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# Intratumoral Application of Standardized Mistletoe Extracts Down Regulates Tumor Weight *via* Decreased Cell Proliferation, Increased Apoptosis and Necrosis in a Murine Model

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**Abstract.** *The cytotoxic in vitro activity of standardized mistletoe extracts (ME) was examined by established assays towards the human ductal breast carcinoma cell line BT474. A dose-dependent (optimum 25 mg/mL medium) and significantly ($p<0.05$) enhanced cytotoxic activity towards the BT474 cells was demonstrated. In vivo experiments on the antitumor activity of ME-A and ME-M were performed in a BALB/c-mouse / BT474 ductal breast carcinoma model. ME-A and ME-M were intratumorally administered according to an application schedule which was found to be optimal concerning dosage and time of administration. Standardized intratumoral application of ME-A and ME-M induced a significantly ($p<0.05$) decreased tumor weight in experimental mice. Histological investigations were performed comprising analysis of mitosis and proliferation rates (Ki67 expression), as well as necrosis and apoptosis induction (ssDNA detection). As compared to tumors of control mice with intratumoral phosphate-buffered saline (PBS) injections, tumors of the ME-A and ME-M treated groups showed a decreased cell proliferation rate, as well as an increased cell necrosis and apoptosis rate. Standardized mistletoe extracts, interfering with defined tumor cell functions, e.g., proliferation, necrosis and apoptosis, may have an impact on local cancer treatment.*

Treatment of cancer patients with extracts from *Viscum album* L. (mistletoe extract) has been recently suggested as a complementary cancer therapy (1-5). Mistletoe extracts (ME) have been used in the treatment of cancer patients since the beginning of the twentieth century and were introduced into oncology by Rudolf Steiner (1). Although there are many reports on experimental and clinical efficacy of mistletoe extracts, the evidence of these results is still discussed controversially because of a lack of adequate methodology of earlier studies in evaluating the safety and efficacy of complementary medicine (1, 3, 4).

Current evidence-based complementary immunotherapies in oncology are performed with subcutaneous applications of standardized mistletoe extracts (6-10). Preclinical research using mistletoe extracts or their defined components (lectins, viscotoxins, carbohydrates) have demonstrated immunomodulation, cytotoxicity, apoptosis induction, DNA stabilization, anti-tumor, anti-metastatic, anti-angiogenic and anti-infectious effects (6, 11-13).

Initial evidence-based medicine level I and II clinical studies on subcutaneous mistletoe application showed beneficial effects in certain cancer types, *e.g.* breast, colorectal and ovarian carcinomas, non-small cell lung cancer, malignant melanoma and glioblastoma, such as reduction in side-effects with simultaneous improvement in quality of life and reproducible immune system stimulation during chemotherapy (8, 14-19). However, effects of complementary mistletoe treatment in patients suffering from malignant melanoma or squamous cell carcinomas of the head and neck are discussed controversially (20, 21). In a panel of 24 prospective clinical trials, four studies did not demonstrate superiority of the applied mistletoe preparation and dosage as compared to the untreated control group (1).

*In vitro* cytotoxicity assay findings encouraged intratumoral application of cytotoxic dosages of the mistletoe extracts and enabled histopathological examinations of cell proliferation, necrosis and apoptosis. The current investigations on the direct antitumor efficacy of standardized mistletoe extracts harvested from defined host trees (fir tree Abies, ME-A and apple tree Malus, ME-M)

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and its causative molecular mechanisms were performed in a well-established *in vitro* / *in vivo* model with BALB/c-mice and the BT474 ductal breast carcinoma cell line. To improve insight into immunological mechanisms, the lymphatic infiltrate (TIL; tumor-infiltrating lymphocytes) in the tumor was analyzed immunohistochemically.

## Materials and Methods

*Animals.* Inbred male BALB/c-mice (n=8 per experimental group; Charles River Wiga Breeding Co., Sulzfeld, Germany), 8- to 10-weeks-old, weighing about 20 g were used for these investigations. The animals were kept in plastic cages and allowed free access to food and water.

