Molecular mechanisms of mistletoe plant extract-induced apoptosis in acute lymphoblastic leukemia in vivo and in vitro

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Abstract

Viscum album (Mistletoe) is one of the most widely used alternative cancer therapies. Aqueous mistletoe extracts (MT) contain the three mistletoe lectins I, II and III as one predominant group of biologically active agents. Although MT is widely used, there is a lack of scientifically sound preclinical and clinical data. In this paper, we describe for the first time the in vivo efficacy and mechanism of action of MT in lymphoblastic leukemia. For this purpose, we first investigated both the cytotoxic effect and the mechanism of action of two standardized aqueous MTs (MT obtained from fir trees (MT-A); MT obtained from pine trees (MT-P)) in a human acute lymphoblastic leukemia (ALL) cell line (NALM-6). MT-A, MT-P and ML-I inhibited cell proliferation as determined by Casy/C210 Count analysis at very low concentrations with MT-P being the most cytotoxic extract. DNA-fragmentation assays indicated that dose-dependent induction of apoptosis was the main mechanism of cell death. Finally, we evaluated the efficacy of MT-A and MT-P in an in vivo SCID-model of pre-B ALL (NALM-6). Both MTs significantly improved survival (up to 55.4 days) at all tested concentrations in contrast to controls (34.6 days) without side effects.

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1. Introduction

Cytotoxic substances derived from plants (e.g. vinca alkaloids or paclitaxel (Taxol)) are often used in oncology as highly potent drugs and/or serve as model for synthetic compounds [1]. Extracts from Viscum album I (MT) belong to the most frequently used complementary cancer treatments in Europe and have been used for more than 80 years on a
more or less empirical basis. Indications for the use of MT are the reduction of treatment-associated side effects during chemotherapy and as adjuvant therapy [2]. However, no in vivo data or data from controlled studies on the use of MT in leukemia have been published to date. One reason for the use of whole MT is an assumed synergistic therapeutic effect of the different components contained in the extract. This hypothesis is so far only partly substantiated [3]. However, analysis of the extracts reveals a number of pharmacologically interesting components which are known to possess synergistic effects – both with each other and with conventional antineoplastic drugs [3–5].

The best investigated components of aqueous mistletoe extracts are the mistletoe lectins I, II and III. However, most of the published studies have been with ML-I. The cytotoxic effect produced by ML-I is brought about by its receptor-mediated uptake into the target cell and subsequent cleavage into A and B chains. Inactivation of the 60S ribosomal subunit by the N-glycosylated ML-I-A chain then leads to inhibition of ribosomal protein synthesis. The ML-I-B chain is responsible for the receptor-mediated uptake. The apoptosis inducing action of mistletoe lectin I as type II ribosome inactivating protein results in the inhibition of cytosolic protein biosynthesis with subsequent activation of an only partially identified mitochondrial apoptosis pathway involving receptor independent activation of effector caspases [6–13]. The precise mechanisms by which mistletoe extracts induce apoptosis are however still only rudimentarily understood. The three known MT lectins differ in their monosaccharide specificity as well as in their molecular weight. The A chain of the mistletoe lectins is largely conserved in all three isoforms [14] while the B chain is lectin-specific [15]. In spite of the close kinship of the lectins there are marked differences in their cytotoxic activity. In an assay with the human leukemia cell line Molt-4, for example, the cytotoxicity of ML-III was found to be 10 times greater than that of ML-I [16,17]. Marked differences in the systemic stimulation of cytokine release have also been shown. In addition to ML-I, II and III the clinically used preparations contain numerous other biologically active substances such as viscos, polysaccharides, triterpenes, lipids, amines, phytosterols, flavonoids, phenylpropanes and several enzymes. Various authors have reported a growth-inhibiting action of MT and isolated ML-I both in vitro and in vivo in individual tumors. However, some of the results are still contradictory [18–21].

There are no in vivo data to date on the experimental significance of MT in acute leukemia. The aim of our investigations was to examine the therapeutic efficacy of mistletoe extracts in ALL and identify the relevant mechanisms of action. Since the preparations used in clinical practice are usually whole extracts of mistletoe, and since we still know too little about the main active components and the differences between the components depending on the host tree, we decided to use one mistletoe lectin rich preparation (MT-P (HELIXOR®-P)) and one mistletoe lectin poor preparation (MT-A (HELIXOR®-A)) for our investigations.

