Absence of Tumor Growth Stimulation in a Panel of 26 Human Tumor Cell Lines by Mistletoe (*Viscum album* L.) Extracts Iscador in vitro

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Summary

Mistletoe (*Viscum album* L.) extracts exhibit antitumor activity based on direct inhibition of tumor growth as well as modulation of immune response. Recent reports suggested potential stimulation of tumor growth at low doses of mistletoe extracts, particularly in hematological tumors and tumors responding to immunotherapy. Therefore, the direct effect of the three mistletoe extracts Iscador® M Spezial, Iscador Qu Spezial and Iscador P on tumor growth was investigated in a panel of 26 human tumor cell lines in vitro using cellular proliferation assays. Antitumor activity of the three preparations at high concentrations was investigated in a panel of 12 cell lines.

The results showed no evidence of stimulation of tumor growth by any of the three extracts, in particular the five tumor cell lines previously reported to be sensitive to direct mistletoe lectin stimulation. On the contrary, the lectin containing preparations Iscador M Spezial and Iscador Qu Spezial expressed a pronounced antitumor activity exhibiting a nearly identical antitumor profile compared to isolated mistletoe lectin 1.

Zusammenfassung

Ausschluss einer Tumorstimulation durch Mistel (*Viscum album* L.)-Extrakte Iscador in vitro in einem Panel von 26 humanen Tumorzelllinien

Die antitumorale Wirksamkeit von Mistel (*Viscum album* L.)-Extrakten beruht sowohl auf einer direkten Inhibition des Tumorwachstums als auch auf einer Modulation von immunologischen Reaktionen. In jüngster Zeit wurde allerdings auf das Risiko einer Tumorstimulation durch Mistelextrakte in geringen Konzentrationen, vor allem bei hämatologischen Tumoren sowie Tumoren, die auf eine Immuntherapie ansprechen, hingewiesen. Um den direkten Einfluss von Mistelextrakten auf das Tumorwachstum zu prüfen, wurden die drei Iscador®-Präparate Iscador M Spezial, Iscador Qu Spezial und Iscador P auf ihre wachstums-stimulierenden Eigenschaften an 26 humanen Zelllinien in vitro im Niedrigdosisbereich mittels zellulärer Proliferations-Assays untersucht. Ebenso wurde die antitumorale Wirksamkeit dieser drei Präparate in hohen Konzentrationen an einem Panel von 12 Zelllinien getestet.

Key words

- **Iscador®**
- **Mistletoe extracts, antitumor activity, human tumor cell lines, stimulation of tumor growth**
- ***Viscum album* L.**

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Die Ergebnisse erbrachten keinerlei Hinweise auf eine Stimulation des Tumorwachstums durch die drei Iscador-Präparate, insbesondere auch nicht in den 5 Tumorzelllinien, die als durch Mistellektin 1 stimulierbar beschrieben worden waren. Allerdings zeigten die Lektin-haltigen Präparate Iscador M Spezial und Iscador Qu Spezial eine ausgeprägte antitumorale Wirksamkeit mit nahezu identischen Wirkprofilen wie isoliertes Mistellektin 1.

1. Introduction

Mistletoe (*Viscum album* L.) extracts found a broad application in the adjuvant therapy of malignant tumors mainly in German speaking countries in Europe [1, 2, 3]. A number of preparations are being used clinically which differ with respect to origin of different host trees, harvest time point and extraction procedures, and they demonstrated different pharmacological effects [4]. These extracts are characterized with respect to the main components containing mainly mistletoe lectins, viscotoxins and alcaloids. One of the oldest preparations of mistletoe is Iscador®.

