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IN VITRO INVESTIGATION INTO THE POTENTIAL OF A MISTLETOE EXTRACT TO ALLEVIATE ADVERSE EFFECTS OF CYCLOPHOSPHAMIDE

Jessica Burkhart; Chantal Wälchli, PhD; Peter Heusser, MD, MME; Ulrike Weissenstein, PhD; Stephan Baumgartner, PhD; Anne-Catherine Andres, PhD

Objectives • Mistletoe extracts have been shown to provide deoxyribonucleic acid (DNA)–stabilizing effects in human peripheral blood mononuclear cells (PBMC) in vitro. We investigated the effect of a mistletoe extract on PBMC with and without concomitant treatment with cyclophosphamide and compared mitochondrial activity and replication of normal PBMC with that of a T-cell leukemia cell line.

Design • The experiments were performed with PBMC of healthy blood donors and the T-cell leukemia Jurkat cell line. Cells were pre-incubated with mistletoe extract for 60 to 65 hours. 4-hydroperoxycyclophosphamide (4-hpc, precursor of 4-hydroxycyclophosphamide) was added for 2 hours, after which mitochondrial activity and replication were measured. All experiments were randomized and blinded.

Main Outcome Measures • Cell mitochondrial activity and replication were assessed with spectrophotometric analysis of WST-1 reduction and BrdU incorporation.

Results • The application of 4-hpc consistently reduced mitochondrial activity and replication of PBMC and Jurkat cells. Mistletoe extract strongly enhanced PBMC mitochondrial activity and replication (with or without 4-hpc) and partially inhibited Jurkat cell replication (with 4-hpc only). Compared to mistletoe untreated cells, enhancement of PBMC mitochondrial activity by mistletoe extract was independent of treatment with 4-hpc, but enhancement of PBMC replication by mistletoe extract was stronger when treated with 4-hpc.

Conclusions • Mistletoe extract strongly stimulated healthy PBMC but not malignant Jurkat cells. In addition, mistletoe extract seemed to partially protect healthy PBMC—but not malignant Jurkat cells—from the cytostatic effect of 4-hpc. The results motivate further preclinical and clinical investigations of mistletoe extracts as an adjuvant medication in cancer therapy to alleviate side effects of conventional therapy. (*Altern Ther Health Med*. 2010;16(3):40-48.)

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Extracts of European mistletoe (*Viscum album* L) are widely used as adjuvant medication during cancer therapy. They are reported to reduce side effects of conventional therapy,[1] as well as to increase quality of life[2] and survival time.[3,4] Mistletoe extracts and their active components, mainly mistletoe lectins ML I, ML II, ML III, and viscotoxins, exert immunomodulatory and antiproliferative effects. They increase the number and activity of natural killer cells and neutrophils[5-7] and induce cytokines such as TNFα, IL-1, IL-2, IL-6, IL-10, and IL-1β.[8-10] In cultured tumor cells, extracts of mistletoe induce cytostatic and/or cytotoxic effects,[11-15] whereas a DNA-stabilizing effect on PBMC has been reported for low doses of mistletoe extracts.[16]

Cyclophosphamide is used for the treatment of lymphoproliferative and solid cancer diseases. It is a member of bifunctional alkylating agents that forms DNA cross-links, leading to a misreading of the DNA and impaired replication. Cyclophosphamide markedly inhibits human lymphocyte proliferation.[17]

Mistletoe extracts induced in vitro DNA protective effects in cyclophosphamide-impaired PBMC, from healthy individuals by decreasing sister chromatide exchange[18,19] and from cancer patients by enhancing DNA repair.[20] There is preliminary evidence that Jurkat leukemic cells were not protected from cyclophosphamide damage by mistletoe extracts.[18,19]

In view of these results, one might expect beneficial effects of a simultaneous application of mistletoe extracts and conventional cytostatic drugs. The protective effect should be limited to healthy cells, however, and not extend to cancer cells. We do not know of any investigation comparing PBMC and cancer cells with analogous methods with respect to the effects of a concomitant treatment with cyclophosphamide and mistletoe extracts. In addition, our interest was to determine whether the presumed DNA protective effect would be specific for cyclophosphamide damage, as the available literature is not conclusive regarding this question: mistletoe extracts decreased sister chromatide exchange in unimpaired PBMC[16] similarly as in cyclophosphamide-affected PBMC.[18,19] Thus the effect might be additive and unspecific regarding the damage induced by cyclophosphamide.

Based on these considerations, we set out to investigate the effect of mistletoe extracts on mitochondrial activity and replication of PBMC and Jurkat cancer cells with and without concomitant cyclophosphamide treatment in vitro.

MATERIALS AND METHODS

Ethics

This investigation is in compliance with the declaration of Helsinki. The ethics committee of the canton of Bern was informed about the study. The ethics committee decided that no official submission to the ethics committee was necessary because of the preclinical nature of the study and because of the anonymity of the blood donors. The study furthermore complies with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines where applicable.

