaling” was for viscum treatment less significant than for viscumTT when regarding the FDR value (Table 2). For TT treated cells, enriched GO clusters were “TLR signaling”, “positive regulation of cell death” and “inflammatory response” displaying only a significant p-value (p ≤ 0.05) but no significant FDR value (Table 2).

Table 2: Significantly regulated KEGG pathways in TC-71 cells upon viscum, TT and viscumTT treatment versus untreated control cells.

Extract	Gene Ontology	# Genes	p-Value	FDR
viscum	Response to reactive oxygen species	8	2.59E-06	1.35E-03
	Response to organic substance	19	2.17E-05	7.49E-03
	Positive regulation of cell death	12	8.81E-04	7.34E-02
	MAPK signaling pathway	10	1.75E-03	1.43E-01
TT	Toll-like receptor signaling pathway	3	4.86E-02	7.64E-01
	Positive regulation of cell death	5	4.52E-02	9.95E-01
	Inflammatory response	5	1.79E-02	1.00E+00
viscumTT	MAPK signaling pathway	15	6.84E-06	6.70E-04
	Positive regulation of cell death	15	1.54E-04	1.54E-02
	Toll-like receptor signaling pathway	8	3.24E-04	1.58E-02
	Oxidative stress response	6	1.06E-03	1.95E-02
	Apoptosis	19	4.41E-05	2.84E-02

* p ≤ 0.05 and FDR ≤ 0.1 regarded as significant

Taken together, the transcriptomic results show that the extracts (especially viscum and viscumTT) alter the expression of many genes, mostly in favor of gene upregulation compared to untreated control cells. Viscum and viscumTT seem to involve the same signaling pathways and suggest an upregulation of genes related to cell stress, MAPK signaling and cell death. In line with the other results, TT treatment displays less potency than viscum and viscumTT and indicates the involvement of genes associated with inflammatory response/TLR signaling and cell death. The synergistic pro-apoptotic effect of viscumTT appears to be driven by other mechanisms than by the differential expression of genes compared to the gene expression of the single extracts.

3.11 *Viscum album* L. extracts alter the proteomic profile of TC-71 cells

A proteomic profiling of TC-71 cells was performed to gain global insights into the effects of viscum, TT and viscumTT on the proteome of Ewing sarcoma cells. Therefore, biological triplicates of TC-71 cells were treated with viscum, TT or viscumTT for 24 h in ~ IC50 concentrations in reference to untreated control cells and their protein extracts were analyzed by LC−MS/MS. More than $3x10^4$ MS spectra were identified for each sample belonging to 3565 protein groups in total. The obtained heat map of the proteomic profile displayed a strong alteration of protein expression after treatment with viscum or viscumTT compared to untreated control cells, whereas TT treatment showed less changes compared to control cells (Figure 18A). In line with heat map results, principal component analysis of the samples displayed the biggest differences between viscum or viscumTT treated cells and untreated control cells. Furthermore, it revealed a good compliance of the triplicates (Figure 18 B). Therefore, ion intensities of the triplicates were averaged for further secondary data analyses (pathway and string network analysis), only valid values were used.

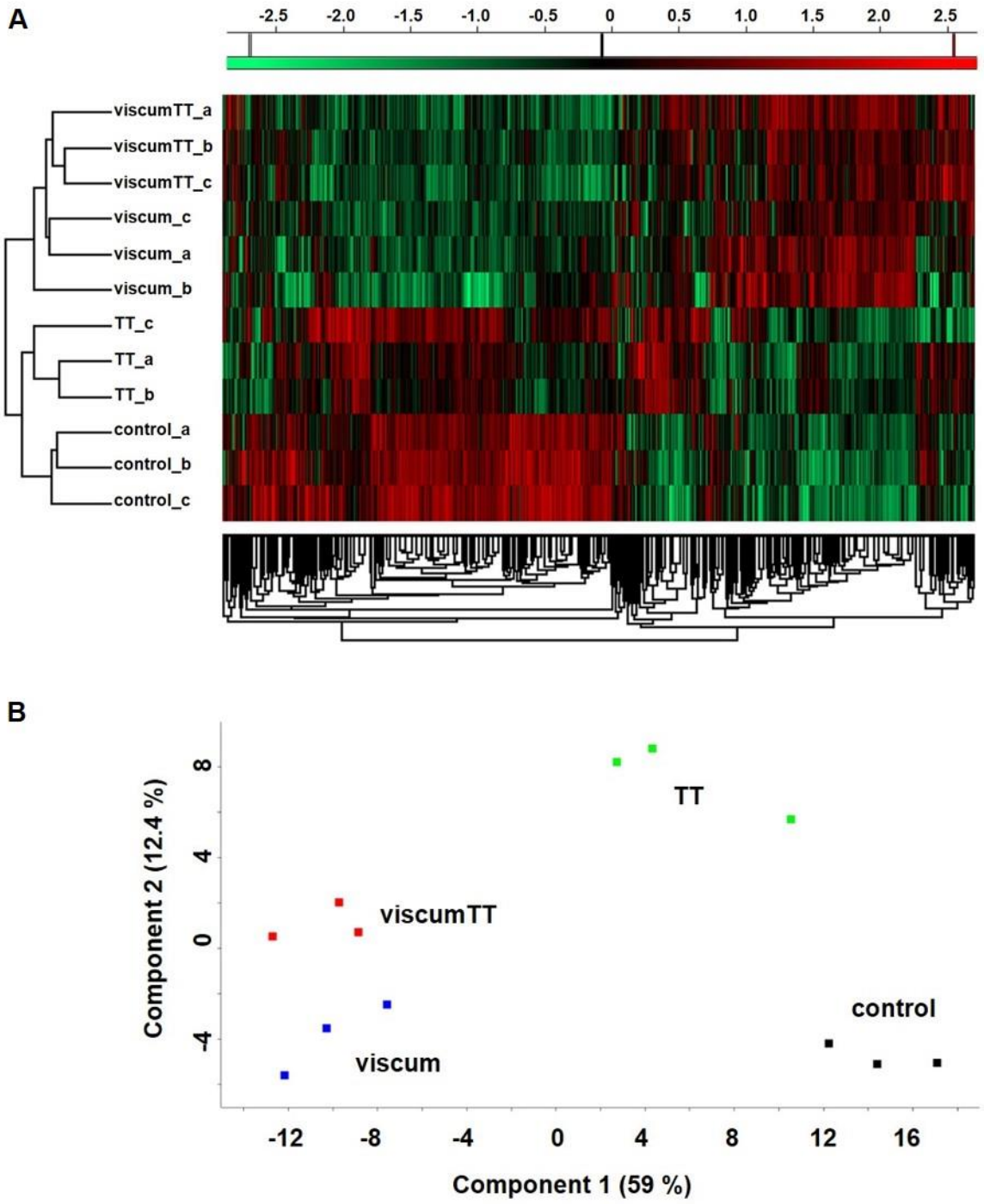

Figure 18: Viscum, TT and viscumTT alter the proteomic profile. TC-71 cells were treated as biological triplicates with viscum, TT or viscumTT (~ IC50) for 24 h in reference to untreated control cells and analyzed by LC−MS/MS. **A.** To display differential protein expression in sample cohorts, a heat map was created by using the z-score (standard score) of log2 transformed ion intensities for normalization showing high protein levels in red and low in green. **B.** Principal component analysis (PCA) was performed to compare the effects of the triplicates after treatment with viscum (blue), TT (green) or viscumTT (red) to each other and to untreated control cells (black).

Table 3a: Top 40 proteins regulated in TC-71 cells by viscum relative to untreated control cells.

Downregulated protein	Fold-change	PEP	Upregulated protein	Fold-change	PEP
PKP1	0.12	6.97E-261	MARCKS	2.44	2.89E-141
ELF1	0.18	3.79E-41	ARL8B	2.44	1.06E-32
CETN2	0.18	3.04E-140	IPO5	2.45	0.00E+00
CKS2	0.18	1.02E-16	DDOST	2.46	2.30E-92
UBE2T	0.19	8.70E-20	S100A13	2.47	8.25E-16
RPS28	0.20	3.73E-80	DDX3X	2.47	1.42E-169
TYMS	0.20	3.48E-05	INTS3	2.47	1.05E-54
HIST1H2AC	0.21	9.28E-285	ENOPH1	2.49	3.29E-15
CCDC159	0.23	0.0013738	PSMA3	2.50	1.31E-38
RPL39P5	0.26	1.75E-12	FNDC3A	2.51	2.20E-31
CCNB1	0.26	1.73E-20	EIF2S1	2.51	6.10E-165
LTV1	0.27	8.41E-48	PSMB5	2.54	7.61E-66
MRPS6	0.27	5.59E-14	TARS	2.56	2.01E-153
MLLT11	0.28	8.49E-234	TROVE2	2.58	2.46E-48
LLPH	0.28	5.66E-26	RPN2	2.60	3.65E-145
SAFB2	0.28	2.37E-272	OTUB1	2.62	7.03E-54
RPL10	0.30	9.60E-149	DIAPH1	2.62	1.45E-37
PDCL	0.30	1.43E-13	THOP1	2.69	2.06E-36
CDCA8	0.31	1.57E-16	PSMB4	2.70	7.33E-82
CNBP	0.31	1.22E-228	PTMA	2.71	7.64E-48
FAM207A	0.31	2.85E-11	TTLL12	2.73	1.69E-150
HIST1H3A	0.31	0.00E+00	RAB11B	2.73	7.88E-62
TOMM6	0.31	4.80E-16	KDSR	2.73	3.23E-45
USP36	0.32	1.89E-15	OGDH	2.89	6.45E-66
CRIP1	0.32	2.74E-42	UAP1	2.91	1.55E-93
UBE2C	0.33	8.04E-26	NAA25	2.95	4.92E-44
CDC42SE2	0.33	3.84E-28	MAP2K1	2.97	1.39E-35
FKBP11	0.33	3.72E-41	AP2B1	3.00	6.05E-65
BRD2	0.34	2.16E-22	DDB1	3.01	1.57E-56
COX7C	0.34	3.08E-19	COPB2	3.04	1.74E-90
MKI67	0.35	1.27E-133	GNB2L1	3.05	1.87E-269
MTHFD2	0.36	3.75E-145	SF3B3	3.07	3.08E-116
FDX1	0.36	6.20E-36	CLTC	3.12	0.00E+00
HIST2H3A	0.36	0.00E+00	NUCKS1	3.13	5.37E-09
SELH	0.36	3.66E-11	TBCD	3.19	1.13E-20
NDUFB6	0.37	2.25E-13	PRKDC	3.23	0.00E+00
BTF3	0.37	0.00E+00	IPO7	3.24	5.23E-119
HSPB1	0.37	0.00E+00	ASNS	3.28	2.00E-88
UBE2S	0.37	3.37E-43	VDAC3	3.31	1.99E-71
MED15	0.38	3.43E-23	KMO	3.42	2.26E-62

*FDR≤ 0.01, PEP = posterior error probability,

Table 3b: Top 40 proteins regulated in TC-71 cells by TT relative to untreated control cells.