*Test substances.* Biologically and biochemically standardized aqueous mistletoe extracts (ME) harvested from defined host trees (fir Abies: ME-A, Helixor® A, Ch.-B. 030788, containing 3.6 ng/mL ML-I and 354 ng/mL ML-III and apple tree Malus: ME-M, Helixor® M, Ch.-B. 030389, containing 23.0 ng/mL ML-I and 500 ng/mL ML-III; measured using ELISA) were supplied by Helixor Heilmittel GmbH & Co. KG, Rosenfeld, Germany. The isotonic solutions of ME-A and ME-M were aqueous extracts of 50 mg fresh plant material/mL.

*Tumor.* The human ductal breast carcinoma cell line BT474 was purchased from the American Type Culture Collection (ATCC) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemicals Co., Heidelberg, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Boehringer, Mannheim, Germany), 2 mM Glutamin (Gibco, Grand Island, USA) and 1% P+S solution (penicillin, 100 U/mL and streptomycin, 100 μg/mL). Cells were cultivated for 18-24 h (37˚C, 5% $CO_2$, 95% air) in FALCON plates (Becton-Dickinson, Heidelberg, Germany), supernatant was decanted and cells were washed in phosphate-buffered saline (PBS, Gibco). Adherent cells were solved from the plates with 2-4 mL 0.25% trypsin-EDTA solution (Gibco) and washed. Supernatant was decanted and the cell sediment was suspended in appropriate medium for the *in vitro* and *in vivo* experiments.

BALB/c-mice (n=8 per experimental group) were subcutaneously inoculated with $1x10^6$ BT474 cells in the abdominal region. On days 5, 6, 7, 12, 13 and 14 intratumoral injections (100 μL per application) of PBS (control group) or ME-A and ME-M (5 mg/100 μL per injection; treatment groups) were administered. This dosage and treatment schedule proved to be optimal in preceeding experiments. Experiments were terminated on day 16, mice were painlessly killed, tumors explanted, weighed and prepared for histological analysis.

*Immunohistochemistry for proliferation and apoptosis.* Formalin-fixed tissue was embedded in paraffin and histological slides were produced according to standard procedures. For assessment of proliferation, the detection of Ki67 antigen (TEC3 DAKO, Glostrup, Denmark) was used. Antigen retrieval was achieved by microwave treatment (two times for 7 min at 650 W in 0.01 M citrate buffer, pH 6.0). After blocking of endogenous peroxidase and endogenous biotin, the slides were incubated with the primary antibody in a dilution of 1:50 overnight. A goat-rabbit antibody (DAKO) was used as a secondary antibody (30 min, room temperature). Visualization was performed with avidin/biotin complex with horseradish peroxidase using DAB Vector (Vectastain ABC Kit, Vector Laboratories, Bulingame, USA) as substrate. Apoptosis detection was carried out by measuring the formation of single-stranded (ss)DNA using the ssDNA apoptosis ELISA kit (Bender Med Systems, Vienna, Austria).

*Immunohistochemistry for immunological investigations*. Cryostat sections (4-μm thick) of tumor tissue were fixed in ice-cooled acetone for 10 min. After air drying, the sections were stained with monoclonal antibodies by an indirect immunohistochemical method. In brief, after rehydrating in TBS buffer, the sections were incubated with an avidin/biotin block in powdered milk and rat normal serum in order to block endogenous biotin and non-specific antibody binding. The primary antibodies (antiCD-3, antiCD-4, antiCD-8 and antiCD-45; Becton-Dickinson) were incubated overnight at 4˚C in a humified chamber. The slides were washed and incubated with the secondary biotinylated rabbit anti-mouse antibody (dilution 1:200; DAKO). After washing in TBS buffer, avidin-biotin complex with alkaline phosphatase (Vectastain ABC-Kit, Vector Laboratories) using fast red with levamisol (DAKO) served for visualization.

*Evaluation.* Randomly selected high power fields (HPF) were investigated and positive cells were counted using an ocular grid (22, 23). The mean number of positive cells was calculated.

*Cytotoxicity assays.* Cytotoxicity investigations comprised the internationally recommended colorimetric tests, crystal violet and tetrazolium salt uptake/cleavage (WST-1 test; Boehringer Mannheim) as proposed by the manufacturers. Time and dosage kinetics were performed, as described elsewhere (24), to determine optimal cytotoxic concentrations of the test substances ME-A and ME-M and the control substance tumor necrosis factor (TNF)-α (Beckton-Dickinson).

