

## Paediatric Medulloblastoma Cells are Susceptible to *Viscum Album* (Mistletoe) Preparations

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**Abstract.** *Background:* Medulloblastoma constitute more than 20% of all paediatric brain tumours and are the most common malignant brain tumours in children. Adjuvant chemotherapy has seen a strong increase in the use of complementary medicine for cancer treatment. Evidence for cytotoxic and apoptotic effects of *Viscum album* (Mistletoe) *in vitro* is available, however, no data concerning paediatric tumours, especially paediatric brain tumours, has been provided so far. *Materials and Methods:* In order to compare the receptiveness of medulloblastoma cells to different *Viscum album* preparations, *in vitro* cytotoxic effects of eight *Viscum album* extracts on four different paediatric medulloblastoma cell lines were analysed by MTT-Tests. Lectin contents of the extracts were determined to correlate them with the mitochondrial activity of mistletoe-treated cells. Flowcytometric analyses with Annexin V-FITC staining were carried out to quantify the amount of apoptotic cells compared to necrotic and viable cells. *Results:* Data obtained with the medulloblastoma cell lines, Daoy, D342, D425 and UW-288-2, treated with *Viscum album* preparations from eight dissimilar host trees (*Iscucin Abietis*, *Pini*, *Populi*, *Mali*, *Salicis*, *Crataegi*, *Quercus* and *Tiliae*), indicated a significant growth-inhibition of all cell lines, yet the cell susceptibility was dissimilar against the different extracts. The decrease in mitochondrial activity and increase in apoptosis correlated with the lectin content of the used preparation in a dose-dependent manner. *Conclusion:* These *in vitro* results show that paediatric medulloblastoma cells respond to *Viscum album* preparations, by undergoing cell death through apoptosis and that this growth-inhibition correlates with the lectin content of the used preparation.

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Medulloblastoma are the most common malignant brain tumours in children, constituting more than 20% of all paediatric brain tumours (1). Medulloblastoma are characterised by their aggressive clinical behaviour and high risk of leptomeningeal dissemination. A majority of metastatic and most recurrent childhood medulloblastoma are resistant to current therapeutic approaches, including high-dose chemotherapy with autologous haematopoietic stem cell rescue (2-4). Survival rates have been nearly static for two decades (5). In addition, standard treatment including craniospinal radiotherapy induces cognitive deficiencies, which are particularly severe in young children (6-8).

Whereas the development of new therapies remains the major goal, the establishment of effective complementary therapies may lead to a reduction of side-effects caused by the conventional therapy and to enhanced quality of life (9). The use of complementary medicine for paediatric cancer treatment has strongly increased, with 42-73% of the cancer patients using it (10, 11). The latest survey across a number of European countries shows that 35.9% (range 14.8% to 73.1%) of all cancer patients use some kind of complementary and alternative medicine (CAM). Within complementary cancer treatment, the aqueous extracts from *Viscum album* (mistletoe) are best known and widely used (12). There is an obvious discrepancy between the popularity of mistletoe extracts and their classification as an unconventional treatment modality with statistically unproven efficacy in oncology. Reliable evaluation of the effects of extract preparations is seriously hampered by the fact that the extract composition is complex and markedly depends on the method of preparation, type of host tree and harvest time. In search of the biologically active constituents of the extracts, mistletoe lectins have gained increasing attention and have been documented to exert a remarkable immunomodulating capacity both *in vitro* and *in vivo*. Furthermore, lectins have been shown to have cytotoxic and apoptosis-inducing properties against a large number of human tumour cell lines *in vitro* (13, 14). *In vitro* results suggest that mistletoe extracts have a direct cytotoxic effect on tumour cells, which is not only caused by ribosome inactivating properties of the A

chain of mistletoe lectins, but also depends on the direct induction of apoptosis (15). It has recently been shown that mistletoe caused early cell cycle inhibition followed by apoptosis in a dose-dependent manner. Apoptosis was induced by activating the mitochondrial, but not the death receptor-dependent pathway (16).

