Anti-Cancer Drugs 2002, **13,** pp. 373–379

Preclinical report

Absence of tumor growth stimulation in a panel of 16 human tumor cell lines by mistletoe extracts *in vitro*

Gerhard Maier[1] and Heinz-Herbert Fiebig[1]
[1]Institute of Experimental Oncology, Oncotest GmbH, 79108 Freiburg, Germany.

Extracts of *Viscum album* (mistletoe) are widely used as complementary cancer therapies in Europe. The mistletoe lectins have been identified as the main active principle of mistletoe extracts. They have been shown to exhibit cytotoxic effects as well as immunomodulatory activities. The latter is exemplified by induction of cytokine secretion and increased activity of natural killer cells. Recent reports, however, indicated possible tumor growth stimulation by mistletoe extracts. Therefore, the three aqueous mistletoe extracts (Iscador M special, Iscador Qu special and Iscador P) were evaluated for antiproliferative and/or stimulatory effects in a panel of 16 human tumor cell lines *in vitro* using a cellular proliferation assay. The results show no evidence of stimulation of tumor growth by any of the three Iscador preparations, comprising central nervous system, gastric, non-small cell lung, mammary, prostate, renal and uterine cancer cell lines, as well as cell lines from hematological malignancies and melanomas. On the contrary, Iscador preparations containing a high lectin concentration (Iscador M special and Iscador Qu special) showed antitumor activity in the mammary cancer cell line MAXF 401NL at the 15 μg/ml dose level with a more than 70% growth inhibition compared to untreated control cells. In addition, a slight antitumor activity (growth inhibition 30–70%) was found in three tumor cell lines for Iscador M special and in seven tumor cell lines for Iscador Qu special, respectively. Iscador P, which contains no mistletoe lectin I, showed no antiproliferative activity. [© 2002 Lippincott Williams & Wilkins.]

Key words: Antiproliferative activity, human tumor cell lines, mistletoe extracts, stimulation of tumor growth.

Introduction

Aqueous extracts of the European mistletoe (*Viscum album* L.) have been widely used for decades as alternative treatment and adjuvant cancer therapy, particularly in Germany, Austria and Switzerland.[1–3] The main components of mistletoe extract are lectins, viscotoxins and alkaloids. The mechanism of action is probably 2-fold. On the one hand, mistletoe lectins can stimulate immunological relevant effector cells like macrophages, natural killer cells, and B and T lymphocytes with subsequent release of cytokines [interleukin (IL)-1, IL-6, IL-10, tumor necrosis factor- α and granulocyte macrophage colony stimulating factor];[4–8] on the other hand, mistletoe lectins have shown direct growth inhibitory effects on tumor cells. Depending on the concentration, treatment with mistletoe lectins results in death via apoptosis or necrosis.[9–13] Moreover, preclinical activity of aqueous mistletoe extracts has been shown in transplantable murine tumor models *in vivo*.[14]

In particular, the stimulation of immunological effector cells could be also associated with a potential growth stimulatory effect on hematological malignancies which are derived from the immune system like non-Hodgkin's or Hodgkin's lymphomas as well as acute leukemias. There is a case report that mistletoe extracts can enhance the growth of non-Hodgkin's lymphomas.[15] The majority of patients, however, appear to have benefited from an additional therapy with mistletoe.[1–3] Furthermore, Gabius *et al.* described stimulation of melanoma and sarcoma cell lines by a purified mistletoe lectin *in vitro*.[16]

One of the oldest mistletoe preparations is Iscador. Iscador is extracted from mistletoe plants growing on different host trees like apple (Iscador M special), oak (Iscador Qu special) and pine (Iscador P). The aqueous extracts are biologically and biochemically standardized. Iscador M special contains 250 ng total lectins/ml, Iscador Qu special contains 375 ng total lectins/ml, whereas Iscador P contains only trace amounts of lectins.