Test Substances

For our investigations, an aqueous, fermented total extract derived from *Viscum album* L grown on the host tree pine was used. This extract was chosen because Stein et al observed the strongest proliferative response of PBMC in vitro after application of this extract compared to all other mistletoe preparations investigated.[21] Additionally, we wanted to avoid strong pro-apoptotic effects of mistletoe lectins on PBMC and to study potential lectin-independent effects. The concentration of the extract used corresponds to 200 mg fresh plant material per mL extract. This preparation is used to prepare the commercially available mistletoe extract Iscador Pini by dilution in physiological saline solution. Two lots were used (N° 0504/5094 and 0504/5095; Verein für Krebsforschung, Arlesheim, Switzerland). The following amounts of mistletoe lectins and viscotoxins were found in the extract by enzyme-linked immunosorbent assay[22] and high-performance liquid chromatography,[23] respectively: 40 ± 4 ng/mL mistletoe lectins and 89 ± 4 µg/mL viscotoxins (0504/5094); 36 ± 4 ng/mL mistletoe lectins and 107 ± 5 µg/mL viscotoxins (0504/5095). For the experiments, mistletoe extracts were diluted in the medium used for cell culture (see below). 4-hydroperoxycyclophosphamide (4-hpc; Niomech, Bielefeld, Germany) was used as precursor of 4-hydroxycyclophosphamide, which is the primary metabolite of cyclophosphamide. In aqueous solutions 4-hpc rapidly generates 4-hydroxycyclophosphamide. The powdery 4-hpc was stored at –20°C. A working solution was freshly prepared with sterile distilled water directly before use.

Blood Donors and Isolation of PBMC

Human PBMC were prepared from buffy-coats (leukocyte-enriched peripheral blood) obtained from the Blood Center of Bern, Switzerland. The blood donors (10 women and 11 men) were regular donors at the Blood Center. They were healthy according to the criteria of the Blood Center, and none of them had a known allergy.

The PBMC were isolated from buffy-coats based on previously described procedures.[24] Briefly, the PBMC were isolated by centrifugation on a Histopaque-1077 density gradient (Sigma-Aldrich, St Louis, Missouri) on the day of blood donation. PBMC were cultured without mitogen in 96-well plates (Becton Dickinson, Heidelberg, Germany) directly after isolation.

Cell Culture

Apart from the PBMC, we used the human T-cell leukemia Jurkat cell line (DSMZ, Braunschweig, Germany). The cells were cultured in RPMI 1640 medium supplemented with 2 mmol/L glutamine (Oxoid AG, Basel, Switzerland) and 5% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere with 5% CO_2. Jurkat cells in exponential growth phase were transferred to 96-well plates for each experiment.

Pretreatment With Mistletoe Extracts

Cells were distributed (100 µL/well, $2x10^6$ cells/mL for PBMC and $1x10^5$ cells/mL for Jurkat) into wells containing the mistletoe extract (100 µL/well, final concentrations of fresh plant material: 10 µg/mL, corresponding to 1.9 pg/mL mistletoe lectins and 4.9 ng/mL viscotoxins, and 100 µg/mL, corresponding to 19 pg/mL mistletoe lectins and 49 ng/mL viscotoxins). The two mistletoe lots were investigated in parallel. Mistletoe extracts and the control (medium) were coded (blinded) by a person not involved in the experiments and newly randomized for each experiment. Cells were incubated for 60 to 65 hours with mistletoe extract.

Main Experimental Procedure

Apoptosis and cytotoxicity were assessed after 60 to 65 hours mistletoe preincubation (without 4-hpc treatment) for PBMC and Jurkat cells by an Annexin V-PE Apoptosis Detection assay in 6 independent experiments each.

For analysis of 4-hpc effects, 4-hpc was added at concentra-

tions depending on cell type and on subsequent test after incubating cells with mistletoe extract for 60 to 65 hours. Two concentrations of 4-hpc were tested for each cell type and test (see below). 4-hpc concentrations were chosen to obtain an inhibition of approximately 50% according to preliminary tests. Incubation was continued for 2 hours. Cell mitochondrial activity was assessed by the WST-1 test and cell replication by BrdU incorporation. Five wells were used for each experimental condition. The outer wells of the plate were filled with cell culture medium and not used for measurements in order to avoid possible edge effects. PBMC were tested in ten and eleven independent experiments for WST-1 and BrDU, respectively (gender was randomized). Thirteen independent experiments were performed with Jurkat cells for WST-1 and BrDU each.

Determination of Apoptosis

To quantitatively determine the percentages of apoptotic cells and of cytotoxicity after mistletoe preincubation, the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen, Heidelberg, Germany) was used. The assay was performed according to manufacturer's protocol: After 60 to 65 hours incubation of cells with mistletoe extract, cells were washed with cold PBS and then resuspended in 1X Binding Buffer at a concentration of 1 x 10^6 cells/mL. Next, 100 µL of the solution was transferred to a 5-mL culture tube, and 5 µL of PE Annexin V and 5 µL 7-AAD were added. Cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. Four hundred µL of 1X Binding Buffer was added to each tube, and cells were analyzed by flow cytometry using a BD FACScan Flow Cytometer (Becton Dickinson) within 1 hour.

Early apoptotic cells were defined as Annexin V positive and 7-AAD negative. Cells that were Annexin V negative and 7-AAD positive, or Annexin V and 7-AAD double positive were defined as necrotic or late apoptotic cells. Cytotoxicity was calculated by totaling the percentages of necrotic, late, and early apoptotic cells.

