Multiple anti-tumoral properties of a whole mistletoe extract viscumTT in osteosarcoma

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For my grandparents

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Summary

Osteosarcoma is the most primary bone tumor and its peak incidence occurs in the second decade in children and adolescents. Diseases like retinoblastoma or Li-Fraumeni syndrome predisposed osteosarcoma at higher risk. Generally, high grade osteosarcoma is associated with bad chemotherapy response, rising chemotherapy resistances, poor prognosis and outcome. New therapy approaches, and drug discovery are essential for the improvement of survival. Complementary mistletoe therapy is widespread in the European countries and is applied mainly as adjuvant therapy in adult tumors. Commercial *Viscum album* preparations are water-based, contain mainly mistletoe lectins and viscotoxins but hydrophobic triterpene acids are nearly not included. These triterpene acids were solubilized by 2-hydroxypropyl-ß-cyclodextrins for our studies. Both hydrophilic as well as hydrophobic substances possess high anti-tumoral properties. By combining an aqueous (viscum) and a triterpene acid (TT) extract a whole mistletoe extract (viscumTT) was recreated and their unified anti-tumoral potential was investigated in pediatric osteosarcoma.

In this work, viscum, TT and viscumTT inhibited proliferation and induced apoptosis in a dose- and cell line-dependent manner. Additionally, viscumTT-mediated effect was always synergistic and nearly same effective in each tested cell line by seemingly non-essential role of TP53 *in vitro*. Mistletoe extracts mainly induced caspase-dependent apoptosis by triggering intrinsic and extrinsic components. High anti-tumoral effect was confirmed in an osteosarcoma xenograft model *in vivo* as viscumTT was also more effective than the single extracts. A closer look to alterations in cell cycle progression gave insights into molecular mechanism of action. Viscum arrested cell cycle either in G1 phase in *TP53* wild-type and null-mutant cells or in S phase in *TP53* mutant cells, whereby TT led to G1 arrest in each cell line. ViscumTT did not change arrested stadium by single extracts except in *TP53* mutant cells as no distinct cell cycle arrest was detectable. ViscumTT-mediated alterations resulted from induced gene expression by single extracts by up-regulation of *GADD45A* and *CDKN1A* as well as down-regulation of *SKP2* whereas the effect was lower in *TP53* null mutant cells. Further investigations indicated the role of *GADD45A* and *CDKN1A* also in induction of apoptosis. Additionally, viscumTT triggered environmental stress response via MAPK8 activation as well as inactivation of survival associated pathways MAPK1/3 and STAT3. Especially, BIRC5 and MYC that are often associated with poor prognosis were down-regulated. Moreover, viscumTT-mediated synergistic effect might be based on a complex interaction of targets that were affected by viscum and TT as single extracts. All findings of this work could qualify viscumTT as high potential promising therapy approach for osteosarcoma patients.

Zusammenfassung

Das Osteosarkom ist der häufigste primäre Knochentumor mit einem erhöhten Vorkommen im zweiten Lebensjahrzehnt bei Kindern und jungen Erwachsenen. Genetische Prädispositionen, wie das Retinoblastom oder Li-Fraumeni Syndrom, erhöhen das Risiko, an einem Osteosarkom zu erkranken. Es ist mit einem schlechten Ansprechen auf Chemotherapie, erhöhtem Risiko für Chemotherapieresistenzen und schlechter Prognose assoziiert. Das macht die Entwicklung neuer Medikamente und Therapieansätze essentiell. Die Misteltherapie wird in der komplementären Medizin in Europa bei Erwachsenentumoren häufig angewendet. Kommerzielle Präparate beinhalten hauptsächlich hydrophile Bestandteile, wie die Mistellektine und Viscotoxine. Hydrophobe Substanzen, wie die Triterpensäuren, die durch 2-Hydroxypropyl-ß-Cyclodextrin für unsere Studien solubilisiert wurden, sind so gut wie nicht enthalten. Allerdings besitzen sowohl die hydrophilen als auch die hydrophoben Bestandteile eine hohe antitumorale Wirksamkeit. Durch die Kombination eines wässrigen (viscum) und eines lipophilen (TT) Extraktes wurde ein Gesamtmistelextrakt (viscumTT) hergestellt und auf dessen antitumorale Eigenschaften im Osteosarkom untersucht.

In dieser Arbeit zeigten viscum, TT und viscumTT eine konzentrations- und zelllinienabhängige Inhibierung der Proliferation sowie Induktion der Apoptose. Darüberhinaus erzielte viscumTT immer einen synergistischen und nahezu gleichen Effekt in den hier getesteten Zellen, in dem TP53 scheinbar keine essentielle Rolle spielte. Die Mistelextrakte induzierten unter der Beteiligung von intrinsischen und extrinsischen Komponenten Caspase-abhängig Apoptose. Der hohe antitumorale Effekt wurde in einem Osteosarkom Modell *in vivo* bestätigt, in dem viscumTT auch eine höhere Wirksamkeit als die Einzelextrakte aufwies. Untersuchungen des Zellzyklus zeigten, dass viscum *TP53*-Wildtyp und -Nullmutante Zellen in G1 oder *TP53*-Mutante Zellen in S Phase arrestierte, während TT in allen Zelllinien einen G1 Arrest induzierte. ViscumTT führte ebenfalls zu einem G1 Arrest, allerdings zu keinem eindeutigen Zellzyklusarrest in *TP53*-Mutante Zellen. Die durch viscumTT induzierten Zellzyklusveränderungen basierten auf der Wirkung der Einzelextrakte durch die Hochregulation der Gene *GADD45A* und *CDKN1A* sowie durch die Herunterregulation von *SKP2*. Außerdem waren *GADD45A* und *CDKN1A* auch an der Induktion der Apoptose beteiligt. ViscumTT aktivierte die stressinduzierte Signalantwort über MAPK8 und inaktivierte zellwachstumsstimulierende Signalwege wie MAPK1/3 und STAT3. BIRC5 und MYC, die mit einer schlechten Prognose assoziiert sind, wurden herunterreguliert. Der Synergismus von viscumTT resultierte vermutlich aus einem komplexen Zusammenspiel verschiedener Zielproteine, ausgelöst durch die Einzelextrakte viscum und TT. Alle Ergebnisse dieser Arbeit verdeutlichen das hohe therapeutische Potential von viscumTT als ein möglicher, neuer Therapieansatz für Osteosarkom Patienten.

1. Introduction

1.1 *Viscum album*

Viscum album, also known as the European white berry mistletoe, is a species of the hemi-parasitic, evergreen plant mistletoe in the family of *Santalaceae*. It grows in different host trees, such as apple (*Malus*), ash (*Fraxinus*), oak (*Quercus*), fir (*Abietis*), and pine (*Pinus*). Mistletoe attaches and penetrates branches to absorb water and nutrients, but is able to carry out photosynthesis by itself (Figure 1) [1].

Figure 1 ***Viscum album.*** Infested host tree (left, https://pixabay.com/de/mistel-baum-der-parasit-natur-2684556/) white berries and new shoots (right, https://pixabay.com/de/mistel-schmarotzer-heilpflanze-229837/).

The founder of anthroposophic medicine, Rudolf Steiner, recognized parallels of mistletoe growth and tumor growth. Together with Ita Wegmann, he established mistletoe as cancer therapy approach in 1917 [2]. Since then, different mistletoe preparations have been developed, which have found their way into complementary cancer therapy. Today they are most widely used in Europe [3]. Mistletoe therapy is clinically relevant for the improvement of quality of life and the reduction of side effects associated with standard therapies, such as chemotherapy, surgery, and irradiation. This effectiveness has been demonstrated in several studies [4-6]. Besides, the positive effects on general state of health in patients, mistletoe has specifically anti-tumoral properties based on its large number of effective ingredients [7]. Therefore, a widespread range of biologically active substances is already known. The composition of active substances is depending on host trees, harvesting period, and manufacturing process [7]. Mistletoe contains hydrophilic as well as lipophilic substances, including mistletoe lectins (MLs), viscotoxins (VTs), phenolic acids, flavonoids, oligo- and polysaccharides, alkaloids, and triterpene acids [8-13]. Today, commercially applied *Viscum album* preparations are water-based and mainly contain hydrophilic substances, such as MLs and VTs that represent the best investigated ingredients in mistletoe [8, 9]. Commercial

anthroposophic *Viscum album* preparations (AbnobaViscum®, Helixor®, Iscador®, Iscucin®) are commonly applied subcutaneously (s.c.), but as off label use intravenously (i.v.), intratumorally (i.t.) and intrapleurally (i.p.) are also possible routes of administration [14]. These preparations differ in manufacturing process, type of mistletoe lectin, and host tree and their application depends on tumor type and clinical staging [15]. In the following sub-chapters the main ingredients of *Viscum album* will be characterized in detail.

1.1.1 Mistletoe lectins

MLs are the main compounds of *Viscum album* and belong to the group of glycoproteins. In mistletoe plants, three different MLs (ML I-III) and the chitin-binding mistletoe lectin (with over 20 isoforms) were identified. The type of ML and its amount depends on the host tree as well as the season. Therefore, ML I mainly appears in mistletoes grown in deciduous trees, whereas ML III is often found in mistletoes, which infested coniferous trees [12]. A higher content of ML I can be found in winter on deciduous trees [16]. ML I-III are heterodimeric and composed of a catalytic A and a binding B chain, which are disulfide-linked (Figure 2). They differ in their molecular weight and binding to different carbohydrate structures on cell membrane receptors [17]. Therefore, ML I binds specifically to D-galactose, ML III to N-acetyl-galactosamine and ML II to both carbohydrates [8, 18]. MLs are classified as type II ribosome inactivating proteins (RIP type II) due to their ribosomal ribonucleic acid (rRNA)-cleaving enzyme activity of the A chain, whereby the uptake mechanism is similar to that of other RIP type II toxins like ricin [19]. Especially, the uptake is initiated via binding of B chain to carbohydrate structure on the cell surface and internalization is mediated via clathrin-dependent and clathrin-independent endocytosis [20]. Furthermore, the uptake takes place superficially in small vesicles in the cell periphery, including lamellapodia and cell contact regions [21, 22]. Dissociation of ML into catalytic and binding subunit and intracellular trafficking through cytoplasm are central steps of mechanism [23, 24] and are responsible for unfolding of biological effects, e.g., immunomodulatory [18, 25]. Especially, enzymatic activity is exerted by A chain by inactivating ribosomes (Figure 2) [17, 26, 27], particularly, by removing the essential adenine residue (adenine 4324) of the 28s rRNA subunit [28]. Finally, protein biosynthesis stops and affected cells undergo apoptosis [29, 30]. These properties make MLs medical important for cancer therapy and will described below in more detail.

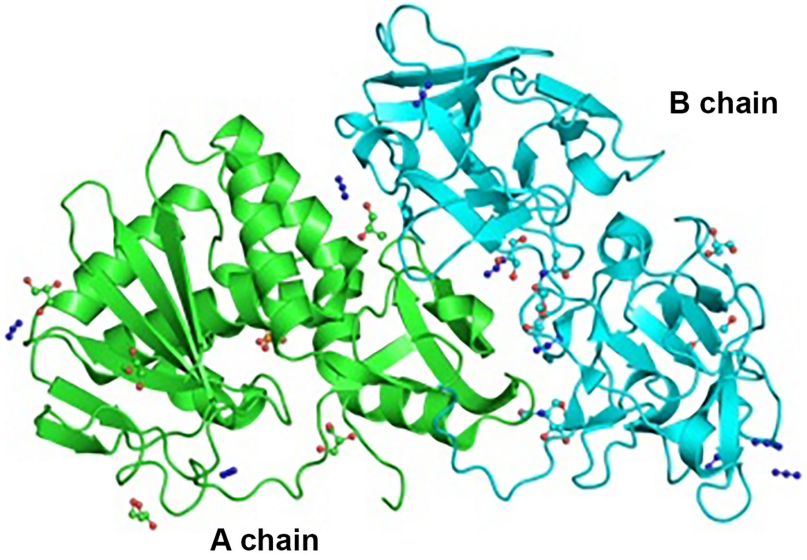


Figure 2 Chrystal structure from ML I from *Viscum album*. ML I is classified as RIP type II protein with catalytic A and binding B chain. ML I binds to D-galactose by its B chain on the cell surface and is internalized by endocytosis. Inside the cells A chain is responsible for enzymatic properties. Protein data bank in Europe [31], modified.

1.1.2 Viscotoxins

VTs are small cysteine-rich, cationic proteins consisting of 46 amino acid residues stabilized by disulfide bridges (Figure 3) [12, 32]. So far, six different isoforms of VTs have been described: VT A1, A2, A3, B, 1-PS, U-PS [33], whereby A2, A3, and B are the most common [34]. However, VT A3 reveals the highest cytotoxic potential, whereas VT B demonstrates the least (Figure 3) [9, 35]. VTs interact with membranes, have high affinity to DNA, and induce permeabilization of the cell membrane [36-38]. These properties underlie cytotoxic potential of VTs, mean an additional benefit in effectiveness of *Viscum album* preparations and will be elucidated in the section below.

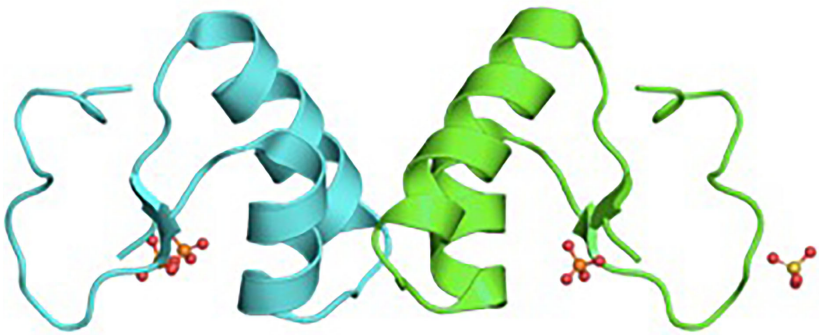
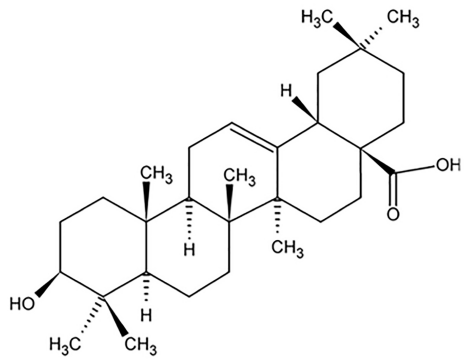


Figure 3 Chrystal structure of VT A3 from *Viscum album*. VT A3 is one of the most occurred VTs in the European mistletoe and has the highest cytotoxic potential. Protein data bank in Europe [39].

1.1.3 Triterpene acids

Triterpene acids are the lipophilic fraction of mistletoe extracts. The main compounds, oleanolic- and betulinic acid (OA, BA), belong to the pentacyclic triterpenes and consist of six isoprene units (Figure 4). Because of the resulting C-30 backbone, triterpene acids are strongly water insoluble. In 1987 Fukunaga *et al.* were able to isolate OA and BA out of *Viscum album* for the first time using ß-amyrin acetate [40]. In commercial water-soluble mistletoe preparations triterpene acids are nearly not included [41] and a new extraction method for triterpene acids was established. Therefore, mistletoe herb was hackled and isolated with n-heptane under pressure at 120°C. After cooling and drying, OA and BA were solubilized with 2-hydroxypropyl-ß-cyclodextrins (CD) and sodium phosphate buffer (pH 7.3) and a yield of OA up to 80 % could be reached [42, 43]. Due to their high biological potential, triterpene acids represent an interesting field in research. Especially, they possess anti-inflammatory [44, 45], anti-oxidant, immunomodulatory [46] and cytotoxic properties [47], which qualify triterpene acids also as anti-cancer drugs and is annotated in the next paragraph.

Oleanolic acid



Betulinic acid

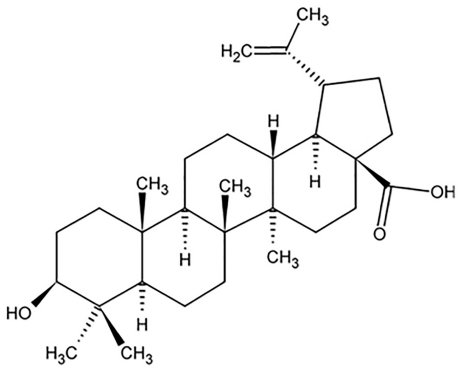


Figure 4 Structure of OA and BA. OA and BA are as lipophilic compounds nearly not included in water-soluble commercial mistletoe extracts [41], modified.

1.2 Main effective compounds of *Viscum album* extracts and their mechanistic effects against cancer

Due to mistletoe-containing main active compounds as described above, it represents a promising therapeutic in different fields. The following paragraph depicts chosen knowledge of mistletoe-containing compounds MLs, VTs and triterpene acids and their mechanistic action especially against cancer after the current state of research.

Mistletoe is well known for its anti-cancer effects based on its valuable ingredients. Commercial *Viscum album* preparations inhibit proliferation and induce apoptosis in various cancer cell

lines, such as leukemia [48], lymphoma, and melanoma [49] as well as head and neck squamous cell carcinoma [50]. In human lymphocytes MLs are responsible for induction of apoptosis, whereby ML III possesses the highest potential followed by ML II and ML I [51]. Only the holoprotein is able to implement apoptosis and activates caspase-3 (CASP3). Isolated A or B chain do not have such ability, their effect is rather based on the interaction [52]. Induction of apoptosis by Korean mistletoe as well as recombinant ML is based on triggering extrinsic and intrinsic pathway in a TP53-independent mechanism with the involvement of mitochondrial anti- and pro-apoptotic proteins [53-55]. Further, an inactivation of survival phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway [50, 56] and an activation of c-Jun-N-terminal kinases/p38 mitogen-activated protein kinase (MAPK8/MAPK14) pathway was demonstrated [57]. Additionally, cytotoxic and anti-tumoral effects were also observed in lymphoblastic leukemia [58], Ehrlich ascites carcinoma [59], and melanoma *in vivo* [60]. However, besides the relevance to re-activate the apoptotic function in the fight against cancer, also triggering of the immune system is considered to be highly important. Multiple studies have shown that mistletoe extracts are able to stimulate the specific and unspecific immune response, for instance, by increasing the number of CD4+ and CD8+ T helper-cells in patients, activation of macrophages and monocytes like-cells as well as production of pro-inflammatory cytokines *in vitro* [61-67].

On the other hand, VTs, as second compound in water-based extracts, have no apoptotic but cytotoxic properties [51]. Cytotoxicity of different VTs was demonstrated in several tumor cells, such as cervix carcinoma, myeloid and lymphoid leukemia [68, 69]. VTs mainly induce necrotic cell death with intermediate production of reactive oxygen species [35]. Furthermore, immunomodulatory effects have been also described. As VTs lead to a slightly increase of granulocytes activation, phagocytosis, and burst activity [70, 71] and increased natural killer-cell mediated cytotoxicity [72]. Klein *et al.* investigated different sera from patients with various tumors and were able to prove immunoglobulins against VTs and ML I, which indicate the induction of a specific immune response against mistletoe extracts consequently a pro-inflammatory stimulation [73].

Moreover, OA and BA, as lipophilic compounds in mistletoe and their synthetic derivatives also show apoptotic potential in a variety of tumor entities. They lead to inhibition of cell proliferation and apoptosis, for instance, in leukemia [74, 75], hepatocarcinoma [76], ovarian cancer [77], and hepatoblastoma cells [78] *in vitro*. An OA derivative, 3-oxo-OA, inhibits tumor growth in melanoma xenografts [79] and ursolic acid (UA), another triterpene acid, enhances anti-tumoral effectiveness of oxaliplatin in colorectal cancer *in vivo* [80]. On mechanistic level, the TP53-dependent activation of extracellular signal-regulated kinase (MAPK1/3, ERK), activation of mitochondria-mediated apoptosis as well as inhibition of AKT pathway was described for OA [81, 82]. CDDO-Me, an OA derivative, is able to activate the MAPK8 pathway

[83]. OA also down-regulates inhibitor of apoptosis protein (IAP) X-linked inhibitor of apoptosis (XIAP) in hepatocellular carcinoma cells [84]. Additionally, an induction of cell cycle arrest in Gap 1 (G1) phase was multiple described in leukemia [85], prostate cancer [82], and hepatocellular carcinoma [86]. Furthermore, CDDO-Me inhibits the signal transducer and activator of transcription 3 (STAT3) pathway in ovarian cancer cells and BA induces apoptosis via mitochondrial associated B-cell lymphoma 2 (BCL-2) proteins TP53-independently in melanoma cells [87].

1.3 Osteosarcoma

Osteosarcoma is a rare disease, but it is the most common type of primary malignant bone tumor with a worldwide incidence of four to five cases annually per million [88]. Incidence is bimodally distributed with a first peak in the age between ten to 14 years and a smaller second peak in older adults over 60 years [89]. Higher number of cases in children and young adults correlate with pubertal growth spurt and boys are slightly more affected than girls with a worldwide male-to-female ratio in ages zero to 24 years of 1.43:1 (Figure 5) [90]. Osteosarcoma mainly occurs in the long bones of the limbs, including femur (42 %), tibia (19 %) and humerus (10 %) at the metaphyseal growth plate [91]. Osteosarcoma is diagnosed and defined by malignant osteoblastic cells producing osteoid and immature bone [92]. The cell of origin of osteosarcoma is unknown, but mesenchymal stem cells as well as cells along the differentiation lineage and osteoblastic precursors are possible [93]. Osteosarcoma is classified in primary and secondary osteosarcomas, whereas the primary conventional osteosarcoma is the most common form. The World Health Organization classified primary osteosarcoma in further sub-types: intramedullary/central (osteoblastic, chondroblastic, fibroblastic, small cell, telangiectatic, low grade central) and surface osteosarcomas (parosteal, periosteal) [88]. Risk factors for primary osteosarcoma are related to physiological growth in metaphyseal area [90]. However, secondary osteosarcoma often correlates with pre-existing conditions, for instance, irradiation or chemotherapy [94]. Osteosarcoma has an aggressive clinical course with local bone and soft tissue destruction followed by metastasis primarily in lungs and other organs. Since the introduction of combined chemotherapy survival rates for localized osteosarcoma patients raised up to 78 %. Despite further treatment options and improved monitoring prognostic outcome and survival rates stagnated in the last two to three decades. Prognosis highly depends on the stage of diagnosis. High-grade osteosarcoma patients have a survival rate up to 66 %, whereas patients with advanced or metastatic stage have a poor prognosis with only a survival rate of 20 to 30 % [95-97]. Until today, the standard therapy is surgery with ideally complete tumor resection followed by neoadjuvant or adjuvant chemotherapy [98]. Current treatment of osteosarcoma based on different combined

chemotherapeutic drugs of high-dose methotrexate with leucovorin rescue, doxorubicin, cisplatin, cyclophosphamide, and ifosfamide [99]. Unfortunately, these therapies are always accompanied with severe side effects and often limited by increasing chemotherapeutic drug resistances [100]. Therefore, novel agents are urgently needed to improve the survival of patients suffering from osteosarcoma, especially for those in metastatic stage.

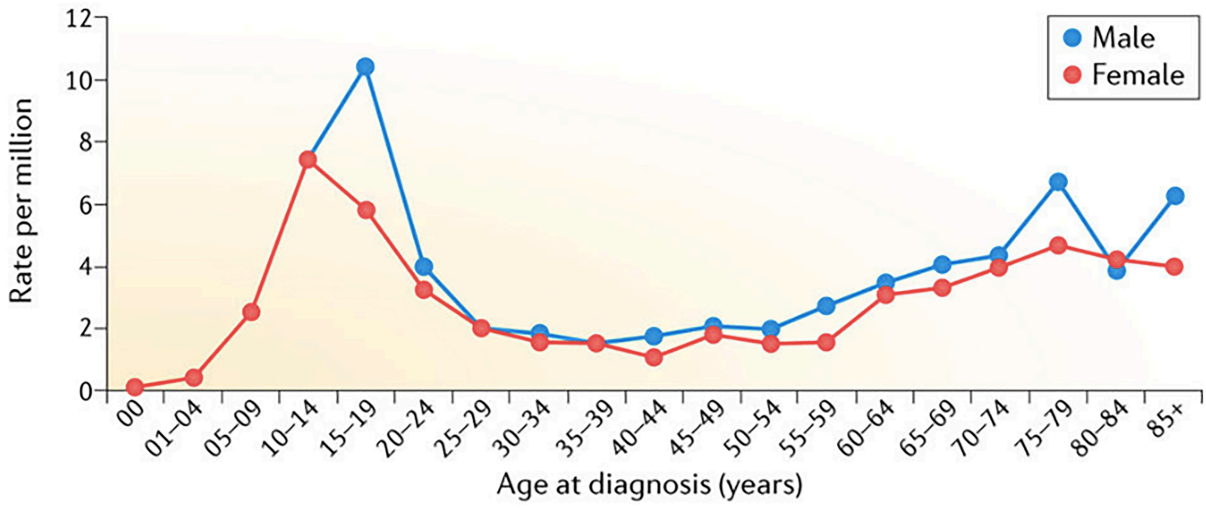


Figure 5 Worldwide incidence of osteosarcoma. Incidence of osteosarcoma is distributed in a first peak in the children and young adults and a second peak in the older adults. Boys are slightly more affected than girls [101], modified.

1.3.1 Molecular Pathogenesis

In general, osteosarcomas are characterized by a high level of genetic instability and heterogeneity. Often osteosarcomas are predisposed in patients with hereditary familiar syndromes, such as retinoblastoma and Li-Fraumeni. Alterations in syndromes associated genes (retinoblastoma 1 (*RB1*), tumor protein TP53 (*TP53*), respectively) may play an important role in pathogenesis [102, 103]. *RB1*, located at chromosome 13q14, is the best characterized gene found in osteosarcoma. Patients suffering from retinoblastoma have a 1000 times higher risk to be affected by osteosarcoma compared to healthy population. Additional, loss of function of *RB1* is associated with high-grade osteosarcoma and poor prognosis [104]. Alterations of any kind that are associated with inactivation of *RB1* occur in about 50 % of osteosarcomas [105]. *RB1* encoded RB1, functioned as tumor suppressor and blocks the transition from G1 to synthesis (S) phase after DNA damage [106]. Thereby, active RB1 (dephosphorylated state) binds and suppresses members of E2F transcription factor family, which further blocks the progression from G1 to S phase. Phosphorylation and inactivation of RB1 is driven by cyclin-dependent kinase 4 (CDK4)/cyclin D (CCND), cyclin-dependent kinase 6 (CDK6)/CCND and subsequently cyclin-dependent kinase 2

(CDK2)/cyclin E (CCNE) complex that leads to release of E2F and progression into S phase [107]. Also, dysregulation of other components of RB pathway, including CDKs and/or cyclin-dependent kinase inhibitors (CDKNs) are often described as genetic alterations in osteosarcoma. Amplifications of CDK4 and CCNE as well as deletions of cyclin-dependent kinase inhibitor 2A (CDKN2A, p16) or 2B (CDKN2B, p15) promote osteosarcoma growth [108-110].

TP53 is located at the short arm of chromosome 17 at 13.1 and somatic mutations often occur in several cancer types ,e.g., ovarian, lung, colorectal, and pancreatic cancer as well as sarcomas [111]. Germline mutation results in Li-Fraumeni syndrome, which is strongly associated with tumor development, including an up to 12 % of osteosarcomas [112, 113]. TP53 is like RB1 a tumor suppressor and involved in many cellular processes, including cell cycle regulation, DNA repair, senescence, and apoptosis [114]. In response to DNA damage wild-type *TP53* leads to up-regulation of *CDKN1A* (p21), which further inhibits CDK4/CCND, CDK6/CCND or CDK2/CCNE complexes, resulting in cell cycle arrest. Prolonged and severe DNA damage induce TP53-dependent apoptosis [115, 116]. Alterations in TP53 itself and in its regulators or effectors, such as mouse double minute 2 (MDM2) are found in osteosarcoma and are associated with tumor progression by dysfunctional cell cycle and apoptosis mechanism [103, 117]. MDM2 inactivates RB1 and TP53 and is in turn transcriptionally up-regulated by TP53. MDM2 also functions as E3 ubiquitin ligase, targets TP53 for its ubiquitination and blocks its translocation into the nucleus [118]. Overexpressed MDM2 is associated with metastasis in osteosarcoma [119]. Besides dysregulated RB1 and TP53 many other molecular pathological mechanisms are related to the development of osteosarcoma. Therefore, aberrations in run-related transcription factor 2 (RUNX2) or wingless-type MMTV integration site family (WNT) signaling, which play a role in normal osteogenesis, dysregulation of diverse signaling pathways like PI3/AKT, MAPK1/3 and Janus kinase (JAK)/STAT and abnormal expression of oncogenes like Aurora A kinase (AURKA) and MYC proto-oncogene (MYC) are found [120]. High heterogeneity and demonstrated characteristics in osteosarcoma pathogenesis reveal many potential targets for new strategies in therapeutic approaches.

1.4 Apoptosis versus cell cycle

Tissue homeostasis is maintained by regulated cell proliferation and programmed cell death. Imbalance of both processes can result in either tissue atrophy or tissue growth, therefore cell cycle and apoptosis are strictly controlled. Development of tumors can result from decrease in cell death or increase in proliferation. On the one hand, mitotic and apoptotic cells display several similar morphological features, including lose substrate attachment, become rounded, cell shrinkage, condensed chromatin and membrane blebbing. On the other hand, there are

distinct differences in both processes, for instance, DNA degradation of approximately 180bp fragments or multiples thereof as property of late apoptosis and DNA segregation in mitotic cells. Finally, apoptotic cells are phagocytosed, whereas mitosis ends with cytokinesis and cell division in two identical daughter cells [121]. A direct link between cell cycle and apoptosis was evidenced by manipulation of the cell cycle, which further led to induction or prevention of apoptosis [122]. Tumor suppressors, such as TP53, RB1, E2F, MYC and various CDKs have been shown to participate in both processes as molecular linkage [122].

1.4.1 Cell cycle regulation

The cell cycle is an event in which cellular components are first doubled and second accurately segregated into two daughter cells. It is divided into four phases: G0/G1, G2, S and mitosis (M). Many postmitotic cells enter into a quiescence stadium, the so called G0 phase, which can be prolonged for various time intervals. Most of these cells are able to re-entry into the cell cycle. In G1 phase cells grow and are prepared for the following S phase, where DNA replication takes place. Then cells progress into G2 phase, where protein synthesis occurs, and cells are prepared for M. In more molecular detail, key regulators of cell cycle progression are CDKs and cyclins (CCNs, Figure 6). Over the whole cell cycle, CDK protein levels remain stable, whereas their activators CCNs occur periodically [123]. CDKs are a family of serine/threonine protein kinases and until now nine have been identified, whereof five are involved in the cell cycle. Moreover, 16 CCNs are described of which not all are related to the cell cycle. Three CCNDs are known (CCND1, CCND2, CCND3) that form complexes with CDK4 and CDK6 in early G1 phase [124]. In contrast to the other CCNs, CCND is not expressed periodically [125]. Activation of these complexes leads to inactivation of RB proteins (RB1, RB transcriptional corepressor like 1, 2 (RBL1, RBL2)). CCNE is expressed, binds, and activates CDK2 in later G1 phase [126]. This complex regulates G1 to S progression and is completely inactivated by the phosphorylation of RB proteins [127]. The level of CDK2/CCNE complex reaches its maximum in G1/S and promotes S phase entry [128]. Negative regulators of CDKs are the CDKNs that are divided into two families and also periodically expressed. The families are: the inhibitors of CDK4 family (INK4) with CDKN2B, CDKN2A, CDKN2C (p18), CDKN2D (p19) as members and CDK inhibitors of Cip/Kip family, including CDKN1A, CDKN1B (p27) and CDKN1C (p57) [129]. Inhibitors of INK4 family block CDK4 and CDK6 during G1 phase, whereas Cip/Kip inhibitors are able to inhibit CDK activity in each phase of the cell cycle. These inhibitors play a regulatory role in G1/S transition. The cell cycle progression is tightly controlled via checkpoints that decide to pass or fail the next phase. They strictly monitor the order and recognize defects during DNA synthesis or segregation and prevent progression into S or M phase, respectively. Activated checkpoints lead to cell cycle arrest and allow cells to repair failures. In case of genotoxic stress in healthy cells, CDKN1A

is TP53-dependently induced, binds and inactivates CDK4, 6/CCND and CDK2/CCNE complex, which further results in RB inactivation and G1/S arrest [130, 131]. After successful DNA repair cells re-entry into the cell cycle. In case of prolonged or severe damage, cells undergo apoptosis. CDK2/CCNA complex is essential during S phase [132]. The replication checkpoint (S to G2 transition) prevents mitosis in presence of unsuccessful DNA replication or arrested replication forks. This checkpoint differs from the others as it is more tolerant in response to DNA damage leading to a delay of the cell cycle [133]. After complete DNA replication in S phase, CDK1/CCNA complex in G2 promotes entry into M phase [134]. As next essential key transition, cells have to pass G2 checkpoint. DNA damage at this state of the cell cycle leads to TP53-dependent or -independent G2 arrest by inhibition of CDK1. Therefore, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are activated, which can directly phosphorylate TP53. Activation of checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) by ATM results in phosphorylation and simultaneously inactivation of cell division cycle 25 (CDC25), which further maintained CDK1 in its inhibited form [135]. TP53 is also able to initiate dissociation of CDK1/cyclin B complex by induction of growth arrest and DNA damage inducible alpha (GADD45A) [136]. Cells in G2 arrest are affected by the DNA repair machinery. If successful, cells pass M phase, which is regulated by CDK1/CCNB complex [137]. The whole cell cycle is accompanied by the ubiquitination machinery for proteolysis of CCNs, CDKs and their inhibitors as posttranslational process [138]. The ubiquitination cascade consisting of E1 ubiquitin-activating, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. Especially, the E3s, SKP1-cullin 1-F-box (SCF) and anaphase-promoting complex/cyclosome (APC/C), are known for cell cycle control [139]. SCF mainly regulates S phase and mitotic entry by particularly ubiquitination of CDKNs, G1 and S phase CCNs and mitotic inhibitors [140]. In this complex F-box proteins, 70 of that are known, are variable and possess substrate specificity [141]. S phase kinase-associated protein 2 (SKP2) is one of that F-box proteins and targets, for instance, CDKN1B [142], CDKN1A and CCND1 [143] and MYC [144]. APC/C regulates mitosis progression and subsequent G1 and targets, e.g., CCNA, B and Aurora kinases A, B (AURKA, B) [140]. Both complexes control each other and especially APC/C mediates SKP2/cyclin-dependent kinase regulatory subunit 1 (CKS1) degradation in G1phase [145] and prevents S phase entry.

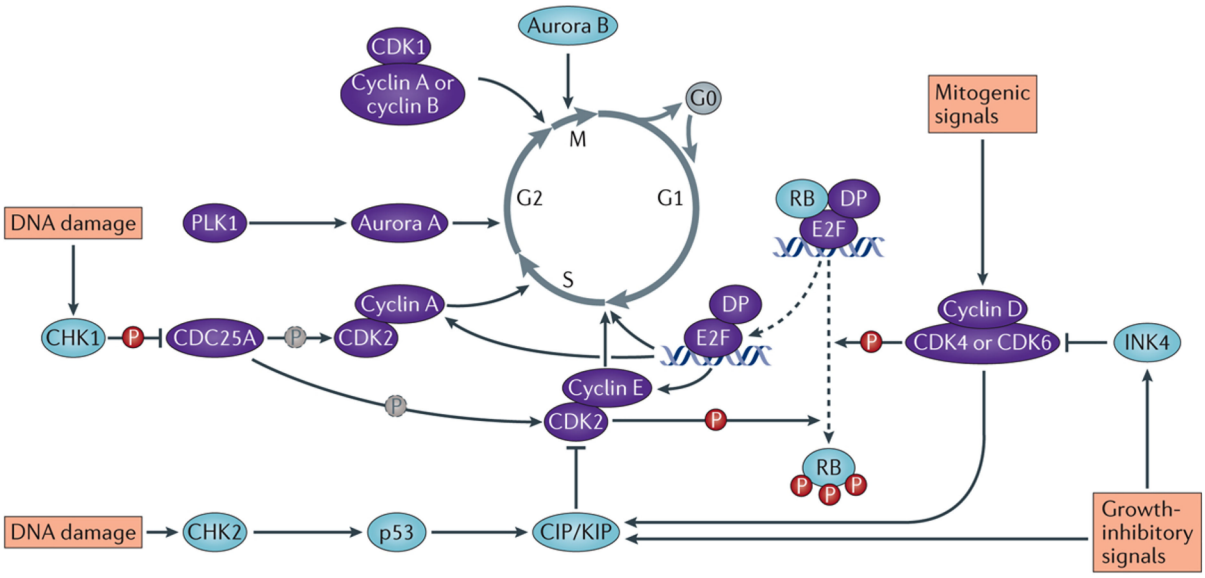


Figure 6 Cell cycle progression via major key regulators. Mainly cells occur in G0 phase of the cell cycle and re-enter the cell cycle via mitogenic signals that activate cascades of CDKs and CCNs (cyclins). CDK4/6/CCND promote transition from G1 to S phase by phosphorylation of targets like RB. Hyperphosphorylation of RB leads to activation of E2F family of transcription factors and growth suppressive function is attenuated. CDK2/CCNE is essential for S phase progression. G2 and M phases are also controlled by CDKs/CCN complexes and other proteins, such as Polo-like kinase 1 (PLK1) and Aurora A. The cell cycle is regulated by members of INK4 and CIP/KIP family inhibitors. DNA damage induces TP53 (p53) and initiates cell cycle arrest. CHK1/2, checkpoint kinase 1/2; CDC25, cell division cycle 25; DP, dimerization partner transcription factor [146], modified. Black arrows: activation, P: phosphorylation, blue ovals: negative regulators, violet ovals: positive regulators.

1.4.2 Apoptosis

Apoptosis, as one form of programmed cell death, is necessary for the maintenance of balance in different cell processes, including normal cell turnover, proper functioning of the immune system and chemical-induced cell death. Characteristically morphologic changes during this form of cell death were long known before it firstly termed apoptosis in 1972 [147]. Apoptosis is a highly complex and energy-dependent process triggered by extrinsic or intrinsic signals. Therefore, two main signaling pathways of apoptosis were described as the intrinsic or mitochondrial and extrinsic or receptor-mediated pathway (Figure 7). Briefly, various cell death receptors, for instance, FAS cell surface death receptor (FAS), tumor necrosis factor receptor 1 (TNFR1) and death receptor 5 (DR5, TRAILR2) are involved in triggering extrinsic apoptosis when binding its ligand. Via adapter molecule, FAS-associated protein with death domain (FADD), caspase cascade is initiated, starting with recruiting of proCASP8, followed by its activation and initiation of proteolytic processing of proCASP3 in its active form CASP3. The extrinsic process ends with substrate cleavage, for instance, poly (ADP-ribose)-polymerase (PARP). The intrinsic pathway can be activated by multiple non-receptor-mediated stimuli, including radiation, DNA damage, hypoxia, hyperthermia, and free radicals. All of these stimuli

lead to changes in the mitochondrial membrane and initiate mitochondria outer membrane permeabilization (MOMP). Consequently, pro-apoptotic signals are released into cytoplasm, e.g., cytochrome c, direct inhibitor of apoptosis-binding protein (DIABLO), and HtrA-serine peptidase 2 (HTRA2). Cytochrome c binds the adaptor protein apoptotic protease activation factor 1 (APAF1) and forms the apoptosome leading to the activation of CASP3 and cleavage of PARP. Both signaling pathways are linked by mitochondria as crosstalk organelles via BH3 interacting-domain death agonist (BID) cleavage mediated by CASP8 [148]. MOMP is tightly regulated by pro- and anti-apoptotic members of B-cell lymphoma 2 (BCL-2) family proteins. The balance between anti-apoptotic proteins like BCL-2, B-cell lymphoma like protein 1, (BCL2L1, BCLXL) or myeloid leukemia cell differentiation protein 1 (MCL-1) (to mention a few) and pro-apoptotic BCL2 associated X (BAX) and BCL2 homologous antagonist killer (BAK) is crucial for initiating apoptosis. Often the BCL-2/BAX ratio is determined for the cellular apoptotic fate [149]. Another subgroup of BCL-2 protein family, so called BCL2-homology 3 (BH3-family), including TP53 up-regulated modulator of apoptosis (BBC3, Puma), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1, NOXA), and BID binds and regulates function of BCL-2 family proteins to promote apoptosis [150]. Furthermore, proteins, which are important for the tightly regulation of apoptosis, are the group of inhibitor of apoptosis proteins (IAPs) that are able to inhibit initiator and effector caspases. Eight mammalian members are known baculoviral inhibitor of apoptosis repeat-containing 1-8 (BIRC1-8) [151] and are further controlled by mitochondria proteins like DIABLO and HTRA2 [152, 153]. Besides caspase-dependent cell death, a caspase-independent programmed cell death exists, which is characterized by signals that normally induce apoptosis receptor- as well as mitochondrial-mediated, but without caspase activation [154]. Therefore, different processes are found that are involved in caspase-independent cell death. The mature form of HTRA2 binds directly to X chromosome-linked inhibitor of apoptosis (XIAP), inhibits its caspase inhibition and induces cell death that cannot block by caspase inhibitors [155]. Furthermore, increase of calcium ions by mitochondria stimulation led to activation of calcium-activated enzymes, including calpains, which are related to caspase regulation during apoptosis. Calpains directly inactivate CASP7, -8 and -9 preventing CASP3 activation and cytochrome c release [156], but initiate apoptosis-like death program ,e.g., cell shrinkage, membrane blebbing, and phosphatidylserine externalization [157]. In apoptosis TP53 is defined as key regulator [158, 159]. Under normal conditions, TP53 is low expressed and becomes stabilized after stressful stimuli. Then it initiates direct transcription of genes related to apoptosis, e.g., FAS, DR5, BAX, BBC3, BID to name a few. Since, calpain seems to be also responsible for activation or proteolytic cleavage of TP53 [160, 161] and further its stabilization indicates a regulatory role in both programmed cell death events by TP53.

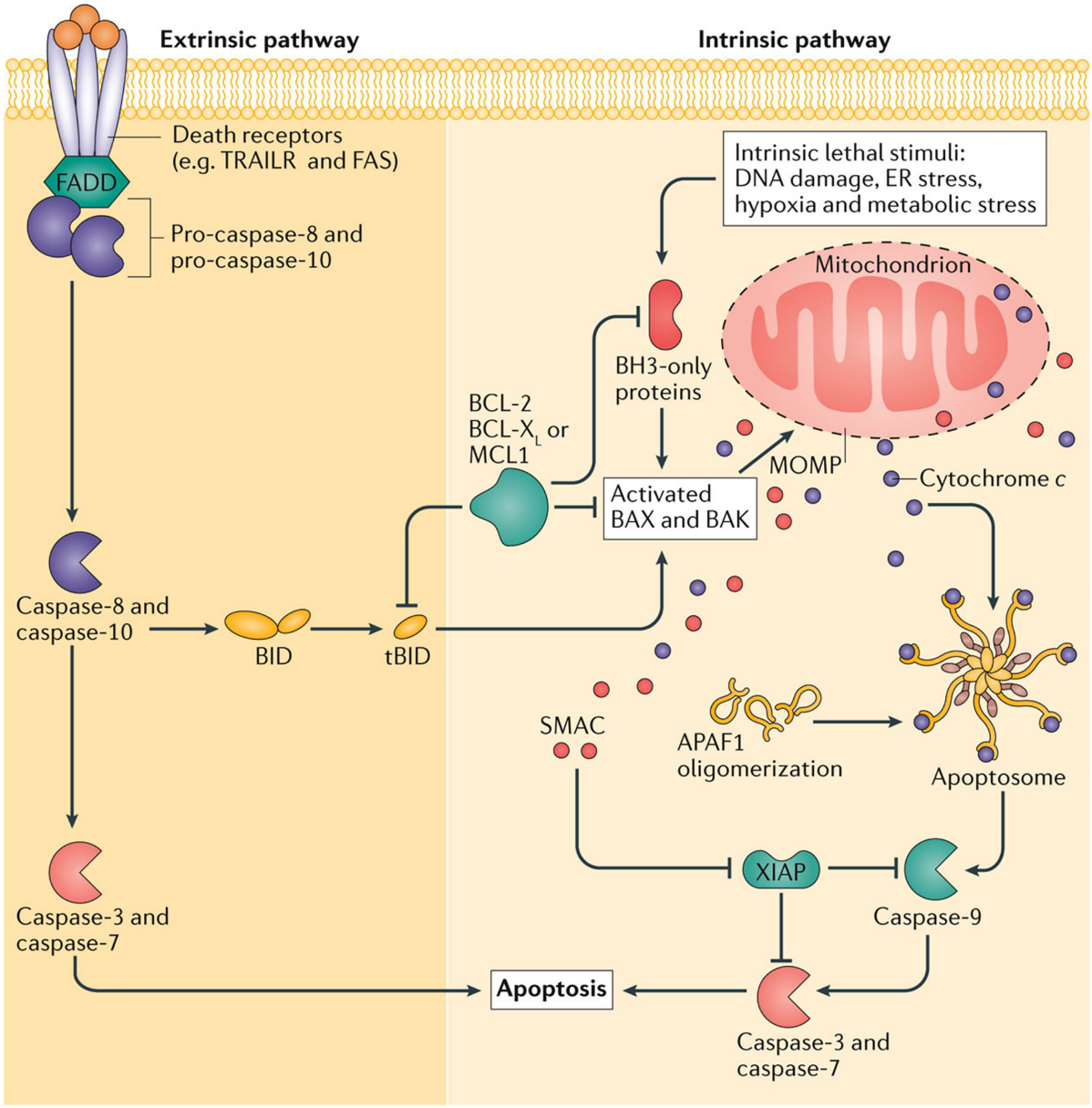


Figure 7 Intrinsic and extrinsic signaling pathway of apoptosis. In the extrinsic pathway binding of ligands to death receptors (TNFR1, TRAILR1/2, and FAS) lead to activation of initiator caspases. Proteolytic processing of proCASP8 and proCASP10 activate effector CASP3 and CASP7 and initiate apoptosis. Cellular stresses stimulate intrinsic pathway via shifting the members of BCL2 proteins and BH3 proteins towards MOMP. This lead to cytochrome c release that forms with APAF1 the apoptosome and activates CASP9. Further CASP3 and CASP7 are activated and apoptosis is induced. Apoptotic process is regulated via inhibitors of apoptosis like XIAP and in turn via its inhibitor second mitochondria-derived activator of caspases (SMAC) which is also released following MOMP. Both processes are crosslinked via MCL1, BID and truncated BID (tBID), BAX, BCLXL. ER, endoplasmic reticulum [162]. Black arrows: activation, blocked lines: inhibition.

1.5 Aim of the work

It is quite known that *Viscum album* has high anti-tumoral potential and is already used in adult oncologic complementary medicine as adjuvant therapy. Therefore, classical *Viscum album* preparations are mainly administered for improvement of the quality of life and reduction of side effects of standard therapies in diverse tumor entities. These applied extracts have hardly included lipophilic triterpene acids. Earlier studies with a mixture of water-based viscum extract (viscum) and a CD solubilized triterpene extract (TT), the so called viscumTT has shown an enhanced anti-tumoral effect *in vitro* and *in vivo* in different pediatric tumors. Some insights of mechanism of action are already known, but direct impact on osteosarcoma was still not investigated.

The focus of this work is the examination of viscumTT regarding its anti-tumoral properties in comparison to its single extracts (viscum, TT) in osteosarcoma *in vitro* as well as *in vivo*. Furthermore, especially the direct effect on three different osteosarcoma cell lines that possess various types of *TP53* should to be analyzed. Viscum, TT and viscumTT are to be investigated regarding their anti-proliferative and apoptotic potential and moreover their effect on the cell cycle. Additionally, this work should give more insights into the mechanistic action and define new direct targets of mistletoe extract in osteosarcoma cells. All in all, this work should provide more knowledge about mistletoe effectiveness as well as confirm the high therapeutic potential in pediatric solid tumors as osteosarcoma.

