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Human cancer cells exhibit *in vitro* individual receptiveness towards different Mistletoe extracts

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In vitro cytotoxic effects of three aqueous mistletoe extracts on cell physiology against different human tumor cell lines and primary cancer cells were investigated in order to compare the receptiveness of different cancer cells against different mistletoe products. Therefore cell proliferation (BrdU-incorporation assay), mitochondrial activity (MTT-testing) and necrotic cell toxicity (LDH assay) were assayed over serial dilutions of the test products. Data obtained with HELA-S3, MOLT-4, MFM-223, COR-L51, KPL-1 and VM-CUB1 tumor cell lines and Iscador®M (20 mg/ml), Iscador®Q (20 mg/ml) and Abnobaviscum®Fraxini −2 (20 mg/ml) indicated significant growth-inhibition of all cell lines, but also different cell susceptibilities against the different extracts. These variations were not only monitored on established cell lines but also on primary mamma carcinoma cells from surgical resectates. Concerning cell proliferation and mitochondrial activity Abnobaviscum®Fraxini exhibits stronger inhibitory effects compared to products from the Iscador® series. In case the evaluation was standardized on the active contents of VAA-I within the different products, the Iscador® extracts possess higher cytotoxic activity. Pure viscotoxins and mistletoe lectins exhibited less effects than the extracts. The simultaneous presence of pure mistletoe lectins and mistletoe polysaccharides diminished the VAA-mediated cytotoxic effects. The presence of fetal calf serum (FCS) in cultivation media during *in vitro* testing diminished the cytotoxic effects of mistletoe extracts. It was shown that *in vivo* application of mistletoe preparations led to the formation of antibodies against unknown compounds of the extracts, diminishing the cytotoxic effect.

1. Introduction

Within cancer treatment the adjuvant therapy with aqueous extracts of the european mistletoe (*Viscum album* L.) is included more and more into strategic, personalized therapy regimes. Especially the bifunctional mode of action by simultaneous immune activation by mistletoe polysaccharides (Berg and Stein 2002) and the cytotoxic effects by viscotoxins (leading to necrotic cell toxicity) and *Viscum album* agglutinins VAA (leading to apoptotic cell death) accumulate to a valuable combination (Büssing et al. 1996, 1999). Beside potential curative aspects especially the increased quality of life of cancer patients is clinically well documented (for review see Kienle et al. 2003). The immunmodulation is seen predominantly at low-dose therapy, while cytotoxic effects are monitored during higher dose regimes and/or application within areas around the tumor.

Within the classical therapy with mistletoe extracts different preparations can be found on the market, differing by the choice of the *Viscum album* host tree, the respective manufacturing procedure, the dilution factors etc. Therefore it may be obvious that these different extracts are slightly different concerning the spectrum of ingredients. Consequently, this can lead to different clinical effects, but can provide also the opportunity to select special extracts with unique properties for the special need of an individual patient. On the other hand cancer cells of different patients – even from the same organs – exhibit great individual variability, depending on the individuum, age and differentiation of tumor, etc. (Slonim et al. 2001). Therefore pharmacogenomic variability and differences between the mistletoe extracts will sum up to a multi-variate system. While pharmacogenomic preinvestigations prior to a classical chemotherapy ("anti-oncogramm") are getting more and more public (McLeod and Evans 2001) such investigations are not known for adjuvant therapies. In the present study we examined three different mistletoe extracts against a variety of tumor cell lines and primary tumor cells in order to clarify the question concerning different *in vitro* growth inhibitory effects versus the respective tumor cell type.

2. Investigations and results

2.1. Inhibition of proliferation of tumor cell lines

Various human tumor cell lines (HELA-S3 cervix carcinoma, MOLT-4 leukaemia, MFM-223 and KPL-1 mamma carcinoma, COR-L51 lung carcinoma and VM-CUB1