Correspondence to H-H Fiebig, Institute of Experimental Oncology, Oncotest GmbH, Am Flughafen 12–14, 79108 Freiburg, Germany.
Tel: (+49) 761 51559 11; Fax: (+49) 761 51559 55;
E-mail: fiebig@oncotest.de

In order to study direct effects of Iscador preparations on the growth of tumor cells *in vitro*, a panel of 16 human tumor cell lines was investigated in a cellular proliferation assay.

Materials and methods

Cell lines

Characteristics of the 16 tumor cell lines are shown in Table 1. Ten cell lines were established from human tumor xenografts as described by Roth *et al.*[17] The origin of the donor xenografts was described by Fiebig *et al.*[18] They comprise the following tumor types: GXF 251L (gastric), LXFA 629L, LXFL 529L and LXFE 66NL (lung, non-small-cell), MAXF 401NL (mammary), MEXF 462NL and MEXF 514L (melanoma), RXF 944L and RXF 393NL (renal), and UXF 1138L (uterine). The cell lines SF268 (glioblastoma), H460 (lung, non-small-cell), MCF7 (mammary) and PC3M (prostate), as well as the hematological cell lines HL60 (promyelocytic leukemia) and RPMI 8226 (myeloma) were kindly provided by the US National Cancer Institute. Cells were routinely passaged once or twice weekly. They are maintained no longer than 20 passages in culture. All cells were grown at 37°C in a humidified atmosphere (95% air, 5% CO_2) in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany) and 0.1% gentamicin (Invitrogen).

Cell proliferation assay

A modified propidium iodide assay was used to assess the effects of Iscador extracts on the growth of the human tumor cell lines.[19]

Briefly, cells were harvested from exponential phase cultures by trypsinization (not for hematological cells), counted and plated in 96-well flat-bottomed microtiter plates at a cell density dependent on the cell line (5–12 000 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 was added to the wells. Each concentration was plated in triplicate. Iscador preparations were applied in five concentrations ranging from 0.0015 to 15 μg plant extract/ml (Iscador M special and Qu special) and 0.003 to 30 μg plant extract/ml (Iscador P), respectively. Following 4 days of continuous drug exposure, cell culture medium with or without drug was replaced by 200 μl of an aqueous propidium iodide (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 the Cytofluor 4000 microplate reader (excitation 530 nm/emission 620 nm), giving a direct relationship to the total cell number. The assay included untreated and positive controls (doxorubicin and vindesine).

Table 1. Human tumor cell lines used for testing Iscador extracts

Tumor type	Cell line	Histology in nude mice	Doubling time (h)	Tumor formation *in vivo*
CNS	SF268	undifferentiated glioblastoma	ND	yes
Gastric	GXF 251L	poorly differentiated adenocarcinoma	32	yes
Hematologic	RPMI 8226	myeloma	ND	yes, in *scid* mice
	HL60	acute promyelocytic leukemia	ND	yes
Lung,	H460	large cell	18	yes
non-small cell	LXFA 629L	adenocarcinoma	31	yes
	LXFL 529L	large cell	25	yes
	LXFE 66NL	squamous cell carcinoma	41	yes
Mammary	MCF7	adenocarcinoma	30	yes
	MAXF 401NL	papillary adenocarcinoma, well differentiated	45	yes
Melanoma	MEXF 462NL	amelanotic	28	yes
	MEXF 514L	melanotic	29	yes
Prostate	PC3M	poorly differentiated adenocarcinoma	ND	yes
Renal	RXF 944L	clear cell carcinoma	30	yes
	RXF 393NL	clear cell carcinoma	ND	yes
Uterine	UXF 1138L	carcinosarcoma	31	yes

Cell lines developed in Freiburg were described by Roth *et al.*[17]

Growth inhibition/stimulation was expressed as treated/control × 100 (%T/C). Antitumor activity was defined as inhibition of tumor growth to less than 30% compared to the medium-treated control cells. Coefficient of variation [SD/mean × 100 (%)] was below 20% in nearly all experiments. Experiments were performed 3 times and T/C values are shown as mean of three experiments (Figures 1–3).