2. Material and Methods

2.1 Material

2.1.1 Equipment

Biological Safe Cabinet Maxi Safe	Thermo Scientific, Dreieich, Germany
ChemiDoc™ Molecular imager	Bio-Rad, Munich, Germany
Heraeus™ Centrifuge MEGAFUGE™ 8	Thermo Scientific, Dreieich, Germany
Centrifuge MIKRO™ 22R	Hettich, Tuttlingen, Germany
Centrifuge Rotanta™ 460 R	Hettich, Tuttlingen, Germany
Cytoperm™ CO_2 incubator	Thermo Scientific, Dreieich, Germany
Desk centrifuge	NeoLab, Heidelberg, Germany
Excelsior™ ES Tissue Processor	Thermo Scientific, Dreieich, Germany
FACS Calibur™	BD Bioscience, Heidelberg, Germany
Freezing Container Mr. Frosty™	Thermo Scientific, Dreieich, Germany
HistoStar™ Embedding Workstation	Thermo Scientific, Dreieich, Germany
Light Microscope Nikon TMS	Niko, Tokio, Japan
Microscope Axiostar plus	Zeiss, Göttingen, Germany
Microtom HM340E	Thermo Scientific, Dreieich, Germany
Mastercycler	Eppendorf, Hamburg, Germany
Microplate Reader Multiskan Ascent	Thermo Scientific, Dreieich, Germany
NanoDrop™ 2000 spectrophotometer	Thermo Scientific, Dreieich, Germany
Pipetboy	Integra Bioscience, Fernwald, Germany
Pipettes Eppendorf Research Plus	Eppendorf, Hamburg, Germany
pH meters	WRW, Weilheim, Germany
StepOnePlus™ Real Time PCR System	Applied Biosystems, Darmstadt, Germany
Spectrophotometer	Bio-Rad, Munich, Germany
TC20™ cell counter	Bio-Rad, Munich, Germany
Trans-Blot® Turbo™ Transfer System	Bio-Rad, Munich, Germany
Vortex Genie 1 Touchmixer	Scientific Industries, New York, USA

2.1.2 Consumables

Cannulas	B. Braun Melsungen AG, Melsungen, Germany
Cell Counting slides	BD Bioscience, Heidelberg, Germany
Cell culture flasks (T25, T75, T175)	BD Bioscience, Heidelberg, Germany
Cover Slides	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
CryoPure tubes, 1.6 mL	Sarstedt, Nümbrecht, Germany
FACS tubes	BD Bioscience, Heidelberg, Germany
Falcon tubes (15 mL, 50 mL)	BD Bioscience, Heidelberg, Germany
MicroAmp Fast Optical 96-well Reaction Plate	Applied Biosystems, Darmstadt, Germany
MicroAmp Optical Adhesive Film	Applied Biosystems, Darmstadt, Germany
Microscope slides, superfrost	R. Langenbrinck GmbH, Emmendingen, Germany
Microtiter Plates (96-, 12-, 6-well), flat bottom for adherent cells	BD Bioscience, Heidelberg, Germany
Microtome disposable blades	Thermo Scientific, Dreieich, Germany
Millex Syringe Filter Units, 0.22 µm, polyvinylidene difluoride (PVDF) membrane	Merck Millipore, Darmstadt, Germany
PCR Single Cap 8er Soft Strips	Biozym Scientific, Hessisch Oldendorf, Germany
Pipette Tips (10, 100, 1000 µL)	Sarstedt, Nümbrecht, Germany
Serological Pipettes (5, 10, 25 mL)	BD Bioscience, Heidelberg, Germany
Safe Seal Reaction Tube (0.5, 1.5, 2.0 mL)	Sarstedt, Nümbrecht, Germany
Safe Seal SurPhob Pipette Filter Tips (10, 100, 1000 µL)	Biozym Scientific, Hessisch Oldendorf, Germany
Syringe, luer lock, 5 mL	BD Bioscience, Heidelberg, Germany
Syringe, 5 mL	B. Braun Melsungen AG, Melsungen, Germany
Trans-Blot® Turbo transfer packs, nitrocellulose	Bio-Rad, Munich, Germany

2.1.3 Chemicals and reagents

4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate [(Water soluble tetrazolium (WST-1)]	Roche Diagnostics, Mannheim, Germany
4-hydroperoxyifosfamide (4-OOH)	Niomech, Bielefeld, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich, Seelze, Germany
5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1)	AAT Bioquest, Sunnyvale, CA, USA
AEC High Sensitivity Substrate Chromogen	Dako, Santa Clara, CA, USA
Annexin V APC	BD Bioscience, Heidelberg, Germany
Ammoniumpersulfate	Carl Roth, Karlsruhe, Germany
Bovines serum albumin	Sigma-Aldrich, Seelze, Germany
Bradford Protein Assay	Bio Rad, Munich, Germany
Calciumchloride dehydrate ($CaCl*2H_2O$)	Sigma-Aldrich, Seelze, Germany
Carbonyl cyanide 3- chlorophenylhydrazone (CCCP)	Sigma-Aldrich, Seelze, Germany
Chloral hydrate ($C_2H_3Cl_3O_2$)	Fisher Scientific, Schwerte, Germany
Citric acid ($C_6H_8O_7$)	Fisher Scientific, Schwerte, Germany
Citric acid monohydrate ($C_6H_8O_7*H_2O$)	Merck, Darmstadt, Germany
cOmplete™ Protease Inhibitor Cocktail	Roche Diagnostics, Mannheim, Germany
DAB Peroxidase Substrate Kit	Vector Laboratories, Burlingame, CA, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Seelze, Germany
Doxorubicin (Doxo-Cell 150 mg)	Cell Pharm GmbH, Hannover, Germany
Dithiothreitol (DTT)	Fluka Feinchemikalien GmbH, Neu-Ulm, Germany
Dulbecco´s PBS	Gibco, Darmstadt, Germany
Eosin phloxine	Merck, Darmstadt, Germany
Ethanol, absolute	Fisher Scientific, Schwerte, Germany
Ethanol 70 %	Carl Roth, Karlsruhe, Germany
Ethanol 96 %, denatured	Carl Roth, Karlsruhe, Germany
Etoposide (VP16)	Sigma-Aldrich, Seelze, Germany
FACS Flow	BD Bioscience, Heidelberg, Germany

Fetal calve serum (FCS)	Biochrom, Berlin, Germany
Glacial acetic acid (CH_3COOH)	Carl Roth, Karlsruhe, Germany
Goat serum	Invitrogen, Karlsruhe, Germany
Hematoxylin	Merck, Darmstadt, Germany
Hydrogen peroxide	Merck, Darmstadt, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Kaiser's Glycerine Gelatine	Merck, Darmstadt, Germany
Laemmli sample buffer 4x	Bio Rad, Munich, Germany
Lipofectamine™ RNAiMAX transfection reagent	Invitrogen, Karlsruhe, Germany
Lysis Buffer 17	R&D Systems, Minneapolis, MN, USA
McCoys	Gibco, Darmstadt, Germany
Mounting medium	Sigma-Aldrich, Seelze, Germany
Paraffin Paraplast Plus	Leica Biosystems, Nussloch, Germany
Pierce ECL Western Blotting Substrate	Life Technologies, Darmstadt, Germany
Penicillin-Streptomycin	Biochrom, Berlin, Germany
Pepstatin A	Sigma-Aldrich, Seelze, Germany
PHOSstop™	Roche Diagnostics, Mannheim, Germany
Potassium aluminium sulfate ($KaI(SO_4)_{2)}$	Merck, Darmstadt, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Potassium hydrogen phosphate (KH_2PO_4)	Sigma-Aldrich, Seelze, Germany
Power SYBR® Green Master Mix	Applied Biosystems, Darmstadt, Germany
Precision Plus Protein Dual Color	Bio Rad, Munich, Germany
Propidium iodide (PI)	Sigma-Aldrich, Seelze, Germany
Recombinant desoxyribonuclease (rDNAse)	Macherey-Nagel, Düren, Germany
Recombinant ribonuclease (rRNAse)	Roche Diagnostics, Mannheim, Germany
RIPA Buffer	Sigma-Aldrich, Seelze, Germany
Rotiphorese Gel-40 (Acrylamide)	Carl Roth, Karlsruhe, Germany
RPMI 1640	Gibco, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe, Germany
Sodium azide (NaN_3)	Sigma-Aldrich, Seelze, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium citrate ($Na_3C_6H_5O_7$)	Carl Roth, Karlsruhe, Germany

Sodium iodate ($NaIO_3$)	Merck, Darmstadt, Germany
Sodium hydrogen phosphate (Na_2HPO_4)	Arcos Organics, Geel, Belgium
SP600125 (MAPK8 Inhibitor)	Sigma-Aldrich, Seelze, Germany
Tetramethylethylenediamine (TEMED)	Bio Rad, Munich, Germany
Trypsin/EDTA, 0.05 %	Gibco, Darmstadt, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
Xylene	Carl Roth, Karlsruhe, Germany
H_2O, nuclease free	Thermo Scientific, Dreieich, Germany
zVAD-FMK	R&D Systems, Minneapolis, MN, USA

2.1.4 *Viscum album* extracts

All *Viscum album* extracts (lot # 154) in this work were prepared from mistletoe harvested from apple trees (*Malus*) and were kindly provided by Birken AG (Niefern-Öschelbronn, Germany). Therefore, viscum was manufactured from collected one year old young shoots and TT from mistletoe herb (*Herba Visci Albi*, Kottas Heilkräuter, Vienna, Austria). The extraction process of *Viscum album* extracts was described previously [42, 163, 164]. Included intact ML I (A+B chain) in the viscum extract was quantified by ELISA as described earlier [165]. Quantification of OA and BA in the lyophilized TT (containing CD) extract was carried out by gas chromatography-flame ionization detector [42]. Both lyophilized extracts (viscum, TT) were reconstituted in PBS to a final concentration of 1 µg/mL intact ML in viscum and 4000 µg/mL OA in TT (Table 1). ML for viscum and OA for TT were used as marker substances.

Table 1 Composition of mistletoe extracts.

Extract	CD mg/mL	OA µg/mL	BA µg/mL	ML I ng/mL	VT µg/mL
viscum	230	/	/	570	22.13
TT	230	3600	270	/	/
viscumTT	230	3600	270	570	22.13
CD	230	/	/	/	/

2.1.5 Triterpene acid standards

Both triterpene acid standards OA (lot #153.1) and BA (lot #156) were purchased from Birken AG (Niefern-Öschelbronn, Germany) as pure substances from Extrasynthese (Lyon, France). Then standards were lyophilized and solubilized in CD like TT extract by them and kindly provided.

2.1.6 Chemotherapeutic drugs

VP16 was purchased from Sigma-Aldrich (Seelze, Germany) and 4-OOH (active metabolite of ifosfamide) was obtained from Niomech (Bielefeld, Germany). VP16 and 4-OOH stock solutions were diluted in DMSO and stored at -20 °C. Doxo was produced from Doxo-Cell 150 mg and was kindly provided by the hospital pharmacy of Charité and stored at 4 °C. Working solutions were prepared freshly for every chemotherapeutic drug immediately before treatment.

2.1.7 Buffer

Annexin binding buffer, 10 x	0.1 M HEPES, 1.4 M NaCl, 25 mM $CaCl*2H_2O$, pH 7.4
Blocking buffer histological analyses	1 x TBST, 2 % goat serum
Blocking buffer histological analyses	1 x TBST, 0.5 % BSA
Blocking buffer western blot	1 x TBST, 5 % BSA, pH 7.6
Citrate buffer	0.1 M $C_6H_8O_7$, 0.1 M $Na_3C_6H_5O_7$, mix 1:5.5 + 450 mL dmH2O, pH 6.0
Phosphate-buffered saline (PBS), 10 x	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 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, 10 x	250 mM Tris base, 1 % SDS (v/v), 2 M Glycine (w/v)
Tris-buffered saline with Tween, (TBST), 10 x	0.5 M Tris base, 1.5 M NaCl, 1 % Tween 20 (v/v), pH 7.6

2.1.8 Staining solutions

Mayer's hematoxylin solution:

Hematoxylin solution	3.3 mM Hematoxylin, 1 mM $NaIO_3$, 5 M $KaI(SO_4)_2$, 0.3 M $C_2H_3Cl_3O_2$, 4 mM $C_6H_8O_7*H_2O$, add 1 L dmH_2O
Eosin solution	96 % Ethanol 770 mL, Eosin phloxine 110 mL, CH_3COOH 10 mL, add 1 L dmH_2O

2.1.9 Kits

Green Caspase Staining Kit	Roche Diagnostics, Mannheim, Germany
High Capacity RNA-to-cDNA Kit	Applied Biosystems, Darmstadt, Germany
NucleoSpin® RNA Kit	Macherey-Nagel, Düren, Germany
MycoAlert™	Lonza AG, Basel, Switzerland
RT² Profiler™ PCR Array Human Cell Cycle	

2.1.10 SDS-Page gels

Separating gel 12.5 %	3.75 mL Rotiphorese gel
	3.00 mL Separating gel buffer
	5.20 mL dmH_2O
	96 µL 12.5 % APS (w/v)
	7.5 µL TEMED
Stacking gel 4.5 %	0.83 mL Rotiphorese gel
	1.85 mL Stacking gel buffer
	4.80 mL dmH_2O
	60 µL 12.5 % APS
	7.5 µL TEMED

2.1.11 Antibodies

ß-Actin (ACTB) #A3854	Sigma-Aldrich, Hamburg, Germany
BCL2 #2870	Cell signaling Technology, Danvers, MA, USA
BIRC5 #2803	Cell signaling Technology, Danvers, MA, USA
CASP3 #9662	Cell signaling Technology, Danvers, MA, USA
CDK2 sc-163	Santa Cruz Biotechnology, Heidelberg, Germany
CDK4 sc-260	Santa Cruz Biotechnology, Heidelberg, Germany
Cleaved-CASP3 RBK009-05	Zytomed, Berlin, Germany
MYC #9402	Cell signaling Technology, Danvers, MA, USA
CCNA sc-239	Santa Cruz Biotechnology, Heidelberg, Germany
CCND1 ab134175	Abcam, Cambridge, GB
CCNE sc-247	Santa Cruz Biotechnology, Heidelberg, Germany
GADD45A #9662	Cell signaling Technology, Danvers, MA, USA
GAPDH sc-25778	Santa Cruz Biotechnology, Heidelberg, Germany
MKI-67 ab16667	Abcam, Cambridge, GB
CDKN1A #2947	Cell signaling Technology, Danvers, MA, USA

pMAPK1/3 (ERK1/2) #4370	Cell signaling Technology, Danvers, MA, USA
PARP #9542	Cell signaling Technology, Danvers, MA, USA
pMAPK8 sc-6254	Cell signaling Technology, Danvers, MA, USA
pSTAT3 Ser727 #94994	Cell signaling Technology, Danvers, MA, USA
pSTAT3 Tyr705 #9145	Cell signaling Technology, Danvers, MA, USA
SKP2 sc-7164	Santa Cruz Biotechnology, Heidelberg, Germany
STAT3 #4904	Cell signaling Technology, Danvers, MA, USA
TP53 sc-73566	Santa Cruz Biotechnology, Heidelberg, Germany

2.1.12 Primer

All primers were predesigned and purchased from Integrated DNA Technologies (IDT, Leuven, Belgium).

B2M	Hs.PT.58v.18759587
CDKN1A	Hs.PT.58.40874346.g
GADD45A	Hs.PT.58.38974274
GAPDH	Hs.PT.39a.22214836
SKP2	Hs.PT.58.39891597.g

2.1.13 siRNA

De-salted siRNA was purchased from Eurofins Genomics GmbH, Ebersberg, Germany. *Silencer*™ Negative Control No.1 siRNA was used as non-targeting siRNA (AM4611, Thermo Scientific, Dreieich, Germany).

CDKN1A	Sense 5'-GAUGGAACUUCGACUUUGU-3' Antisense 5'-ACAAAGUCGAAGUUCCAUC-3'
GADD45A	Sense 5'-AAAGUCGCUACAUGGAUCAAU-3' Antisense 5'-AUUGAUCCAUGUAGCGACUUU-3'

2.1.14 Cell lines

The human osteosarcoma cell lines U2OS, 143B, and Saos-2 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and human foreskin fibroblastic cell line (VH7) was kindly provided by AG Eggert (Charité, Berlin, Germany). All cell lines were routinely tested for mycoplasma and mycoplasma free cells were used for experiments. 143B is a human osteosarcoma cell line established from the bone of a 13 years old Caucasian girl. Saos-2 cells are human osteosarcoma cells from a bone of an eleven years old Caucasian girl. U2OS osteosarcoma cell lines were established from the bone of a 15 years old Caucasian girl.

2.2 Methods

2.2.1 Cell culture

Cells were cultured at 37 °C in a humidified atmosphere at 5 % CO_2. 143B, Saos-2, U2OS and VH7 cells were split every two or three days. Therefore, cells were washed with PBS, treated with 0.05 % Trypsin/EDTA for 2-4 min at 37 °C and fresh media were added. For subculture, cells were centrifuged (220 g, 5 min), resuspended and splitted in defined ratios.

2.2.1.1 Cell lines

143B cells were cultured in RPMI 1640 medium with L-glutamine, Saos-2 and U2OS cells were maintained in McCoys medium with L-glutamine and VH7 cells were cultured in DMEM high glucose medium. Media were supplemented with 10 % heat-inactivated FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (1 % P/S). All cell lines were cultured up to 90-100 % confluency and were used for experiments to a maximum of 20 passages.

2.2.1.2 Cryopreservation of cells

For cryopreservation, cells were washed with PBS, treated with 0.05 % Trypsin/EDTA for 2-4 min at 37 °C and counted using TC20^{TM} cell counter. Then, cells were centrifuged at 220 g and resuspended in 90 % FCS and 10 % DMSO and stored at -80°C for at least 90 min in freezing container. For long-term storage, cells were transferred into liquid nitrogen.

2.2.1.3 Treatment with *Viscum album* extracts

2x10^5/mL 143B and U2OS cells, 5x10^5/mL Saos-2 cells and 3x10^5/mL VH7 cells were seeded onto 6-well plates (2 mL), 12-well plates (1 mL) or 96-well plates (100 µL), T75 cell culture flasks (15 mL) or T175 cell culture flasks (35 mL) depending on experimental set-up. Cells were allowed 24 h for attachment. Then, after media change, cells were treated for 24 h with rising concentrations of viscum (ML), TT (OA) and the combination of both, viscumTT. Untreated control cells were used as reference. CD as solvent control was excluded as no influence to the cells was detected previously [163].

2.2.1.4 Chemotherapeutic drugs assays

For analyzing whether viscum, TT and viscumTT have additional effect on chemotherapeutic drug-treated cells, treatment combination experiments were performed. Therefore, cells were seeded onto 12-well plates in respective cell counts. After 24 h of attachment, cells were treated with Doxo, VP16 and 4-OOH alone and in combination with viscum, TT and viscumTT in rising concentrations for additional 48 h.

2.2.2 Cell biological analyses

2.2.2.1 Measurement of cell proliferation/WST-1 Assay

143B, Saos-2, U2OS and VH7 cells were seeded in respective cell counts onto 96-well plates. After 24 h of attachment, cells were treated with viscum, TT and viscumTT in rising concentrations for further 24 h. Afterwards, cells were treated with WST-1 reagent for 2 h at 37 °C in a humidified atmosphere at 5 % CO_2. Because of mitochondria active dehydrogenases, WST-1 is cleaved to formazan. Increase of formazan correlates directly with the number of metabolically active cells in the culture. The absorbance of formazan is measured at 450 nm against 690 nm as reference wavelength with a spectrophotometer.

2.2.2.2 Measurement of apoptosis

Apoptotic cells were stained with Annexin V/PI and analyzed by flow cytometry. After treatment with viscum, TT and viscumTT in rising concentrations for 24 h in 6-well plates, cells were harvested and washed twice with PBS (220 g, 5 min, 4 °C), resuspended in 100 µL Annexin V binding buffer and stained with 5 µL APC-conjugated Annexin V for 25 min and 4 °C in the dark. Immediately before measurement, 1 µL PI (1 mg/mL in PBS) was added and cells were analyzed by flow cytometry using FL2 for PI fluorescence and FL4 for APC fluorescence. The results were evaluated with FlowJo® Software (FlowJo, Ashland, OR, USA).

2.2.2.3 Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential ($\Delta\Psi m$) were measured by JC-1 staining and analyzed by flow cytometry. After 24 h of incubation with viscum, TT and viscumTT in increased concentrations 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. 1 µL CCCP (50 mM in DMSO), a mitochondria membrane disrupter, was added to untreated cells as positive control. After staining, cells were washed twice with PBS (220 g, 5 min, 21 °C) and analyzed by flow cytometry. Intact $\Delta\Psi m$ was displayed in FL2 (red) and disrupted $\Delta\Psi m$ was measured in FL1 (green). The results were evaluated with FlowJo® Software.

2.2.2.4 Measurement of active caspases

Active CASP8 and CASP9 were measured using the Green Caspase Staining Kit with the nontoxic, irreversibly caspase-binding cell-permeable FITC-IETD-FMK and FITC-DEVD-FMK. After the treatment with viscum, TT and viscumTT 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 FITC-IETD-FMK (CASP8) and FITC-DEVD-FMK (CASP9) for 30 min, 37 °C, in the

dark. Subsequently, cells were washed twice with the kit's washing buffer and analyzed by flow cytometry. CASP activity was detected in FL1. The results were evaluated with FlowJo® Software.

2.2.2.5 Inhibitor assays

143B and Saos-2 cells were preincubated for 1 h with pan-caspase inhibitor zVAD-FMK (100 µM) and 143B and U2OS cells with SP600125 (MAPK8 inhibitor, 5 µM) before treated with the highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (ML 5 ng/mL+ OA 50 µg/mL) concentration in 12-well microtiter plates for 24 h. DMSO was carried along as solvent control. Afterwards, apoptotic cell death was measured by flow cytometry after Annexin V/PI staining. The results were evaluated with FlowJo® Software.

2.2.2.6 Cell cycle analyses

For cell cycle studies, ethanol fixed cells were stained with PI and analyzed by flow cytometry. Therefore, Saos-2 and U2OS cell line were cultured in FCS-reduced (0.04 %) McCoys and 143B cells in FCS-reduced RPMI 1640 media in 6-well microtiter plates for 24 h for synchronization in G1 phase. Afterwards, cells were treated with viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (ML 5 ng/mL+ OA 50 µg/mL) for 3-24 h. After incubation time, cells were harvested and washed with ice-cold PBS (220 g, 5 min, 4 °C). $1x10^6$ cells were fixed with dribs and drabs -20 °C cold 70 % ethanol under gently vortexing and following stored at - 20 °C until analysis. Within two weeks, DNA fragmentation was stained with PI. Briefly, fixed cells were centrifuged (220 g, 5 min, 4°C) and subsequently washed with ice-cold PBS (220 g, 5 min, 4 °C). Then, cells were treated with 100 µL RNAse (50 µg/mL) for 30 min at 37 °C. Finally, cells were stained with 200 µL of PI (50 µg/mL) for further 30 min at 4 °C in the dark. DNA content was analyzed after exclusion of cell duplexes (FL2-A against FL2-W). Cell cycle phases were evaluated by FlowJo® Software includes Dean Jett Fox method [166].

2.2.3 Molecular biological analyses

2.2.3.1 RNA isolation

For RNA isolation by spin columns using NucleoSpin® RNA Kit, cells were incubated with 10 ng/mL ML for viscum, 50 µg/mL OA for TT and 5 ng/mL ML+50 µg/mL OA for viscumTT for 3-24 h in T175 cell culture flasks. After incubation time, cells were harvested and RNA was isolated according to the manufacturer's protocol. Afterwards, concentration and purity were determined by OD260/280 and OD260/230 using NanoDrop™ 2000 spectrophotometer. On a denaturing agarose gel, RNA integrity was checked. RNA was stored in -80 °C freezer.

2.2.3.2 RNA to cDNA transcription

As preparation for RT-qPCR analyses, RNA was transcribed into cDNA by using High Capacity RNA-to-cDNA Kit. Therefore, 0.5 µg RNA from cells were used and cDNA was produced according to the manufacturer´s protocol.

2.2.3.3 RT² Profiler™ PCR Array

Alterations in 84 cell cycle genes were analyzed by RT² Profiler™ PCR Array Human Cell Cycle (Qiagen, Hilden, Germany) after 24 h treatment. Array was performed with all three cell lines according to the manufacturer's protocol. Briefly, cDNA template were prepared in SYBR® Green master mix and 25 µL of each sample were given to the SYBR® Green optimized primers assay. Then standard RT-qPCR program was used (10 min, 95 °C; 15 s, 95 °C and 60 s, 60 °C, 40 x). Array was performed once for every cell line. The threshold was manually set to 1 and CT values were uploaded as an easy-to-use Excel-based file. For further analysis software from Qiagen Analysis Center was used. Data analysis is based on the ΔΔCT-method with normalization of the raw data to either housekeeping genes. Fold-change higher than one indicated an up-regulation. Fold-changes higher/lower than two were interpreted as significant by the software. The fold-regulation is equal to the fold-change. Fold-change less than one indicates a down-regulation. The fold-regulation is the negative inverse of fold-change. Calculation of fold-regulation correlates with fold-change values in a biologically meaningful way.

2.2.3.4 RT-qPCR analyses

To confirm over-expressed genes (*GADD45A, CDKN1A*) and down-regulated gene (*SKP2*) in RT² Profiler™ PCR Array, gene expression was validated by RT-qPCR on a StepOnePlus™ System in 96-well fast plates with Power SYBR® Green Master Mix, including ROX as passive reference. RT-qPCR was performed under standard conditions (10 min, 95 °C; 15 sec, 95 °C and 60 sec, 60 °C, 40 x). Threshold was manually set to 1. A "no template control" and a "no reverse transcriptase control" were carried along. Quantitative RT-qPCR reaction was set up in total volume of 20 µl containing 5 ng cDNA and 500 nM primers. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) levels for 143B and Saos-2 cells or ß-2-microglobulin (*B2M*) levels for U2OS cells were used as housekeeping genes and for normalization. The relative expression of genes was calculated by ΔΔCT method with further reckoning the fold-regulation (see above).

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

Formula 1 Calculation of ΔΔCT for quantification of RT-qPCR results.

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

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

Formula 2 Calculation of fold-change for quantification of RT-qPCR results.

2.2.3.5 Western blot analyses

To check alterations of apoptotic- as well as cell cycle-associated proteins, western blotting was performed. Therefore, cells were incubated in T175 cell culture flasks with viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (ML 5 ng/mL+OA 50 µg/mL) for different time points depending on proteins of interest. After incubation, cells were harvested and washed with 4 °C-cold PBS three times. Cells were lysed for 30 min either with Lysis Buffer 17 or RIPA buffer, both containing cOmplete™ Protease Inhibitor Cocktail, pepstatin A and PHOSstop™. Then, cells were centrifuged for 5 min at 24100 g. Afterwards, protein concentration was measured by Bradford assay reagent regarding to BSA standard series or protein lysates were stored in -80 °C freezer for later use. SDS-PAGE was performed with 30 µg protein per lane and separation of proteins passed off 4.5 % stacking gel and 12.5 % or 10 % separating gel in 1 x SDS running buffer at 80 V for 20 min followed by 120 V for 60 min. Subsequently, proteins were transferred from gel 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 (RT). Next, blots were incubated with primary antibodies diluted in TBST containing 5 % BSA overnight at 4 °C. Afterwards, blots were washed three times for 10 min in TBST and incubated 1 h with HRP-conjugated secondary antibodies. After further three washing steps, protein expression was visualized by enhanced chemiluminescence (ECL) solution on a Molecular Imager ChemiDoc™. ACTB or GAPDH were used as loading controls.

2.2.3.6 siRNA knockdown

For siRNA knockdown experiments, U2OS and 143B cells were reverse transfected with Lipofectamine™ RNAiMAX transfection reagent and P/S-free, 0.04 % FCS containing McCoys or RPMI 1640 media for 48 h. Non-targeting negative control siRNA was carried along. Afterwards, media was changed into P/S-free, 10 % FCS containing RPMI or McCoys and cells were incubated with viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (ML 5 ng/mL+OA 50 µg/mL) for further 24 h. siRNA knockdown were confirmed by western blotting. After transfection and treatment, cells were analyzed for induction of apoptosis and cell cycle phase distribution. Therefore, cells were stained as described above. Data were evaluated with FlowJo® Software.

2.2.4 Osteosarcoma xenografts

Mice experiments were performed by EPO Berlin-Buch GmbH, but planning the study, interpretation of the results and statistical analysis were performed independently. Eight-weeks-old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac mice were obtained from Taconic (Biosciences GmbH, Cologne, Germany). Animals were housed in a pathogen-free facility under pathogen-free conditions and fed autoclaved standard diet (Sniff, Soest, Germany) with acidified drinking water *ad libitum*. 1x10^7 Saos-2 cells were s.c. injected into the left flank. When tumor was palpable (day 21), treatment started. Viscum (0.75/1.25/1.75 µg/kg ML), TT (50/70/90 mg/kg OA) and the combination thereof, viscumTT, were intratumorally (i. t.) applied twice weekly, whereby each concentration was giving two times. Control mice were treated with CD. To assess health and side effects or symptoms of toxicity, mice were daily monitored. Body weight and tumor volume were measured and documented twice a week. For calculation of individual tumor volumes caliper-like instrument was used and volume was evaluated with the following formula related to the values at the first day of the treatment (relative tumor volume):

$$V_{cm^3} = \frac{W^2 * L}{2}$$

Formula 3 Calculation of tumor volume by caliper-like measurement. V_{cm3}: tumor volume, W: width, L: length.

On day 43 mice were sacrificed by cervical dislocation considering when moribund (tumor volume >1.2cm^3 or >10% body weight loss) sacrificed earlier. Animal study was 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 A0452/08).

2.2.5 Histological analyses

2.2.5.1 De-hydration, tissue embedding and sectioning

Tumor tissue of osteosarcoma xenograft was fixed in 4 % formaldehyde. For storing tissue and remove fixation medium, tissue was transferred to 1 x PBS. Before embedding tissue was de-hydrated by Thermo Scientific Excelsior^{TM} ES by standard protocol overnight:

2 x 1 min	Ethanol 50 %
2 x 30 sec	Ethanol 70 %
2 x 1 min	Ethanol 96 %
1 x 1 h	Xylene
2 x 1.5 h	Xylene
1 x 1 h	Paraffin
2 x 1.5 h	Paraffin

After de-hydration tissue was immediately embedded in fresh paraffin by Thermo Scientific HistoStar^{TM}. Paraffin-embedding tumor tissue were cut in 4 µm sections by microtome and dried on microscope slides overnight in drying cabinet at 37 °C. Samples were stored at RT.

2.2.5.2 Preparation for staining: de-paraffinization and antigen retrieval

To perform tissue staining, paraffin-embedded tissue had to de-paraffinized in decrease alcohol-series as follows.

2 x 5 min	Xylene
2 x 5 min	Ethanol 96 %
1 x 3 min	Ethanol 80 %
1 x 3 min	Ethanol 70 %
1 x 3 min	Ethanol 50 %
1 x 5 min	dmH_2O

For immune histological staining, antigens were demasked by heat induced epitope retrieval (HIER). De-paraffinized tissue was dipped into citrate buffer (pH 6.0) and heated in microwave for 4 x 5 min. Subsequently, slides were immediately cooled down for 30 min before staining.

2.2.5.3 H&E staining

As overview, hematoxylin and eosin staining (H&E) was performed. Therefore, de-paraffinized tissue was dipped in Mayer's hematoxylin into histological glas cuvettes for 3 min, rinsed in tap water for 10 min and counterstained by eosin solution for 1 min. Stained tissue was embedded in Kaiser's glycerin gelatin.

2.2.5.4 MKI67 and cleaved CASP3 immunostaining

De-paraffinized tumor tissue was used and de-masked by HIER before the marker of proliferation KI-67 (MKI67) and apoptotic marker cleaved CASP3 were stained. Therefore, non-specific background staining was reduced by pre-treatment with 2 % H_2O_2 solution. Furthermore, tissue was blocked by 0.5 % BSA for MKI67 and 2 % goat serum for cleaved CASP3 in 1 x TBST for 1 h at RT and stained with primary antibodies MKI67 (1:100) or cleaved CASP3 (1:50) overnight at RT. Then, stained tissue was washed with 1 x PBS three times and was incubated with secondary antibodies diluted in 0.5 % BSA TBST (1:100) for 1 h at RT. After further three washing steps with 1 x PBS, tissues were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water for 10 min before embedding with Kaiser's glycerin gelatin and analyzed by Olympus BX43 microscope. Pictures were made by Olympus cellSens™ 17 software and quantification was performed with Image J software by fix thresholding for area with positive events related to total area in five representative pictures of each sample of each tumor of each group.

2.3 Statistical analyses

All *in vitro* experiments were independently performed thrice, except RT² Profiler™ PCR Array was performed once for each cell line. For all the other experiments, means ± standard deviation (SD) was calculated. Two-way ANOVA was used for calculating significance. Tukey's multiple comparison test was used for significant difference between all groups. All significant results are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.001related to untreated control, non-significant results are displayed as ns. Chosen p-values for group effect are listed in supplementary data section (Sec. 6). For mice experiments, two-way ANOVA and Bonferroni correction was used for determination of significance as well as differences between all xenograft groups.

For synergistic effect, combination index (CI) of Bliss-independence model was calculated. Synergism (CI<1), additive effect (CI=1), antagonism (CI>1) [167]. Therefore, following formula was used:

$$E_{a,b}[\%] = \frac{apoptotic\ cells_{\ treated} - apoptotic\ cells_{\ untreated}}{100}$$

$$CI = \frac{E_a + E_b + \ldots + E_n - E_a * E_b - E_a * E_n - E_b * E_n - \ldots - E_a * E_b * \ldots * E_n}{E_{ab\ldots n}}$$

Formula 4 Calculation of CI of two or more compounds after Bliss-independence model: Ea: effect of substance a, Eb: effect of substance b, Eab: combinatory effect of substance a and substance b, CI: combination index.

3. Results

3.1 Viscum, TT and viscumTT lead to morphological changes and inhibit proliferation

For a first overview, cells were evaluated under the light microscope after viscum, TT and viscumTT treatment. After 24 h incubation time untreated controls revealed 100 % confluency in each cell line. Already morphological changes and reduced cell numbers were observed after viscum, TT and viscumTT treatment in tumor cells (Figure 8 a, b, c), but not in VH7 healthy foreskin fibroblasts (Sec. 6.1, Figure S1a). Also differences in sensitivity towards the extracts were obvious. Therefore, U2OS (*TP53* wild-type) and 143B (*TP53* mutant) cells seemed to be more sensitive to viscum than Saos-2 (*TP53* null-mutant) and observations to TT were similar in each cell line. However, in all three cell lines, stronger effectiveness of viscumTT was suggested in microscopic consideration and is illustrated as an example in Figure 8a-c.

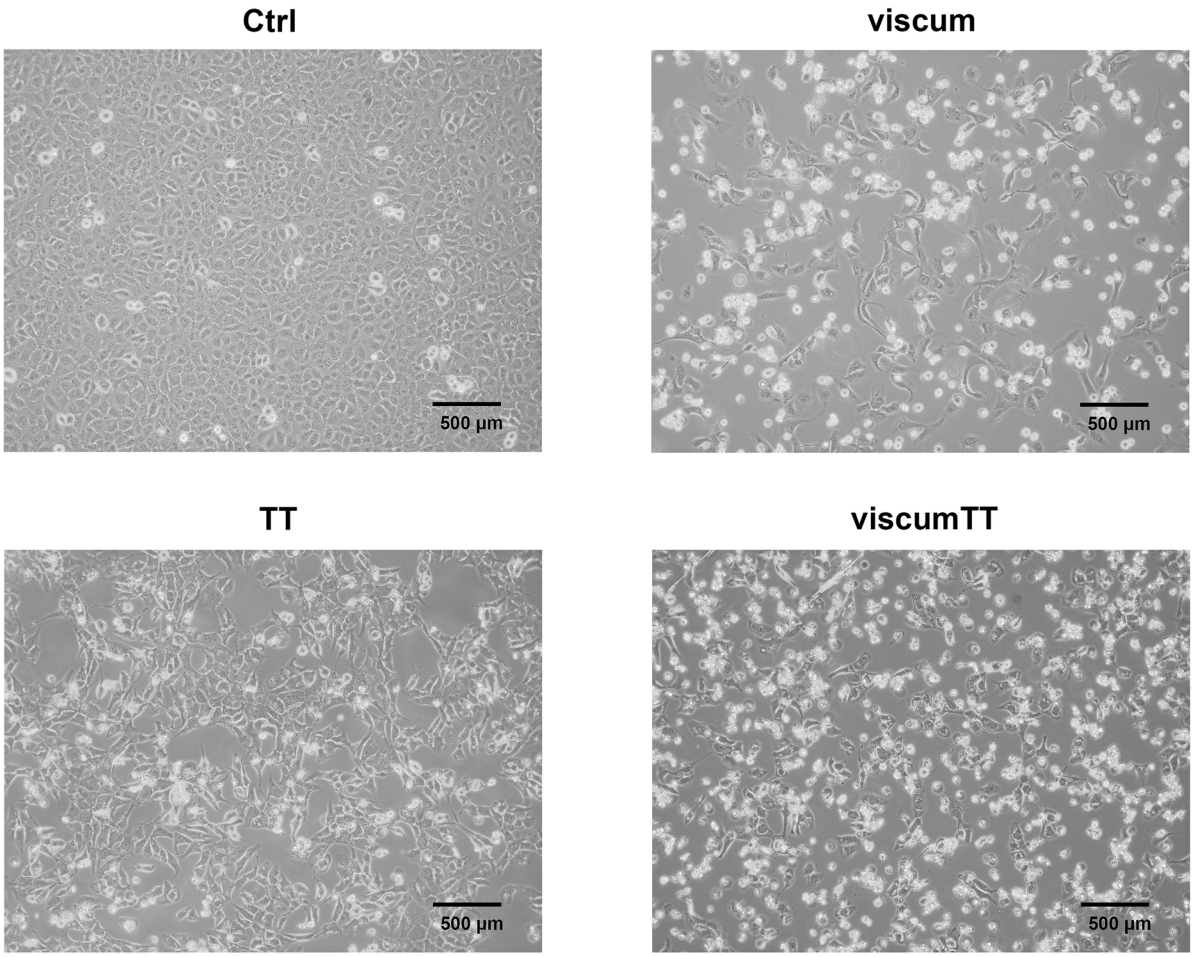


Figure 8a Morphological changes in U2OS cells. Cells were evaluated under the light microscope. Represented are viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (10 ng/mL+60 µg/mL) as an example after 24 h of treatment.

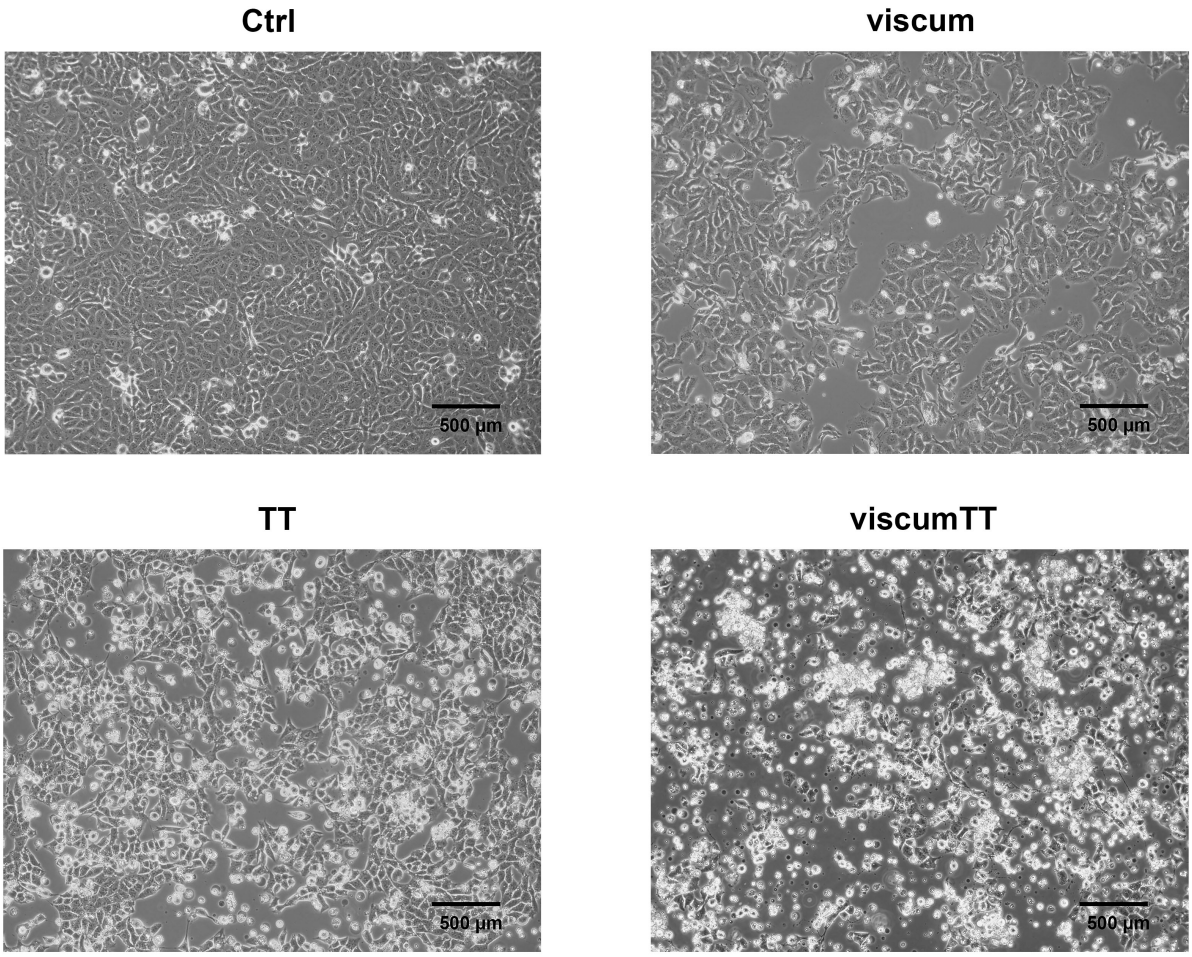


Figure 8b Morphological changes in 143B cells. After 24 h of viscum, TT and viscumTT treatment, cells were evaluated under the light microscope. Pictures illustrate viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (10 ng/mL+60 µg/mL) as an example.

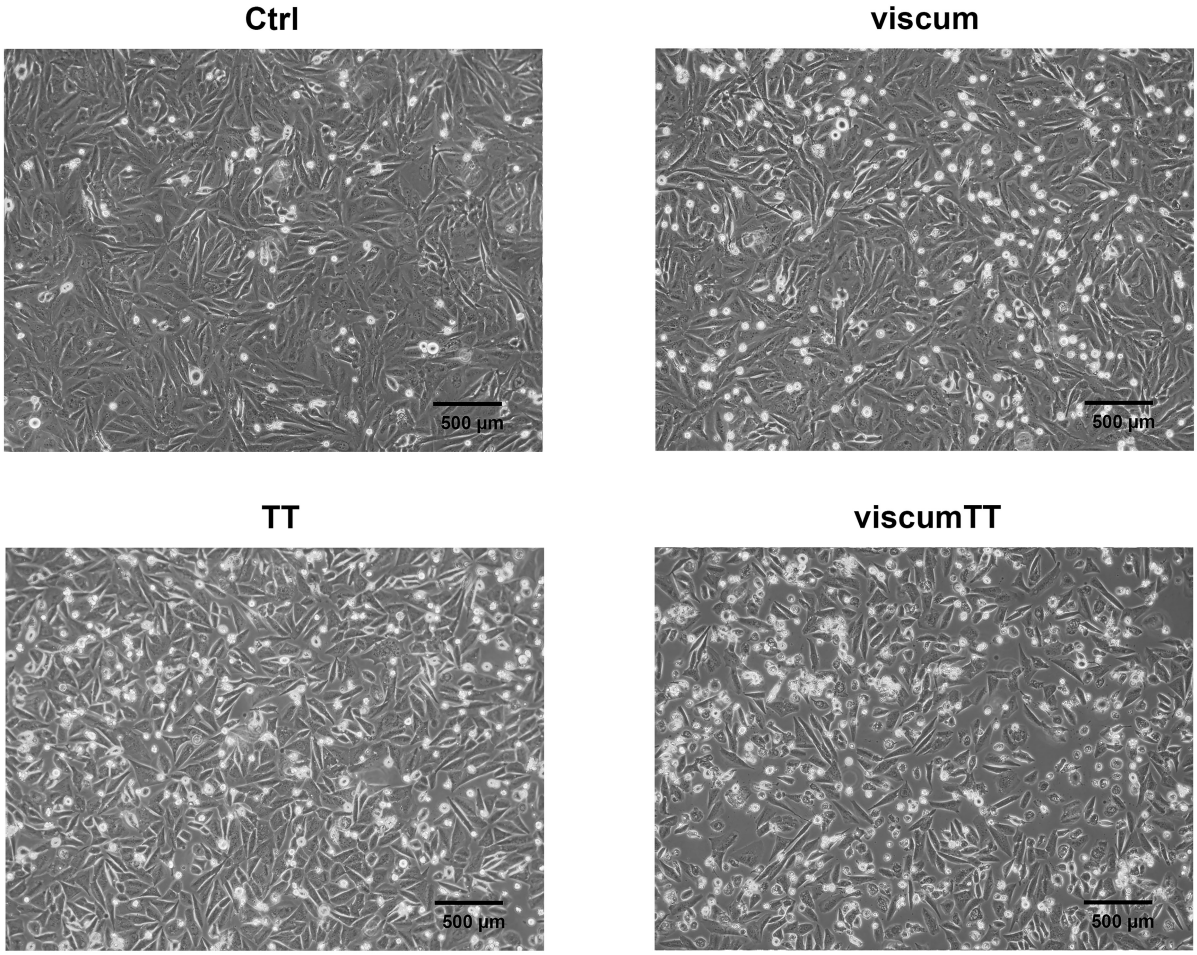


Figure 8c Morphological changes in Saos-2 cells. After the treatment, cells were evaluated under the light microscope. Images demonstrate viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (10 ng/mL+60 µg/mL) after 24 h as an example.

To investigate whether the extracts led to inhibition of proliferation, cells were incubated with viscum, TT and viscumTT in rising concentrations for 24 h and analyzed by WST-1 proliferation assay. In all three cell lines a dose-dependent inhibition of proliferation was observed. However, *TP53* wild-type U2OS as well as *TP53* mutant 143B cells were more sensitive in lower concentrations to viscum than *TP53* null-mutant Saos-2 cells and confirmed microscopic evaluation (Figure 9). On the other hand, TT was more effective in U2OS and Saos-2 cells than in 143B cells. In higher concentrations each single extract revealed to be significant in each cell line. No changes were demonstrated in VH7 cells for the single extracts (Sec. 6.1, Figure S1b, left).

Interestingly, viscumTT led to an equally enhanced inhibition of proliferation in all three osteosarcoma cell lines (Figure 9), which corresponded to the observations under the light microscope. ViscumTT-mediated effect was proved as significant already in the lowest combination of concentrations, but not in healthy fibroblasts (Figure S1b, left). Synergistic effect for viscumTT was pointed out as additive (CI=1) or synergistic (CI<1) and was seen in

each cell line at each combination of concentrations (Table 2). For healthy fibroblasts (VH7 cells) neither additive nor synergistic effect was observed for inhibition of proliferation. Taken together, viscum, TT and viscumTT initiated inhibition of proliferation in tumor cells and effectiveness of the single extracts (viscum, TT) seemed to be influenced by *TP53* status of the cells. Interestingly, efficacy of viscumTT was the same in each cell line.

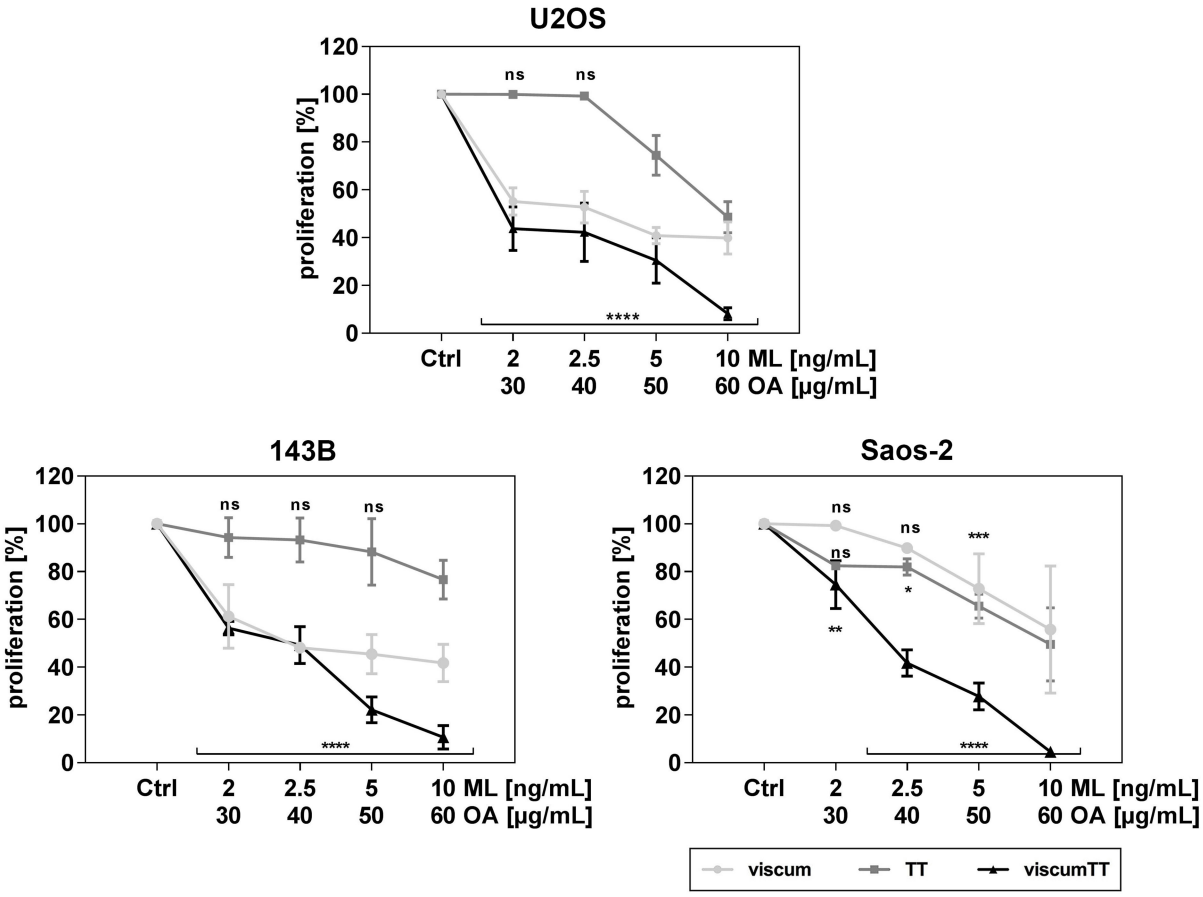


Figure 9 ViscumTT synergistically inhibits proliferation. U2OS, 143B, and Saos-2 cells were treated with viscum, TT and viscumTT in rising concentrations for 24 h and inhibition of proliferation was measured by WST-1 assay. The results are presented as percentage of untreated control (Ctrl). Ctrl was set to 100 %. Mean ± SD of at least three independent experiments are displayed. Significant results are indicated: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to Ctrl, ns: non-significant.

Table 2 Combination index (CI) of viscumTT for inhibition of proliferation.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Cell line	viscumTT (ML ng/mL+OA µg/mL)			
	2+30	**2.5+40**	**5+50**	**10+60**
U2OS	0.80	0.82	1.00	0.88
143B	0.97	1.08	0.77	0.76
Saos-2	0.59	0.45	0.80	0.86

3.2 ViscumTT induces stronger apoptosis than its single extracts

Whether viscum, TT and viscumTT were also able to induce apoptosis, Annexin V/PI staining was performed. To exclude necrosis, lactate dehydrogenase (LDH) release was measured in the supernatant of 143B and Saos-2 cells, but no early toxicity was seen [168]. For detection of apoptosis, cells were treated with increasing concentrations with each extract for 24 h. The single components of TT, OA and BA, were analyzed separately and results are demonstrated and discussed in supplementary data section (Sec. 6.2, Figure S2 a, b).

In each cell line a dose-dependent induction of apoptosis was observed after the treatment with viscum, TT and viscumTT. Moreover, at the highest viscum concentration (ML 10 ng/mL) U2OS (*TP53* wild-type) cells were more sensitive to viscum (up to 50 % apoptotic) in comparison to 143B (*TP53* mutant, 16 %) and Saos-2 (*TP53* null-mutant, 20 %) cells (Figure 10). Additionally, U2OS and Saos-2 cells were more resistant to TT in lower concentrations than 143B cells. At highest TT concentration (OA 60 µg/mL) cells were approximately 40 % (U2OS, Saos-2) and 60 % (143B) apoptotic (Figure 10). Already both single extracts led to significant effects starting from lowest or mean concentration cell line-dependent. No significant induction of apoptosis was observed in healthy fibroblasts (VH7 cells, Figure S1b, right).

ViscumTT showed independent from the cell line an equal, significant and dose-dependent increase of apoptotic cells. At highest concentration (ML 10 [ng/mL]+OA 60 [µg/mL]) all three cell lines were apoptotic by 77 to 87 %. VH7 cells showed only a weak significant induction of apoptosis (15 %, Figure S1b, right). Each viscumTT concentration revealed to be synergistic (CI<1) and is depicted in Table 3. To conclude the results, viscum and TT led to different apoptotic responses, whereas viscumTT initiated an equally induction of apoptosis in every tested cell line.