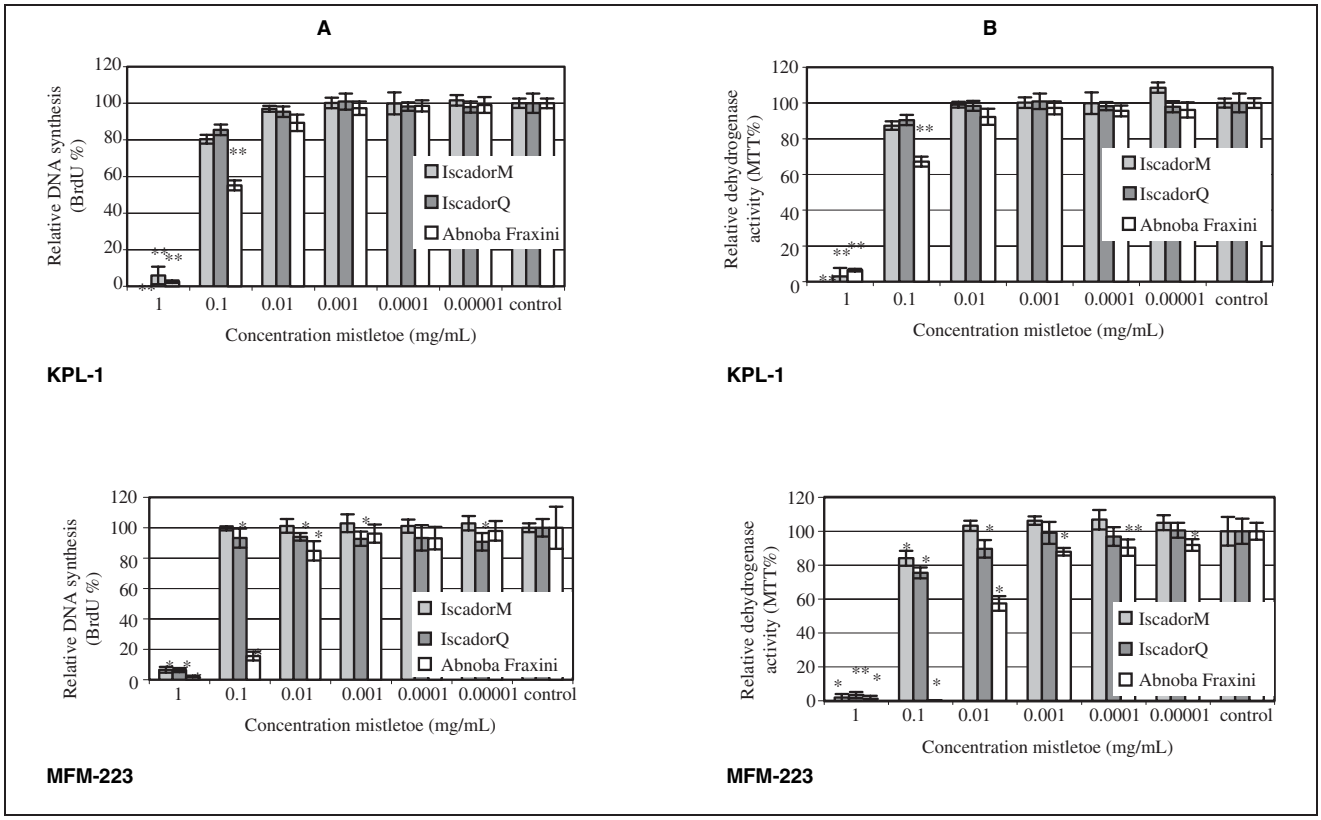

Fig. 1: Influence of three different mistletoe extracts in serial dilutions on proliferation rates (column A) and mitochondrial activity (column B) against mamma carcinoma cells KPL-1 and MFM-223. Incubation of cells for 48 h; determination of proliferation rates by BrdU-incorporation ELISA and mitochondrial cell activity by MTT test; %-values are correlated to the untreated control (100%); * $p < 0.05$, ** $p < 0.01$, $n = 6$

bladder carcinoma) were cultured in the presence or absence of serial dilutions of mistletoe extracts. Two principally different extract preparations were used: mistletoe from the Iscador® series is manufactured from fermented extracts, while the respective Abnobaviscum® preparations are obtained as a multi-vesicular, unfermented extract. Tumor cell growth was monitored by incorporation of bromo-deoxy-uridin (BrdU) into cellular DNA with subsequent quantification of labelled DNA by ELISA technique. Additionally, mitochondrial activity was determined by MTT test.

Principally all mistletoe extracts exhibited a strong, dose-depended *in vitro* cytotoxicity on all cell types tested. Obviously, this growth inhibition, as monitored during BrdU incorporation test and MTT test, was quantitatively different between the various cell types and the different mistletoe preparations.

Fig. 1 shows the exemplary differences of growth inhibitory effects of Iscador®Mali, Iscador®Quercus and Abnobaviscum Fraxini against two different tumor cell lines. While no great differences were visible for the three mistletoe extracts against the mamma carcinoma cell line KPL-1, strong growth inhibitory effects were induced by Abnobaviscum Fraxini against the mamma carcinoma MFM-223. The Iscador® extracts had less cytotoxic effects. The effects of Iscador®series were seen also between two different batches of test solutions, indicating no dependence on batch-variation (no similar tests performed on Abnobaviscum series). The anti-proliferative properties of the different mistletoe preparations were also reflected when monitoring the mitochondrial activity, which can directly be correlated with the cellular activity. Table 1 comprises the inhibitory effects of the three mistletoe extracts