The antitumoral properties of the extracts is mainly founded on mistletoe lectins acting at high concentrations by a direct cytotoxic inhibition of the tumor growth. At low dosages mistletoe lectins modulate a number of immunological effector cells. The cell death of tumor cells is a result of a dose dependent apoptosis or necrosis [5–8]. In transplantable murine tumor models antitumor efficacy could be demonstrated in vivo [9]. At low dosages mistletoe lectin stimulated immunologically relevant effector cells such as macrophages, natural killer cells, and B- and T-lymphocytes [10, 11, 12], which is associated with the release of cytokines, mainly Interleukin-6, Tumor necrosis factor-α and interferon-γ [13, 14, 15]. In recent years it has been reported that mistletoe lectins can eventually stimulate the tumor growth. This could be the result of a direct stimulation of the tumor cells or of the release of the cytokines. Hematological malignancies and solid tumors which respond to an immune therapy like melanomas and renal cell carcinomas could present the highest risk of a tumor stimulation [16, 17]. In vitro a slight stimulation of the tumor growth has been reported in permanent cell lines being derived from soft tissue sarcomas and melanomas by isolated ML-1 (mistletoe lectin 1) [18].

In the present investigation we addressed the question of a potential tumor stimulation and investigated the effect of three aqueous mistletoe extracts (Iscador M Spezial, Iscador Qu Spezial and Iscador P) on the tumor growth in a panel of 26 permanent human tumor cell lines. The test models included cell lines of hematological origin, derived from kidney cancers and melanomas as well as the five cell lines which were stimulated by mistletoe lectin ML-1 at low doses according to Gabius et al. [18]. In addition, the antitumor efficacy of the three preparations was investigated at high dose levels in a panel of 12 human tumor cell lines.

2. Materials and methods

2.1. Cell lines

The cell lines and their origin are shown in Table 1. Ten cell lines were derived from established human tumor xenografts [19]. Eleven cell lines were obtained from the US National Cancer Institute (Bethesda, MD, USA). Five cell lines were obtained from ATCC (Rockville, MD, USA). Cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2 in RPMI 1640 tissue culture medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal calf serum (Sigma, Deisenhofen, Germany) and 0.1 mg/ml gentamicin (Invitrogen).

Table 1: Origin of the 26 tumor cell lines.

Type	Cell Line	Origin	Type	Cell Line	Origin
CNS[1)]	SF268	NCI[2)]	Melanoma	HT144	ATCC[4)]
Gastric	GXF 251L	Xenograft, FR[3)]		MALME-3M	NCI
Lung	H460	NCI		SK-MEL28	ATCC
	LXFA 629L	Xenograft, FR		MEXF 462NL	Xenograft, FR
	LXFE 66NL	Xenograft, FR		MEXF 514L	Xenograft, FR
	LXFL 529L	Xenograft, FR	Prostate	PC3M	NCI
Leukemia and lymphoma	CCRFCEM	NCI	Renal	RXF 393NL	Xenograft, FR
	MOLT-4	NCI		RXF 944L	Xenograft, FR
	HL-60	NCI	Sarcoma	Hs729	ATCC
	K562	NCI		SK-LMS-1	ATCC
	U937	NCI		SK-UT-1B	ATCC
	RPMI 8226	NCI	Uterus	UXF 1138L	Xenograft, FR
Mammary	MCF7	NCI			
	MAXF 401NL	Xenograft, FR			

[1)] CNS: Central nervous system.
[2)] National Cancer Institute, Bethesda, MD, USA.
[3)] Cell line established in Freiburg from a human tumor xenograft; Roth et al. [19].
[4)] ATCC: American Type Culture Collection, Rockville, MD, USA.

2.2. Cell proliferation assay

A modified propidium iodide (PI) assay [20] was used to assess the effects of Iscador preparations on the growth of the human tumor cell lines derived from solid tumors (adherent growing cells) and a sulforhodamine B (SRB) assay [21] for the hematological tumor lines (suspension cultures).

