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Differences in the apoptosis-inducing properties of *Viscum album* L. extracts

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***Viscum album* L. (mistletoe) extracts are widely used in adjuvant cancer therapy. In contrast to purified components, such as mistletoe lectins and viscotoxins, whole plant extracts of mistletoe resulted in DNA stabilization in cyclophosphamide-treated lymphocytes but also provided cytotoxicity in tumour cells and lymphocytes. The killing capacities of mistletoe extracts were host tree-specific and not correlated with mistletoe lectin or viscotoxin content. In human lymphocytes, only mistletoe lectins induced a pathway of apoptotic killing. Within 72 h, the lectin B chains also increased the number of lymphocytes undergoing apoptosis. This finding suggests that inhibition of protein synthesis by the A chain of the hololectin may accelerate a receptor-mediated killing pathway induced by the B chains. An unexpected finding was related to the mistletoe-mediated killing, which was more effective against CD8+T cells with an activated phenotype than CD19+ B cells and CD4+T cells. *In vitro* treatment of human neutrophils with mistletoe resulted in a slight decrease of phagocytosis and burst activity. The observed dose-dependent occurrence of two neutrophil subsets with different burst activities indicates differences in their susceptibility to mistletoe and suggests the implication of an induction of the apoptotic killing pathway.**

Keywords: Viscum album L., DNA stabilization, cytotoxicity, apoptosis, granulocytes, phagocytosis, oxidative burst, lymphocytes.

The antimutagenic effects of *Viscum album* L.

Viscum album L. (mistletoe) extracts are widely used as adjuvant cancer therapy. Research suggests that mistletoe stimulates the immune system non-specifically and exerts cytostatic/cytotoxic activities. In addition, mistletoe contains DNA stabilizing properties for mononuclear cells in the peripheral blood. In cultured peripheral blood mononuclear cells, the whole plant extract *Helixor* A (10 μg/ml) significantly reduced the number of spontaneous and cyclophosphamide-induced sister chromatid exchange-inducing DNA lesions [1–3]. In rapidly proliferating amniotic fluid cells, however, only very high concentrations of mistletoe extract Iscador P significantly reduced sister chromatid exchange [4]. The sister chromatid exchange assay is considered to be a sensitive and specific indicator of cytogenetic damage and mutagenicity and is used as a sensitive and rapid method for the detection of agents that damage DNA [5,6]. Based on these findings, our results clearly reject a proposed mutagenic potential of mistletoe.

If these results reflect a stabilization of DNA with mistletoe, one expects a concomitant improvement of protein synthesis and surface molecule expression. Indeed, the drug extract *Helixor* A protected expression of activation-associated surface molecules, specifically interleukin-2 receptor α chains (CD25) and transferrin receptors (CD71) on T cells, against cyclophosphamide-mediated depression [7]. These results indicate an improved functional competence with the addition of the whole plant extract. However, purified components, such as mistletoe lectins I–III and viscotoxins, did not prevent the activation marker depression on T cells mediated by cyclophosphamide [7]. These findings suggest that the whole plant extract, not the purified compounds, is effective.

Our results were confirmed by others who observed an improved incorporation of [^{3}H]-thymidine in the DNA of ultra violet-damaged lymphocytes from breast cancer patients after treatment with mistletoe (Iscador) [8]. Based on sedimentation of DNA strand breaks on an alkaline sucrose gradient, mistletoe treatment showed an increased repair of radiation- and cyclophosphamide-induced DNA damage in lymphocytes of breast cancer patients [9,10]. Murine models illustrated the radioprotective effects of mistletoe for cyclophosphamide- and γ-radiated mice and resulted in increased survival and reduced leukocytopenia [11,12]. The results suggest that mistletoe modifies DNA damage associated with carcinogens. However, in Jurkat T cells, the simultaneous addition of cyclophosphamide and mistletoe resulted in a more severe decline of cell number than cyclophosphamide alone. In mice infected with mammary carcinoma cells and treated with cyclophosphamide and mistletoe (*Isorel*), the number of lung metastases was severely reduced compared to cyclophosphamide or mistletoe alone [3]. Thus, in the murine model and in cultured leukaemic cells, mistletoe produced no protective effects [2,3,13].