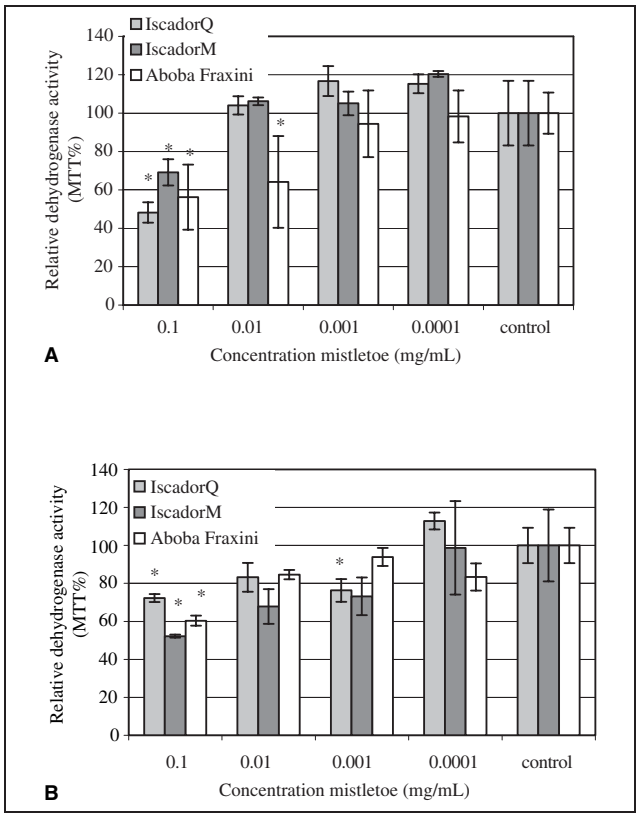

Fig. 2: Influence of three different mistletoe extracts in serial dilutions on mitochondrial activity of primary mamma carcinoma cells from two different patients. Incubation of cells for 48 h; determination of cell activity by MTT test; %-values are correlated to the untreated control (100%); * $p < 0.05$, $n = 3$

Table 1: Inhibition of proliferation (A) and mitochondrial activity (B) of different cancer cell lines under in vitro conditions by different mistletoe extracts

A Inhibition (%) of cell proliferation

Conc. (mg/ml)	Iscador® M				Iscador® Q				Abnobaviscum®Fraxini			
	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}
MFM-223	94 ± 2**	0 ± 1	−1 ± 5	−3 ± 6	−1 ± 4	94 ± 1**	7 ± 6*	6 ± 3*	7 ± 5*	7 ± 8*	98 ± 1**	84 ± 3**	15 ± 6*	4 ± 6	7 ± 7
MOLT-4	97 ± 1**	95 ± 2**	5 ± 12	−1 ± 10	0 ± 8	96 ± 7**	92 ± 14**	4 ± 7	4 ± 9	8 ± 9	100 ± 1**	100 ± 1**	100 ± 3**	71 ± 27	45 ± 34
COR-L51	93 ± 5**	83 ± 6**	31 ± 7**	64 ± 12*	39 ± 20	96 ± 5**	32 ± 27**	−8 ± 15	−25 ± 15	−20 ± 40	54 ± 3**	45 ± 10	2 ± 5**	−20 ± 9	−8 ± 13
KPL-1	98 ± 1**	54 ± 2**	30 ± 11**	18 ± 6**	15 ± 7**	98 ± 2**	66 ± 4**	18 ± 14**	20 ± 9**	12 ± 9**	91 ± 3**	60 ± 7**	21 ± 19**	25 ± 14**	12 ± 10
HELA	15 ± 10**	n.d.	78 ± 10	55 ± 8*	40 ± 4*	n.d.	n.d.	n.d.	n.d.	n.d.	30 ± 10**	30 ± 8**	58 ± 8**	41 ± 4	35 ± 7**
CUB-1	100 ± 2**	13 ± 3	1 ± 2	0 ± 3	0 ± 6	97 ± 5**	10 ± 3	2 ± 3	−1 ± 4	2 ± 2	94 ± 1**	33 ± 3**	8 ± 4	3 ± 4	4 ± 3

B Inhibition (%) of cellular mitochondrial activity

MFM-223	98 ± 2**	16 ± 4**	−3 ± 3	−6 ± 3	−7 ± 6	97 ± 2**	25 ± 3**	10 ± 5**	1 ± 6	3 ± 5	99 ± 2**	100 ± 1**	43 ± 4**	12 ± 2**	10 ± 5**
MOLT-4	100 ± 1**	100 ± 1**	9 ± 6**	0 ± 5	2 ± 2	100 ± 1**	100 ± 1**	6 ± 2**	10 ± 4**	5 ± 4	100 ± 1**	100 ± 1**	100 ± 1**	33 ± 5**	4 ± 7
COR-L51	95 ± 2**	67 ± 4**	27 ± 5**	6 ± 6	3 ± 4	100 ± 1**	68 ± 4**	32 ± 10**	14 ± 9**	8 ± 8*	69 ± 4**	49 ± 9**	31 ± 6**	−7 ± 19	−9 ± 19
KPL-1	98 ± 1**	−5 ± 9**	−4 ± 9	−4 ± 5	−5 ± 6	98 ± 2**	6 ± 4**	−2 ± 6	−1 ± 4	0 ± 6	56 ± 6**	−7 ± 23	−1010	−10 ± 6	−3 ± 13
HELA	87 ± 2**	18 ± 14**	2 ± 9	0 ± 3	5 ± 8	98 ± 2**	7 ± 6	11 ± 15	24 ± 18	25 ± 6	97 ± 4**	16 ± 9**	3 ± 5	0 ± 3	0 ± 5
CUB-1	95 ± 1**	11 ± 7	3 ± 3	0 ± 1	−3 ± 3	97 ± 5**	10 ± 3	2 ± 3	−1 ± 4	2 ± 2	97 ± 3**	66 ± 2**	25 ± 2	−14 ± 1	−6 ± 2