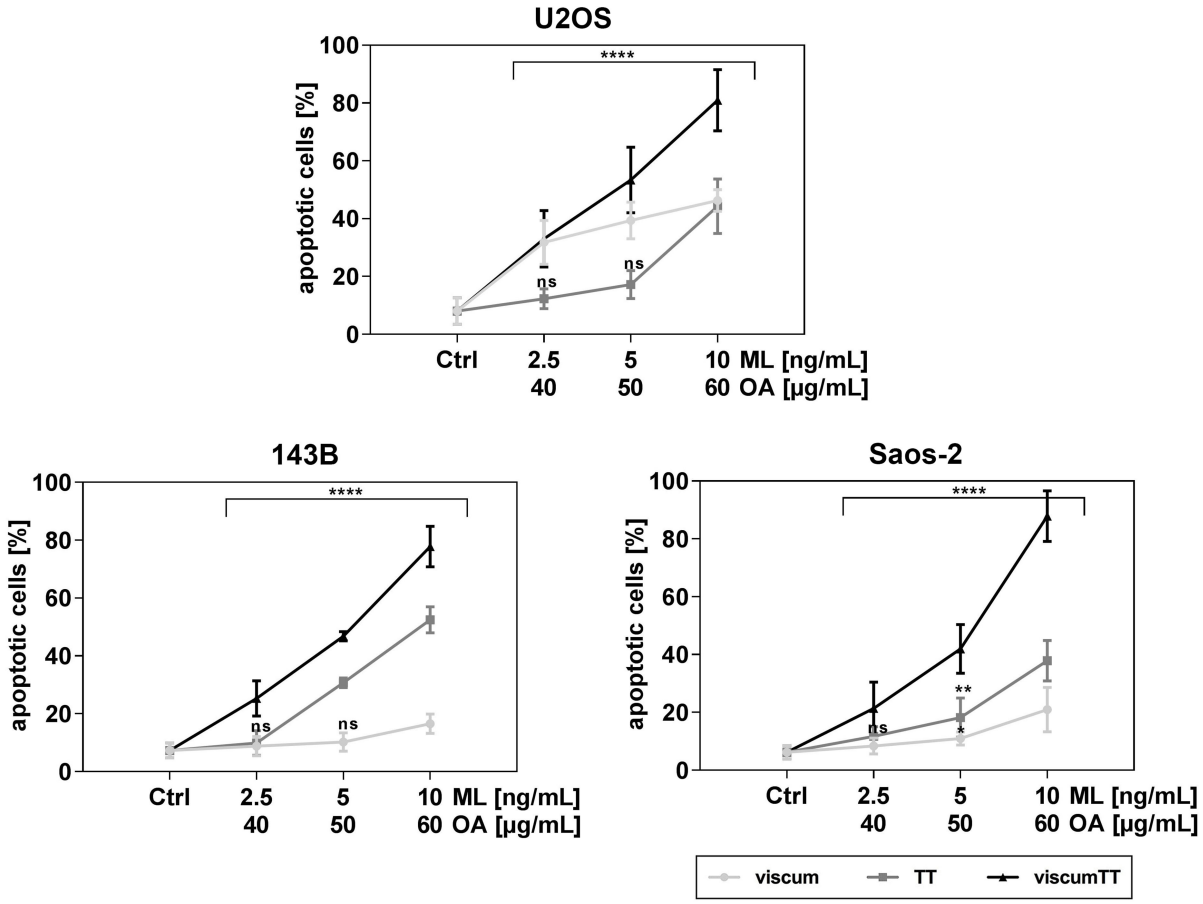


Figure 10 Viscum, TT and viscumTT induce apoptosis. Cells were incubated with viscum, TT and viscumTT in rising concentrations for 24 h, stained with Annexin V/PI and analyzed by flow cytometry. Mean of apoptotic cells are presented in percentage ± SD of at least three independent experiments. Significant results are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to untreated cells (Ctrl), ns: non-significant.

Table 3 Combination index (CI) of viscumTT for induction of apoptosis.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Cell line	viscumTT (ML ng/mL+OA µg/mL)		
	2.5+40	5+50	10+60
U2OS	0.95	0.86	0.81
143B	0.22	0.65	0.71
Saos-2	0.90	0.65	0.58

To confirm induction of apoptosis cells were incubated with viscum, TT as well as viscumTT and whole cell lysates were analyzed by western blotting. Activation of CASP3 and cleavage of PARP were used as classical apoptotic markers after 24 h of treatment with highest concentration of viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean concentration of viscumTT (ML 5 ng/mL+OA 50 µg/mL). In each cell line decrease of proCASP3 and cleaved PARP were detected (Figure 11). U2OS and 143B cells showed no active CASP3 and slight

cleavage of PARP in comparison to Saos-2 cells. Nevertheless, protein levels supported observed induction of apoptosis.

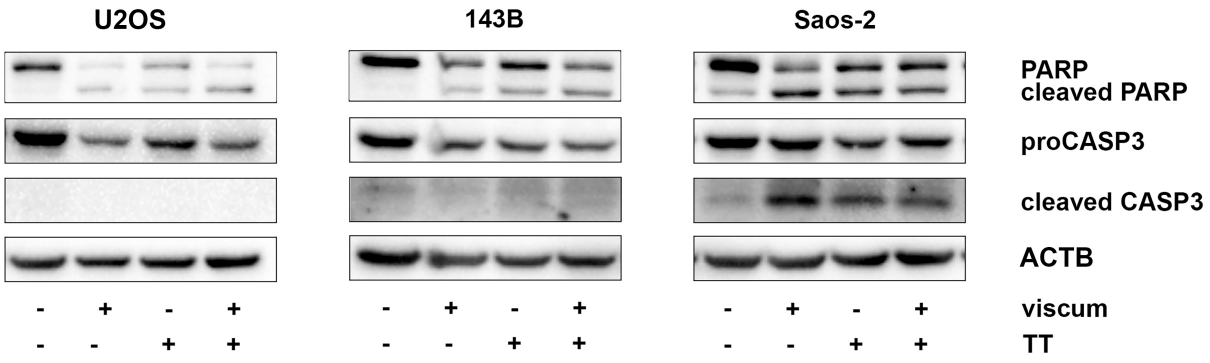


Figure 11 Western blots confirm induction of apoptosis. U2OS, 143B, and Saos-2 cells were treated with viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (ML 5 ng/mL+OA 50 µg/mL) for 24 h. Protein expression was analyzed in whole cell lysates using western blotting. Representative blots are shown from three independent experiments. ACTB was used as loading control.

3.3 Viscum, TT and viscumTT alter TP53 protein expression in wild-type and mutant cells

Regarding *TP53* status of the cells, alterations of protein level were analyzed by western blotting. Viscum, TT and viscumTT almost completely down-regulated wild type TP53 in U2OS cells whereby the level of mutant TP53 in 143B cells were distinctively reduced after viscum and viscumTT, but not after TT treatment. Null-mutant Saos-2 cells showed no TP53 protein expression (Figure 12).

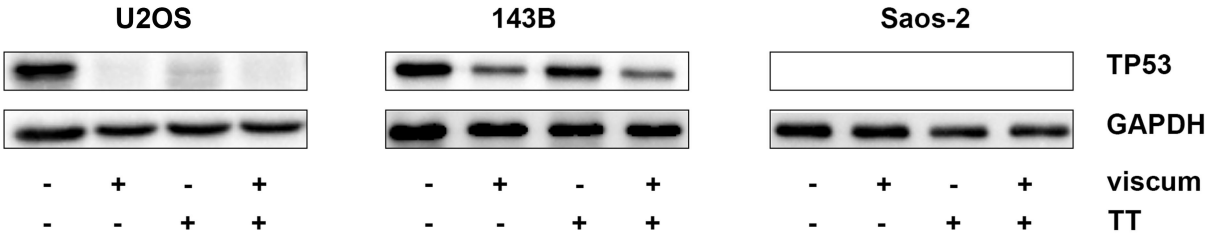


Figure 12 Down-regulation of TP53 protein expression after visum, TT and viscumTT treatment. Blots demonstrate TP53 protein level after the treatment with viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (5 ng/mL+50 µg/mL). Representative blots are shown from three independent experiments. GAPDH was used as loading control.

3.4 Viscum, TT and viscumTT facilitate induction of apoptosis of chemotherapeutic drugs

It is already known that conventional *Viscum album* preparations are able to improve the effect of standard chemotherapeutic drugs. In order to investigate enhanced induction of apoptosis, cell lines were in additional to viscum, TT and viscumTT co-treated with Doxo, VP16 or 4-OOH in lower rising concentrations for 48 h.

0.05 µg/mL Doxo in combination with viscum, TT and viscumTT showed a slightly additional apoptotic effect in U2OS cells (5-10 %, Figure 13a). Each co-treatment was significant to untreated control, but also a significant combinatory effect within the groups was revealed for viscumTT (ML 2 ng/mL+30 µg/mL) co-treated with Doxo ($p \leq 0.05$, Sec. 6.3, Table S1a). In all, a weaker effect was shown, when cells were co-treated with VP16 (0.5 µg/mL) and within the groups no significance was found. Furthermore, 0.5 µg/mL 4-OOH in combination with viscum, TT and viscumTT led dose-dependently to 5-15 % higher induction of apoptosis when compared to each single treatment. Here a significant group effect was evaluated for viscumTT (ML 2 ng/mL+OA 30 µg/mL) and 4-OOH ($p \leq 0.001$) and induction of apoptosis was approximately 25 % enhanced (Figure 13a). Additionally, several of the co-treatments were calculated even as synergistic (CI<1, Table 4a).

In 143B cells 0.01 µg/mL Doxo induced 29 % apoptosis, thus they were the most sensitive cells to Doxo compared to the other cell lines. In comparison to each single treatment, induction of apoptosis was enhanced up to 30 % for viscum, TT and Doxo as well as up to 43 % for viscumTT and Doxo (Figure 13b). Furthermore, 0.05 µg/mL VP16 led only to 9 % apoptotic cells, but an increase was seen for viscum, TT and viscumTT co-treated with VP16 (up to 28 %, 40 %, 35 %, respectively and dose-dependent). Moreover, in both treatments the number of apoptotic cells increased in a dose-dependent manner and combinatory effect revealed significance to control as well as within some co-treated groups ($p \leq 0.01$, $p \leq 0.0001$, Table S1b). On the other hand, such additional effect was not seen for co-treatment with 4-OOH. Here, no significant group effect was calculated, but in highest TT (OA 40 µg/mL) and viscumTT (ML 2.5+OA 40 µg/mL) concentration addition of 4-OOH led to 15 % increase of apoptosis. A synergistic effect (CI<1) was evaluated for Doxo, VP16 and 4-OOH in nearly each combination of concentrations with viscum and TT and in individual ones with viscumTT (Table 4b).

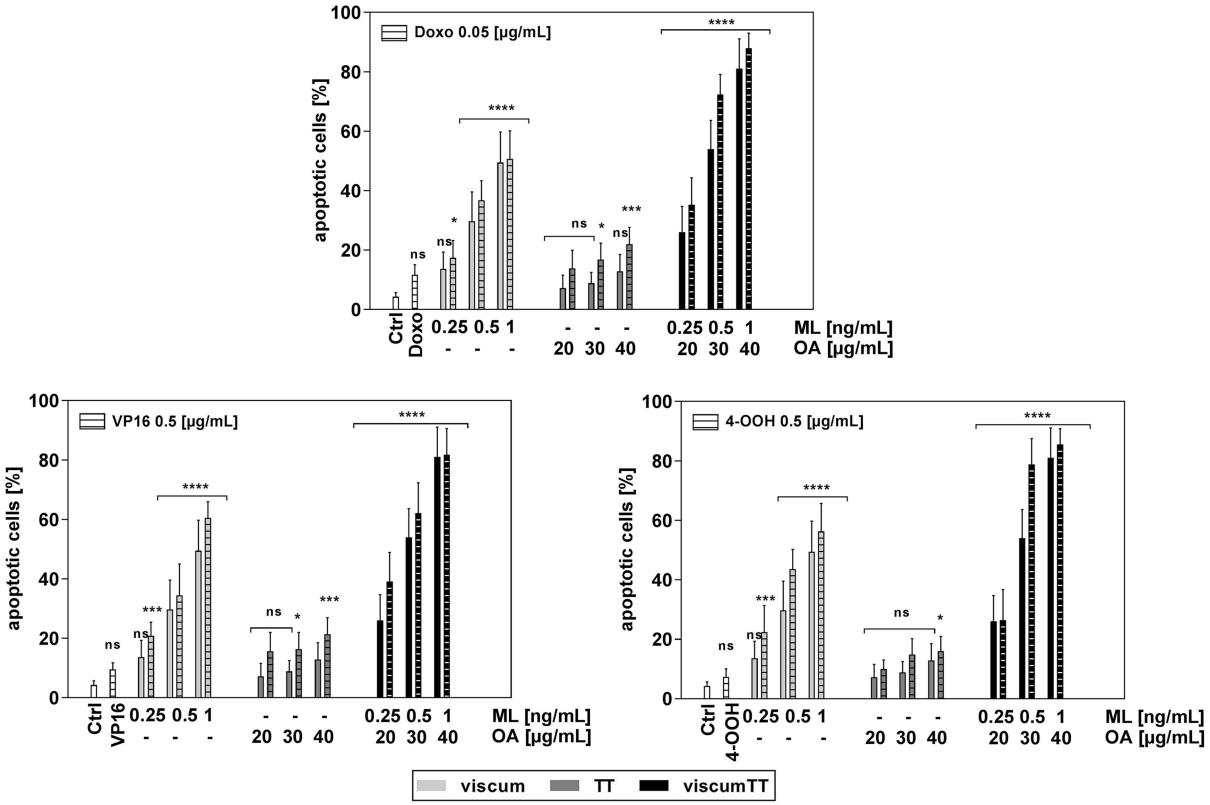


Figure 13a Viscum, TT and viscumTT co-treated with Doxo, VP16 and 4-OOH enhance induction of apoptosis in U2OS cells. Viscum, TT and viscumTT-treated cells were co-treated with Doxo, VP16 and 4-OOH for 48 h. Apoptotic cells were stained with Annexin V/PI and analyzed by flow cytometry. Mean percentage of apoptotic cells ± SD are displayed of at least three independent experiments. Significant results are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to untreated control (Ctrl), ns: non-significant.

Table 4a Combination index (CI) of viscum, TT and viscumTT co-treated with chemotherapeutic drugs for induction of apoptosis in U2OS cells.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Drug	viscum (ML ng/mL)			TT (OA µg/mL)			viscumTT		
(µg/mL)	0.25	0.5	1	20	30	40	0.25+20	0.5+30	1+40
							0.60	0.60	0.66
Doxo 0.05	1.21	0.95	1.06	1.04	0.91	0.85	0.65	0.59	0.81
VP16 0.5	0.85	0.98	0.86	0.52	0.72	0.80	0.52	0.65	0.84
4-OOH 0.5	0.81	0.76	0.93	1.31	0.93	1.12	0.97	0.57	0.86

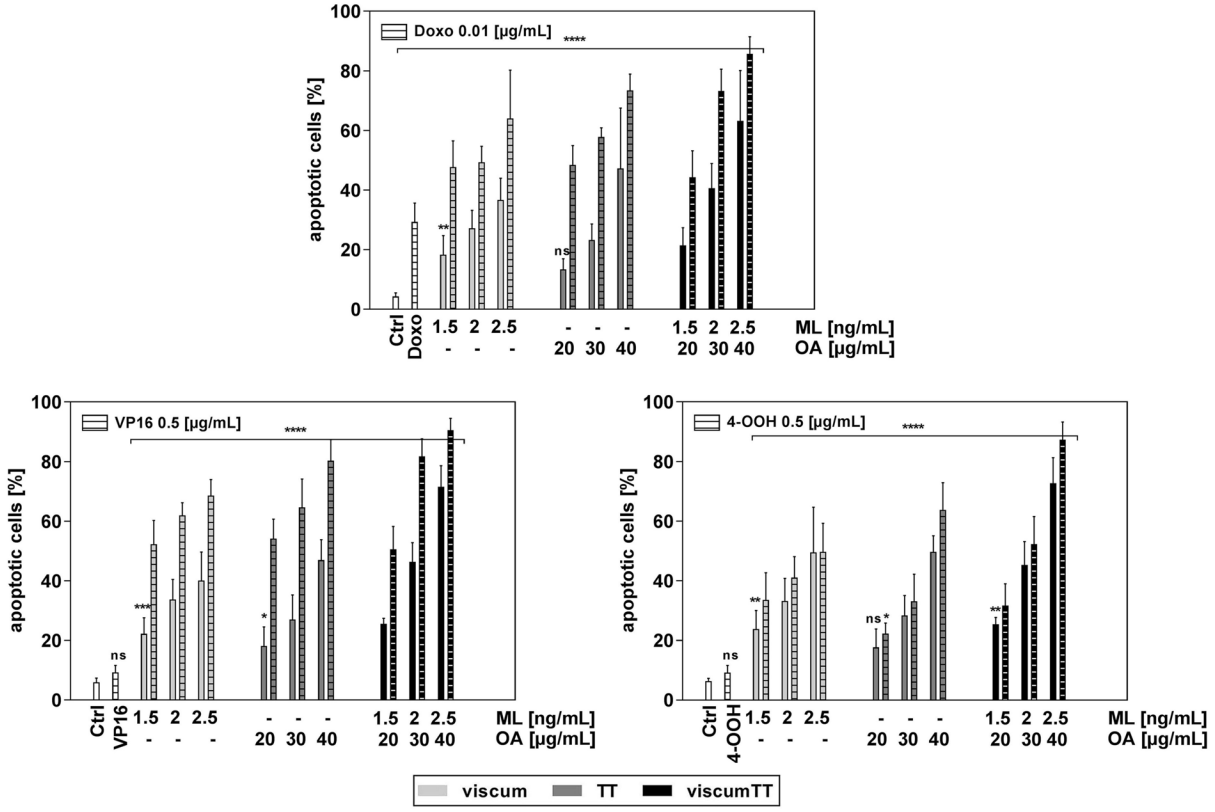


Figure 13b Co-treatment with Doxo and VP16 strongly increase apoptotic response in 143B cells. 143B cells were incubated with viscum, TT and viscumTT in low rising concentrations and co-treated with Doxo, VP16 and 4-OOH for 48 h. Induction of apoptosis was analyzed by Annexin V/PI staining and flow cytometry. Graphs display mean percentage of apoptotic cells ± SD of at least three independent experiments. Significant results are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to untreated control, ns: non-significant.

Table 4b Combination index (CI) of viscum, TT and viscumTT co-treated with chemotherapeutic drugs for induction of apoptosis in 143B cells.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Drug	viscum (ML ng/mL)			TT (OA µg/mL)			viscumTT		
(µg/mL)	0.25	0.50	1.00	20	30	40	0.25+20	0.5+30	1+40
							1.34	1.06	1.01
Doxo 0.01	0.82	0.93	0.90	0.72	0.73	0.76	1.27	1.04	1.28
VP16 0.5	0.41	0.54	0.58	0.31	0.40	0.58	0.76	0.77	1.09
4-OOH 0.5	0.73	0.84	1.03	0.87	0.91	0.78	1.33	1.26	1.34

Contrary to the other cell lines, Saos-2 cells were in all more resistant to TT and viscumTT treatment in low concentrations, but a moderate dose-dependent induction of apoptosis was still observed. Especially towards viscum treatment, cells demonstrated a prolonged resistance also after 48 h of incubation (Figure 13c). However, Saos-2 cells showed a better apoptotic response to Doxo than U2OS and to VP16 as well as 4-OOH compared to both other tested cell lines. Nevertheless, only in individual combinations of concentrations slightly additional effects were seen for TT and viscumTT when combined with Doxo or VP16 (up to 5 %, 10 %, respectively) and referred to be significant within the treatment groups regarding

to viscum, TT and viscumTT (p≤0.01, Sec. 6.3, Table S1c). Such weak, but significant enhanced apoptosis was also observed for TT in lowest concentration (OA 20 µg/mL) co-treated with 4-OOH (p≤0.001, CI<1), but not for viscum or viscumTT (Figure 13c, Table 4c). Taken these co-treatment experiments together, cell lines showed differences in apoptotic response depending on chemotherapeutic drug as well as administered concentration. 143B cells were the most sensitive ones followed by U2OS and Saos-2 cells regarding to Doxo and VP16 co-treatment. Additionally, the best enhanced apoptotic effect for co-treatment was also observed for 143B cells. However, demonstrated results indicated good combinatory effectiveness for viscum, TT and viscumTT and tested chemotherapeutic drugs.

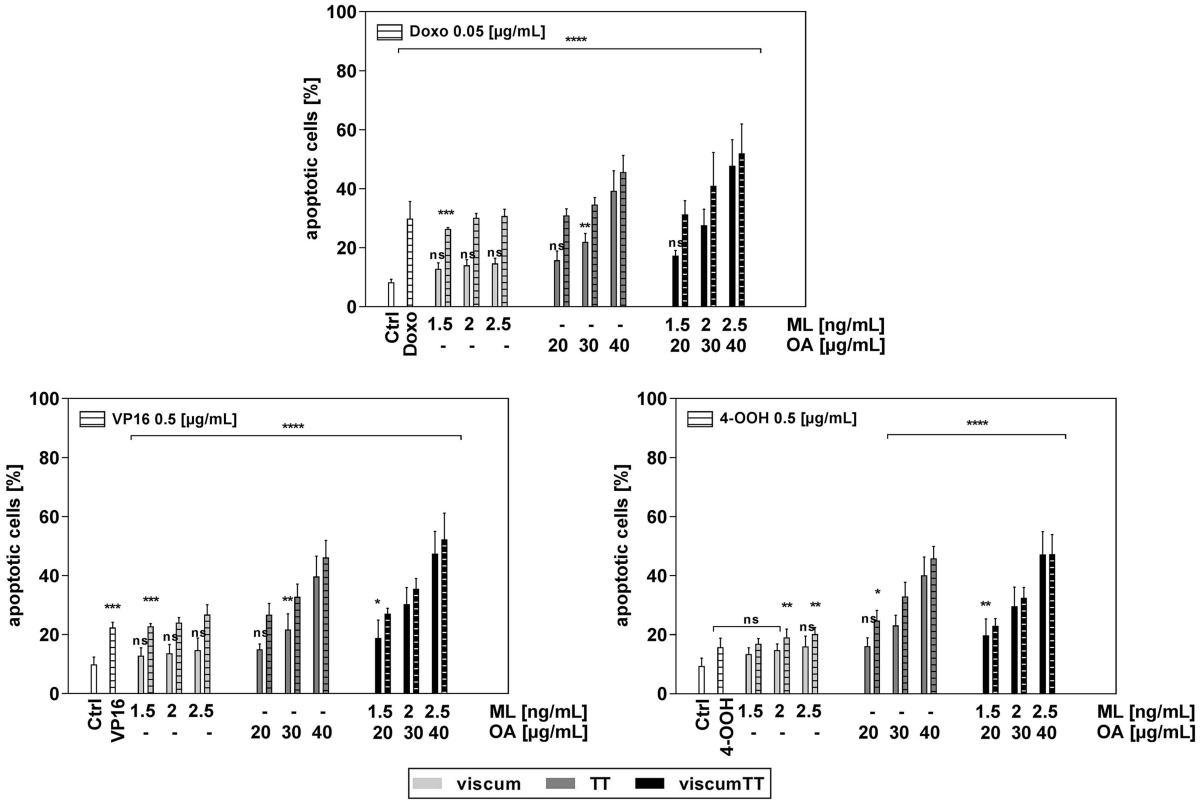


Figure 13c Viscum, TT and viscumTT co-treated with Doxo, VP16 and 4-OOH slightly increase apoptosis in Saos-2 cells. Cells were incubated with Doxo, VP16 and 4-OOH for 48 h, stained with Annexin V/PI and analyzed by flow cytometry. Mean percentage of apoptotic cells ± SD are illustrated of at least three independent experiments. Significant results are proved as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to untreated control (Ctrl), ns: non-significant.

Table 4c Combination index (CI) of viscum, TT and viscumTT co-treated with chemotherapeutic drugs for induction of apoptosis in Saos-2 cells.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Drug	viscum (ML ng/mL)			TT OA (µg/mL)			viscumTT		
(µg/mL)	0.25	0.50	1.00	20	30	40	0.25+20	0.5+30	1+40
							1.07	0.87	0.90
Doxo 0.05	1.39	1.19	1.18	1.21	1.23	1.23	1.45	1.23	1.27
VP16 0.5	1.17	1.12	0.99	1.01	1.00	1.06	1.20	1.08	1.07
4-OOH 0.5	1.34	1.17	1.16	0.82	0.81	0.96	1.26	1.10	1.17

3.5 ViscumTT depolarizes mitochondria membrane potential

To get more insights into the apoptotic action of viscumTT, ΔΨm was measured by JC-1 staining with following flow cytometry analysis relative to untreated control cells. All three cell lines were treated with viscum, TT and viscumTT in rising concentrations for 24 h. CCCP, a mitochondrial membrane disrupter, was used as positive control. Single treatment of viscum as well as TT significantly dose-dependently induced loss of ΔΨm in U2OS (up to 47, 49 %, respectively) and Saos-2 cells (up to 30, 39.7 %, respectively) (Figure 14). In 143B cells effect on mitochondria was less (up to 15 %) for each single treatment.

ViscumTT enhanced depolarization in all three cell lines and approximately 80 % of U2OS and Saos-2 cells lost their ΔΨm at the highest concentration. A synergistic effect (CI<1) was reached for Saos-2 and 143B for each and for U2OS cells at the highest concentrations (Figure 14, Table 5). These results indicated an involvement of intrinsic apoptosis pathway in mechanism of apoptotic action of viscumTT and validated synergistic induction of apoptosis.

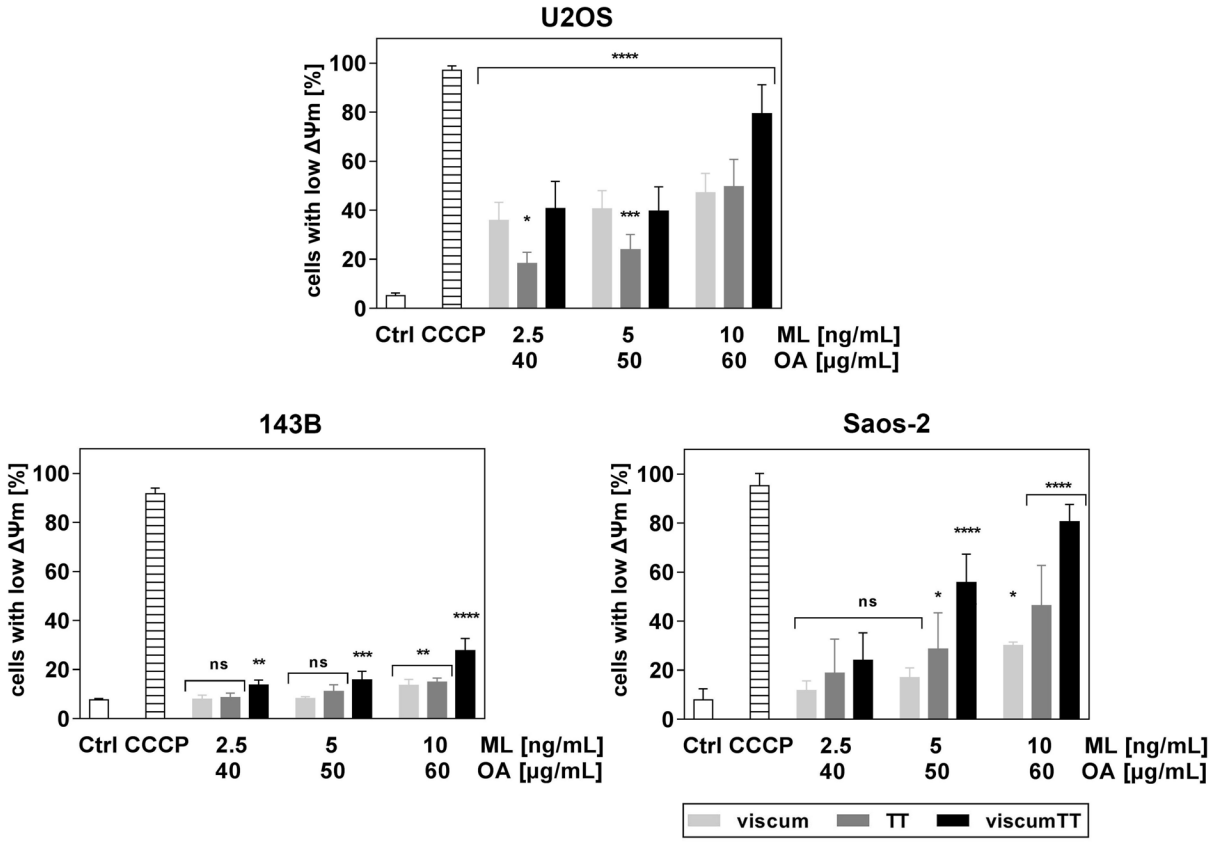


Figure 14 ViscumTT induces apoptosis via depolarization of mitochondrial membrane potential in U2OS, 143B, and Saos-2 cells. Cells were incubated with increasing concentrations of viscum, TT and viscumTT and loss of mitochondrial membrane potential (ΔΨm) was measured by JC-1 staining and flow cytometry. CCCP, a mitochondria disrupter, was used as positive control. Bars show the mean percentage of cells with low ΔΨm ± SD of at least three independent experiments. Significant results are indicated: *p≤0.05, **p≤0.01, ***p≤0.0001, ****p≤0.001 related to untreated control (Ctrl), ns: non-significant.

Table 5 Combination index (CI) of viscumTT for loss of mitochondrial membrane potential.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Cell line	viscumTT (ML ng/mL+OA µg/mL)		
	2.5+40	5+50	10+60
U2OS	1.12	1.38	0.91
143B	0.10	0.49	0.63
Saos-2	0.49	0.46	0.65

3.6 ViscumTT activates CASP8 and CASP9

For further investigations of the apoptotic mechanism, CASP8 and CASP9 activity was analyzed in all three cell lines. Therefore, cells were treated with viscum, TT as well as viscumTT in increasing concentrations for 24 h. After staining with FITC-LEHD-FMK and FITC-IETD-FMK flow cytometry was performed. All three cell lines demonstrated a similar significantly dose-dependent increase of CASP8 activity regarding viscum, whereby the strongest activity was observed in U2OS cells (up to 52 %). TT led also to significant and dose-dependent activation of CASP8, but stronger in Saos-2 (up to 57 %) than in U2OS and 143B cells (Figure 15a). On the other hand, viscumTT equally activated CASP8 in each cell line (up to 77 % in U2OS, 60 % in 143B and 74 % in Saos-2 cells). Additionally, a synergistic effect (C<1) was reached nearly for every concentration of viscumTT (Table 6a). These findings indicated an involvement of the initiator caspase, CASP8, of the extrinsic signaling pathway of apoptosis.

Alike, a dose-dependent increase of CASP9 activity was detected in U2OS cells after viscum, TT and viscumTT treatment (up to 51, 39, 81 %, respectively), whereas in 143B and Saos-2 cells only TT and viscumTT caused such effect (Figure 15a). Furthermore, 143B cells showed no and Saos-2 cells only at highest concentration (ML 10 ng/mL) CASP9 activity after viscum treatment (19.7 %). ViscumTT led to a stronger activation of CASP9 in all three cell lines and effect was calculated as synergistic in almost each concentration (CI<1, Table 6b). With these experiments the involvement of CASP9, the initiator caspase of the intrinsic apoptosis pathway, was elucidated for viscumTT-induced apoptosis and further validated the results of loss of $\Delta\Psi m$.

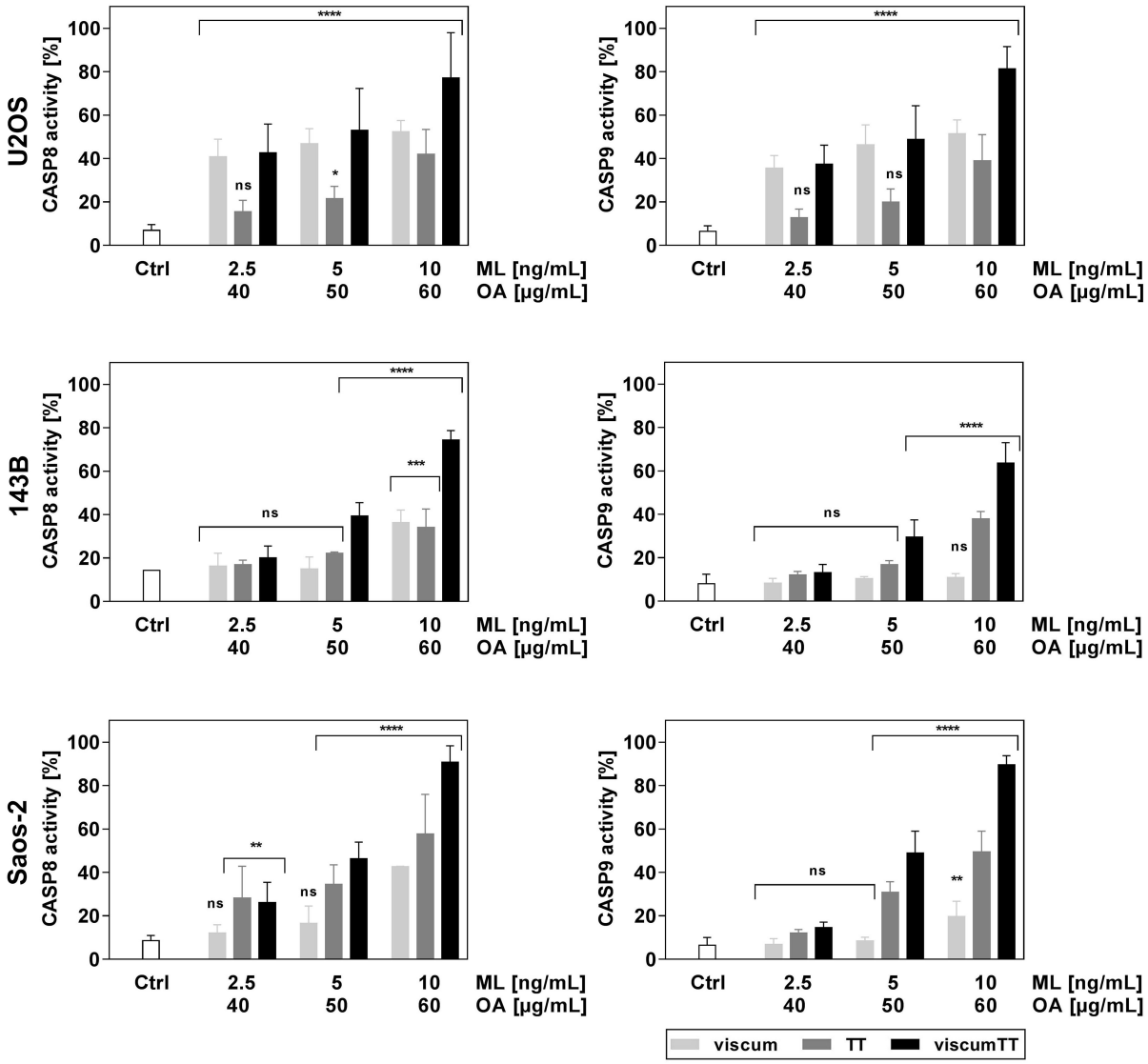


Figure 15a ViscumTT induces apoptosis via CASP8 and CASP9 activation. U2OS, 143B, and Saos-2 cells were treated with rising concentrations of viscum, TT and viscumTT and CASP8 and CASP9 activity was analyzed using FITC-LEHD-FMK and FITC-IETD-FMK staining. Graphs display mean activity ± SD of at least three independent experiments. Significant results are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to untreated control (Ctrl), ns: non-significant.

Table 6a Combination index (CI) of viscumTT for CASP8 activity.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Cell line	viscumTT (ML ng/mL+OA µg/mL)		
	2.5+40	5+50	10+60
U2OS	0.92	0.87	0.87
143B	0.80	0.34	0.63
Saos-2	1.28	0.85	0.81

Table 6b Combination index (CI) of viscumTT for CASP9 activity.
Synergism (CI<1), additive effect (CI=1), antagonism (CI>1)

Cell line	viscumTT (ML ng/mL+OA µg/mL)		
	2.5+40	5+50	10+60
U2OS	1.09	1.19	0.86
143B	0.86	0.51	0.57
Saos-2	0.84	0.65	0.62

To investigate whether the activation of caspases play an important role in viscum, TT and viscumTT-induced apoptosis, 143B and Saos-2 cells were preincubated with zVAD-FMK, a pan-caspase inhibitor, for 1 h. After 24 h of treatment induction of apoptosis was analyzed in absence or presence of zVAD-FMK. In 143B and Saos-2 cells viscum-mediated apoptosis was significantly inhibited by approximately 14 % and TT-induced apoptosis up to 15 % (143B) and 25 % (Saos-2). Moreover, zVAD-FMK prevented viscumTT-mediated apoptosis by 26 % in 143B and 31.3 % in Saos-2 cells (Figure 15b). These results confirmed an essential role of caspases in viscum, TT and viscumTT-induced apoptosis.

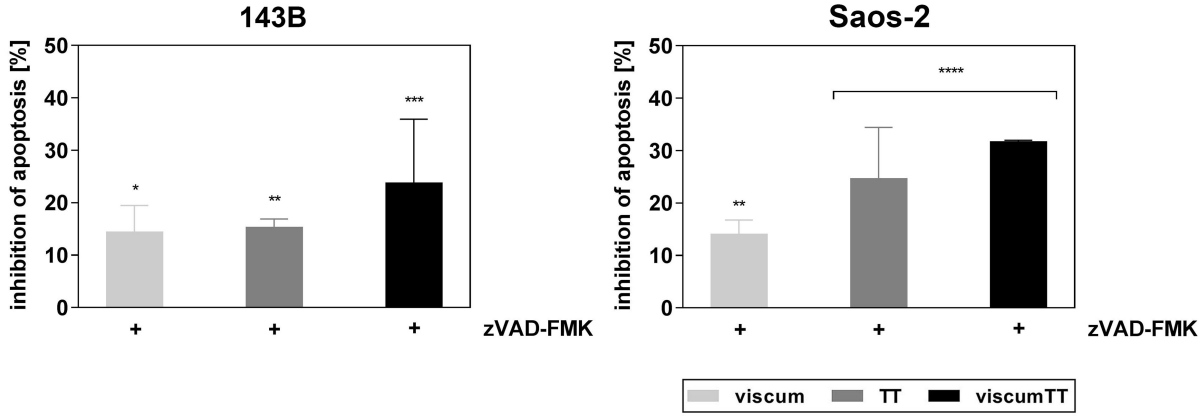


Figure 15b zVAD-FMK pan-caspase inhibitor reduces induction of apoptosis in 143B and Saos-2 cells. 143B and Saos-2 cells were 1 h pretreated with zVAD-FMK and additional incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean concentration of viscumTT (5 ng/mL+50 µg/mL) for 24 h before stained with Annexin V/PI and flow cytometry measurements. Bars represent mean inhibition of apoptosis ± SD of at least three independent experiments. Significant results are displayed as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 between treated cells related to untreated control (Ctrl), ns: non-significant.

3.7 Viscum, TT and viscumTT induce cell cycle arrest cell line-dependent

To analyze whether viscum, TT and viscumTT may have an influence on the cell cycle, synchronized, treated U2OS, 143B, and Saos-2 cells were fixed with ice-cold ethanol after different time points and analyzed for cell cycle distribution by PI staining and flow cytometry. In all three cell lines an influence on the cell cycle was observed after viscum, TT as well as viscumTT treatment. 60-80 % of the cells of each cell line were synchronized in G1 phase at the beginning of the examinations (Figure 16a-c). Untreated U2OS cells (*TP53* wild-type) passed periodically the cell cycle (Figure 16a). Therefore, cells went from G1 to S phase after about 14 h and simultaneously the number of cells increased in G2/M phase. After the treatment with all three extracts, the majority of the cells (approximately 60 %) starting from 9/14 h were not able to progress in S, but rather the cells in G1 phase increased in comparison to control. U2OS cells were significantly arrested in G1 phase after viscum, TT and viscumTT incubation over the whole tested time points (Figure 16a). However, treated cells showed a weak, but not significantly enhanced S phase after 14 h, which indicated a not fully stagnation. A slight increase of number of cells in G2/M phase were observed after 48 h for TT treatment (Figure 16a).

In contrast, synchronized 143B (*TP53* mutant) control cells went into S phase already after 3-6 h and prolonged in this stage around 20 h (Figure 16b). 143B control cells revealed a faster growth rate compared to the other cell lines. After treatment with viscum, TT and viscumTT cells arrested in different phases of the cell cycle. Here, viscum treatment led to prolonged S phase, which was referred as significant after 48 h. On the other hand, TT significantly arrested cells in G1 phase already after 3/6 h, whereas the number of cells in S phase decreased drastically from 18 h (Figure 16b). Incubation with viscumTT showed no clear cell cycle arrest, neither in G1 nor in S phase, but rather an enhanced cell number in both phases was significantly demonstrated after 14 h in G1 and after 48 h in S phase.

The Saos-2 (*TP53* null-mutant) control cells passed the cell cycle similar to U2OS cells. Each extract led to drastic G1 arrest starting from 14 h, which was proved as significant. 20 % of the Saos-2 cells were still able to progress in S phase, but the number was significant lower than in control cells (Figure 16c). Additionally, number of cells in G2/M phase were significant lower to control, but suggested a not complete stagnation of the cell cycle after viscum, TT and viscumTT treatment.

These results showed an influence of viscum, TT and viscumTT to mainly G1 or S phase of the cell cycle depending on cell type, which might be resulting from the TP53 status of the cells. The increase of cell number in G2/M phase in some individual cases indicated that the cell cycle did not undergo complete arrest, but rather a delay in progression.

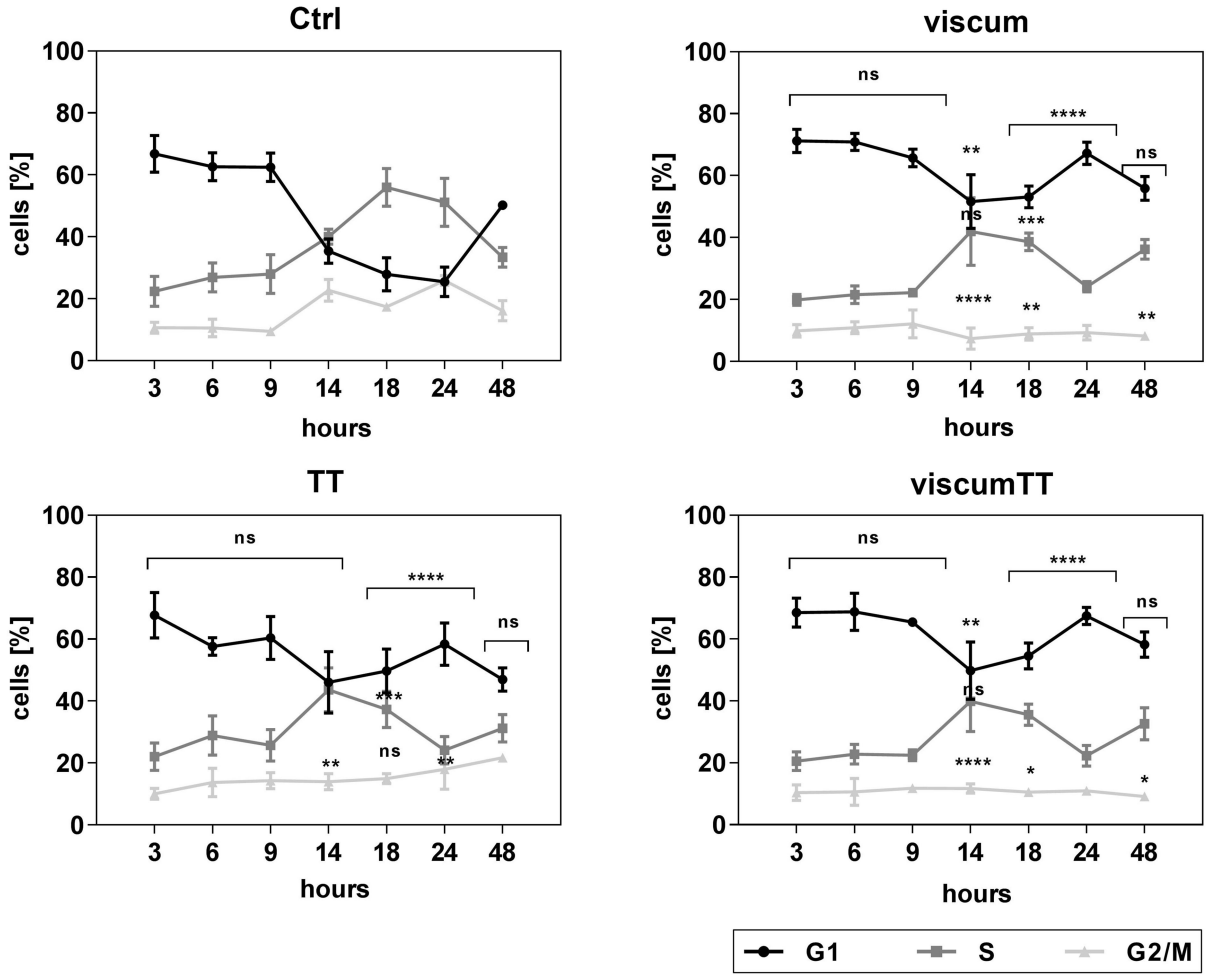


Figure 16a Viscum, TT and viscumTT arrest U2OS cells in G1 phase. Synchronized U2OS cells were treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 3-48 h. Cell cycle analysis was performed by PI staining and flow cytometry. Graphs display mean cells [%] ± SD of at least three independent experiments and their cell cycle progression after treatment over indicated time period. Significant results are revealed as *p≤0.05, **p≤0.01, ***p≤0.0001, ****p≤0.001 related to control (Ctrl), ns: non-significant.

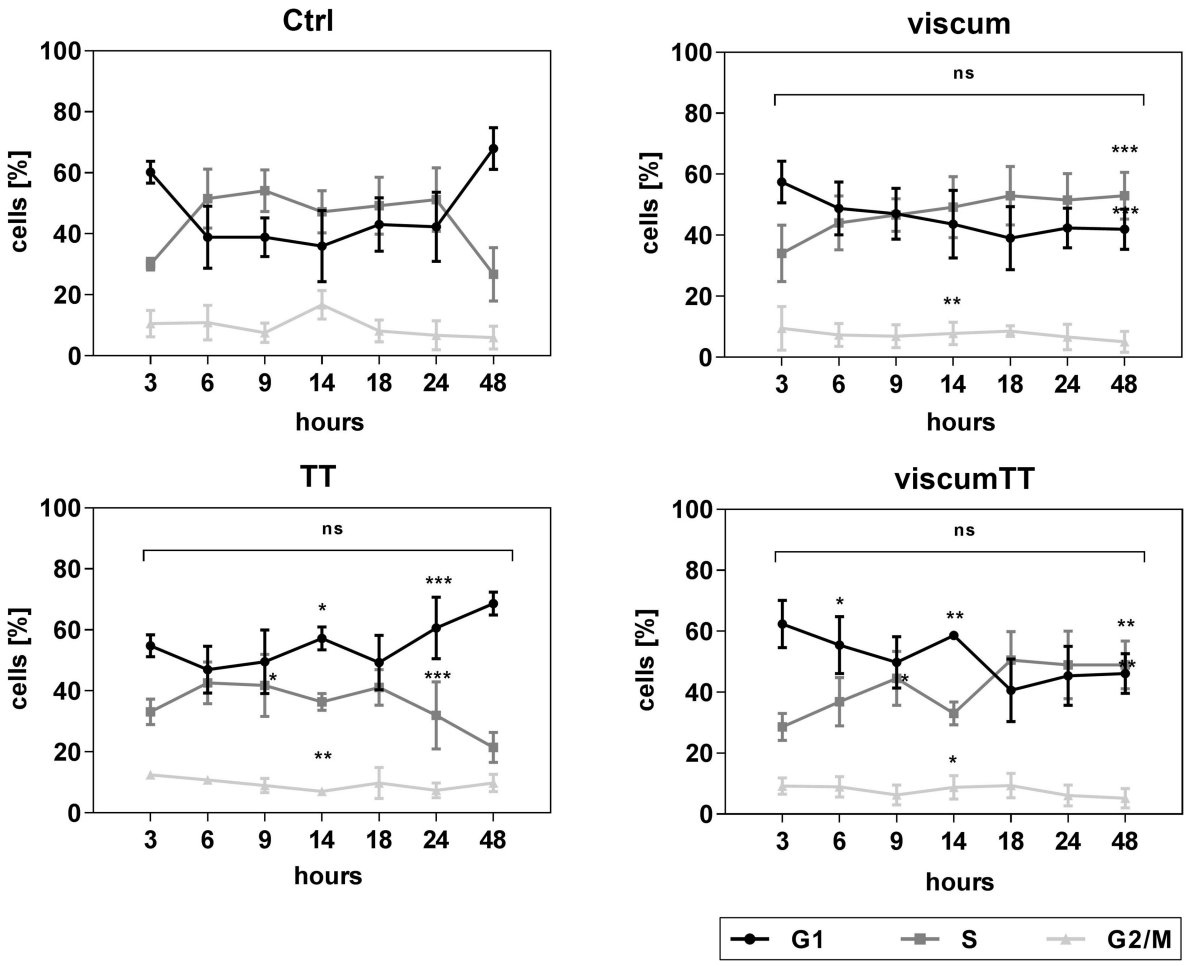


Figure 16b Viscum, TT and viscumTT block cell cycle progression in 143B cells. 143B cells were synchronized and incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for indicated time points. Cell cycle distribution was analyzed by PI staining and flow cytometry. Images illustrate mean cells [%] in cell cycle phase ± SD of at least three independent experiments. Significances are displayed as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control (Ctrl), ns: non-significant.

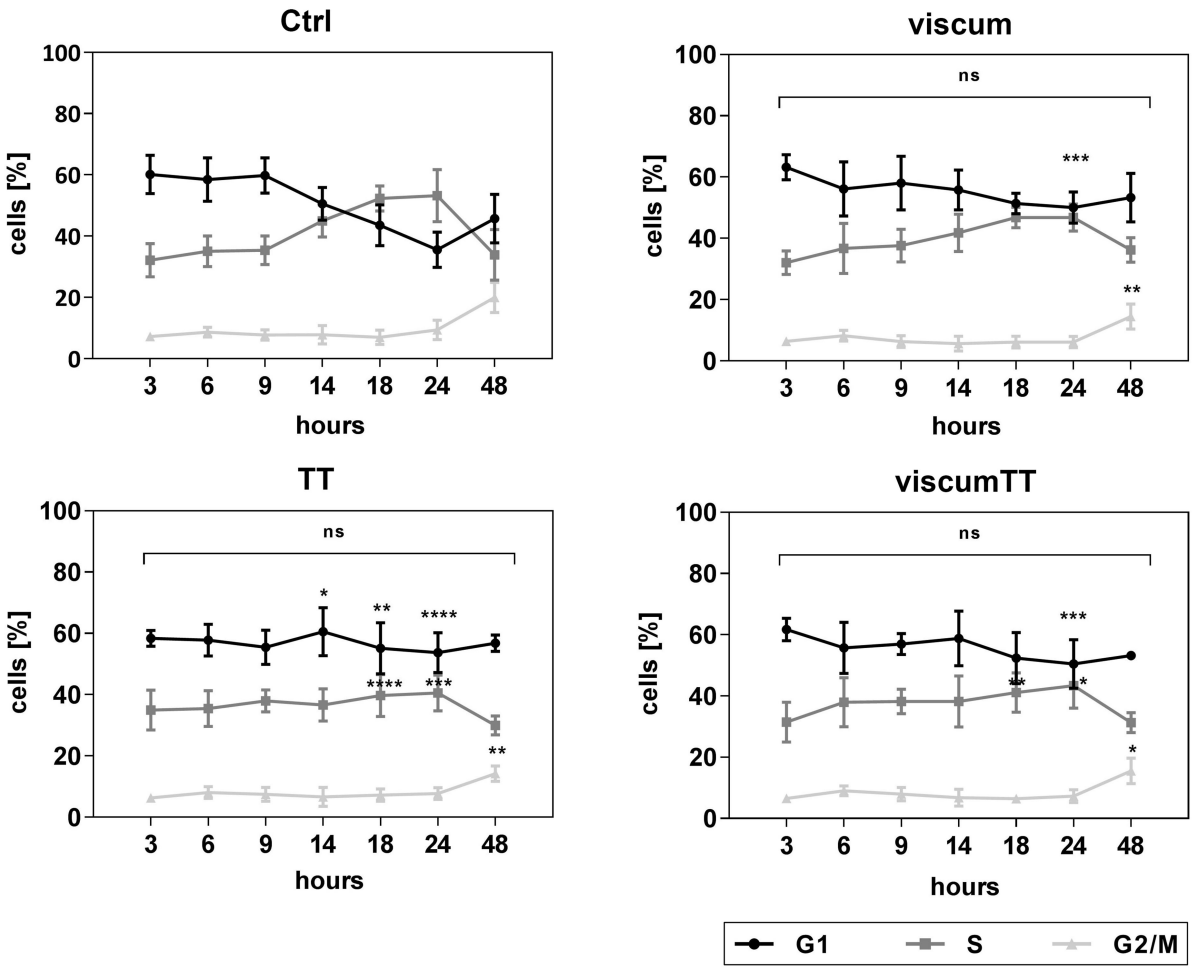


Figure 16c Viscum, TT and viscumTT lead to G1 arrest in Saos-2 cells. Saos-2 cells were synchronized and treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 3-48 h. Cell cycle analyses were performed by PI staining and flow cytometry. Graphs represent mean cells in cell cycle phase [%] ± SD of at least three independent experiments. Significant results are displayed as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control (Ctrl), ns: non-significant.

