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Antiproliferative Activity of an Aqueous Mistletoe Extract in Human Tumor Cell Lines and Xenografts in vitro

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Summary

The in vitro antiproliferative activity of an aqueous mistletoe extract (AME) with a defined content of bioactive mistletoe lectin (ML) was tested in 25 human tumor cell lines, including 20 solid and 5 hematological malignancies and 47 human tumor xenografts. The antiproliferative activity of AME was compared to that of the standard cytotoxic agent doxorubicin (CAS 23214-92-8, adriamycin, ADR) using the sulforhodamin B, propidium iodide and soft agar colony forming assays, respectively. AME was highly cytotoxic in solid human tumors with mean IC_{70} values in the range of 0.17 – 1 ng ML/ml (2.8–17 pmol bioactive ML). On a molar basis, AME was 3 to 4 logs more potent than ADR and showed differential cytotoxicity towards tumors of the breast, small cell and non-small cell lung, prostate and renal cell cancers. AME was also highly active in hematological malignancies with steep dose response curves resulting in mean IC_{70} values of 0.12 ng ML/ml (2 pmol). The acute lymphoblastic leukemia cell line HL-60 was the most sensitive, the histiocytic lymphoma cell line U937 the most resistant hematological malignancy. It is important to stress that AME did not induce a biologically relevant increase of cell proliferation in any of the tumor cell lines tested.

Our data suggest that AME has in vitro antitumor profiles similar to those of classical anticancer agents. Clear dose-response relationships were found in all of the performed experiments and interesting differential cytotoxicity patterns were observed. Experiments with sensitive tumor types identified in these in vitro studies are currently ongoing in order to demonstrate the anticancer activity of AME in different animal tumor models.

Key words

- **CAS 23214-92-8**
- **Doxorubicin**
- **Lektinol**
- **Mistletoe extract, antiproliferative activity in vitro, human tumor cell lines, xenografts**
- **Mistletoe lectin**

Arzneim.-Forsch./Drug Res. 51 (II), 748–757 (2001)

Zusammenfassung

Antiproliferative Wirkung eines Mistelextraktes auf menschliche Tumorzell-Linien und Xenografts in vitro

Die antiproliferative Wirkung eines wäßrigen Mistelextraktes (AME) mit definiertem Mistellektin-Gehalt wurde an 25 verschiedenen humanen Tumorzell-Linien und 47 Xenografts im Vergleich zu Doxorubicin (CAS 23214-92-8, Adriamycin, ADR) in vitro geprüft. Dabei erwies sich AME als hoch zytotoxisch mit mittleren IC_{70}-Werten im Bereich von 0.17-1 ng Mistellektin (ML)/ml (2,8 bis 17

pmol bezogen auf aktives ML). AME war auf molarer Basis um ca. 3 bis 4 Zehnerpotenzen aktiver als ADR. Verschiedene Tumorzell-Linien oder Xenografts wie Mamma-, Lungen-, Prostata- und Nierenzellkarzinome waren gegen eine Behandlung mit AME besonders sensitiv.

Vergleichbare Effekte zeigten sich für AME auch bei diversen hämatologischen Tumorzell-Linien mit mittleren IC_{70}-Werten im Bereich von 0,12 ng ML/ml (2 pmol). Eine relevante Begünstigung des Tumorzellwachstums wurde in keinem Fall festgestellt. Die Bedeutung der hier geschilderten Zellkulturbefunde für die In-vivo-Situation wird derzeit in entsprechenden Tiermodellen geprüft.

1. Introduction

Aqueous extracts of the European mistletoe (*Viscum album* L.) are widely used for complementary therapy in cancer patients [1]. Despite more than 70 years of clinical experience with different mistletoe extracts in German speaking countries, data from controlled clinical trials and systematic preclinical investigations are still rare [2].