*Statistics.* The *in vivo* data were analyzed within an ANOVA model. In case of overall effects, Dunnett's *t*-test was applied which controls type-1 experimental error for multiple comparisons of active treatments against the control. Statistical analysis was performed with SAS version 6.12. The *in vitro* data were analyzed using the student's *t*-test.

## Results

The *in vitro* cytotoxicity of the test substances ME-A and ME-M towards BT474 cells (h-ductal breast carcinoma cells) was assessed using colorimetric assays, including crystal violet uptake (pre-evaluation) and tetrazolium salt cleavage, the internationally recommended confirmation test for cytotoxicity testing. These data were compared to the activity of TNF-α, the cytotoxic standard for *in vitro* tests. Dose kinetics were performed (6.0 μg – 25 mg/mL medium for ME-A and ME-M; 0.001 – 5 ng/mL medium for TNF-α, these ranges were found to be optimal in literature and preceding investigations). As representatively shown in Figure 1, all test substances exert a dose dependent cytotoxic effect towards BT474 cells. However, cytotoxicity was

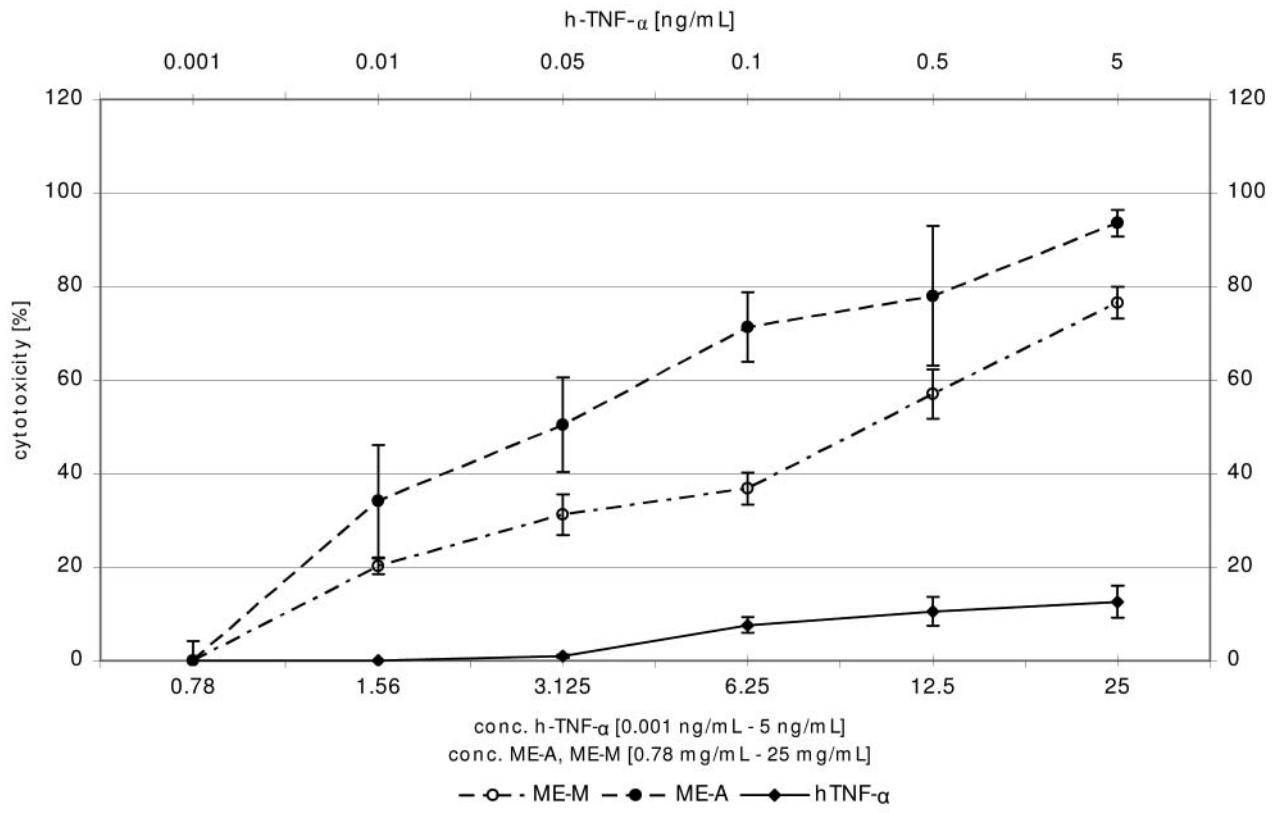


Figure 1. *In vitro cytotoxicity of ME-A and ME-M 0.78 – 25 mg/mL medium and h-TNF-α 0.001 – 5 ng/mL medium towards BT474 cells in WST-1 (tetrazolium salt cleavage) colorimetric assay.*

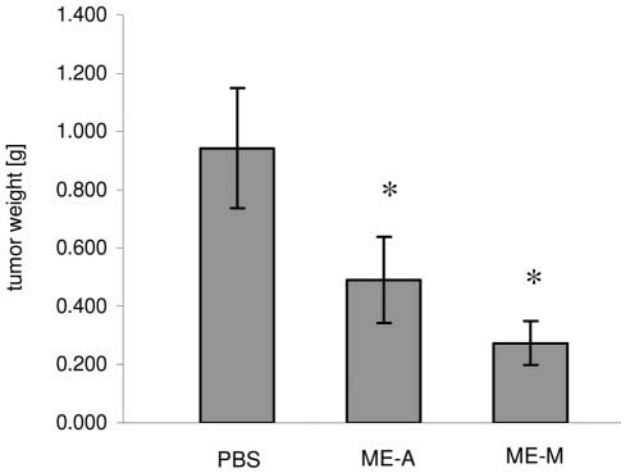