Cell cycle progression is regulated by CDKs and CCNs. In order to viscum, TT and viscumTT-mediated cell cycle arrest, western blotting were used to detect alterations of different CCNs and CDKs, especially those which are essential in G1 and S phase progression and transition, after 24 h. CDK4 and CCND1 that are required for G1 progression were drastically down-regulated in each tested cell line independent from the treatment. Protein levels of CDK2 and CCNE that are mainly involved in G1/S transition were distinctively reduced in U2OS and 143B cells after viscum, TT and viscumTT incubation, whereas Saos-2 cells still showed more expressed CCNE. CCNA, which in complex with CDK2, is essential for S phase progression, was also strongly down-regulated after each treatment (Figure 17). Reduction of these proteins confirmed the observed influence on G1 and S phase by the extracts and corroborated the block of cell cycle progression.

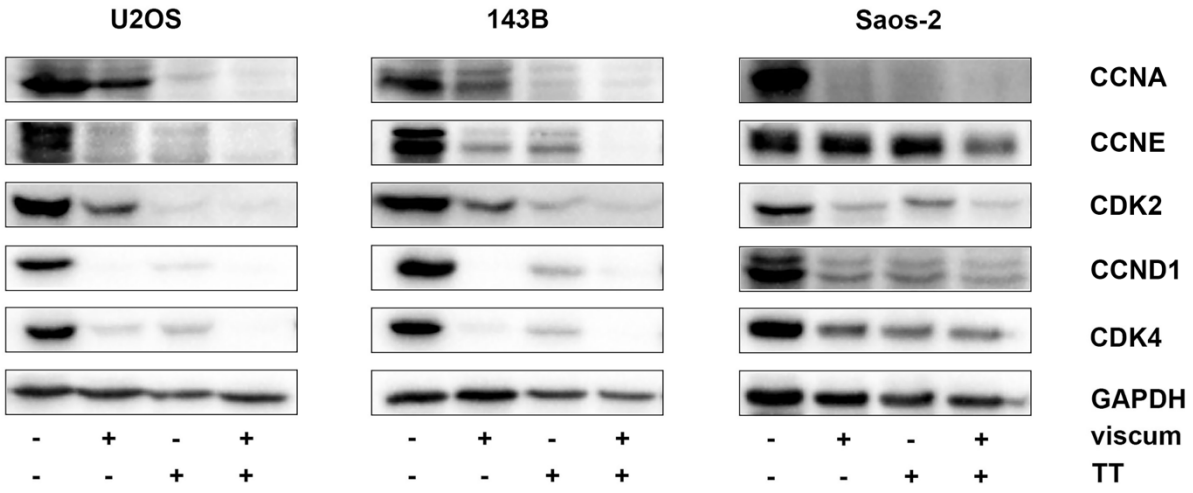


Figure 17 Viscum, TT and viscumTT down-regulate key regulators of G1/S transition. Synchronized cells were incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean concentration of viscumTT (5 ng/mL+50 µg/mL) for 24 h. Whole cell lysates were analyzed by western blotting regarding protein expression. Representative blots are shown from three independent experiments. GAPDH was used as loading control.

3.8 Viscum, TT and viscumTT alter expression of cell cycle-associated genes

For further investigations of the molecular alterations of cell cycle regulation, a RT² Profiler™ PCR Array, including 84 human cell cycle genes, was performed. At first glance, gene expression pattern was completely different in every cell line (Figure 18a-c). No gene was noticeable exclusively expressed in viscumTT-treated cells. After merging the results, genes were selected according to different criteria. On the one hand, the gene had to be up- or down-regulated in each tested cell line and on the other hand, genes should have been relevant for all treatments (viscum, TT and viscumTT). Based on this, three different cell cycle genes were found. These genes were not expressed at the same level, but were detected in all three cell lines. The first gene *GADD45A* was strongly up-regulated in U2OS and 143B cells predominantly after viscum (U2OS) or viscumTT (143B) treatment by approximately 20-30-fold increase related to control (Figure 18a, b). However, in Saos-2 cells *GADD45A* was not as strong over-expressed, but still an up-regulation of up to 6-fold was documented after viscum treatment (Figure 18c). In none of the three cell lines *GADD45A* expression was detected after TT incubation.

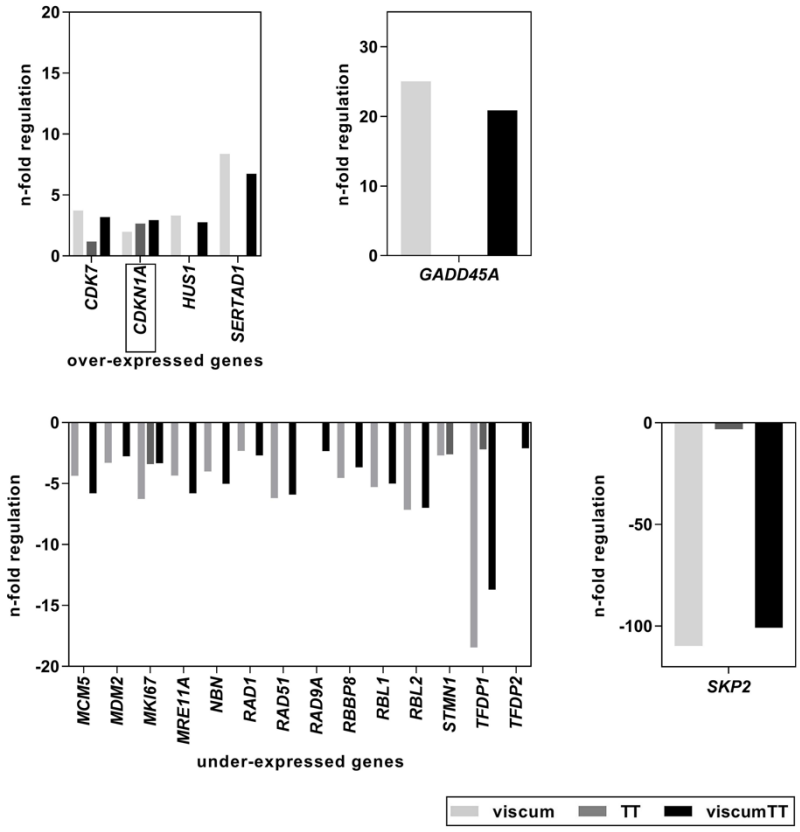


Figure 18a Viscum, TT and viscumTT alter expression of cell cycle-associated genes in U2OS cells. U2OS cells were incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h. Gene expression of 84 cell cycle genes were analyzed by RT² Profiler™ qPCR Array. Bars represent ± fold-regulation related to untreated control. For evaluation ± 2-fold regulation and p≤0.05 was set as cut off.

As second gene *CDKN1A* was chosen and its up-regulation was found in each cell line, but superficially observed in TT-treated cells (U2OS 2.61-fold, in 143B 6.47-fold and in Saos-2 cells 3.49-fold increase). Moreover, viscum led to no significant alteration of *CDKN1A* in U2OS and Saos-2 cells, but increased 5.06-fold to untreated control in 143B cells. The highest expression was detected for viscumTT (>10-fold-increase) in143B cells (Figure 18b).

SKP2 was identified as third gene and was predominantly down-regulated by viscum and viscumTT-treated U2OS (109, 100-fold decrease, respectively), 143B (18, 20-fold decrease, respectively) and Saos-2 cells (viscumTT: 2.2-fold decrease). The exact values for every significant up- or down-regulated gene with corresponding clustergrams are represented in supplementary data section (Sec. 6.4, Figure S3a-c, Table S2a-c) of this work for each tested cell line. These findings indicated transcriptionally differences in affected cell cycle-associated genes by viscum, TT and viscumTT. Similarities were found in *GADD45A* and *CDKN1A* up-regulation as well as *SKP2* down-regulation and further investigations are demonstrated in the next paragraph in detail.

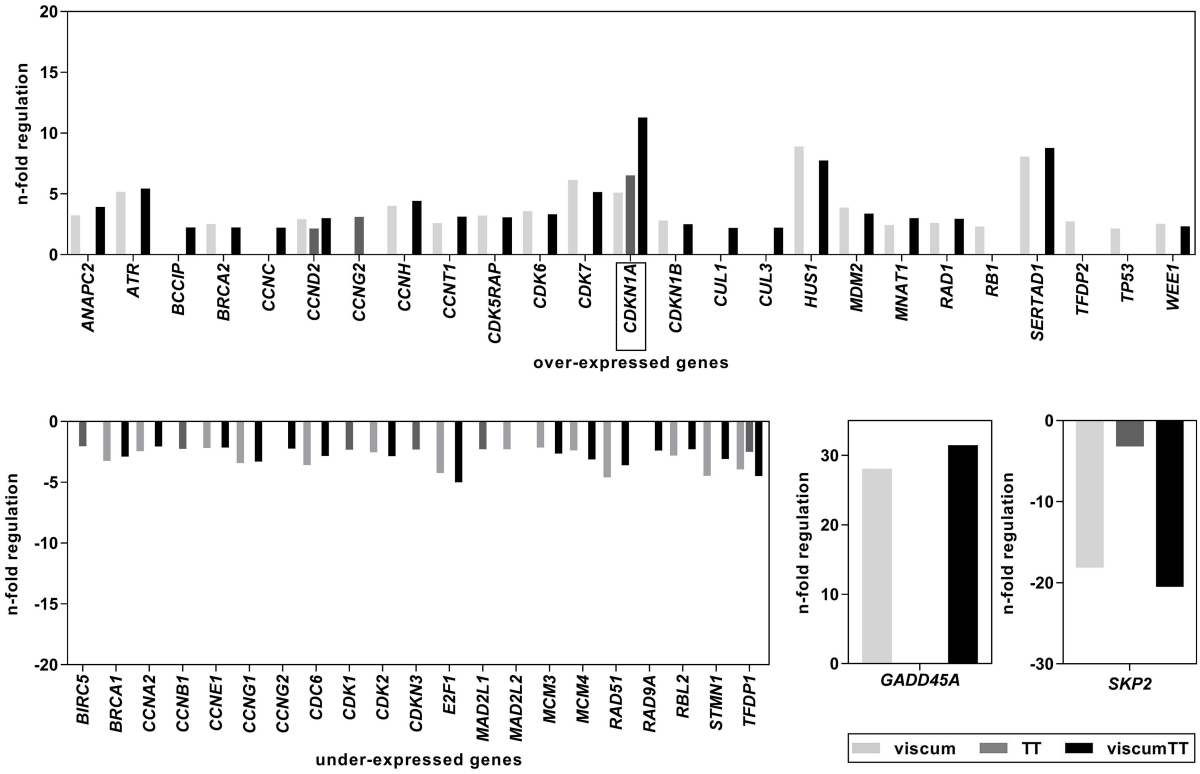


Figure 18b Viscum, TT and viscumTT lead to up- and down-regulation of cell cycle-associated genes in 143B cells. Cells were treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) as well as mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h and alteration of cell cycle-related genes were analyzed by RT² Profiler™ Human cell cycle qPCR Array. Illustrated are genes with ± fold-regulation >2 that were defined as significant change related to ontrol ($p \leq 0.05$).

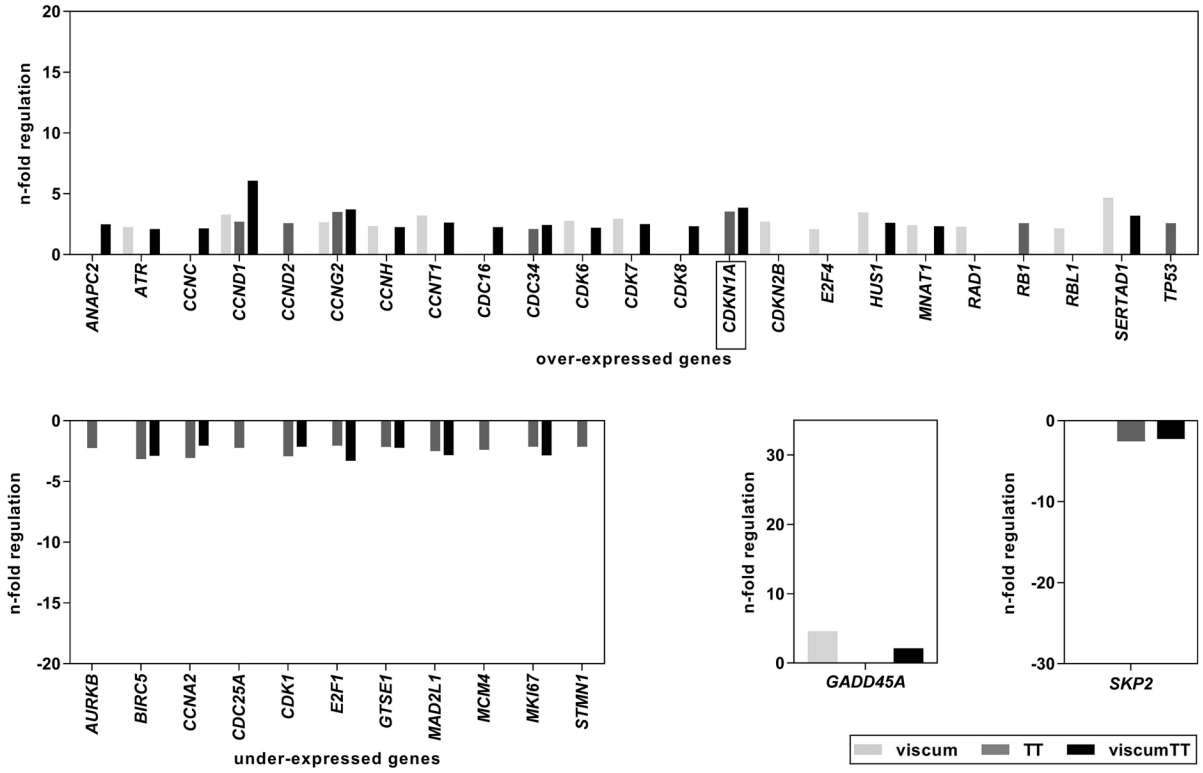


Figure 18c Viscum, TT and viscumTT alter expression pattern of cell cycle-related genes in Saos-2 cells. Synchronized Saos-2 cells were incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h and 84 cell cycle-associated genes were examined using RT² Profiler™ Human cell cycle qPCR Array. Represented are ± fold-regulation of genes related to untreated control, (cut off was set to ± 2-fold, p≤0.05).

3.9 ViscumTT up-regulates *CDKN1 and GADD45A* and down-regulates *SKP2* cell line-dependent

For the validation of the RT² Profiler™ Human cell cycle qPCR Array results, expression level of *CDKN1A*, *GADD45A* and *SKP2* were analyzed at different time points of treatment in synchronized U2OS, 143B, and Saos-2 cells by RT-qPCR. U2OS and 143B cells showed a comparable expression pattern, whereas that of Saos-2 cells was completely different (Figure 19). In detail, *CDKN1A* expression significantly increased even after 6 h (approximately 3-fold), peaked at 14 h (approximately 6-7-fold) and decreased again after both, viscum and TT treatment, in a similar manner in U2OS and 143B cells. This was also the case for TT, but not for viscum-treated Saos-2 cells as the highest expression was reached at 7.6-fold after 24 h. Interestingly, the treatment of viscumTT triggered a stronger *CDKN1A* expression than the single extracts, viscum and TT, in U2OS and 143B cells, but not in Saos-2 cells.

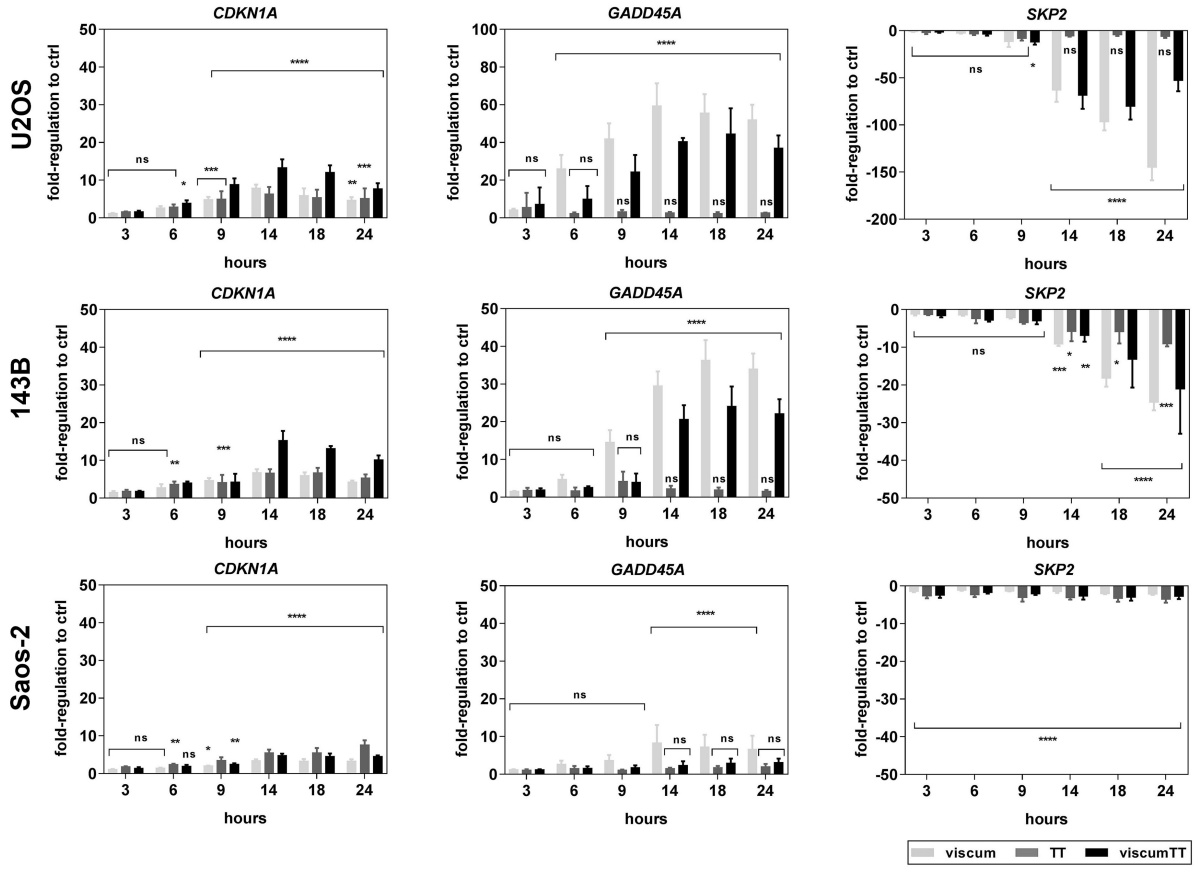


Figure 19 Viscum, TT and viscumTT alter *CDKN1A*, *GADD45A* and *SKP2* expression cell line-dependent. Synchronized cells were incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 3-24 h and gene expression was analyzed using RT-qPCR. Bars represent mean fold-regulation ± SD normalized to housekeeping genes related to untreated control of at least three independent experiments. Significant results are displayed as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control, ns: non-significant.

Furthermore, the experiments demonstrated a significant *GADD45A* up-regulation predominantly in viscum-treated cells that peaked at 14 h (59-fold in U2OS, 8-fold in Saos-2 cells) or 18 h (36-fold in 143B). The single treatment of TT led to no significant up-regulation in all three cell lines and *GADD45A* expression was less induced by viscumTT than by viscum (Figure 19).

Additionally, *SKP2* was strongly, time-dependently down-regulated after viscum treatment in U2OS cells (up to 150-fold decrease related to control) and 143B (about 25-fold decrease related to control), but not in Saos-2 cells. This expression level was attenuated after the addition of TT. TT itself triggered down-regulation of *SKP2* up to 8-fold decrease in U2OS after 9 h, 9-fold decrease in 143B and 3.6-fold decrease in Saos-2 cells after 24 h. These observations were in line with the demonstrated RT² Profiler™ qPCR Array results, apart from some variations in the amount of up- or down-regulation. Nevertheless, *CDKN1A*, *GADD45A*

and *SKP2* seemingly functioned as transcriptional targets and are further involved in alterations in cell cycle progression mediated by viscum, TT and viscumTT.

3.10 ViscumTT up-regulates GADD45A and down-regulates CDKN1A and SKP2 at protein level

Western blotting was used to analyze protein expression of CDKN1A, GADD45A and SKP2 after different time points. 6 h of viscum incubation CDKN1A was still highly expressed with afterwards stronger decreased level in all three cell lines, exempted from Saos-2 cells in comparison to control. TT triggered down-regulation after 14 h (U2OS, Saos-2) or 18 h of treatment (143B), whereas after viscumTT application distinctly lower expression of CDKN1A was detected starting from 6 h (U2OS, 143B) compared to control. In Saos-2 cells nearly no alteration was demonstrated after viscumTT treatment in comparison to control cells (Figure 20).

In U2OS cells GADD45A was low expressed in untreated control, whereas its level increased after 6 h of viscum treatment before it decreased again after 18 h. TT led to no change in GADD45A protein expression, but viscumTT showed the highest expression after 6 h in U2OS cells. In 143B cells GADD45A was lower expressed 6 and 9 h after viscum treatment, but then increased to similar level and achieved higher level at 24 h compared to control cells. Alike control TT showed an GADD45A expression that decreased after 18 h, whereas viscumTT led to prolonged protein level over the whole 24 h. On the other hand, in relation to control viscum and viscumTT equally increased and TT reduced GADD45A expression in Saos-2 cells. (Figure 20).

Depending on the cell line SKP2 was expressed in a similar way starting from 6 or 14 h after termination of synchronization. Incubation with viscum led to its down-regulation in U2OS and 143B, but less in Saos-2 cells, while TT decreased SKP protein level in 143B and Saos-2, but in a lower manner in U2OS cells (Figure 20). In viscumTT-treated cells an enhanced decrease was demonstrated in 143B and Saos-2 than in U2OS cells.

These results correlated with gene expression experiments except for CDKN1A. It was increased at transcriptional level, whereas it was decreased at protein level at equal time points. However, protein level analyses of affected targets confirmed their involvement in viscum, TT and viscumTT action.

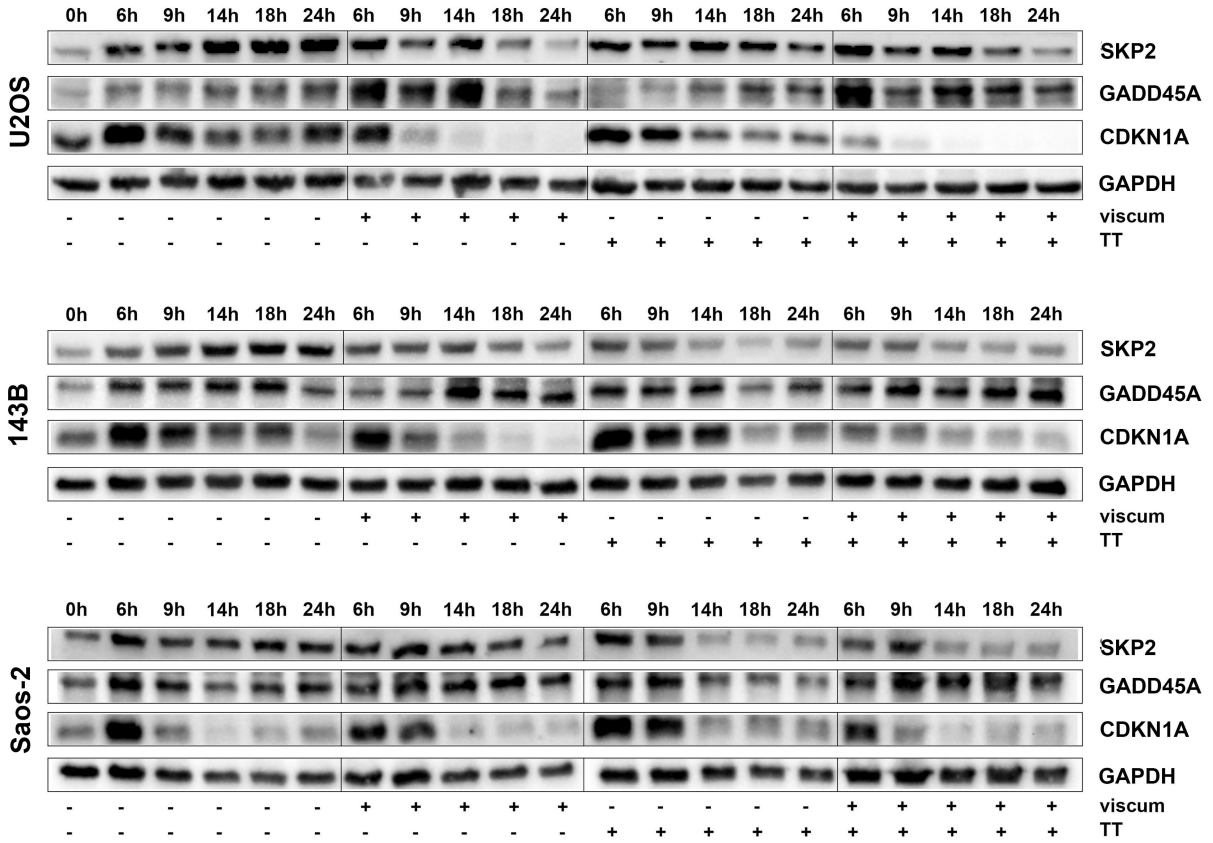


Figure 20 ViscumTT induces up-regulation of GADD45A and down-regulation of CDKN1A and SKP2 at protein level. Synchronized cells were treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean concentration of viscumTT (5 ng/mL+50 µg/mL) for indicated time points. Whole cell lysates were analyzed for protein expression using western blotting. Representative blots are shown from three independent experiments. GAPDH was used as loading control.

3.11 Knockdown of *CDKN1A* and *GADD45A* attenuate viscum, TT or viscumTT-mediated cell cycle arrest and induction of apoptosis

To evaluate the crucial role of CDKN1A and GADD45A for viscumTT induced cell cycle arrest and apoptosis, siRNA experiments were performed. Therefore, cells were transfected with both, single siRNAs and their combination, and further analyzed regarding cell cycle distribution and induction of apoptosis (Figure 21, 22). siRNA knockdown was confirmed by western blotting (Figure 22). Neither siRNAs nor non-targeting negative control siRNA influenced untreated control U2OS cells (Sec. 6.5, Figure S4).

After 24 h, 37.5 % of control U2OS cells were in G1, 34.2 % in S and 28.5 % in G2/M phase. All three treatments led to significant G1 arrest, whereas TT arrested cells stronger in G1 phase (64.64 %) than viscum (55.22 %) or viscumTT (55.38 %). With the knockdown of *GADD45A* a significant decrease ($p \leq 0.05$) of G1-arrested cells after viscum and viscumTT treatment was

obtained, whereas TT-induced G1 arrest was not prevented. However, transfection with siCDKN1A led to a reduction of cells in G1 phase after all three treatments, whereby revealed as significant for TT and viscumTT ($p \leq 0.01$). After simultaneously knockdown of *GADD45A* and *CDKN1A*, viscum-treated cells were nearly equally distributed, such as untreated control cells and number of cells in G1 phase were significantly reduced ($p \leq 0.05$). The siRNA combination led significant prevention of G1 arrest ($p \leq 0.0001$) in TT-treated cells, but not to complete phase distribution of untreated control cells. In viscumTT-treated cells knockdown of *GADD45A* and *CDKN1A* resulted in significant ($p \leq 0.05$) less number of cells in G1 and led to nearly the same distribution after single knockdown of *CDKN1A* or *GADD45A* (Figure 21). Consequently, each knockdown variant prevented viscumTT-induced G1 arrest in U2OS cells, but was accompanied with increase of cells in S phase.

In untreated control were 50 % of 143B cells in G1 phase, 33.7 % in S and 16.3 % in G2/M phase. Viscum led to a weak, but non-significant increase of cells in S phase (41.7 %) after 24 h. *GADD45A* knockdown prevented such enhanced S phase and led to nearly equally distribution of untreated control cells. siCDKN1A as well as combination of both siRNAs had not such attenuating effect and cell cycle distribution remained almost unaffected in viscum-treated cells with a slight increase of cells in S phase ($p \leq 0.05$ for siRNA combination). In contrast, TT led to significant G1 arrest in 143B cells (62.64 %). This effect was significantly strengthened by *GADD45A* knockdown ($p \leq 0.001$, 71.69 %, Figure 21). Interestingly, siCDKN1A and the combination of both siRNAs nearly prevented the complete TT-induced G1 arrest (51.35 %, 47.83 %, respectively), whereby the siRNA combination was proven as significant ($p \leq 0.01$). No clear cell cycle arrest was seen after 24 h of viscumTT treatment. All p-values within groups are listed in supplementary data section (Sec. 6.5, Table S3a, b).

Taken together, these results demonstrated prominant roles of CDKN1A and GADD45A in viscum, TT or viscumTT-mediated cell cycle arrest.

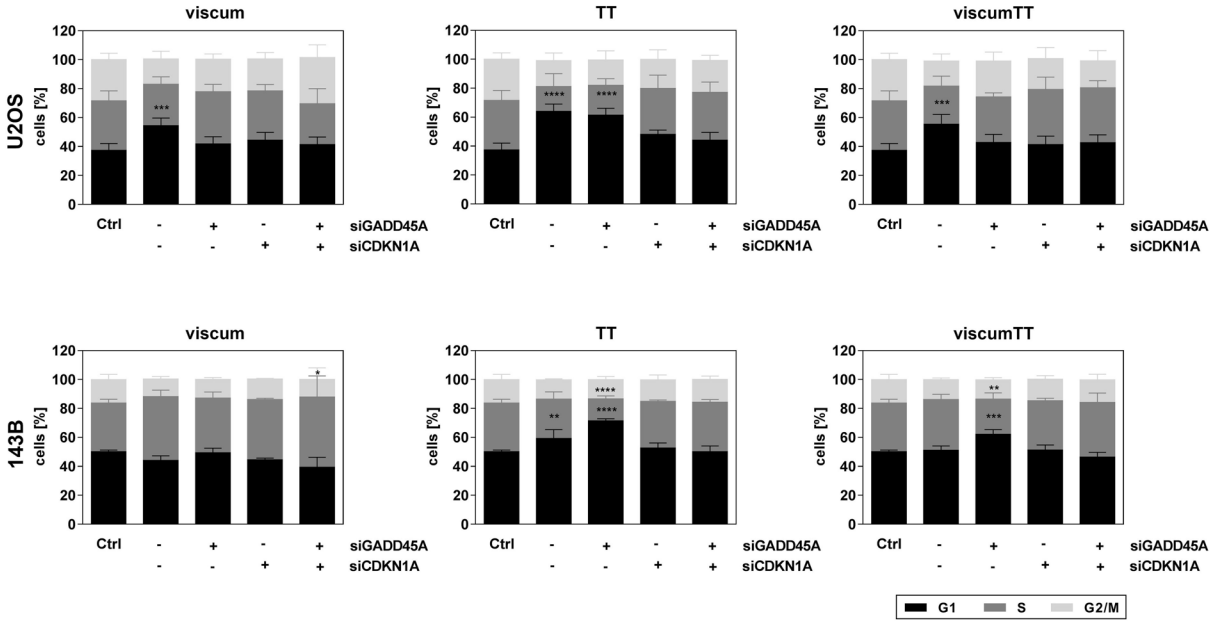


Figure 21 Viscum, TT and viscumTT-mediated cell cycle arrest is prevented by *CDKN1A* and G*ADD45A* knockdown cell line-dependent. U2OS and 143B cells were reverse transfected with different siRNA variants for 48 h and further treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration. Cell cycle distribution was analyzed by PI staining and flow cytometry. Bars display cells [%] in cell cycle phase ± SD of at least three independent experiments. Significances are indicated as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control (Ctrl).

To clarify whether *GADD45A* and *CDKN1A* play a further role in induction of apoptosis, transfected U2OS and 143B cells were analyzed after 24 h of treatment. As described above (Figure 10), 143B cells were more resistant to viscum than U2OS cells, whereas U2OS cells were more resistant to TT in lower concentrations, but both cell lines were equally sensitive towards viscumTT. U2OS cells showed induction of apoptosis of 52 % when treated with viscum (ML 10 ng/mL). After *GADD45A* knockdown apoptosis was significantly inhibited by 27 %. siCDKN1A and siRNA combination led to weaker inhibition of apoptosis (Figure 22, left). TT induced apoptosis in 41.5 % of U2OS cells and *GADD45A* knockdown prevented this effect by 41 %. Surprisingly, knockdown of *CDKN1A* as well as the combination of both siRNAs enhanced induction of apoptosis by TT. Moreover, viscumTT-initiated apoptotic effect was inhibited by 25 % by *GADD45A* knockdown, 14.6 % by *CDKN1A* knockdown and 11.6 % by both siRNAs.

In 143B cells viscum induced only 19.3 % apoptosis, which was not rescued by siGADD45A. By contrast, knockdown of *CDKN1A* prevented apoptosis induction by 56.2 % and the combination of both siRNAs led to almost complete inhibition of apoptosis. TT-induced apoptosis (62.2 %) was inhibited by knockdown of *GADD45A* as well as *CDKN1A* and the combination thereof by approximately 50 % (Figure 22, right). Knockdown of *CDKN1A*

prevented stronger apoptosis (65 %) in viscumTT-treated 143B cells, whereas siGADD45A and the combination of both siRNAs showed a similar inhibitory effect.

To conclude demonstrated findings and comparing both cell lines, both GADD45A and CDKN1A, are also involved in apoptotic process mediated by viscum, TT and viscumTT.

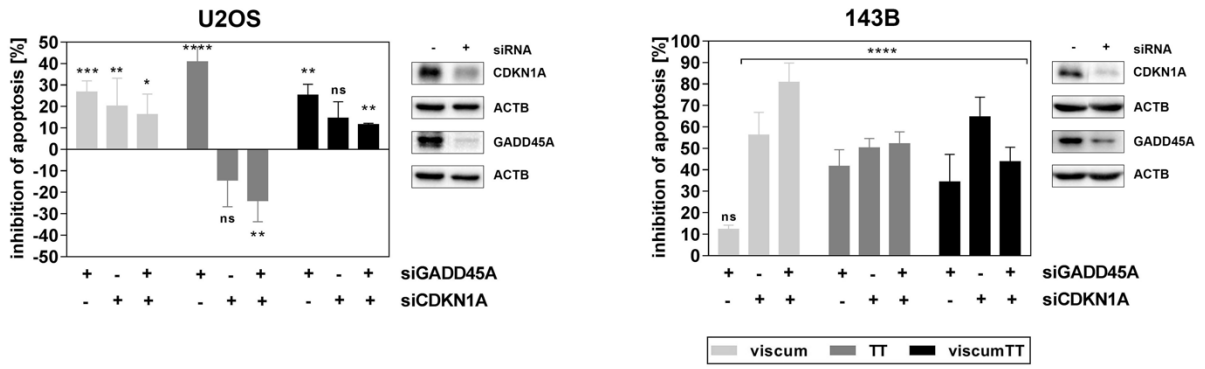


Figure 22 ViscumTT-induced apoptosis is attenuated by *CDKN1A* and *GADD45A* knockdown. Knockdown experiments by siRNA were performed and cells treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h. Cells were stained by Annexin V/PI and analyzed by flow cytometry. Bars represent mean inhibition of apoptosis [%] ± SD of at least three independent experiments. Significant inhibition is displayed as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control, ns: non-significant.

3.12 ViscumTT activates MAPK8 and inactivates MAPK1/3

Due to the direct interaction of GADD45A and MAPK pathways, protein expression and phosphorylation of MAPK8 and MAPK1/3 was investigated. Therefore, cells were treated with highest viscum, TT and mean viscumTT concentration for 24 h and cell lysates were analyzed by western blotting. In U2OS and 143B control cells, MAPK8 isoform 46 was weakly activated through phosphorylation in comparison to Saos-2 untreated control cells. However, in each cell line, MAPK8 was phosphorylated after viscum and viscumTT treatment (Figure 23a). TT also increased MAPK8 activation slightly in U2OS, higher 143B cells, but in Saos-2 cells phosphorylated MAPK8 was decreased. As opposed to phosphorylation status of MAPK8 in control cells, MAPK1/3 was strongly activated. After the treatment with viscum, TT and viscumTT, pMAPK1/3 was drastically dephosphorylated in all three cell lines, except in 143B cells. Here TT did not alter the phosphorylation status (Figure 23a). For closer look to MAPK8 and its role in induction of apoptosis, U2OS and 143B cells were preincubated with the MAPK8 inhibitor SP600125. Interestingly, only viscum-induced apoptosis was inhibited by SP600125 by approximately 40 % in U2OS cells. In 143B cells weak apoptosis induction by viscum treatment was also suppressed. On the other hand, SP600125 led to no inhibition of apoptosis

in TT-treated cells, whereas even an enhanced induction of apoptosis was observed in viscumTT-treated U2OS cells. In 143B cells SP600125 increased the number of apoptotic cells mediated by TT as well as viscumTT (Figure 23b). These results led to the assumption that viscum, TT as well as viscumTT initiated their apoptotic properties via involvement of MAPK pathway signaling.

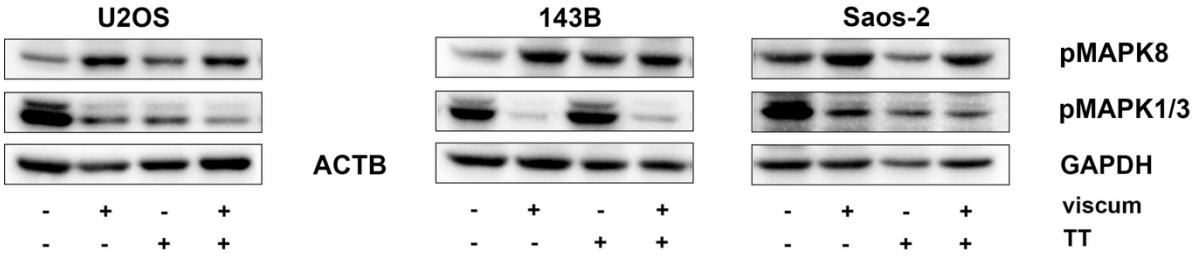


Figure 23a Viscum, TT and viscumTT activate MAPK8 and inactivate MAPK1/3 pathway. U2OS, 143B, and Saos-2 cells were treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean concentration of viscumTT (5 ng/mL+50 µg/mL) for 24 h. Protein expression was analyzed by western blotting. Representative blots are shown from three independent experiments. ACTB and GAPDH were used as loading controls.

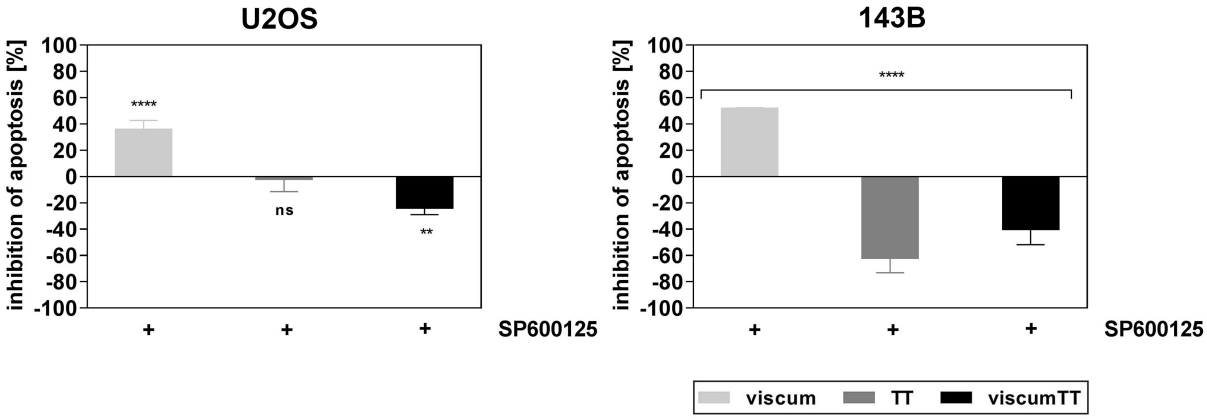


Figure 23b Inhibition of MAPK8 leads to decrease or increase of viscum, TT and viscumTT-mediated apoptosis. U2OS and 143B cells were pretreated with MAPK8 inhibitor SP600125 and further incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h. Cells were stained with Annexin V/PI and analyzed by flow cytometry. Bars display mean percentage of inhibition of apoptosis ± SD of at least three independent experiments. Significance is indicated with *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 related to control, ns: non-significant.

3.13 ViscumTT inactivates STAT3 pathway

In order to further determine the mechanistic impacts of viscum, TT and viscumTT, STAT3 and its two different activation sites were investigated. The classical STAT3 Tyr705 phosphorylation site is mainly regulated by JAK1 and often constitutively activated in osteosarcoma. The second phosphorylation site at Ser727 seems to be regulated by MAPKs. All cells lines were treated with highest viscum, TT and mean viscumTT concentration for 24 h and cell lysates were examined by western blotting. STAT3 and its phosphorylation at both sites occurred in each of the control cells, whereas in 143B cells Ser727 phosphorylation was less. In U2OS cells all three extracts completely dephosphorylated and consequently inactivated both STAT3 Tyr705 and Ser727 (Figure 24) with simultaneously down-regulation of total STAT3. Total STAT3 level was distinctively higher in viscum-treated 143B and Saos-2 cells, but STAT3 Tyr705 and Ser727 was nearly not detectable after each treatment. Furthermore, BIRC5 and MYC, two proteins, which are often over-expressed in osteosarcoma and are direct targets of STAT3, were completely down-regulated by viscum, TT and viscumTT in U2OS. In 143B cells viscum and viscumTT led to stronger down-regulation of BIRC5 than TT and MYC was also slightly affected by viscum and viscumTT. In Saos-2 cells BIRC5 and MYC were down-regulated by all three extracts (Figure 24). In all, *TP53* wild-type (U2OS) cells showed a better response to inhibition of BIRC5 and MYC in comparison to *TP53* mutant (143B) and *TP53* null-mutant (Saos-2) cells. However, and to sum up these findings a strong prevention of STAT3 signaling pathway by viscum, TT and viscumTT extract was seen and revealed another involved mechanism of action.

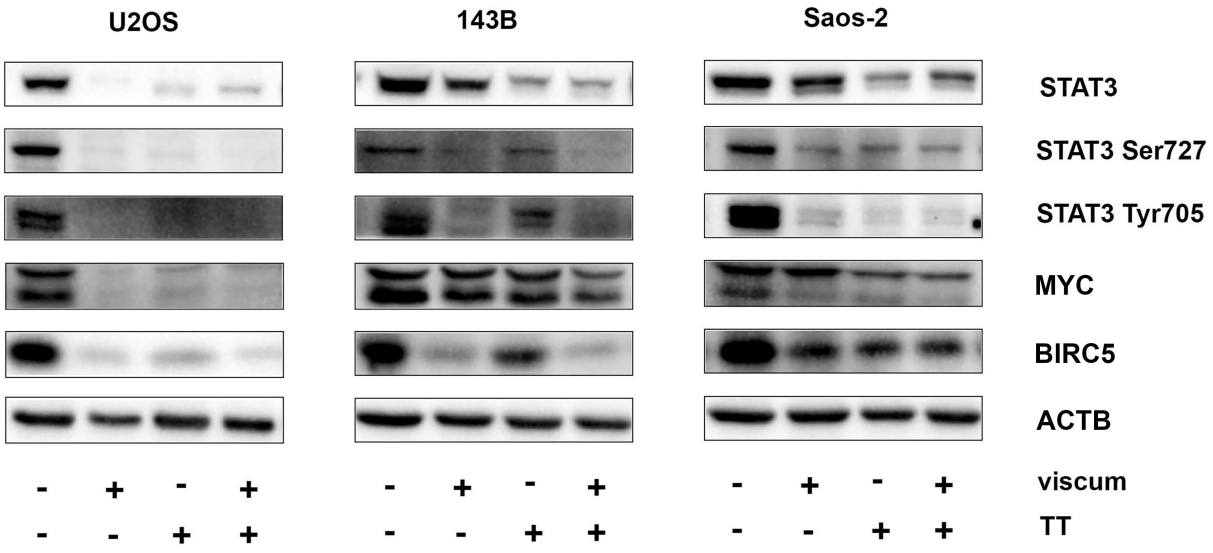


Figure 24 ViscumTT inactivates STAT3 pathway. Cells were treated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and mean viscumTT (5 ng/mL+50 µg/mL) concentration for 24 h. Protein expression was analyzed using western blotting. Representative blots are shown from three independent experiments. ACTB was used as loading control.

3.14 Viscum, TT and viscumTT reduce tumor volume in osteosarcoma xenograft

To investigate a cytotoxic effect of viscum, TT and viscumTT *in vivo*, an osteosarcoma xenograft with Saos-2 cells was established. Tumors were grown s.c. in female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac mice and were treated two to three times per week with viscum (0.75/1.25/1.75 µg/kg ML), TT (50/70/90 mg/kg OA) and a combination thereof (viscumTT). Each dose was applied twice i.t.. As control group mice were treated with CD. Figure 25 displays the reduction of tumor volume (left) at the end of the study and the course of the mean body weight (right). Viscum as well as TT significantly reduced the tumor volume approximately by 30 % compared to control group. ViscumTT led to an even enhanced reduction of tumor volume compared to viscum, TT and reduced tumor volume by almost 50 % in comparison to control group. Each treatment group showed a slightly variance and viscumTT treatment revealed significant results to control and viscum group (p-values for group effect are listed in Sec. 6.7, Table S4). Each applied extract was tolerated well with no side effects, toxicity symptoms or significant body weight loss. These observations led to the assumption that viscum and TT as single treatment effectively inhibited tumor growth and further viscumTT administration suppressed osteosarcoma growth even stronger in mice xenografts.

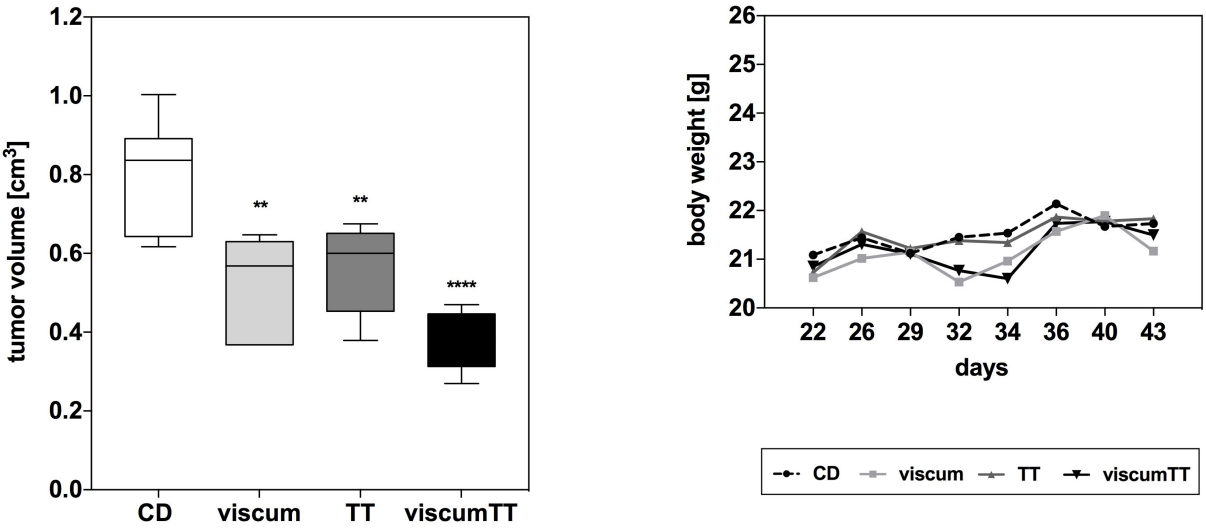


Figure 25 Viscum, TT and viscumTT reduce tumor volume. Xenografts were i.t. treated with CD, viscum (ML 0.75/1.25/1.75 µg/kg), TT (OA 50/70/90 mg/kg) or a combination thereof (viscumTT). Box plots represent tumor volume ± SD relative to control group (CD, left) and lines represent the mean body weight (right). Significance are displayed related to CD group (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

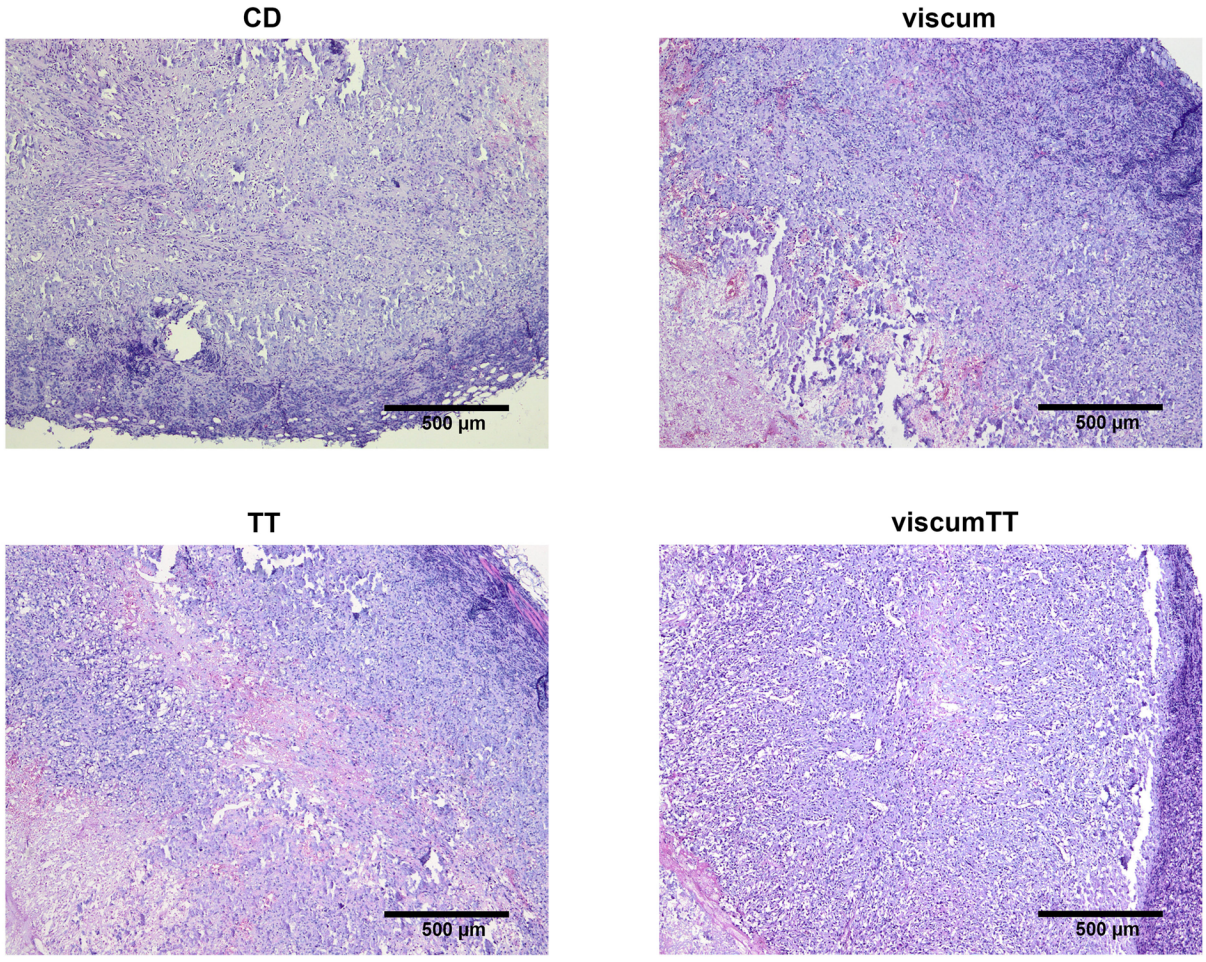


Figure 26a H&E staining as overview of Saos-2 xenograft tissue. Images illustrate tumor tissue after treatment and H&E staining by 4 x magnification.