In addition, there is a wide variety of commercially available mistletoe preparations, but differences in extraction and manufacturing processes are likely to produce different pharmacological effects [3]. The mistletoe lectins (ML) have been identified as the main active principle of mistletoe extracts [4]. ML I has a broad range of affinity for galactopyranosyl residues. ML II is specific for D-galactose and N-acetyl-D-galactosamine and ML III recognizes N-acetyl-D-galactosamine only. These heterodimetric glycoproteins with molecular weights between 55 and 63 kDa belong to a group of type II ribosome-inactivating proteins (RIP). They are composed of a lectinic B subunit, which mediates cellular uptake of the disulfide-bonded hololectin, and a cytotoxic A subunit, which inhibits protein biosynthesis enzymatically. Depending on the concentration, this results in cell death via apoptosis involving caspases or necrosis [5–10].

Besides the cytotoxic potential, mistletoe extracts and ML have further been demonstrated to possess immunomodulatory potencies by enhancing the secretion of cytokines and activity of natural killer cells [11–15]. Moreover, the standardized mistletoe extract (AME) used in the present in vitro studies has previously been shown to inhibit the pulmonary colonization of B16 melanoma cells and the growth of the murine MB49 urinary bladder carcinoma in syngeneic mice [16, 17].

Due to their strong cytotoxic properties, we examined whether mistletoe extracts could be a candidate for the treatment of human cancers. Thus, the aim of the present study was to systematically examine the in vitro cytotoxic/antiproliferative activity of an aqueous mistletoe extract (AME) in a panel of human tumor cell lines and xenografts representing fresh patient explants. This procedure should aid the identification of the most sensitive human target tumors for the investigation of anticancer effects in animal models and patients.

2. Materials and methods

2.1. Drugs

The aqueous mistletoe extract (1 : 1.1–1.5)[1)], batch no. 511128, was provided by Madaus AG (Cologne, Germany) and is designated as AME in the following text. The content of active (galactoside-specific) ML was 405 µg/ml. All concentrations given for AME reflect bioactive ML content determined by measuring the carbohydrate binding activity to asialofetuin in relation to ML by an enzyme-linked lectin assay (ELLA) [18]. Fresh plant material of *Viscum album* L. without lignified twigs and berries, grown on poplar trees, was extracted with water and filtered to obtain a sterile extract. The extract was stored at 2–8 °C. Dilutions were prepared freshly using Povidone K17 PF (pyrogen-free poly(1-vinyl-2-pyrrolidone; BASF, Ludwigshafen, Germany) in phosphate buffered saline.

Contamination of AME by potential bacterial endotoxins (lipopolysaccharides, LPS) was assessed using the chromogenic Limulus amebocyte lysate (LAL) assay kit (Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany) with a detection limit of 0.06 EU/ml. The sample of AME used in the present investigations were found to be endotoxin-free (< 0.06 EU/ml).

Doxorubicin (CAS 23214-92-8, adriamycin, ADR) was purchased from Sigma Chemicals Co. (St Louis, USA). Stock solutions were prepared in DMSO and aliquots stored at –20 °C. Final dilutions were prepared in culture medium prior to use.

5-Fluorouracil (CAS 51-21-8, 5-FU) was obtained as commercial preparation from Lederle (Münster, Germany) and was stored at room temperature in the dark. The drug was further diluted in culture media.

2.2. Clonogenic assay

The in vitro activity of AME in human tumor xenografts was studied using a modification of the two-layer soft agar culture system introduced by Hamburger and Salmon [20]. The target cell population in this assay are tumor stem cells, responsible for unlimited growth of a tumor. Single cell suspensions of solid human tumor xenografts (Table 1) were obtained by mechanical disaggregation and subsequent incubation with an enzyme cocktail consisting of collagenase (123 U/ml) (Worthington Biochemical Corp, Freehold, USA), DNAse (375 U/ml) and hyaluronidase (290 U/ml) (Boehringer Mannheim, Germany) in Iscove's medium (Life Technologies, Karlsruhe,

[1)] Active substance of Lektinol; manufacturer: Madaus AG, Cologne (Germany).

Germany) at 37 °C for 30 min. The cells were washed twice and passed through sieves of 200 and 50 µm mesh size to remove any remaining clumps. The percentage of viable cells was determined in a hemocytometer using trypan blue exclusion.