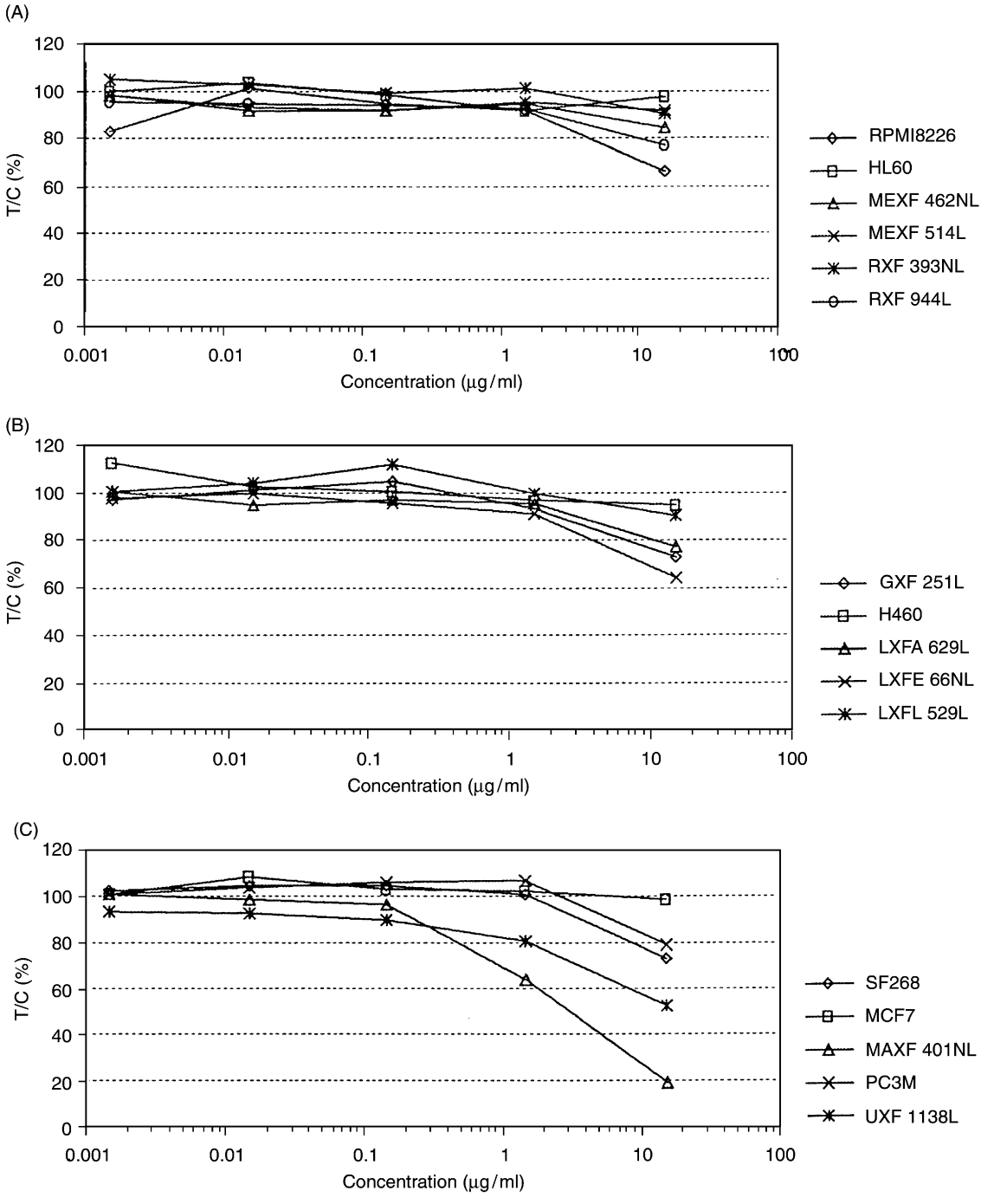

Figure 1. *In vitro* growth effects of Iscador M in a panel of 16 tumor cell lines. Growth inhibition/stimulation was expressed as treated/control × 100 (% T/C). Results were presented as mean of three experiments. (A) Hematologic, melanoma and renal. (B) Gastric and lung. (C) Central nervous system, mammary, prostate and uterine.