Cells were incubated for 48 h with the respective extracts at different dilutions (concentration mistletoe mg/ml), * $p < 0.05$, ** $p < 0.01$, n = 6

on proliferation and mitochondrial activity against six different tumor cell lines, indicating clear differences in receptiveness of different cells against the different extracts.

2.2. Inhibition of proliferation of primary tumor cells

Isolation and short-time cultivation of primary tumor cells from different human mamma carcinomas was performed either after trypsinisation or as explantates. Sufficient growth was observed only at high cell density and the cell proliferation significantly decreased after several passages. Therefore tests on cytotoxic activity of mistletoe extracts were performed after the first passage. Fig. 2 comprises exemplary data on inhibition of mitochondrial activity of two primary tumor cells (A: mamma carcinoma, histology: slightly differentiated, invasive, lobular, alveolar subtyp; B: mamma carcinoma, invasive, G2, pT2) by different mistletoe extracts. These data again indicate different inhibitory effects of the various mistletoe extracts.

2.3. Influence of VAA, visotoxins and mistletoe polysaccharides on tumor cell growth

In a previous report *in vitro* interaction of mistletoe polysaccharides with VAA I was described (Edlund et al. 2000). Consequently this could result theoretically in a decrease of cytotoxic functionality of mistletoe extracts which contain both, lectins as well as the respective rhamnogalacturonans. To answer the question if such an interaction has an influence on the cytotoxic potential pure mistletoe lectin (VAA I) and pure viscotoxin were tested concerning their cytotoxic effects against MOLT-4 cells. Additionally purified mistletoe polysaccharides (Edlund et al. 2000) were added to the VAA-resp. viscotoxin-containing media. As shown in Fig. 3 lectins caused extensive growth inhibition also in low concentrations (Fig. 3A), while viscotoxins were less active (Fig. 3B). These data are congruent with results published by Jansen et al. (1983). Cotreatment of cells with VAA resp. viscotoxin together with mistletoe polysaccharides resulted in a significant reduction of cytotoxicity of VAA, while the proliferation inhibitory effect of viscotoxins was not influenced. This means that polysaccharides within mistletoe preparations can diminish the VAA-mediated cytotoxic capacity of the respective preparations while the viscotoxin-induced toxicity is not influenced by exogenous polysaccharides.

2.4. Influence of serum additives on MOLT-4 cells

Because glycosylated compounds are capable to interact with mistletoe lectins it seemed interesting to investigate the influence of serum on cytotoxic effects of mistletoe extracts and VAA. This may be important because of two different reasons: on the one hand *in vitro* studies are mainly based on cells cultivated in media containing fetal calf sera (FCS). Because FCS is a crude mixture of mainly proteins and glycoproteins a potential interaction of glycosylated compounds with VAA may be possible. Secondly, a potential interaction of mistletoe agglutinins with human serum components may also occur under *in vivo* therapy when applying the respective mistletoe extracts by the usual parenteral route. For these reason MOLT-4 cells were cultivated with and without addition of serum in TurboDoma® medium and then incubated with different mistletoe extracts. Fig. 4 indicates that ser-