Downregulated protein	Fold-change	PEP	Upregulated protein	Fold-change	PEP
MKI67	0.08	1.27E-133	STX6	1.95	6.92E-43
KIAA0101	0.08	1.89E-32	FNDC3A	1.96	2.20E-31
TPX2	0.19	3.44E-62	ANXA1	1.96	0.00E+00
UBE2C	0.22	8.04E-26	ESYT1	1.97	1.68E-131
CDCA8	0.22	1.57E-16	IDI1	1.97	1.26E-127
INCENP	0.24	5.60E-17	ROBO2	1.98	6.45E-14
RPL39P5	0.26	1.75E-12	WARS	1.99	0.00E+00
MRFAP1	0.27	3.06E-60	SLC3A2	1.99	0.00E+00
KIF11	0.31	1.8793E-42	PSMB6	2.00	1.91E-45
RIC8A	0.32	4.01E-09	DTD1	2.04	8.61E-24
EP400	0.35	6.39E-07	ZFPL1	2.04	1.12E-16
TMSB15B	0.35	1.97E-10	MVD	2.04	1.06E-28
KPNA2	0.35	0.00E+00	YWHAE	2.05	1.68E-296
POLR2D	0.36	4.85E-40	MAP1LC3B	2.05	1.02E-16
WASH3P	0.36	1.53E-13	TYSND1	2.10	1.49E-19
ZFAND5	0.37	1.78E-17	LONP1	2.15	0.00E+00
BAIAP2	0.38	5.63E-08	SLC16A1	2.16	3.58E-42
BRD2	0.38	2.16E-22	B2M	2.18	1.87E-83
PIK3C2A	0.39	2.81E-08	KDSR	2.20	3.23E-45
PKP1	0.39	6.97E-261	GARS	2.20	4.07E-299
ZNF593	0.40	1.48E-35	ARMCX3	2.22	1.87E-69
PPIL4	0.40	1.4216E-40	ATP5C1	2.23	7.40E-30
WAC	0.41	1.23E-09	APOC3	2.24	7.17E-115
EDF1	0.41	3.08E-34	HAX1	2.24	6.18E-23
SLAIN1	0.41	1.83E-66	CALCOCO2	2.25	3.15E-132
USP36	0.42	1.89E-15	TIMP1	2.25	6.20E-115
LTV1	0.42	8.41E-48	TAX1BP1	2.25	1.48E-37
CRIP1	0.42	2.74E-42	MARCKS	2.25	2.89E-141
GINS4	0.43	6.72E-26	AP2B1	2.26	6.05E-65
RBM7	0.43	4.46E-23	LEPRE1	2.28	1.10E-15
C9orf78	0.43	1.23E-23	ECM29	2.31	2.41E-53
PBK	0.44	8.85E-74	SEC61A1	2.32	2.99E-11
MRPL27	0.44	4.38E-15	DIAPH1	2.35	1.4516E-37
EIF4ENIF1	0.45	2.9617E-07	NUCB2	2.41	7.35E-41
WHSC1	0.45	4.14E-18	RAB11B	2.47	7.88E-62
RPL10	0.46	9.60E-149	SLC38A2	2.57	1.2781E-67
IRF2BP1	0.46	1.5905E-29	ASS1	2.69	2.22E-65
RCHY1	0.46	3.2311E-07	GFPT1	2.72	1.17E-108
PDCL	0.46	1.43E-13	UTS2	2.76	1.22E-13
ARHGEF1	0.46	2.47E-21	IARS	2.82	9.08E-147

*FDR≤ 0.01, PEP = posterior error probability,

Table 3c: Top 40 proteins regulated in TC-71 cells by viscumTT relative to untreated control cells.

Downregulated protein	Fold-change	PEP	Upregulated protein	Fold-change	PEP
PKP1	0.07	6.97E-261	RRP12	2.69	6.82E-138
RPL39P5	0.15	1.75E-12	DDX39A	2.69	8.53E-224
HIST1H2AC	0.18	9.28E-285	GNB2L1	2.70	1.8697E-269
RPS28	0.19	3.73E-80	OSBPL8	2.74	6.28E-12
HIST1H3A	0.20	0.00E+00	CLTC	2.77	0.00E+00
CKS2	0.22	1.02E-16	KMO	2.79	2.26E-62
EDF1	0.23	3.08E-34	DIS3	2.83	6.79E-98
CHCHD2	0.26	4.35E-38	DHCR24	2.85	3.05E-42
CNBP	0.27	1.2174E-228	CS	2.85	1.30E-283
POLR2D	0.27	4.85E-40	ACBD3	2.90	2.79E-41
RPL10	0.27	9.60E-149	TMEM41B	2.91	8.15E-10
BRD2	0.27	2.16E-22	SEL1L	2.91	1.84E-06
NDUFB10	0.27	5.19E-48	PRPF8	2.93	1.42E-206
TPX2	0.28	3.44E-62	RAB2A	2.94	6.27E-63
CETN2	0.28	3.04E-140	PRPS1	2.98	2.94E-30
CD248	0.29	1.48E-33	NAA25	3.01	4.92E-44
NOB1	0.30	3.93E-14	USP7	3.03	8.74E-61
UBE2C	0.31	8.04E-26	MARCKS	3.04	2.89E-141
PDCL	0.32	1.43E-13	PSMB4	3.07	7.33E-82
CCDC159	0.33	1.37E-03	SLC25A3	3.08	1.09E-86
ELF1	0.33	3.79E-41	PRKDC	3.13	0.00E+00
MLLT11	0.33	8.4901E-234	APEH	3.21	1.21E-28
CDCA8	0.33	1.57E-16	ENOPH1	3.29	3.29E-15
POLR2L	0.33	4.97E-30	TTLL12	3.30	1.69E-150
LLPH	0.33	5.66E-26	PTMA	3.32	7.64E-48
RBM19	0.34	2.10E-13	PSMB5	3.34	7.61E-66
KIAA0101	0.34	1.89E-32	OTUB1	3.40	7.03E-54
CCDC137	0.35	2.58E-58	RAB11B	3.42	7.88E-62
NTPCR	0.35	2.06E-25	DDB1	3.45	1.57E-56
TRMT112	0.35	2.49E-54	TM9SF2	3.51	5.37E-27
CRIP1	0.35	2.74E-42	DIAPH1	3.52	1.45E-37
POU3F1	0.36	8.66E-97	BCAP31	3.55	1.02E-36
LYRM7	0.36	8.10E-24	DYNC1H1	3.55	0.00E+00
HIBADH	0.37	9.7111E-53	MAP2K1	3.63	1.39E-35
COA4	0.37	4.56E-22	IPO7	3.66	5.23E-119
MESDC2	0.37	2.80E-11	HMGN3	3.86	7.2052E-30
SRP14	0.37	2.6107E-99	FTL	4.07	4.86E-72
FAM207A	0.38	2.8546E-11	APOB	4.07	4.89E-19
NDUFA4	0.38	4.42E-17	TBL3	4.08	4.01E-20
PPP4R2	0.38	8.96E-33	SPCS2	4.16	3.50E-14

*FDR≤ 0.01, PEP = posterior error probability,

Treatment with viscum resulted in 638 down- and 291 upregulated proteins by at least 1.5-fold and 183 down- and 115 upregulated proteins by at least 2-fold relative to untreated control cells. The top differentially expressed proteins after treatment with viscum were PKP1, which is involved in apoptotic cleavage of cell adhesion proteins, and the PKC substrate MARCKS (Table 3a). TT treatment, by contrast, resulted in 370 down- and 194 upregulated proteins by at least 1.5-fold and 64 down- and 41 upregulated proteins by at least 2-fold relative to untreated control cells. Top differentially expressed proteins after TT treatment were MKI67 (involved in cell proliferation) and STX6, which is involved in protein transport (Table 3b). Treatment with the combinatory viscumTT extract led to the downregulation of 651 and upregulation of 319 proteins by at least 1.5-fold and a downregulation of 163 and upregulation of 128 proteins by at least 2-fold relative to untreated control cells. Treatment with viscumTT displayed as top differentially expressed proteins PKP1 and the RNA binding protein RRP12 (Table 3c).

Pathway analysis showed a downregulation of proteins related to the “ribosome”/”translation” and “transcription” after treatment with viscum, TT or viscumTT (Table 4). Protein-protein interaction network analysis of at least 2-fold downregulated proteins displayed proteins involved in the “ribosome” and “spliceosome” after treatment with viscum or viscumTT, whereas TT showed no significant network results (Figure 19). Furthermore, pathway analysis revealed an upregulation of proteins involved in “aminoacyl-tRNA biosynthesis” and the “proteasome” after treatment with viscum, TT or viscumTT. Moreover, viscum and viscumTT treatment displayed the upregulation of proteins associated with the “regulation of apoptosis”, “protein folding”, “PERK regulated gene expression”, “MAP kinase activation in TLR cascade” and the “immune system” (Table 4) indicating cellular stress and immune response. Protein-protein interaction network analysis of at least 2-fold upregulated proteins displayed the networks “aminoacyl-tRNA synthesis” for viscum, “protein processing in ER” for viscumTT and “proteasome” for viscum and viscumTT treatment (Figure 19).

The data shows that viscumTT and the both single extracts decrease protein expression related to the ribosome and spliceosome and increase protein expression associated with the proteasome. In addition, viscum and viscumTT trigger the upregulation of proteins related to apoptosis, protein folding and MAPK signaling. Like the tran-

scriptomic profile, the proteomic profile indicates similar effects of viscum and viscumTT suggesting that other mechanisms are responsible for the synergistic pro-apoptotic effect of viscumTT.

Table 4: Significantly regulated KEGG and Reactome pathways in TC-71 cells treated with viscum, TT or viscumTT versus untreated control cells.