Tumor cells were harvested from exponential phase cultures by trypsination (monolayer cultures) or centrifugation (suspension cultures), counted and plated in 96-well flat-bottom micrometer plates at a cell density dependent on the cell line (5,000–12,000 viable cells/well). After 24 h recovery to allow the cells to resume exponential growth, 10 µl of culture medium (six control wells per plate) or culture medium containing Iscador extracts were added to the wells. Each concentration was plated in triplicate. Iscador preparations were applied at five concentrations ranging from 0.0015 to 15 µg plant extract/ml (Iscador M Spezial and Qu Spezial) and 0.003 to 30 µg plant extract/ml (Iscador P), respectively, followed by 4 days of continuous drug exposure. For the investigations of antitumor efficacy in the 12 cell line panel dose levels of Iscador preparations were increased up to 300 µg plant extract/ml.

For the PI assay, cell culture medium with or without drug was replaced by 200 µl of an aqueous PI solution (7 µg/ml). Since PI only passes leaky or lysed cell membranes, DNA of dead cells can be stained and measured, while living cells will not be stained. To measure the proportion of living cells, cells were permeabilized by freezing the plates, resulting in death of all cells. After thawing of the plates fluorescence was measured using a Cytofluor 4000 microplate reader (excitation 530 nm, emission 620 nm), giving a direct relationship to the total cell number.

For the SRB assay, the cell suspensions were centrifuged to the bottom of the micrometer plate at 1000 g for 5 min after 4 days of continuous drug exposure. Cells were fixed with 50 % trichloroacetic acid, kept at 4 °C for 1 h and washed 5 times with tap water. Cells were stained by addition of 100 µl of an aqueous SRB solution (0.4 %) containing 1 % acidic acid. Micrometer plates were then kept at room temperature for 30 min and washed 4 times with 1 % acidic acid. The plates were air-dried overnight and the protein-bound stain solubilized in 200 µl of 10 mmol Tris pH 10.4 while shaking until the precipitates were fully dissolved. The purple color was quantified using a microplate reader (490 nm Dynatech MR 5000, DPC Biermann GmbH, Bad Nauheim, Germany).

Both assays included untreated and positive controls (doxorubicin).

Growth inhibition/stimulation was expressed as treated/control × 100 (T/C %) in the PI and SRB assays. Antitumor activity was defined as inhibition of tumor growth to less than 30 % compared with the medium-treated control cells (T/C < 30 %), and slight antitumor activity as T/C 30–70 %. Tumor growth stimulation was defined as tumor cell proliferation of more than 120 % (T/C > 120 %). Experiments were performed three times and T/C values are shown as mean of 3 experiments.

Antitumor activity and tumor selectivity were displayed as the IC_{70}-mean graph presentations. These show the distribution of the individual IC_{70} values of a compound in each cell line relative to the mean IC_{70} value for that compound as determined from the median T/C values obtained from the 3 experiments per cell line. Deviations of individual IC_{70} values from the mean IC_{70} value are expressed as bars on a logarithmically scaled axis. Bars to the left represent IC_{70} values lower than the mean IC_{70} (sensitive cell lines), bars to the right show higher individual IC_{70} values (resistant cell lines).

2.3. Mistletoe extracts

Iscador M Spezial, Iscador Qu Spezial and Iscador P were provided by Weleda AG (Schwäbisch Gmünd, Germany). The ampoules contained 1 ml of an aqueous extract of 5 mg plant material from *Viscum album* (Malus) with 250 ng/ml total mistletoe lectin, 5 mg plant material of *Viscum album* (Quercus) with 375 ng/ml total mistletoe lectin and 10 mg plant material from *Viscum album* (Pinus). Doxorubicin (adriamycin) was purchased from Medac (Hamburg, Germany). Isolated ML-1 was kindly provided from the "Institut für Phytochemie", private University Witten/Herdecke (Witten, Germany).

3. Results

In order to investigate the potential growth stimulating properties the effect of the three Iscador preparations was investigated in 26 permanent human tumor cell lines *in vitro* in a proliferation inhibition assay at low concentrations.