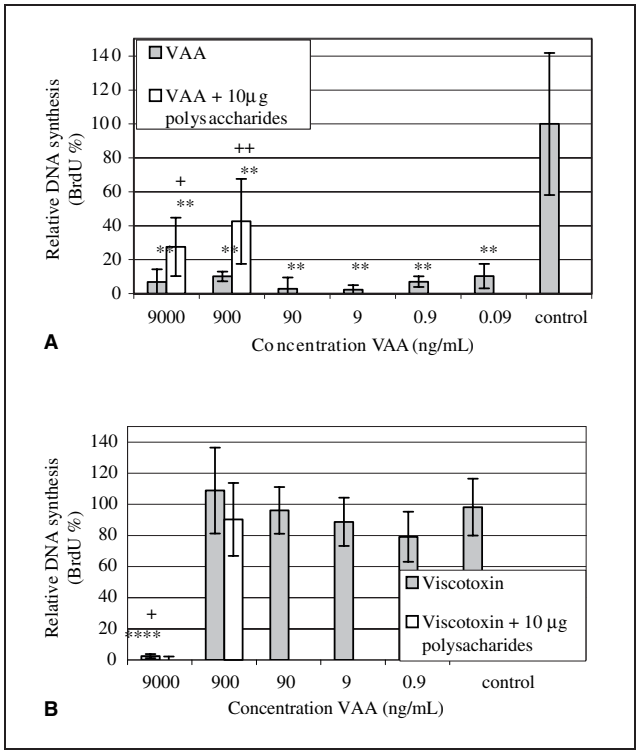

Fig. 3: Influence of *Viscum album* agglutinin VAA I (A) and viscotoxin (B) in serial dilutions on proliferation rates of serum-free cultured MOLT-4 cells. Within the test concentrations of 9000 and 900 ng/ml *Viscum album* polysaccharides were added at 10 µg/ml for testing a potential influence of interactions on cytotoxic capacity. Incubation of cells for 48 h; determination of proliferation rates by BrdU-incorporation ELISA; %-values are correlated to the untreated control (100%) * $p < 0.05$, ** $p < 0.01$ and to the respective non-polysaccharide treated groups $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $n = 6$

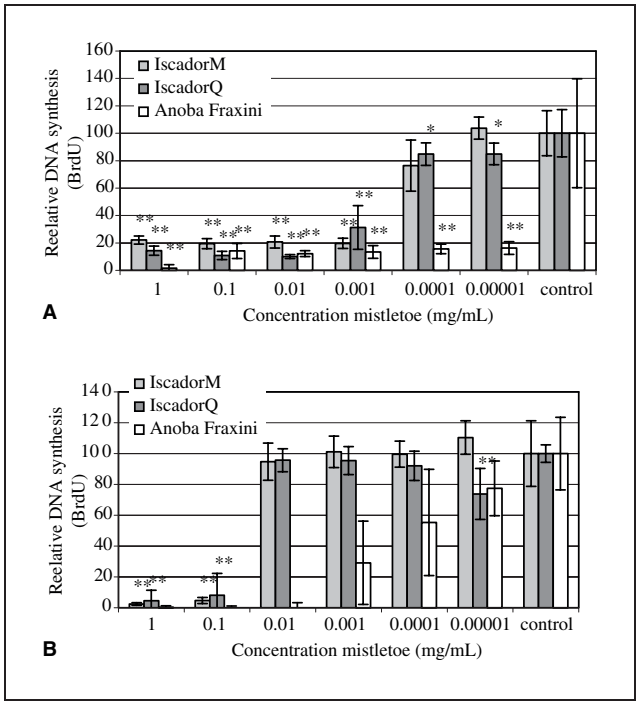

Fig. 4: Influence of different mistletoe extracts in serial dilutions on proliferation rates of MOLT-4 cells cultured under serum-free conditions (A) and by addition of 10% FCS (B). Incubation of cells for 48 h; determination of proliferation rates by BrdU-incorporation ELISA; %-values are correlated to the untreated control (100%); * $p < 0.05$, ** $p < 0.01$, $n = 6$

um free cultivated cells (Fig. 4A) were much more sensitive against mistletoe extracts than cells conditioned by FCS (Fig. 4B). Additionally it was obvious that the inhibition of extract toxicity by serum additives is quantitatively different between the three test preparations which means different extracts react quantitatively different against the respective serum components.

2.5. Serum antibodies against mistletoe reduce cytotoxic effects

Because mistletoe extracts contain high-molecular compounds and especially VAA – a group of immunologically recognizable proteins (Klein et al. 2002a and 2002b) – it seemed appropriate to investigate the influence of a human antibody containing serum on *in vitro* cell toxicity of mistletoe preparations. Therefore sera were used from a person previously undergoing a standard mistletoe therapy with Abnobaviscum Mali 5,10,20, Iscador®P and Abnobaviscum Fraxini 30 and from a control patient definitely having never been in contact with parenteral mistletoe. Initially both pooled sera were tested on presence resp. absence of mistletoe antibodies by dot-blots. Therefore nitrocellulose blot membranes were coated with mistletoe extracts and subsequently with the human sera. Using POD-labelled-anti-human-IgG-antibodies antibodies from the sera coupled to the dot membranes were detected. Serum of the mistletoe-treated patient showed the presence of antibodies against mistletoe. This antibody was not directed against VAA or viscotoxins as was shown in control experiments with immobilized pure compounds bound to nitrocellulose membranes. The respective control serum was negative.

To investigate if antibody-containing human serum can influence the cytotoxic *in vitro* activity of mistletoe extracts MOLT-4 cells were cultivated either with standard cell culture medium containing 10% FCS or under addition of the respective medium supplemented with human serum from the mistletoe-treated patient resp. human control serum. Fig. 5 indicates that the cell proliferation was diminished in case of antibody-containing serum. The inhibition of mitochondrial activity at mistletoe concentrations of 0.01 mg/ml was determined with 58% by incubation with antibody-containing serum. Cells incubated with FCS or the human control serum were much more inhibited at