H&E staining gave a first overview of texture and differences of the tumor tissue of the respective group. Here, a damaged structure of the tissue was observed nearly in each tumor of each group and is demonstrated in Figure 26a as a representative example. In almost every tumor an augmented presence of erythrocytes was found. Necrotic areas were also seen in CD control tissue as well as extract-treated groups certainly amount varied depending on tumor size. As distinct evaluation of differences between groups was not to be assessed with this staining. Microscopic 20x magnification of H&E-stained tumors are illustrated in Figure 26b.

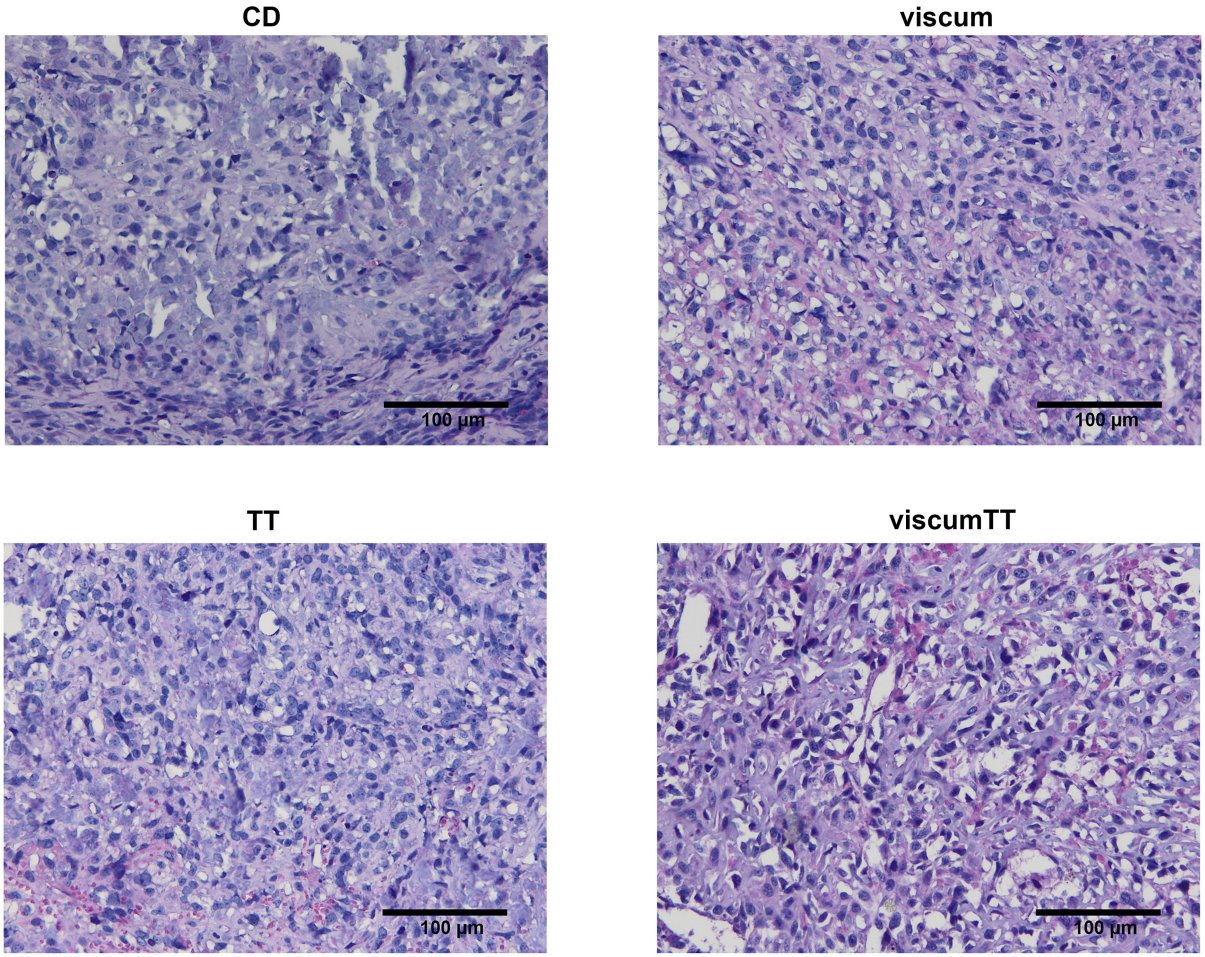


Figure 26b Tumor tissue of Saos-2 xenograft after H&E staining. Images illustrate tumor tissue after treatment after H&E staining by 20 x magnification.

3.15 ViscumTT does not affect weakly expressed MKI67 *in vivo*

For further histological investigations, proliferation marker MKI67 was analyzed using immunohistochemical staining. After detailed consideration only a small amount of MKI67 positive cells were found. Already in CD-treated control tissue MKI67 protein was not strongly expressed. After the evaluation of five representative pictures of each tumor of each group a

slight, but not significant tendency of decreased MKI67 was observed (Figure 27 a, b left). Nevertheless, no clear statement regarding alteration of MKI67 expression after viscum, TT and viscumTT treatment was possible, because of its faint occurrence in that tissue.

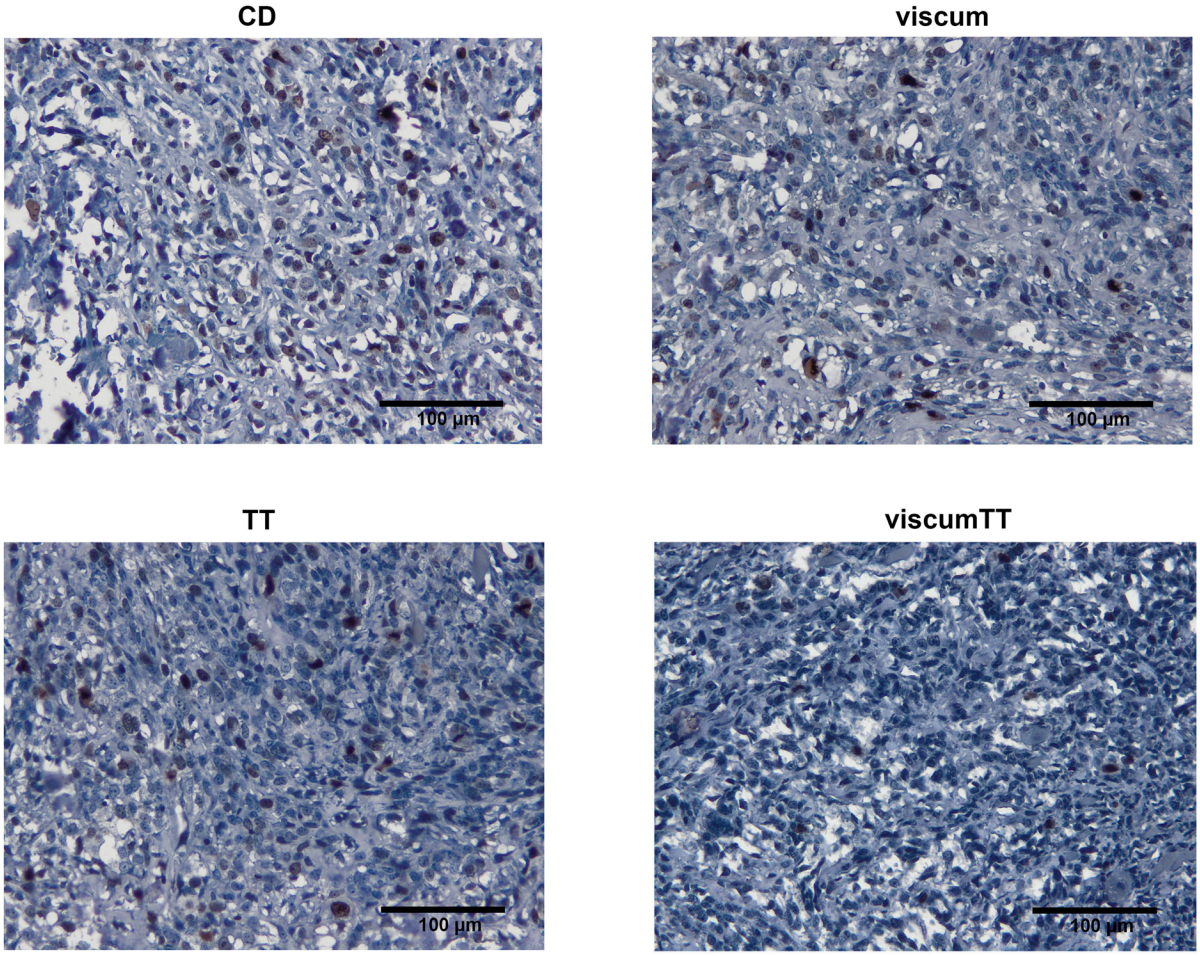


Figure 27a MKI67 immunostaining of Saos-2 xenograft tissue. Images demonstrate 20 x magnified tumor tissue after treatment and MKI67 immunostaining.

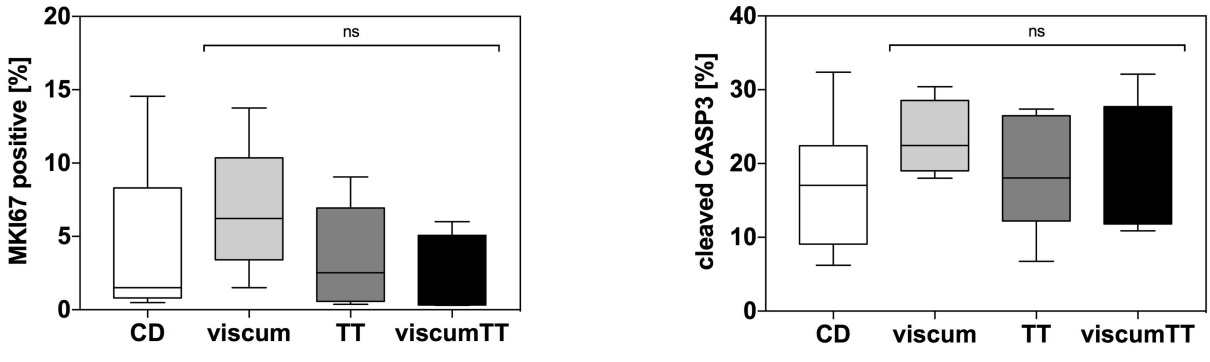


Figure 27b Quantification of MKI67 and cleaved CASP3 immunostaining. Five representative images out of one sample of each tumor of each group were quantified by positive area in relation to whole tissue. Box plots represent percentage of positive stained area ± SD. ns: non-significant.

3.16 ViscumTT does not increase CASP3 activity *in vivo*

For analyzing induction of apoptosis *in vivo*, cleaved CASP3 was stained by immunohistochemistry. Already, the first consideration of stained tissue showed a distinct CASP3 activity in each treatment group. Against all expectations, in CD-treated group up to a quarter of tumor tissue was stained positive (Figure 28, 27b right). The viscum, TT and viscumTT-treated groups revealed only a weak, but no significant increase.

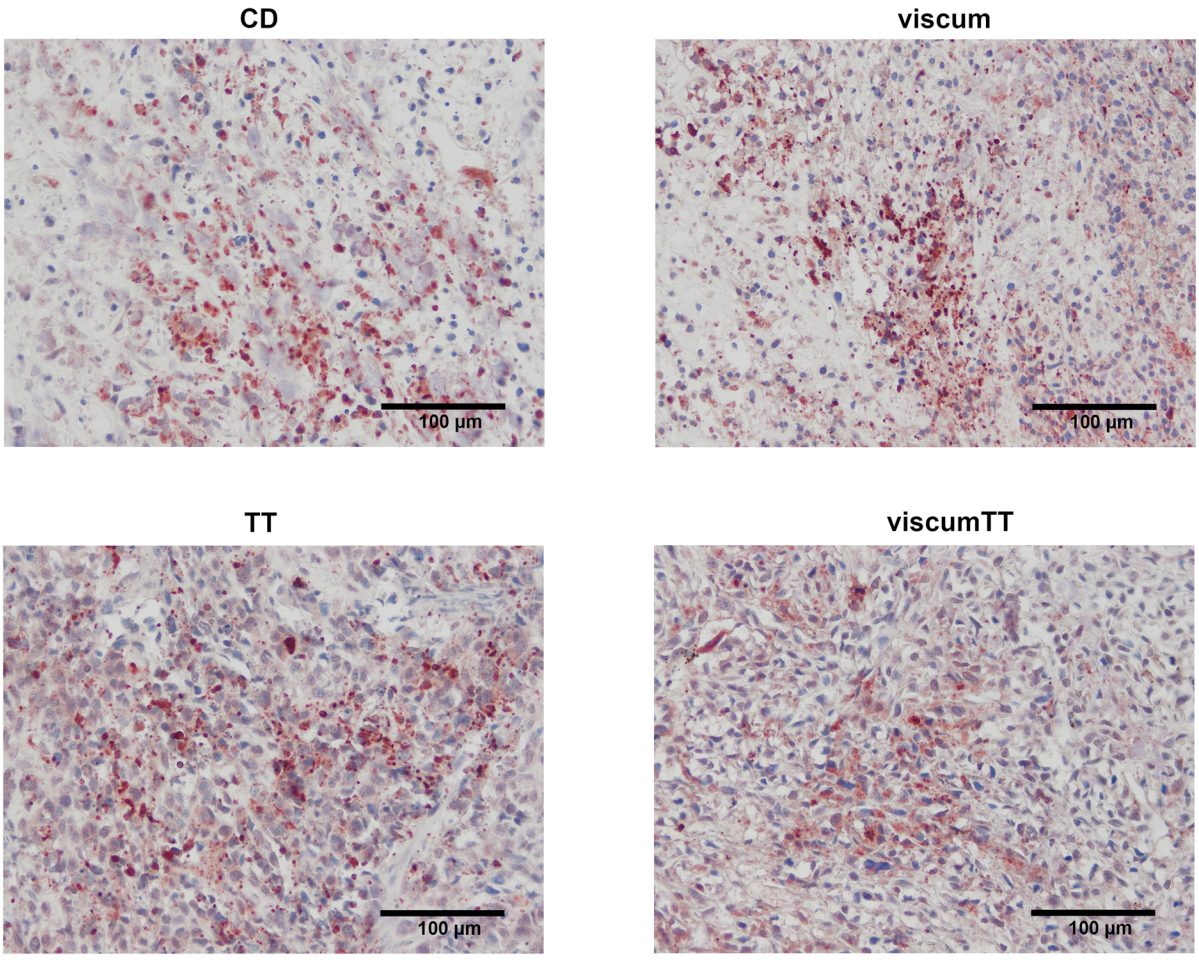


Figure 28 Viscum, TT and viscumTT did not higher activate CASP3 *in vivo*. Images demonstrate 20 x magnified tumor tissue after treatment and cleaved CASP3 immunostaining.

In conclusion H&E staining showed bad tissue quality which made the assessment more difficult and based poor requirements for MKI67 and cleaved CASP3 immunostaining. None of that proved to be suitable for confirmation the good response in reduction of tumor volume

4. Discussion

The results of this work demonstrated the high potential of a combined mistletoe extract, so called viscumTT, in pediatric osteosarcoma. It possesses distinct anti-tumoral properties regarding inhibition of proliferation, induction of apoptosis, and alteration in cell cycle progression *in vitro*. An enhanced reduction of tumor volume was observed after viscumTT treatment in comparison to its single extracts *in vivo*. Multiple signaling pathways and targets, which crosslinked apoptosis and cell cycle were affected and emphasized its potential as new therapy approach for pediatric osteosarcoma.

4.1 VisumTT synergistically induces inhibition of proliferation and apoptosis

The single extracts, viscum and TT, affected cells differently, whereas viscumTT showed nearly the same potential in inhibition of proliferation and induction of apoptosis in the tested cells. Therefore, viscum led to dose-dependent inhibition of proliferation in *TP53* wild-type (U2OS), mutant (143B) and null-mutant (Saos-2) cells. But, only *TP53* wild-type cells were sensitive regarding apoptosis. 143B cells harbor a point mutation at DNA-binding domain of *TP53* at Arg156Pro, leading to high, but non-functional protein expression [169, 170]. In Studies with recombinant ML, ML isolated from Korean mistletoe as well as ML III extracted from European mistletoe an induction of apoptosis independent from *TP53* status of the cells were described [53, 55, 171], whereby further down-regulation of total and nuclear TP53 was described probably due to the protein synthesis-inhibiting subunit inactivation by the A chain [171]. Inhibitors of protein synthesis suppressed the increase of TP53 after DNA damage [172]. Also in this work, viscum (including ML I) decreased total TP53 in *TP53* wild-type (U2OS) and *TP53* mutant (143B) cells as well as in leukemia cells in earlier study (HL-60, *TP53* mutant) [164] indicating a TP53-independent mechanism of action. Additionally, in two Ewing's sarcoma cell lines, harboring different TP53 mutant forms, a suppression of protein expression was also seen, but cells were not resistant to viscum [173]. Furthermore, surface characteristics of the cells play a role for variable sensitivity [8] and could be another cause for viscum resistance in tested osteosarcoma cell lines.

On the other hand, TT showed a stronger growth inhibitory effect in *TP53* wild-type (U2OS) and null-mutant (Saos-2) cells than in *TP53* mutant (143B) cells, whereas the apoptotic response was vice versa certainly dose-dependent. Contrary, studies with OA described a mitochondrial and TP53-dependent apoptosis mechanism by translational up-regulation of TP53 in gallbladder carcinoma [174] and transcriptional increase of *TP53* in murine melanoma cells [175]. However, BA induced in neuroectodermal tumors and UA in melanoma cells

intrinsic apoptosis pathway without changes in TP53 level [176, 177]. In this present work, TT also decreased TP53 at protein level stronger in wild-type than in mutant osteosarcoma cells, whereas in Ewing's sarcoma cells suppression correlated with higher sensitivity and is seemingly dependent from type of mutation [173]. However, this does not exclude a participation of functional TP53 in inhibition of proliferation and apoptotic response but suggesting a secondary role for its involvement and has to be further investigated.

Interestingly, for viscumTT these differences between the cell lines were not observed, neither in inhibition of apoptosis nor in induction of apoptosis, but rather led always to a synergistic effect and overcame potential TP53 dependence. Furthermore, investigations with a whole soy plant extract showed also higher apoptotic potential than its single extracts in pancreatic cancer cells [178]. Hong-Fang Ji *et al.* reviewed such enhanced effects of natural products in drug combinations not only in cancer therapy [179].

The exact mechanism of action of viscum, TT and viscumTT has not been completely understood yet. Apoptosis induced by both single extracts was mediated via CASP8 and CASP9 activation, suggesting participation of components of extrinsic- and intrinsic apoptosis pathway in *TP53* wild-type (U2OS) cells. Furthermore, *TP53* mutant and null-mutant cells showed no loss of $\Delta\Psi m$ and CASP9 activation in response to viscum, whereas null-mutant cells demonstrated the strongest cleavage of PARP and CASP3 activation, which evidenced a stronger DNA fragmentation by absence of TP53 [180]. Induction of apoptosis via extrinsic and intrinsic components was also seen in other pediatric tumors, including leukemia and Ewing's sarcoma [163, 164, 173] seemingly independent from TP53 status. A CASP9 activation was also initiated in leukemia cells treated with a commercial *Viscum album* preparation [48].

Since ML I activates CASP8 without death receptor stimulation [181], triggering extrinsic apoptosis pathway by viscum and viscumTT is possible via a feedback mechanism. Here, cytochrome c and activated CASP3 were able to activate CASP8 [182, 183]. Additionally, CASP8 activation was also evidenced in ML I-treated FAS-resistant or FADD negative mutants [181]. Hence, other death receptors may play a role in mistletoe extrinsic apoptosis activation. Antagonizing TNFR1 attenuate Korean mistletoe-induced cytotoxicity in colorectal adenocarcinoma cells [54] and DR4 as well as DR5 were enhanced after viscum and viscumTT treatment in AML cells [164]. Another study reported that CASP10 played a role in viscum and viscumTT-induced apoptosis in rhabdomyosarcoma cells [184] and gave grounds for assumption that extrinsic pathway is mediated by CASP10 FADD-dependent, but independent of death receptor stimulation [185]. However, an exact interaction with death receptors has not been demonstrated so far, consequently, triggering extrinsic pathway by mistletoe extracts remains unclear.

OA, its synthetic derivatives, and BA themselves induced apoptosis in a caspase-dependent manner in different tumor entities, such as neuroectodermal tumors [186], leukemia [74], breast cancer [187], hepatocarcinoma [76], and osteosarcoma [188]. Ito *et al.* demonstrated that the OA derivative CDDO induced CASP8 and CASP3 in BCL2L1 ((BCL-X(L)) overexpressing osteosarcoma and leukemia cells and mediated cytochrome c release via BID cleavage [188, 189]. A crosstalk between extrinsic and intrinsic apoptosis pathway mediated by BID cleavage into tBID initiate by CASP8 is also conceivable for TT mechanism of action. BID cleavage was recently evidenced in TT, but not in viscumTT-treated 143B and Saos-2 cells [168]. A second indication for a crosstalk function of TT is the induction of nearly the same activation level of CASP8 and CASP9, especially in U2OS cells. Partially inhibition of CASP-dependent apoptosis by zVAD-FMK in viscum, TT and viscumTT-treated 143B and Saos-2 cells supposed other forms of cell death might be triggered by mistletoe extracts. Previously, spontaneous necrosis was excluded [168].

CDDO as well as UA induced caspase-independent cell death by apoptosis-inducing factor (AIF) [190, 191] that released from mitochondria and directly induced chromatin condensation and large-scale DNA fragmentation [192]. Since TT and viscumTT increased the cellular autophagy marker microtubule-associated protein 1A/1B light chain 3B (LC3B-II) in Ewing's sarcoma cell lines [193], an additional event was revealed as mechanistic action. UA induced autophagy in different cell lines [194]. Transcriptome and proteome analyses of Ewing's sarcoma cell line gave more insights of mechanism of action of viscum, TT and viscumTT [193], but basic molecular processes for synergistic action of viscumTT has not been fully clarified. ViscumTT led to synergism in CASP8, -9 activation and loss of $\Delta\Psi m$ also in viscum resistant cells. One study with 143B cells indicated that TT improved viscum uptake [195] possibly as result of the property of OA and other triterpene acids to embed into lipid membranes [196]. Explicit knowledge about target molecules of each drug component in a combination preparation is required to understand combinatory processes of action. Therefore, synergistic effects often resulted from triggering the same or different targets in same or related pathways by the single components [197]. Further insights to this are given in the following paragraphs.

4.2 ViscumTT sensitizes osteosarcoma cells to chemotherapeutic drugs

Chemotherapy resistances are a rising problem in the treatment of cancer and limit the therapeutic success, also in osteosarcoma. In general, osteosarcoma is considered as relatively chemotherapy resistant. Classical therapies, for instance, vincristine and 5-fluorouracil, which are common applied in solid pediatric tumors, are not effective in osteosarcoma [198]. The most common administered chemotherapeutic drugs with moderate

to good responding are high dose methotrexate, Doxo, ifosfamide, and cisplatin [199]. However, nearly 50 % of osteosarcoma patients are priori resistant or acquire chemotherapy resistance [200]. Several clinical trials and case reports described an improvement of the quality of life resulting from reduction of side effects when chemotherapy is combined with commercial *Viscum album* preparations in various tumor entities [4, 201, 202]. Such results were also reported for osteosarcoma patients treated with oral etoposide and s.c. classical *Viscum album* preparation [203]. In this work, a stronger induction of apoptosis when chemotherapeutic drugs were co-treated with viscum or TT was shown, but achieved its highest effectiveness with viscumTT. The best combinatory effect was reached with viscum, TT or viscumTT with Doxo or VP16 in 143B cells. Generally, the combinatory benefit was lowest when co-treated with 4-OOH, suggesting mistletoe extracts are more effective in combination with topoisomerase II inhibitors than with DNA intercalating agents like ifosfamide *in vitro*. This is in line with other studies that demonstrated enhanced cytotoxicity of Doxo in breast and pancreatic cancer and T-cell leukemia cells after the treatment with commercial *Viscum album* extracts and Korean mistletoe preparation [204-206]. The combination with sub-apoptotic Doxo concentrations achieved the best anti-tumoral effect in leukemia cells [207] and isolated ML I was shown to increase VP16-induced apoptosis in T-cell leukemia cells [181]. Meanwhile, many molecular chemotherapeutic drug resistance mechanisms are known, but not fully understood. Often drug resistances are accompanied by over-expressed ABC-transporters, such as ABCB1 (MDR1, P-gp) on tumor cell surface [208], which are also associated with Doxo resistance in osteosarcoma [209]. ABCB1 and other members of the ABC-subfamily act as an energy-dependent efflux pump of different chemotherapeutic drugs [210]. Valentiner *et al.* showed that ML I–III similarly inhibit cell proliferation in HT29 $^{mdr+}$ as well as HT29 $^{mdr-}$ colorectal cancer cells independent of the presence of the ABCB1 transporter. High sensitivity of HT29 $^{mdr+}$ cells are accompanied with increase glycosylation, which consequently results in higher density of ML binding sites [29], since binding to cell surface determined ML-induced cytotoxicity [8, 211]. ML I binds directly to sugar structures of ABCC5 (MRP5), another protein of the ABC-transporters, which is also related to developing multi-drug resistance when over-expressed [212]. Furthermore, UA sensitizes colon cancer cells by inhibiting ABCB1 [213]. OA decreases ABCC1 (MRP1), but not ABCB1 activity [214]. Since constitutively activation of MAPK1/3 as consequence of non-functional TP53 sensitizes to Doxo [215], supports the observation that highest combinatory effect was achieved in 143B cells. Consequently, the underlying molecular mechanism and mutation status determine the successful increase of induction of apoptosis in viscum, TT and viscumTT co-treatment with chemotherapeutic drugs. However, *in vitro* results of this and other works emphasize a combinatory effect of mistletoe extracts and different chemotherapeutic agents beyond the improvement of quality of life, but rather regarding direct enhanced anti-tumoral effects. To

improve these effects, it will be of advantage to optimize the concentration of each member of the co-treatment in the future.

4.3 GADD45A and CDKN1A are involved in cell cycle modulatory effects and induction of apoptotis by viscumTT

Children with retinoblastoma or Li-Fraumeni syndrome have a higher risk to develop osteosarcoma, which is often accompanied by dysregulation of the cell cycle and uncontrolled cell growth caused by dysfunction in the RB and TP53 pathway. Viscum, TT and viscumTT initiated distinct alterations in cell cycle-associated genes and proteins resulting in cell cycle arrest. Each treatment led to distinct down-regulation of CDKs and CCNDs that are involved in G1/S transition. After viscum treatment cells were arrested in G1 (U2OS, Saos-2) or in S phase (143B), which led to the conclusion that cell cycle arrest is cell-type dependent. In line to this, commercial *Viscum album* preparations arrested cells in different phases, for instance, G2/M arrested in breast cancer cells [216] as well as none, G1, S or G2/M arrest in a panel of cell lines, including small cell lung cancer, adenocarcinoma from the lung, breast or colon cancer [217]. Another standardized *Viscum album* extract was able to induce cell cycle arrest in every phase dependent on the type of myeloma cell line [218]. *Viscum album* agglutinins also initiate none cell cycle arrest in leukemia cell line U937 [219]. The underlying mechanisms of induction of cell cycle arrest of mistletoe extracts in different phases are relatively unknown. OA affected the cell cycle and arrested hepatocellular carcinoma cells [86], gallbladder cancer cells [220], and colon cancer cells [221] in G0/G1 phase and pancreatic cancer cells in G2/M phase [222]. G1 arrest can be even induced by BA [223, 224]. The majority of these studies are in line with the induction of G1 arrest after TT treatment in this work.

Cell cycle progression is driven by periodically ubiquitin-dependent degradation of key cell cycle regulators. Especially, the SCF protein complex is responsible for regulation of G1/S transition. SKP2, as F-box protein in this complex, targets among others CDKN1A [225], CCND [143] and CCNE [226] for protein degradation. High expression levels of SKP2 are observed in osteosarcoma and associated with metastasis and poor prognosis [227]. Recent studies demonstrated that an SKP2 up-regulation promoted osteosarcoma growth and inhibited apoptosis [228], whereas its down-regulation suppressed proliferation and invasion [229]. In this work, a strong down-regulation of SKP2 by viscum and viscumTT at gene and protein level was demonstrated that prevented cell cycle progression of each cell line.

In healthy cells *GADD45A* is highly expressed during G1, decreased in S phase and also interacts directly with *CDKN1A* [230, 231]. *GADD45A* as well as *CDKN1A* are induced in response to cellular stress and DNA damage in a TP53-dependent mechanism [232, 233]. In

this case, GADD45A initiates G2/M [136] and CDKN1A G1 arrest [233]. This is contrary to the results of *TP53* wild-type U2OS cells in connection to GADD45A.

In general, cells with dysfunctional *TP53* or *CDKN1A* actually lose their G1/S checkpoint and are not able to arrest in G1 phase [234]. In contrast, *TP53* mutant 143B and *TP53* null-mutant Saos-2 cells arrested mainly in G1 phase (expect 143B in response to viscum and viscumTT (S)), which supports the theory of TP53-independent mechanism of action of the mistletoe extracts via induction of *GADD45A* and *CDKN1A*. TP53-independent G1 arrest is often accompanied by up-regulation of CDKN1A [235, 236]. Besides, other natural compounds also induced G1 arrest with simultaneous increase of *GADD45A* expression [237] in a TP53-independent manner [238]. Another study illustrated that expression of *GADD45A* after DNA damage is associated with *CDKN1A* expression and results then either in G1 or in G2 arrest [239]. Both, GADD45A and CDKN1A, mediated their function in a TP53-dependent and - independent mechanism [240, 241]. Therefore, *CDKN1A* is able to induce TP53-independent G1 [242] and S arrest [243], which may explain viscum-mediated observed S arrest with increased *CDKN1A* expression in *TP53* mutant 143B cells. Additionally, an observation of prolonged G1/S transition is possible to result in S arrest [244]. At protein level, CDKN1A was faster degraded in viscum-treated than in TT-treated cells, which reinforce the message that TT-mediated G1 arrest is primarily driven by CDKN1A. G1 arrest is associated to CDKN1A expression, which was also described for OA and UA [245, 246]. ViscumTT initiated distinct G1 arrest in *TP53* wild-type U2OS and null-mutant Saos-2 cells, whereas in *TP53* mutant 143B cells first G1 followed by slight S arrest was observed, which might have resulted from the combination of the single extract effects. The increased number of cells in G2/M phase of the cell cycle in this study indicated a not complete stagnation, but delay of the cell cycle. In arrested stadium cells had time for DNA repair, which finally allowed cells to continue cell cycle progression, whereby prolonged cell cycle arrest often results in apoptosis.

In further consideration, both GADD45A and CDKN1A, played a role in induction of apoptosis by viscum, TT and viscumTT as siRNA attenuated apoptotic effect. Knockdown of CDKN1A in U2OS cells increased apoptotic response, which was already described in colon cancer cells [247] and prevented in combination with TT CDKN1A pro-survival function. In addition to UA, CDKN1A was induced in *TP53* wild-type and mutant colon cancer cells and compromised apoptotic effect via MCL1 up-regulation. Further inhibition of CDKN1A led to a pro-apoptotic effect [245]. Pro-survival effect of CDKN1A is prevented via TP53-dependent and - independent fashion, but based mechanisms are mostly unknown. So up-regulation of pro-apoptotic BAX [248] and enhanced apoptotic effect of cisplatin in ovarial carcinoma [249] correlated with CDKN1A expression. In case of GADD45A, its pro-apoptotic function under involvement of MAPK14/8 was recently shown in lymphocytes by riboflavin and ultraviolet light

[250] and previously in TP53-independent response to breast cancer 1 (*BRCA1)* expression [251].

Taken together, viscum, TT and viscumTT mediated their cell cycle modulatory and pro-apoptotic effect via involvement of CDKN1A and GADD45A under secondary role of TP53 particiption. Therfore, they are qualified for osteosarcoma treatment, especially for such osteosarcomas with occurred dysfunction in TP53 and RB1 resulting from retinoblastoma and Li-Fraumeni syndrome. Finally, the roles of *GADD45A* and *CDKN1A* differ versus cell cycle alterations and induction of apoptosis initiated by viscum, TT and viscumTT, but might depend on cell line and their TP53 status.

4.4 ViscumTT activates stress-induced MAPK8 and inactivates survival-associated MAPK1/3 pathway

Regarding the above mentioned mechanisms, GADD45A directly interacts with MAP3K4 (MEKK4) and initiates MAPK14/MAPK8 pathway in environmental stress response [252]. As central regulation cascade, MAPK pathways are involved in similar cellular processes, including proliferation, differentiation, survival, apoptosis, and stress response. Key players are three kinases that activate sequential kinase within the cascade and finally the regulatory target protein. Four mammalian MAPK components are known, named MAPK1/MAPK3, MAPK8, MAPK14, and MAPK7 (ERK5). In this present work, activation of MAPK1/MAPK3 and MAPK8 were investigated after viscum, TT and viscumTT treatment. MAPK1/3 is mainly involved in proliferation and survival, whereby MAPK8 is initiated after several stress signals, such as ER stress [253], oxidative stress [254] irradiation [255], and DNA damage [256]. Several studies demonstrated a MAPK8 pathway activation after mistletoe treatment with various extracts [57, 193, 257], which is in line with present results. Viscum and viscumTT strongly activated (phosphorylated) MAPK8 in each cell line. This correlated to GADD45A expression of the extracts as discussed in the previous section.

For BA and OA also MAPK8 activation is reported certainly in controversy discussion. On the one hand, activation resulted in apoptosis [258, 259] and on the other hand, it mediated cancer cell protection by autophagy [260]. Surprisingly, activation of MAPK8 was not prevented by its inhibitor SP600125, but rather enhanced apoptosis induction was seen in TT and viscumTT-treated cells. This could be explained with the inhibition of the OA-induced autophagy effect, resulting in stronger apoptosis [260, 261]. Such enhanced apoptosis was also shown by inhibition of autophagy mediated by UA [262, 263]. Interestingly, autophagy is mainly induced in G1, less in S and never in G2/M phase when TP53 is dysfunctioned [264]. Furthermore, autophagy is induced in TT and viscumTT, but not in viscum-treated Ewing's sarcoma cells [193] and led to the assumption that apoptosis as well as autophagy are simultaneously

induced also in osteosarcoma. Viscum, TT and viscumTT dephosphorylated MAPK1/3 in U2OS, but not in TT-treated 143B. Opposing results were also shown for the activation of MAPK1/3 by OA derivative [265] or inactivation by UA [266, 267], but both induced apoptosis. TP53 plays different roles in MAPK signaling and deals with its activation as well as inactivation to maintain cell death and survival in response to DNA damage [268]. In conclusion, viscum, TT and viscumTT results suggest again TP53-independent action by involvement of MAPK1/3 and MAPK8 pathway in triggering apoptosis induction.

4.5 ViscumTT inhibits survival-associated STAT3 pathway and down-regulates its anti-apoptotic downstream targets

STAT3 is one of seven cytoplasmic transcription factors that receives signals, mainly via interleukin-6 (IL-6) and JAK2, from the cell membrane and relays them into the nucleus to regulate transcription of different genes involved, for instance, in cellular survival and proliferation. In osteosarcoma and other tumors, STAT3 is often over-expressed and constitutively activated by its phosphorylation on Tyr705 and promotes tumor growth [269, 270]. STAT3 can also be phosphorylated at Ser727, but its role is discussed controversial. Studies suggested that Ser727 phosphorylation is necessary for full activity of Tyr705-phosphorylated STAT3 [271]. It was found that MAPK1/3 and MAPK8 are responsible for that, but MAPK8 led simultaneously to its negative regulation [272]. Today, Ser727 phosphorylation seems to play a more complex role in tumorigenesis [273, 274]. In this work, STAT3 was phosphorylated at both sites in untreated control cells. Viscum, TT and viscumTT treatment dephosphorylated STAT3 at Ser727 as well as Tyr 705 with simultaneously total STAT3 degradation. A study with gastric cancer cells has shown that depletion of STAT3 simultaneously led to SKP2 down-regulation with further increase of CDKN1A and CDKN1B [275]. Since *CCND1* is revealed as transcriptional target of STAT3, it indicates a direct crosstalk of STAT3 pathway and cell cycle progression [276]. Therefore, strong down-regulation of STAT3 correlated with those of SKP2 and confirmed targeting STAT3 by viscum and viscumTT. Down-regulation of STAT3 was previously described for a fermented commercial *Viscum album* preparation in gliomas [277] and for OA in colorectal cancer [278]. Furthermore, STAT3 down-stream targets, MYC and BIRC5, were also decreased in this study. Moreover, down-regulation of BIRC5 was recently published for AML and Ewing's sarcoma [164, 173]. IAP family member BIRC5 is often over-expressed in several cancer types and is associated with chemotherapy resistance and poor prognosis [279]. MYC over-expression in osteosarcoma is associated with cell invasion [280], but promotes survival depending on E2F transcription factor activity [281]. Taken together, viscum, TT and viscumTT prevented the STAT3-mediated survival benefit with further depletion of BIRC5 and MYC. Due

to their negative-associated function in tumor development when overexpressed, they represent additional interesting targets for treating osteosarcoma.

4.6 ViscumTT is more effective in Saos-2 xenograft than its single extracts

Observed anti-tumoral effects of viscum, TT and viscumTT *in vitro* were confirmed *in vivo* as tumor volume was significantly reduced. The high effective potential *in vivo* of these extracts was multiple shown in Ewing's sarcoma, leukemia, and murine melanoma [164, 173, 282]. In an AML study viscumTT has shown a similar therapeutic effectiveness like cytarabine, a standard chemotherapeutic drug in leukemia treatment. The single effects of both treatments were additionally enhanced when combined administered [164]. On the other hand, viscumTT application either i.t. or i.v. showed no significant difference in Ewing's sarcoma xenograft and was nearly as effective as Doxo [173]. In contrast to another study with rhabdomyosarcoma patient-derived xenografts as viscumTT showed higher effectiveness when administered i.t. than i.v.. However, in the same study, an additional reduction of tumor volume was seen for viscumTT compared to viscum or TT single treatment, but Doxo had no effect [184]. In this present work, a significant stronger reduction of tumor volume was observed for viscumTT to control and viscum group and confirmed higher therapeutic effectiveness of viscumTT also *in vivo*.

However, implementation of mice experiments can be assessed critical. On the one hand, calculation of tumor volume is less meaningful than tumor weight, because of subjective measurement and non-invasive technics. On the other hand, tumor volume should be approved by endpoint histological stainings that correlate in best case with assessed tumor volume. Unfortunately, neither decreased MKI67 nor increased CASP3 activity were significantly observed, which was also seen in CASP3 activity results in murine melanoma study [282]. Mills *et al.* analyzed different sarcoma cell lines, including proliferative and invasive behavior of Saos-2 cells *in vitro* and *in vivo,* respectively. Interestingly, they documented that classical *in vitro* markers like MKI67 do not correlate with histopathology in s.c. xenografts [283]. Consequently, MKI67 seems to be a poor marker for assessment of Saos-2 proliferation in xenografts. In addition to this, seemingly i.t. application vastly influences tissue, which could explain the bad tissue texture and difficulties to stain the samples. Also, the relatively high amount of necrotic areas and the positive cleaved CASP3 staining in control group could result from this route of administration. Moreover, this study should not to be interpreted as evident result due to the small and unequal sample size (eight mice in control and five mice in each treatment group).

Nevertheless, the *in vivo* results indicated a high anti-tumoral effectiveness of viscum and TT that was additionally enhanced by viscumTT with regard to the remarkable reduction of tumor

volume and good tolerability as potential therapy for osteosarcoma considering poor chosen histological markers.

4.7 The whole context – a hypothesis for synergistic action of viscumTT and its high potential as new therapeutic approach in osteosarcoma

To conclude the results of this work, the high potential of a whole mistletoe extract, viscumTT, combined the advantages of its single extracts thus enhanced their anti-tumoral effects and led to a higher efficiency in osteosarcoma.

Studies with recombinant ML I and Korean mistletoe extract described a TP53-independent induced apoptosis [53, 55]. In this present work, *TP53* mutant (143B) and null mutant (Saos-2) cells were more resistant to viscum treatment than wild-type (U2OS) cells, regarding to induction of apoptosis, but not to inhibition of proliferation. Reasons for TP53-independent mechanism of action of mistletoe extracts were revealed when *TP53* mutant and null mutant cells were able to arrest in G1 phase and when distinct down-regulation of TP53 was detected in wild-type and mutant cells. In healthy cells TP53 is low expressed, possesses a half-life of a few minutes, but is rapidly transcriptionally induced and increasingly translated in response to DNA damage or stress signals. Then TP53 is stabilized and mediates its function mainly at protein level by transcriptional regulation of target genes or protein-protein interactions [284]. Since 143B cells express non-functional TP53 and Saos-2 cells are TP53 deficient [169], down-stream regulation by TP53 does not occur. However, a wild-type TP53 participation in induction of apoptosis and cell cycle alterations by viscum, TT and viscumTT could not be completely excluded, but its function was not essential in this case.

The observation that the inhibition of MAPK8 led to stronger apoptosis in TT and viscumTT, but not in viscum-treated cells, opened new insights of a possible synergistic mechanism of viscumTT. Since synergistic and antagonistic effects of co-treatments resulted from same or related targets in the same or related pathways, the dual role of MAPK8 activation was of particular interest. Consequently, it can be hypothesized that viscum is able to shift the balance between autophagy and apoptosis towards apoptosis or even functions as inhibitor of autophagy in the context of TT and lead consequently to synergistic induction of apoptosis in MAPK8-dependent mechanism. Different observations gave notes for this assumption.

First, MAPK8 was induced by viscum and viscumTT, but less by TT in *TP53* wild-type and mutant cells and was also involved in the induction of both, autophagy and apoptosis [285]. Triterpene acids, especially UA via phosphorylation of MAPK8, are able to trigger autophagy as well as apoptosis [286]. Furthermore, MAPK8 could not be inhibited in TT or viscumTT-treated cells, rather stronger apoptosis was induced instead. Inhibition of OA-induced autophagy mediated by MAPK8 resulted in stronger apoptosis induction [260]. In this case,

viscum could possess a role as autophagy inhibitor, which might be the reason for viscumTT-mediated action.

Second, *CDKN1A* knockdown was also associated with enhanced induction of apoptosis in TT-treated *TP53* wild-type cells. Some studies have demonstrated that a higher *CDKN1A* expression resulted in autophagy and by its inhibition apoptosis was induced [287, 288]. Additionally, apoptosis after knockdown of CDKN1A was caspase- and MAPK8-dependent [289]. The initiation of autophagy in Ewing's sarcoma cell line after TT and viscumTT, but not after viscum treatment [193], supported the hypothesis that autophagy and apoptosis are similarly triggered after viscumTT treatment. It seemed that viscumTT unifies an inducer of apoptosis (viscum, TT) and an inhibitor or suppressor of autophagy (viscum), which consequently results in synergistic induction of apoptosis. In this case, viscum could shift the balance between apoptosis and autophagy towards apoptosis with MAPK8 as key regulator (Figure 29).

Other molecular events that were found in this work demonstrated the multiple targets in viscumTT treatment. Up-regulation of *GADD45A* and *CDKN1A* seemed to be central players in viscumTT mechanism of action. Additionally, expression of *GADD45A* was also associated with sensitizing osteosarcoma cells to chemotherapeutic drugs [290] and medulloblastoma cells to irradiation [291]. Furthermore, viscumTT-mediated functions were accompanied by inactivation of constitutively activated survival pathways, such as STAT3 and MAPK1/3. The down-regulation of often over-expressed and with poor prognosis associated proteins, such as BIRC5, MYC, and SKP2 revealed viscumTT-possessed high potential in more detail. Additionally, its strong influence on the cell cycle, its synergistic apoptotic effect, its non-essential requirement of TP53 in mechanism of action as well as its good therapeutic effectiveness *in vivo* qualify viscumTT as promising therapeutic approach in pediatric osteosarcoma patients.

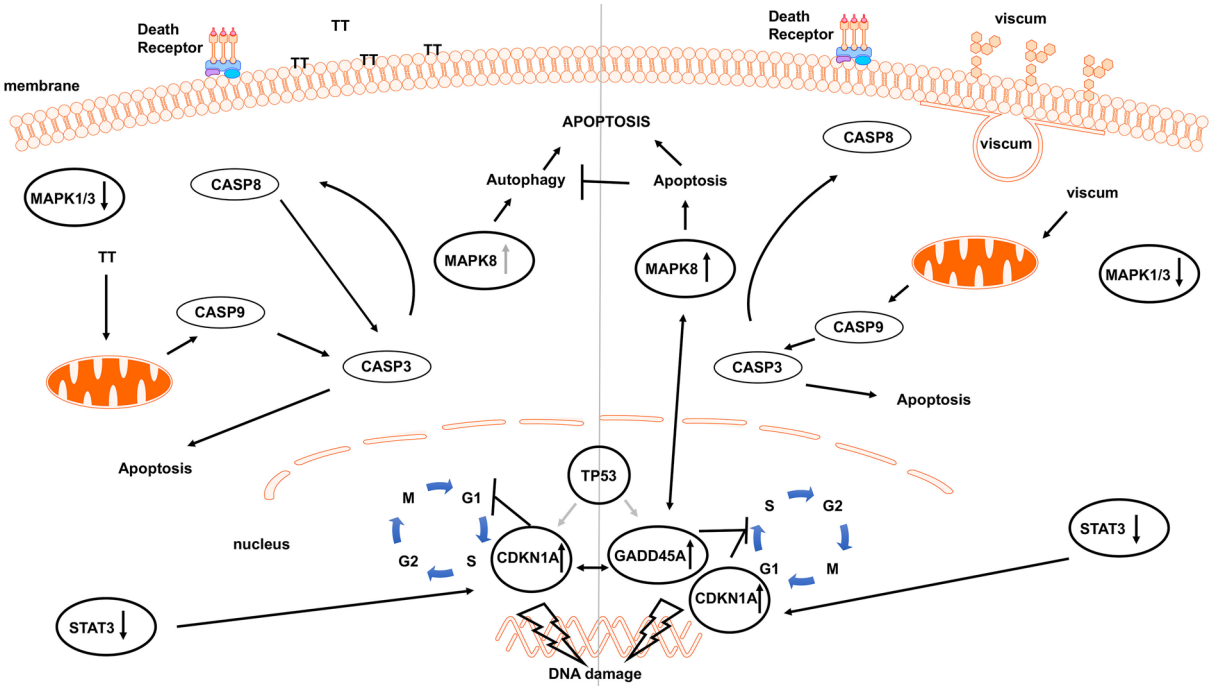


Figure 29 Possible synergistic mechanism of action of viscumTT. Viscum (ML I) binds with its B chain to D-galactose and is internalized by endocytosis. The uptake of TT is mediated by unknown mechanism, but it is able to embed into membranes. Inside the cell both, viscum and TT, initiate cell cycle arrest, inhibition of proliferation and induction of apoptosis via involvement of CDKN1A, GADD45A, CASP8, -9, -3, STAT3 and MAPK pathways. TT may lead also to autophagy whereas viscum induce apoptosis via MAPK8 as key regulator of mechanism of action of viscumTT. Finally, TT functions as an autophagy inducer whereas viscum inhibits this effect, whereby apoptosis is enhanced. Black arrows: activation, up black arrows in circle: up-regulation, down arrows in circle: down-regulation, grey arrows: non-essential role (TP53), up grey arrows in circle: low activation (MAPK8), blocked line: inhibition, double-sided arrow: interaction.

5. Outlook

This present work raised many further questions for future investigations. A central point should be the based mechanism of synergistic effect of viscumTT. It has to be analyzed whether induction of autophagy also occurs in osteosarcoma cells by TT and viscumTT. Further inhibition, knockdown, knockout or over-expression experiments should be carried out to understand the dual role of MAPK8 and to support the hypothesis viscum as inhibitor of autophagy. Therefore, examinations of MAPK8 should be intensified, especially its role in viscumTT-mediated action regarding its activation sites. It is also of importance to understand the function of the single extracts viscum and TT and which affected targets are further involved in MAPK8 activation. Also, examinations of other cell death forms besides apoptosis have to be elucidated in the future. A deeper role of TP53 participation in viscum, TT and viscumTT-initiated processes should be in focus of prospective studies.

For better assessment of therapeutic effectiveness an additional osteosarcoma mice experiment with more mice per group should be performed. Therefore, an alternative route of administration to i.t., for instance, i.v. should be used to include a systemic effect. This would also be an advantage for improvement of the tissue texture. Additionally, other and better histological markers have to be used to confirm actual high therapeutic potential *in vivo*.