The results are summarized in Fig. 1. Each diagram displays the dose-response curves of all 26 cell lines tested for one of the three Iscador preparations. There is no evidence of a tumor stimulation in all three preparations. In no case the test/control value exceeded 120 % which means an increase of tumor growth of more than 20 % compared to the untreated control cells. Interestingly, individual cell lines were inhibited in growth. Iscador M Spezial and Iscador Qu Spezial effected at the concentration of 15 µg/ml total plant extract a marked inhibition of the breast cancer cell line MAXF 401NL.

In a subsequent study the tumor inhibiting properties of the three preparations were tested at higher concentrations in a panel of 12 cell lines. Therefore, Iscador M Spezial and Iscador Qu Spezial were tested at concentrations ranging from 1.5–150 µg/ml total plant extract and Iscador P in concentrations ranging from 3–300 µg/ml total plant extract in half-log increments. Iscador M Spezial and Iscador Qu Spezial effected a marked antitumor efficacy in vitro with a mean IC_{50} value of 30 µg/ml (Table 2). Iscador P, containing only marginal amounts of mistletoe lectins showed no antitumor activity. As apparent from the IC_{70} profile of Iscador M Spezial (Fig. 2A; see p. 439), Iscador M effected a selective antitumor activity (individual IC_{70} value of a cell line < ½ of the mean IC_{70} value of all 12 cell lines) in 3/12 cell lines: the breast line MAXF 401NL, the kidney line RXF 944L and the uterus line UXF 1138L. The activ-

Table 2: Antitumor efficacy of Iscador extracts expressed as mean IC (inhibitory concentrations) values in 12 cell lines.

Mistletoe extract/ standard agent	IC_{50} (µg/ml)	IC_{70} (µg/ml)	IC_{90} (µg/ml)
Iscador M Spezial	30.8	48.4	104
Iscador Qu Spezial	30.1	46.7	135
Iscador P	> 300	> 300	> 300
Doxorubicin	0.05	0.16	2.2