In a systemic review of controlled clinical trials by Kienle GS *et al.*, 23 studies were identified: 16 randomised, 2 quasi-randomised and 5 non-randomised. Among these studies, statistically significant positive outcomes were reported for survival (n=8), tumour remission (n=1), overall quality of life (n=3) and quality of life in relation to side effects during cytoreductive therapy (n=3) (17). In addition, a prospective randomised controlled clinical study has recently shown that complementary treatment with standardised mistletoe extracts can beneficially reduce the side-effects of chemotherapy in cancer patients (breast, ovarian and non-small cell lung) and, thus, improve quality of life (9). However, due to the lack of an adequate methodology in evaluating the efficacy of complementary medicine, the evidence of these results is controversial.

Despite the fact that there is a great body of data about the biochemical and physiological effects of *Viscum album*, systematic investigations of treatment of paediatric tumours (especially brain tumours) are missing. A case report described a patient in whom a large fibrillary astrocytoma of the pons extending into the midbrain was diagnosed at the age of 2 years (18). A therapy with *Viscum album* was set for the duration of three months. After shunting of a hydrocephalus, the clinical symptoms abated without conventional therapy. Repeated MRI studies showed a continuous decrease of the tumour, the latter being no longer visible when the patient was 6.6 years old. Lenartz *et al.* used intracerebral xenograft rat models and found that treatment with lectins from mistletoe resulted in a shrinkage of gliomas (19). The same group performed a prospective clinical trial with 38 patients and claimed that treatment with mistletoe resulted in a prolongation of relapse-free survival for patients with high-grade gliomas (WHO grade III and IV) (20).

To our knowledge, *in vitro* data concerning *Viscum album* sensitivity of brain tumour cells are not available. In the current study, the effects of eight different *Viscum album* preparations were examined on four different medulloblastoma cell lines (Daoy, D341, D425 and UW-228-2). It was investigated whether *Viscum album* is anti-proliferative, whether these effects are dose-dependent, if there are cell-line specific differences and whether the effects are similar to those of doxorubicin and fluorouracil at the same concentrations. Whether *Viscum album* induced cell death by apoptosis was examined by using a flowcytometric assay.

Table I. The employed *Viscum album* preparations from different host trees with the current lectin concentrations.

Iscucin® H	Charge Nr.	Host tree	Concentration of <i>Viscum album</i>	Lectin / extract
Abietis	2066145	Fir	50 mg/ml	0.8 µg/ml
Pini	2116021	Pine	50 mg/ml	1.1 µg/ml
Populi	2116022	Poplar	50 mg/ml	5.3 µg/ml
Mali	3021071	Apple	50 mg/ml	5.3 µg/ml
Salicis	3116088	Willow	50 mg/ml	5.9 µg/ml
Crataegi	2066147	Whitethorn	50 mg/ml	8.4 µg/ml
Quercus	1111019	Oak	50 mg/ml	9.9 µg/ml
Tiliae	2076092	Basswood	50 mg/ml	13.7 µg/ml

## Materials and Methods

*Viscum album* preparation. *Viscum album* extracts (Iscucin Abietis, Pini, Populi, Mali, Salicis, Crataegi, Quercus and Tiliae) were provided by WALA GmbH, Germany (Table I). The determination of the lectin concentrations was performed by Abnoba Heilmittel GmbH, using AC-2-ELISA (21).

*Medulloblastoma cell lines.* The Daoy human medulloblastoma cells were purchased from ATCC (American Type Culture Collection, USA). The D341 and D425 human medulloblastoma cells were the kind gift from Dr. Henry Friedman, Duke University, Durham, NC, USA. The UW 228-2 human medulloblastoma cells were a kind gift from Dr. John R. Silber, University of Washington, WA, USA.

The Daoy, D341, D425 and UW 228-2 cells were maintained in the media recommended by the suppliers and supplemented with the suggested serum concentration. The cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The adherent cells were subcultured twice a week by washing the monolayer with Dulbecco's phosphate buffered saline (PBS) and digesting it with trypsin/EDTA at 37°C for 5 min.