2. Materials and methods

2.1. NALM-6 cells

Human ALL cell lines NALM-6 were obtained from the DSMZ (Bonn, Germany) and was maintained by serial passages in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 25 mM Hepes buffer, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 M glutamine. The cells were grown in a humidified atmosphere of 5% CO2 and air and expanded every other day. Prior to injection into SCID mice, cells were washed once and then resuspended in filter-sterilized phosphate-buffered saline (PBS). Mice received 106 cells via the dorsal tail vein.

2.2. Transplantation of ALL (NALM-6) into SCID mice (CB-17 SCID/SCID)

Ten-week-old male SCID mice (CB-17 SCID/SCID) weighing 20 g were obtained from Charles River WIGA (Sulzfeld, Germany). They were housed and maintained in a specific pathogen-free (SPF) facility. They were maintained under pathogen-free conditions in the animal facility of the Max Delbrück Center of Molecular Medicine (Berlin, Germany). They were fed autoclaved standard diet purchased from Sniff (Soest, Germany) and acidified drinking water ad libitum.

2.3. Assessment of toxicity of aqueous mistletoe extract in SCID mice

SCID mice (CB-17 SCID/SCID) were treated by intraperitoneal injection of MT-P and A at different doses every 4 days a week. Control mice were injected with an equivalent volume of PBS. The mice were carefully monitored for symptoms of toxicity, and weighted twice each week. The mean weight of each group of mice was calculated and used as a parameter of toxicity.
2.4. Mistletoe extracts from *Viscum album* L. *pini* and *abietis*

The biologically and biochemically standardized aqueous mistletoe extracts grown on fir tree (MT-A) and on pine tree (MT-P) both from Helixor Heilmittel GmbH & Co. (Rosenfeld, Germany) were obtained from the pharmacy. The lectin content of MT-A (160 ng/mL) and MT-P (725 ng/mL) 95% ML-II/-III and MT-P (725 ng/mL) ML-II/ML-III. Pine tree mistletoes do not contain ML I was determined by the manufacturer with validated ELLA- and ELISA-methods which are specific for total lectin content (ELLA-ASF) or for ML-I and ML-II/3 (ELISA). For the MT-P and MT-A the contents of ML I and ML II/ III were investigated by using an ELISA test system with monoklonal antibodies (source: Prof. A.G. Tonevitskii, Moscow). The specificity of the test system (cross-reaction of the antibodies) has been evaluated as below 10% (internal Helixor validation report; April 2000). With this ELISA system the average amount of ML-I was measured as not more than 5-10% of the ML II/III content. At the present time, there is no validated method for discerning quantitatively ML-II and ML-III. However, in these extracts the contribution of ML-II to total lectin content can be estimated to be less than 5%.

2.5. LDH release assay

Modified lactate dehydrogenase (LDH) micro-assay, as previously described, was used for determination of MT-P and MT-A effects on NALM-6 cells line. Control or treated NALM-6 cells after centrifugation and medium replacement were transferred to micro-well plates. The LDH release assay was done in 96 micro-well plates (Falcon, Germany) incubated for the next 2 h at 37 °C in humid atmosphere containing 5% CO₂. Subsequently, the plates were centrifuged for 5 min at 1500 rpm and supernatants from each well were transferred into new 96 flat bottom micro-well plates to which 100 µL of the LDH substrate mixture composed of 5.4 x 10⁻² M of (1-)-lactate, 6.6 x 10⁻⁴ M of 2-p-iiodophenyl-3-p-nitropheyl tetrazolium chloride, 2.8 x 10⁻⁴ M of phenazine metosulphate and 1.3 x 10⁻³ of NAD (Boehringer Mannheim, Mannheim, Germany) in 0.2 M Tris-buffer at pH 8.2, was added for determination of LDH release activity. The micro-titer plate reader (Behringer EL-311, Germany) was used for evaluation of the changes in the absorbance using dual filters at 492–630 nm/min.