Extract	Pathway	# Proteins	p-Value	q-Value
	>downregulated			
viscum	Ribosome	75	0.00E+00	0.00E+00
	Transcription	57	0.00E+00	9.64E+03
	Translation	122	7.95E-03	1.80E-01
TT	Ribosome	76	0.00E+00	8.58E-03
	Transcription	59	0.00E+00	3.18E-02
	Translation	123	7.11E-03	2.10E-01
viscumTT	Ribosome	76	0.00E+00	0.00E+00
	Transcription	59	0.00E+00	0.00E+00
	Translation	124	5.95E-03	1.20E-01
	>upregulated			
viscum	Aminoacyl-tRNA biosynthesis	22	0.00E+00	0.00E+00
	Proteasome	35	2.96E-03	9.81E-02
	Protein folding	28	2.98E-03	3.89E-02
	PERK regulated gene expression	10	1.27E-02	6.36E-02
	MAP kinase activation in TLR cascade	8	5.64E-02	1.15E-01
	Immune system	175	0.00E+00	1.51E-01
TT	Aminoacyl-tRNA biosynthesis	22	0.00E+00	0.00E+00
	Proteasome	35	1.08E-02	3.48E-02
viscumTT	Aminoacyl-tRNA biosynthesis	22	0.00E+00	0.00E+00
	Proteasome	35	0.00E+00	5.72E-02
	Regulation of apoptosis	38	0.00E+00	3.10E-02
	PERK regulated gene expression	10	1.80E-02	6.42E-02
	Immune system	175	3.00E-03	7.35E-02
	Protein folding	28	1.42E-02	7.91E-02
	MAP kinase activation in TLR cascade	8	3.90E-02	7.95E-02
	Apoptosis	69	2.85E-02	1.59E-01

*$p \leq 0.05$ and $q \leq 0.25$ regarded as significant

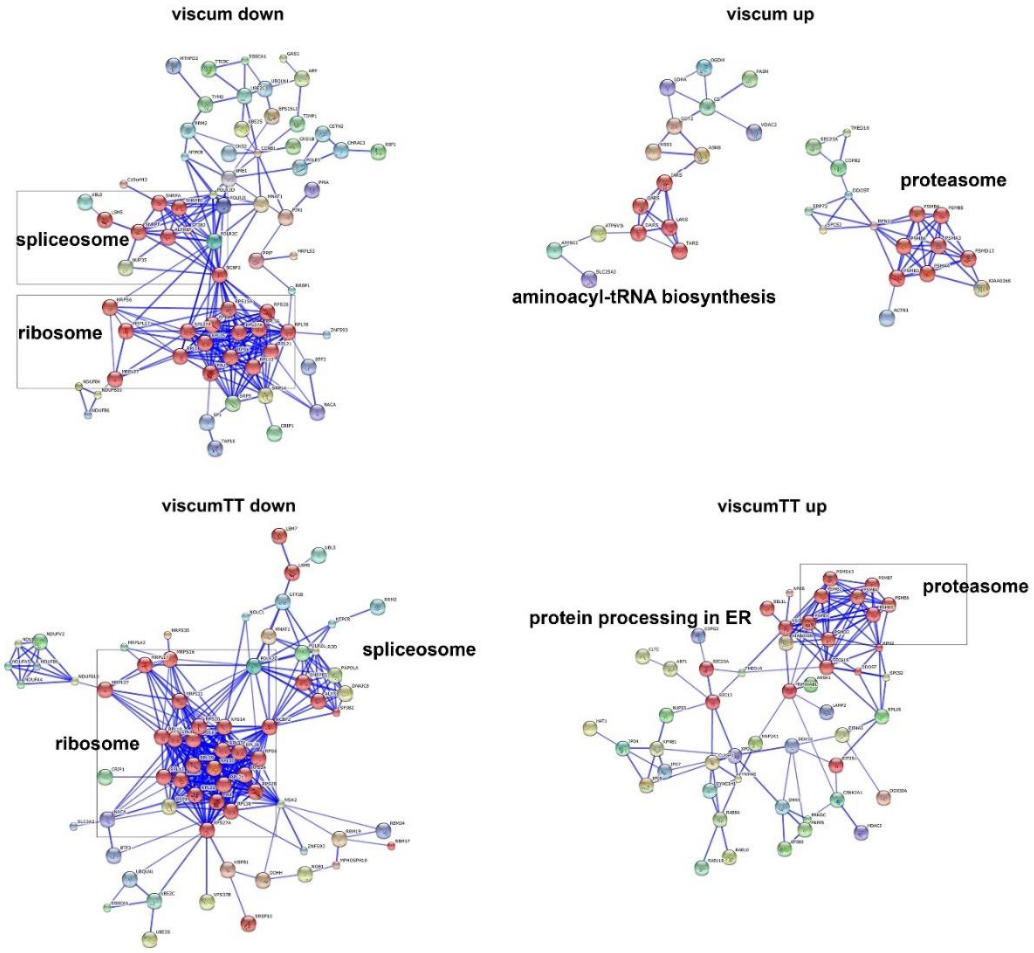

Figure 19: Viscum and viscumTT show enriched protein-protein interaction networks. TC-71 cells were treated as biological triplicates with viscum, TT or viscumTT (~ IC50) for 24 h and analyzed by LC−MS/MS. String network analysis of the averaged replicates was performed to detect up- and downregulated protein-protein interaction networks. Indicated proteins in red belong to a specified KEGG pathway.

3.12 *Viscum album* L. extracts induce cellular stress and activate the unfolded protein response *in vitro*

In order to validate the transcriptomic and proteomic results referring to MAPK signaling and response to oxidative stress, western blots were performed of TC-71 and MHH-ES-1 cells after treatment with increasing concentrations of viscum, TT or viscumTT for 24 h. A dose-dependent activation of MAPK8 (formerly JNK) and MAPK14 (formerly p38-MAPK), evidenced by an increased phosphorylation, was detected after treatment of both cell lines with viscum or viscumTT (Figure 20). Furthermore, a dose-dependent upregulation of the ER chaperone protein HSPA5 (formerly GRP78) was detected after treatment with each of the extracts indicating response to cellular stress such as oxidative stress or ER stress by activation of the unfolded protein response (Figure 20). Additionally, viscum and viscumTT treatment displayed a dose-dependent decrease of the ER stress sensor protein EIF2AK3 (formerly PERK), whereas TT treatment showed a slight upregulation of EIF2AK3 (Figure 20).

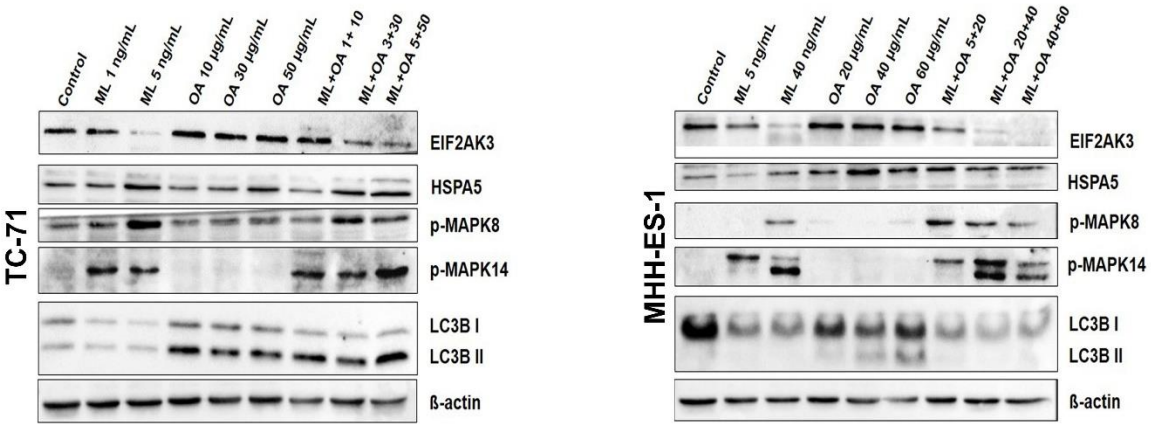

Figure 20: *Viscum album* L. extracts induce cellular stress and activate the unfolded protein response. TC-71 and MHH-ES-1 cells were treated with increasing concentrations of viscum, TT or viscumTT for 24 h in reference to untreated control cells. Western blots were performed to investigate the activation of the unfolded protein response and cellular stress on protein level. β-actin was used as loading control, and images shown are representative for the results in 3 independent experiments Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as a measure of viscum and TT active agent concentration.

Since ER stress is linked to autophagy (Hoyer-Hansen *et al.*, 2007, Heath-Engel *et al.*, 2008), the expression of LC3B (LC3 is a subunit of microtubule-associated proteins 1A and 1B termed MAP1LC3) was analyzed, whose conversion from LC3B-I to LC3B-II is indicative for autophagy activity. In the more sensitive TC-71 cells, LC3B-II expression was increased by TT and viscumTT treatment, whereas the more resistant MHH-ES-1 cells displayed an enhanced expression of LC3B-II only after TT treatment (Figure 20) indicating enhanced autophagy.

To further analyze the role of MAPK8/MAPK14 and oxidative stress in mistletoe-mediated apoptosis of Ewing sarcoma cells, TC-71 cells were treated with viscum, TT or viscumTT for 24 h in the absence or presence of the MAPK14 inhibitor SB203580, the MAPK8 inhibitor SP600125 or the antioxidant NAC (Table 5). Additionally, TC-71 cells were also treated with the TLR4 inhibitor LPS-RS, since the proteomic and transcriptomic data revealed the involvement of TLR signaling. The applied MAPK8 and MAPK14 inhibitors were not able to prevent apoptosis induction by the extracts. Higher concentrations of the used inhibitors increased apoptosis (Table 5). Also in MHH-ES-1 cells both inhibitors were not able to rescue cells from apoptosis induction, and higher concentrations reduced viability.

Table 5: Tested inhibitors

Inhibitor	Concentration	Tolerance
SB203580	5-50 µM	10 µM
SP600125	10 nM-25 µM	5 µM
LPS-RS	0.1-10 µg/mL	0.1 µg/mL
NAC	1-10 mM	10 mM

Western blot analyses of TC-71 cell lysates after treatment with SB203580 and SP600125 revealed no reduced phosphorylation of MAPK8 and MAPK14 and rather an enhanced phosphorylation of MAPK14 (Figure 21A). NAC pretreatment, however, significantly reduced apoptosis induction about 21 % after viscum treatment and about 13 % in viscumTT treated cells (Figure 21B). The TLR4 antagonist LPS-RS, which binds to TLR4 without activating the receptor, significantly reduced TT-mediated apoptosis induction about 20 % (Figure 21B).