*MTT assay.* Cells were seeded at a density of 10<sup>5</sup> cells/ml (or 10,000 cells/well in 100 µl) in a 96-well polycarbonate plate (Orange Scientific, BioConcept, Switzerland) and incubated for 24 h at 37°C and 5% CO<sub>2</sub> before being exposed to different concentrations of appropriate dilutions of *Viscum album* or blank medium (control) for 72 h. Then, 20 µl of a MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution, prepared in Dulbecco's phosphate-buffered saline (PBS) at the concentration of 5 mg/ml, were added to each well. The cells were then incubated for 4 h at 37°C. Mitochondrial dehydrogenase activity reduced the yellow MTT dye to purple formazan crystals, which were solubilised with 200 µl DMSO (Fluka, Switzerland) per well. The plates were gently shaken for 10 min. Then, 25 µl Soerensen's buffer (0.1 M Glycin, 0.1 M NaCl, pH 10.5) were added and the absorbance was measured at 570 nm on a microplate reader (MRX, Dynatech Laboratories). The absorbance value provided a direct indication of viable cells. The cytotoxic response was graded by the percentage of viable cells, which were treated with *Viscum album* and was compared to untreated control cells.

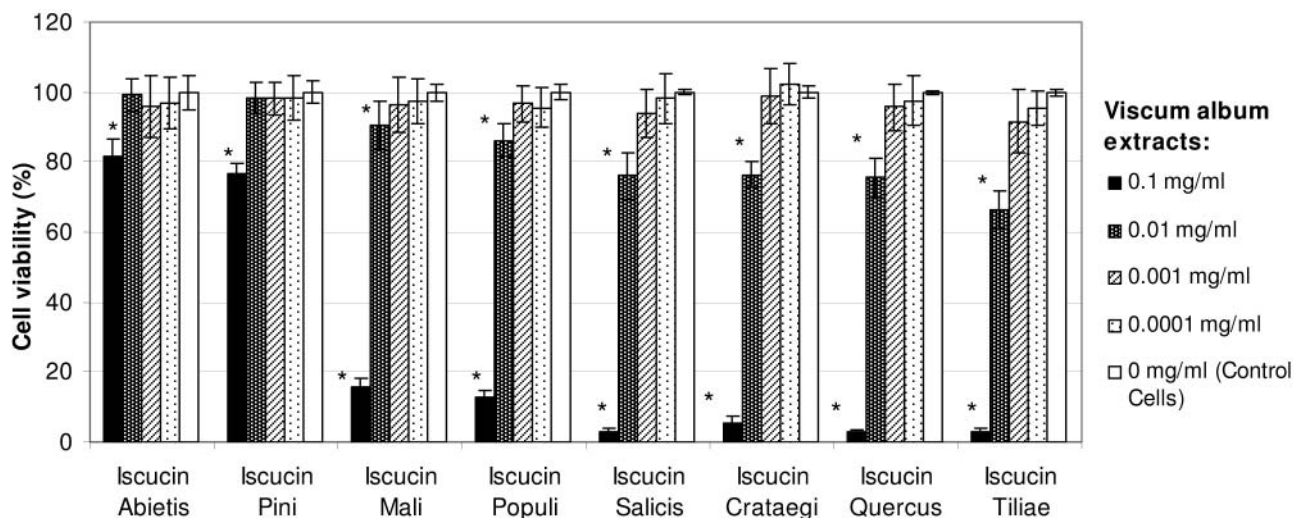


Figure 1. Dose-dependent cytotoxic effects of different mistletoe extracts on Daoy cells. Daoy cells were incubated for 48 h in four different concentrations (0.1, 0.01, 0.001 and 0.0001 mg *Viscum album* extract /ml). The relative mitochondrial activity (MTT) is given as a percent of the optical density of *Viscum album* extract-treated cells compared to untreated ones (control=100%) (n=24). \*Significant decrease of mitochondrial activity ( $p < 0.001$ ).