2.6. Measurement of DNA fragmentation

Apoptotic cell death was determined by a modified cell cycle analysis, which detects DNA fragmentation on the single cell level. For measurement of DNA fragmentation cells were seeded at a density of 1 x 10⁵ cells/ml and treated with different concentrations of MT-A and MT-P as well as isolated ML-I. After 72 h of incubation, cells were collected by centrifugation at 1500 rpm for 5 min, washed with PBS at 4 °C, and fixed in PBS/2% (v/v) formaldehyde on ice for 30 min. After fixation, cells were incubated with ethanol/PBS (2:1, v/v) for 15 min, pelleted, and resuspended in PBS containing 50 µg/ml RNase A. After incubation for 30 min at 37 °C, cells were pelleted again and finally resuspended in PBS containing 50 µg/ml propidium iodide. Nuclear DNA fragmentation was then quantified by flow cytometric determination of hypodiploid DNA. Data were collected and analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with the CELLQuest software. Data are given in percentage of hypoploidy (subG1), which reflects the number of apoptotic cells.

2.7. Determination of cell concentration and cell viability

Cell viability was determined by CASY® Cell Counter + Analyzer System of Schaefere System GmbH (Reutlingen, Germany). Settings were specifically defined for the requirements of the used cells. With this system the cell concentration is analyzed simultaneously in three different size ranges: cell debris, dead cells, and viable cells were determined in one measurement. NALM-6 cells were seeded at a density of 1 x 10⁵ cells/ml and treated with different concentrations of the MT-A, and MT-P, non treated cells served as controls. After 24 h of incubation, cells were resuspended properly and 100 µl of each well was diluted in 10 ml CASYton® (ready-to-use isotonic saline solution) for an immediate automated count of the cells.

2.8. Annexin V–propidium iodide binding assay

Early apoptotic rates were assessed with flow cytometry using the annexin V–fluorescein isothiocyanate/propidium iodide (PI) kit (BD Pharmingen, San Diego, CA, USA), in which annexin V bound to exposed phosphatidylserine of the early apoptotic cells, whereas PI stained the cells that had an increased membrane permeability, i.e., the late apoptotic cells. Samples were prepared according to the manufacturer’s instructions. Flow cytometry analysis was performed using a FACS-Calibur cytometer (Becton Dickinson, Heidelberg, Germany). The annexin V+/PI-cells were defined as early apoptotic cells.

2.9. Measurement of the mitochondrial permeability transition (ΔΨₘ)

After incubation with different concentrations of MT-A and P or ML-I, NALM-6 cells were collected by centrifugation at 1500 rpm, 4 °C for 5 min. Mitochondrial permeability transition was then determined by staining the cells with 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimidazolylcarb-
oceanin iodide (JC-1; Molecular Probes, Leiden, The Netherlands). Cells $(1 \times 10^5)$ were resuspended in 500 µl phenol red-free RPMI 1640 without supplements and JC-1 was added to give a final concentration of 2.5 µg/ml. The cells were incubated for 30 min at 37 °C and moderate shaking. Control cells were likewise incubated in the absence of JC-1 dye. The cells were harvested by centrifugation at 1500 rpm, 4 °C for 5 min, washed with ice-cold PBS and resuspended in 200 µl PBS at 4 °C. Mitochondrial permeability transition was then quantified by flow cytometric determination of cells with decreased fluorescence, i.e. with mitochondria displaying a lower membrane potential. Data were collected and analyzed using a FACSscan (Becton Dickinson) equipped with the CELL Quest software. Data are given in % cells with low $\Delta \Psi_m$, which reflects the number of cells undergoing mitochondrial apoptosis.

2.10. Experimental design of the in vivo experiments

Systemic leukemia was induced in SCID mice by injecting $10^6$ NALM-6 human ALL-cells intravenously. Groups of 8 mice (10 in the control group) with an average weight of 25 g were injected intraperitoneally with MT-A or MT-P 1, 50 and 100 mg/kg (100 mg only MT-A) bodyweight per day. The injections were given four consecutive days a week (day 1–4; 7–11; 14–18) starting one day after tumor cell injection. The control groups received cyclophosphamid 100 mg/kg bodyweight on day 3 or phosphate-buffered saline, respectively. Bodyweight was measured on day 10 and 24 and peripheral blood count was performed 10 days after leukemia injection. Mice were observed daily for the onset of hind leg paralysis and were killed when they developed paresis or paralysis. Kaplan–Meier plots were constructed from the data.