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Table 1. Phagocytic capacity and oxidative burst of *Escherichia coli*-stimulated human neutrophils after treatment with a *Viscum album* L. extract

	VaPR (μg/ml)		
	0	10	100
Phagocytosis (%)†	87.2 ± 13.45	78.6 ± 12.98*	81.1 ± 11.87**
Burst activity(%)†	92.5 ± 11.79	84.4 ± 16.00*	87.7 ± 11.21
R123 fluorescence‡	667.6 ± 47.88	639.8 ± 32.21**	650.5 ± 41.43

The phagocytic capacity of the neutrophils treated with *Viscum album* Pini R (VaPR) was measured by analysing the green fluorescence of incorporated *E. coli* labelled with fluorescein isothiocyanate (FITC; Phagotest, Orpegen, Heidelberg, Germany) incubated for 10 min in a water bath at 37°C. The oxidative burst of human neutrophils was determined by flow cytometric analysis of green fluorescence of oxidized dihydrorhodamine 123 (DHR 123; Bursttest, Orpegen, Heidelberg, Germany). Opsonized *Escherichia coli* (20 μl; 1 × 10^9/ml) and the mistletoe extract VaPR were added to the blood samples and preincubated for 10 min in a water bath at 37°C. After that, the non-fluorescent compound DHR 123 was added (10 min at 37°C). In the presence of hydrogen peroxide, released by activated neutrophils, the non-fluorescent DHR 123 is rapidly oxidized to the highly fluorescent rhodamine 123 (R 123). †Percentage of neutrophils; ‡mean channel of R 123 fluorescence in neutrophils with burst activity. *$P \leq 0.004$, **$P \leq 0.03$, versus control by Wilcoxon sign rank test.

Induction of apoptosis by the mistletoe lectins

Mistletoe exerted strong cytotoxic effects towards tumour cells and lymphocytes. Despite the well-known fact that mistletoe lectins, specifically their catalytic A chain, inhibit protein synthesis [14], mistletoe-mediated cytotoxicity is shown to result from induction of apoptosis [15–18]. Among the toxic proteins from mistletoe, such as the D-galactose-specific mistletoe lectin I, the *N*-acetyl-D-galactosamine-specific mistletoe lectin II and mistletoe lectin III, and the viscotoxins, only the mistletoe lectins induced the apoptotic killing pathway [16]. The killing capacities of mistletoe extracts differed with host tree, however, but were not correlated with the mistletoe lectin content of the plant extract [17]. Within 72 h, lectin B chains of mistletoe lectin I also increased the number of lymphocytes undergoing apoptosis [18], suggesting that inhibition of protein synthesis by the catalytic A chains of the hololectin probably accelerates a receptor-mediated killing pathway induced by the high concentrations of B chains.

This model of mistletoe lectin-mediated cytotoxicity is in agreement with that of Metzner *et al.* [19] who reported that the incorporation of [^{3}H]-thymidine in the DNA of 72 h cultured cells was inhibited by mistletoe lectin I and its lectin B chain (with mistletoe lectin I 30 times more active than the B chain), whereas the enzymatic A chain induced a blastogenic transformation of lymphocytes. Furthermore, only the hololectin and its lectin B chain increased intracellular calcium content in cultured lymphocytes; the enzymatic A chain did not [16].

The apoptotic killing pathway, however, has no relevance in the case of subcutaneous or intravenous application of mistletoe extracts. Drug concentrations as high as 600 mg mistletoe administered intravenously to cancer patients did not produce immunosuppressive side effects or peripheral cellular depletion; however, an increase of juvenile granulocytes and monocytes was observed [20]. The missing cytotoxicity of the mistletoe lectins in a clinical situation might be due to the induction of antimistletoe lectin-antibodies during therapy [21] and inhibition of mistletoe lectins by serum glycoproteins/lipids [22]. Thus, a clinically relevant apoptotic killing might be induced only in the case of intratumoral injection.

Affect of mistletoe on granulocyte function

To test the possibility that the apoptosis-inducing mistletoe lectins affect phagocytosis and the oxidative burst of neutrophil granulocytes, human neutrophils were treated *in vitro* with high concentrations of mistletoe [23]. As shown in Table 1, the phagocytic capacity and the *Escherichia coli*-stimulated oxidative burst of these cells slightly decreased with the addition of the drug. However, within the population with oxidative burst, this activity significantly decreased with the very potent extract *Viscum album* Pini R (10 μg/ml; $P = 0.023$), as measured by the mean channel of R 123 fluorescence (Table 1). This effect was mainly due to a dose-dependent occurrence of two populations with different activities, clearly distinguishable in the population histograms. The worst situation, involving up to 30% of neutrophils with low R 123 fluorescence, is shown in Fig. 1. Preincubation of the bacteria with the drug markedly decreased phagocytosis, while preincubation of blood with the drug did not change the phagocytic capacity of neutrophils (data not shown). Thus, one might suggest an interference of lectins or carbohydrates of mistletoe with surface molecules on the bacterial and/or phagocyte cell surface.