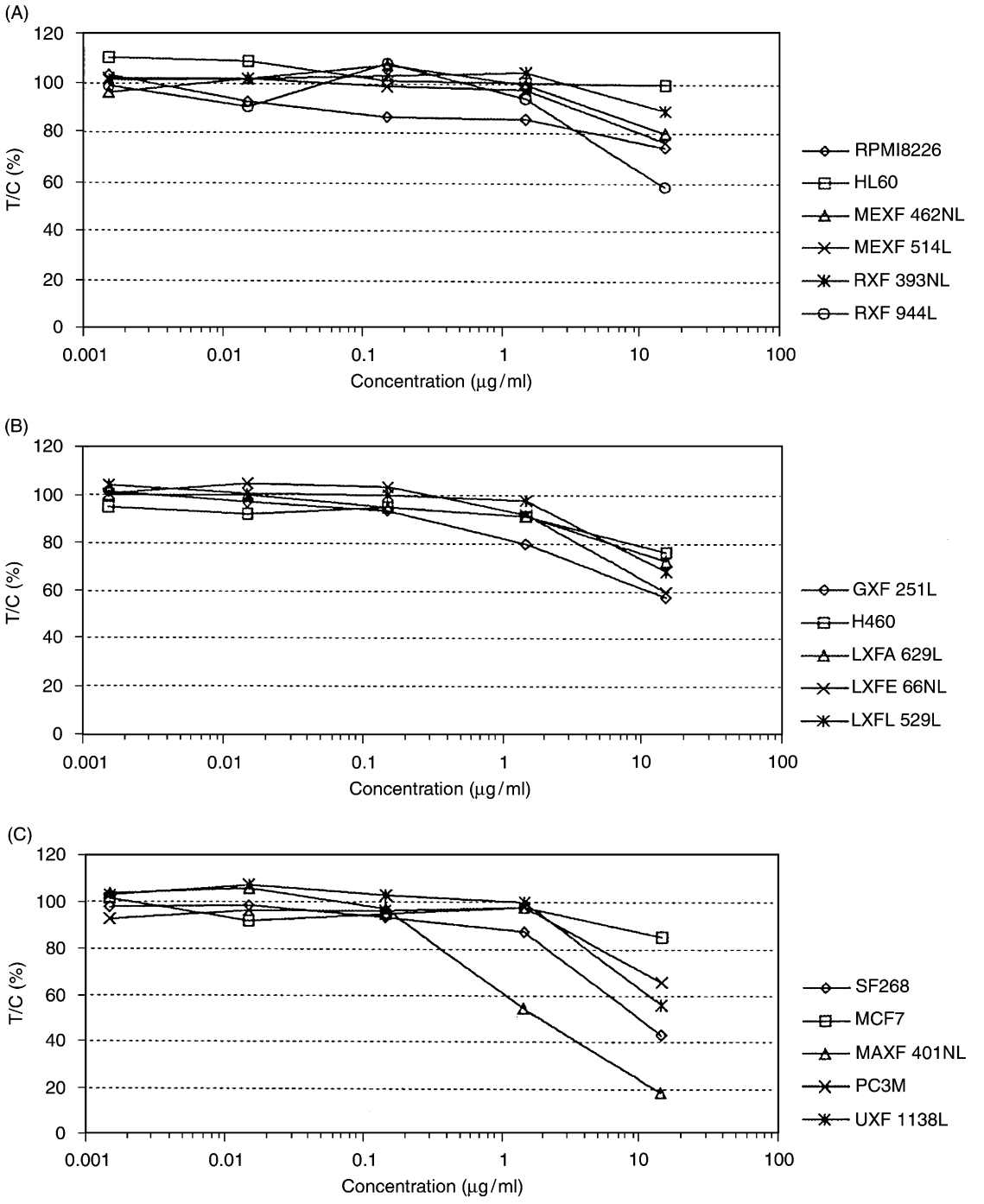

Figure 2. *In vitro* growth effects of Iscador Qu in a panel of 16 tumor cell lines. Growth inhibition/stimulation was expressed as treated/control × 100 (% T/C). Results were presented as mean of three experiments. (A) Hematologic, melanoma and renal. (B) Gastric and lung. (C) Central nervous system, mammary, prostate and uterine.

Mistletoe preparations

Iscador M special, Iscador Qu special and Iscador P were kindly provided by Weleda (Schwäbisch Gmünd, Germany). The ampoules contain 1 ml of an aqueous extract from 5 mg total plant of *V. album* (Mali) with 250 ng total lectins/ml, 5 mg total plant of *V. album* (Quercus) with 375 ng total lectins/ml or 10 mg total plant of *V. album* (Pini), respectively. Referring to these original amounts of plant material,