**Detection of apoptosis by flow cytometry.** Apoptotic cells were detected by staining with Annexin V-FITC (Roche Diagnostics, Rotkreuz, Switzerland) and quantified with a FACScan from Becton Dickinson, while necrotic cells were stained with propidium iodide. At the early stage of apoptosis, phosphatidylserin (PS) is translocated from the inner part of the plasma membrane to the outer layer, becoming exposed at the external surface of the cell. The PC-binding Annexin-V can, hence, be used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and can, therefore, be used to detect apoptotic cells. In this study,  $5 \times 10^6$  cells were seeded in a 25 cm<sup>2</sup>-culture flask and cultivated at 37°C for 24 h. The cells were then exposed to *Viscum album* extracts for 3 h and subsequently washed with PBS, trypsinated and diluted to  $1 \times 10^5$  cells/ml. The cells were then incubated in the presence of Annexin V-FITC and propidium iodide (PI) for 15 min. The staining was measured at 10,000 cells using a flowcytometer FACScan from Becton Dickinson at a wavelength of 615 (PI) and 530 nm (FITC). Living cells were PI- and FITC-negative, dead cells PI- and FITC-positive and apoptotic cells FITC-positive but PI-negative.

## Results

**Dose-dependent cytotoxic effects of different *Viscum album* preparations on Daoy cells.** Iscucin preparations of eight different host trees (Iscucin Abietis, Pini, Populi, Mali, Crataegi, Quercus, Tiliae and Salicis) were used to examine the cytotoxic effect on Daoy cells. The Daoy cells were incubated for 48 h in the presence of *Viscum album* at four different concentrations (0.1, 0.01, 0.001 and 0.0001 mg extract /ml) before determining the mitochondrial activity and comparing it to untreated control cells. The relative mitochondrial activity of Daoy was significantly affected by all *Viscum album* extracts at 0.1 mg/ml (Figure

1). A lower, but still significant, decrease in mitochondrial activity was also observed for Daoy cells incubated in the presence of 0.01 mg/ml of Iscucin Mali (90%), Populi (86%), Salicis (76%), Crataegi (76%), Quercus (75%) and Tiliae (66%). Iscucin Abietis (99%) and Pini (98%) showed no effect at this concentration. Treatment with 0.001 and 0.0001 mg/ml *Viscum album* caused no significant cytotoxic effects on Daoy cells. These results indicate that the tested *Viscum album* preparations are cytotoxic and that cytotoxicity is dose-dependent. The effects of mistletoe extracts from the different host trees differ considerably. Iscucin Tiliae was the most effective, followed by Iscucin Quercus, Salicis and Crataegi.

**Cytotoxic effects of *Viscum album* in comparison to doxorubicin and fluorouracil.** In order to evaluate the cytotoxicity of *Viscum album* on medulloblastoma cells in comparison with currently used chemotherapeutic drugs (22, 23), the cytotoxic effects of the three *Viscum album* preparations, Iscucin Abietis, Mali and Tiliae from three different host trees (fir, apple and linden tree) were compared to those of doxorubicin and fluorouracil (Figure 2). Daoy cells were incubated for 48 h at four different concentrations (0.1, 0.01, 0.001 and 0.0001 mg/ml). Subsequently, their relative mitochondrial activity was determined. The growth inhibition of Daoy cells was low after treatment with Iscucin Abietis, moderate with Iscucin Mali and strong with Iscucin Tiliae. The relative mitochondrial activity of Daoy cells treated with doxorubicin increased very rapidly from 43% at 0.1 mg/ml to 86% at 0.01 mg/ml, 97% at 0.001 mg/ml and 99% at