Figure 2. *Tumor weight (BT474 h-ductal breast carcinoma induced by inoculation of 1x10$^6$ cells per mouse) in BALB/c-mice (n=8 per experimental group) after intratumoral application of ME-A and ME-M (treatment groups; 5 mg per application) and PBS (control group). The mean weights±standard deviations of the tumors were 942 mg ± 206 mg (control group/PBS), 490 mg ± 147 mg (ME-A group) and 274 mg ± 75 mg (ME-M group). *p<0.05 = statistically different from control.*

Table I. *Evaluation of histologic analyses of BALB/c-mice tumors (h-BT474; ductal breast carcinoma) after intratumoral application of PBS (control group) or ME-A and ME-M (treatment groups).*

| BALB/-mice treated with | Mitosis/ HPF | Ki67/ HPF | Necrosis | ssDNA/ HPF |
|---|---|---|---|---|
| PBS | 11 | 71.2 | nd | 0.2 |
| ME-A | 13 | 39.4 | + | 0.9 |
| ME-M | 10 | 56.0 | +++ | 2.4 |

nd: not detected; +: moderate, +++: very high number of positive cells counted microscopically; HPF: high power field; PBS: phosphate-buffered saline. Ki67: proliferation rate; ssDNA: apoptosis rate and mitosis rate were counted by HPF microscopy using an ocular grid.

significantly more pronounced for all tested concentrations of ME-A and ME-M as compared to TNF-α. Concerning the test substances ME-A and ME-M, no relevant difference in *in vitro* cytotoxicity was found between them.

The convincing *in vitro* cytotoxicity of ME-A and ME-M towards BT474 cells encouraged *in vivo* investigations in