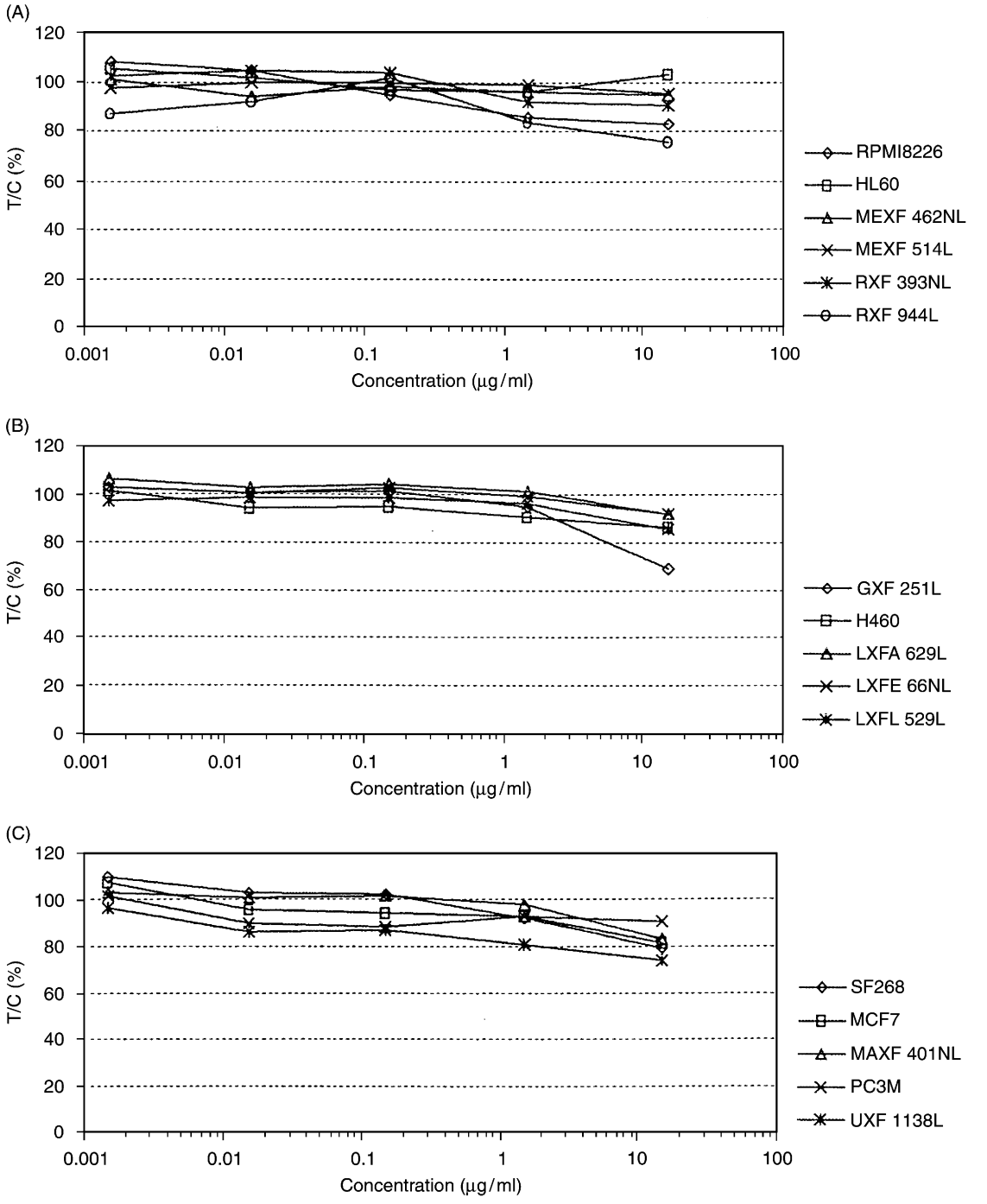

Figure 3. *In vitro* growth effects of Iscador P in a panel of 16 tumor cell lines. Growth inhibition/stimulation was expressed as treated/control × 100 (% T/C). Results were presented as mean of three experiments. (A) Hematologic, melanoma and renal. (B) Gastric and lung. (C) Central nervous system, mammary, prostate and uterine.

maximum final concentrations of 15 μg plant material/ml (Iscador M special and Iscador Qu special) and 30 μg plant material/ml (Iscador P) were administered in the *in vitro* proliferation assay.

Results

The effects of the three mistletoe extracts on tumor growth were investigated in a panel of 16 human

tumor cell lines comprising nine different tumor types. Iscador M special and Iscador Qu special were applied in dose levels ranging from 1.5 ng/ml to 15 μg/ml total plant extract under continuous drug exposure for 4 days. Iscador P was added to the cells in dose levels from 3 ng/ml to 30 μg/ml total plant extract. The results are summarized in Figures 1–3 for Iscador M special, Iscador Qu special and Iscador P, respectively. There was no evidence of stimulation of tumor growth by any of the three Iscador preparations. Independent on the dose level of Iscador preparations, none of the cells lines demonstrated a T/C > 120% compared to the medium-treated control cells.

On the contrary, at the highest dose level, the mistletoe extracts containing standardized lectins (Iscador M special and Iscador Qu special) showed antitumor activity (T/C < 30%) in the mammary cancer cell line MAXF 401NL (Figures 1 and 2). In addition, a