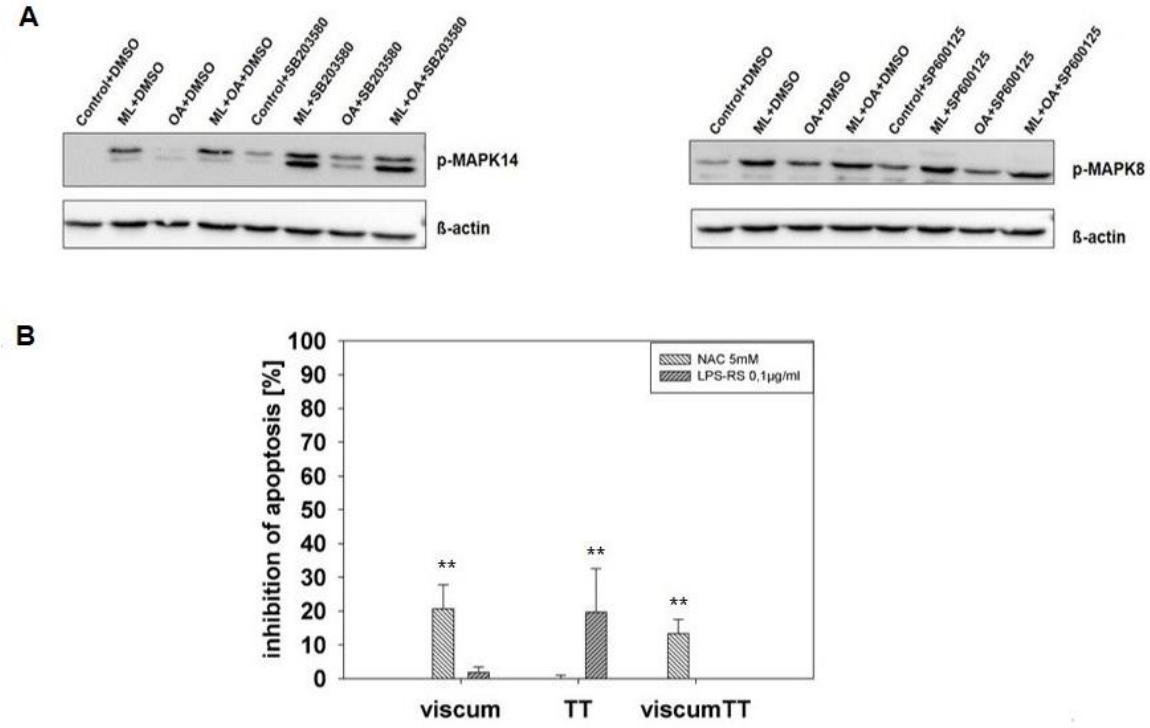

Figure 21: Involvement of MAPK8, MAPK14, TLR4 and oxidative stress in *Viscum album* L. mediated apoptosis A. TC-71 cells were treated with the extracts in ~ IC50 concentrations for 24 h in the presence of 10 µM MAPK14 inhibitor SB203580 or 5 µM MAPK8 inhibitor SP600125. Western blotting was used to assess the inhibition of MAPK activation in whole-cell protein lysates from treated TC-71 cells and untreated control cells. β-actin was used as loading control, and images shown are representative for the results in 3 independent experiments. **B.** TC-71 cells were treated with viscum, TT or viscumTT in ~ IC50 concentrations for 24 h in the absence or presence of the TLR4 inhibitor LPS-RS or the antioxidant N-acetylcysteine (NAC) followed by annexin V/ PI staining and flow cytometry to assess the number of apoptotic cells. The percentage of apoptosis inhibition is shown in bars (± SD) from 3 independent experiments. Kruskal-Wallis one-way analysis revealed a significant treatment effect in apoptosis inhibition ($p \leq 0.05$) and Dunn´s post hoc test comparing all groups with all groups showed significant effects (**FDR ≤ 0.1) between the untreated control group and treatment groups (viscum, TT and viscumTT).

Taken together, these data shows that viscum, TT and viscumTT activate the unfolded protein response. Moreover, treatment with the TT extract results in the activation of TLR4 signaling and suggests autophagy induction. Viscum and viscumTT induce oxidative stress and activate the MAPK8 and MAPK14 signaling pathway, commonly termed as stress-mediated MAPK pathway. Since the extracts trigger these many pathways sensitizing cells to apoptosis, inhibition of single pathways appears to be difficult to rescue cells from apoptosis induction.

4 Discussion

The analyzed *Viscum album* L. extracts displayed anti-proliferative and pro-apoptotic properties in Ewing sarcoma *in vitro, ex vivo and in vivo*. *In vitro* studies showed a strong alteration of the transcriptomic and proteomic profile after treatment with the extracts and the involvement of several apoptosis-related signaling pathways.

4.1 *Viscum album* L. extracts inhibit proliferation and induce apoptosis in Ewing sarcoma cells *in vitro* and *ex vivo*

The *in vitro* and *ex vivo* experiments showed that viscum and viscumTT inhibit proliferation and invoke the intrinsic and extrinsic apoptosis pathway, evidenced by a loss of mitochondria membrane potential and the activation of CASP8 and CASP9. Treatment of TC-71 cells with a pan-caspase inhibitor verified that apoptosis was caspase-dependent. Furthermore, there was no meaningful cell death via necrosis *in vitro*. This is in line with previous data from hepatocellular carcinoma and leukemia (Bantel *et al.*, 1999, Kim *et al.*, 2000, Yang *et al.*, 2012b, Delebinski *et al.*, 2012). Notably, MHH-ES-1 cells and also the primary cells were more resistant to viscum and viscumTT extracts than TC-71 cells, and required ~15-fold higher ML concentrations to reach IC50. For MHH-ES-1 cells, this is unjustifiable only by the doubled cell number in cultures due to the smaller cell size compared to TC-71 cells. It rather demonstrates the variable responsiveness of different cell populations to viscum and viscumTT *in vitro* and *ex vivo*. Variable sensitivity could result from different cell surface characteristics regarding the glycocalyx, because mistletoe lectins specifically bind to D-galactose (Franz *et al.*, 1981). Furthermore, it should be mentioned, that cell death via necrosis was indeed detected in only small amounts, but the results indicated a dose- and time-dependent effect, which may result from the viscotoxins in the viscum and viscumTT extract, since viscotoxins interact with cell membranes (Coulon *et al.*, 2003).

TT proved less cytotoxic than either viscum or viscumTT against Ewing sarcoma cells *in vitro* and *ex vivo*. TT also did not significantly activate CASP8, CASP9 or CASP3 and did not cause mitochondrial membrane depolarization, indicating that other mechanisms are responsible for the moderate TT-mediated apoptosis and proliferation inhibition in TC-71 cells. Triterpene acids, such as UA and OA and its derivatives, have

previously been shown to induce apoptosis in osteosarcoma, breast and pancreatic cancers via extrinsic or intrinsic pathways caspase-dependently (Ito *et al.*, 2001, Shyu *et al.*, 2010, Yan *et al.*, 2010, Chakravarti *et al.*, 2012, Wei *et al.*, 2013, Akl *et al.*, 2014). Furthermore, OA and its derivative have also been shown to induce apoptosis without caspase participation in pancreatic cancer cells and acute myelogenous leukemia (Konopleva *et al.*, 2004, He *et al.*, 2012), suggesting that the triterpene effect is cell type-dependent. Interestingly, in comparison with the TT extract, high concentrations of OA standard alone induced apoptosis in TC-71 cells more effectively and resulted in the depolarization of mitochondria membrane. Furthermore, the comparison of OA and BA standard alone and in combination revealed that their combination is less potent than OA alone, but more effective than BA alone suggesting that BA antagonizes the effect of OA, but OA enhances the effect of BA (additivity). Contrary to this result it has been reported that the anti-tumoral effects of UA and BA are comparable to OA (Gheorgheosu *et al.*, 2014, Soica *et al.*, 2014a). Moreover, the combination of OA and UA has been shown to act synergistically against melanoma cells *in vitro* and *in vivo* (Soica *et al.*, 2014b).

In conclusion, viscum and even more, viscumTT proved effective against Ewing sarcoma cell lines and primary cells inhibiting proliferation and inducing the intrinsic and extrinsic apoptosis pathway. The moderate apoptosis-inducing and anti-proliferative effect of TT *in vitro* might be related to the combination of OA and BA and does not involve the intrinsic and extrinsic apoptosis pathway. However, the presence of TT potentiated the viscum effect creating the synergistic effect of viscumTT. ViscumTT represents a mistletoe total extract and its effects correlate with the well-known phytopharmacological phenomenon that a naturally existing combination is often more effective than the single compounds (Foungbé *et al.*, 1991, Ji *et al.*, 2009).

4.2 Viscum and viscumTT are effective in Ewing sarcoma xenografts *in vivo*

The anticancer effects observed for viscum, TT and viscumTT *ex vivo* and *in vitro* were confirmed *in vivo* in a Ewing sarcoma xenograft mouse model by measurement of tumor volume. However, the *in vivo* study was not performed ideally because measurement of tumor volume is less meaningful than measurement of tumor weight. Tumor volume is measured superficially in living mice, while tumor weight is measured completely after sacrificing mice. But since three mice from intratumorally treated cyclodextrin control group had to be taken of the study on day 21 due to tumor size, day 21 had to be set as endpoint measurement. Unfortunately, it was not possible to sacrifice all mice on day 21 (a Friday) for organizational reasons. Thus, the tumor weights and volumes raised enormously to day 24 because of the aggressive tumor making the comparison of tumor volume and weight of all mice impossible.

Still, regarding tumor volume on day 21, viscum injected intratumorally significantly inhibited xenograft tumor growth. By contrast, TT or viscumTT injected intratumorally revealed no significant effect. While the *in vivo* results for TT treatment were in line with the *in vitro* and *ex vivo* results, the results for viscumTT were contrary. But since the application of agents on cell cultures is different than the application in an organism or tumor, the results are not transferable one-to-one. Furthermore, because the number of cases was small (eight mice per group), the results have to be interpreted as an exploratory study rather than an absolute and evident result. Moreover, intravenous injection of viscumTT displayed a significant effect on tumor growth inhibition and had a comparable effect on xenografts as doxorubicin treatment. However, doxorubicin was administered only once due to low tolerability when applied more often, while viscumTT was applied 5 times in total (subdivided into injections every two to three days). But this also emphasizes the good tolerability of intravenously administered viscumTT compared to doxorubicin. Anticancer effects of mistletoe lectins have also been reported in mouse models of lymphosarcoma, melanoma and pre-B ALL (Braun *et al.*, 2002, Duong Van Huyen *et al.*, 2006, Seifert *et al.*, 2008). For OA and its derivatives, anticancer effects were shown in mouse models of osteosarcoma, lung cancer and melanoma (Hua *et al.*, 2009, Liby *et al.*, 2009, Lucio *et al.*, 2011). Furthermore, the *in vivo* efficacy of viscumTT has been previously demonstrated against mouse models of acute lymphoblastic and myeloid leukemia and melanoma (Delebinski *et al.*, 2012,

Strüh *et al.*, 2012, Delebinski *et al.*, 2015). Taken together, viscum and viscumTT indicate a good tolerability and therapeutic efficacy against Ewing sarcoma *in vivo*.