The bottom layer consisted of 0.2 ml Iscove's medium with 20 % fetal calf serum (Sigma, Deisenhofen, Germany) and 0.75 % agar and was plated in 24-well plates. 4×10^4 to 8×10^4 viable cells (the density was adjusted to the growth rate) were added in a volume of 0.2 ml of the same culture medium and 0.4 % agar over the base layer. Drugs were given in 0.2 ml medium (drug overlay). The control group received the vehicle only. Each plate contained six untreated control wells, three vehicle controls and six different drug concentrations in triplicate. 5-FU (100, 300 and 1000 µg/ml) and endotoxin (0.04 µg/ml) were used as positive or negative control, respectively.

Cell cultures were incubated at 37 °C and 7 % CO_2 in a humidified atmosphere for 5 to 15 days and monitored closely for colony growth using an inverted microscope. Within this period, in vitro tumor growth led to the formation of colonies (diameter of > 50 µm). At the time of maximum colony formation, colonies > 50 µm were counted with an automated image analysis system (Omnicon FAS IV, BioSys GmbH, Karben, Germany). 24 h prior to evaluation, viable colonies were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/ml, 100 µl/well) [19].

Drug effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls according to the formula

$T/C\% = (\text{colony count}_{\text{Treated Group}} / \text{colony count}_{\text{Control Group}}) \times 100\ \%$.

A compound was considered active, if it reduced colony formation to 50 % or less of the control group (G/C < 50 %).

IC_{50} and IC_{70} values being the drug concentration required to inhibit colony formation by 50 % (T/C = 50 %) and 70 % (T/C = 30 %), respectively, were determined by plotting compound concentrations versus T/C% values. Mean IC_{50} and IC_{70} values were calculated according to the formula:

$$\text{Mean } IC_{50/70} = 10 \left(\frac{\sum_{x=1}^{n} \log (IC_{50/70})\ x}{10} \right)$$

where x = value of specific tumor xenograft and n = total number of xenografts studied.

If an IC_{50} or IC_{70} could not be determined within the examined dose range, the lowest or highest concentration studied was used for the calculation.

2.3. Monolayer cell proliferation assays

Human tumor cells were grown at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2) as monolayer cultures in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10 % heat inactivated fetal calf serum, 300 mg/l L-glutamine and 0.1 % gentamycin (Sigma, Deisenhofen, Germany). Cells were passaged weekly.

Exponentially growing cells were harvested by trypsination (not for hematological tumors), washed, spun down and resuspended in fresh culture medium, counted and plated in 96-well flat-bottomed microtitre plates at densities of 0.6 to 1×10^4 viable cells per 45 µl per well. Additional 50 µl of culture medium were added per well. After a 24 h recovery to allow cells to resume exponential growth, 100 µl culture medium (6 control wells per plate) or culture medium containing the drug were added to the wells. Each drug concentration was plated in triplicate.

Drug effects were assessed by means of a modified propidium iodide (PI) assay [21] for hematological tumor cell lines and the sulphorhodamine B (SRB) assay [22] for human tumor cell lines growing as monolayer cultures (Table 1).

Hematological tumor cells were incubated for 7 to 9 days, depending on cell doubling time, after which the culture medium was replaced by 50 µl fresh medium containing propidium iodide (25 µg/ml, Aldrich Chemie, Steinheim, Germany). Before each washing step, suspension cultures were spun at 2000 rpm for 5 min. Fluorescence (FU_1) was measured using a Millipore Cytofluor 2350-microplate reader (excitation 530 nm, emission 620 nm). Microplates were then kept at −18 °C for 24 h, resulting in a total cell kill. After thawing of the plates and a 2nd fluorescence measurement (FU_2), the amount of viable cells was calculated by FU_2-FU_1. The assay included untreated and positive controls (5-FU).

Permanent human tumor cell lines derived from solid tumor types were maintained for 4 to 5 days under continuous exposure to the drug, depending on the growth rate of the specific tumor cell line. The remaining attached cells were fixed with 50 % trichloroacetic acid, kept at 4 °C for 1 h and were then washed 5 times with tap water. Cells were stained by addition of 100 µl of an aqueous SRB solution (0.4 %) containing 1 % acetic acid. Microplates were then kept at room temperature for 30 min and washed 4 times with 1 % acetic acid using an automated plate washing device to remove excess stain. 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).