slight inhibition of growth (T/C=30–70%) was found in three tumor cell lines following treatment with Iscador M special, i.e. leukemia RPMI 8226, non-small cell lung cancer LXFE 66NL and uterine cancer UXF 1138L (Figure 1). Iscador Qu special showed a slight inhibition of growth in seven tumor cell lines, comprising central nervous system cancer SF268, gastric cancer GXF 251L, non-small cell lung cancers LXFE 66NL and LXFL 529L, prostate cancer PC3M, renal cancer RXF 944L, and uterine cancer UXF 1138L (Figure 2). No growth inhibition was observed with Iscador P (Figure 3).

Discussion

Aqueous extracts from leaves and branches of the European mistletoe (*V. album* L.) exert antitumor activity via cytotoxic and immunological mechanisms.[4–13] To exclude possible direct growth stimulatory effects on tumor cells, the activity of three standardized mistletoe extracts, Iscador M special, Iscador Qu special and Iscador P, was investigated in a panel of 16 human tumor cell lines *in vitro* by using a cellular proliferation assay. Doubling times of the cell lines ranged widely from 18–45 h and their chemosensitivity profiles were also diverse. For example, the slowly growing, chemosensitive cell line MAXF 401NL and the faster growing, resistant cell line RXF 944L were included in the studies.[17,18,20,21]

Our results showed no evidence for direct stimulation of tumor growth *in vitro* by all three Iscador extracts, comprising central nervous system, gastric, non-small cell lung, mammary, prostate, renal and uterine cancer cell lines as well as cell lines from hematological malignancies and melanomas.