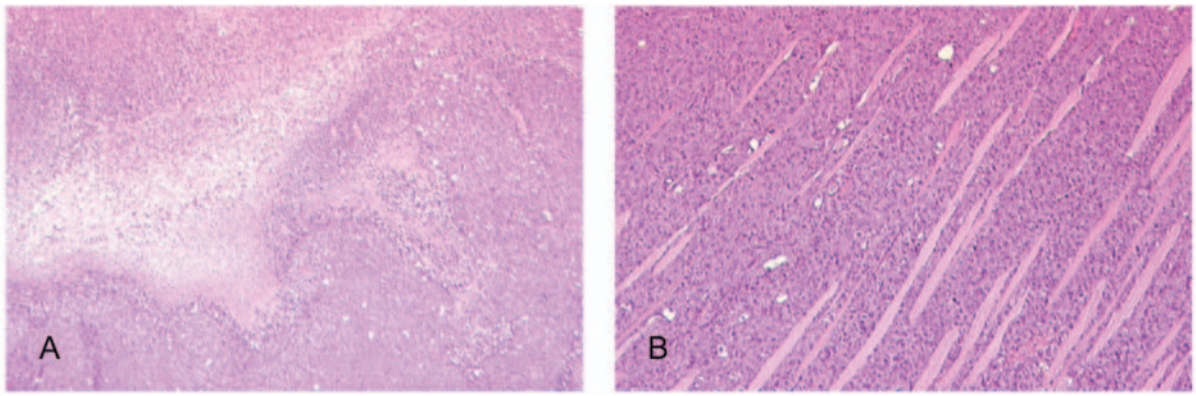


Figure 3. *In vivo necrosis induction in BT474 breast cancers after intratumoral injection of ME-M (A; treatment group) and PBS (B; control group) to BALB/c-mice. The necrotic area is extensive and confluent in ME-M treated tumors (A) and missing in control tumors after PBS injection (B) (magnitude 1:40).*

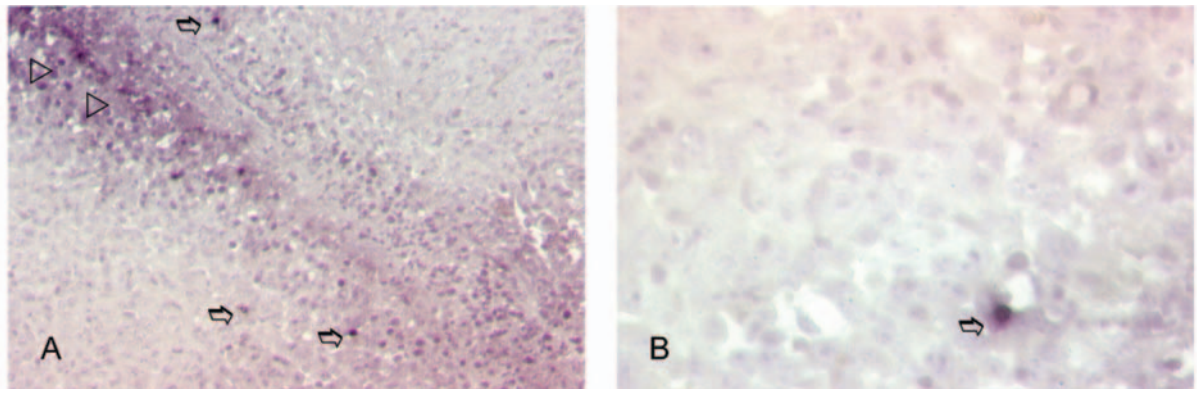


Figure 4. *In vivo expression of ssDNA (apoptosis induction) in BT474 breast cancer cells after intratumoral injection of ME-M (A; treatment group) and PBS (B; control group) to BALB/c-mice. ssDNA expression is higher in ME-M treated tumors as compared to control tumors after PBS injection. Arrows indicate antibody-coated ssDNA in apoptotic cells (Figure 4a: magnitude 1:100, Figure 4b: magnitude 1:200).*

the BALB/c-mouse / BT474 model. Subcutaneous inoculation of BT474 cells into BALB/c-mice induced reproducible local tumor growth, which was investigated and standardized in the preceding experiments. To investigate the *in vivo* cytotoxicity of ME-A and ME-M, both substances were intratumorally administered according to an application scheme, which was found to be optimal in preceding time and dosage kinetics. The treatment seemed to be free of side-effects. It did not influence the behavior or appearance of the animals, *e.g.,* agility, body weight, fur. As shown in Figure 2, intratumoral application of optimal concentrations of ME-A and ME-M (5 mg per application) induced significantly ($p<0.05$) reduced tumor weight in BALB/c-mice as compared to PBS-treated control animals. The mean weights±standard deviations of the tumors were 942 mg ± 206 mg (control group), 490 mg ± 147 mg (ME-A group) and 274 mg ± 75 mg (ME-M group).