2.11. Statistical analysis

Student’s $t$ test was applied to evaluate the differences between treated and control animal groups with respect to life span. For all the tests, the level of significance was set at $P < 0.05$.

3. Results

3.1. In vitro inhibition of proliferation by MT-A and MT-P

In order to rule out unwanted early cytotoxicity the toxicity after 2 h was measured using an LDH assay with PBS as control. After 2 h no significant release of LDH was measured for all substances, so that an unspecific toxic effect on the cells could be excluded (Fig. 1).

The ability of MT-A and MT-P to inhibit proliferation of NALM-6 cells was determined by measuring cell count. Cells were incubated with the two extracts MT-A and MT-P at increasing concentrations (60, 125 and 250 µg plant extract/ml) for 24 h and the cell vitality and cell count were measured. Concentration-dependent inhibition of proliferation was found for both MT extracts (Fig. 3). MT-P and MT-A have the same antileukemic activity adjusted to their ML-III content.

3.2. Induction of apoptosis in NALM-6 by MT

Using the same concentrations of MT-A and MT-P flow cytometric measurements were performed to differentiate between the early and late phases of apoptosis of NALM-6 cells using annexin V and propidium iodide staining after 48 h. This identified apoptosis as major cause of the inhibition of proliferation – shown by the fact that even low concentrations of MT-A and MT-P lead to dose-dependent induction of early apoptotic markers in NALM-6 cells (Figs. 2–4).

The annexin V results were confirmed by cell cycle measurements (Fig. 5), which again showed that MT-P is more toxic in vitro than MT-A and there is a concentration-dependent specific induction of apoptosis.

3.3. JC-1

The breakdown of the mitochondrial potential, a process located above caspase 9 in the cascade, was determined by flow cytometry with a JC-1 stain. We measured membrane potential changes in NALM-6 cells treated with the same concentrations as in the previous experiments. A significant loss of the mitochondrial membrane potential of NALM-6 cells was found after 48 h in 32.8% of the cells treated with 250 µg/ml MT-A and 62.1% of the cells treated with 250 µg/ml MT-P (Fig. 2).

3.4. Toxicity of MT-A and P in mice

Weight loss was used as a parameter to quantify toxicity. The results demonstrated that at higher concentrations just MT-P had a significant effect on the weight of mice when administered at a dose of 50 mg/kg or higher. There was no significant change in body weight in all MT-A groups.

3.5. Effect of MT on the survival of mice with systemic with human ALL

The Kaplan and Meier survival curve of SCID mice bearing systemic human acute lymphoblastic leukemia (Nalm/6) treated with MT-A show an impressive therapeutic potential not only in the MT-A 50 mg/kg group. Mice receiving saline had a mean survival time of 34.6 days and mice receiving cyclophosphamid had a mean survival time of 60 days ($P < 0.05$). MT-A 1 mg/kg prolonged mean survival time to 46.5 days ($P < 0.05$). MT-A 50 mg/kg nearly doubled the life span to a mean survival time of 55.5 days ($P < 0.05$). MT-P (1 mg/50 mg per kg BW) prolonged mean survival time up to 46.3 days.
Fig. 1. LDH release. NALM-6 cells were treated with different concentrations of MT-A and MT-P for 2 h. Then, viability was determined by the LDH release assay. Values are given as % of control ± SD (n = 3). There was no relevant induction of LDH release after 2 h (<10% compared to controls) by the MT.