Our results conflict to some extent with the findings of Timoshenko and Gabius [24], who observed an induction of superoxide anion release in human neutrophils. However, they employed very high (toxic) concentrations of purified lectin to stimulate the neutrophils, while

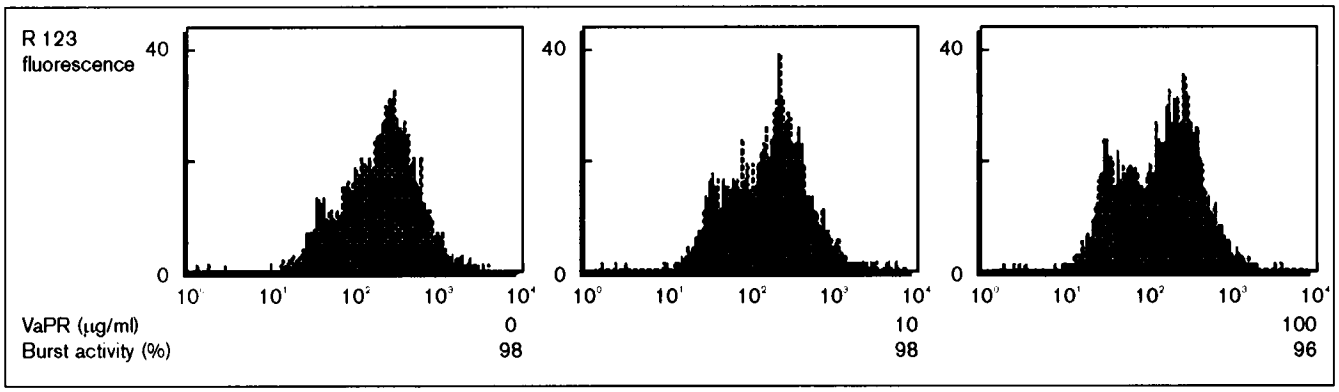

Figure 1. The oxidative burst of human neutrophils as determined by flow cytometric analysis of green fluorescence of oxidized dihydrorhodamine 123 (DHR 123; Bursttest, Orpegen, Heidelberg, Germany). Opsonized *Escherichia coli* (20 µl; 1 x 10^9/ml) and the mistletoe extract *Viscum album* Pini R (VaPR) at final concentrations of 0, 10 and 100 µg/ml were added to the blood samples and preincubated for 10 min in a water bath at 37°C. After that, the non-fluorescent compound DHR 123 was added (10 min at 37°C). In the presence of hydrogen peroxide, released by activated neutrophils, the non-fluorescent DHR 123 is rapidly oxidized to the highly fluorescent rhodamine 123 (R 123; x-axis of the dot plot histograms). Values represent the relative amount of neutrophils with burst activity.

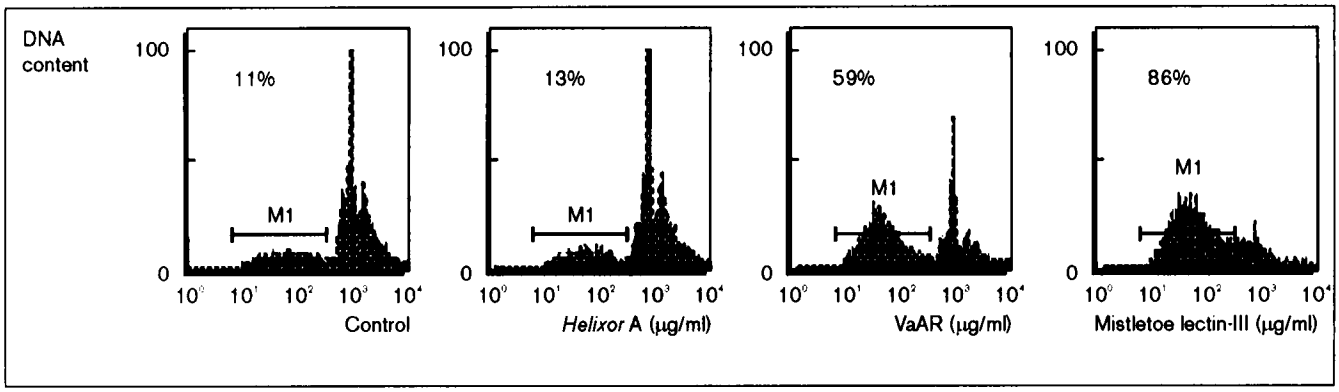

Figure 2. Representative DNA fluorescence profiles of 72 h cultured lymphocytes. Apoptotic DNA is represented as a hypodiploid sub-G1 peak (M1). Per cent DNA fragmentation is indicated to the left of each histogram. Histograms of propidium iodide fluorescence (x-axis) of cultured cells are represented for three independent experiments. The mistletoe extracts *Helixor* A and *Viscum album* Abietis R (VaAR) were added at final concentrations of 100 µg/ml, while the purified mistletoe lectin III was added at a final concentration of 0.1 µg/ml.