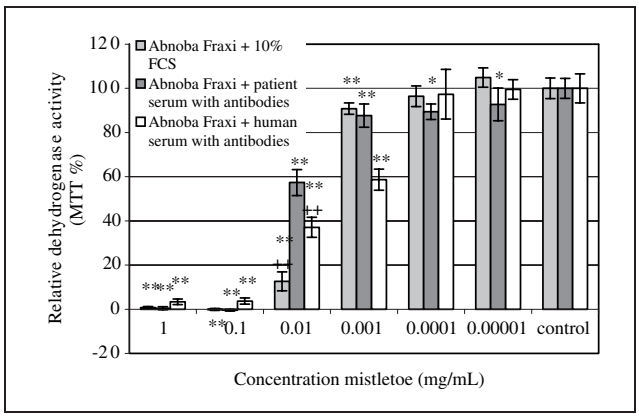

Fig. 5: Influence of mistletoe extract on mitochondrial activity (MTT test) of Molt-4 cells, cultivated with standard fetal calf serum (FCS) or adult human sera from a mistletoe-treated patient (containing anti-mistletoe antibodies) resp. a non-treated patient. * $p < 0.05$, ** $p < 0.01$ against untreated control, ++ $p < 0.01$ against sample cultivated with antibody-containing serum at concentration 0.01 mistletoe extract; $n = 6$

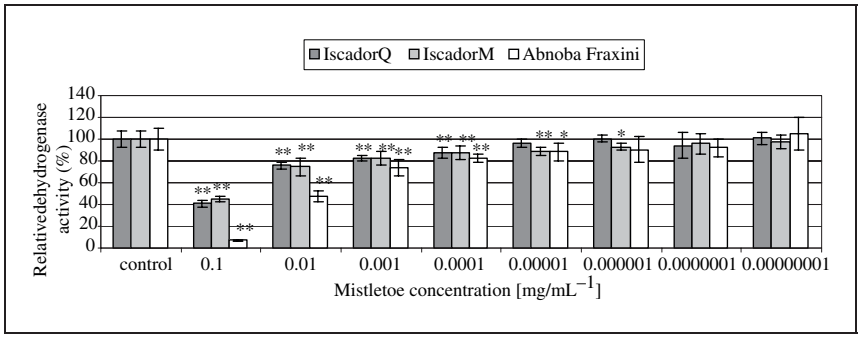

Fig. 6:
Influence of different mistletoe extracts in serial high dilutions on mitochondrial activity of MFM-223 cells. Potential stimulatory effects on cell activity are not detectable. Determination by MTT test; %-values are correlated to the untreated control (100%); * $p < 0.05$, ** $p < 0.01$, $n = 6$

this concentration range (98% resp. 88%). Therefore we can assume that mistletoe treatment will lead to antibody production which may cause an influence on cytotoxicity.

2.6. Mistletoe extracts do not exhibit stimulatory effects on cell growth

I*n vitro* studies are known in which mistletoe lectins in low doses (pg-level) are described to stimulate tumor cell proliferation (Gabius et al. 2001). These data could not been reproduced in further studies using fermented mistletoe extracts (Maier and Fiebig 2002).
In further experiments, three different *Viscum album* preparations were tested concerning such a potential growth promoting effect on MFM-223 mamma carcinoma cells and MOLT-4 cells by monitoring cell vitality by MTT-test. Fig. 6 shows exemplary data for MFM-223 cells, indicating no sign of cell stimulation even at low dose level. Similar data were obtained with MOLT-4 cells. In further studies cell density (6×10^4, 8×10^4, 1×10^4 cells/ml) and incubation time (24, 48, 72 h) was varied in order to get hints for a potential stimulation under variable conditions. At no point of any experiments significant and reproducible stimulatory effects were observed. For this reason we assume that the results obtained by previous studies cannot be reproduced.

3. Discussion

The present study indicates that therapeutically administered mistletoe extracts inhibit the *in vitro* proliferation of cancer cell lines of different origin as well as primary cancer cells. Nevertheless, this growth inhibition does not seem to be due to unspecific cytotoxic effects because of pronounced different sensitivities of different tumor cells towards the various extracts. This different receptiveness can be seen on the one hand between cancer cells originating from different organ origins but on the other hand it is also detectable between tumor cells derived from the same organ target (e.g. Fig.1 mamma carcinoma cells MFM-223 and KPL-1).
This different receptiveness of tumor cells is confronted with the different aqueous mistletoe extracts used successfully in adjuvant cancer therapy. Because of the different way of manufacture of the preparations it is obvious that the composition and therefore the clinical efficacy may be different between the respective market products. Considering the polysaccharide fraction to be responsible for the immunomodulation and the agglutinins and viscotoxins appropriate for cytotoxic effects, shifts between the efficacy of distinct preparations must occur when different extracts are used in therapy. This potential variation provides an unique opportunity to enlarge the therapeutical potential by an individual selection of respective preparations for a specific individual patient. In the present study only the cytotoxic properties of the mistletoe extracts were considered for reasons of simplification, no focus was laid on the immunomodulating action. When monitoring the cytotoxic data obtained from the different extracts, at equal mistletoe concentration a slightly higher cytotoxic tendency was detectable for the multi-vesicle Abnobaviscum preparations compared with the fermented Iscador series. Using two different batches of Iscador® no significant differences were observed, indicating batch-to-batch homogeneity of the preparations. In case the cytotoxic effects are not referred to the mistletoe concentration but to the respective VAA content, the Iscador® preparations are more effective compared to the Abnobaviscum extracts. Table 2 shows this discrepancy at the example of MFM-223 cells, where the evaluation "cytotoxicity versus mistletoe concentration" and "cytoxicity versus lectin concentration" are confronted, indicating that a comparison of cytotoxic properties of different extracts has to be seen very differentiated. It seems interesting that Abnobaviscum Fraxini exhibits cytotoxic effects even at very low concentrations which do not correlate with the respective VAA titers indicating that some other cytotoxic ingredients than VAA must additionally be present in this extract. On the other hand the anti-proliferative effects of extracts from the Iscador® series is dominated mainly by the content of VAA.
These results ultimately should lead to the assessment that the selection of a special mistletoe preparation for an individual patient should be done very carefully. It may be worth to discuss the possibility to evaluate the optimal