6. Supplementary Data

6.1 Viscum, TT and viscumTT do not affect healthy fibroblastic VH7 cells

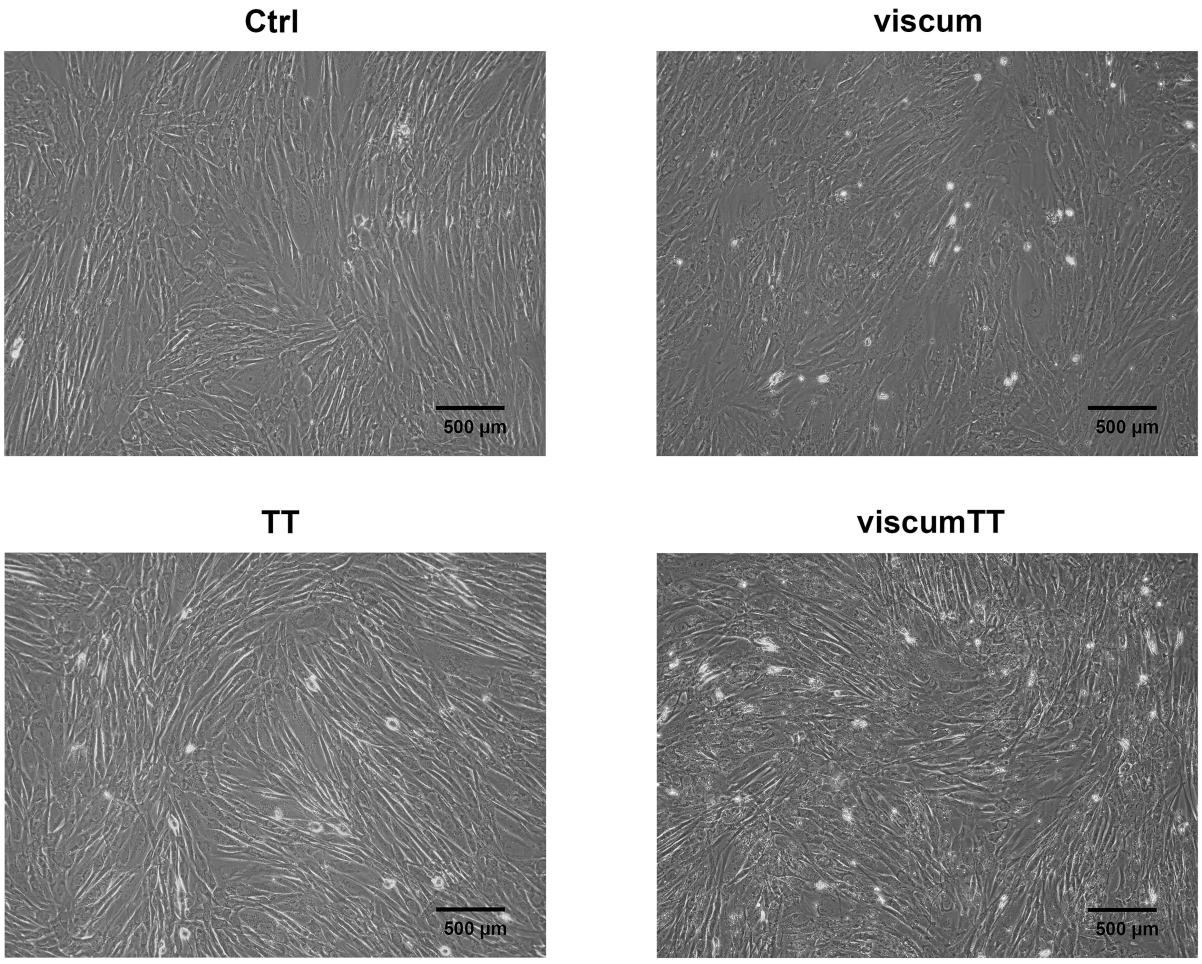


Figure S1a Morphological changes in VH7 healthy fibroblastic cells. Cells were incubated with highest viscum (ML 10 ng/mL), TT (OA 60 µg/mL) and viscumTT (10 ng/mL+60 µg/mL) concentrations for 24 h and were evaluated under light microscope.

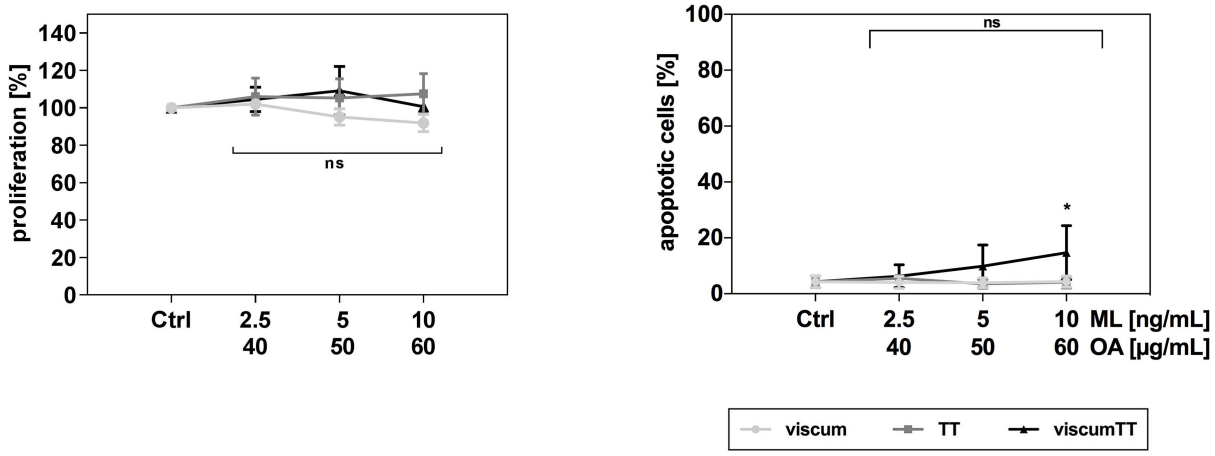


Figure S1b Viscum, TT and viscumTT neither inhibit proliferation nor induce apoptosis in VH7 cells. Cells were treated with increasing concentrations of viscum, TT and viscumTT for 24 h and analyzed either by WST-1 proliferation reagent (left) or stained by Annexin V/PI (right). Graphs display mean percentages of cells ± SD of at least three independent experiments. Significance is indicated with *p≤0.05 related to control; ns, non-significant.

6.2 TT-mediated apoptosis is based on active compounds oleanolic and betulinic acid

The apoptotic effect of TT is based on the main active compounds, OA and BA. In order to this, induction of apoptosis was analyzed in U2OS and 143B cells regarding to OA and BA standards, the combination thereof as well as combined with viscum and compared to TT. OA as well as BA standards were applied in concentrations as included in TT. After the treatment of 24 h, cells were stained with Annexin V/PI and analyzed by flow cytometry. OA led to stronger induction of apoptosis than TT, but was dose-dependent in both cell lines. The treatment of OA at 60 µg/mL showed 69 % (U2OS) and 75.6 % (143B) apoptotic cells, whereas BA at 5 µg/mL had no apoptotic potential (Figure S2 above). Surprisingly, when OA was combined with BA, induction of apoptosis was strongly reduced by almost 50 %. On the other hand, TT was able to trigger approximtately 50 % apoptosis in comparison to the standards at same concentrations. The combinatorial treatment of OA at 60 µg/mL and viscum at 10 ng/mL ML reached 80 %, whereas OA and BA at 5 ng/mL in combination with viscum only led to 30 % apoptotic cells (Figure S2 below). However, viscumTT led to approximately 80 % apoptosis in 143B cells and in U2OS cells viscum plus the standard substances had a slightly stronger effect than viscumTT. These results indicated that OA and BA are responsible for apoptotic effect of TT although single treatment of OA was more potent than OA and BA in combination. Nevertheless, viscumTT was more effective than viscum combined with OA and BA in 143B cells.

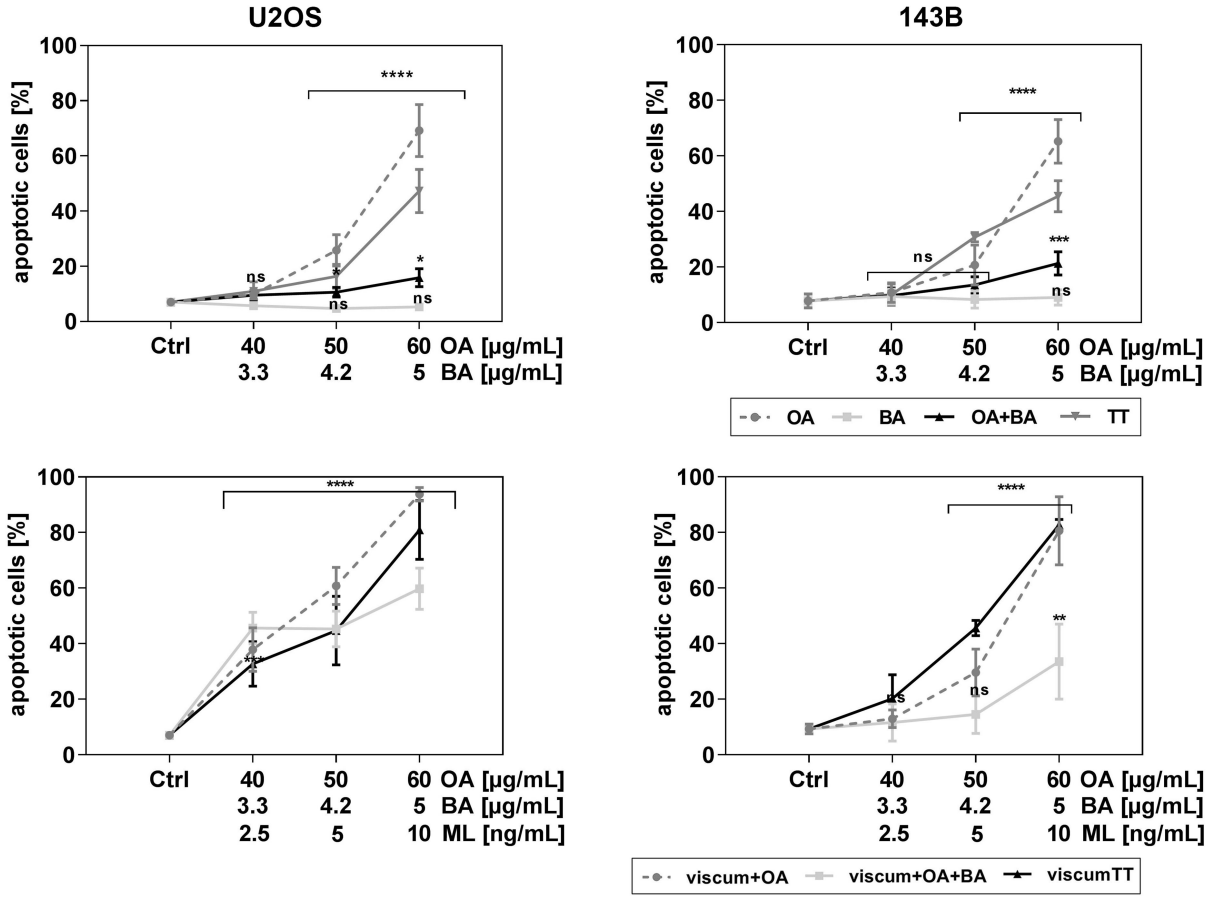


Figure S2a TT-induced apoptosis is predominantly mediated by OA. U2OS and 143B cells were incubated with OA and BA in concentrations as included in TT for 24 h (above). Cells were additionally treated with viscum for 24 h (below). Mean percentage ± SD of apoptotic cells of at least three independent experiments are presented. Significant results are indicated: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.001 between treated cells related to untreated control (Ctrl); ns, non-significant.

In order to TT-containing main compounds, OA and BA, apoptotic potential was tested with their standards. Contrary to our expectations, OA itself induced apoptosis stronger than the combination with BA. The amount of BA is to less for induction of apoptosis, but certainly sufficient to prevent OA effectiveness. Contrarily, combination of OA and UA synergizes the inhibition of proliferation in melanoma cells [292]. However, triterpene acids, including OA, BA and UA possess autophagic and apoptotic properties [261, 293]. Since autophagy is discussed controversial due to its balanced role in protecting cells by preventing apoptosis or promoting cell death [294], antagonistic action by combined OA and BA treatment could be explained. Autophagy promotor rapamycin enhanced the number of OA-mediated autophagosomes, compromised its apoptotic effect and increased cell viability [295]. Potze *et al.* have been shown an autophagic rescue function already at low BA concentrations (<7.5 µg/mL) and autophagy as well as apoptosis was blocked by inhibition of MOMP in HeLa and MCF-7 cells [296]. Additionally, BA was able to trigger apoptotis by inhibition of autophagy in myeloma cells

at higher concentration (20 µg/mL) [297], leading to assumption underlining decision between cell survival and cell death is dose-dependent. Therefore, autophagy keeps the balance in a dose-dependent manner until reached threshold shifting to apoptosis. Consequently, low dose BA may prevented induction of apoptosis by OA causing enhanced autophagic potential. Nevertheless, TT induced stronger apoptosis than combined OA and BA indicating additional substances within the TT extract. Hence, autophagic effect was attenuated, which was further enhanced by viscum and finally results in viscumTT effect.

The lower effectiveness of TT in comparison to OA standard could be further explain due to TT manufactured from mistletoe herb used in this work (TT154). Comparative data revealed higher apoptotic potential by TT extract produced from one-year new shoots (TT167, TT172) are more potent than TT lots from mistletoe herb (Figure S2b).

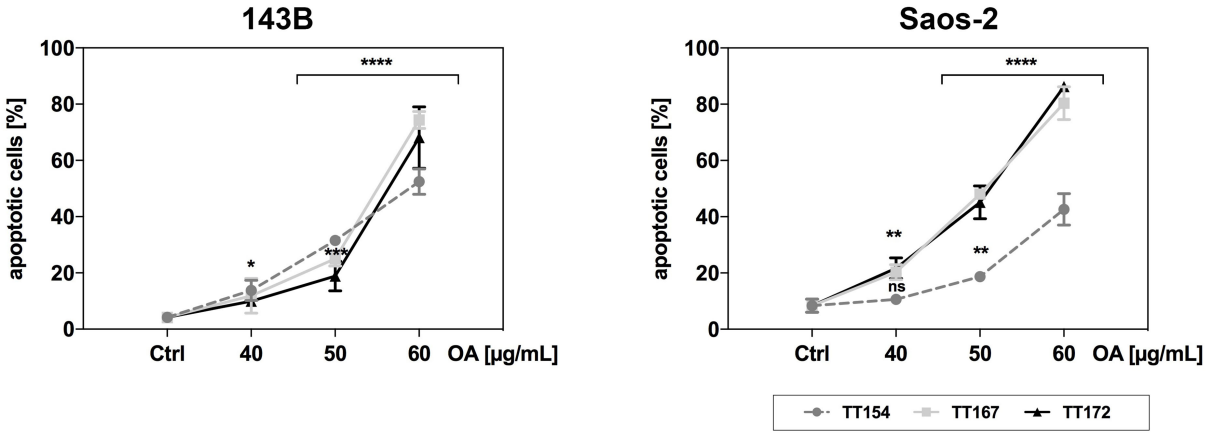


Figure S2b TT from one-year new shoots is more effective than TT from mistletoe herb. 143B and Saos-2 cells were incubated with different TT lots (154, 167, 172) and apoptosis was analyzed by Annexin V/PI staining and flow cytometry. TT154 was produced from mistletoe herb and TT167, 172 was provided by one-year old new shoots. Mean percentage ± SD of apoptotic cells of at least three independent experiments are presented. Significant results are indicated: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.001 between treated cells related to untreated control (Ctrl); ns, non-significant.

6.3 Viscum, TT and viscumTT significantly enhance chemotherapeutic drug treatment

Table S1a P-values of co-treatment with Doxo of U2OS cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. Doxo	-7.414	-18.32 to 3.496	No	ns	0.4209
Ctrl vs. viscum	-9.33	-20.65 to 1.992	No	ns	0.1872
Ctrl vs. viscum+Doxo	-13.09	-24.41 to -1.768	Yes	*	0.0119

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Ctrl vs. TT	-2.993	-14.32 to 8.328	No	ns	0.9918
Ctrl vs. TT+Doxo	-9.542	-20.86 to 1.78	No	ns	0.1654
Ctrl vs. viscumTT	-21.79	-34.45 to -9.132	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-30.96	-42.28 to -19.63	Yes	****	<0.0001
Doxo vs. viscum	-1.916	-12.83 to 8.994	No	ns	0.9994
Doxo vs. viscum+Doxo	-5.676	-16.59 to 5.234	No	ns	0.7442
Doxo vs. TT	4.421	-6.489 to 15.33	No	ns	0.9137
Doxo vs. TT+Doxo	-2.127	-13.04 to 8.782	No	ns	0.9988
Doxo vs. viscumTT	-14.38	-26.67 to -2.085	Yes	*	0.0105
Doxo vs. viscumTT+Doxo	-23.54	-34.45 to -12.63	Yes	****	<0.0001
viscum vs. viscum+Doxo	-3.76	-15.08 to 7.562	No	ns	0.9693
viscum vs. TT	6.337	-4.985 to 17.66	No	ns	0.6675
viscum vs. TT+Doxo	-0.2117	-11.53 to 11.11	No	ns	>0.9999
viscum vs. viscumTT	-12.46	-25.12 to 0.198	No	ns	0.057
viscum vs. viscumTT+Doxo	-21.63	-32.95 to -10.3	Yes	****	<0.0001
viscum+Doxo vs. TT	10.1	-1.225 to 21.42	No	ns	0.1173
viscum+Doxo vs. TT+Doxo	3.548	-7.773 to 14.87	No	ns	0.9778
viscum+Doxo vs. viscumTT	-8.7	-21.36 to 3.958	No	ns	0.4059
viscum+Doxo vs. viscumTT+Doxo	-17.87	-29.19 to -6.545	Yes	****	<0.0001
TT vs. TT+Doxo	-6.548	-17.87 to 4.773	No	ns	0.6293
TT vs. viscumTT	-18.8	-31.45 to -6.139	Yes	***	0.0003
TT vs. viscumTT+Doxo	-27.96	-39.29 to -16.64	Yes	****	<0.0001
TT+Doxo vs. viscumTT	-12.25	-24.91 to 0.4097	No	ns	0.0654
TT+Doxo vs. viscumTT+Doxo	-21.42	-32.74 to -10.09	Yes	****	<0.0001
viscumTT vs. viscumTT+Doxo	-9.167	-21.82 to 3.491	No	ns	0.3374
Row 2					
Ctrl vs. Doxo	-7.414	-18.32 to 3.496	No	ns	0.4209
Ctrl vs. viscum	-25.47	-37.34 to -13.59	Yes	****	<0.0001
Ctrl vs. viscum+Doxo	-32.47	-45.13 to -19.81	Yes	****	<0.0001
Ctrl vs. TT	-4.595	-15.92 to 6.727	No	ns	0.9131
Ctrl vs. TT+Doxo	-12.56	-23.89 to -1.242	Yes	*	0.0187
Ctrl vs. viscumTT	-49.72	-63.59 to -35.85	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-68.07	-81.94 to -54.2	Yes	****	<0.0001
Doxo vs. viscum	-18.05	-29.53 to -6.569	Yes	***	0.0001
Doxo vs. viscum+Doxo	-25.05	-37.34 to -12.76	Yes	****	<0.0001
Doxo vs. TT	2.819	-8.091 to 13.73	No	ns	0.9928
Doxo vs. TT+Doxo	-5.149	-16.06 to 5.761	No	ns	0.827
Doxo vs. viscumTT	-42.31	-55.84 to -28.77	Yes	****	<0.0001
Doxo vs. viscumTT+Doxo	-60.66	-74.19 to -47.12	Yes	****	<0.0001
viscum vs. viscum+Doxo	-7.002	-20.16 to 6.153	No	ns	0.7216
viscum vs. TT	20.87	8.997 to 32.75	Yes	****	<0.0001
viscum vs. TT+Doxo	12.9	1.028 to 24.78	Yes	*	0.0232
viscum vs. viscumTT	-24.25	-38.57 to -9.933	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum vs. viscumTT+Doxo	-42.6	-56.92 to -28.28	Yes	****	<0.0001
viscum+Doxo vs. TT	27.87	15.21 to 40.53	Yes	****	<0.0001
viscum+Doxo vs. TT+Doxo	19.9	7.246 to 32.56	Yes	***	0.0001
viscum+Doxo vs. viscumTT	-17.25	-32.23 to -2.275	Yes	*	0.0125
viscum+Doxo vs. viscumTT+Doxo	-35.6	-50.58 to -20.63	Yes	****	<0.0001
TT vs. TT+Doxo	-7.968	-19.29 to 3.353	No	ns	0.3745
TT vs. viscumTT	-45.13	-58.99 to -31.26	Yes	****	<0.0001
TT vs. viscumTT+Doxo	-63.48	-77.34 to -49.61	Yes	****	<0.0001
TT+Doxo vs. viscumTT	-37.16	-51.02 to -23.29	Yes	****	<0.0001
TT+Doxo vs. viscumTT+Doxo	-55.51	-69.37 to -41.64	Yes	****	<0.0001
viscumTT vs. viscumTT+Doxo	-18.35	-34.36 to -2.339	Yes	*	0.0132
Row 3					
Ctrl vs. Doxo	-7.414	-18.32 to 3.496	No	ns	0.4209
Ctrl vs. viscum	-45.2	-57.08 to -33.33	Yes	****	<0.0001
Ctrl vs. viscum+Doxo	-46.38	-58.25 to -34.5	Yes	****	<0.0001
Ctrl vs. TT	-8.613	-19.94 to 2.708	No	ns	0.2759
Ctrl vs. TT+Doxo	-17.78	-29.1 to -6.455	Yes	***	0.0001
Ctrl vs. viscumTT	-76.78	-88.66 to -64.91	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-83.67	-96.33 to -71.01	Yes	****	<0.0001
Doxo vs. viscum	-37.79	-49.27 to -26.31	Yes	****	<0.0001
Doxo vs. viscum+Doxo	-38.96	-50.45 to -27.48	Yes	****	<0.0001
Doxo vs. TT	-1.199	-12.11 to 9.711	No	ns	>0.9999
Doxo vs. TT+Doxo	-10.36	-21.27 to 0.5475	No	ns	0.0754
Doxo vs. viscumTT	-69.37	-80.85 to -57.89	Yes	****	<0.0001
Doxo vs. viscumTT+Doxo	-76.26	-88.55 to -63.97	Yes	****	<0.0001
viscum vs. viscum+Doxo	-1.174	-13.58 to 11.23	No	ns	>0.9999
viscum vs. TT	36.59	24.72 to 48.46	Yes	****	<0.0001
viscum vs. TT+Doxo	27.43	15.55 to 39.3	Yes	****	<0.0001
viscum vs. viscumTT	-31.58	-43.98 to -19.18	Yes	****	<0.0001
viscum vs. viscumTT+Doxo	-38.47	-51.62 to -25.31	Yes	****	<0.0001
viscum+Doxo vs. TT	37.76	25.89 to 49.64	Yes	****	<0.0001
viscum+Doxo vs. TT+Doxo	28.6	16.73 to 40.48	Yes	****	<0.0001
viscum+Doxo vs. viscumTT	-30.41	-42.81 to -18	Yes	****	<0.0001
viscum+Doxo vs. viscumTT+Doxo	-37.29	-50.45 to -24.14	Yes	****	<0.0001
TT vs. TT+Doxo	-9.163	-20.49 to 2.158	No	ns	0.2058
TT vs. viscumTT	-68.17	-80.04 to -56.3	Yes	****	<0.0001
TT vs. viscumTT+Doxo	-75.06	-87.72 to -62.4	Yes	****	<0.0001
TT+Doxo vs. viscumTT	-59.01	-70.88 to -47.13	Yes	****	<0.0001
TT+Doxo vs. viscumTT+Doxo	-65.9	-78.55 to -53.24	Yes	****	<0.0001
viscumTT vs. viscumTT+Doxo	-6.888	-20.04 to 6.266	No	ns	0.7379

Table S1b P-values of co-treatment with VP16 of U2OS cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. VP16	-5.298	-17.23 to 6.638	No	ns	0.8674
Ctrl vs. viscum	-9.33	-21.27 to 2.606	No	ns	0.2439
Ctrl vs. viscum+VP16	-16.59	-28.52 to -4.651	Yes	***	0.001
Ctrl vs. TT	-2.993	-14.93 to 8.943	No	ns	0.994
Ctrl vs. TT+VP16	-11.34	-23.86 to 1.179	No	ns	0.1057
Ctrl vs. viscumTT	-21.79	-35.13 to -8.445	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-34.83	-46.76 to -22.89	Yes	****	<0.0001
VP16 vs. viscum	-4.032	-15.97 to 7.904	No	ns	0.9663
VP16 vs. viscum+VP16	-11.29	-23.22 to 0.6476	No	ns	0.0778
VP16 vs. TT	2.305	-9.631 to 14.24	No	ns	0.9988
VP16 vs. TT+VP16	-6.042	-18.56 to 6.477	No	ns	0.8097
VP16 vs. viscumTT	-16.49	-29.84 to -3.147	Yes	**	0.0053
VP16 vs. viscumTT+VP16	-29.53	-41.46 to -17.59	Yes	****	<0.0001
viscum vs. viscum+VP16	-7.257	-19.19 to 4.679	No	ns	0.5673
viscum vs. TT	6.337	-5.599 to 18.27	No	ns	0.7238
viscum vs. TT+VP16	-2.01	-14.53 to 10.51	No	ns	0.9997
viscum vs. viscumTT	-12.46	-25.8 to 0.8848	No	ns	0.0855
viscum vs. viscumTT+VP16	-25.5	-37.43 to -13.56	Yes	****	<0.0001
viscum+VP16 vs. TT	13.59	1.657 to 25.53	Yes	*	0.0142
viscum+VP16 vs. TT+VP16	5.247	-7.272 to 17.77	No	ns	0.8983
viscum+VP16 vs. viscumTT	-5.203	-18.55 to 8.141	No	ns	0.9285
viscum+VP16 vs. viscumTT+VP16	-18.24	-30.18 to -6.304	Yes	***	0.0002
TT vs. TT+VP16	-8.347	-20.87 to 4.172	No	ns	0.4461
TT vs. viscumTT	-18.8	-32.14 to -5.452	Yes	***	0.0008
TT vs. viscumTT+VP16	-31.83	-43.77 to -19.9	Yes	****	<0.0001
TT+VP16 vs. viscumTT	-10.45	-24.32 to 3.418	No	ns	0.2873
TT+VP16 vs. viscumTT+VP16	-23.49	-36.01 to -10.97	Yes	****	<0.0001
viscumTT vs. viscumTT+VP16	-13.04	-26.38 to 0.3082	No	ns	0.0606
Row 2					
Ctrl vs. VP16	-5.298	-17.23 to 6.638	No	ns	0.8674
Ctrl vs. viscum	-25.47	-37.98 to -12.95	Yes	****	<0.0001
Ctrl vs. viscum+VP16	-30.14	-43.49 to -16.8	Yes	****	<0.0001
Ctrl vs. TT	-4.595	-16.53 to 7.341	No	ns	0.9329
Ctrl vs. TT+VP16	-12.12	-24.05 to -0.179	Yes	*	0.044
Ctrl vs. viscumTT	-49.72	-64.34 to -35.1	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-57.85	-70.37 to -45.33	Yes	****	<0.0001
VP16 vs. viscum	-20.17	-32.69 to -7.649	Yes	****	<0.0001
VP16 vs. viscum+VP16	-24.84	-38.19 to -11.5	Yes	****	<0.0001
VP16 vs. TT	0.7033	-11.23 to 12.64	No	ns	>0.9999
VP16 vs. TT+VP16	-6.817	-18.75 to 5.119	No	ns	0.6439
VP16 vs. viscumTT	-44.42	-59.04 to -29.8	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
VP16 vs. viscumTT+VP16	-52.55	-65.07 to -40.03	Yes	****	<0.0001
viscum vs. viscum+VP16	-4.677	-18.54 to 9.192	No	ns	0.9666
viscum vs. TT	20.87	8.352 to 33.39	Yes	****	<0.0001
viscum vs. TT+VP16	13.35	0.8324 to 25.87	Yes	*	0.0279
viscum vs. viscumTT	-24.25	-39.35 to -9.156	Yes	****	<0.0001
viscum vs. viscumTT+VP16	-32.38	-45.46 to -19.31	Yes	****	<0.0001
viscum+VP16 vs. TT	25.55	12.2 to 38.89	Yes	****	<0.0001
viscum+VP16 vs. TT+VP16	18.03	4.683 to 31.37	Yes	**	0.0015
viscum+VP16 vs. viscumTT	-19.58	-35.37 to -3.788	Yes	**	0.0051
viscum+VP16 vs. viscumTT+VP16	-27.71	-41.57 to -13.84	Yes	****	<0.0001
TT vs. TT+VP16	-7.52	-19.46 to 4.416	No	ns	0.5212
TT vs. viscumTT	-45.13	-59.74 to -30.51	Yes	****	<0.0001
TT vs. viscumTT+VP16	-53.25	-65.77 to -40.73	Yes	****	<0.0001
TT+VP16 vs. viscumTT	-37.61	-52.22 to -22.99	Yes	****	<0.0001
TT+VP16 vs. viscumTT+VP16	-45.73	-58.25 to -33.21	Yes	****	<0.0001
viscumTT vs. viscumTT+VP16	-8.128	-23.23 to 6.97	No	ns	0.7094
Row 3					
Ctrl vs. VP16	-5.298	-17.23 to 6.638	No	ns	0.8674
Ctrl vs. viscum	-45.2	-57.72 to -32.69	Yes	****	<0.0001
Ctrl vs. viscum+VP16	-56.26	-70.88 to -41.64	Yes	****	<0.0001
Ctrl vs. TT	-8.613	-20.55 to 3.323	No	ns	0.3416
Ctrl vs. TT+VP16	-17.15	-29.08 to -5.211	Yes	***	0.0006
Ctrl vs. viscumTT	-76.78	-89.3 to -64.27	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-77.47	-89.4 to -65.53	Yes	****	<0.0001
VP16 vs. viscum	-39.91	-52.42 to -27.39	Yes	****	<0.0001
VP16 vs. viscum+VP16	-50.96	-65.58 to -36.34	Yes	****	<0.0001
VP16 vs. TT	-3.315	-15.25 to 8.621	No	ns	0.9889
VP16 vs. TT+VP16	-11.85	-23.78 to 0.08764	No	ns	0.0532
VP16 vs. viscumTT	-71.49	-84 to -58.97	Yes	****	<0.0001
VP16 vs. viscumTT+VP16	-72.17	-84.1 to -60.23	Yes	****	<0.0001
viscum vs. viscum+VP16	-11.06	-26.15 to 4.042	No	ns	0.3229
viscum vs. TT	36.59	24.07 to 49.11	Yes	****	<0.0001
viscum vs. TT+VP16	28.06	15.54 to 40.58	Yes	****	<0.0001
viscum vs. viscumTT	-31.58	-44.66 to -18.5	Yes	****	<0.0001
viscum vs. viscumTT+VP16	-32.26	-44.78 to -19.74	Yes	****	<0.0001
viscum+VP16 vs. TT	47.65	33.03 to 62.27	Yes	****	<0.0001
viscum+VP16 vs. TT+VP16	39.11	24.49 to 53.73	Yes	****	<0.0001
viscum+VP16 vs. viscumTT	-20.52	-35.62 to -5.426	Yes	**	0.0014
viscum+VP16 vs. viscumTT+VP16	-21.21	-35.82 to -6.586	Yes	***	0.0005
TT vs. TT+VP16	-8.533	-20.47 to 3.403	No	ns	0.3537
TT vs. viscumTT	-68.17	-80.69 to -55.65	Yes	****	<0.0001
TT vs. viscumTT+VP16	-68.85	-80.79 to -56.92	Yes	****	<0.0001
TT+VP16 vs. viscumTT	-59.64	-72.16 to -47.12	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
TT+VP16 vs. viscumTT+VP16	-60.32	-72.25 to -48.38	Yes	****	<0.0001
viscumTT vs. viscumTT+VP16	-0.681	-13.2 to 11.84	No	ns	>0.9999

Table S1c P-values of co-treatment with 4-OOH of U2OS cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. 4-OOH	-3.066	-15.04 to 8.908	No	ns	0.9931
Ctrl vs. viscum	-9.33	-20.75 to 2.086	No	ns	0.1947
Ctrl vs. viscum+4OOH	-18.13	-30.89 to -5.361	Yes	***	0.0007
Ctrl vs. TT	-2.993	-14.41 to 8.423	No	ns	0.992
Ctrl vs. TT+4OOH	-5.695	-17.11 to 5.721	No	ns	0.7805
Ctrl vs. viscumTT	-21.79	-34.55 to -9.026	Yes	****	<0.0001
Ctrl vs. viscumTT+4OOH	-22.15	-36.13 to -8.165	Yes	****	<0.0001
4-OOH vs. viscum	-6.264	-18.24 to 5.71	No	ns	0.7365
4-OOH vs. viscum+4OOH	-15.06	-28.32 to -1.794	Yes	*	0.0148
4-OOH vs. TT	0.07267	-11.9 to 12.05	No	ns	>0.9999
4-OOH vs. TT+4OOH	-2.629	-14.6 to 9.345	No	ns	0.9973
4-OOH vs. viscumTT	-18.72	-31.99 to -5.459	Yes	***	0.0008
4-OOH vs. viscumTT+4OOH	-19.08	-33.52 to -4.64	Yes	**	0.0022
viscum vs. viscum+4OOH	-8.795	-21.56 to 3.969	No	ns	0.4003
viscum vs. TT	6.337	-5.08 to 17.75	No	ns	0.6743
viscum vs. TT+4OOH	3.635	-7.781 to 15.05	No	ns	0.9753
viscum vs. viscumTT	-12.46	-25.22 to 0.3038	No	ns	0.0609
viscum vs. viscumTT+4OOH	-12.82	-26.8 to 1.165	No	ns	0.0972
viscum+4OOH vs. TT	15.13	2.368 to 27.9	Yes	**	0.009
viscum+4OOH vs. TT+4OOH	12.43	-0.3338 to 25.19	No	ns	0.0621
viscum+4OOH vs. viscumTT	-3.665	-17.65 to 10.32	No	ns	0.9921
viscum+4OOH vs. viscumTT+4OOH	-4.022	-19.12 to 11.08	No	ns	0.9913
TT vs. TT+4OOH	-2.702	-14.12 to 8.715	No	ns	0.9957
TT vs. viscumTT	-18.8	-31.56 to -6.033	Yes	***	0.0004
TT vs. viscumTT+4OOH	-19.15	-33.14 to -5.171	Yes	**	0.0013
TT+4OOH vs. viscumTT	-16.1	-28.86 to -3.331	Yes	**	0.0042
TT+4OOH vs. viscumTT+4OOH	-16.45	-30.43 to -2.47	Yes	**	0.0099
viscumTT vs. viscumTT+4OOH	-0.3567	-15.46 to 14.75	No	ns	>0.9999
Row 2					
Ctrl vs. 4-OOH	-3.066	-15.04 to 8.908	No	ns	0.9931
Ctrl vs. viscum	-25.47	-37.44 to -13.49	Yes	****	<0.0001
Ctrl vs. viscum+4OOH	-39.35	-52.11 to -26.58	Yes	****	<0.0001
Ctrl vs. TT	-4.595	-16.01 to 6.821	No	ns	0.9153
Ctrl vs. TT+4OOH	-10.63	-22.04 to 0.7897	No	ns	0.0872
Ctrl vs. viscumTT	-49.72	-63.7 to -35.74	Yes	****	<0.0001
Ctrl vs. viscumTT+4OOH	-74.61	-88.59 to -60.62	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
4-OOH vs. viscum	-22.4	-34.91 to -9.894	Yes	****	<0.0001
4-OOH vs. viscum+4OOH	-36.28	-49.54 to -23.01	Yes	****	<0.0001
4-OOH vs. TT	-1.529	-13.5 to 10.44	No	ns	>0.9999
4-OOH vs. TT+4OOH	-7.561	-19.53 to 4.413	No	ns	0.5162
4-OOH vs. viscumTT	-46.65	-61.09 to -32.21	Yes	****	<0.0001
4-OOH vs. viscumTT+4OOH	-71.54	-85.98 to -57.1	Yes	****	<0.0001
viscum vs. viscum+4OOH	-13.88	-27.14 to -0.6144	Yes	*	0.0336
viscum vs. TT	20.87	8.897 to 32.84	Yes	****	<0.0001
viscum vs. TT+4OOH	14.84	2.866 to 26.81	Yes	**	0.0052
viscum vs. viscumTT	-24.25	-38.69 to -9.813	Yes	****	<0.0001
viscum vs. viscumTT+4OOH	-49.14	-63.58 to -34.7	Yes	****	<0.0001
viscum+4OOH vs. TT	34.75	21.99 to 47.51	Yes	****	<0.0001
viscum+4OOH vs. TT+4OOH	28.72	15.95 to 41.48	Yes	****	<0.0001
viscum+4OOH vs. viscumTT	-10.38	-25.48 to 4.727	No	ns	0.4043
viscum+4OOH vs. viscumTT+4OOH	-35.26	-50.36 to -20.16	Yes	****	<0.0001
TT vs. TT+4OOH	-6.032	-17.45 to 5.385	No	ns	0.7267
TT vs. viscumTT	-45.13	-59.11 to -31.14	Yes	****	<0.0001
TT vs. viscumTT+4OOH	-70.01	-83.99 to -56.03	Yes	****	<0.0001
TT+4OOH vs. viscumTT	-39.09	-53.08 to -25.11	Yes	****	<0.0001
TT+4OOH vs. viscumTT+4OOH	-63.98	-77.96 to -50	Yes	****	<0.0001
viscumTT vs. viscumTT+4OOH	-24.89	-41.03 to -8.742	Yes	***	0.0002
Row 3					
Ctrl vs. 4-OOH	-3.066	-15.04 to 8.908	No	ns	0.9931
Ctrl vs. viscum	-45.2	-57.18 to -33.23	Yes	****	<0.0001
Ctrl vs. viscum+4OOH	-52.03	-64 to -40.06	Yes	****	<0.0001
Ctrl vs. TT	-8.613	-20.03 to 2.803	No	ns	0.2844
Ctrl vs. TT+4OOH	-11.76	-23.18 to -0.3437	Yes	*	0.0387
Ctrl vs. viscumTT	-76.78	-88.76 to -64.81	Yes	****	<0.0001
Ctrl vs. viscumTT+4OOH	-81.23	-93.99 to -68.46	Yes	****	<0.0001
4-OOH vs. viscum	-42.14	-54.64 to -29.63	Yes	****	<0.0001
4-OOH vs. viscum+4OOH	-48.96	-61.47 to -36.46	Yes	****	<0.0001
4-OOH vs. TT	-5.547	-17.52 to 6.426	No	ns	0.8383
4-OOH vs. TT+4OOH	-8.694	-20.67 to 3.28	No	ns	0.3321
4-OOH vs. viscumTT	-73.72	-86.22 to -61.21	Yes	****	<0.0001
4-OOH vs. viscumTT+4OOH	-78.16	-91.42 to -64.89	Yes	****	<0.0001
viscum vs. viscum+4OOH	-6.826	-19.33 to 5.68	No	ns	0.6927
viscum vs. TT	36.59	24.62 to 48.56	Yes	****	<0.0001
viscum vs. TT+4OOH	33.44	21.47 to 45.42	Yes	****	<0.0001
viscum vs. viscumTT	-31.58	-44.09 to -19.07	Yes	****	<0.0001
viscum vs. viscumTT+4OOH	-36.02	-49.29 to -22.76	Yes	****	<0.0001
viscum+4OOH vs. TT	43.42	31.44 to 55.39	Yes	****	<0.0001
viscum+4OOH vs. TT+4OOH	40.27	28.3 to 52.24	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum+4OOH vs. viscumTT	-24.75	-37.26 to -12.25	Yes	****	<0.0001
viscum+4OOH vs. viscumTT+4OOH	-29.2	-42.46 to -15.93	Yes	****	<0.0001
TT vs. TT+4OOH	-3.147	-14.56 to 8.27	No	ns	0.9893
TT vs. viscumTT	-68.17	-80.14 to -56.2	Yes	****	<0.0001
TT vs. viscumTT+4OOH	-72.61	-85.38 to -59.85	Yes	****	<0.0001
TT+4OOH vs. viscumTT	-65.02	-77 to -53.05	Yes	****	<0.0001
TT+4OOH vs. viscumTT+4OOH	-69.47	-82.23 to -56.7	Yes	****	<0.0001
viscumTT vs. viscumTT+4OOH	-4.441	-17.71 to 8.824	No	ns	0.9674

Table S1d P-values of co-treatment with Doxo of 143B cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. Doxo	-24.97	-34.63 to -15.31	Yes	****	<0.0001
Ctrl vs. viscum	-14.05	-24.41 to -3.688	Yes	**	0.0012
Ctrl vs. viscum+Doxo	-43.4	-54.08 to -32.72	Yes	****	<0.0001
Ctrl vs. TT	-9.078	-19.44 to 1.282	No	ns	0.1337
Ctrl vs. TT+Doxo	-44.09	-55.63 to -32.54	Yes	****	<0.0001
Ctrl vs. viscumTT	-17.17	-27.53 to -6.812	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-39.98	-52.14 to -27.82	Yes	****	<0.0001
Doxo vs. viscum	10.92	0.558 to 21.28	Yes	*	0.0309
Doxo vs. viscum+Doxo	-18.43	-29.12 to -7.754	Yes	****	<0.0001
Doxo vs. TT	15.89	5.528 to 26.25	Yes	***	0.0001
Doxo vs. TT+Doxo	-19.12	-30.67 to -7.573	Yes	****	<0.0001
Doxo vs. viscumTT	7.794	-2.566 to 18.15	No	ns	0.2966
Doxo vs. viscumTT+Doxo	-15.01	-27.17 to -2.858	Yes	**	0.0049
viscum vs. viscum+Doxo	-29.35	-40.67 to -18.04	Yes	****	<0.0001
viscum vs. TT	4.97	-6.045 to 15.99	No	ns	0.8648
viscum vs. TT+Doxo	-30.04	-42.18 to -17.9	Yes	****	<0.0001
viscum vs. viscumTT	-3.124	-14.14 to 7.891	No	ns	0.9885
viscum vs. viscumTT+Doxo	-25.93	-38.65 to -13.21	Yes	****	<0.0001
viscum+Doxo vs. TT	34.32	23.01 to 45.64	Yes	****	<0.0001
viscum+Doxo vs. TT+Doxo	-0.6846	-13.1 to 11.73	No	ns	>0.9999
viscum+Doxo vs. viscumTT	26.23	14.91 to 37.55	Yes	****	<0.0001
viscum+Doxo vs. viscumTT+Doxo	3.421	-9.56 to 16.4	No	ns	0.9926
TT vs. TT+Doxo	-35.01	-47.15 to -22.87	Yes	****	<0.0001
TT vs. viscumTT	-8.094	-19.11 to 2.921	No	ns	0.3266
TT vs. viscumTT+Doxo	-30.9	-43.62 to -18.18	Yes	****	<0.0001
TT+Doxo vs. viscumTT	26.91	14.78 to 39.05	Yes	****	<0.0001
TT+Doxo vs. viscumTT+Doxo	4.106	-9.597 to 17.81	No	ns	0.9841
viscumTT vs. viscumTT+Doxo	-22.81	-35.53 to -10.09	Yes	****	<0.0001
Row 2					
Ctrl vs. Doxo	-24.97	-34.63 to -15.31	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Ctrl vs. viscum	-22.88	-33.24 to -12.52	Yes	****	<0.0001
Ctrl vs. viscum+Doxo	-45.05	-55.41 to -34.69	Yes	****	<0.0001
Ctrl vs. TT	-18.94	-28.8 to -9.08	Yes	****	<0.0001
Ctrl vs. TT+Doxo	-53.49	-64.17 to -42.81	Yes	****	<0.0001
Ctrl vs. viscumTT	-36.31	-46.99 to -25.63	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-68.99	-81.15 to -56.84	Yes	****	<0.0001
Doxo vs. viscum	2.089	-8.271 to 12.45	No	ns	0.9986
Doxo vs. viscum+Doxo	-20.08	-30.44 to -9.723	Yes	****	<0.0001
Doxo vs. TT	6.026	-3.834 to 15.89	No	ns	0.5721
Doxo vs. TT+Doxo	-28.53	-39.21 to -17.85	Yes	****	<0.0001
Doxo vs. viscumTT	-11.34	-22.02 to -0.66	Yes	*	0.0287
Doxo vs. viscumTT+Doxo	-44.03	-56.18 to -31.87	Yes	****	<0.0001
viscum vs. viscum+Doxo	-22.17	-33.19 to -11.16	Yes	****	<0.0001
viscum vs. TT	3.937	-6.609 to 14.48	No	ns	0.9464
viscum vs. TT+Doxo	-30.62	-41.93 to -19.3	Yes	****	<0.0001
viscum vs. viscumTT	-13.43	-24.75 to -2.113	Yes	**	0.0083
viscum vs. viscumTT+Doxo	-46.12	-58.84 to -33.4	Yes	****	<0.0001
viscum+Doxo vs. TT	26.11	15.56 to 36.66	Yes	****	<0.0001
viscum+Doxo vs. TT+Doxo	-8.444	-19.76 to 2.873	No	ns	0.307
viscum+Doxo vs. viscumTT	8.742	-2.575 to 20.06	No	ns	0.2641
viscum+Doxo vs. viscumTT+Doxo	-23.94	-36.66 to -11.23	Yes	****	<0.0001
TT vs. TT+Doxo	-34.55	-45.41 to -23.69	Yes	****	<0.0001
TT vs. viscumTT	-17.37	-28.23 to -6.506	Yes	****	<0.0001
TT vs. viscumTT+Doxo	-50.05	-62.37 to -37.74	Yes	****	<0.0001
TT+Doxo vs. viscumTT	17.19	5.576 to 28.8	Yes	***	0.0003
TT+Doxo vs. viscumTT+Doxo	-15.5	-28.48 to -2.519	Yes	**	0.0077
viscumTT vs. viscumTT+Doxo	-32.69	-45.67 to -19.71	Yes	****	<0.0001
Row 3					
Ctrl vs. Doxo	-24.97	-34.63 to -15.31	Yes	****	<0.0001
Ctrl vs. viscum	-32.39	-43.46 to -21.32	Yes	****	<0.0001
Ctrl vs. viscum+Doxo	-59.7	-70.77 to -48.63	Yes	****	<0.0001
Ctrl vs. TT	-42.98	-53.66 to -32.3	Yes	****	<0.0001
Ctrl vs. TT+Doxo	-69.15	-80.69 to -57.6	Yes	****	<0.0001
Ctrl vs. viscumTT	-58.9	-70.45 to -47.36	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-81.38	-92.93 to -69.84	Yes	****	<0.0001
Doxo vs. viscum	-7.421	-18.49 to 3.647	No	ns	0.4489
Doxo vs. viscum+Doxo	-34.73	-45.8 to -23.66	Yes	****	<0.0001
Doxo vs. TT	-18.01	-28.7 to -7.334	Yes	****	<0.0001
Doxo vs. TT+Doxo	-44.18	-55.73 to -32.63	Yes	****	<0.0001
Doxo vs. viscumTT	-33.94	-45.48 to -22.39	Yes	****	<0.0001
Doxo vs. viscumTT+Doxo	-56.42	-67.96 to -44.87	Yes	****	<0.0001
viscum vs. viscum+Doxo	-27.31	-39.63 to -15	Yes	****	<0.0001
viscum vs. TT	-10.59	-22.56 to 1.375	No	ns	0.1253

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum vs. TT+Doxo	-36.76	-49.51 to -24.01	Yes	****	<0.0001
viscum vs. viscumTT	-26.52	-39.26 to -13.77	Yes	****	<0.0001
viscum vs. viscumTT+Doxo	-49	-61.74 to -36.25	Yes	****	<0.0001
viscum+Doxo vs. TT	16.72	4.749 to 28.69	Yes	***	0.0007
viscum+Doxo vs. TT+Doxo	-9.447	-22.19 to 3.301	No	ns	0.3157
viscum+Doxo vs. viscumTT	0.7959	-11.95 to 13.54	No	ns	>0.9999
viscum+Doxo vs. viscumTT+Doxo	-21.68	-34.43 to -8.937	Yes	****	<0.0001
TT vs. TT+Doxo	-26.16	-38.58 to -13.75	Yes	****	<0.0001
TT vs. viscumTT	-15.92	-28.33 to -3.509	Yes	**	0.0029
TT vs. viscumTT+Doxo	-38.4	-50.81 to -25.99	Yes	****	<0.0001
TT+Doxo vs. viscumTT	10.24	-2.923 to 23.41	No	ns	0.2556
TT+Doxo vs. viscumTT+Doxo	-12.24	-25.4 to 0.9284	No	ns	0.0895
viscumTT vs. viscumTT+Doxo	-22.48	-35.65 to -9.314	Yes	****	<0.0001

Table S1e P-values of co-treatment with VP16 of 143B cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. VP16	-3.286	-14.58 to 8.011	No	ns	0.9855
Ctrl vs. viscum	-16.27	-27.04 to -5.496	Yes	***	0.0002
Ctrl vs. viscum+VP16	-46.37	-57.67 to -35.07	Yes	****	<0.0001
Ctrl vs. TT	-12.17	-22.94 to -1.401	Yes	*	0.0154
Ctrl vs. TT+VP16	-48.26	-59.55 to -36.96	Yes	****	<0.0001
Ctrl vs. viscumTT	-19.66	-30.43 to -8.893	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-44.64	-56.69 to -32.6	Yes	****	<0.0001
VP16 vs. viscum	-12.98	-24.28 to -1.684	Yes	*	0.0128
VP16 vs. viscum+VP16	-43.08	-54.88 to -31.29	Yes	****	<0.0001
VP16 vs. TT	-8.886	-20.18 to 2.411	No	ns	0.237
VP16 vs. TT+VP16	-44.97	-56.77 to -33.17	Yes	****	<0.0001
VP16 vs. viscumTT	-16.38	-27.67 to -5.081	Yes	***	0.0005
VP16 vs. viscumTT+VP16	-41.36	-53.87 to -28.84	Yes	****	<0.0001
viscum vs. viscum+VP16	-30.1	-41.4 to -18.81	Yes	****	<0.0001
viscum vs. TT	4.095	-6.676 to 14.87	No	ns	0.9372
viscum vs. TT+VP16	-31.99	-43.29 to -20.69	Yes	****	<0.0001
viscum vs. viscumTT	-3.397	-14.17 to 7.374	No	ns	0.977
viscum vs. viscumTT+VP16	-28.38	-40.42 to -16.34	Yes	****	<0.0001
viscum+VP16 vs. TT	34.2	22.9 to 45.49	Yes	****	<0.0001
viscum+VP16 vs. TT+VP16	-1.886	-13.68 to 9.913	No	ns	0.9997
viscum+VP16 vs. viscumTT	26.71	15.41 to 38	Yes	****	<0.0001
viscum+VP16 vs. viscumTT+VP16	1.726	-10.79 to 14.24	No	ns	0.9999
TT vs. TT+VP16	-36.08	-47.38 to -24.79	Yes	****	<0.0001
TT vs. viscumTT	-7.492	-18.26 to 3.279	No	ns	0.3901
TT vs. viscumTT+VP16	-32.47	-44.51 to -20.43	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
TT+VP16 vs. viscumTT	28.59	17.3 to 39.89	Yes	****	<0.0001
TT+VP16 vs. viscumTT+VP16	3.612	-8.903 to 16.13	No	ns	0.9862
viscumTT vs. viscumTT+VP16	-24.98	-37.02 to -12.94	Yes	****	<0.0001
Row 2					
Ctrl vs. VP16	-3.286	-14.58 to 8.011	No	ns	0.9855
Ctrl vs. viscum	-27.81	-38.58 to -17.03	Yes	****	<0.0001
Ctrl vs. viscum+VP16	-56.04	-66.81 to -45.27	Yes	****	<0.0001
Ctrl vs. TT	-21.1	-31.87 to -10.33	Yes	****	<0.0001
Ctrl vs. TT+VP16	-58.68	-69.45 to -47.91	Yes	****	<0.0001
Ctrl vs. viscumTT	-40.49	-51.26 to -29.72	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-75.8	-87.09 to -64.5	Yes	****	<0.0001
VP16 vs. viscum	-24.52	-35.82 to -13.22	Yes	****	<0.0001
VP16 vs. viscum+VP16	-52.75	-64.05 to -41.46	Yes	****	<0.0001
VP16 vs. TT	-17.82	-29.11 to -6.521	Yes	***	0.0001
VP16 vs. TT+VP16	-55.39	-66.69 to -44.1	Yes	****	<0.0001
VP16 vs. viscumTT	-37.2	-48.5 to -25.9	Yes	****	<0.0001
VP16 vs. viscumTT+VP16	-72.51	-84.31 to -60.71	Yes	****	<0.0001
viscum vs. viscum+VP16	-28.24	-39.01 to -17.46	Yes	****	<0.0001
viscum vs. TT	6.702	-4.069 to 17.47	No	ns	0.538
viscum vs. TT+VP16	-30.88	-41.65 to -20.1	Yes	****	<0.0001
viscum vs. viscumTT	-12.68	-23.45 to -1.911	Yes	**	0.0097
viscum vs. viscumTT+VP16	-47.99	-59.29 to -36.7	Yes	****	<0.0001
viscum+VP16 vs. TT	34.94	24.17 to 45.71	Yes	****	<0.0001
viscum+VP16 vs. TT+VP16	-2.64	-13.41 to 8.131	No	ns	0.9948
viscum+VP16 vs. viscumTT	15.55	4.783 to 26.32	Yes	***	0.0005
viscum+VP16 vs. viscumTT+VP16	-19.76	-31.05 to -8.461	Yes	****	<0.0001
TT vs. TT+VP16	-37.58	-48.35 to -26.81	Yes	****	<0.0001
TT vs. viscumTT	-19.38	-30.15 to -8.613	Yes	****	<0.0001
TT vs. viscumTT+VP16	-54.69	-65.99 to -43.4	Yes	****	<0.0001
TT+VP16 vs. viscumTT	18.19	7.423 to 28.96	Yes	****	<0.0001
TT+VP16 vs. viscumTT+VP16	-17.12	-28.41 to -5.821	Yes	***	0.0002
viscumTT vs. viscumTT+VP16	-35.31	-46.61 to -24.01	Yes	****	<0.0001
Row 3					
Ctrl vs. VP16	-3.286	-14.58 to 8.011	No	ns	0.9855
Ctrl vs. viscum	-34.19	-45.48 to -22.89	Yes	****	<0.0001
Ctrl vs. viscum+VP16	-62.67	-73.97 to -51.37	Yes	****	<0.0001
Ctrl vs. TT	-41.05	-52.34 to -29.75	Yes	****	<0.0001
Ctrl vs. TT+VP16	-74.37	-85.67 to -63.08	Yes	****	<0.0001
Ctrl vs. viscumTT	-65.62	-76.39 to -54.85	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-84.66	-95.96 to -73.37	Yes	****	<0.0001
VP16 vs. viscum	-30.9	-42.7 to -19.1	Yes	****	<0.0001
VP16 vs. viscum+VP16	-59.38	-71.18 to -47.59	Yes	****	<0.0001
VP16 vs. TT	-37.76	-49.56 to -25.96	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
VP16 vs. TT+VP16	-71.09	-82.89 to -59.29	Yes	****	<0.0001
VP16 vs. viscumTT	-62.33	-73.63 to -51.03	Yes	****	<0.0001
VP16 vs. viscumTT+VP16	-81.38	-93.18 to -69.58	Yes	****	<0.0001
viscum vs. viscum+VP16	-28.48	-40.28 to -16.69	Yes	****	<0.0001
viscum vs. TT	-6.86	-18.66 to 4.939	No	ns	0.6231
viscum vs. TT+VP16	-40.19	-51.99 to -28.39	Yes	****	<0.0001
viscum vs. viscumTT	-31.43	-42.73 to -20.13	Yes	****	<0.0001
viscum vs. viscumTT+VP16	-50.48	-62.28 to -38.68	Yes	****	<0.0001
viscum+VP16 vs. TT	21.62	9.825 to 33.42	Yes	****	<0.0001
viscum+VP16 vs. TT+VP16	-11.7	-23.5 to 0.09485	No	ns	0.0535
viscum+VP16 vs. viscumTT	-2.947	-14.24 to 8.35	No	ns	0.9924
viscum+VP16 vs. viscumTT+VP16	-21.99	-33.79 to -10.2	Yes	****	<0.0001
TT vs. TT+VP16	-33.33	-45.13 to -21.53	Yes	****	<0.0001
TT vs. viscumTT	-24.57	-35.87 to -13.27	Yes	****	<0.0001
TT vs. viscumTT+VP16	-43.62	-55.42 to -31.82	Yes	****	<0.0001
TT+VP16 vs. viscumTT	8.757	-2.54 to 20.05	No	ns	0.2537
TT+VP16 vs. viscumTT+VP16	-10.29	-22.09 to 1.509	No	ns	0.1351
viscumTT vs. viscumTT+VP16	-19.05	-30.34 to -7.75	Yes	****	<0.0001