Fig. 2. Mistletoe-induced mitochondrial permeability. Mistletoe-induced mitochondrial permeability transition precedes DNA fragmentation. NALM-6 cells were incubated with different concentrations of MT-A and MT-P for 48 h. After incubation, mitochondrial permeability transition was measured by flow cytometric analysis on the single cell level as described in Section 2. Values of mitochondrial permeability transition and DNA fragmentation are given as percentages of cells with low ΔΨm ± SD (n = 3) and as percentages of cells with hypodiploid DNA ± SD (n = 3), respectively. Mitochondrial permeability transition and DNA fragmentation of the medium control (time 0 h) did not change over time. Values of mitochondrial permeability transition are given as percentages of cells with low mitochondrial permeability m ± SD (n = 3).
and showed no significant benefit compared to the corresponding dose MT-A (Fig. 6b). There were at least three long-term survivors (LTS, healthy beyond 60 days) amongst the animals treated with MT-A, all of them from two of the three groups (2 LTS in 50 mg MT-A and 1 LTS in 100 mg/kg BW). These animals showed no evidence of leukemia or lymphoma at autopsy. The tolerability of MT-A was very good. There was no evidence of toxicity on the basis of the weight and hematological parameters (Fig. 6b). On the contrary, in the group treated with MT-A 100 mg/kg there was a trend towards an increase in the leukocyte (not significant in group MT-A 50 mg 4.84 ± 1.06 but significant in group MT-P 50 mg vs. controls 3.22 ± 1.25) and platelet counts (1161 ± 187 vs. controls 885 ± 151).

4. Discussion

The development of paclitaxel from the bark of the tree *Taxus baccata* and of the vinca alkaloids from the periwinkle plant *Catharanthus rosea* document impressively the great therapeutic potential that lies in the realm of natural, plant-derived substances [1]. Ricin, a plant poison from *Ricinus communis*, is likewise the basis for immunotoxins, which have been shown to be clinically effective [22]. Mistletoe contains a similar group of active substances. These are the mistletoe lectins in the form of RIP type 2 as described above [6]. However, mistletoe extracts, or their components such as individual lectins, have not so far received much attention as potential first-line drugs. Although there is some preclinical evidence of efficacy only one clinical phase-II study has been published to date [23]. The only published phase-I study with ML-I indicates good tolerability and shows that considerably higher concentrations of ML-I i.v. are tolerated without problems [24]. It is still open whether the concentrations and dosage forms used in the published clinical studies were within a therapeutic range, however there is some evidence of the clinical anti-tumor efficacy [25] of the low-dose subcutaneous mistletoe therapy used in empirical medicine and the mentioned trials.

There are as yet no in vivo data and few in vitro data on therapeutic effects in hematological malignancies and on the responsible substances. Nothing has been published about the efficacy in ALL in vivo. ML-III has been identified as the most important known detectable substance in the MT-A and MT-P extracts used. The difference between...

Fig. 3. Apoptosis induction by MT-A and MT-P in NALM-6 cells. By flow cytometry, we measured early and late apoptosis when the NALM-6 cell line was incubated with different concentrations of MT-A and MT-P. With MT-A, the percentage of apoptosis was up to 40%. Incubation with MT-P resulted in up to 59% apoptotic cell death. The data demonstrated that MT-A and MT-P augment antileukemic effects by induction of apoptosis. After 48 h of incubation, phosphatidylserine exposure and DNA fragmentation were likewise measured. Values of phosphatidylserine exposure and DNA fragmentation are given as percentages of annexin V-positive/PI-negative cells ± SD (n = 3) and as percentages of cells with hypodiploid DNA ± SD (n = 3), respectively.
Fig. 4. MT-A and MT-P inhibit the proliferation of NALM-6 cells in a concentration-dependent manner. NALM-6 cells were treated with different concentrations of MT-A and MT-P. After incubation for 24 h, the concentration and viability of the cells were determined by using the CASY® Cell Counter System. The figures show a significant inhibition of the cell proliferation up to 63.55% 24 h treatment with MT-P. Data points represent the mean of two determinations from separate cultures with an error less than 3%. The experiments were repeated twice and yielded similar results.

Fig. 5. DNA fragmentation by MT-A and MT-P. NALM-6 cells were treated with different concentrations of MT-A and MT-P. After 72 h of incubation, DNA fragmentation was measured by flow cytometric analysis by assessing the cellular DNA content. Values are given as percentages of cells with hypodiploid DNAs (n = 3).

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ML-III and ML-I lies in the sugar-binding B chain the structure of which has recently been described [15,26–28]. The majority of the few available publications on ML-III show greater cytotoxicity of ML-III against tumor cells compared with ML-I. However, results showing exactly the opposite have also been published, which may indicate that the different toxicity for different tumor cells depends on the binding to the tumor cell, i.e. on the B chain of the respective mistletoe lectin. However, the precise mechanism of uptake of the lectins into tumor cells is not yet known.