Table 2: Inhibition rates (% related to the untreated controls = 100%) of cell growth of MFM-223 cell line by serial dilutions of mistletoe extracts referred to the respective mistletoe concentration and to the respective lectin (VAA) content

VAA (ng/ml)	600	60	6	0.6	0.06
Mistletoe extract					
Iscador®M	100	90	85	87	87
Iscador®Q	100	85	87	88	88
Abnobaviscum Fraxini	96	98	92	46	10

Mistletoe content (mg/ml)	1	0.1	0.01	0.001	0.0001
Mistletoe extract					
Iscador®M	99	16	0	0	0
Iscador®Q	98	22	3	0	0
Abnobaviscum Fraxini	97	98	96	50	18

Determination of proliferation rates by BrdU-incorporation assay

extract for an individual patient by a quick test of cytotoxic power of several *Viscum* preparations against tumor cells obtained from chirurgical cancer resection; the final selection should be based on these data. We have shown in our investigations that such a procedure is possible in practice: after surgical resection of tumor material a small piece of this tissue is used for the isolation of tumor cells; the cells are to be propagated and after one or two passages the testing of potential preparations is performed by the quite efficient MTT test which allows a fast and cost-saving statement. Such an individualized selection of cancer therapeutics may also be done within the same "oncogramm" for different classical chemotherapeutics in order to achieve a rational therapeutic regime considering the relevant pharmacogenomic properties of the patient.

Nevertheless it has to be kept in mind that the cytotoxic capacity of mistletoe extracts is only one mechanistic aspect of mistletoe efficacy and also the immunmodulating aspects have to be assessed within such an individualized therapy.

It is interesting that no stimulation of tumor cell proliferation capacity was detected within the studies, as it was claimed by Gabius et al. (2000) in earlier presentations. Within the broad screening of extracts against a variety of tumor cells, sometimes at low concentrations proliferation rates and mitochondrial activities were seen at a level higher than the control. But in all cases a clear reproducibility in further independent experiment was not possible. Therefore we assume that "stimulation effects" can be deduced more by a statistical fact – we are quite sure that an increase in test number will eliminate these deviations so that potential stimulatory activities will not be on a significant level. Our data for this problem are in accordance with previous investigations of Maier and Fiebig (2001).

Other aspects of mistletoe therapy in our studies lead to interesting results concerning diminishing effects on the cytostatic properties of the mistletoe preparations tested: it was shown that mistletoe polysaccharides are capable to interact not only under *in vitro* conditions (Edlund et al. 2000) with VAA but that this interaction also leads to a decreased cytotoxicity. This interaction with glycosylated compounds was expanded to serum ingredients: the cytotoxic mistletoe effects were clearly diminished by FCS-addition to the culture media, while the viscotoxin-mediated effects were not affected. From these data we can speculate that parenteral application of mistletoe preparations could lead to an immediate complexation of agglutinins. If these complexes can dissociate under *in vivo* conditions the formation of sustained-actives has to be investigated in further studies. In this context it has to be discussed if preparations containing only purified mistletoe agglutinin would not have less effects in therapy because of a potential quick complexation of the active ingredient after parenteral application.

During mistletoe therapy also a potential antibody production has to be considered (Kaiser et al. 2002). In our preliminary study we observed IgG-antibodies against mistletoe, which were not directed against VAA or viscotoxins. These antibodies were shown to be capable to diminish the cytotoxic properties of the extracts. From this point of view interval treatments with mistletoe preparations or alternating the different extracts as it is done in clinically practice would make a rational sense.

Summarizing quite a strong cytotoxicity can be assigned to the mistletoe extracts used in this study, whereby it has to be kept in mind that the different products can operate in different manners and to different extents concerning their cytotoxic power. Therefore an individualized therapy may be a future goal in order to minimize the influences of pharmacogenomic differences between tumours and patients.