WST-1 Test

Cell mitochondrial activity was measured by use of a colorimetric assay based on the reduction of the tetrazolium salt WST-1 (Roche, Mannheim, Germany). WST-1 is cleaved by mitochondrial enzymes to a soluble formazan dye, the amount of which is proportional to the amount of viable cells and can be measured spectrophotometrically.

After 2 hours incubation with 4-hpc (PBMC: 70 and 120 µg/mL; Jurkat: 50 and 70 µg/mL), 10 µL WST-1 were added into 200 µL cell suspension. Absorbance at 450 nm (reference 690 nm) was measured after 4 hours with a υmax kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, California). Background absorbance of wells only containing medium was subtracted from the experimental values.

A possible interference of the mistletoe extract with the WST-1 assay was excluded by a test in a cell-free system (data not shown).

BrdU Test

Cell replication was measured by a colorimetric immunoassay based on the incorporation of the pyrimidine-analogue BrdU into the DNA during replication (BrdU-Kit; Roche, Mannheim, Germany).

After 2 hours' incubation with 4-hpc (PBMC: 15 and 20 µg/mL; Jurkat: 20 and 30 µg/mL), the cells were pulsed with 20 µL BrdU/well (containing 200 µL cell suspension) during 23 to 24 hours. The BrdU ELISA was performed according to the manufacturer's instructions. Absorbance at 450 nm (reference 690 nm) was measured with a υmax kinetic microplate reader (Molecular Devices Corporation). Background absorbance of wells containing only medium was subtracted from the experimental values.

Data Reduction and Statistics

Data analysis of apoptotic cells and of cytotoxicity after mistletoe preincubation was effectuated by one-way analysis of variance with the independent variable mistletoe concentration with global F-tests and subsequent Bonferroni tests for pair wise comparisons.

For BrdU and WST-1 data, percentage ratios were calculated for every experiment by referring OD (optical density) values to the 4-hpc and mistletoe-untreated cells (set to 100%). Data analysis was effectuated by a full 4-way analysis of variance with the four independent variables (1) mistletoe lot, (2) mistletoe concentration, (3) 4-hpc concentration, and (4) experiment number, with global F-tests and subsequent Bonferroni tests for pair-wise comparisons.

All data analysis was performed with the statistics software "Statistica 6.0" (Statsoft, Inc, Tulsa, Oklahoma).

RESULTS

Apoptosis and Cytotoxicity

After pretreatment of PBMC and Jurkat cells with different concentrations of mistletoe extract, apoptosis and cytotoxicity was detected using the Annexin V-PE apoptosis detection kit I.

PBMC

There was no significant difference observed between average proportions of 14% ± 8% of early apoptotic cells in PBMC without mistletoe pretreatment and 12% ± 5% and 14% ± 5% in cells pretreated with mistletoe extract (10 µg/mL and 100 µg/mL, respectively) (mean effect ± 2SE). Cytotoxicity values were also unaffected by mistletoe preincubation with amounts of 26% ± 10% without mistletoe pretreatment and 24% ± 7% and 27% ± 7% for 10 and 100 µg/mL mistletoe pretreated cells.

Jurkat

The amount of early apoptotic cells in Jurkat cells ranged between 3.5% ± 0.8% and 4.2% ± 0.6% in cells without mistletoe pretreatment or pretreated with 10 µg/mL or 100 µg/mL mistletoe extract. Cytotoxicity was 8% ± 3% for cells without mistletoe pretreatment and 9% ± 3% and 10% ± 2% for cells pretreated with 10µg/mL and 100 µg/mL of mistletoe extract (mean effect ± 2SE). There were no statistically significant differences between any treatments.

TABLE 1 Cell Mitochondrial Activity and Cell Replication of PBMC and Jurkat Cells*

PBMC Cells: Mitochondrial Activity (WST-1 Test)

		4-hpc concentration		
		0 µg/mL	70 µg/mL	120 µg/mL
Mistletoe concentration	0 µg/mL	0.32 ± 0.01	0.26 ± 0.01	0.21 ± 0.01
	10 µg/mL	0.56 ± 0.01	0.44 ± 0.01	0.34 ± 0.01
	100 µg/mL	0.66 ± 0.01	0.54 ± 0.01	0.43 ± 0.01

Jurkat Cells: Mitochondrial Activity (WST-1 Test)

		4-hpc concentration		
		0 µg/mL	50 µg/mL	70 µg/mL
Mistletoe concentration	0 µg/mL	1.15 ± 0.02	0.65 ± 0.02	0.49 ± 0.02
	10 µg/mL	1.16 ± 0.02	0.66 ± 0.02	0.49 ± 0.02
	100 µg/mL	1.12 ± 0.02	0.69 ± 0.02	0.49 ± 0.02

PBMC cells: replication (BrdU test)

		4-hpc concentration		
		0 µg/mL	15 µg/mL	20 µg/mL
Mistletoe concentration	0 µg/mL	0.59 ± 0.02	0.19 ± 0.02	0.11 ± 0.02
	10 µg/mL	0.72 ± 0.02	0.33 ± 0.02	0.17 ± 0.02
	100 µg/mL	0.62 ± 0.02	0.33 ± 0.02	0.20 ± 0.02