4.3 *Viscum album* L. extracts alter the expression of many apoptosis-related proteins *in vitro*

The deeper assessment of the apoptotic mechanism of mistletoe extract treatment revealed an altered expression of BCL2 family proteins, IAP family proteins and proteins involved in cell cycle control, shifting the balance towards apoptosis. Treatment with viscum or viscumTT effectively reduced expression of the anti-apoptotic protein, MCL1. The MCL1 downregulation in both Ewing sarcoma cell lines is in line with previous data demonstrating MCL1 downregulation by mistletoe lectins in leukemia cells (Park *et al.*, 2012) and by an OA derivative in osteosarcoma cells (Ryu *et al.*, 2010). TT strongly upregulated the pro-apoptotic protein, PMAIP1, in both Ewing sarcoma cell lines, but did not affect MCL1 expression, possibly resulting in less effective apoptosis induction. PMAIP1 upregulation together with downregulation of the anti-apoptotic BCL2L1 protein have been correlated with increased apoptosis in pancreatic tumor cells treated with an OA derivative (Leal *et al.*, 2013). Ehrhardt *et al*. reported that PMAIP1 is involved in apoptotic induction by drug combinations, such as doxorubicin and BA in diverse cancer cell lines, including breast, colon and leukemia (Ehrhardt *et al.*, 2012). The combinatory viscumTT extract contains defined concentrations of OA, BA and mistletoe lectins, which together produce a phytopolychemotherapeutic effect. But unlike TT, viscumTT did not upregulate PMAIP1 expression in Ewing sarcoma cells, indicating PMAIP1 regulation is not involved in the synergistic effect produced by viscumTT.

In addition, PMAIP was shown to be involved in ER stress-induced apoptosis in MEF cells (Li *et al.*, 2006). ER stress caused by the triterpene extract is conceivable. ER stress was shown to be important for the activation of MAPK8 and induction of apoptosis by an OA derivative in non-small-cell lung cancer cells (Zou *et al.*, 2008). An ER stress mediated apoptosis induction was also shown for an OA derivative and BA in HeLa cells (Won *et al.*, 2010, Xu *et al.*, 2014). Furthermore, TT treatment also upregulated HSPD1 expression in TC-71 cells supporting the ER stress hypothesis. Heat shock proteins are crucial for maintaining protein homeostasis during physiological and

stress conditions (Walter *et al.*, 2002). HSPD1 has been shown to possess prosurvival and pro-apoptotic functions depending on cellular context (Chandra *et al.*, 2007, Campanella *et al.*, 2008). Moreover, HSPD1 has been suggested to act as a barrier to pharmacologically induced oxidative stress mediated apoptosis in rat histiocytoma and human neuroblastoma cells (Sarangi *et al.*, 2013). In TT-treated TC-71 cells, HSPD1 may provide prosurvival properties, resulting in less effective apoptosis induction.

Interestingly, the mistletoe extracts also suppressed the expression of tumor suppressor protein TP53 in Ewing sarcoma cell lines. But since TC-71 cells express an inactive TP53 mutant protein (May *et al.*, 2013) and MHH-ES-1 cells have an in-frame deletion of Ser215 in the TP53 transactivation domain, this effect is less meaningful. Still, the downregulation suggests signaling that would trigger downregulation of wildtype TP53. However, this must not mean it would have an anti-apoptotic effect, because mistletoe lectins were also shown to suppress wildtype TP53 expression and induce apoptosis in human lymphocytes (Büssing *et al.*, 1998). Other reports did not analyze TP53 expression directly, but they showed that *Viscum album* L. induced apoptosis in cell lines (SMMC7721 hepatocellular carcinoma and NALM-6 acute lymphoblastic leukemia cells) harboring wildtype *TP53* (Yang *et al.*, 2012b, Delebinski *et al.*, 2012). The expression of CLSPN, a checkpoint function protein, was also decreased in both cell lines after treatment with viscum, TT or viscumTT. CLSPN is required for efficient DNA replication during a normal S phase and has been shown to promote cancer cell survival (Choi *et al.*, 2014). It is an essential upstream regulator of checkpoint kinase 1, and triggers cell cycle arrest in response to replicative stress or DNA damage. In a previous study, it was demonstrated that viscum, TT and viscumTT similarly reduced CLSPN expression in acute myeloid leukemia cells, where the extracts also induced apoptosis and inhibited proliferation (Delebinski *et al.*, 2015). These results suggest an important role for CLSPN in mistletoe-mediated apoptosis and inhibition of proliferation independent of cancer entity.

The mistletoe extracts further reduced expression of the IAP family members BIRC5 and XIAP. The IAP family is involved in cell death, cell cycle and migration by inhibiting caspases or the assembly of pro-apoptotic protein complexes, and mediating the expression of anti-apoptotic proteins (Lopez *et al.*, 2010, de Almagro *et al.*, 2012). As BIRC5 is expressed by many cancer cell types but not in differentiated normal tissue, it has been suggested as an attractive target for new anti-cancer agents (Coumar *et al.*, 2013). But also the IAP family member XIAP is overexpressed in many cancer

types and associated with drug resistance and therefore a promising therapeutic target (Schimmer *et al.*, 2005, Kashkar, 2010, Obexer *et al.*, 2014). Furthermore, BIRC5 expression is associated with radio- and chemoresistance in Ewing sarcoma and poor patient prognosis (Greve *et al.*, 2012, Hingorani *et al.*, 2013). Therefore, especially BIRC5 downregulation by *Viscum album* L. extracts makes it an interesting candidate for targeting Ewing sarcoma. A downregulation of BIRC5 and XIAP by viscum, TT and viscumTT has been previously also achieved in acute myeloid leukemia cells (Delebinski *et al.*, 2015). Furthermore, OA and UA were reported to induce apoptosis and to downregulate XIAP in hepatocellular carcinoma cells (Shyu *et al.*, 2010). OA was also shown to decrease BIRC5 expression in human ovarian cancer and non-small cell lung cancer cells (Duan *et al.*, 2009, Lucio *et al.*, 2011). Moreover, BA derivatives were demonstrated to downregulate BIRC5 in glioblastoma cell lines (Bache *et al.*, 2014).

In conclusion, the results indicate that the cytotoxic compounds derived from mistletoe involve several signaling pathways shifting the balance of apoptosis-related proteins towards apoptosis. The diverse involved signaling pathways might explain the synergistic effect of the combined mistletoe extract viscumTT.

4.4 *Viscum album* L. extracts alter the transcriptomic profile of TC-71 cells

Viscum and viscumTT displayed an impressive impact on the transcriptome of Ewing sarcoma cells resulting in a similar profile. The TT extract, by contrast, revealed a lower impact on the transcriptome. This data is in line with the previous *in vitro* and *ex vivo* results, demonstrating a very potent apoptosis induction and inhibition of proliferation in Ewing sarcoma by viscum and viscumTT, but a low effect of the TT extract alone. The transcriptomic profile of TC-71 cells treated with viscum or viscumTT indicated the upregulation of genes (as the top candidate *JUN*) associated with the positive regulation of cell death, response to oxidative stress and MAPK signaling, whereas TT treatment altered the expression of genes involved in inflammatory response/TLR signaling and positive regulation of cell death. Since mRNA sequencing was performed once without replicates, the results reporting the upregulation of *JUN*, *DDIT3* and the downregulation of *MAP2K6* were confirmed by qPCR. Yang *et al*. also reported about several differentially expressed genes of the MAPK signaling cascade after treatment of

hepatocellular carcinoma cells with recombinant ML (Yang *et al.*, 2012b). Furthermore, a similar transcriptomic profile was shown in breast cancer cells using DNA microarray chips after treatment with a commercial aqueous mistletoe extract involving apoptosis, MAPK and TLR signaling pathways (Eggenschwiler *et al.*, 2006). Interestingly, that work showed that aqueous mistletoe extracts from the hosts oak and apple tree have a bigger impact on immune defense and stress response, whereas aqueous extracts from the host tree white fir affect cell-cell adhesion and cytoskeleton pathways. In line with this, the transcriptomic results of TC-71 cells after treatment with the extracts from the apple tree as host displayed the involvement of stress responses and immune defense.

Taken together, the transcriptomic data of TC-71 cells revealed that viscum and viscumTT have a bigger impact on the transcriptome than TT and display similar transcriptomic profiles. Enriched deregulated genes after treatment with viscum, TT or viscumTT are related to cellular stress responses and immune defense. The similar transcriptomic profiles and deregulated pathways of viscum or viscumTT treated TC-71 cells suggest that the synergistic effect of viscumTT is created on a different level.

4.5 *Viscum album* L. extracts alter the proteomic profile of TC-71 cells

Like the transcriptomic results, viscum and viscumTT showed a massive and similar impact on the proteomic profile of Ewing sarcoma cells, whereas TT revealed a lower impact on the proteome. This data also correlates with the previous *in vitro* and *ex* vivo results, displaying a potent effect by viscum and viscumTT, but a low effect by the TT extract alone. The proteomic profile of TC-71 cells treated with viscum, TT or viscumTT showed the downregulation of proteins involved in the ribosome/translation and spliceosome/transcription. Furthermore, treatment with all extracts revealed an upregulation of proteins involved in aminoacyl-tRNA biosynthesis and the proteasome. For the single viscum extract and the combinatory viscumTT these effects are explainable, as they both contain mistletoe lectins. Mistletoe lectins are classified as ribosome-inactivating proteins type-II and thus they provoke a breakdown of translation (Olsnes *et al.*, 1982, Franz *et al.*, 1982, Ye *et al.*, 2006). When the ribosome is inhibited, it may result in the accumulation of aminoacyl-tRNA and the activation of the proteasome, which degrades misfolded or unfolded proteins. Ribosome-inactivating proteins type-II

have n-glycosidase activity removing single adenines from rRNA. They were originally thought to act exclusively on ribosomes, but there is evidence growing that they are also able to inactivate non-ribosomal nucleic acid substrates (Barbieri *et al.*, 1997, Barbieri *et al.*, 2001). Therefore, it is conceivable that they also inactivate the spliceosome, which consists of snRNAs and protein complexes.