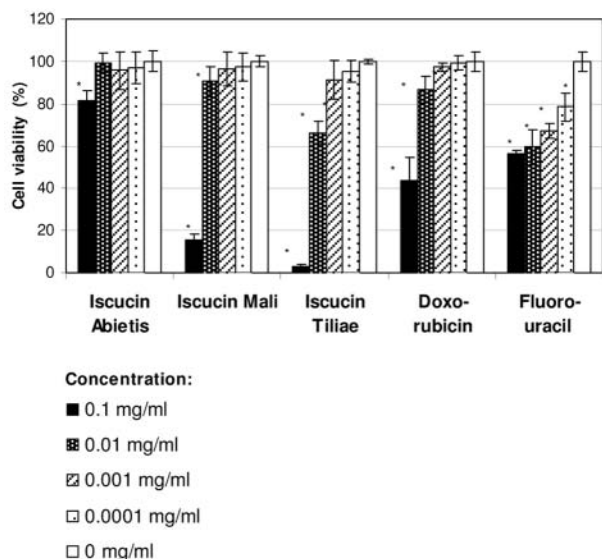


Figure 2. Cytotoxic effects of *Viscum album* extracts, doxorubicin and fluorouracil on Daoy cells. The cells were incubated with the three different mistletoe extracts Iscucin Abietis, Mali and Crataegi, as well with doxorubicin and fluorouracil at 0.1, 0.01, 0.001 and 0.0001 mg/ml for 48 h. The relative mitochondrial activity was given by comparing the absorbance of cells treated with the cytotoxic agent to untreated ones (n=24). \*Significant decrease of mitochondrial activity (p<0.001).

0.0001 mg/ml. At lower concentrations (55% at 0.1 mg/ml, 59% at 0.01 mg/ml, 67% at 0.001 mg/ml, 78% at 0.0001 mg/ml, respectively), significant cytotoxic effects of fluorouracil were still observed. The cells treated with high concentrations of fluorouracil showed a relatively low decrease in mitochondrial activity at 0.1 mg/ml. In spite of a dose-dependent response significantly slower than that with doxorubicin, even the low concentration of 0.0001 mg/ml still resulted in a reduction of 22%. Interestingly, the cells treated at low concentration (0.0001 mg/ml) showed still a relatively high decrease in mitochondrial activity (22%). These results show that the cytotoxic effects of *Viscum album* preparations are comparable to those of well-known chemotherapeutics, a phenomenon that has already been observed in other tumour models (22, 23). To exclude inhibiting effects caused by the buffer used to produce the *Viscum album* extract, the buffer was used as an additional control. The buffer did not influence the relative mitochondrial activity (data not shown).

*Cytotoxicity of Viscum album extracts on different medulloblastoma cell lines.* The effects of *Viscum album* were investigated on four different medulloblastoma cell lines: Daoy, D341, D425 and UW-228-2, incubated at different concentrations of *Viscum album* preparations (0.1, 0.01, 0.001, 0.0001 mg/ml) (Figure 3). The relative mitochondrial

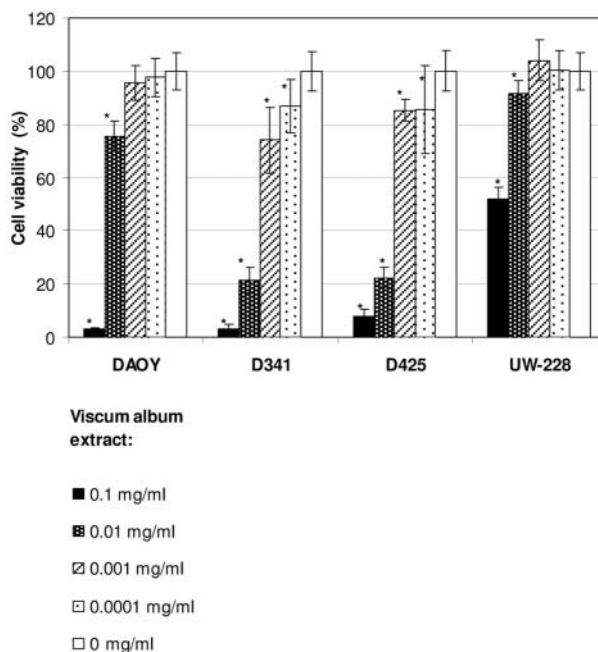


Figure 3. Cytotoxic effects of Iscucin Quercus on the four different medulloblastoma cell lines Daoy, D341, D425 and UW-228-2. The cells were incubated for 48 h at 0.1, 0.01, 0.001 and 0.0001 mg Iscucin Quercus/ml and the mitochondrial activity was determined and compared to untreated cells (control = 100%) (n=24). \*Significant decrease of mitochondrial activity (p<0.001).