Jurkat cells: replication (BrdU test)

		4-hpc concentration		
		0 µg/mL	20 µg/mL	30 µg/mL
Mistletoe concentration	0 µg/mL	1.75 ± 0.02	0.94 ± 0.03	0.36 ± 0.03
	10 µg/mL	1.77 ± 0.02	0.92 ± 0.03	0.35 ± 0.03
	100 µg/mL	1.78 ± 0.02	0.81 ± 0.03	0.30 ± 0.03

*Cell mitochondrial activity (assessed with WST-1) and cell replication (assessed with BrdU) of PBMC and Jurkat cells, respectively. Cells were incubated with mistletoe extracts (10 or 100 µg/mL), and subsequently treated with 4-hydroperoxycyclophosphamide (4-hpc) at different concentrations (see table). Mean OD values (± double standard error).

Cell Mitochondrial Activity (WST-1 Test)

To assess mitochondrial activity, we analyzed the conversion of WST-1 by mitochondrial enzymes (descriptive statistics of mean OD values; Table 1). Results of the global F-tests are summarized in Table 2. The comparison of the two lots of mistletoe extracts revealed no statistically significant differences. Thus, the data of both lots were pooled for further evaluation. We observed strong and concentration dependent effects of 4-hpc and mistletoe extracts. The significant effects for experiment number are due to variations in absolute OD values from experiment to experiment, reflecting natural variability of mitochondrial activity.

PBMC

Incubation of PBMC with mistletoe extracts resulted in an average higher mitochondrial activity of 197% ± 3% with 10 µg/mL and 225% ± 3% with 100 µg/mL mistletoe extract (mean effect ± 2SE) compared to a 100% mitochondrial activity in mistletoe untreated control (Figure 1A).

Incubation with 4-hpc only resulted in a reduction of mitochondrial activity to 78% ± 5% with 70 µg/mL and 68% ± 5% with 120 µg/mL 4-hpc (mean effect ± 2SE; Figure 1A).

In cells incubated in the presence of mistletoe extract followed by 4-hpc-treatment (70 µg/mL), a mitochondrial activity of 147% ± 5% with 10 µg/mL mistletoe extract and of 176% ± 5% with 100 µg/mL mistletoe extract (mean effect ± 2SE; Figure 1A) compared to a mitochondrial activity of 78% ± 5% in the control sample without mistletoe supplement could be detected. A similar effect of mistletoe extract was also seen after increasing 4-hpc concentration to 120 µg/mL: mitochondrial activity was 123% ± 5% with 10 µg/mL mistletoe extract and 154% ± 5% with 100 µg/

TABLE 2 Results of Analysis of Variance Global F-tests for PBMC and Jurkat Cells*

	PBMC		Jurkat	
	WST-1	BrDU	WST-1	BrDU
(1) Mistletoe lot	.051	.169	.944	.504
(2) Mistletoe concentration	<.001	<.001	.676	<.001
(3) 4-hpc concentration	<.001	<.001	<.001	<.001
(4) Experiment number	<.001	<.001	<.001	<.001
Interaction (1)*(2)	.142	.714	.361	.669
Interaction (1)*(3)	.942	.116	.811	.040
Interaction (2)*(3)	<.001	<.001	<.001	<.001
Interaction (1)*(4)	<.001	.209	.200	<.001
Interaction (2)*(4)	<.001	<.001	.023	<.001
Interaction (3)*(4)	<.001	<.001	<.001	<.001
Interaction (1)*(2)*(3)	.400	.052	.913	.027
Interaction (1)*(2)*(4)	<.001	.091	<.001	<.001
Interaction (1)*(3)*(4)	.559	.669	.019	.037
Interaction (2)*(3)*(4)	<.001	<.001	.044	<.001
Interaction (1)*(2)*(3)*(4)	.188	.925	.125	<.001

*Results of analysis of variance (ANOVA) global F-tests for PBMC and Jurkat cells, assessed with WST-1 and BrdU, for corresponding OD values (referred to the mistletoe and 4-hpc untreated cells [set to 100%], as plotted in Figures 1 and 2). The experimental design included 4 independent variables: (1) mistletoe lot, (2) mistletoe concentration, (3) 4-hpc concentration, and (4) experiment number. Significant ($P < .01$) values are bolded.