Recently, Gabius *et al.*[16] reported a slight enhancement of tumor growth by a purified mistletoe lectin in human tumor cell lines comprising the sarcoma Hs729, SK-UT-1B and SK-LMS-1 cell lines as well as the melanoma SK-MEL-28 and HT-144 cell lines. At a dose level of 50 pg/ml galactoside-binding mistletoe lectin they have found a very weak growth enhancement of 10 to maximal 40% compared to growth of untreated cells, in most cases at one incubation time point only, without a clear dose or time dependency. In our studies, Iscador M special was applied at dose levels ranging from 1.5 ng/ml up to 15 μg/ml plant extract, corresponding to a concentration of total lectin from 0.075 to 750 pg/ml. The lectin dose levels of the other lectin-containing product, Iscador Qu special, ranged from 0.11 to 1100 pg/ml in our experiments. Neither preparation showed any stimulatory effect at a lectin concentration of 50 pg/ml. We plan to examine the above-mentioned sarcoma and melanoma cell lines in our cell proliferation assay.

At the highest dose level studied, 15 μg/ml total plant extract, corresponding to a lectin concentration of 0.75 ng/ml (Iscador M special) and 1.1 ng/ml (Iscador Qu special), respectively, both extracts showed antiproliferative activity in distinct cell lines, which is in agreement with results reported by other groups for a variety of aqueous mistletoe preparations standardized for bioactive mistletoe lectins.[9–13] Moreover, Siegle *et al.*[22] reported recently on the enhancement of cytotoxicity of purified mistletoe lectin in combination with standard therapeutic drugs, including doxorubicin, cisplatin and taxol in the human lung carcinoma cell line A549 *in vitro*, suggesting new clinical perspectives for mistletoe therapy.

Conclusion

In conclusion, the standardized mistletoe extracts Iscador M special, Iscador Qu special and Iscador P showed no tumor stimulatory properties in a panel of 16 human tumor cells lines. In contrast, the mistletoe extracts containing high amounts of lectins (Iscador M special and Qu special) showed antitumor activity in the mammary cancer cell line MAXF 401NL, which is an encouraging result that should be further exploited and confirmed in *in vivo* animal models.