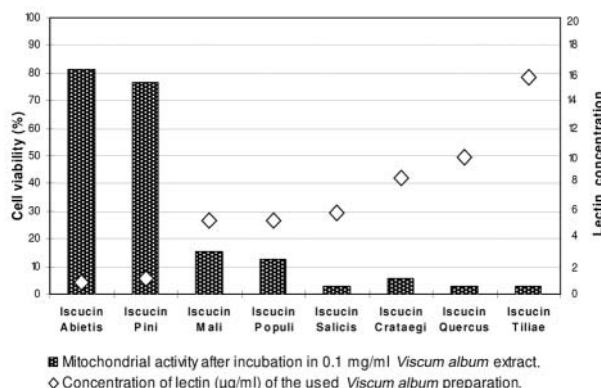


Figure 4. Current lectin concentrations of the *Viscum album* preparations used were correlated to the corresponding relative mitochondrial activity of Daoy cells, which were incubated for 48 h at 0.1 mg/ml extract.

activity of Daoy cells after treatment with Iscucin Quercus was 3%, 75%, 95% and 97%, respectively, (concentrations of 0.1, 0.01, 0.001, 0.0001 mg/ml) compared to the untreated control cells. The proliferation rate of D341 cells was 3%, 21%, 74% and 86%, respectively, that of D425 cells was 7%, 22%, 85% and 85%, respectively and that of the UW-228 cells was 52%, 91%, 103% and 100%, respectively. The

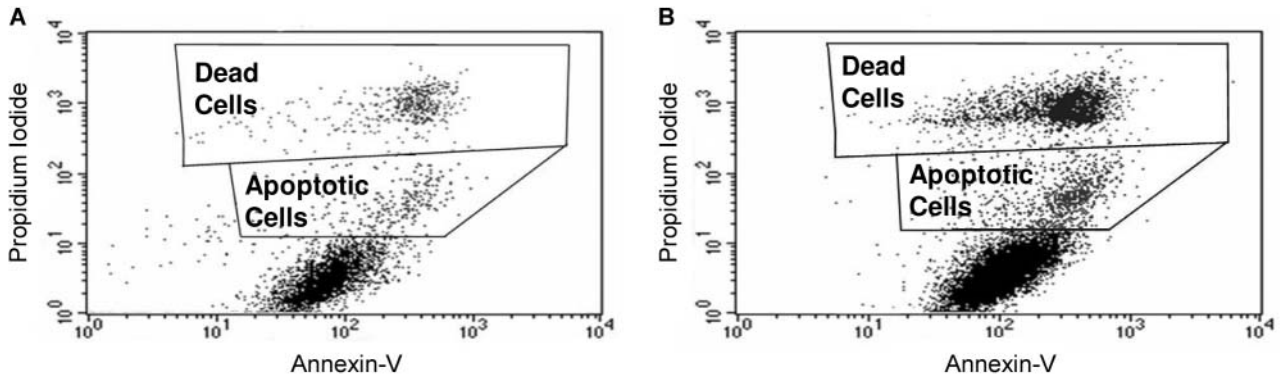


Figure 5. Induction of apoptosis by 1 mg *Iscucin Quercus*/ml on Daoy cells. A) Control cells without mistletoe extract were analysed. B) Mistletoe-treated cells. Apoptotic cells were detected and quantified by staining with Annexin V-FITC using FACScan. Apoptotic cells were stained with FITC-labelled annexin-V (FITC +/ PI -), dead cells with propidium iodide (FITC +/ PI +) as well. Live cells were neither FITC- nor propidium iodide-positive (FITC -/ PI -).

most susceptible cell line to *Iscucin Quercus* at 0.01 and 0.001 mg/ml was D341, followed by D425 and Daoy. In comparison to the other cell lines, UW-228-2 responded relatively weakly. The results indicate that the sensitivity to *Viscum album* varies between different cell lines of the same tumour entity.