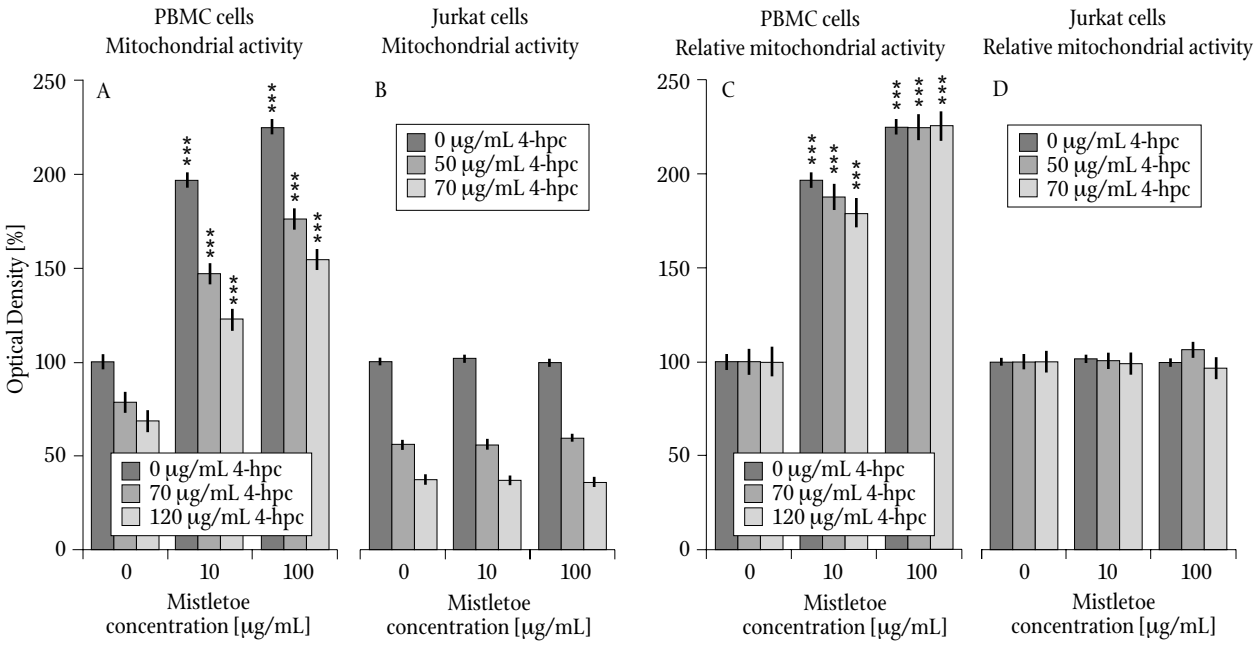

FIGURE 1 Left side: Cell mitochondrial activity (assessed with WST-1) of PBMC (A) and Jurkat (B) cells, respectively. Cells were incubated with mistletoe extract (10 or 100 µg/mL) and subsequently treated with 4-hydroperoxycyclophosphamide (4-hpc) at a concentration of 70 or 120 µg/mL for PBMC and 50 or 70 µg/mL for Jurkat cells, respectively. OD values are referred to the Viscum album and 4-hpc untreated cells (%). Right side: Relative mitochondrial activity (C, D) is expressed relative to the mistletoe untreated cells set to 100%. Mean OD values ± double standard error. Significance levels (Bonferroni test) are given compared to the mistletoe untreated cells with analogous 4-hpc-treatment (*$P < .05$, **$P < .01$, ***$P < .001$).

mL mistletoe extract (mean effect ± 2SE; Figure 1A) compared to 68% ± 5% in the control sample.

Normalization of the data to the appropriate mistletoe untreated controls (set to 100%; Figure 1C) shows that the enhancing effect of the mistletoe extracts on mitochondrial activity was essentially independent of the concomitant treatment with 4-hpc (average increase to 188% and 225% for 10 µg/mL and 100 µg/mL mistletoe extract, respectively).

Jurkat

In Jurkat cells, the mistletoe extracts had no relevant effect on mitochondrial activity, neither when applied alone nor after treatment with 4-hpc (Figure 1B). With 4-hpc alone, a reduction to 56% ± 2% (for 50 µg/mL) and 37% ± 2% (with 70 µg/mL) was observed (mean effect ± 2SE; Figure 1B). This reduction of mitochondrial activity was not affected by any concentrations of the mistletoe extracts added (Figure 1D).

Thus, the strong enhancing effect of the mistletoe extracts found in PBMC from healthy individuals could not be seen in the Jurkat cancer cell line.

Cell Replication (BrdU Test)

As an assessment of cellular replication, cell proliferation was analyzed by measuring BrdU incorporation into DNA during S-phase (descriptive statistics of mean OD values; Table 1). Results of the global F-tests are summarized in Table 2. The comparison of the two lots of mistletoe extracts revealed no statistically significant differences. Thus, the data of both lots were pooled for further evaluation. We observed strong and concentration-dependent effects of 4-hpc and mistletoe extracts. The significant effects for experiment number are due to variations in absolute OD values from experiment to experiment reflecting natural variability of cell replication.

PBMC

Incubation with the mistletoe extracts resulted in an increased cell proliferation of 134% ± 3% (10 µg/mL) and 124% ± 3% (100 µg/mL), respectively (mean effect ± 2SE; Figure 2A).

Incubation with 15 µg/mL and 20 µg/mL of 4-hpc without pre-treatment with mistletoe extract led to an inhibition of cell replication down to 30% ± 5% and 19% ± 5%, respectively (mean effect ± 2SE; Figure 2A).

Preincubation of cells with mistletoe extracts resulted in a highly statistically significant alleviation of the inhibition by 4-hpc treatment (15 µg/mL): a cell proliferation rate of 58% ± 5% with 10 µg/mL and of 73% ± 5% with 100 µg/mL mistletoe extract (mean effect ± 2SE; Figure 2A) was determined compared to 30% ± 5% in the control sample without mistletoe supplement. Also at a 20 µg/mL 4-hpc concentration, an analogous impact of mistletoe extracts was observed: the proliferation rate was 33% ± 5% in cells pretreated with 10 µg/mL and 47% ± 5% with 100 µg/mL mistletoe extract (mean effect ± 2SE) vs 19% ± 5% in the control sample (Figure 2A).