In order to investigate mechanisms of the antitumor activity of ME-A and ME-M, histological analyses of tumor tissues were performed and tumor cell necrosis and apoptosis (ssDNA detection), as well as tumor cell mitosis and proliferation (Ki67 expression) were assessed. As shown in Table I, tumors of ME-A and ME-M treated mice presented a decreased tumor cell proliferarion rate and an increased cell necrosis and apoptosis rate, as compared to PBS-treated control mice (Figures 3 and 4). It might be, thus, anticipated that defined tumor cell functions (*e.g.,* proliferation, apoptosis and necrosis) were altered after intratumoral ME-A and ME-M treatment, resulting in significantly reduced tumor weight. These data confirm current literature demonstrating that decreased tumor cell necrosis and apoptosis rate, as well as more than two mitotic figures per HPF correlate to less favourable prognosis (23). There was no significant increase of CD-3, CD-4, CD-8 and CD-45 expression and no significant immigration of leukocytes into the tumor tissue.

## Discussion

Since the early 1950s, various mistletoe constituents have been isolated from the whole extract (1, 2, 4, 5), including low molecular weight substances, such as phenolic acids, flavonoids, and high molecular weight compounds, such as viscotoxins, polysaccharides, glycoproteins/lectins. At the beginning of the 1980s, active substances have been isolated in larger quantities and at least some of their pharmacological activities have been evaluated (1, 5). Immunological, as well as cytotoxic effects of mistletoe extracts partly depend on their lectin content, influenced by interaction with other mistletoe substances (6, 24, 25).

Some years ago, aqueous mistletoe extracts were classified by evidence-based medicine (EBM) as therapeutics that have a supposed but not yet proved clinical efficacy and, thus, have been rejected from mainstream oncology. Recently, in several EBM-relevant studies (levels I and II) the immunoactive and quality of life (QoL) stabilizing effects of subcutaneously applicated standardized mistletoe preparations were demonstrated unambiguously (8, 14-18). Improvement of QoL was shown to result from the significantly reduced side-effects of standard treatment protocols (8) and from enhanced plasma levels of β-endorphin (2, 7).

Mistletoe extracts were shown to induce apoptosis of tumor cells and of non-malignant cells *in vitro* (5, 6). The apoptosis-inducing potentials of the mistletoe extracts derived from different host trees correlated closely with the content of cytotoxic lectins, while necrotic cell death was rather caused by viscotoxins, as becomes evident when testing extracts with high concentrations of viscotoxins (5, 6).

Clinically relevant mistletoe extract-mediated cytotoxic, cytostatic and apoptotic effects are most likely expected with high concentrations over time by intratumoral applications, which have been described in some case reports (26-28). However, scientifically evaluated data on the intratumoral administration of standardized mistletoe extracts are rare and limited to initial documentations of tumor size and weight in an experimental xenograft model (29).

The present investigations confirmed the dose-dependent *in vitro* / *in vivo* cytotoxic activity of standardized mistletoe extracts ME-A and ME-M. *In vitro*, the cytotoxic activity of ME-A and ME-M towards BT474 (h-ductal breast carcinoma cells) was significantly ($p<0.05$) more pronounced as compared to TNF-α. *In vivo* (BALB/c-mouse / BT474 cell model) intratumoral application of the optimal ME-A and ME-M dosages induced a significant reduction of tumor weight ($p<0.05$) as compared to a PBS-treated control group of mice. This antitumor activity of intratumoral ME-A and ME-M application was shown to correlate with a decreased proliferation rate of the tumor cells and an enhanced necrosis/apoptosis induction. Thus, the antitumor activity in this animal model was due to cytotoxic and apoptotic mechanisms, excluding major immunomodulatory effects which should be investigated in an allogenic tumor model.

Mistletoe extracts induce apoptosis by activating death pathways, possibly through activation of the caspase-8/FLICE independent death receptor signalling, breakdown of mitochondrial transmembrane potential activating caspase-3 cascade and by promoting and down regulating nuclear p53 and Bcl-2 protein and telomeric associations (6). Only intratumoral high-dosage mistletoe application results in such impressive tumor apoptosis or necrosis (9, 30). Considering previous work on a large panel of malignant cell lines (31) and the present results, the efficacy of intratumoral application is dose-dependent.

The presented data are promising and should stimulate further experimental and clinical studies on local intralesional treatment in an attempt to optimize cancer therapy.

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