The TT extract contains mainly OA and BA. Therefore, the downregulation of ribosomal and spliceosomal proteins together with an upregulation of proteins involved in aminoacyl-tRNA biosynthesis by TT is new and hard to explain. Regarding the upregulation of proteasomal proteins, BA was shown to increase protein degradation in prostate cancer cells i*n vitro* (Reiner *et al.*, 2013). However, BA derivatives as well as the triterpene celastrol were shown to inhibit the proteasome (Yang *et al.*, 2006, Huang *et al.*, 2007, Qian *et al.*, 2011). The proteasome is a multicatalytic protease complex being involved in many essential cellular functions as regulation of cell cycle, stress signaling and apoptosis (Huang *et al.*, 2009). Therefore, its inhibition or activation might be useful as therapeutic strategy for cancer treatment, as successfully demonstrated by the approved proteasome inhibitor Bortezomib in multiple myeloma treatment.

Furthermore, the proteomic data of viscum or viscumTT treated cells displayed the upregulation of proteins involved in protein folding, PERK regulated gene expression, regulation of apoptosis, immune system and MAP kinase activation correlating with the transcriptomic results and suggesting that viscum and viscumTT have an impact on MAPK signaling and protein folding. The role of MAPK signaling and protein folding in mistletoe-treated cells will be discussed in the next section.

In conclusion, the proteomic data of TC-71 cells showed that viscum and viscumTT have a bigger impact on the proteome than TT and display similar proteomic profiles. Enriched deregulated proteins after treatment with the extracts were associated with translation, transcription and protein degradation leading to apoptosis. Additionally, viscum and viscumTT upregulated proteins involved in cellular stress and immune defense correlating with the transcriptomic results. The similar proteomic profiles and involved pathways of TC-71 cells treated with viscum or viscumTT indicate that the synergistic effect of viscumTT is created on a different level.

4.6 *Viscum album* L. extracts induce cellular stress and activate the unfolded protein response *in vitro*

The deeper assessment of the involved signaling pathways revealed the activation (phosphorylation) of MAPK8 and MAPK14 by viscum and viscumTT, but not TT, in both cell lines. The MAPK signaling cascade consists of three subfamilies with several isoforms: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (MAPK8) and p38-MAPKs (MAPK14). ERKs are important for cell survival, while MAPK8 and MAPK14 are stress-mediated being activated in response to diverse cellular and environmental stresses as DNA damage, inflammatory cytokines or oxidative stress resulting in apoptosis. But since MAPK8 and MAPK14 were also shown to have oncogenic functions depending on cellular context, the role of stress-mediated MAPKs is complex and controversial (Wada *et al.*, 2004, Dhillon *et al.*, 2007). In line with the present results, the activation of MAPK8 has been described in leukemia and hepatocarcinoma cells after treatment with a commercial aqueous European mistletoe extract (Abnobaviscum) and an aqueous Korean mistletoe extract (Kim *et al.*, 2000, Kim *et al.*, 2004, Park *et al.*, 2012). Moreover, other ribosome-inactivating proteins as ricin or Shiga toxins were also shown to activate MAPK8 and MAPK14 in human monocytes/macrophages (Gonzalez *et al.*, 2006, Lindauer *et al.*, 2010, Tesh, 2012). Contrary to the present results, there have been also reports about the activation of MAPK8 and MAPK14 by OA and BA in melanoma, pancreatic cancer and osteosarcoma cells (Tan *et al.*, 2003, Liu *et al.*, 2014a) and by OA in hypertrophic scar fibroblasts (Chen *et al.*, 2014).

The activation of MAPK8 and MAPK14 upon treatment with mistletoe extracts appears to be a stress response resulting in apoptosis induction. Additionally, the transcriptomic results also displayed response to oxidative stress for viscum and viscumTT and TLR signaling (an immune defense response) for TT treatment. Therefore, the apoptosis-preventive impact of specific inhibitors targeting MAPK8, MAPK14, TLR4 and oxidative stress was investigated after treatment with the extracts. Pretreatment with the TLR4 antagonist LPS-RS reduced TT-mediated apoptosis pointing out the involvement of TLR4 signaling after TT treatment. The involvement in TLR4 signaling has been already demonstrated in mouse splenocytes for an OA derivative (Auletta *et al.*, 2010). Regarding oxidative stress, NAC pretreatment slightly reduced the apoptotic cell death induced by viscum and viscumTT indicating that oxidative stress is involved in viscum-

and viscumTT-mediated apoptosis. In line with this, the involvement of mistletoe-mediated oxidative stress resulting in apoptosis was shown in leukemia and hepatocarcinoma cells treated with aqueous mistletoe extracts (Kim *et al.*, 2004, Park *et al.*, 2012). But also an OA derivative was reported to induce oxidative stress in ovarian cancer cells (Petronelli *et al.*, 2009a).

Considering stress-mediated MAPK signaling pathway, the applied MAPK8 and MAPK14 inhibitors (up to 5 µM SP600125, 10 µM SB203580) were not able to inhibit the activation (phosphorylation) of the kinases and to prevent the induction of apoptosis by the extracts. Higher concentrations of the inhibitors resulted in a significant loss of cell viability demonstrating the cytotoxic potential of the inhibitors in Ewing sarcoma cells. Others have used superior concentrations (up to 30 µM SB203580 or SP600125) to achieve a block in mistletoe-mediated apoptosis induction without a loss of cell viability in leukemia and hepatocarcinoma cells suggesting that higher concentrations might be necessary to effectively block apoptosis (Pae *et al.*, 2001, Kim *et al.*, 2004, Park *et al.*, 2012).

Ewing sarcoma cells consistently express IGF-1R and insulin receptor and the oncogenic function of EWS-FLI1 is dependent on IGF-1 signaling (Silvany *et al.*, 2000, Benini *et al.*, 2004, Cironi *et al.*, 2008). MAPK is downstream of IGF pathway and plays an important role in Ewing sarcoma exhibiting permanent activation of ERK1/2 (Silvany *et al.*, 2000, Benini *et al.*, 2004, Chandhanayingyong *et al.*, 2012) leading to proliferation. SB203580 is a pyridinyl imidazole that inhibits MAPK14. This inhibition depends on the presence of threonine at residue 106, which almost all protein kinases do not have except few as MAPK14 and Raf-1. Raf-1 is a serine/threonine-protein kinase that acts as a regulatory link between the membrane-associated Ras GTPases and the MAPK/ERK cascade resulting in ERK activation. But paradoxically, SB203580 does not suppress activation of the classical MAPK cascade because it also triggers the activation of Raf-1 (Hall-Jackson *et al.*, 1999). Consequently, Ewing sarcoma cells treated with both, SB203580 and mistletoe extracts, receive simultaneously opposing signals referring to MAPK signaling. Furthermore, the MAPK8 inhibitor SP600125 is a reversible ATP-competitive inhibitor with more than 20-fold selectivity for MAPK8 versus a range of kinases and enzymes (Bennett *et al.*, 2001) but this does not exclude the inhibition of other protein kinases resulting in cell death. Maybe, treatment with MAPK8/14 inhibitors causes additional cellular stress in Ewing sarcoma cells, whose proliferation is depend on several protein kinase signaling as IGF or ERK, resulting in

cell death and not in prevention of it. On the other hand, especially viscum and viscumTT displayed an impressive impact on several apoptosis-related proteins as well as on the proteomic and transcriptomic profile indicating that stress-mediated MAPK signaling and oxidative stress are not the only reason for the apoptosis induction by the extracts.

Since there is a crosstalk between oxidative stress and the unfolded protein response and ER stress is also linked to MAPK8 activation (Urano *et al.*, 2000, Nishitoh *et al.*, 2002, Kim *et al.*, 2009), next the protein expression of the ER chaperone protein HSPA5 (Wang *et al.*, 2009) and the ER stress sensor protein EIF2AK3 (Chakrabarti *et al.*, 2011, Jäger *et al.*, 2012) was analyzed. Treatment with the extracts resulted in an upregulation of HSPA5 in both cell lines suggesting response to oxidative or ER stress by activation of the unfolded protein response. The unfolded protein response takes care of ER protein homeostasis and functionality. This is achieved by reduction of protein translation in order to decrease the load of newly synthesized proteins on the ER and by transcriptional activation of genes associated with restoring normal ER function (Ron *et al.*, 2007, Chakrabarti *et al.*, 2011). In line with the present results, the induction of HSPA5 and DDIT3 (formerly CHOP) leading to ER stress was reported for a commercial aqueous extract in leukemia cells (Park *et al.*, 2012). Concordantly, a transcriptional upregulation of *DDIT3* after treatment with viscum, TT or viscumTT was also detected in TC-71 cells. Ricin was also shown to activate the unfolded protein response in breast and colon cancer cell lines (Horrix *et al.*, 2011) and Shiga toxins were reported to activate the unfolded protein response in a rat renal tubular epithelial cell line (Zhao *et al.*, 2011). Furthermore, the naturally occurring triterpenoid celastrol was shown to induce the unfolded protein response in head and neck cancer cell lines (Fribley *et al.*, 2015). Interestingly, viscum and viscumTT treatment concurrently displayed a decrease of EIF2Ak3, whereas TT treatment showed a slight upregulation matching response to ER stress. Others demonstrated for an OA derivative the induction of apoptosis by ER stress in HeLa cells (Won *et al.*, 2010). Proteomic investigation of BA induced apoptosis in HeLa cells also displayed the induction of ER stress (Xu *et al.*, 2014). Moreover, it was demonstrated that an OA derivative triggered ER stress leading to MAPK8-dependent apoptosis (Zou *et al.*, 2008).

As ER stress is connected to autophagy (Hoyer-Hansen *et al.*, 2007, Heath-Engel *et al.*, 2008, Schleicher *et al.*, 2010, Appenzeller-Herzog *et al.*, 2012), next the expression

of the autophagy marker LC3B was analyzed. Autophagy is a self-degradative process, which is generally seen as a survival mechanism (Glick *et al.*, 2010). The TT extract increased LC3B-II expression in both cell lines indicating enhanced autophagic activity. Consistent with these results, triterpenes as UA, BA or OA and its derivatives were reported to induce autophagy in human embryonic kidney 293 cells (Yore *et al.*, 2011), myeloma cells (Yang *et al.*, 2012a), breast cancer cells (Zhao *et al.*, 2013, Lisiak *et al.*, 2014), glioblastoma (Shen *et al.*, 2014) and KRAS transfected MCF10A breast epithelial cells (Liu *et al.*, 2014b). Others, by contrast, reported autophagy induction by Korean ML in placenta-derived mesenchymal stem cells (Choi *et al.*, 2012).