in our experiments the influence of a whole plant extract on the burst activity of neutrophils stimulated with opsonized bacteria was measured. The explanation for the apparent conflict between these data is not certain, but the following might provide a reasonable hypothesis. By the addition of mistletoe lectin I, an increase in intracellular calcium and H_2O_2 was observed in rat thymocytes [25]. Since an increase in intracellular calcium is reported to be one of the key factors causing advanced apoptotic changes [26,27], we suggest that the described effects are the implication of an induction of the apoptotic killing pathway by high drug concentrations, as observed in cultured lymphocytes [18]. The significance of these *in vitro* effects for the clinical situation are speculative, however.

Differences in the killing potencies of mistletoe extracts produced from fir trees

To further characterize the properties of mistletoe, two viscotoxin-free, mistletoe lectin II/III-rich extracts produced from mistletoes grown on fir trees were investigated, a drug extract termed *Viscum album* Abietis R and the commercially available *Helixor* A, both provided by Helixor, Rosenfeld, Germany. Both underwent the same pharmaceutical process but differed in that a large amount of mistletoe berries were processed in *Viscum album* Abietis R. As a consequence of this, *Viscum album* Abietis R is characterized by a relatively higher amount of carbohydrates (0.6 versus 0.3 mg/ml), proteins (30 versus 15 µg/ml) and mistletoe lectin II/III (3.6

Table 2. Flow cytometric analysis of lymphocyte subsets from cultured peripheral blood mononuclear cells

	n	Medium control	*Helixor* A (100 μg/ml)	VaAR (100 μg/ml)	Mistletoe lectin-III (100 ng/ml)
Lymphocytes (counts)		4780 ± 303	4421 ± 412***	2247 ± 498***	673 ± 350***
CD3+ T cells	10	70.4 ± 6.3	70.4 ± 7.2	69.0 ± 7.3	66.5 ± 13.8
CD19+ B cells*	10	7.2 ± 3.4	7.5 ± 3.7	13.2 ± 5.5**	16.8 ± 6.1**
CD4+ T-helper cells	10	41.1 ± 8.4	41.2 ± 9.5	46.4 ± 11.2*	44.3 ± 13.5
CD8+ Ts/c cells	10	34.7 ± 9.8	33.3 ± 9.7	26.2 ± 8.6***	22.0 ± 11.0***
CD28+ CD8+ Tc cells	10	16.2 ± 5.9	14.4 ± 5.0*	12.3 ± 4.7**	14.1 ± 8.0
CD28– CD8+ Ts cells	10	18.4 ± 6.6	18.8 ± 7.2	14.1 ± 6.5***	7.3 ± 6.2***
CD4/CD8 ratio	10	1.39 ± 0.70	1.47 ± 0.79*	2.21 ± 1.33***	3.18 ± 2.28**
CD25+ in CD3+ (%)	8	68.4 ± 8.7	67.4 ± 8.8	41.1 ± 8.5**	19.1 ± 6.4**
CD71+ in CD3+ (%)	7	50.4 ± 9.5	51.3 ± 9.5	27.7 ± 7.1**	5.8 ± 5.2**

Results are means ± SD of experiments with phytohaemagglutinine-activated lymphocytes (72 h) from healthy individuals and are given as % of lymphocytes, while activation marker expression (CD25, interleukin-2 receptor α chains; CD71, transferrin receptor) is given as % of CD3+ T cells. VaAR, *Viscum album* Abietis R; Ts/c, T-suppressor/cytotoxic. *$P \leq 0.02$, **$P \leq 0.009$, ***$P \leq 0.004$, versus control by Wilcoxon sign rank test.