Growth inhibition was expressed as treated/control × 100 (T/C%) in both the PI and SRB assays. IC_{50} and IC_{70} values were determined by plotting compound concentration versus cell viability.

The PI assay was only considered evaluable if the positive control (5-FU) induced a tumor growth inhibition of T/C > 50 % and if vehicle treated control cells had a fluorescence intensity > 500 units, for the SRB an optical density (O.D. 490 nm) of control cells of > 0.5 was considered evaluable.

3. Results

3.1. Clonogenic assay

AME was tested in vitro in 47 and ADR in 42 evaluable human tumor xenografts mimicking fresh patient explants [23] (Table 1). Drugs were added under continuous exposure in concentrations ranging from 10 pg to 1 µg ML/ml and 100 pg to 10 µg ADR/ml. At the concentration of 10 pg/ml, 47 % of the tumors were sensitive to AME and this percentage increased via 62 % (100 pg/ml) and 91 % (100 ng/ml) to 98 % at the concentration of 1 µg/ml. The IC_{50} and IC_{70} values over 47 xenografts were 0.06 and 0.17 ng/ml, respectively. ADR was active at 100 pg/ml in 14 % of the xenografts, which increased via 50 % (100 ng/ml) and 85 % (1 µg/ml) to 94 % at the concentration of 10 µg/ml. The IC_{50} and IC_{70} values of ADR over 42 xenografts were 6 and 46 ng/ml. Endotoxin, used to check whether possible contamination

of the preparation had any effect, was inactive at its concentration of 0.04 µg/ml.

Comparison of the activities of AME and ADR in the same 42 human tumors for their ability to inhibit human tumor stem cell colony formation showed that the IC_{70} value of AME (0.22 ng/ml) was 200 times lower than that of ADR (46 ng/ml). On a molar basis, the difference in IC_{70} values was even more pronounced: 4 pmol for AME (MW mistletoe lectin = 60 kD) and 80 nmol for ADR (MW = 580 D). Thus, AME was 20,000 times more potent than ADR.

The IC_{70} plots (Fig. 1 and 2) illustrate variations of any individual cell type IC_{70} value from the mean value (n = 47 or 42 xenografts, respectively), which are expressed as bars in logarithmic scale. Bars to the left demonstrate IC_{70} values lower than the mean concentration, bars to the right higher values. The mean graphs represent a fingerprint of the antiproliferative profile of each compound and can be used as a guideline for the selection of appropriate in vivo responding tumor xenografts [19, 23]. It can be seen that breast, prostate, renal, non-small and small cell lung cancer xenografts were generally more sensitive to AME than bladder and colon cancer xenografts, although every tumor type had one or two very sensitive xenografts. The profile of ADR differed from that of AME: small cell lung and breast cancers were the most sensitive tumor types and bladder, colon, and renal cancer the least sensitive. But there were also clear differences in the sensitivity of the individual tumor lines within a tumor type. For instance, BXF 1299 was relatively the most sensitive tumor in bladder cancer for ADR and T24 one of the relatively insensitive tumors, whereas this is quite the opposite for the AME profile.

3.2. SRB assay

The cytotoxicity of AME and ADR was also evaluated in 20 permanent human tumor cell lines (Table 1). The drugs were tested under continuous exposure in the same concentration range as used in the clonogenic assay. 10 % of the cell lines were sensitive to AME at the dose of 10 pg/ml, 20 and 25 % at 0.1 and 1 ng/ml, after which it sharply increased to 60 % (10 ng/ml) reaching 100 % at 100 ng/ml and 1 µg/ml. The dose response to ADR was much steeper. ADR was not active at 0.1 and 1 ng/ml, but at 10 ng/ml 40 % of the cell lines were sensitive, 80 % responded at 100 ng/ml, and 95 and 100 % at 1 and 10 µg/ml, respectively.