**Lectin-dependent cytotoxicity.** To clarify whether cytotoxicity is related to the mistletoe lectin, the lectin content of each extract was analysed by Abnoba Heilmittel GmbH. The determined lectin concentrations are provided in Table I. The extracts from coniferous mistletoes (*Iscucin Abietis* and *Pini*) had a relatively low concentration of lectin with 800 and 1100 ng/ml, respectively, while those of broad-leaved trees ranged between 5300 ng/mg (*Iscucin Mali*) and 13700 ng/ml (*Iscucin Tiliae*). The lectin content was compared with the relative mitochondrial activity. A significant inverse correlation between the lectin content and mitochondrial activity ( $p=0.01$ ; Pearson coefficient  $-0.766$ ) was observed (Figure 4). This result suggests that lectins are predominantly responsible for growth inhibition of cancer cells regarding the effect of coniferous mistletoe extracts compared with those of broad-leaved trees. However, among the *Viscum album* preparations from broad-leaved trees, the differences between the cytotoxic sensitivities could not be explained by the lectin content alone.

**Induction of apoptosis by *Viscum album*.** It has been demonstrated that tumour cells become apoptotic upon treatment with isolated mistletoe lectins (15, 24). *Viscum album* extracts induced cytotoxic effects on tumour cells that are likewise apoptosis-related, accompanied at higher doses with necrotic cell death *in vitro*. Therefore, we examined whether *Iscucin* preparations also caused apoptosis in medulloblastoma cells. The Daoy cells were treated with

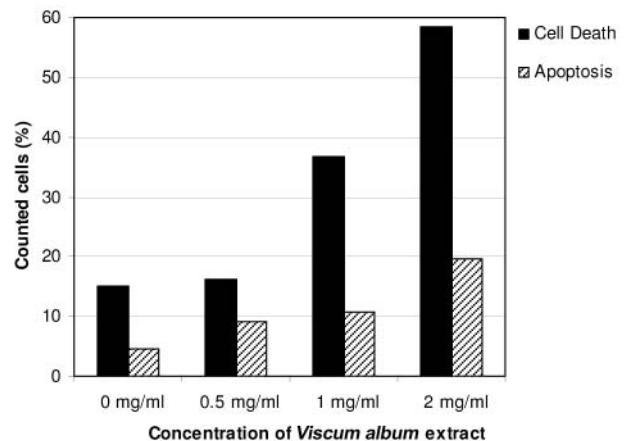


Figure 6. Induction of apoptosis in Daoy cells treated with *Viscum album* (*Iscucin Quercus*). Cells were incubated with varying concentrations of *Viscum album* and stained for cytometric analysis with FITC-labelled Annexin-V and propidium Iodide. The apoptotic and dead cells were correlated to the population of all cells (viable, apoptotic and dead).

*Iscucin Quercus* at concentrations of 0.5, 1 and 2 mg/ml for 4 h. Apoptotic cells were marked with FITC-labelled Annexin-V (FITC +/ PI -) and dead cells additionally with propidium Iodide (FITC +/ PI +). The appropriate percentage of viable, apoptotic and dead cells was determined using FACScan. Living cells were neither FITC- nor propidium iodide-positive (FITC-/ PI-) (Figures 5 and 6).

In the control cell population without *Viscum album*, 5% of apoptotic and 15% of dead cells were detected. We should therefore bear in mind that a considerable fraction underwent spontaneous apoptosis without mistletoe treatment. After treatment with 0.5 mg/ml *Viscum album* extract, 9% apoptotic and 16% dead cells were measured in the total cell population. Treatment with 1 mg/ml resulted

in 11% apoptotic and 36% dead cells and treatment with 2 mg/ml in 20% apoptotic and 58% dead cells. The percentage of cells undergoing apoptosis has, thus, been shown to be dose-dependent.

## Discussion

The aim of this study was to investigate the effects of *Viscum album* extracts from eight different host trees (Iscucin Abietis, Pini, Populi, Mali, Salicis, Crataegi, Quercus, Tiliae) on four distinct medulloblastoma cell lines. Although there are substantial data regarding the biological efficacy of mistletoe extracts, this is the first investigation showing *in vitro* effects on children's brain tumour cells.