An enhanced proliferative response of at least 20% com-

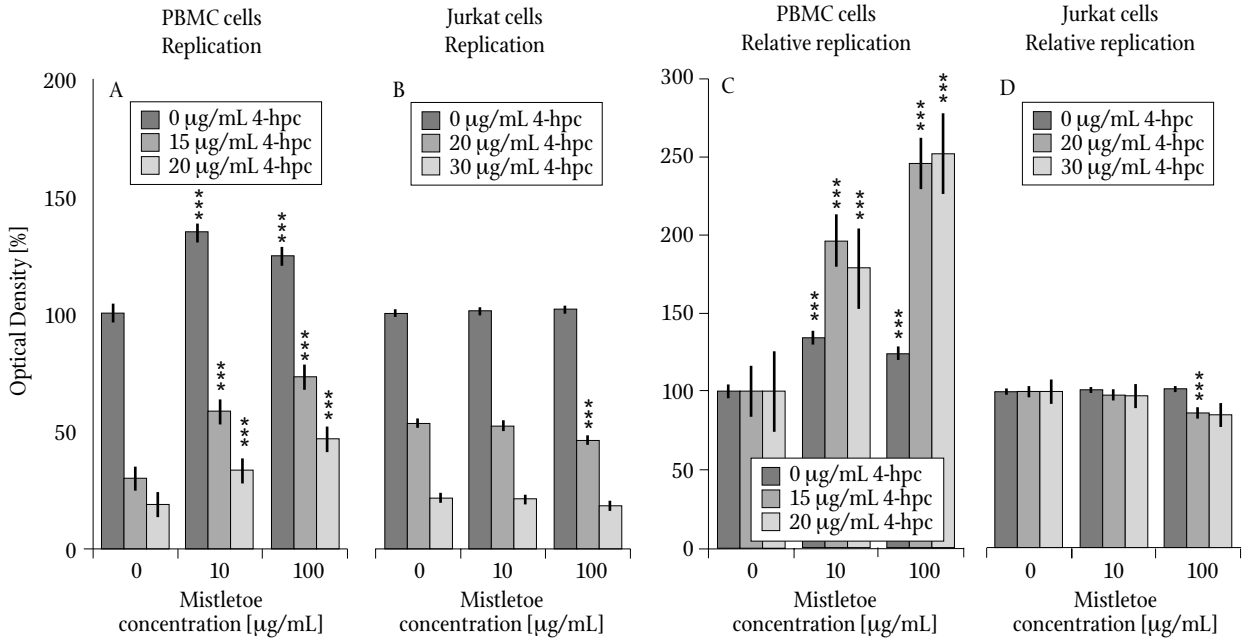

FIGURE 2 Left side: Cell replication (assessed with BrdU) of PBMC (A) and Jurkat (B) cells, respectively. Cells were incubated with mistletoe extract (10 or 100 µg/mL), and subsequently treated with 4-hydroperoxycyclophosphamide (4-hpc) at a concentration of 15 or 20 µg/mL for PBMC, and 20 or 30 µg/mL for Jurkat cells, respectively. OD values are referred to the mistletoe and 4-hpc untreated cells (%). Right side: Relative replication (C, D) is expressed relative to the mistletoe untreated cells set to 100%. Mean OD values ± double standard error. Significance levels (Bonferroni test) are given compared to the mistletoe untreated cells with analogous 4-hpc-treatment (**P*<.05, ***P*<.01, ****P*<.001).

pared to the mistletoe untreated control was detected in a frequency of eleven of eleven donors after preincubation with 10 µg/mL mistletoe extract and 15 µg/mL 4-hpc, in nine of eleven donors for 10 µg/mL mistletoe extract and 20 µg/mL 4-hpc, in eight of eleven donors for 100 µg/mL mistletoe extract and 15 µg/mL 4-hpc and in eight of eleven donors for 100 µg/mL mistletoe extract and 20 µg/mL 4-hpc. In control samples without 4-hpc treatment, the frequency of individuals with a proliferation above 120% was six of eleven after preincubation with 10 µg/mL and six of eleven with 100 µg/mL mistletoe extract, respectively.

Normalization of the data to the appropriate mistletoe-untreated controls (set to 100%, Figure 2C) shows that the enhancing effect of mistletoe extracts on cell proliferation was considerably stronger when it was applied concomitantly with 4-hpc, compared to 4-hpc–untreated cells. This result indicates that the mistletoe extracts exerted a specific protective effect on PBMC cells exposed to the cytotoxic drug.

Jurkat

In Jurkat cells treated with mistletoe extracts only, no effect of the extract on BrdU incorporation could be shown (Figure 2B).

After treatment with 4-hpc only, an inhibition of cell replication down to 53% ± 1% and 21% ± 1% with 20 µg/mL and 30 µg/mL 4-hpc, respectively, was observed (mean effect ± 2SE; Figure 2B).