The IC_{70} values were 1 and 32 ng/ml for AME and ADR and on a molar basis 0.017 and 54 nmol, respectively. AME was thus about 3,200 times more potent than ADR in this assay. The IC_{70} profiles of both compounds were different (Fig. 3 and 4) as seen in the clonogenic assay indicative for different modes of action of these drugs. ADR was most active in non-small cell lung cancer and AME in prostate cancer. The most sensitive tumor cell lines for AME were breast cancer

Table 1: Characteristics of human tumor cell lines and human tumor xenografts used in the clonogenic assay.

Tumor type	Tumor designation	Histology
Solid human tumors		
Urinary bladder	BXF 1299	transitional cell carcinoma
	XF 1301	rhabdomyosarcoma
	BXF 1352	transitional cell carcinoma
	RT 112[a)]	transitional cell carcinoma
	T24[b)]	transitional cell carcinoma
Breast	MAXF MX1	adenocarcinoma
	MAXF 401[b)]	well differentiated papill. adenocarcinoma
	MCF-7[b)]	adenocarcinoma, pleural effusion
	MCF-7/ADR[a)]	MCF7 ADR resistant (from our own lab)
	MAXF 1162	invasive ductal carcinoma, poorly diff.
	MAXF 449	solid ductal carcinoma
	MDA-MB-231[b)]	adenocarcinoma, pleural effusion
Colon	CXF 280	Poorly diff. adenocarcinoma
	CXF 1103	adenocarcinoma
	HT29[b)]	adenocarcinoma, grade II
	HCT116LX	carcinoma
	CXF 158	adenocarcinoma, poorly diff.
	CXF 609	adenocarcinoma, poorly diff.
	DLD-1LX	adenocarcinoma
	SW620[b)]	adenocarcinoma, lymph node metastasis
Gastric	GXF 251	undifferentiated carcinoma
	GXF 209	adenocarcinoma, poorly diff.
	GXF 97	adenocarcinoma, moderately diff.
Lung	LXFA 289	adenocarcinoma
	LXFA 526[b)]	adenocarcinoma
	LXFA 629	adenocarcinoma
	LXFS 650	small cell carcinoma, intermediate
	DMS 114LX	small cell carcinoma
	DMS 273[a)]	small cell carcinoma
	LXFL 529[b)]	undiff. large cell carcinoma
	LXFL 1072[b)]	large cell carcinoma
Melanoma	MEXF 989	amelanotic melanoma
	MEXF 514	melanotic melanoma
	MEXF 276[b)]	amelanotic melanoma
	MEXF 462[b)]	amelanotic melanoma
Ovarian	OVXF 899[b)]	adenocarcinoma
	OVXF 1353	adenoid carcinoma
	OVXF 1023	adenocarcinoma, poorly diff.
	SKOV3[a)]	adenocarcinoma, malignant ascites
Pancreas	PAXF 736	adenocarcinoma
	PAXF 546	adenosquamous carcinoma
Prostate	PC3MLX[b)]	adenocarcinoma, metastatic clone
	DU 145[b)]	carcinoma, brain metastasis
	LNCaP	metastatic adenocarcinoma
Renal	RXF 423	well diff. hypernephroma
	RXF 1220	hypernephroma
	RXF 486[b)]	hypernephroma, poorly diff.
	RXF 631	hypernephroma, poorly diff.
	RXF 393	hypernephroma, well diff.
	RXF 944L[a)]	hypernephroma
Testis	TXF 881	teratoma, poorly diff.
Hematological tumors		
Leukemia	K562	chronic myelogenous leukemia
	HL-60	acute lymphoblastic leukemia
Lymphoma	RAJI	Burkitt's lymphoma
	U937	histiocytic lymphoma
Myeloma	RPMI 8226	myeloma

[a)] Tested in the SRB assay. [b)] Tested in the SRB and clonogenic assay.