Table S1f P-values of co-treatment with 4-OOH of 143B cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. 4-OOH	-2.878	-16.03 to 10.27	No	ns	0.9972
Ctrl vs. viscum	-17.5	-31.45 to -3.557	Yes	**	0.0047
Ctrl vs. viscum+4-OOH	-27.26	-41.21 to -13.32	Yes	****	<0.0001
Ctrl vs. TT	-11.99	-25.94 to 1.958	No	ns	0.1446
Ctrl vs. TT+4-OOH	-15.91	-29.86 to -1.962	Yes	*	0.0145
Ctrl vs. viscumTT	-19.04	-32.99 to -5.095	Yes	**	0.0015
Ctrl vs. viscumTT+4-OOH	-25.41	-39.36 to -11.46	Yes	****	<0.0001
4-OOH vs. viscum	-14.63	-28.57 to -0.6792	Yes	*	0.0331
4-OOH vs. viscum+4-OOH	-24.39	-38.33 to -10.44	Yes	****	<0.0001
4-OOH vs. TT	-9.111	-23.06 to 4.836	No	ns	0.4635
4-OOH vs. TT+4-OOH	-13.03	-26.98 to 0.9158	No	ns	0.0842
4-OOH vs. viscumTT	-16.16	-30.11 to -2.217	Yes	*	0.0122
4-OOH vs. viscumTT+4-OOH	-22.53	-36.48 to -8.584	Yes	****	<0.0001
viscum vs. viscum+4-OOH	-9.76	-24.46 to 4.941	No	ns	0.4422
viscum vs. TT	5.515	-9.186 to 20.22	No	ns	0.9375
viscum vs. TT+4-OOH	1.595	-13.11 to 16.3	No	ns	>0.9999
viscum vs. viscumTT	-1.538	-16.24 to 13.16	No	ns	>0.9999
viscum vs. viscumTT+4-OOH	-7.905	-22.61 to 6.796	No	ns	0.7007
viscum+4-OOH vs. TT	15.28	0.5738 to 29.98	Yes	*	0.036
viscum+4-OOH vs. TT+4-OOH	11.36	-3.346 to 26.06	No	ns	0.2519

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum+4-OOH vs. viscumTT	8.223	-6.479 to 22.92	No	ns	0.6576
viscum+4-OOH vs. viscumTT+4-OOH	1.855	-12.85 to 16.56	No	ns	>0.9999
TT vs. TT+4-OOH	-3.92	-18.62 to 10.78	No	ns	0.9906
TT vs. viscumTT	-7.053	-21.75 to 7.649	No	ns	0.8062
TT vs. viscumTT+4-OOH	-13.42	-28.12 to 1.281	No	ns	0.0989
TT+4-OOH vs. viscumTT	-3.133	-17.83 to 11.57	No	ns	0.9976
TT+4-OOH vs. viscumTT+4-OOH	-9.5	-24.2 to 5.201	No	ns	0.4778
viscumTT vs. viscumTT+4-OOH	-6.368	-21.07 to 8.334	No	ns	0.8753
Row 2					
Ctrl vs. 4-OOH	-2.878	-16.03 to 10.27	No	ns	0.9972
Ctrl vs. viscum	-26.91	-40.86 to -12.97	Yes	****	<0.0001
Ctrl vs. viscum+4-OOH	-34.72	-49.9 to -19.54	Yes	****	<0.0001
Ctrl vs. TT	-22.63	-36.58 to -8.682	Yes	****	<0.0001
Ctrl vs. TT+4-OOH	-26.76	-40.71 to -12.82	Yes	****	<0.0001
Ctrl vs. viscumTT	-38.97	-52.92 to -25.02	Yes	****	<0.0001
Ctrl vs. viscumTT+4-OOH	-45.97	-61.16 to -30.79	Yes	****	<0.0001
4-OOH vs. viscum	-24.04	-37.98 to -10.09	Yes	****	<0.0001
4-OOH vs. viscum+4-OOH	-31.84	-47.03 to -16.66	Yes	****	<0.0001
4-OOH vs. TT	-19.75	-33.7 to -5.804	Yes	***	0.0009
4-OOH vs. TT+4-OOH	-23.89	-37.83 to -9.939	Yes	****	<0.0001
4-OOH vs. viscumTT	-36.09	-50.04 to -22.15	Yes	****	<0.0001
4-OOH vs. viscumTT+4-OOH	-43.1	-58.28 to -27.91	Yes	****	<0.0001
viscum vs. viscum+4-OOH	-7.807	-23.69 to 8.072	No	ns	0.7861
viscum vs. TT	4.285	-10.42 to 18.99	No	ns	0.9842
viscum vs. TT+4-OOH	0.15	-14.55 to 14.85	No	ns	>0.9999
viscum vs. viscumTT	-12.06	-26.76 to 2.644	No	ns	0.1878
viscum vs. viscumTT+4-OOH	-19.06	-34.94 to -3.181	Yes	**	0.0082
viscum+4-OOH vs. TT	12.09	-3.787 to 27.97	No	ns	0.2683
viscum+4-OOH vs. TT+4-OOH	7.957	-7.922 to 23.84	No	ns	0.7695
viscum+4-OOH vs. viscumTT	-4.251	-20.13 to 11.63	No	ns	0.9904
viscum+4-OOH vs. viscumTT+4-OOH	-11.25	-28.23 to 5.722	No	ns	0.4442
TT vs. TT+4-OOH	-4.135	-18.84 to 10.57	No	ns	0.9871
TT vs. viscumTT	-16.34	-31.04 to -1.641	Yes	*	0.0189
TT vs. viscumTT+4-OOH	-23.35	-39.22 to -7.466	Yes	***	0.0005
TT+4-OOH vs. viscumTT	-12.21	-26.91 to 2.494	No	ns	0.1758
TT+4-OOH vs. viscumTT+4-OOH	-19.21	-35.09 to -3.331	Yes	**	0.0074
viscumTT vs. viscumTT+4-OOH	-7.003	-22.88 to 8.877	No	ns	0.8648
Row 3					
Ctrl vs. 4-OOH	-2.878	-16.03 to 10.27	No	ns	0.9972
Ctrl vs. viscum	-43.17	-58.35 to -27.99	Yes	****	<0.0001
Ctrl vs. viscum+4-OOH	-43.4	-58.59 to -28.22	Yes	****	<0.0001
Ctrl vs. TT	-43.38	-58.57 to -28.2	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Ctrl vs. TT+4-OOH	-57.45	-72.63 to -42.27	Yes	****	<0.0001
Ctrl vs. viscumTT	-66.4	-80.34 to -52.45	Yes	****	<0.0001
Ctrl vs. viscumTT+4-OOH	-80.95	-94.9 to -67	Yes	****	<0.0001
4-OOH vs. viscum	-40.29	-55.48 to -25.11	Yes	****	<0.0001
4-OOH vs. viscum+4-OOH	-40.53	-55.71 to -25.34	Yes	****	<0.0001
4-OOH vs. TT	-40.51	-55.69 to -25.32	Yes	****	<0.0001
4-OOH vs. TT+4-OOH	-54.57	-69.76 to -39.39	Yes	****	<0.0001
4-OOH vs. viscumTT	-63.52	-77.47 to -49.57	Yes	****	<0.0001
4-OOH vs. viscumTT+4-OOH	-78.07	-92.02 to -64.12	Yes	****	<0.0001
viscum vs. viscum+4-OOH	-0.2333	-17.21 to 16.74	No	ns	>0.9999
viscum vs. TT	-0.2133	-17.19 to 16.76	No	ns	>0.9999
viscum vs. TT+4-OOH	-14.28	-31.26 to 2.695	No	ns	0.1637
viscum vs. viscumTT	-23.23	-39.1 to -7.347	Yes	***	0.0005
viscum vs. viscumTT+4-OOH	-37.78	-53.66 to -21.9	Yes	****	<0.0001
viscum+4-OOH vs. TT	0.02	-16.96 to 17	No	ns	>0.9999
viscum+4-OOH vs. TT+4-OOH	-14.05	-31.02 to 2.929	No	ns	0.1792
viscum+4-OOH vs. viscumTT	-22.99	-38.87 to -7.113	Yes	***	0.0006
viscum+4-OOH vs. viscumTT+4-OOH	-37.55	-53.42 to -21.67	Yes	****	<0.0001
TT vs. TT+4-OOH	-14.07	-31.04 to 2.909	No	ns	0.1778
TT vs. viscumTT	-23.01	-38.89 to -7.133	Yes	***	0.0006
TT vs. viscumTT+4-OOH	-37.57	-53.44 to -21.69	Yes	****	<0.0001
TT+4-OOH vs. viscumTT	-8.946	-24.82 to 6.933	No	ns	0.6493
TT+4-OOH vs. viscumTT+4-OOH	-23.5	-39.38 to -7.619	Yes	***	0.0004
viscumTT vs. viscumTT+4-OOH	-14.55	-29.25 to 0.1487	No	ns	0.0543

Table S1f P-values of co-treatment with Doxo of Saos-2 cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. Doxo	-21.56	-32.85 to -10.27	Yes	****	<0.0001
Ctrl vs. viscum	-4.563	-15.85 to 6.729	No	ns	0.9098
Ctrl vs. viscum+Doxo	-18.09	-29.38 to -6.794	Yes	***	0.0001
Ctrl vs. TT	-7.473	-18.76 to 3.819	No	ns	0.4464
Ctrl vs. TT+Doxo	-22.71	-34 to -11.42	Yes	****	<0.0001
Ctrl vs. viscumTT	-8.995	-20.29 to 2.296	No	ns	0.2178
Ctrl vs. viscumTT+Doxo	-22.99	-34.28 to -11.7	Yes	****	<0.0001
Doxo vs. viscum	17	5.706 to 28.29	Yes	***	0.0003
Doxo vs. viscum+Doxo	3.475	-7.816 to 14.77	No	ns	0.9784
Doxo vs. TT	14.09	2.796 to 25.38	Yes	**	0.0051
Doxo vs. TT+Doxo	-1.15	-12.44 to 10.14	No	ns	>0.9999
Doxo vs. viscumTT	12.57	1.274 to 23.86	Yes	*	0.0187
Doxo vs. viscumTT+Doxo	-1.43	-12.72 to 9.861	No	ns	>0.9999

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum vs. viscum+Doxo	-13.52	-24.81 to -2.231	Yes	**	0.0084
viscum vs. TT	-2.91	-14.2 to 8.381	No	ns	0.9924
viscum vs. TT+Doxo	-18.15	-29.44 to -6.856	Yes	****	<0.0001
viscum vs. viscumTT	-4.433	-15.72 to 6.859	No	ns	0.9216
viscum vs. viscumTT+Doxo	-18.43	-29.72 to -7.136	Yes	****	<0.0001
viscum+Doxo vs. TT	10.61	-0.6786 to 21.9	No	ns	0.0807
viscum+Doxo vs. TT+Doxo	-4.625	-15.92 to 6.666	No	ns	0.9037
viscum+Doxo vs. viscumTT	9.09	-2.201 to 20.38	No	ns	0.2067
viscum+Doxo vs. viscumTT+Doxo	-4.905	-16.2 to 6.386	No	ns	0.8736
TT vs. TT+Doxo	-15.24	-26.53 to -3.946	Yes	**	0.0018
TT vs. viscumTT	-1.523	-12.81 to 9.769	No	ns	0.9999
TT vs. viscumTT+Doxo	-15.52	-26.81 to -4.226	Yes	**	0.0014
TT+Doxo vs. viscumTT	13.72	2.424 to 25.01	Yes	**	0.0071
TT+Doxo vs. viscumTT+Doxo	-0.28	-11.57 to 11.01	No	ns	>0.9999
viscumTT vs. viscumTT+Doxo	-14	-25.29 to -2.704	Yes	**	0.0055
Row 2					
Ctrl vs. Doxo	-21.56	-32.85 to -10.27	Yes	****	<0.0001
Ctrl vs. viscum	-5.785	-17.08 to 5.506	No	ns	0.7491
Ctrl vs. viscum+Doxo	-21.87	-33.16 to -10.58	Yes	****	<0.0001
Ctrl vs. TT	-13.67	-24.96 to -2.381	Yes	**	0.0074
Ctrl vs. TT+Doxo	-26.34	-37.63 to -15.05	Yes	****	<0.0001
Ctrl vs. viscumTT	-19.3	-30.59 to -8.009	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-32.65	-43.94 to -21.36	Yes	****	<0.0001
Doxo vs. viscum	15.78	4.484 to 27.07	Yes	**	0.0011
Doxo vs. viscum+Doxo	-0.3075	-11.6 to 10.98	No	ns	>0.9999
Doxo vs. TT	7.888	-3.404 to 19.18	No	ns	0.3755
Doxo vs. TT+Doxo	-4.783	-16.07 to 6.509	No	ns	0.8874
Doxo vs. viscumTT	2.26	-9.031 to 13.55	No	ns	0.9984
Doxo vs. viscumTT+Doxo	-11.09	-22.38 to 0.2011	No	ns	0.0578
viscum vs. viscum+Doxo	-16.08	-27.37 to -4.791	Yes	***	0.0008
viscum vs. TT	-7.888	-19.18 to 3.404	No	ns	0.3755
viscum vs. TT+Doxo	-20.56	-31.85 to -9.266	Yes	****	<0.0001
viscum vs. viscumTT	-13.52	-24.81 to -2.224	Yes	**	0.0084
viscum vs. viscumTT+Doxo	-26.87	-38.16 to -15.57	Yes	****	<0.0001
viscum+Doxo vs. TT	8.195	-3.096 to 19.49	No	ns	0.3266
viscum+Doxo vs. TT+Doxo	-4.475	-15.77 to 6.816	No	ns	0.9179
viscum+Doxo vs. viscumTT	2.568	-8.724 to 13.86	No	ns	0.9965
viscum+Doxo vs. viscumTT+Doxo	-10.78	-22.07 to 0.5086	No	ns	0.0718
TT vs. TT+Doxo	-12.67	-23.96 to -1.379	Yes	*	0.0172
TT vs. viscumTT	-5.628	-16.92 to 5.664	No	ns	0.7743
TT vs. viscumTT+Doxo	-18.98	-30.27 to -7.686	Yes	****	<0.0001
TT+Doxo vs. viscumTT	7.043	-4.249 to 18.33	No	ns	0.524

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
TT+Doxo vs. viscumTT+Doxo	-6.308	-17.6 to 4.984	No	ns	0.6589
viscumTT vs. viscumTT+Doxo	-13.35	-24.64 to -2.059	Yes	**	0.0097
Row 3					
Ctrl vs. Doxo	-21.56	-32.85 to -10.27	Yes	****	<0.0001
Ctrl vs. viscum	-6.368	-17.66 to 4.924	No	ns	0.6481
Ctrl vs. viscum+Doxo	-22.46	-33.75 to -11.17	Yes	****	<0.0001
Ctrl vs. TT	-30.93	-42.22 to -19.63	Yes	****	<0.0001
Ctrl vs. TT+Doxo	-37.36	-48.65 to -26.06	Yes	****	<0.0001
Ctrl vs. viscumTT	-39.48	-50.77 to -28.19	Yes	****	<0.0001
Ctrl vs. viscumTT+Doxo	-43.69	-54.98 to -32.4	Yes	****	<0.0001
Doxo vs. viscum	15.19	3.901 to 26.48	Yes	**	0.0018
Doxo vs. viscum+Doxo	-0.9025	-12.19 to 10.39	No	ns	>0.9999
Doxo vs. TT	-9.365	-20.66 to 1.926	No	ns	0.1769
Doxo vs. TT+Doxo	-15.8	-27.09 to -4.504	Yes	**	0.001
Doxo vs. viscumTT	-17.92	-29.21 to -6.626	Yes	***	0.0001
Doxo vs. viscumTT+Doxo	-22.13	-33.42 to -10.84	Yes	****	<0.0001
viscum vs. viscum+Doxo	-16.1	-27.39 to -4.804	Yes	***	0.0008
viscum vs. TT	-24.56	-35.85 to -13.27	Yes	****	<0.0001
viscum vs. TT+Doxo	-30.99	-42.28 to -19.7	Yes	****	<0.0001
viscum vs. viscumTT	-33.11	-44.4 to -21.82	Yes	****	<0.0001
viscum vs. viscumTT+Doxo	-37.32	-48.61 to -26.03	Yes	****	<0.0001
viscum+Doxo vs. TT	-8.463	-19.75 to 2.829	No	ns	0.2872
viscum+Doxo vs. TT+Doxo	-14.89	-26.18 to -3.601	Yes	**	0.0024
viscum+Doxo vs. viscumTT	-17.02	-28.31 to -5.724	Yes	***	0.0003
viscum+Doxo vs. viscumTT+Doxo	-21.23	-32.52 to -9.934	Yes	****	<0.0001
TT vs. TT+Doxo	-6.43	-17.72 to 4.861	No	ns	0.6368
TT vs. viscumTT	-8.553	-19.84 to 2.739	No	ns	0.2746
TT vs. viscumTT+Doxo	-12.76	-24.05 to -1.471	Yes	*	0.0159
TT+Doxo vs. viscumTT	-2.123	-13.41 to 9.169	No	ns	0.9989
TT+Doxo vs. viscumTT+Doxo	-6.333	-17.62 to 4.959	No	ns	0.6544
viscumTT vs. viscumTT+Doxo	-4.21	-15.5 to 7.081	No	ns	0.9394

Table S1g P-values of co-treatment with VP16 of Saos-2 cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. VP16	-12.52	-21.25 to -3.798	Yes	***	0.0006
Ctrl vs. viscum	-3.052	-11.78 to 5.672	No	ns	0.9587
Ctrl vs. viscum+VP16	-12.98	-21.7 to -4.256	Yes	***	0.0003
Ctrl vs. TT	-5.152	-13.88 to 3.572	No	ns	0.6015
Ctrl vs. TT+VP16	-16.86	-25.58 to -8.134	Yes	****	<0.0001
Ctrl vs. viscumTT	-9.006	-17.73 to -0.282	Yes	*	0.0379
Ctrl vs. viscumTT+VP16	-17.24	-25.97 to -8.518	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
VP16 vs. viscum	9.47	0.746 to 18.19	Yes	*	0.0236
VP16 vs. viscum+VP16	-0.458	-9.182 to 8.266	No	ns	>0.9999
VP16 vs. TT	7.37	-1.354 to 16.09	No	ns	0.1624
VP16 vs. TT+VP16	-4.336	-13.06 to 4.388	No	ns	0.7837
VP16 vs. viscumTT	3.516	-5.208 to 12.24	No	ns	0.9148
VP16 vs. viscumTT+VP16	-4.72	-13.44 to 4.004	No	ns	0.7021
viscum vs. viscum+VP16	-9.928	-18.65 to -1.204	Yes	*	0.0144
viscum vs. TT	-2.1	-10.82 to 6.624	No	ns	0.9953
viscum vs. TT+VP16	-13.81	-22.53 to -5.082	Yes	***	0.0001
viscum vs. viscumTT	-5.954	-14.68 to 2.77	No	ns	0.413
viscum vs. viscumTT+VP16	-14.19	-22.91 to -5.466	Yes	****	<0.0001
viscum+VP16 vs. TT	7.828	-0.896 to 16.55	No	ns	0.1122
viscum+VP16 vs. TT+VP16	-3.878	-12.6 to 4.846	No	ns	0.8652
viscum+VP16 vs. viscumTT	3.974	-4.75 to 12.7	No	ns	0.8498
viscum+VP16 vs. viscumTT+VP16	-4.262	-12.99 to 4.462	No	ns	0.7982
TT vs. TT+VP16	-11.71	-20.43 to -2.982	Yes	**	0.0017
TT vs. viscumTT	-3.854	-12.58 to 4.87	No	ns	0.8689
TT vs. viscumTT+VP16	-12.09	-20.81 to -3.366	Yes	**	0.0011
TT+VP16 vs. viscumTT	7.852	-0.872 to 16.58	No	ns	0.11
TT+VP16 vs. viscumTT+VP16	-0.384	-9.108 to 8.34	No	ns	>0.9999
viscumTT vs. viscumTT+VP16	-8.236	-16.96 to 0.488	No	ns	0.0787
Row 2					
Ctrl vs. VP16	-12.52	-21.25 to -3.798	Yes	***	0.0006
Ctrl vs. viscum	-3.858	-12.58 to 4.866	No	ns	0.8683
Ctrl vs. viscum+VP16	-14.19	-22.92 to -5.47	Yes	****	<0.0001
Ctrl vs. TT	-11.88	-20.6 to -3.154	Yes	**	0.0014
Ctrl vs. TT+VP16	-22.97	-31.69 to -14.25	Yes	****	<0.0001
Ctrl vs. viscumTT	-20.43	-29.16 to -11.71	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-25.64	-34.36 to -16.91	Yes	****	<0.0001
VP16 vs. viscum	8.664	-0.05999 to 17.39	No	ns	0.053
VP16 vs. viscum+VP16	-1.672	-10.4 to 7.052	No	ns	0.9989
VP16 vs. TT	0.644	-8.08 to 9.368	No	ns	>0.9999
VP16 vs. TT+VP16	-10.45	-19.17 to -1.724	Yes	**	0.008
VP16 vs. viscumTT	-7.912	-16.64 to 0.812	No	ns	0.1045
VP16 vs. viscumTT+VP16	-13.12	-21.84 to -4.392	Yes	***	0.0003
viscum vs. viscum+VP16	-10.34	-19.06 to -1.612	Yes	**	0.0091
viscum vs. TT	-8.02	-16.74 to 0.704	No	ns	0.0952
viscum vs. TT+VP16	-19.11	-27.84 to -10.39	Yes	****	<0.0001
viscum vs. viscumTT	-16.58	-25.3 to -7.852	Yes	****	<0.0001
viscum vs. viscumTT+VP16	-21.78	-30.5 to -13.06	Yes	****	<0.0001
viscum+VP16 vs. TT	2.316	-6.408 to 11.04	No	ns	0.9914
viscum+VP16 vs. TT+VP16	-8.776	-17.5 to -0.05201	Yes	*	0.0476
viscum+VP16 vs. viscumTT	-6.24	-14.96 to 2.484	No	ns	0.3514

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum+VP16 vs. viscumTT+VP16	-11.44	-20.17 to -2.72	Yes	**	0.0024
TT vs. TT+VP16	-11.09	-19.82 to -2.368	Yes	**	0.0037
TT vs. viscumTT	-8.556	-17.28 to 0.168	No	ns	0.0587
TT vs. viscumTT+VP16	-13.76	-22.48 to -5.036	Yes	***	0.0001
TT+VP16 vs. viscumTT	2.536	-6.188 to 11.26	No	ns	0.9853
TT+VP16 vs. viscumTT+VP16	-2.668	-11.39 to 6.056	No	ns	0.9803
viscumTT vs. viscumTT+VP16	-5.204	-13.93 to 3.52	No	ns	0.5891
Row 3					
Ctrl vs. VP16	-12.52	-21.25 to -3.798	Yes	***	0.0006
Ctrl vs. viscum	-4.878	-13.6 to 3.846	No	ns	0.6661
Ctrl vs. viscum+VP16	-16.92	-25.65 to -8.198	Yes	****	<0.0001
Ctrl vs. TT	-29.82	-38.55 to -21.1	Yes	****	<0.0001
Ctrl vs. TT+VP16	-36.28	-45 to -27.55	Yes	****	<0.0001
Ctrl vs. viscumTT	-37.61	-46.33 to -28.88	Yes	****	<0.0001
Ctrl vs. viscumTT+VP16	-42.41	-51.14 to -33.69	Yes	****	<0.0001
VP16 vs. viscum	7.644	-1.08 to 16.37	No	ns	0.1307
VP16 vs. viscum+VP16	-4.4	-13.12 to 4.324	No	ns	0.7708
VP16 vs. TT	-17.3	-26.03 to -8.578	Yes	****	<0.0001
VP16 vs. TT+VP16	-23.75	-32.48 to -15.03	Yes	****	<0.0001
VP16 vs. viscumTT	-25.08	-33.81 to -16.36	Yes	****	<0.0001
VP16 vs. viscumTT+VP16	-29.89	-38.61 to -21.17	Yes	****	<0.0001
viscum vs. viscum+VP16	-12.04	-20.77 to -3.32	Yes	**	0.0011
viscum vs. TT	-24.95	-33.67 to -16.22	Yes	****	<0.0001
viscum vs. TT+VP16	-31.4	-40.12 to -22.67	Yes	****	<0.0001
viscum vs. viscumTT	-32.73	-41.45 to -24	Yes	****	<0.0001
viscum vs. viscumTT+VP16	-37.53	-46.26 to -28.81	Yes	****	<0.0001
viscum+VP16 vs. TT	-12.9	-21.63 to -4.178	Yes	***	0.0004
viscum+VP16 vs. TT+VP16	-19.35	-28.08 to -10.63	Yes	****	<0.0001
viscum+VP16 vs. viscumTT	-20.68	-29.41 to -11.96	Yes	****	<0.0001
viscum+VP16 vs. viscumTT+VP16	-25.49	-34.21 to -16.77	Yes	****	<0.0001
TT vs. TT+VP16	-6.452	-15.18 to 2.272	No	ns	0.3089
TT vs. viscumTT	-7.782	-16.51 to 0.942	No	ns	0.1166
TT vs. viscumTT+VP16	-12.59	-21.31 to -3.864	Yes	***	0.0005
TT+VP16 vs. viscumTT	-1.33	-10.05 to 7.394	No	ns	0.9998
TT+VP16 vs. viscumTT+VP16	-6.136	-14.86 to 2.588	No	ns	0.3733
viscumTT vs. viscumTT+VP16	-4.806	-13.53 to 3.918	No	ns	0.6826

Table S1h P-values of co-treatment with 4-OOH of Saos-2 cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
Ctrl vs. 4-OOH	-6.284	-14.45 to 1.887	No	ns	0.2613
Ctrl vs. viscum	-4.004	-12.17 to 4.167	No	ns	0.7954

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Ctrl vs. viscum+4OOH	-7.508	-15.68 to 0.6626	No	ns	0.0955
Ctrl vs. TT	-6.688	-14.86 to 1.483	No	ns	0.1929
Ctrl vs. TT+4OOH	-15.34	-23.51 to -7.165	Yes	****	<0.0001
Ctrl vs. viscumTT	-10.31	-18.48 to -2.137	Yes	**	0.0041
Ctrl vs. viscumTT+4OOH	-13.55	-21.72 to -5.383	Yes	****	<0.0001
4-OOH vs. viscum	2.28	-5.891 to 10.45	No	ns	0.9884
4-OOH vs. viscum+4OOH	-1.224	-9.395 to 6.947	No	ns	0.9998
4-OOH vs. TT	-0.404	-8.575 to 7.767	No	ns	>0.9999
4-OOH vs. TT+4OOH	-9.052	-17.22 to -0.8814	Yes	*	0.0192
4-OOH vs. viscumTT	-4.024	-12.19 to 4.147	No	ns	0.7913
4-OOH vs. viscumTT+4OOH	-7.27	-15.44 to 0.9006	No	ns	0.1185
viscum vs. viscum+4OOH	-3.504	-11.67 to 4.667	No	ns	0.8854
viscum vs. TT	-2.684	-10.85 to 5.487	No	ns	0.9706
viscum vs. TT+4OOH	-11.33	-19.5 to -3.161	Yes	**	0.001
viscum vs. viscumTT	-6.304	-14.47 to 1.867	No	ns	0.2576
viscum vs. viscumTT+4OOH	-9.55	-17.72 to -1.379	Yes	*	0.0107
viscum+4OOH vs. TT	0.82	-7.351 to 8.991	No	ns	>0.9999
viscum+4OOH vs. TT+4OOH	-7.828	-16 to 0.3426	No	ns	0.0704
viscum+4OOH vs. viscumTT	-2.8	-10.97 to 5.371	No	ns	0.963
viscum+4OOH vs. viscumTT+4OOH	-6.046	-14.22 to 2.125	No	ns	0.3081
TT vs. TT+4OOH	-8.648	-16.82 to -0.4774	Yes	*	0.0301
TT vs. viscumTT	-3.62	-11.79 to 4.551	No	ns	0.867
TT vs. viscumTT+4OOH	-6.866	-15.04 to 1.305	No	ns	0.1672
TT+4OOH vs. viscumTT	5.028	-3.143 to 13.2	No	ns	0.5494
TT+4OOH vs. viscumTT+4OOH	1.782	-6.389 to 9.953	No	ns	0.9974
viscumTT vs. viscumTT+4OOH	-3.246	-11.42 to 4.925	No	ns	0.9205
Row 2					
Ctrl vs. 4-OOH	-6.284	-14.45 to 1.887	No	ns	0.2613
Ctrl vs. viscum	-5.304	-13.47 to 2.867	No	ns	0.4794
Ctrl vs. viscum+4OOH	-9.61	-17.78 to -1.439	Yes	**	0.0099
Ctrl vs. TT	-13.67	-21.84 to -5.497	Yes	****	<0.0001
Ctrl vs. TT+4OOH	-23.56	-31.73 to -15.39	Yes	****	<0.0001
Ctrl vs. viscumTT	-20.19	-28.36 to -12.02	Yes	****	<0.0001
Ctrl vs. viscumTT+4OOH	-23.1	-31.27 to -14.93	Yes	****	<0.0001
4-OOH vs. viscum	0.98	-7.191 to 9.151	No	ns	>0.9999
4-OOH vs. viscum+4OOH	-3.326	-11.5 to 4.845	No	ns	0.9104
4-OOH vs. TT	-7.384	-15.55 to 0.7866	No	ns	0.107
4-OOH vs. TT+4OOH	-17.28	-25.45 to -9.109	Yes	****	<0.0001
4-OOH vs. viscumTT	-13.9	-22.07 to -5.733	Yes	****	<0.0001
4-OOH vs. viscumTT+4OOH	-16.82	-24.99 to -8.649	Yes	****	<0.0001
viscum vs. viscum+4OOH	-4.306	-12.48 to 3.865	No	ns	0.7289
viscum vs. TT	-8.364	-16.53 to -0.1934	Yes	*	0.0409
viscum vs. TT+4OOH	-18.26	-26.43 to -10.09	Yes	****	<0.0001

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum vs. viscumTT	-14.88	-23.05 to -6.713	Yes	****	<0.0001
viscum vs. viscumTT+4OOH	-17.8	-25.97 to -9.629	Yes	****	<0.0001
viscum+4OOH vs. TT	-4.058	-12.23 to 4.113	No	ns	0.7841
viscum+4OOH vs. TT+4OOH	-13.95	-22.12 to -5.783	Yes	****	<0.0001
viscum+4OOH vs. viscumTT	-10.58	-18.75 to -2.407	Yes	**	0.0029
viscum+4OOH vs. viscumTT+4OOH	-13.49	-21.66 to -5.323	Yes	****	<0.0001
TT vs. TT+4OOH	-9.896	-18.07 to -1.725	Yes	**	0.007
TT vs. viscumTT	-6.52	-14.69 to 1.651	No	ns	0.2197
TT vs. viscumTT+4OOH	-9.436	-17.61 to -1.265	Yes	*	0.0122
TT+4OOH vs. viscumTT	3.376	-4.795 to 11.55	No	ns	0.9038
TT+4OOH vs. viscumTT+4OOH	0.46	-7.711 to 8.631	No	ns	>0.9999
viscumTT vs. viscumTT+4OOH	-2.916	-11.09 to 5.255	No	ns	0.954
Row 3					
Ctrl vs. 4-OOH	-6.284	-14.45 to 1.887	No	ns	0.2613
Ctrl vs. viscum	-6.64	-14.81 to 1.531	No	ns	0.2003
Ctrl vs. viscum+4OOH	-10.78	-18.95 to -2.605	Yes	**	0.0022
Ctrl vs. TT	-30.66	-38.83 to -22.49	Yes	****	<0.0001
Ctrl vs. TT+4OOH	-36.43	-44.6 to -28.26	Yes	****	<0.0001
Ctrl vs. viscumTT	-37.75	-45.92 to -29.58	Yes	****	<0.0001
Ctrl vs. viscumTT+4OOH	-37.84	-46.5 to -29.17	Yes	****	<0.0001
4-OOH vs. viscum	-0.356	-8.527 to 7.815	No	ns	>0.9999
4-OOH vs. viscum+4OOH	-4.492	-12.66 to 3.679	No	ns	0.6846
4-OOH vs. TT	-24.37	-32.54 to -16.2	Yes	****	<0.0001
4-OOH vs. TT+4OOH	-30.15	-38.32 to -21.98	Yes	****	<0.0001
4-OOH vs. viscumTT	-31.46	-39.63 to -23.29	Yes	****	<0.0001
4-OOH vs. viscumTT+4OOH	-31.55	-40.22 to -22.89	Yes	****	<0.0001
viscum vs. viscum+4OOH	-4.136	-12.31 to 4.035	No	ns	0.7673
viscum vs. TT	-24.02	-32.19 to -15.85	Yes	****	<0.0001
viscum vs. TT+4OOH	-29.79	-37.96 to -21.62	Yes	****	<0.0001
viscum vs. viscumTT	-31.11	-39.28 to -22.94	Yes	****	<0.0001
viscum vs. viscumTT+4OOH	-31.2	-39.86 to -22.53	Yes	****	<0.0001
viscum+4OOH vs. TT	-19.88	-28.05 to -11.71	Yes	****	<0.0001
viscum+4OOH vs. TT+4OOH	-25.66	-33.83 to -17.49	Yes	****	<0.0001
viscum+4OOH vs. viscumTT	-26.97	-35.14 to -18.8	Yes	****	<0.0001
viscum+4OOH vs. viscumTT+4OOH	-27.06	-35.73 to -18.39	Yes	****	<0.0001
TT vs. TT+4OOH	-5.778	-13.95 to 2.393	No	ns	0.366
TT vs. viscumTT	-7.09	-15.26 to 1.081	No	ns	0.1386
TT vs. viscumTT+4OOH	-7.18	-15.85 to 1.486	No	ns	0.1808
TT+4OOH vs. viscumTT	-1.312	-9.483 to 6.859	No	ns	0.9996
TT+4OOH vs. viscumTT+4OOH	-1.403	-10.07 to 7.264	No	ns	0.9996
viscumTT vs. viscumTT+4OOH	-0.0905	-8.757 to 8.576	No	ns	>0.9999

6.4 Viscum, TT and viscumTT alter expression of cell cycle-associated genes

Table S2a Up- and down-regulated genes by viscum, TT and viscumTT in U2OS cells.

RefSeq Number	Gene	Description	Fold-regulation viscum
NM_000051	*ATM*	Ataxia telangiectasia mutated	-7.28
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	-2.09
NM_003600	*AURKA*	Aurora kinase A	-5.3
NM_004217	*AURKB*	Aurora kinase B	-7.28
NM_016567	*BCCIP*	BRCA2 and CDKN1A interacting protein	-2.69
NM_000633	*BCL2*	B-cell CLL/lymphoma 2	-9.37
NM_001168	*BIRC5*	Baculoviral IAP repeat containing 5	-9.05
NM_007294	*BRCA1*	Breast cancer 1, early onset	-10.83
NM_000059	*BRCA2*	Breast cancer 2, early onset	-3.19
NM_001237	*CCNA2*	Cyclin A2	-20.01
NM_031966	*CCNB1*	Cyclin B1	-5.57
NM_004701	*CCNB2*	Cyclin B2	-3.17
NM_053056	*CCND1*	Cyclin D1	-2.52
NM_001759	*CCND2*	Cyclin D2	-3.72
NM_001760	*CCND3*	Cyclin D3	-3.19
NM_001238	*CCNE1*	Cyclin E1	-8.09
NM_001761	*CCNF*	Cyclin F	-12.84
NM_004060	*CCNG1*	Cyclin G1	-3.1
NM_003903	*CDC16*	Cell division cycle 16 homolog ((Saccharomyces (S.). cerevisiae))	-2.19
NM_001255	*CDC20*	Cell division cycle 20 homolog (S. cerevisiae)	-10.89
NM_001789	*CDC25A*	Cell division cycle 25 homolog A (S. pombe)	-8.18
NM_004359	*CDC34*	Cell division cycle 34 homolog (S. cerevisiae)	-3.12
NM_001254	*CDC6*	Cell division cycle 6 homolog (S. cerevisiae)	-8.14
NM_001786	*CDK1*	Cyclin-dependent kinase 1	-5.09
NM_001798	*CDK2*	Cyclin-dependent kinase 2	-3.39
NM_000075	*CDK4*	Cyclin-dependent kinase 4	-3.73
NM_003885	*CDK5R1*	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	-5.02
NM_001799	*CDK7*	Cyclin-dependent kinase 7	3.68
NM_004936	*CDKN2B*	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	3.73
NM_005192	*CDKN3*	Cyclin-dependent kinase inhibitor 3	-3.76
NM_001826	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	-3.53
NM_003592	*CUL1*	Cullin 1	-2.11
NM_003591	*CUL2*	Cullin 2	-2.06
NM_005225	*E2F1*	E2F transcription factor 1	-9.34
NM_001950	*E2F4*	E2F transcription factor 4, p107/p130-binding	-2.02
NM_013376	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	24.79
NM_016426	*GTSE1*	G-2 and S-phase expressed 1	-10.9
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. Pombe)	3.26
NM_014708	*KNTC1*	Kinetochore associated 1	-2.27
NM_002266	*KPNA2*	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	-4.09

RefSeq Number	Gene	Description	Fold-regulation viscum
NM_002358	*MAD2L1*	MAD2 mitotic arrest deficient-like 1 (yeast)	-7.17
NM_004526	*MCM2*	Minichromosome maintenance complex component 2	-5.44
NM_002388	*MCM3*	Minichromosome maintenance complex component 3	-9.69
NM_006739	*MCM5*	Minichromosome maintenance complex component 5	-4.31
NM_002392	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	3.82
NM_002417	*MKI67*	Antigen identified by monoclonal antibody Ki-67	-6.21
NM_005590	*MRE11A*	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	-4.28
NM_002485	*NBN*	Nibrin	-3.96
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	-2.28
NM_002875	*RAD51*	RAD51 homolog (S. cerevisiae)	-6.14
NM_002894	*RBBP8*	Retinoblastoma binding protein 8	-4.49
NM_002895	*RBL1*	Retinoblastoma-like 1 (p107)	-5.24
NM_005611	*RBL2*	Retinoblastoma-like 2 (p130)	-7.09
NM_013376	*SERTAD1*	SERTA domain containing 1	8.33
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-109.43
NM_005563	*STMN1*	Stathmin 1	-2.64
NM_007111	*TFDP1*	Transcription factor Dp-1	-18.4
NM_002046	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	-3.05
NM_000194	*HPRT1*	Hypoxanthine phosphoribosyltransferase 1	-3.23

RefSeq Number	Gene	Description	Fold-regulation TT
NM_003600	*AURKA*	Aurora kinase A	-3.6
NM_004217	*AURKB*	Aurora kinase B	-2.89
NM_001168	*BIRC5*	Baculoviral IAP repeat containing 5	-4.5
NM_001237	*CCNA2*	Cyclin A2	-4.33
NM_031966	*CCNB1*	Cyclin B1	-3.33
NM_004701	*CCNB2*	Cyclin B2	-3.7
NM_001761	*CCNF*	Cyclin F	-4.7
NM_001255	*CDC20*	Cell division cycle 20 homolog (S. cerevisiae)	-4.93
NM_001790	*CDC25C*	Cell division cycle 25 homolog C (S. pombe)	-3.64
NM_001786	*CDK1*	Cyclin-dependent kinase 1	-3.32
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	-2.48
NM_004064	*CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.61
NM_005192	*CDKN3*	Cyclin-dependent kinase inhibitor 3	-2.62
NM_005225	*E2F1*	E2F transcription factor 1	-2.44
NM_016426	*GTSE1*	G-2 and S-phase expressed 1	-3.33
NM_002266	*KPNA2*	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	-2.84
NM_002358	*MAD2L1*	MAD2 mitotic arrest deficient-like 1 (yeast)	-2.27
NM_002388	*MCM3*	Minichromosome maintenance complex component 3	-2.17
NM_005914	*MCM4*	Minichromosome maintenance complex component 4	-2.5
NM_002417	*MKI67*	Antigen identified by monoclonal antibody Ki-67	-3.37
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	-2.28

RefSeq Number	Gene	Description	Fold-regulation TT
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-2.87
NM_005563	*STMN1*	Stathmin 1	-2.56
NM_007111	*TFDP1*	Transcription factor Dp-1	-2.15
NM_001101	*ACTB*	Actin, beta	-2.79

RefSeq Number	Gene	Description	Fold-regulation viscumTT
NM_013366	*ANAPC2*	Anaphase promoting complex subunit 2	-2.11
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	-2.47
NM_003600	*AURKA*	Aurora kinase A	-4.23
NM_004217	*AURKB*	Aurora kinase B	-3.84
NM_016567	*BCCIP*	BRCA2 and CDKN1A interacting protein	-3.09
NM_000633	*BCL2*	B-cell CLL/lymphoma 2	-2.24
NM_001168	*BIRC5*	Baculoviral IAP repeat containing 5	-5.55
NM_007294	*BRCA1*	Breast cancer 1, early onset	-9.87
NM_000059	*BRCA2*	Breast cancer 2, early onset	-2.23
NM_001237	*CCNA2*	Cyclin A2	-11.78
NM_031966	*CCNB1*	Cyclin B1	-4.2
NM_004701	*CCNB2*	Cyclin B2	-3.11
NM_053056	*CCND1*	Cyclin D1	-2.21
NM_001759	*CCND2*	Cyclin D2	-5.57
NM_001760	*CCND3*	Cyclin D3	-3.37
NM_001238	*CCNE1*	Cyclin E1	-5.67
NM_001761	*CCNF*	Cyclin F	-8.3
NM_004060	*CCNG1*	Cyclin G1	-3.59
NM_004354	*CCNG2*	Cyclin G2	3.75
NM_003903	*CDC16*	Cell division cycle 16 homolog (S. cerevisiae)	-2.75
NM_001255	*CDC20*	Cell division cycle 20 homolog (S. cerevisiae)	-9.57
NM_001789	*CDC25A*	Cell division cycle 25 homolog A (S. pombe)	-6.15
NM_004359	*CDC34*	Cell division cycle 34 homolog (S. cerevisiae)	-3.64
NM_001254	*CDC6*	Cell division cycle 6 homolog (S. cerevisiae)	-6.35
NM_001786	*CDK1*	Cyclin-dependent kinase 1	-4.04
NM_001798	*CDK2*	Cyclin-dependent kinase 2	-4.16
NM_000075	*CDK4*	Cyclin-dependent kinase 4	-2.72
NM_003885	*CDK5R1*	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	-5.12
NM_016408	*CDK5RAP1*	CDK5 regulatory subunit associated protein 1	-2.78
NM_001799	*CDK7*	Cyclin-dependent kinase 7	3.14
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.89
NM_004936	*CDKN2B*	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.93
NM_005192	*CDKN3*	Cyclin-dependent kinase inhibitor 3	-3.97
NM_001274	*CHEK1*	CHK1 checkpoint homolog (S. pombe)	-3.23
NM_007194	*CHEK2*	CHK2 checkpoint homolog (S. pombe)	-3.16

RefSeq Number	Gene	Description	Fold-regulation viscumTT
NM_001826	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	-2.81
NM_003591	*CUL2*	Cullin 2	-2.22
NM_005225	*E2F1*	E2F transcription factor 1	-9.67
NM_001950	*E2F4*	E2F transcription factor 4, p107/p130-binding	-2.3
NM_013376	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	20.79
NM_016426	*GTSE1*	G-2 and S-phase expressed 1	-6.51
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. Pombe)	2.71
NM_014708	*KNTC1*	Kinetochore associated 1	-2.77
NM_002266	*KPNA2*	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	-3.22
NM_004526	*MCM2*	Minichromosome maintenance complex component 2	-4.98
NM_002388	*MCM3*	Minichromosome maintenance complex component 3	-11.55
NM_005914	*MCM4*	Minichromosome maintenance complex component 4	-7.91
NM_006739	*MCM5*	Minichromosome maintenance complex component 5	-5.75
NM_002392	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	-2.71
NM_002417	*MKI67*	Antigen identified by monoclonal antibody Ki-67	-3.28
NM_005590	*MRE11A*	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	-5.76
NM_002485	*NBN*	Nibrin	-4.97
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	-2.64
NM_002875	*RAD51*	RAD51 homolog (S. cerevisiae)	-5.86
NM_004584	*RAD9A*	RAD9 homolog A (S. pombe)	-2.28
NM_002894	*RBBP8*	Retinoblastoma binding protein 8	-3.62
NM_002895	*RBL1*	Retinoblastoma-like 1 (p107)	-4.97
NM_005611	*RBL2*	Retinoblastoma-like 2 (p130)	-6.94
NM_013376	*SERTAD1*	SERTA domain containing 1	6.69
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-100.51
NM_007111	*TFDP1*	Transcription factor Dp-1	-13.66
NM_006286	*TFDP2*	Transcription factor Dp-2 (E2F dimerization partner 2)	-2.06
NM_002046	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	-3.05
NM_000194	*HPRT1*	Hypoxanthine phosphoribosyltransferase 1	-2.5

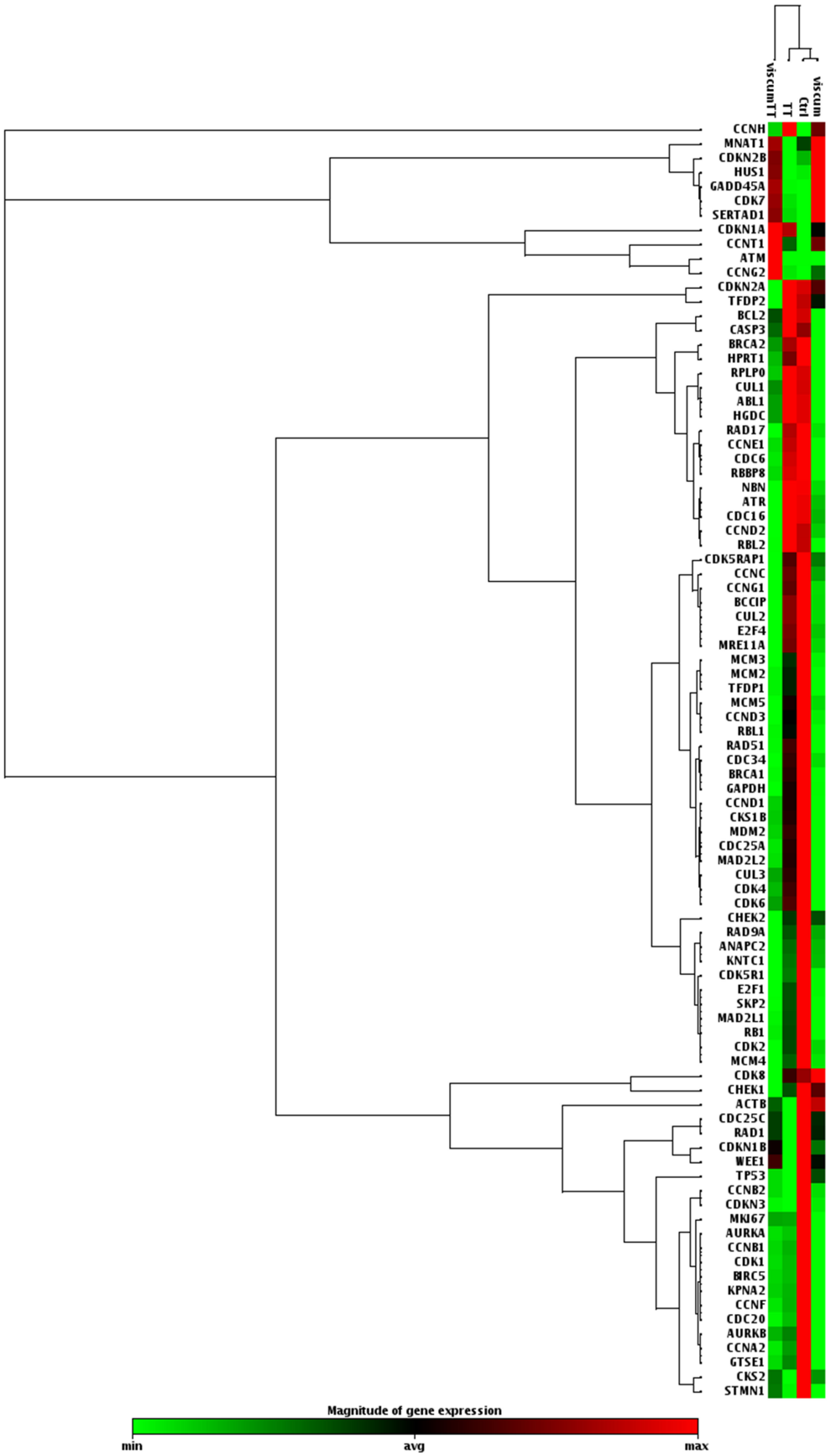


Figure S3a Clustergram of U2OS cells after viscum, TT and viscumTT treatment.