In conclusion, viscum, TT and viscumTT induce diverse cellular stresses and influence protein folding by activating the unfolded protein response. Moreover, the TT extract indicates the induction of autophagy and the involvement of TLR4 signaling. As viscum and viscumTT appear to activate the same signaling pathways differing from TT-activated pathways, the synergistic effect of viscumTT cannot be explained so far. However, the results suggest that the synergistic effect of viscumTT is created by other mechanisms.

4.7 Final conclusion

Taken together, the *in vitro* and *ex vivo* data revealed that *Viscum album* L. extracts, especially viscum and viscumTT, activate various signaling pathways resulting in apoptosis. The *in vivo* study indicated a good therapeutic efficacy of viscum and viscumTT against Ewing sarcoma. Viscum, TT and viscumTT induce diverse cellular stress and immune responses and have a strong impact on the transcriptomic and proteomic profile of Ewing sarcoma cells. However, viscum and viscumTT appear to activate the same or similar signaling pathways *in vitro*. Therefore, the synergistic effect of viscumTT might be created on a different level. For saponins, which are triterpene derivatives (triterpenoids) consisting of a steroid or triterpene skeleton attached to one or more sugar side chains (Osbourn *et al.*, 2011, Hill *et al.*, 2013), it has been shown that they enhance the activity of ribosome-inactivating proteins as saporin by modulation of endocytosis (Hebestreit *et al.*, 2006, Weng *et al.*, 2008). The same effect is conceivable for the viscumTT extract containing the triterpenes OA and BA and mistletoe lectins. Thus, it should be investigated in future. Nevertheless, viscumTT combines the effects of hydrophilic and hydrophobic mistletoe compounds creating a potent anticancer effect. Consequently, it may represent a phytopolychemotherapeutic option as a promising adjuvant therapy for pediatric patients with Ewing sarcoma.

5 Summary

Ewing sarcoma is the second most common bone cancer in children and adolescents. It has a poor prognosis and outcome. Therefore, drug discovery of new anti-tumor agents is still crucial to improve survival of Ewing sarcoma patients. Combining active substances may be beneficial since some drug combinations are able to enhance each other creating synergistic effects. Natural substances in plant extracts contain diverse cytotoxic compounds and provide potential active ingredients for tumor therapy. The European mistletoe (*Viscum album* L.) contains hydrophobic triterpene acids and hydrophilic lectins and viscotoxins, which all possess anticancer properties. Standardized commercial *Viscum album* L. extracts are aqueous, excluding the insoluble triterpenes. However, triterpene acids can be solubilized by using cyclodextrins. By combining a solubilized mistletoe triterpene extract (TT) with an aqueous mistletoe extract (viscum) a total mistletoe effect was obtained (viscumTT) and the mechanism of action of viscum, TT and viscumTT was analyzed in Ewing sarcoma cells.

In vitro and *ex vivo* treatment of Ewing sarcoma cells with viscum inhibited proliferation and induced apoptosis in a dose-dependent manner, while the TT extract displayed only moderate pro-apoptotic properties. Importantly, viscumTT combination treatment generated a synergistic effect. Viscum- and viscumTT-induced apoptosis occurred via the intrinsic and extrinsic apoptotic pathway, evidenced by the depolarization of mitochondria membrane and the activation of caspase 8 and 9. Additionally, the antitumor activity of viscum and viscumTT was validated in a Ewing sarcoma mouse model *in vivo*. A deeper assessment of the molecular mechanisms of viscum, TT and viscumTT treatment leading to apoptosis *in vitro* revealed a balance shift of anti-apoptotic/ pro-survival regulatory proteins towards the pro-apoptotic state, mainly via MCL1, CLSPN, BIRC5 and XIAP downregulation. Moreover, the transcriptomic and proteomic profile of Ewing sarcoma cells showed strong alterations evoking a transcriptional upregulation of stress-activated and apoptosis-associated genes accompanied with an upregulation of proteasomal proteins and a downregulation of ribosomal and spliceosomal proteins. Furthermore, the extracts triggered the activation of the unfolded protein response. While viscum and viscumTT displayed also response to oxidative stress and the activation of stress-mediated MAPK signalling, the TT extract indicated the involvement of TLR signalling and autophagy.

Since the combinatory viscumTT extract displayed phytopolychemotherapeutic properties and demonstrated a potent pro-apoptotic impact, it may represent an adjuvant therapy option for paediatric patients with Ewing sarcoma. In any case, this work contributes to an improved understanding on the mechanism of action of *Viscum album* L. extracts.

6 Zusammenfassung

Das Ewing-Sarkom stellt den zweithäufigsten Knochentumor bei Kindern und Jugendlichen dar und ist mit einer ungünstigen Prognose assoziiert. Somit ist die Erforschung und Entwicklung neuer therapeutischer Agenzien weiterhin essentiell, um das Überleben von Patienten mit einem Ewing-Sarkom zu verbessern. Die Kombination mehrerer Wirkstoffe kann vorteilhaft sein, da einige Kombinationen sich gegenseitig verstärken können und so synergistische Effekte hervorrufen. Pflanzen enthalten diverse natürlich vorkommende zytotoxische Substanzen und liefern dadurch potentielle Wirkstoffe für die Tumortherapie. Die europäische Mistel (*Viscum album* L.) enthält als anti-kanzerogene Substanzen die lipophilen Triterpensäuren sowie die hydrophilen Mistellektine und Viscotoxine. Standardisierte, kommerziell erhältliche *Viscum album* L. Extrakte sind jedoch auf wässriger Basis und enthalten deshalb keine Triterpensäuren. Diese können aber durch die Verwendung von Cyclodextrinen solubilisiert werden. Durch die Kombination eines triterpenhaltigen Mistelextrakts (TT) mit einem wässrigen Mistelextrakt (viscum) wurde ein Mistel-Gesamtextrakt (viscumTT) erhalten und der Wirkmechanismus von viscum, TT und viscumTT im Ewing-Sarkom-Modell untersucht.
Die Behandlung der Ewing-Sarkom Zellen mit den Extrakten *in vitro* und *ex vivo* zeigte, dass viscum konzentrationsabhängig die Proliferation inhibierte und die Apoptose induzierte, während TT nur schwache pro-apoptotische Eigenschaften aufwies. Zudem zeigte sich, dass der kombinatorische Extrakt viscumTT synergistisch die Apoptose-Induktion bewirkt. Die durch viscum und viscumTT vermittelte Apoptose erfolgte dabei sowohl durch den intrinsichen als auch den extrinsichen Apoptoseweg, was durch die Absenkung des Mitochondrienmembranpotentials und die Aktivierung der Caspase-8 und Caspase-9 belegt wurde. Die anti-kanzerogenen Eigenschaften von viscum und viscumTT wurden auch in einem Ewing-Sarkom-Mausmodell *in vivo* nachgewiesen. Tiefer gehende Untersuchungen des molekularen Wirkmechanismus von viscum, TT und viscumTT *in vitro* zeigten eine Verschiebung von anti-apoptotischen Proteinen zugunsten der Apoptose, die sich in der Abnahme der Proteinexpression von MCL1, CLSPN, BIRC5 und XIAP äußerte. Zudem führten die Extrakte zu einer starken Veränderung des Transkriptom- und Proteom-Profils, wobei sie die transkriptionelle Hochregulation von Stress-aktivierten und Apoptose-assoziierten Genen bewirkten, sowie die Zunahme von proteasomalen Proteinen und die Abnahme von ribosomalen und spliceosomalen Proteinen auslösten. Außerdem lösten die Extrakte eine Antwort auf

ungefaltete Proteine (*unfolded protein response)* aus. Weiterhin wurde gezeigt, dass viscum und viscumTT oxidativen Stress und die Aktivierung des Stress-vermittelten MAPK Signalwegs verursachen, während die Behandlung mit dem TT Extrakt auf die Beteiligung des TLR Signalwegs und der Autophagie hindeutet.

Da vor allem der kombinatorische Extrakt viscumTT potente pro-apoptotische Eigenschaften in Ewing-Sarkom Zellen zeigte, könnte dieser als eine Art Phytopolychemotherapie eine zusätzliche, adjuvante Therapieoption für pädiatrische Patienten mit einem Ewing-Sarkom darstellen. In jedem Fall trägt die vorliegende Arbeit zu einem besseren Verständnis des Wirkmechanismus von *Viscum album* L. Extrakten bei.

7 Supplementary data

Table S1: Comparison of mean measurement values of untreated control (Ctrl) and with cyclodextrins (CD) treated control cells.

	TC-71		MHH-ES-1	
Measurement [%]	Ctrl	3.96 mg/mL CD*	Ctrl	4.76 mg/mL CD*
Apoptosis	14.06	14.40	7.33	7.18
Low ΔΨm	15.30	9.00	9.90	9.00
Proliferation	100.00	89.00	100.00	100.00
LDH release	0.00	0.50	0.00	0.00
active CASP8	9.30	8.40	7.42	6.60
active CASP9	8.50	9.00	7.83	8.32
active CASP3	10.60	10.10	8.05	8.96

*The analyzed CD concentration is equivalent to the CD concentration in the highest used OA concentration.

Table S2: TC-71 statistics (Dunn´s test, treatment versus untreated control cells).

				FDR			
Treatment	Apoptosis	JC-1	Proliferation	LDH	CASP8	CASP9	CASP3
OA 10 µg/mL	0.478	0.318	0.338	-	0.405	0.439	0.384
OA 20 µg/mL	0.466	0.238	0.342	-	0.436	0.462	0.493
OA 30 µg/mL	0.304	0.293	0.283	-	0.321	0.286	0.316
OA 40 µg/mL	0.193	0.366	0.214	-	0.210	0.179	0.202
OA 50 µg/mL	0.093	0.459	0.161	-	0.099	0.099	0.133
ML 1 ng/mL	0.229	0.353	0.091	-	0.300	0.318	0.279
ML 2 ng/mL	0.087	0.217	0.034	-	0.129	0.155	0.156
ML 3 ng/mL	0.025	0.134	0.012	-	0.062	0.065	0.047
ML 4 ng/mL	0.013	0.062	0.006	-	0.025	0.018	0.017
ML 5 ng/mL	0.005	0.034	0.002	-	0.013	0.012	0.017
1 ng/mL + 10 µg/mL	0.047	0.262	0.039	-	0.034	0.030	0.049
2 ng/mL + 20 µg/mL	0.006	0.075	0.004	-	0.005	0.006	0.008
3 ng/mL + 30 µg/mL	0.002	0.019	0.005	-	0.003	0.002	0.003
4 ng/mL + 40 µg/mL	0.001	0.011	0.003	-	0.001	0.001	0.001
5 ng/mL + 50 µg/mL	0.001	0.007	0.001	-	0.001	0.002	0.001

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S3: MHH-ES-1 statistics (Dunn´s test, treatment versus untreated control cells).