The Daoy human medulloblastoma cells were incubated with *Viscum album* preparations at four different concentrations. The results revealed a dose-dependent decrease of relative mitochondrial activity for all preparations. The cytotoxicity of *Viscum album* from the different host trees differed markedly, which is in agreement with other investigations (15, 25, 26). While the influence of Iscucin Abietis and Pini was relatively weak, the cytotoxicity of Iscucin Salicis, Crataegi, Quercus and Tiliae was very strong. Interestingly, *Viscum album* preparations from willow (Iscucin Salicis), whitethorn (Iscucin Crataegi) and basswood (Iscucin Tiliae) have not yet been tested *in vitro*, and are still rather unknown. Yet, due to their obviously strong cytotoxicity at higher concentrations, a local (intra/peritumoral) or high-dose systemic application might be of benefit to cancer patients. This was already established in a case of bladder cancer (27).

*Viscum album* extracts exhibited a strong, dose-dependent *in vitro* cytotoxicity on all four medulloblastoma cell lines tested. Sensitivity to *Viscum album* varied considerably among the different cell lines. Whereas the influence on the Daoy, D341 and D425 cells was similar, the UW-228 cells were less sensitive to *Viscum album*. Such different effects of the same *Viscum album* preparation on different tumour cell lines from the same tumour entity have already been observed in other tumour systems (16, 28). Microarray-based approaches to test the individual susceptibility of primary tumours are in progress (29).

Several groups have examined the biochemical and physiological effects of defined substances isolated from *Viscum album*, such as mistletoe lectins 1-3, viscotoxins and polysaccharides (15, 24, 30, 31). Experimental results showed that lectins play a predominant role in producing the cytotoxic effects of *Viscum album* preparations. Therefore, the lectin contents of the used preparations were determined. A significant correlation between lectin amount and growth-inhibitory effects was found. This is in agreement with results of cytotoxicity assays which determined the effect of mistletoe lectins on carcinoma cells. Still, other substances are likely to have contributed to these findings.

The fact that lectins – probably in combination with several other components – are responsible for the mode of action, suggests that *Viscum album* mediates cell death predominantly by causing apoptosis (32). The results in medulloblastoma cells showed a dose-dependent increase in apoptotic cells, which implicated the important role of lectins in causing apoptotic cell death. From the data of this work, it cannot be deduced clearly whether the necrotic cells represented secondary necrosis as a result of the disintegration of apoptotic cells, or whether they represented primary necrosis, which would indicate a high sensitivity to viscotoxins and other membrane damaging compounds.

Although these first investigations on children's brain tumour cells are encouraging, we should bear in mind that the cytotoxic capacity of *Viscum album* is only one mechanistic aspect of mistletoe efficacy, and the immunomodulating aspects also need to be assessed within such an individual therapy. Clinically relevant immunomodulation, such as a significant increase in immune cell counts, an increase in cytokine-induced acute phase reactants or an increase in B-endorphins found in adult cancer patients after regular (low dose) subcutaneous application was described (33-36). Chernyshov *et al.* reported immunomodulatory and clinical responses to *Viscum album* in 92 children with respiratory infection, as a result of the Chernobyl nuclear accident (37). Although there are several reports concerning the use of complementary therapies in children, data about *in vivo* effects, side-effects or compliance of *Viscum album* preparations in children with cancer are largely lacking.

To verify *Viscum album* extracts as a therapeutic approach in paediatric brain tumours, it has to be clarified whether substances of *Viscum album* preparations, especially lectins, could pass the blood-brain barrier. This is a condition for direct cytotoxic effects in the brain. Histochemical analyses showed a high affinity of microglia in brain tissue to mistletoe lectins (38, 39). However, the question whether lectins pass the blood-brain barrier *in vivo* still needs to be clarified. Passing the blood-brain barrier could possibly be avoided by an intrathecal application with an implanted subcutaneous reservoir in children, an approach which is currently used for chemotherapy in young children with medulloblastoma (40).

Several authors assumed that phytopharmaceuticals are often used by paediatric cancer patients (10, 11). Nevertheless, therapies should be based on scientific research derived from *in vitro* and *in vivo* results and be carried out very carefully. The findings of this work suggest that every type of tumour has to be analysed individually to determine its individual response profile to mistletoe extracts. Furthermore, research in brain tumour is characterised by an additional biochemical challenge – the blood-brain barrier – which should be considered in the

evaluation of new therapies. Therefore, additional preclinical investigations are needed before proceeding to *in vivo* experiments.

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