References

1. Stein GM, Schietzel M, Bussing A. Mistletoe in immunology and the clinic. *Anticancer Res* 1998; **18**: 3247–9.
2. Beuth J. Clinical relevance of immunoactive mistletoe lectin I. *Anti-Cancer Drugs* 1997; **8**(suppl 1): 53–5.
3. Grossarth-Maticek R, Kiene H, Baumgartner SM, Ziegler R. Use of Iscador, an extract of European mistletoe (*Viscum album*), in cancer treatment: prospective nonrandomized and randomized matched-pair studies nested within a cohort study. *Altern Health Med* 2001; **7**: 57–66, 68–72, 74–6.
4. Hajto T, Lanzrein C. Natural killer and antibody-dependent cell-mediated cytotoxicity activities and large granular lymphocyte frequencies in *Viscum album*-treated breast cancer patients. *Oncology* 1986; **43**: 93–7.
5. Hajto T, Hostanska K, Gabius HJ. Modulatory potency of the β-galactoside-specific lectin from mistletoe extracts (Iscador) on the host defense system *in vivo* in rabbits and patients. *Cancer Res* 1989; **49**: 4803–8.
6. Hajto T, Hostanska K, Frei K, *et al*. Increased secretion of tumor necrosis factor-α , interleukin-1, and interleukin-6 by human mononuclear cells exposed to the β-galactoside-specific lectin from clinically applied mistletoe extract. *Cancer Res* 1990; **50**: 3322–6.
7. Hostanska K, Hatjo T, Spagnoli GC, Fischer J, Lentzen H, Herrmann R. A plant lectin derived from *Viscum album* induces cytokine gene expression and protein production in cultures of human peripheral blood mononuclear cells. *Nat Immun* 1995; **14**: 295–304.
8. Schink M. Mistletoe therapy for human cancer: the role of natural killer cells. *Anti-Cancer Drugs* 1997; **8**(suppl 1): 47–51.
9. Stirpe F, Sandvig K, Olsnes S, Pihl A. Action of viscumin, a toxic lectin from mistletoe, on cells in culture. *J Biol Chem* 1982; **257**: 13271–7.
10. Janssen O, Scheffler A, Kabelitz D. *In vitro* effects of mistletoe extracts and mistletoe lectins. Cytotoxicity towards tumor cells due to induction of programmed cell death (apoptosis). *Arzneimittelforschung* 1993; **43**: 1221–7.
11. Bussing A, Suzart K, Schweizer K. Differences in the apoptosis-inducing properties of Viscum album L. extracts. *Anti-Cancer Drugs* 1997; **8**(suppl 1): 9–14.
12. Bussing A, Schietzel M. Apoptosis-inducing properties of *Viscum album* L. extracts from different host trees, correlate with their content of toxic mistletoe lectins. *Anticancer Res* 1999; **19**: 23–8.
13. Burger AM, Mengs U, Schuler JB, Fiebig HH. Antiproliferative activity of an aqueous mistletoe extract in human tumor cell lines and xenografts *in vitro*. *Arzneimittelforschung* 2001; **51**: 748–57.
14. Burger AM, Mengs U, Schuler JB, Fiebig HH. Anticancer activity of an aqueous mistletoe extract (AME) in syngeneic murine tumor models. *Anticancer Res* 2001; **21**: 1965–8.
15. Hagenah W, Dorges I, Gafumbegete E, Wagner T. Subcutaneous manifestations of a centrocytic non-Hodgkin lymphoma at the injection site of a mistletoe preparation. *Dtsch Med Wochenschr* 1998; **123**: 1001–4.
16. Gabius HJ, Darro F, Remmelink M. Evidence for stimulation of tumor proliferation in cell lines and histotypic cultures by clinically relevant low doses of the galactoside-binding mistletoe lectin, a component of proprietary extracts. *Cancer Invest* 2001; **19**: 114–26.
17. Roth T, Burger AM, Dengler WA, Willmann H, Fiebig HH. Human tumor cell lines demonstrating the characteristics of patient tumors as useful models for anticancer drug screening. In: Fiebig HH, Burger AM, eds. *Relevance of tumor models for anticancer drug development. Contrib oncol.* Basel: Karger 1999; **54**: 145–56.
18. Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR. Combined *in vitro/in vivo* test procedure with human tumor xenografts. In: Fiebig HH, Berger DP, eds. *Immunodeficient mice in oncology. Contrib oncol.* Basel: Karger 1992; **42**: 321–51.
19. Dengler WA, Schulte J, Berger DP, Mertelsmann R, Fiebig HH. Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. *Anti-Cancer Drugs* 1995; **6**: 522–32.
20. Fiebig HH, Dengler WA, Roth T. Human tumor xenografts: predictivity, characterization, and discovery of new anticancer agents. In: Fiebig HH, Burger AM, eds. *Relevance of tumor models for anticancer drug development. Contrib oncol.* Basel: Karger 1999; **54**: 29–50.
21. Roth T, Burger AM, Dengler WA, Willmann H, Fiebig HH. Human tumor cell lines demonstrating the characteristics of patient tumors as useful models for anticancer drug screening. In: Fiebig HH, Burger AM, eds. *Relevance of tumor models for anticancer drug development. Contrib oncol.* Basel: Karger 1999; **54**: 145–56.
22. Siegle I, Fritz P, McClellan M, Gutzeit S, Murdter TE. Combined cytotoxic action of *Viscum album* agglutinin-1 and anticancer agents against human A549 cancer cells. *Anticancer Res* 2001; **21**: 2687–91.

(Received 2 January 2002; accepted 21 January 2002)