				FDR			
Treatment	Apoptosis	JC1	Proliferation	LDH	CASP8	CASP9	CASP3
OA 20 µg/mL	0.391	0.394	0.448	0.352	0.462	0.370	0.428
OA 30 µg/mL	0.410	0.439	0.439	-	0.385	0.278	0.226
OA 40 µg/mL	0.312	0.480	0.376	0.221	0.455	0.424	0.416
OA 50 µg/mL	0.256	0.409	0.456	-	0.470	0.374	0.316
OA 60 µg/mL	0.193	0.399	0.343	0.079	0.407	0.447	0.459
ML 5 ng/mL	0.193	0.229	0.224	0.011	0.210	0.255	0.310
ML 10 ng/mL	0.082	0.156	0.188	-	0.119	0.173	0.238
ML 20 ng/mL	0.026	0.055	0.070	0.026	0.050	0.072	0.104
ML 30 ng/mL	0.008	0.023	0.032	-	0.025	0.034	0.058
ML 40 ng/mL	0.003	0.009	0.015	0.006	0.018	0.022	0.032
5 ng/mL + 20 µg/mL	0.071	0.158	0.226	-	0.103	0.170	0.161
10 ng/mL + 30 µg/mL	0.017	0.076	0.015	0.054	0.035	0.053	0.059
20 ng/mL + 40 µg/mL	0.004	0.010	0.039	-	0.007	0.013	0.016
30 ng/mL + 50 µg/mL	0.001	0.003	0.005	0.115	0.003	0.006	0.008
40 ng/mL + 60 µg/mL	0.001	0.002	0.007	0.015	0.003	0.003	0.004

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S4: *Ex vivo* statistics (Dunn´s test, treatment versus untreated control cells).

	FDR
Treatment	Apoptosis
OA 20 µg/mL	0.401
OA 40 µg/mL	0.444
OA 60 µg/mL	0.464
ML 10 ng/mL	0.153
ML 20 ng/mL	0.065
ML 40 ng/mL	0.015
10 ng/mL + 20 µg/mL	0.047
20 ng/mL + 40 µg/mL	0.004
40 ng/mL + 60 µg/mL	0.004

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S5: OA and BA standard statistics (Dunn´s test, treatment versus untreated control cells).

	FDR	
Treatment	Apoptosis	JC-1
OA 10 µg/mL	0.478	0.321
OA 20 µg/mL	0.493	0.192
OA 30 µg/mL	0.187	0.405
OA 40 µg/mL	0.058	0.395
OA 50 µg/mL	0.022	0.319
BA 0.9 µg/mL	0.481	0.420
BA 1.9 µg/mL	0.485	0.333
BA 2.6 µg/mL	0.427	0.300
BA 3.5 µg/mL	0.210	0.204
BA 4.4 µg/mL	0.066	0.065
10 µg/mL + 0.9 µg/mL	0.324	0.196
20 µg/mL + 1.8 µg/mL	0.288	0.213
30 µg/mL + 2.6 µg/mL	0.098	0.362
40 µg/mL + 3.5 µg/mL	0.045	0.373
50 µg/mL + 4.4 µg/mL	0.028	0.477

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S6a: *In vivo* statistics (Dunn´s test, treatment versus control group intratumorally (i.t.) treated with cyclodextrins).

Treatment	FDR
viscum i.t,	0.0956
TT i.t.	0.3146
viscumTT i.t.	0.3015

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S6b: *In vivo* statistics (Dunn´s test, treatment versus control group intravenously (i.v.) treated with cyclodextrins).

Treatment	FDR
viscumTT i.v.	0.0138
doxorubicin i.v.	0.0119

*FDR = false discovery rate, **≤** 0.1 considered as significant

Table S7: TC-71 inhibitors statistics (Dunn´s test, treatment versus untreated control cells).

		FDR	
Treatment	Z-VAD-FMK	NAC	LPS
OA 50 µg/mL	0.183	0.500	0.012
ML 2 ng/mL	0.026	0.020	0.154
1 ng/mL + 10 µg/mL	0.018	0.046	0.500

*FDR = false discovery rate, **≤** 0.1 considered as significant

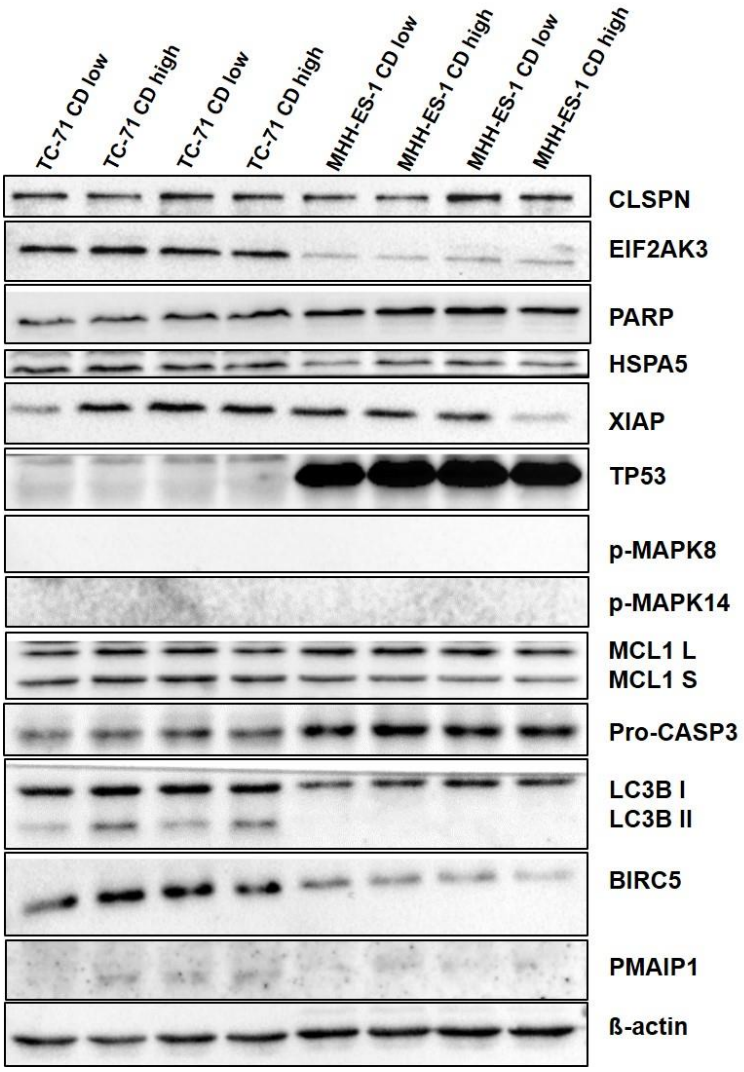

Figure S1: Cyclodextrins have no impact on apoptosis-related proteins. TC-71 and MHH-ES-1 cells were treated with increasing concentrations of ß-cyclodextrins (CD, low = 0.79 µg/mL, high = 4.7 µg/mL) in two independent experiments. Western blots were performed to investigate their impact on apoptosis- and cellular stress-related proteins. β-actin was used as loading control.

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9 Publications

9.1 Research articles

Twardziok M, Rolff J, Jäger S, Eggert A, Seifert G, Delebinski CI (2015). Multiple active compounds from *Viscum album L.* synergistically converge to promote apoptosis in Ewing sarcoma. *Submitted to PLoS ONE*.

Twardziok M, Meierhofer D, Börno S, Timmermann B, Jäger S, Eggert A, Delebinski CI, Seifert G (2015). Defined European mistletoe total extract activates stress-mediated MAPK pathway and the unfolded protein response in Ewing sarcoma. *In preparation*.

Delebinski CI, **Twardziok M**, Kleinsimon S, Hoff F, Mulsow K, Rolff J, Jäger S, Eggert A, Seifert G (2015). A Natural Combination Extract of Viscum album L. Containing Both Triterpene Acids and Lectins Is Highly Effective against AML In Vivo. *PLoS ONE*, 10: e0133892.

Delebinski CI, Georgi S, Kleinsimon S, **Twardziok M**, Kopp B, Melzig MF, Seifert G (2015). Analysis of proliferation and apoptotic induction by 20 steroid glycosides in 143B osteosarcoma cells in vitro. C*ell Proliferation*, published ahead of print.

9.2 Oral presentations

Dejewska* M, Delebinski CI, Jäger S, Seeger K, Seifert G.
Viscum album L. extracts composed of triterpene acids and mistletoe lectins exhibit synergistic apoptosis induction in pediatric Ewing´s sarcoma cells *in vitro*. 8th International Congress of Complementary Medicine Research, 11th – 13th April 2013, London, UK.

Twardziok M, Delebinski CI, Jäger S, Seeger K, Seifert G.
Viscum album L. extracts composed of triterpene acids and mistletoe lectins exhibit synergistic apoptosis induction in pediatric Ewing´s sarcoma cells *in vitro*. 6th European Congress for Integrative Medicine, 4th – 5th October 2013, Berlin, Germany.

9.3 Poster

Delebinski CI, **Dejewska* M,** Jäger S, Seeger K, Seifert G.
A new development of triterpene acid containing extract from *Viscum album* L. displays synergistic induction of apoptosis in acute myeloid leukemia. 8th International Congress of Complementary Medicine Research, 11th – 13th April 2013, London, UK.

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A combination of triterpene acids and mistletoe lectins induces highly effective apoptosis in Ewing´s sarcoma. International Research Congress on Integrative Medicine and Health, 13th – 16th May 2014, Miami, USA.

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Survivin is down-regulated by *Viscum album* L. in pediatric Ewing´s sarcoma cells *in vitro.* 9th International Conference of Anticancer Research, 6th – 10th October 2014, Sithonia, Greece.

**Twardziok (nee Dejewska)*