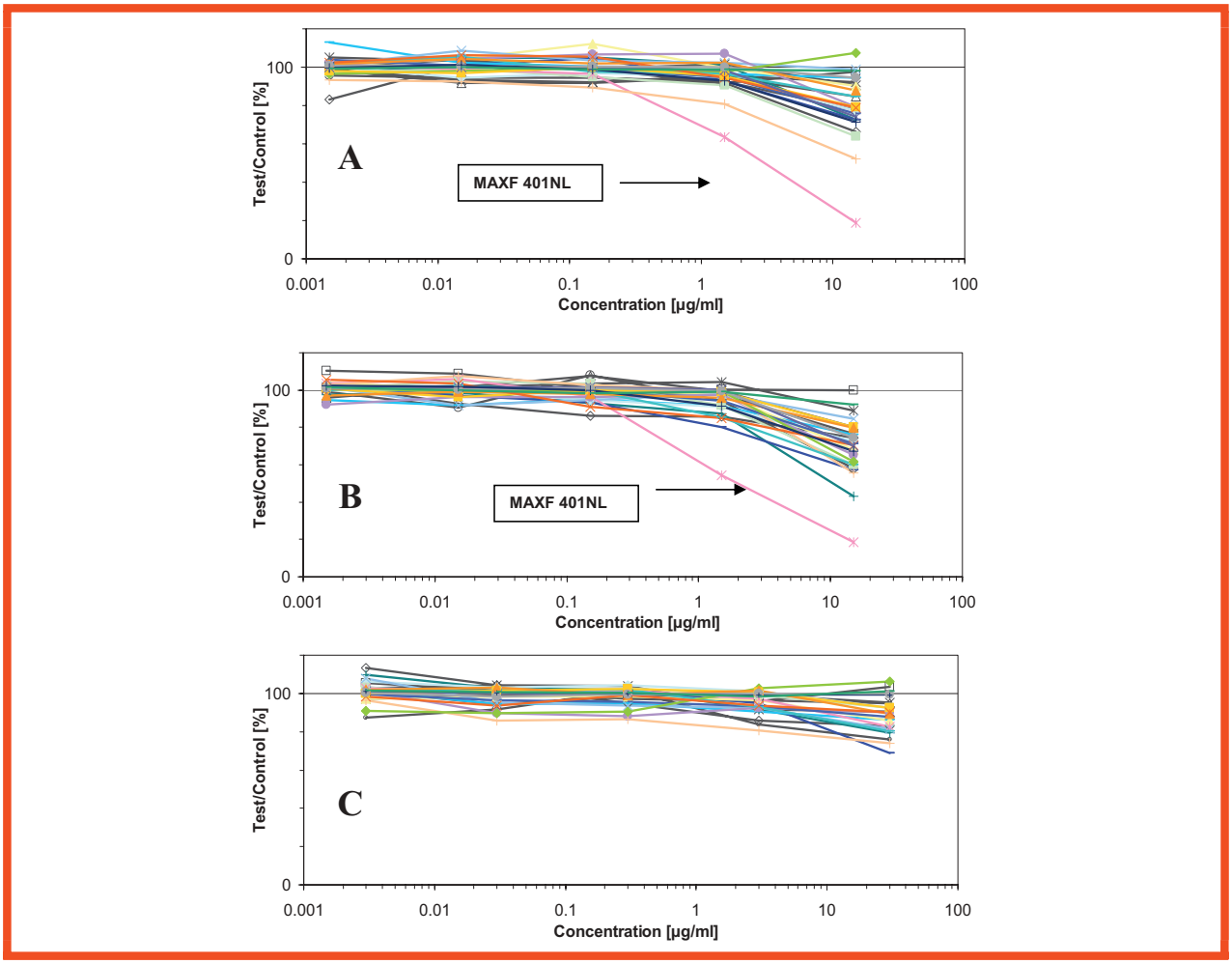

Fig. 1: Effect of Iscador M Spezial (A), Iscador Qu Spezial (B) and Iscador (P) on the growth of 26 human tumor cell lines in vitro. The cells were incubated for 96 h with the extracts. The growth was determined after incubation with propidium iodide or the SRB-assay (hematological cell lines). The test/control value for each test concentration is displayed. The mean of three independent experiments is shown. The arrow marks the dose-response curve of the most sensitive cell line MAXF 4001NL.

ity profile of Iscador Qu is almost identical (data not shown). Importantly, the IC_{70} profile of Iscador M and Iscador Qu were nearly identical to the profile of pure ML-1 (Fig. 2B) suggesting a similar mechanism of action and/or similar active ingredients.

4. Discussion

Mistletoe extracts from leafs and branches of the European mistletoe effect antitumor activity via cytotoxic and immunological mechanisms. In recent years two reports provoked a controversial discussion about potential tumor stimulating properties due to direct effect on the tumor cells [18] and via indirect immune modulation of the mistletoe [17]. In order to investigate a direct tumor stimulation we studied the effect of the three standardized Iscador preparations Iscador M Spezial, Iscador Qu Spezial and Iscador P in a panel of 26 human tumor cell lines in vitro. Cell lines derived from hematological malignancies as well as cell lines, which by Gabius et al. [18] had been described as being stimulated, were included. Our results clearly demonstrate that no cell lines could be stimulated by one of the three mistletoe extracts, especially in low concentrations. Gabius et al. [18] had previously reported tumor stimulating activity. In the present study, no evidence of tumor stimulation with these five cell lines has been found.

Overall, we could not confirm the stimulation of tumor growth as described by Gabius et al. [18]. Gabius observed a marginal tumor stimulation of 10 % to 20 % compared to untreated cells at the concentration of 50 pg/ml galactose-binding ML-1. In 4 out of the 5 described cell lines, this stimulation was observed at only one incubation period. In our study Iscador M was investigated at concentration ranging from 1.5 ng/ml up to 15 µg/ml total plant extract. This corresponds to a total lectin content of 0.075–750 pg/ml. The Iscador Qu Spezial preparation showed a concentration of total ML between 0.11 and 1100 pg/ml. Especially the low dose range of ML was covered in our study.