In our experiments we were able to show that the inhibition of proliferation of NALM-6 cells can be attributed to effective induction of apoptosis by MT-A and MT-P at very low concentrations. It was indicated by the loss of mitochondrial membrane potential ($\Delta \psi_{m}$) and DNA Fragmentation as hallmarks for apoptosis. The difference in the efficacy between MT-A and MT-P correlates with the different ML-III concentrations depending on the host tree. Altogether the effective concentrations are at a very low concentration range and it was found in an LDH assay after 2 h and in the

Fig. 6. (a and b) In vivo efficacy of MT-A and MT-P. (a) Effect of MT-A and MT-P therapy in systemic leukemia xenografts. Kaplan and Meier survival curve of SCID mice bearing systemic human acute lymphoblastic leukemia (Nalm/6) treated with MT-A 1 mg/kg, 50 mg/kg and 100 mg/kg, five days per week for three weeks post-transplantation of cells. Control mice received PBS or cyclophosphamide, respectively. (b) All doses of MT-A had no significant effect on the body weight. The increased platelet count is not dose-dependent. Compared to MT-A, MT-P had no therapeutic advantage regarding the survival time. MT-P was more toxic than MT-A as indicated by the body weight change in the MT-P 50 mg group.

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<td>1148 ±131*</td>
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*significant vs. PBS Control, p < 0.05
dose-finding studies in vivo that the toxicity of MT-A and MT-P is relatively low. Interestingly there is a marked difference between MT-A and MT-P as regards the in vivo tolerability. This difference in the maximum tolerated dose correlates with the content of ML-III which is about four times higher in MT-P than in MT-A. However, the antileukemic efficacy of MT-A appears to be the same as – if not better than – that of MT-P despite its lower ML-III content. This means that it is apparently not the absolute concentration of ML-III alone, which is responsible for the therapeutic effect but potentially host-tree specific differences in the extracts resulting in a greater cytotoxicity or immunomodulatory effects. They could be mediated by unimpaired NK-cell compartment of the mice model. Various in vitro studies have shown that MT also has immunomodulating action. This is brought about by induction and activation of the transcription and secretion of pro-inflammatory cytokines such as IL-1, IL-12 and TNF-alpha in human mononuclear cells of the blood and endothelial cells [29–32]. In addition, MT is able to increase NK cell activity [33–36]. This action may not be attributed to the mistletoe lectins alone [37,36] but the responsible mechanisms are still largely obscure. Other MT components such as polysaccharides or vesicles from MT, for which synergistic effects are also discussed [4,38–40], probably also play a role. However, in our experiments the apoptotic effect appears to be more important than the mistletoe induced immunomodulation.

Furthermore, the results of the in vivo experiments show a trend to higher white blood-cell (WBC) counts. Because we took just one sample of five animals, the increase of the WBC was only in the MT-P 50 mg/kg statistically significant.

It was shown in our experiments that mistletoe extracts have therapeutically interesting effects and contain hitherto little studied components the further investigation and further development of which could be of clinical interest.

5. Conclusion

We were able to show that the investigated mistletoe extracts containing ML-III as one of the most important components lead to very effective, concentration-dependent induction of apoptosis via the mitochondrial pathway in a caspase independent manner in human B-cell precursor lymphoblastic leukemia cells. In vitro MT-P was found to be superior to MT-A in line with its higher ML-III content. In vivo, however, despite its considerably lower ML-III content MT-A was more effective and less toxic. The in vivo efficacy thus appears to depend not only on the absolute ML-III concentration. The pharmacological explanation for these differences has not yet been found. However, we can say in summary that in an experimental in vivo setting of these substances, which are currently used chiefly for adjuvant treatment, show impressive therapeutic potential in hematological malignancies. Issues of particular interest here are also the incompletely understood mechanisms of induction of apoptosis and the differences in the efficacy of MT-A and MT-P or ML-III whose clinical therapeutic value is not yet known. These investigations thus provide an interesting basis for further experimental studies of the efficacy of mistletoe extracts, individual components and synergistic effects in ALL.

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