When cells were incubated with mistletoe extracts followed by 4-hpc treatment, cell proliferation could not be recovered by any dose of the extract (Figures 2B and 2D). Interestingly, even an opposite effect was observed: at a concentration of 20 µg/mL 4-hpc and 100 µg/mL mistletoe extract, the proliferation rate dropped significantly from 53% to 46% ± 1% (mean effect ± 2SE; Figure 2B).

Thus, the enhancing effect of extracts from mistletoe found in PBMC from healthy individuals could not be seen in the Jurkat cell line. On the contrary, the mistletoe extract seems to reinforce the inhibitory effects of 4-hpc.

DISCUSSION

Initially, a concentration-dependent proapoptotic and cytotoxic effect of the mistletoe extracts themselves was excluded. It was shown that the pretreatment of PBMC and Jurkat with different concentrations of mistletoe extract used in our experiments does not influence the amount of early apoptotic cells and of cytotoxicity. As already shown by Büssing et al, extracts of mistletoe plants grown on pine trees that are almost devoid of lectins are not able to induce cell death in PBMC of healthy donors, while the induction of apoptosis by other mistletoe extracts correlates with their mistletoe lectin contents.[25] Induction of apoptosis by the isolated mistletoe lectin I (ML-I) from apple trees at concentrations between 1 ng/mL and 10 µg/mL in 24-hour to 72-hour cultures of human PBL was demonstrated by Hostanska et al.[26, 27]

Our study observed a strong impact of an extract from *Viscum album* L (grown on the host tree pine) on human PBMC from healthy donors. Mitochondrial activity was clearly enhanced in mistletoe-treated samples as compared with the untreated con-

trol. Because apoptosis and cytotoxicity are not specifically affected by mistletoe preincubation, we suppose a stimulation of lymphocytes and a subsequently augmented proliferation as the underlying effect. In principle, the enhanced mitochondrial activity could also be caused by augmented metabolic activity without increased proliferation. Whether the presumed proliferation is a direct effect of mistletoe components on PBMC or it is a consequence of mistletoe-induced cytokines has to be proven.

Similar results have been obtained previously when different mistletoe extracts were applied to PBMC.[9,10,21,28] In a study by Stein et al, a fermented mistletoe extract from the host tree pine was found to be the only extract out of six tested which was capable to induce cell proliferation in PBMC of mistletoe naive individuals, supporting our present results.[21] Huber et al revealed a significant increase of the proliferative response by PBMC after stimulation with mistletoe extracts from the host trees pine or oak in vivo and in vitro.[28] Furthermore, a strong increase in the production of macrophage/monocyte related and T helper type 1 and type 2 related cytokines was observed. Similarly, a stimulatory effect was observed in PBMC treated with an extract of mistletoe grown on poplar.[10] Vehmeyer et al demonstrated a costimulatory effect of mistletoe lectin I on the cytokine-induced proliferation of haematopoietic progenitor cells.[29]

Stimulation of lymphocyte proliferation by low concentration of mistletoe extracts also has been described in animal studies. Weber et al demonstrated a positive impact of a mistletoe preparation on leukopoiesis after cyclophosphamide-induced leukopenia in mice.[30] Hajto et al observed a significant elevation of thymocyte numbers in mice after a single injection of mistletoe extract (from apple host tree) given in a low dose.[31] Higher doses did not induce significant enhancement. Moreover, mistletoe treatment on long-term therapy significantly protected mice from dexamethasone-induced thymocyte reduction. Proliferation of murine splenocytes stimulated by submitogenic concentrations of the T cell mitogen Concanavalin A or anti-CD3 antibodies was further enhanced by low concentrations of purified mistletoe lectin (4-32 pg/mL) whereas higher doses (1-8 ng/mL) inhibited the proliferation. Nonstimulated splenocytes, however, were not affected.[32]

We did not detect any stimulatory effect of the mistletoe extracts investigated on the human T-cell leukemia cell line Jurkat at the concentrations tested. This result is in line with other investigations of different mistletoe extracts with a multitude of cell lines.[33,34] Thus, it seems from our results that the mistletoe extract used is able to stimulate healthy PBMC, whereas malignant Jurkat cells do not respond similarly. This finding cannot be generalized to cancer cells in general. Other cancer cell lines should be investigated in an analogous experimental setting. Huber et al postulated the existence of either natural immunity toward components of mistletoe extract acting as "recall antigens" or of some mitogenic activity of mistletoe components as possible underlying effects for the stimulation of PBMC.[28] Another disparity of the mistletoe impact on PBMC and Jurkat might be due in part to the difference in the low innate proliferative activity of resting PBMC and the fast dividing cancer cells. Indeed, mistletoe extract was shown not to affect proliferation of rapidly proliferating amniotic fluid cells,[35] supporting the hypothesis that its stimulating effect is limited to slowly proliferating cells. In other reports, mistletoe extracts were shown to display cytotoxic and antiproliferative effects in different human cancer cell lines.[11-15] We did not observe strong cytotoxic effects of the mistletoe extract on Jurkat cells, most probably due to the low concentrations administered.