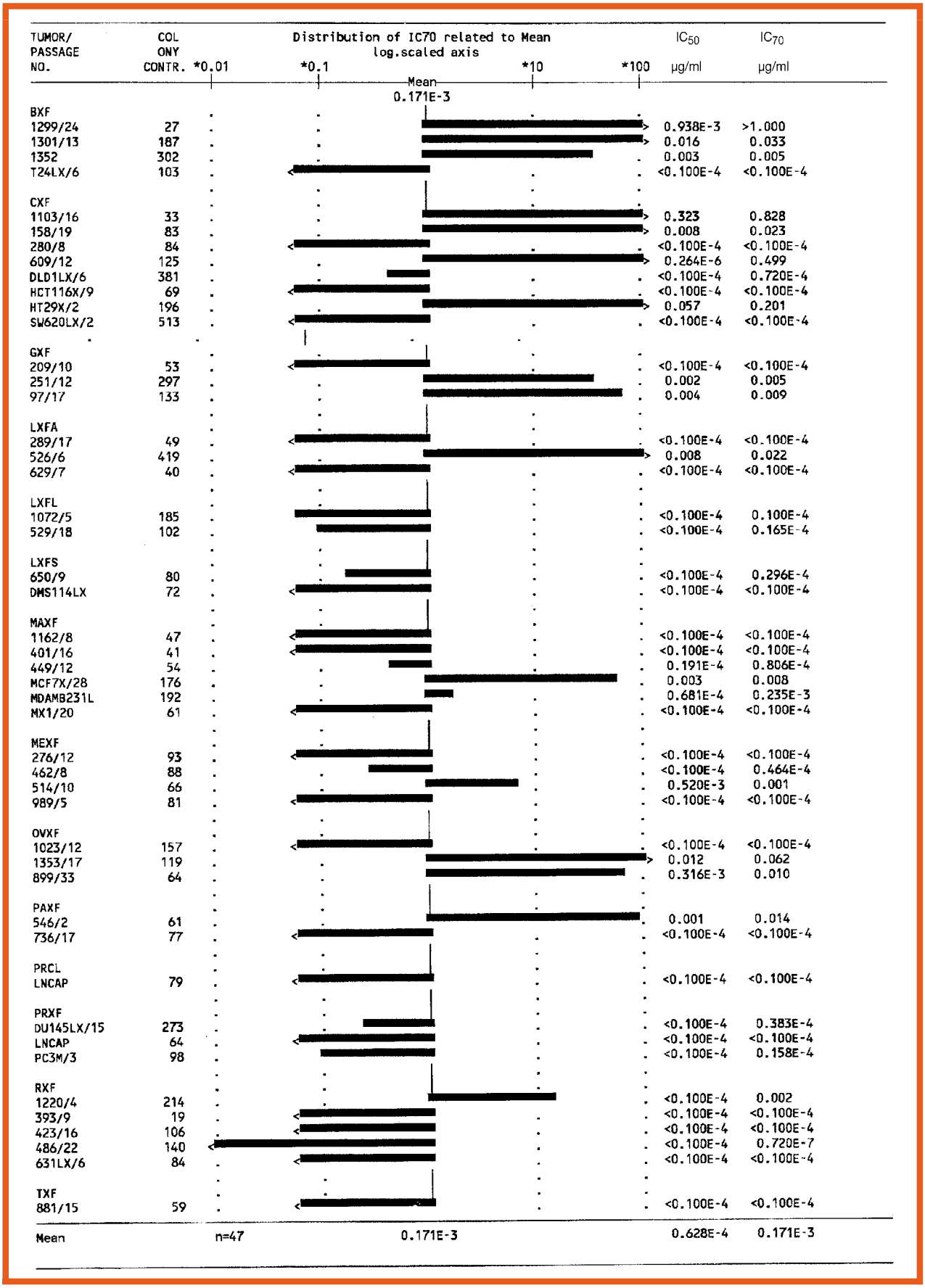

Fig. 1: IC_{70} plot of AME, clonogenic assay results. Shown are the deviation of individual tumors tested from the mean IC_{70} value. Bars to the left present the more sensitive tumor types, bars to the right indicate the more resistant tumors. The tumor designation and histologies are listed in Table 1. Colony contr. = mean number of colonies in a control well (set 100 %); IC_{50} (IC_{70}) = inhibitory concentration 50 % (70 %).