Table 3. Flow cytometric analysis of CD8+ lymphocytes from cultured peripheral blood mononuclear cells

	n	Medium control	*Helixor* A (100 μg/ml)	VaAR (100 μg/ml)	Mistletoe lectin-III (100 ng/ml)
CD28+ in CD8+ (%)	10	48.2 ± 10.4	44.7 ± 13.0	47.1 ± 12.5	65.5 ± 20.9*
CTLA4+ in CD8+ (%)	5	32.6 ± 15.0	36.9 ± 5.6	27.0 ± 4.2	11.4 ± 0.4
CD38+ in CD8+ (%)	8	59.5 ± 13.8	52.0 ± 11.4*	35.5 ± 10.2**	22.2 ± 14.6**
CD25+ in CD8+ (%)	5	48.2 ± 12.2	48.7 ± 10.2	36.6 ± 5.2	14.7 ± 6.4
CD71+ in CD8+ (%)	3	30.0 ± 3.2	38.2 ± 11.1	28.9 ± 0.2	14.6 ± 0.3

Results are means ± SD of experiments with phytohaemagglutinine-activated lymphocytes (72 h) from healthy individuals. Surface molecule expression is given as % of CD8+ cells. VaAR, *Viscum album* Abietis R. *$P < 0.02$; **$P = 0.006$, versus control by Wilcoxon sign rank test.

versus 1.4 μg/ml). While *Helixor*A did not induce apoptosis, even at a final concentration of 100 μg/ml, *Viscum album* Abietis R was found to be a potent inducer of the apoptotic killing pathway (Fig. 2). Within the surviving lymphocytes, the sister chromatid exchange number was significantly lower [1,3]. Thus, although both extracts differed in their capacity to induce apoptosis, both were effective in reducing the number of sister chromatid exchange-inducing DNA lesions.

By analysing surviving cells, we found that *Viscum album* Abietis R and mistletoe lectin III significantly reduced the number of CD25+ and CD71+ (proliferating) CD3+ T cells but *Helixor* A did not affect these cells (Table 2). However, predominantly CD8+ T-suppressor/cytotoxic cells died while the amount of CD4+ T-helper cells remained almost unchanged, suggesting CD8+ T cells are more sensitive towards the induction of *Viscum album* Abietis R/mistletoe lectin III-mediated apoptosis than CD4+ T cells or CD19+ B cells (100 ng/ml mistletoe lectin III > 100 μg/ml *Viscum album* Abietis R > 100 μg/ml *Helixor* A > controls). Analysis of the surface expression of CD8+ T cells (Table 3) indicated a predominant decline of cells with an activated phenotype (CD38, CD25, CD71, CTLA-4). However, CD8+ cells that lacked the CD28 molecule, which is an important coligand for T-cell activation, were significantly more sensitive towards the *Viscum album* Abietis R and mistletoe lectin III-mediated killing (Table 2). This finding confirms the observations of Monteiro *et al.* [28] showing that the telomeric length was significantly shorter in the CD28– CD8+ subset, indicating an exhaustion of their replicative capacity. Since these cells have undergone many more rounds of replication than their CD28+ counterparts, presumably in response to antigenic exposure, one may expect this subset to be a 'memory' population. In a recent paper it was confirmed that naive T cells require strong T-cell receptor signals and high levels of multiple synergizing costimulatory signals, while effector cells responded efficiently to anti-CD3 alone [29]. Thus, it is conceivable that the CD28– CD8+ subset represents 'memory'/effector cells responding to lower doses of toxic lectins from mistletoe than the other subsets.

Thus, both drug extracts contain high concentrations of toxic mistletoe lectin II/III. However, although the mistletoe lectin III is reported to be the most potent mistletoe lectin [16,30], high levels of mistletoe lectins are not necessarily associated with a high killing potency of the plant extract. These results indicate that only the cytotoxic activities of mistletoe are mediated by the mistletoe lectins, probably influenced by various interacting components present in the whole plant extract, but not the protective effects.

Conclusion

At present, the antimutagenic properties are observed only in peripheral blood mononuclear cells treated with whole plant extracts of mistletoe, while purified components, such as mistletoe lectins and viscotoxins, are ineffective. The therapeutic significance of the protecting effects is currently under investigation. The cytotoxicity of mistletoe is due to the induction of apoptosis, mediated by the mistletoe lectins. Surprisingly, CD8+ cells with an activated phenotype are more sensitive to the mistletoe lectin III-mediated killing. Also, in human granulocytes treated with mistletoe *in vitro*, we observed differences in the susceptibility to the drug. It is, however, important to fully understand the balance between immune cell activation and killing. At low drug concentrations, mistletoe provides protecting and stimulating effects. At higher drug concentrations, mistletoe induces killing. These findings highlight the need for further research on the two properties of mistletoe, both of which are important for adjuvant cancer treatment.

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