When mistletoe extract and cyclophosphamide were consecutively applied to PBMC, the stimulating effect of the mistletoe extracts regarding mitochondrial activity (WST-1 test) was additive (linear). That implies that the effect of mistletoe extract was independent of the damaging effect of cyclophosphamide.

Regarding replication (BrdU-test), however, the effect induced by mistletoe extract was over-additive (nonlinear): compared to undamaged cells, the relative stimulatory impact on DNA synthesis was more frequent in the individual samples investigated as well as at least twice as strong in cyclophosphamide treated cells. Thus, the mistletoe extract seems to have counteracted the cytostatic effect of cyclophosphamide when the cytotoxic drug was added to mistletoe-pretreated PBMC. An interaction with the enzymes that activate cyclophosphamide in vivo seems to be unlikely because we worked with 4-hpc, which spontaneously generates the main active metabolite of cyclophosphamide in aqueous solutions. We cannot exclude that cell-cycle blocks occurred, however, meaning that cells might have accumulated in S or G2/M phase without further dividing. Future studies should investigate the presumed cellular protection processes induced by mistletoe extracts in more detail.

The mistletoe extracts used seem to have exerted a protective effect in normal PBMC from damage of cyclophosphamide. Two studies corroborate this finding: Sister chromatide exchanges induced by exposure to cyclophosphamide were reduced by concomitant application of mistletoe extracts in PBMC.[18] Likewise, DNA repair was found to be enhanced by a mistletoe extract in PBMC exposed to cyclophosphamide, the effect being accompanied by an increase in IFN-gamma production.[20] However, only our study directly compared unimpaired and impaired PBMC with and without mistletoe extracts within the same experiment and was therefore able to document a specific protection effect of PBMC by mistletoe extracts regarding the damage induced by cyclophosphamide.

Taken together, the data obtained indicate that mistletoe extracts may exert a DNA-protective effect in normal PBMC. This effect could be due to DNA stabilization, to enhancement of DNA repair, or to other so far undefined mechanisms. We could not find a similar protective effect of mistletoe extract in Jurkat cancer cells. On the contrary, the inhibitory effect of cyclophosphamide on DNA synthesis was slightly but significantly increased by the concomitant application of mistletoe extract. Similarly, Büssing et al reported an aggravation of the cytostatic effect of cyclophosphamide in Jurkat cells simultaneously treated with mistletoe extract.[18] The results obtained with Jurkat cells cannot be general-

ized to cancer cells in general. Other cancer cell lines should be investigated in an analogous experimental setting.

Because mistletoe extracts are a mixture of components (proteins, glycoproteins like lectins, oligo- and polysaccharides, etc) and the compositions as well as the amounts of components depend on the host tree and on manufacturing processes, mistletoe effects can vary extensively and may differ from individual to individual. The mechanisms underlying the effects of the mistletoe extract found in the present study are at present unclear, as are the active components responsible for these effects. It is known that the cytotoxic activity of mistletoe extracts in lymphocytes and cancer cell lines is based mainly on the apoptotic effect of mistletoe lectins and necrosis induced by viscotoxins.[36-38] The stimulating and protective effects on PBMC described here could be triggered by mistletoe lectins at very low concentrations like 1.9 pg/mL and 19 pg/mL, respectively, as described, eg, for similar concentrations of isolated mistletoe lectin (4-32 pg/mL).[32] But stimulating effects also could be due to nonlectin components, as already postulated by Stein and Berg.[21] They also demonstrated that only in the presence of homologous anti-ML-1 rich plasma proliferation of PBMC was induced by Abnobaviscum Mali, suggesting other extract components as lectins to be responsible for the stimulation.[39] Elsässer-Beile et al described increased cell viability in PBMC cultures of five of ten blood donors at mistletoe lectin concentrations between 10 and 1000 pg/mL.[10] This effect could not be inhibited with goat-anti-mistletoe lectin antibodies and was not induced by pure lectins. Therefore, they suggested a lectin-independent cell proliferation.[10] In contrast to the addition of 10 µg/mL of mistletoe total extract, isolated mistletoe lectins and viscotoxins at high concentrations of 1 ng/mL and 10 ng/mL were found to be ineffective in reverting damaging effect of cyclophosphamide on surface molecule expression in PBMC.[18] The influence of lower concentrations of isolated mistletoe lectins and viscotoxins was not investigated. Whether isolated components, and which components, are capable to protect cellular DNA from damage induced by cytostatic drugs or whether the whole extract is necessary for DNA protection also remains to be demonstrated.

In conclusion, our in vitro results are in line with the assumption based on clinical data[1,4] that mistletoe extracts may be an adequate adjuvant medication for cancer patients under chemotherapy to alleviate side effects of conventional therapy. We observed that under cyclophosphamide treatment a mistletoe extract protected healthy PBMC in vitro and slightly enhanced the effect of the cytostatic drug in cancer cells in vitro. The results motivate further preclinical investigations of mistletoe extracts (eg, with other common cytostatic drugs such as 5-fluorouracil) as well as with cultured cells of neoplastic disorders that in practice are typically treated with cyclophosphamide (eg, breast and lung cancer cells). The results also motivate further clinical investigations of mistletoe extracts as a potential adjuvant medication in cancer therapy to alleviate side effects of conventional therapy like leukopenia.

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