Table S2b Up- and down-regulated genes by viscum, TT and viscumTT in 143B cells.

RefSeq Number	Gene	Description	Fold-regulation viscum
NM_013366	*ANAPC2*	Anaphase promoting complex subunit 2	3.19
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	5.12
NM_007294	*BRCA1*	Breast cancer 1, early onset	-3.2
NM_000059	*BRCA2*	Breast cancer 2, early onset	2.48
NM_001237	*CCNA2*	Cyclin A2	-2.39
NM_001759	*CCND2*	Cyclin D2	2.86
NM_001238	*CCNE1*	Cyclin E1	-2.12
NM_001239	*CCNG1*	Cyclin G1	-3.38
NM_001240	*CCNH*	Cyclin H	3.96
NM_001259	*CCNT1*	Cyclin T1	2.54
NM_001254	*CDC6*	Cell division cycle 6 homolog (S. cerevisiae)	-3.53
NM_001798	*CDK2*	Cyclin-dependent kinase 2	-2.5
NM_016408	*CDK5RAP1*	CDK5 regulatory subunit associated protein 1	3.16
NM_001259	*CDK6*	Cyclin-dependent kinase 6	3.52
NM_001799	*CDK7*	Cyclin-dependent kinase 7	6.09
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5.06
NM_004064	*CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.76
NM_005225	*E2F1*	E2F transcription factor 1	-4.2
NM_013376	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	27.99
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. Pombe)	8.86
NM_006341	*MAD2L2*	MAD2 mitotic arrest deficient-like 2 (yeast)	-2.25
NM_002388	*MCM3*	Minichromosome maintenance complex component 3	-2.1
NM_005914	*MCM4*	Minichromosome maintenance complex component 4	-2.33
NM_002392	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	3.82
NM_002431	*MNAT1*	Menage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)	2.39
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	2.55
NM_002875	*RAD51*	RAD51 homolog (S. cerevisiae)	-4.55
NM_000321	*RB1*	Retinoblastoma 1	2.26
NM_005611	*RBL2*	Retinoblastoma-like 2 (p130)	-2.74
NM_013376	*SERTAD1*	SERTA domain containing 1	8.01
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-18.07
NM_005563	*STMN1*	Stathmin 1	-4.42
NM_007111	*TFDP1*	Transcription factor Dp-1	-3.9
NM_006286	*TFDP2*	Transcription factor Dp-2 (E2F dimerization partner 2)	2.69
NM_000546	*TP53*	Tumor protein p53	2.1
NM_003390	*WEE1*	WEE1 homolog (S. pombe)	2.49

RefSeq Number	Gene	Description	Fold-regulation TT
NM_001168	*BIRC5*	Baculoviral IAP repeat containing 5	2
NM_031966	*CCNB1*	Cyclin B1	-2.2
NM_001759	*CCND2*	Cyclin D2	2.1

RefSeq Number	Gene	Description	Fold-regulation TT
NM_001239	*CCNG2*	Cyclin G2	3.05
NM_001786	*CDK1*	Cyclin-dependent kinase 1	-2.27
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6.47
NM_005192	*CDKN3*	Cyclin-dependent kinase inhibitor 3	-2.26
NM_016426	*GTSE1*	G-2 and S-phase expressed 1	-2.08
NM_002358	*MAD2L1*	MAD2 mitotic arrest deficient-like 1 (yeast)	-2.25
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-3.1
NM_007111	*TFDP1*	Transcription factor Dp-1	-2.45

RefSeq Number	Gene	Description	Fold-regulation viscumTT
NM_013366	*ANAPC2*	Anaphase promoting complex subunit 2	3.88
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	5.39
NM_016567	*BCCIP*	BRCA2 and CDKN1A interacting protein	2.19
NM_007294	*BRCA1*	Breast cancer 1, early onset	-2.85
NM_000059	*BRCA2*	Breast cancer 2, early onset	2.19
NM_001237	*CCNA2*	Cyclin A2	-2.01
NM_005190	*CCNC*	Cyclin C	2.18
NM_053056	*CCND2*	Cyclin D1	2.95
NM_001238	*CCNE1*	Cyclin E1	-2.09
NM_001239	*CCNG1*	Cyclin G1	-3.25
NM_004354	*CCNG2*	Cyclin G2	-2.17
NM_001239	*CCNH*	Cyclin H	4.38
NM_001240	*CCNT1*	Cyclin T1	3.07
NM_003903	*CDC6*	Cell division cycle 16 homolog (S. cerevisiae)	-2.79
NM_001254	*CDK2*	Cell division cycle 6 homolog (S. cerevisiae)	-2.81
NM_001798	*CDK5RAP1*	Cyclin-dependent kinase 2	3.02
NM_001259	*CDK6*	Cyclin-dependent kinase 6	3.28
NM_001799	*CDK7*	Cyclin-dependent kinase 7	5.1
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	11.23
NM_004064	*CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.46
NM_003592	*CUL1*	Cullin 1	2.15
NM_003590	*CUL3*	Cullin 3	2.17
NM_005225	*E2F1*	E2F transcription factor 1	-4.96
NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	31.38
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. Pombe)	7.69
NM_002388	*MCM3*	Minichromosome maintenance complex component 3	-2.6
NM_005914	*MCM4*	Minichromosome maintenance complex component 4	-3.07
NM_002392	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	3.32
NM_002431	*MNAT1*	Menage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)	2.96
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	2.91

RefSeq Number	Gene	Description	Fold-regulation viscumTT
NM_005611	*RBL2*	Retinoblastoma-like 2 (p130)	-2.24
NM_013376	*SERTAD1*	SERTA domain containing 1	8.76
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-20.43
NM_005563	*STMN1*	Stathmin 1	-3.04
NM_007111	*TFDP1*	Transcription factor Dp-1	-4.45
NM_003390	*WEE1*	WEE1 homolog (S. pombe)	2.28
NM_001101	*ACTB*	Actin, beta	-2.65
NM_004048	*B2M*	Beta-2-microglobulin	2.36

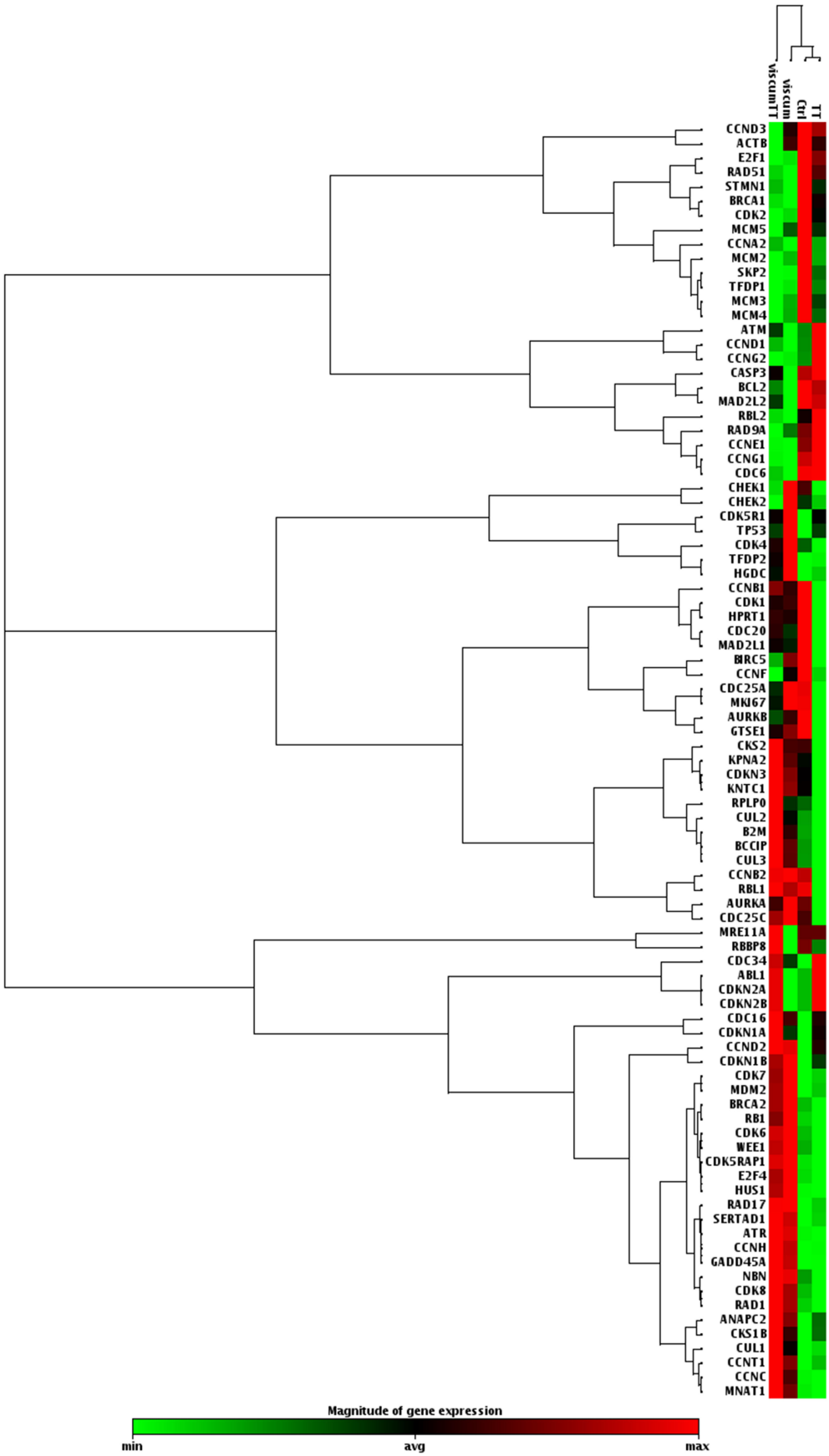


Figure S3b Clustergram of 143B cells after viscum, TT and viscumTT treatment.

Table S2c Up- and down-regulated genes by viscum, TT and viscumTT in Saos-2 cells.

RefSeq Number	Gene	Description	Fold-regulation viscum
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	2.21
NM_053056	*CCND1*	Cyclin D1	3.24
NM_004354	*CCNG2*	Cyclin G2	2.61
NM_001239	*CCNH*	Cyclin H	2.29
NM_001240	*CCNT1*	Cyclin T1	3.17
NM_001259	*CDK6*	Cyclin-dependent kinase 6	2.73
NM_001799	*CDK7*	Cyclin-dependent kinase 7	2.9
NM_004936	*CDKN2B*	Cyclin-dependent kinase inhibitor 2B (p15)	2.66
NM_001950	*E2F4*	E2F transcription factor 4	2.02
NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	4.51
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. pombe)	3.4
NM_002431	*MNAT1*	Menage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)	2.37
NM_002853	*RAD1*	RAD1 homolog (S. pombe)	2.24
NM_002895	*RBL1*	Retinoblastoma-like 1 (p107)	2.11
NM_013376	*SERTAD1*	SERTA domain containing 1	4.63

RefSeq Number	Gene	Description	Fold-regulation TT
NM_003600	*AURKB*	Aurora kinase A	-2.21
NM_001168	*BIRC5*	Baculoviral IAP repeat containing 5	-3.11
NM_001237	*CCNA2*	Cyclin A2	-3.03
NM_053056	*CCND1*	Cyclin D1	2.65
NM_001759	*CCND2*	Cyclin D2	2.52
NM_001259	*CCNG2*	Cyclin G2	2.45
NM_001789	*CDC25A*	Cell division cycle 25 homolog A (S. pombe)	-2.17
NM_004359	*CDC34*	Cell division cycle 34 homolog (S. cerevisiae)	2.06
NM_001786	*CDK1*	Cyclin-dependent kinase 1	-2.88
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.49
NM_005225	*E2F1*	E2F transcription factor 1	-2.01
NM_016426	*GTSE1*	G-2 and S-phase expressed 1	-2.11
NM_002358	*MAD2L1*	MAD2 mitotic arrest deficient-like 1 (yeast)	-2.44
NM_005914	*MCM4*	Minichromosome maintenance complex component 4	-2.35
NM_002417	*MKI67*	Antigen identified by monoclonal antibody Ki-67	-2.1
NM_000321	*RB1*	Retinoblastoma 1	2.52
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-2.46
NM_005563	*STMN1*	Stathmin 1	-2.09
NM_000546	*TP53*	Tumor protein p53	2.52
NM_001101	*ACTB*	Actin, beta	-2.54

RefSeq Number	Gene	Description	Fold-regulation viscumTT
NM_013366	*ANAPC2*	Anaphase promoting complex subunit 2	2.44
NM_001184	*ATR*	ATR Ataxia telangiectasia and Rad3 related	2.04
NM_005190	*CCNC*	Cyclin C	2.1
NM_053056	*CCND1*	Cyclin D1	6.02
NM_004354	*CCNG2*	Cyclin G2	3.66
NM_001239	*CCNH*	Cyclin H	2.2
NM_001240	*CCNT1*	Cyclin T1	2.58
NM_003903	*CDC16*	Cell division cycle 16 homolog (S. cerevisiae)	2.21
NM_004359	*CDC34*	Cell division cycle 34 homolog (S. cerevisiae)	2.39
NM_001259	*CDK6*	Cyclin-dependent kinase 6	2.16
NM_001799	*CDK7*	Cyclin-dependent kinase 7	2.46
NM_001260	*CDK8*	Cyclin-dependent kinase 8	2.27
NM_000389	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.8
NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, alpha	2.05
NM_004507	*HUS1*	HUS1 checkpoint homolog (S. Pombe)	2.56
NM_002431	*MNAT1*	Menage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)	2.28
NM_013376	*SERTAD1*	SERTA domain containing 1	3.15
NM_005983	*SKP2*	S-phase kinase-associated protein 2 (p45)	-2.2

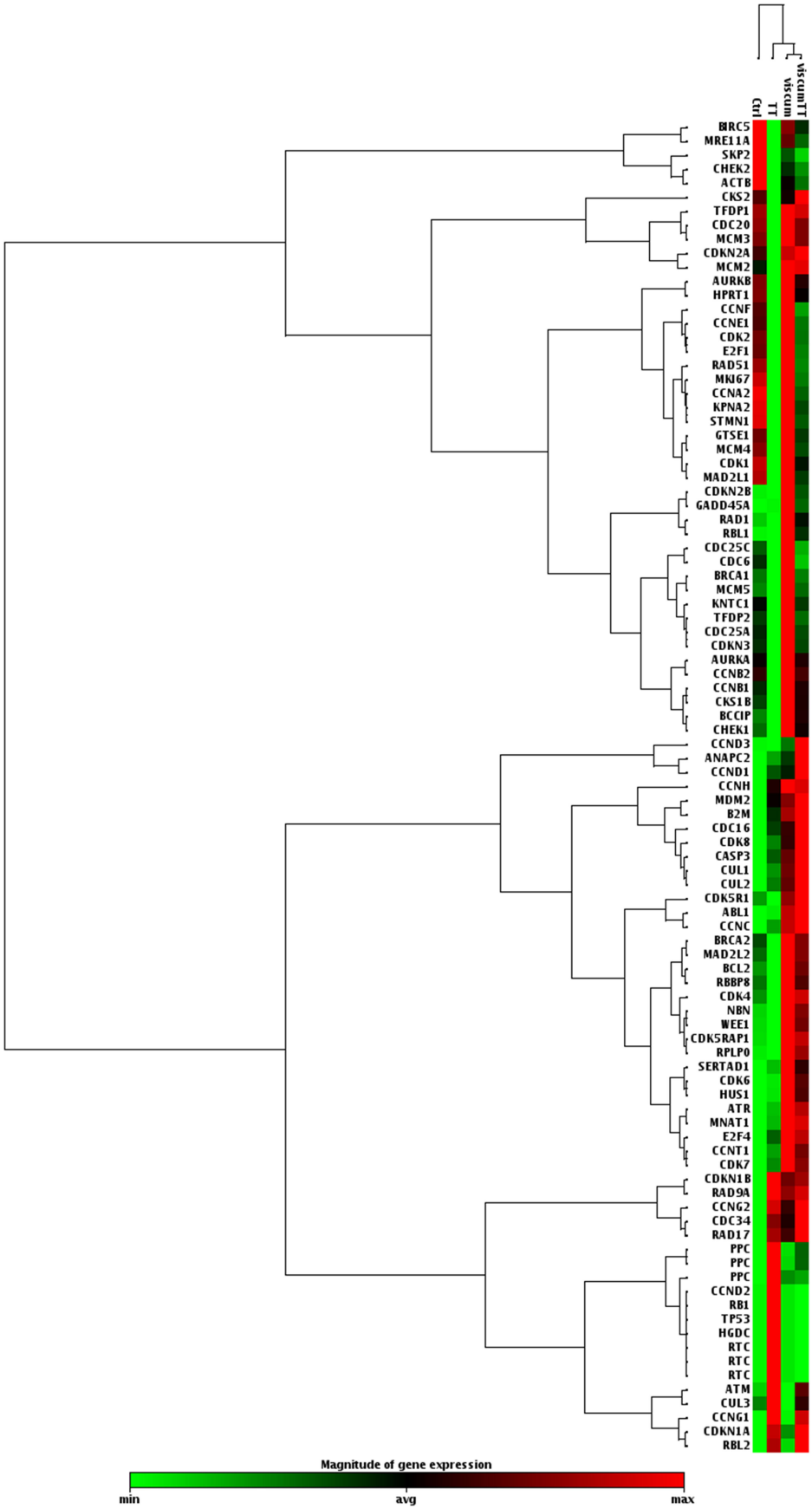


Figure S3c Clustergram of Saos-2 cells after viscum, TT and viscumTT treatment.

6.5 siRNA knockdown does not alter cell cycle phase distribution in control U2OS cells

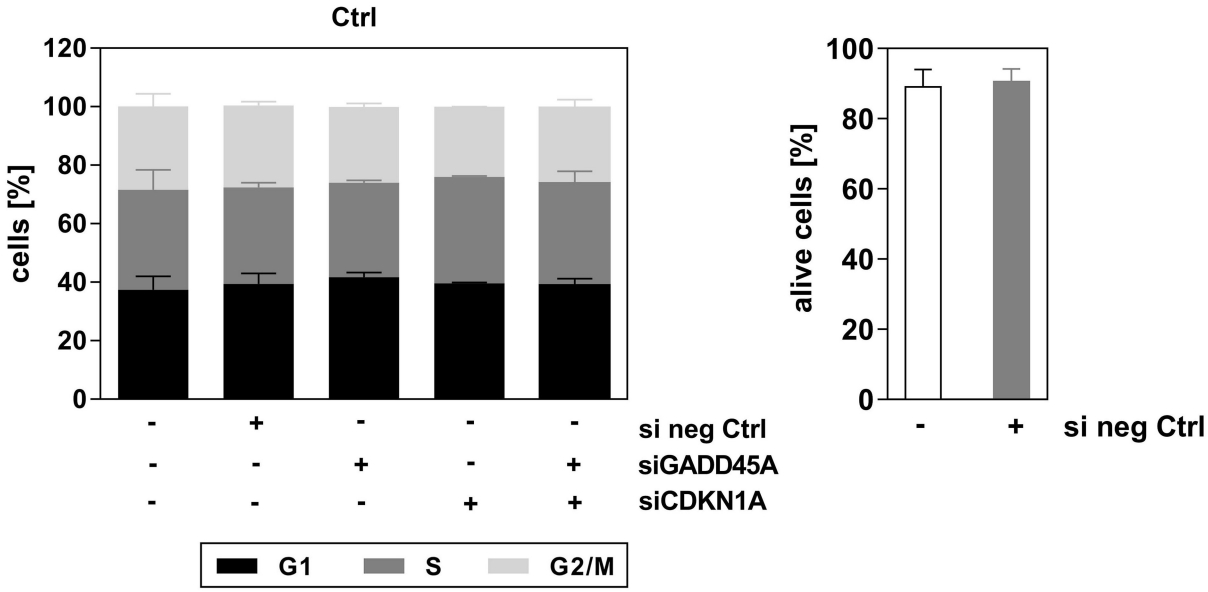


Figure S4 siRNA knockdown experiments did not influence cell cycle distribution in U2OS control cells. U2OS cells were reverse transfected with different siRNA variations and non-targeting negative siRNA (si neg Ctrl) and not further treated with viscum, TT and viscumTT. Cell cycle distribution was analyzed by PI staining and flow cytometry. Bars display cells [%] in cell cycle phase ± SD of at least three independent experiments.

6.6 Knockdown of *CDKN1A* and *GADD45A* significantly attenuate cell cycle arrest

Table S3a P-values of siRNA experiments of the cell cycle in U2OS cells.

Tukey's multiple comparisons test viscum	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G1					
Ctrl vs. viscum	-17	-27.11 to -6.89	Yes	***	0.0002
Ctrl vs. +siGADD45A	-4.366	-15.09 to 6.359	No	ns	0.7754
Ctrl vs. +siCDKN1A	-6.894	-18.57 to 4.782	No	ns	0.4576
Ctrl vs. +siGADD45A+siCDKN1A	-3.987	-15.66 to 7.689	No	ns	0.867
viscum vs. +siGADD45A	12.64	1.91 to 23.36	Yes	*	0.0136
viscum vs. +siCDKN1A	10.11	-1.568 to 21.78	No	ns	0.1183
viscum vs. +siGADD45A+siCDKN1A	13.01	1.339 to 24.69	Yes	*	0.022
+siGADD45A vs. +siCDKN1A	-2.528	-14.74 to 9.683	No	ns	0.9762
+siGADD45A vs. +siGADD45A+siCDKN1A	0.3792	-11.83 to 12.59	No	ns	>0.9999
+siCDKN1A vs. +siGADD45A+siCDKN1A	2.907	-10.15 to 15.96	No	ns	0.9689

Tukey's multiple comparisons test viscum	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
S					
Ctrl vs. viscum	5.638	-4.474 to 15.75	No	ns	0.5149
Ctrl vs. +siGADD45A	-1.913	-12.64 to 8.813	No	ns	0.9863
Ctrl vs. +siCDKN1A	0.1033	-11.57 to 11.78	No	ns	>0.9999
Ctrl vs. +siGADD45A+siCDKN1A	6.033	-5.643 to 17.71	No	ns	0.5879
viscum vs. +siGADD45A	-7.551	-18.28 to 3.175	No	ns	0.2824
viscum vs. +siCDKN1A	-5.535	-17.21 to 6.141	No	ns	0.6639
viscum vs. +siGADD45A+siCDKN1A	0.3953	-11.28 to 12.07	No	ns	>0.9999
+siGADD45A vs. +siCDKN1A	2.016	-10.2 to 14.23	No	ns	0.9897
+siGADD45A vs. +siGADD45A+siCDKN1A	7.946	-4.265 to 20.16	No	ns	0.3592
+siCDKN1A vs. +siGADD45A+siCDKN1A	5.93	-7.124 to 18.98	No	ns	0.6981
G2/M					
Ctrl vs. viscum	11.06	0.9444 to 21.17	Yes	*	0.0258
Ctrl vs. +siGADD45A	6.028	-4.697 to 16.75	No	ns	0.507
Ctrl vs. +siCDKN1A	6.361	-5.315 to 18.04	No	ns	0.5376
Ctrl vs. +siGADD45A+siCDKN1A	-3.359	-15.03 to 8.317	No	ns	0.9239
viscum vs. +siGADD45A	-5.028	-15.75 to 5.697	No	ns	0.673
viscum vs. +siCDKN1A	-4.695	-16.37 to 6.981	No	ns	0.7832
viscum vs. +siGADD45A+siCDKN1A	-14.41	-26.09 to -2.739	Yes	**	0.0087
+siGADD45A vs. +siCDKN1A	0.3333	-11.88 to 12.54	No	ns	>0.9999
+siGADD45A vs. +siGADD45A+siCDKN1A	-9.387	-21.6 to 2.824	No	ns	0.2043
+siCDKN1A vs. +siGADD45A+siCDKN1A	-9.72	-22.77 to 3.334	No	ns	0.2314

Tukey's multiple comparisons test TT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G1					
Ctrl vs. TT	-26.59	-37.1 to -16.08	Yes	****	<0.0001
Ctrl vs. +siGADD45A	-24.03	-34.54 to -13.52	Yes	****	<0.0001
Ctrl vs. +siCDKN1A	-10.62	-21.77 to 0.5237	No	ns	0.0688
Ctrl vs. +siGADD45A+siCDKN1A	-6.569	-17.72 to 4.579	No	ns	0.465
TT vs. +siGADD45A	2.564	-7.946 to 13.07	No	ns	0.9582
TT vs. +siCDKN1A	15.97	4.82 to 27.12	Yes	**	0.0015
TT vs. +siGADD45A+siCDKN1A	20.02	8.875 to 31.17	Yes	****	<0.0001
+siGADD45A vs. +siCDKN1A	13.4	2.256 to 24.55	Yes	*	0.0109
+siGADD45A vs. +siGADD45A+siCDKN1A	17.46	6.311 to 28.61	Yes	***	0.0004
+siCDKN1A vs. +siGADD45A+siCDKN1A	4.055	-7.696 to 15.81	No	ns	0.8658
S					
Ctrl vs. TT	16.98	6.47 to 27.49	Yes	***	0.0003
Ctrl vs. +siGADD45A	13.67	3.164 to 24.18	Yes	**	0.0048
Ctrl vs. +siCDKN1A	2.418	-8.73 to 13.57	No	ns	0.9726
Ctrl vs. +siGADD45A+siCDKN1A	0.99	-10.16 to 12.14	No	ns	0.9991

Tukey's multiple comparisons test TT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
TT vs. +siGADD45A	-3.306	-13.82 to 7.204	No	ns	0.9002
TT vs. +siCDKN1A	-14.56	-25.71 to -3.415	Yes	**	0.0046
TT vs. +siGADD45A+siCDKN1A	-15.99	-27.14 to -4.842	Yes	**	0.0015
+siGADD45A vs. +siCDKN1A	-11.26	-22.4 to -0.1088	Yes	*	0.0467
+siGADD45A vs. +siGADD45A+siCDKN1A	-12.68	-23.83 to -1.536	Yes	*	0.0182
+siCDKN1A vs. +siGADD45A+siCDKN1A	-1.428	-13.18 to 10.32	No	ns	0.9969
G2/M					
Ctrl vs. TT	10.56	0.0538 to 21.07	Yes	*	0.0483
Ctrl vs. +siGADD45A	11	0.4918 to 21.51	Yes	*	0.0359
Ctrl vs. +siCDKN1A	8.376	-2.772 to 19.52	No	ns	0.2266
Ctrl vs. +siGADD45A+siCDKN1A	6.451	-4.697 to 17.6	No	ns	0.4834
TT vs. +siGADD45A	0.438	-10.07 to 10.95	No	ns	>0.9999
TT vs. +siCDKN1A	-2.189	-13.34 to 8.959	No	ns	0.981
TT vs. +siGADD45A+siCDKN1A	-4.114	-15.26 to 7.034	No	ns	0.835
+siGADD45A vs. +siCDKN1A	-2.627	-13.77 to 8.521	No	ns	0.963
+siGADD45A vs. +siGADD45A+siCDKN1A	-4.552	-15.7 to 6.596	No	ns	0.778
+siCDKN1A vs. +siGADD45A+siCDKN1A	-1.925	-13.68 to 9.826	No	ns	0.9903

Tukey's multiple comparisons test viscumTT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G1					
Ctrl vs. viscumTT	-17.93	-28.56 to -7.306	Yes	***	0.0001
Ctrl vs. +siGADD45A	-5.258	-15.88 to 5.368	No	ns	0.6315
Ctrl vs. +siCDKN1A	-3.974	-15.24 to 7.296	No	ns	0.8558
Ctrl vs. +siGADD45A+siCDKN1A	-5.196	-16.47 to 6.074	No	ns	0.6906
viscumTT vs. +siGADD45A	12.67	2.048 to 23.3	Yes	*	0.0119
viscumTT vs. +siCDKN1A	13.96	2.688 to 25.23	Yes	**	0.0082
viscumTT vs. +siGADD45A+siCDKN1A	12.74	1.465 to 24.01	Yes	*	0.0194
+siGADD45A vs. +siCDKN1A	1.284	-9.986 to 12.55	No	ns	0.9976
+siGADD45A vs. +siGADD45A+siCDKN1A	0.0615	-11.21 to 11.33	No	ns	>0.9999
+siCDKN1A vs. +siGADD45A+siCDKN1A	-1.223	-13.1 to 10.66	No	ns	0.9984
S					
Ctrl vs. viscumTT	7.842	-2.784 to 18.47	No	ns	0.2418
Ctrl vs. +siGADD45A	2.6	-8.026 to 13.23	No	ns	0.9575
Ctrl vs. +siCDKN1A	-3.853	-15.12 to 7.418	No	ns	0.8691
Ctrl vs. +siGADD45A+siCDKN1A	-3.843	-16.11 to 8.426	No	ns	0.9011
viscumTT vs. +siGADD45A	-5.242	-15.87 to 5.384	No	ns	0.6342
viscumTT vs. +siCDKN1A	-11.69	-22.96 to -0.4243	Yes	*	0.0384
viscumTT vs. +siGADD45A+siCDKN1A	-11.69	-23.95 to 0.5841	No	ns	0.069
+siGADD45A vs. +siCDKN1A	-6.453	-17.72 to 4.818	No	ns	0.4931
+siGADD45A vs. +siGADD45A+siCDKN1A	-6.443	-18.71 to 5.826	No	ns	0.5773
+siCDKN1A vs. +siGADD45A+siCDKN1A	0.009167	-12.82 to 12.84	No	ns	>0.9999

Tukey's multiple comparisons test viscumTT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G2/M					
Ctrl vs. viscumTT	11.1	0.4724 to 21.72	Yes	*	0.0366
Ctrl vs. +siGADD45A	3.574	-7.052 to 14.2	No	ns	0.8756
Ctrl vs. +siCDKN1A	7.041	-4.23 to 18.31	No	ns	0.4044
Ctrl vs. +siGADD45A+siCDKN1A	9.848	-2.421 to 22.12	No	ns	0.1718
viscumTT vs. +siGADD45A	-7.524	-18.15 to 3.102	No	ns	0.28
viscumTT vs. +siCDKN1A	-4.058	-15.33 to 7.213	No	ns	0.8462
viscumTT vs. +siGADD45A+siCDKN1A	-1.25	-13.52 to 11.02	No	ns	0.9984
+siGADD45A vs. +siCDKN1A	3.467	-7.804 to 14.74	No	ns	0.9069
+siGADD45A vs. +siGADD45A+siCDKN1A	6.274	-5.995 to 18.54	No	ns	0.602
+siCDKN1A vs. +siGADD45A+siCDKN1A	2.808	-10.02 to 15.64	No	ns	0.9715

Table S3b P-values of siRNA experiments of the cell cycle in 143B cells.

Tukey's multiple comparisons test viscum	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G1					
Ctrl vs. viscum	5.897	-6.365 to 18.16	No	ns	0.6355
Ctrl vs. +siGADD45A	0.6967	-11.57 to 12.96	No	ns	0.9998
Ctrl vs. +siCDKN1A	5.453	-6.809 to 17.72	No	ns	0.6992
Ctrl vs. +siGADD45A+siCDKN1A	10.58	-1.682 to 22.84	No	ns	0.1169
viscum vs. +siGADD45A	-5.2	-17.46 to 7.062	No	ns	0.7342
viscum vs. +siCDKN1A	-0.4433	-12.71 to 11.82	No	ns	>0.9999
viscum vs. +siGADD45A+siCDKN1A	4.683	-7.579 to 16.95	No	ns	0.8011
+siGADD45A vs. +siCDKN1A	4.757	-7.505 to 17.02	No	ns	0.7921
+siGADD45A vs. +siGADD45A+siCDKN1A	9.883	-2.379 to 22.15	No	ns	0.161
+siCDKN1A vs. +siGADD45A+siCDKN1A	5.127	-7.135 to 17.39	No	ns	0.7441
S					
Ctrl vs. viscum	-10.39	-22.66 to 1.869	No	ns	0.1276
Ctrl vs. +siGADD45A	-4.223	-16.49 to 8.039	No	ns	0.8537
Ctrl vs. +siCDKN1A	-8.04	-20.3 to 4.222	No	ns	0.3382
Ctrl vs. +siGADD45A+siCDKN1A	-14.87	-27.13 to -2.608	Yes	*	0.0114
viscum vs. +siGADD45A	6.17	-6.092 to 18.43	No	ns	0.5955
viscum vs. +siCDKN1A	2.353	-9.909 to 14.62	No	ns	0.9802
viscum vs. +siGADD45A+siCDKN1A	-4.477	-16.74 to 7.785	No	ns	0.8256
+siGADD45A vs. +siCDKN1A	-3.817	-16.08 to 8.445	No	ns	0.8935
+siGADD45A vs. +siGADD45A+siCDKN1A	-10.65	-22.91 to 1.615	No	ns	0.1132
+siCDKN1A vs. +siGADD45A+siCDKN1A	-6.83	-19.09 to 5.432	No	ns	0.4994
G2/M					
Ctrl vs. viscum	4.087	-8.175 to 16.35	No	ns	0.8678
Ctrl vs. +siGADD45A	3.417	-8.845 to 15.68	No	ns	0.926
Ctrl vs. +siCDKN1A	2.237	-10.03 to 14.5	No	ns	0.9836
Ctrl vs. +siGADD45A+siCDKN1A	4.153	-8.109 to 16.42	No	ns	0.861
viscum vs. +siGADD45A	-0.67	-12.93 to 11.59	No	ns	0.9998

Tukey's multiple comparisons test TT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
viscum vs. +siCDKN1A	-1.85	-14.11 to 10.41	No	ns	0.992
viscum vs. +siGADD45A+siCDKN1A	0.06667	-12.2 to 12.33	No	ns	>0.9999
+siGADD45A vs. +siCDKN1A	-1.18	-13.44 to 11.08	No	ns	0.9986
+siGADD45A vs. +siGADD45A+siCDKN1A	0.7367	-11.53 to 13	No	ns	0.9998
+siCDKN1A vs. +siGADD45A+siCDKN1A	1.917	-10.35 to 14.18	No	ns	0.9908
G1					
Ctrl vs. TT	-9.323	-16.58 to -2.071	Yes	**	0.0066
Ctrl vs. +siGADD45A	-21.45	-28.71 to -14.2	Yes	****	<0.0001
Ctrl vs. +siCDKN1A	-2.643	-9.895 to 4.609	No	ns	0.8265
Ctrl vs. +siGADD45A+siCDKN1A	-0.003333	-7.255 to 7.249	No	ns	>0.9999
TT vs. +siGADD45A	-12.13	-19.38 to -4.878	Yes	***	0.0003
TT vs. +siCDKN1A	6.68	-0.572 to 13.93	No	ns	0.0825
TT vs. +siGADD45A+siCDKN1A	9.32	2.068 to 16.57	Yes	**	0.0067
+siGADD45A vs. +siCDKN1A	18.81	11.56 to 26.06	Yes	****	<0.0001
+siGADD45A vs. +siGADD45A+siCDKN1A	21.45	14.2 to 28.7	Yes	****	<0.0001
+siCDKN1A vs. +siGADD45A+siCDKN1A	2.64	-4.612 to 9.892	No	ns	0.8271
S					
Ctrl vs. TT	6.5	-0.752 to 13.75	No	ns	0.0959
Ctrl vs. +siGADD45A	18.49	11.24 to 25.74	Yes	****	<0.0001
Ctrl vs. +siCDKN1A	1.427	-5.825 to 8.679	No	ns	0.9784
Ctrl vs. +siGADD45A+siCDKN1A	-0.6533	-7.905 to 6.599	No	ns	0.9989
TT vs. +siGADD45A	11.99	4.738 to 19.24	Yes	***	0.0004
TT vs. +siCDKN1A	-5.073	-12.33 to 2.179	No	ns	0.2771
TT vs. +siGADD45A+siCDKN1A	-7.153	-14.41 to 0.09865	No	ns	0.0546
+siGADD45A vs. +siCDKN1A	-17.06	-24.32 to -9.811	Yes	****	<0.0001
+siGADD45A vs. +siGADD45A+siCDKN1A	-19.14	-26.4 to -11.89	Yes	****	<0.0001
+siCDKN1A vs. +siGADD45A+siCDKN1A	-2.08	-9.332 to 5.172	No	ns	0.9185
G2/M					
Ctrl vs. TT	3.063	-4.189 to 10.32	No	ns	0.737
Ctrl vs. +siGADD45A	2.993	-4.259 to 10.25	No	ns	0.7529
Ctrl vs. +siCDKN1A	1.403	-5.849 to 8.655	No	ns	0.9796
Ctrl vs. +siGADD45A+siCDKN1A	0.5	-6.752 to 7.752	No	ns	0.9996
TT vs. +siGADD45A	-0.07	-7.322 to 7.182	No	ns	>0.9999
TT vs. +siCDKN1A	-1.66	-8.912 to 5.592	No	ns	0.9626
TT vs. +siGADD45A+siCDKN1A	-2.563	-9.815 to 4.689	No	ns	0.8417
+siGADD45A vs. +siCDKN1A	-1.59	-8.842 to 5.662	No	ns	0.9679
+siGADD45A vs. +siGADD45A+siCDKN1A	-2.493	-9.745 to 4.759	No	ns	0.8545
+siCDKN1A vs. +siGADD45A+siCDKN1A	-0.9033	-8.155 to 6.349	No	ns	0.9962

Tukey's multiple comparisons test viscumTT	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
G1					
Ctrl vs. viscumTT	-1.133	-8.331 to 6.066	No	ns	0.9913
Ctrl vs. +siGADD45A	-12.14	-19.34 to -4.942	Yes	***	0.0002
Ctrl vs. +siCDKN1A	-1.228	-8.426 to 5.971	No	ns	0.9882
Ctrl vs. +siGADD45A+siCDKN1A	3.685	-3.513 to 10.88	No	ns	0.5941
viscumTT vs. +siGADD45A	-11.01	-17.67 to -4.343	Yes	***	0.0003
viscumTT vs. +siCDKN1A	-0.095	-6.759 to 6.569	No	ns	>0.9999
viscumTT vs. +siGADD45A+siCDKN1A	4.818	-1.847 to 11.48	No	ns	0.2564
+siGADD45A vs. +siCDKN1A	10.91	4.248 to 17.58	Yes	***	0.0003
+siGADD45A vs. +siGADD45A+siCDKN1A	15.83	9.161 to 22.49	Yes	****	<0.0001
+siCDKN1A vs. +siGADD45A+siCDKN1A	4.913	-1.752 to 11.58	No	ns	0.2389
S					
Ctrl vs. viscumTT	-1.365	-8.563 to 5.833	No	ns	0.9825
Ctrl vs. +siGADD45A	9.4	2.202 to 16.6	Yes	**	0.005
Ctrl vs. +siCDKN1A	-0.4675	-7.666 to 6.731	No	ns	0.9997
Ctrl vs. +siGADD45A+siCDKN1A	-4.263	-11.46 to 2.936	No	ns	0.4524
viscumTT vs. +siGADD45A	10.77	4.101 to 17.43	Yes	***	0.0004
viscumTT vs. +siCDKN1A	0.8975	-5.767 to 7.562	No	ns	0.9952
viscumTT vs. +siGADD45A+siCDKN1A	-2.897	-9.562 to 3.767	No	ns	0.7289
+siGADD45A vs. +siCDKN1A	-9.868	-16.53 to -3.203	Yes	**	0.0012
+siGADD45A vs. +siGADD45A+siCDKN1A	-13.66	-20.33 to -6.998	Yes	****	<0.0001
+siCDKN1A vs. +siGADD45A+siCDKN1A	-3.795	-10.46 to 2.869	No	ns	0.4916
G2/M					
Ctrl vs. viscumTT	2.462	-4.736 to 9.66	No	ns	0.8651
Ctrl vs. +siGADD45A	2.862	-4.336 to 10.06	No	ns	0.7882
Ctrl vs. +siCDKN1A	1.417	-5.781 to 8.615	No	ns	0.9799
Ctrl vs. +siGADD45A+siCDKN1A	0.6717	-6.526 to 7.87	No	ns	0.9989
viscumTT vs. +siGADD45A	0.4	-6.264 to 7.064	No	ns	0.9998
viscumTT vs. +siCDKN1A	-1.045	-7.709 to 5.619	No	ns	0.9914
viscumTT vs. +siGADD45A+siCDKN1A	-1.79	-8.454 to 4.874	No	ns	0.939
+siGADD45A vs. +siCDKN1A	-1.445	-8.109 to 5.219	No	ns	0.9714
+siGADD45A vs. +siGADD45A+siCDKN1A	-2.19	-8.854 to 4.474	No	ns	0.881
+siCDKN1A vs. +siGADD45A+siCDKN1A	-0.745	-7.409 to 5.919	No	ns	0.9977

6.7 ViscumTT reveals significant group effect in Saos-2 xenograft

Table S4 P-values of osteosarcoma xenograft.

Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
CD vs. viscum	0.000675	-0.129 to 0.1303	No	ns	>0.9999
CD vs. TT	0.000675	-0.129 to 0.1303	No	ns	>0.9999
CD vs. viscumTT	0.000875	-0.1288 to 0.1305	No	ns	>0.9999
viscum vs. TT	0	-0.1438 to 0.1438	No	ns	>0.9999
viscum vs. viscumTT	0.0002	-0.1436 to 0.144	No	ns	>0.9999
TT vs. viscumTT	0.0002	-0.1436 to 0.144	No	ns	>0.9999
Row 2					
CD vs. viscum	0.01918	-0.1105 to 0.1488	No	ns	>0.9999
CD vs. TT	0.01038	-0.1193 to 0.14	No	ns	>0.9999
CD vs. viscumTT	-0.002225	-0.1319 to 0.1274	No	ns	>0.9999
viscum vs. TT	-0.0088	-0.1526 to 0.135	No	ns	>0.9999
viscum vs. viscumTT	-0.0214	-0.1652 to 0.1224	No	ns	>0.9999
TT vs. viscumTT	-0.0126	-0.1564 to 0.1312	No	ns	>0.9999
Row 3					
CD vs. viscum	0.04943	-0.08022 to 0.1791	No	ns	>0.9999
CD vs. TT	0.03323	-0.09642 to 0.1629	No	ns	>0.9999
CD vs. viscumTT	0.07423	-0.05542 to 0.2039	No	ns	0.7678
viscum vs. TT	-0.0162	-0.16 to 0.1276	No	ns	>0.9999
viscum vs. viscumTT	0.0248	-0.119 to 0.1686	No	ns	>0.9999
TT vs. viscumTT	0.041	-0.1028 to 0.1848	No	ns	>0.9999
Row 4					
CD vs. viscum	0.1113	-0.01834 to 0.2409	No	ns	0.1386
CD vs. TT	0.0947	-0.03494 to 0.2243	No	ns	0.3162
CD vs. viscumTT	0.1453	0.01566 to 0.2749	Yes	*	0.0192
viscum vs. TT	-0.0166	-0.1604 to 0.1272	No	ns	>0.9999
viscum vs. viscumTT	0.034	-0.1098 to 0.1778	No	ns	>0.9999
TT vs. viscumTT	0.0506	-0.09323 to 0.1944	No	ns	>0.9999
Row 5					
CD vs. viscum	0.1068	-0.02289 to 0.2364	No	ns	0.1754
CD vs. TT	0.07675	-0.05289 to 0.2064	No	ns	0.6936
CD vs. viscumTT	0.1184	-0.01129 to 0.248	No	ns	0.0949
viscum vs. TT	-0.03	-0.1738 to 0.1138	No	ns	>0.9999
viscum vs. viscumTT	0.0116	-0.1322 to 0.1554	No	ns	>0.9999
TT vs. viscumTT	0.0416	-0.1022 to 0.1854	No	ns	>0.9999
Row 6					
CD vs. viscum	0.1783	0.04868 to 0.308	Yes	**	0.002
CD vs. TT	0.1729	0.04328 to 0.3026	Yes	**	0.0029
CD vs. viscumTT	0.2775	0.1479 to 0.4072	Yes	****	<0.0001
viscum vs. TT	-0.0054	-0.1492 to 0.1384	No	ns	>0.9999
viscum vs. viscumTT	0.0992	-0.04463 to 0.243	No	ns	0.403

Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
TT vs. viscumTT	0.1046	-0.03923 to 0.2484	No	ns	0.3224
Row 7					
CD vs. viscum	0.3392	0.2096 to 0.4689	Yes	****	<0.0001
CD vs. TT	0.3824	0.2528 to 0.5121	Yes	****	<0.0001
CD vs. viscumTT	0.5264	0.3968 to 0.6561	Yes	****	<0.0001
viscum vs. TT	0.0432	-0.1006 to 0.187	No	ns	>0.9999
viscum vs. viscumTT	0.1872	0.04337 to 0.331	Yes	**	0.0039
TT vs. viscumTT	0.144	0.0001726 to 0.2878	Yes	*	0.0495
Row 8					
CD vs. viscum	0.2825	0.1528 to 0.4121	Yes	****	<0.0001
CD vs. TT	0.2337	0.104 to 0.3633	Yes	****	<0.0001
CD vs. viscumTT	0.4155	0.2858 to 0.5451	Yes	****	<0.0001
viscum vs. TT	-0.0488	-0.1926 to 0.09503	No	ns	>0.9999
viscum vs. viscumTT	0.133	-0.01083 to 0.2768	No	ns	0.0872
TT vs. viscumTT	0.1818	0.03797 to 0.3256	Yes	**	0.0055

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8. Publications

8.1 Research Articles

Kleinsimon S, Longmuss E, Rolff J, Jaeger S, Eggert A, Delebinski C, Seifert G: GADD45A and CDKN1A are involved in apoptosis and cell cycle modulatory effects by viscumTT with further inactivation of STAT3 pathway. *submitted.*

Stammer RM, **Kleinsimon S**, Rolff J, Jaeger S, Eggert A, Seifert G, Delebinski CI: Synergistic Antitumour Properties of ViscumTT in Alveolar Rhabdomyosarcoma. *Journal of Immunology Research* 2017, 2017:13.

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8.2 Oral presentations

Kleinsimon S, Rolff J, Jaeger S, Eggert A, Delebinski C, Seifert G: Well tolerated triterpene–containing mistletoe extract viscumTT reduces tumor volume in pediatric sarcoma xenografs. *9th European Congress for Integrative Medicine (ECIM)* 2016, Budapest, Hungarian.

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Kleinsimon S, Rolff J, Jaeger S, Eggert A, Delebinski C, Seifert G: Whole mistletoe extract viscumTT has anti-cancer effects on osteosarcoma *in vitro* and *in vivo. 28. Jahrestagung der Kind-Philipp Stiftung* 2015, Wilsede, Germany.

8.3 Posters

Kleinsimon S, Kauczor G, Jaeger S, Eggert A, Seifert G, Delebinski C: Whole mistletoe extract viscumTT has multiple anti-cancer effects in osteosarcoma cell lines. *European Association for Cancer Research (EACR) Series 2016 – A matter of life or death: Mechanisms and relevance of cell death for cancer biology and treatment* 2016, Amsterdam, Netherlands.

Kleinsimon S, Kauczor G, Jaeger S, Eggert A, Seifert G, Delebinski C: Multiple active compounds from *Viscum album L.* induce apoptosis synergistically and effect cell cycle. *European Cell Death Organization (ECDO) 23rd Conference* 2015, Geneva, Switzerland.

Kleinsimon S, Delebinski C, Twardziok M, Jaeger S, Rolff J, Eggert A, Seifert G: A triterpenic-containig mistletoe extract *(Viscum album L.)* induces apoptosis via extrinsic and intrinsic pathway in leukemia cells. *9th International Conference of Anticancer Research (ICAR)* 2014, Sithonia, Greece

List of Abbreviations

4-OOH	4-hydroperoxyifosfmide
ABCB1	ABC transporter B1
ACTB	ß-Actin
AML	Acute myeloid leukemia
AKT	Protein kinase B
APAF1	Apoptotic protease activation factor 1
APC	Allophphycocyanin
APC/C	Anaphase promoting complex/cyclosome
APS	Ammoniumpersulfate
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3 related
AURKA	Aurora A kinase
B2M	Beta-2-microglobolin
BA	Betulinic acid
BAK	BCL2 homologous antagonist killer
BAX	BCL2 associated X
BBC3	TP53 up-regulated modulator of apoptosis
BCL2	B-cell lymphoma 2
BCL2L1	B-cell lymphoma like protein 1
BH3	BCL2-homology 3
BID	BH3-interacting domain death agonist
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
CASP	Caspase
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CCN	Cyclin
CD	2-hydroxypropyl-ß-cyclodextrin
CDC25	Cell division cycle 25
CDDO-Me	C-28 methyl ester of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid
CDK	Cyclin-dependen kinase
CDKN	Cyclin-dependent kinase inhibitor
cDNA	Complementary DNA
CHK1/2	Checkpoint kinase 1/2

CI	Combination index
CKS1	SKP2/cyclin-dependent kinase regulatory subunit 1
dmH_2O	Demineralized water
DIABLO	Diablo IAP-binding mitochondrial protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Doxo	Doxorubicin
DR5	Death receptor 5
E2F	E2F transcription factor
ECL	Enhanced chemiluminescence
e.g.	*Exempli gratia*
ER	Endoplasmic reticulum
FADD	FAS-associated death domain
FAS	FAS cell surface death receptor
GADD45A	Growth arrest and DNA damage
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIER	Heat induced epitope retrieval
HRP	Horseradish peroxidase
HTRA2	HtrA serine peptidase 2
IAP	Inhibitor of apoptosis protein
INK4	Inhibitors of cyclin-dependent kinase 4
IL6	Interleukin 6
i.p.	intrapleural
i.t.	intratumoral
i.v.	intraveneous
JAK	Janus kinase
LC3B-II	Microtubule-associated protein 1A/1B light chain 3B
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid leukemia cell differentiation protein 1
MDM2	Mouse double minute 2
ML	Mistletoe lectin
mRNA	Messenger RNA
MYC	MYC proto-oncogene
ns	Non-significant
OA	Oleanolic acid

PARP	Poly(ADP-ribose)-polymerase 1
PI3	Phosphoinositide 3-kinase
PI	Propidium iodide
PBS	Phosphate buffered saline
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
P/S	Penicillin/streptomycin
PVDF	Polyvinylidene difluoride
RB1	Retinoblastoma 1
RBL1	RB transcriptional corepressor like 1
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
RUNX2	Run related transcription factor 2
s.c.	subcutaneous
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	SKP1-cullin-F-box-protein complex
Sec.	Section
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SKP2	S phase kinase protein 2
SMAC	Second mitochondria-derived activator of caspases
STAT3	Signal transducer and activation transcription factor 3
tBID	truncated BID
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEMED	Tetramethylethylenediamine
TNFR1	Tumor necrosis factor receptor 1
TP53	Tumor suppressor 53
TRAILR	TNF-related apoptosis inducing ligand receptor
UA	Ursolic acid
VP16	Etoposide
VT	Viscotoxin
WNT	Wingless-type MMTV integration site family
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Water soluble tetrazolium)
XIAP	X-linked inhibitor of apoptosis