4. Experimental

4.1. Material

HELA-S3 (cervix carcinoma cells), MOLT-4 (leukaemia cells), MFM-223 (mamma carcinoma cells), COR-L51 (lung carcinoma cells), KPL-1 (mamma carcinoma cells) and VM-CUB1 (bladder carcinoma cells) were obtained from German Collection of Microorganisms and Cell Cultures DSMZ (Braunschweig, Germany). Chemicals and reagents were obtained from Fluka (Switzerland) if not stated otherwise in p.a. quality. Iscador®M (Batches 64538, 009/1078), Iscador®Q (Batches 51627, 009/1067) were gifts from Weleda AG, (Arlesheim, Switzerland), Abnobaviscum®Fraxini-2 (Batch 111IMP) and Abnobaviscum Pini-2 (Batch 102ACI) were from Abnoba GmbH (Niefern/Öschelbronn, Germany). VAA (8000 ng/g in ascorbate/phosphate buffer) and viscotoxins (from Fraxini-2) were obtained from Abnoba GmbH. Mistletoe polysaccharides were isolated as described by Edlund et al. (2000).

4.2. Cell culture

Sterilisation of heat-sensitive solutions and media was done by filtration (cellulose nitrate, Protraban BA79, 0.22 μm, Schleicher & Schüll). Incubation of cell cultures was performed at 37 °C in a water-saturated, 5% CO_2 atmosphere in DMEM/Hams F12 medium (AMIMED), DMEM High Glucose medium (AMIMED) and TurboDorma® (Cell Culture TechnologiesGmBH, Switzerland) under addition of 10% heat-inactivated FCS (Sigma, Switzerland) and 1 mM glutamine (AMIMED).

Molt-4, CUB-51 and MFM-223 cells were cultivated in TurboDorma, KPL-1 in DMEM/Hams F12 medium, HeLa in HamsF-12, primary mamma carcinoma cells in TurboDorma® under addition of antibiotics.

Standard long time cultivation of cells was performed in T25 flasks (Orange Scientific) and propagation of cells prior to experiments in T75 ml flasks. Adherent cells were detached from culture flask surface by a 3 to 5 min incubation at 37 °C with a Trypsin-EDTA solution in PBS. Cell tests were performed in 96 well plates, flat-bottomed (Orange Scientific), using cell densities of 6.0×10^5 cells/ml. 100 μl of this cell suspension was transferred into each well and 24 h after this incubation 100 μl of the respective test samples were added. This point was determined prior to the experiments to be within the log-phase.

Test samples were either pure medium as negative control or mistletoe preparations in serial dilutions. The latter were prepared from a stock solution with mistletoe concentrations according batch analysis of the manufacturer of 2 mg/ml. This stock solution was diluted with the respective media to 0.2, 0.02, 0.002, 0.0002 and 0.00002 mg/ml. Final concentration in the test well: 1, 0.01, 0.001, 0.0001, 0.00001, 0.000001 mg/ml. Incubation of cells with the test substances was performed for 2 days.

4.3. Isolation of primary tumor cells

Tumor tissue from fresh surgical resection of mamma carcinomas was obtained from Paracelsus Spital, Richterswil. The ethical committee approved the study. Written informed consent was obtained by the patients. Tumor material was stored until start of the experiment in PBS under addition of antibiotics (penicillin, streptomycin, Fungizol®). After removal of necrotic parts the chopped tumor parts were washed twice with PBS and the pieces were incubated after trypsination (15 to 30 min under stirring) explantated in T25 flasks under addition of 100 U/ml antibiotics. Cultivation over 2 to 5 passages in 6 well plates.

4.4. Investigations on antibodies in human sera

Human serum was obtained from two different human subjects, one treated several months ago with mistletoe extracts, the other one was definitely not in contact with mistletoe before. The respective sera were added at 10% to the respective cell culture systems. For antibody testing a nitrocellulose membrane Protraban BA79 (Schleicher & Schüll Switzerland) was coated with Mistletoe extract, VAA or viscotoxins, blocked with albumin/milk powder and washed 3 times. The coated membrane was incubated for 12 h with the diluted (1 : 10) human sera. After washing the membrane was incubated with a peroxidase-labelled mouse-anti-human-IgG antibody, γ-chain specific (Sigma, Switzerland) for 2 h, washed and detected with Western blotting Detection Reagents (BioRad, Switzerland) by dot-blot technique.

4.5. Analytical evaluation

The proliferation rate of tumor cells was determined by BrdU-incorporation assay, using the methodology described by Porstman et al. (1985), adapted by Deters et al. (2003). The mitochondrial activity was quantified by the MTT-test via succinatdehydrogenase assay (Moosmann 1983). Ne-

crotic cell toxicity was determined by extracellular lactate dehydrogenase assay, using the Cytotoxicity Detection Kit LDH (Sigma, Steinheim). Evaluation on significance was performed by Students-Tests, unpaired, after prove that data were normal distributed (S-Plus®).

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