A. Iscador M Spezial

TUMOR/ PASSAGE NO.	EXP. CTRL FLUOR. UNITS	Distribution of MIC7 related to Mean log.scaled axis *0.01 *0.1 Mean 48.356 *10 *100	IC50 ug/ml	IC70 ug/ml	IC90 ug/ml
Gastric					
GXF 251L (3)	2305		81.351	195.173	507.352
Lung					
H460 * (3)	3623		49.401	69.684	98.294
LXFA 629L (3)	2757		136.407	219.929	354.590
LXFL 529L (3)	2836		18.410	27.655	93.162
Mammary					
MCF7 * (3)	3230		185.842	246.311	326.454
MAXF 401NL(3)	2705		<3.000	<3.000	3.000
Melanoma					
MEXF 462NL(3)	3131		21.576	36.209	76.845
MEXF 514L (3)	3364		69.042	116.993	256.425
Prostate					
PC3M * (3)	3037		19.199	27.063	173.205
Renal					
RXF 486L* (3)	2606		69.831	112.983	254.939
RXF 944L* (3)	3095		9.369	17.320	42.317
Uterus					
UXF 1138L (3)	3550		6.854	11.921	28.710
Mean	n=12	48.356	30.775	48.356	104.549

B. Mistletoe lectin 1

TUMOR/ PASSAGE NO.	EXP. CTRL FLUOR. UNITS	Distribution of MIC7 related to Mean log.scaled axis *0.01 *0.1 Mean 0.004 *10 *100	IC50 ug/ml	IC70 ug/ml	IC90 ug/ml
Gastric					
GXF 251L* (3)	2456		0.062	0.129	0.268
Lung					
H460 * (3)	4281		0.004	0.008	0.050
LXFA 629L (2)	3249		0.008	0.033	0.143
LXFL 529L (3)	3883		0.299E-3	0.636E-3	0.100
Mammary					
MCF7 * (4)	3550		0.003	0.006	0.100
MAXF 401NL(3)	2140		0.353E-4	0.114E-3	0.001
Melanoma					
MEXF 462NL(3)	3346		0.003	0.005	0.011
MEXF 514L (3)	2210		0.070	0.109	0.168
Prostate					
PC3M * (3)	4298		0.001	0.003	0.010
Renal					
RXF 486L* (2)	3070		0.009	0.050	0.278
RXF 944L* (2)	2878		0.165E-3	0.571E-3	0.100
Uterus					
UXF 1138L (3)	3094		0.161E-4	0.788E-4	>0.100
Mean	n=12	0.004	0.001	0.004	0.058

Fig. 2: Antitumor efficacy of Iscador M Spezial (A) and mistletoe lectin 1 (B) in 12 human tumor cell lines in vitro. The cells were incubated for 96 h with test compounds. The growth of the cells was determined with the propidium iodide assay. The effective concentrations were calculated based on median test/control values of three independent experiments per cell line.

On the contrary the mistletoe preparations with a high ML content effected an antitumoral activity at high concentrations. The activity profiles of Iscador M Spezial and Iscador Qu Spezial were identical and both have a remarkable similarity with the profile of isolated ML-1. This suggests that Iscador M and Iscador Qu Spezial contain the same active components, ML-1 seems to be the most important one.

These investigations demonstrate that there are no hints of a tumor stimulation by the standardized mistletoe extracts Iscador M Spezial, Iscador Qu Spezial and Iscador P in vitro in a panel of 26 human tumor cell lines. The preparations with a high lectin content (Iscador M Spezial and Iscador Qu Spezial) showed at high test concentrations an antitumor activity in vitro.

5. Literature

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