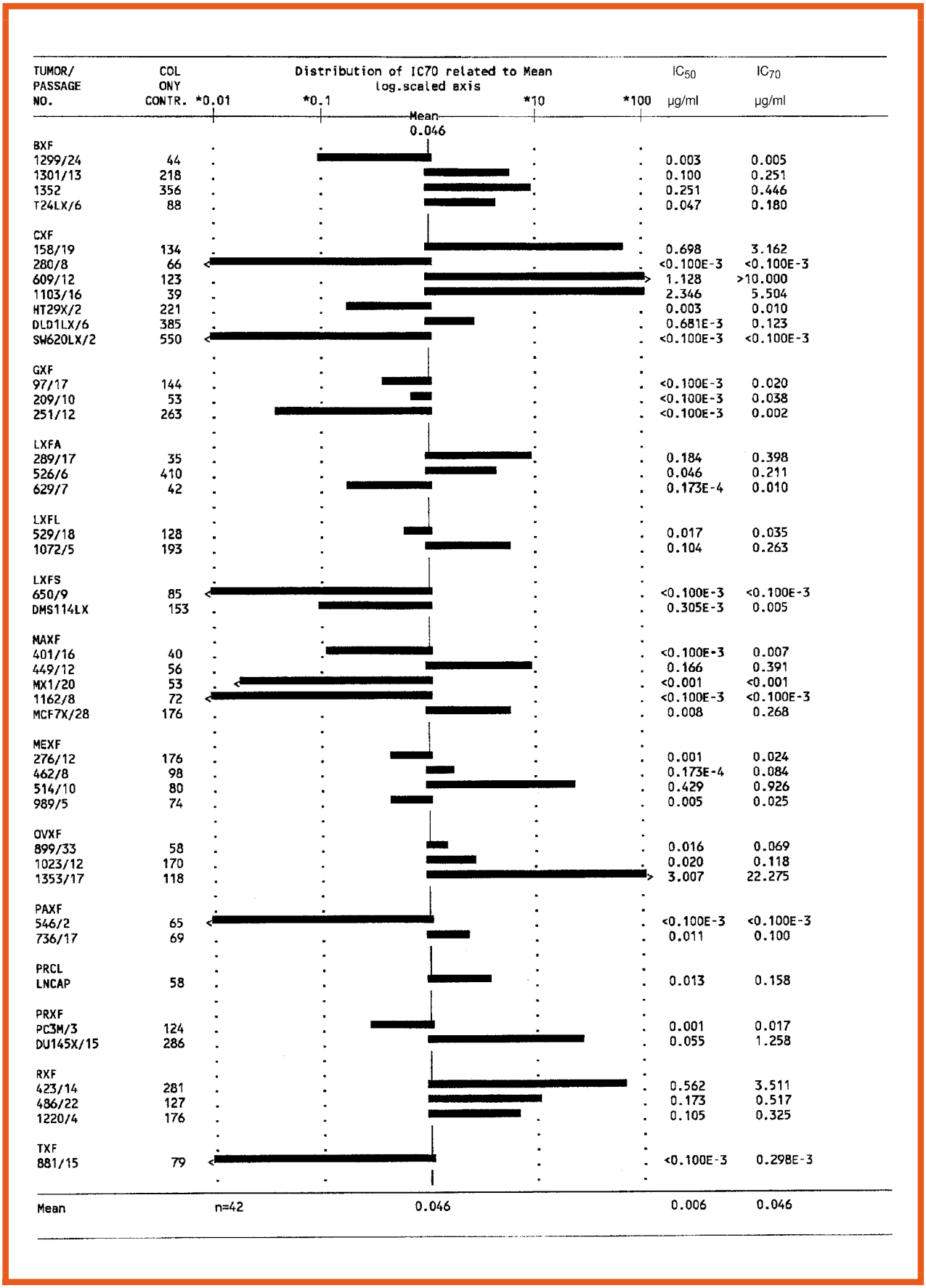

Fig. 2: IC_{70} plot of adriamycin, clonogenic assay results. Shown are the deviation of individual tumors tested from the mean IC_{70} value. Bars to the left present the more sensitive tumor types, bars to the right indicate the more resistant tumors. The tumor designation and histologies are listed in Table 1. Colony contr. = mean number of colonies in a control well (set 100 %); IC_{50}) = inhibitory concentration 50 % (70 %).

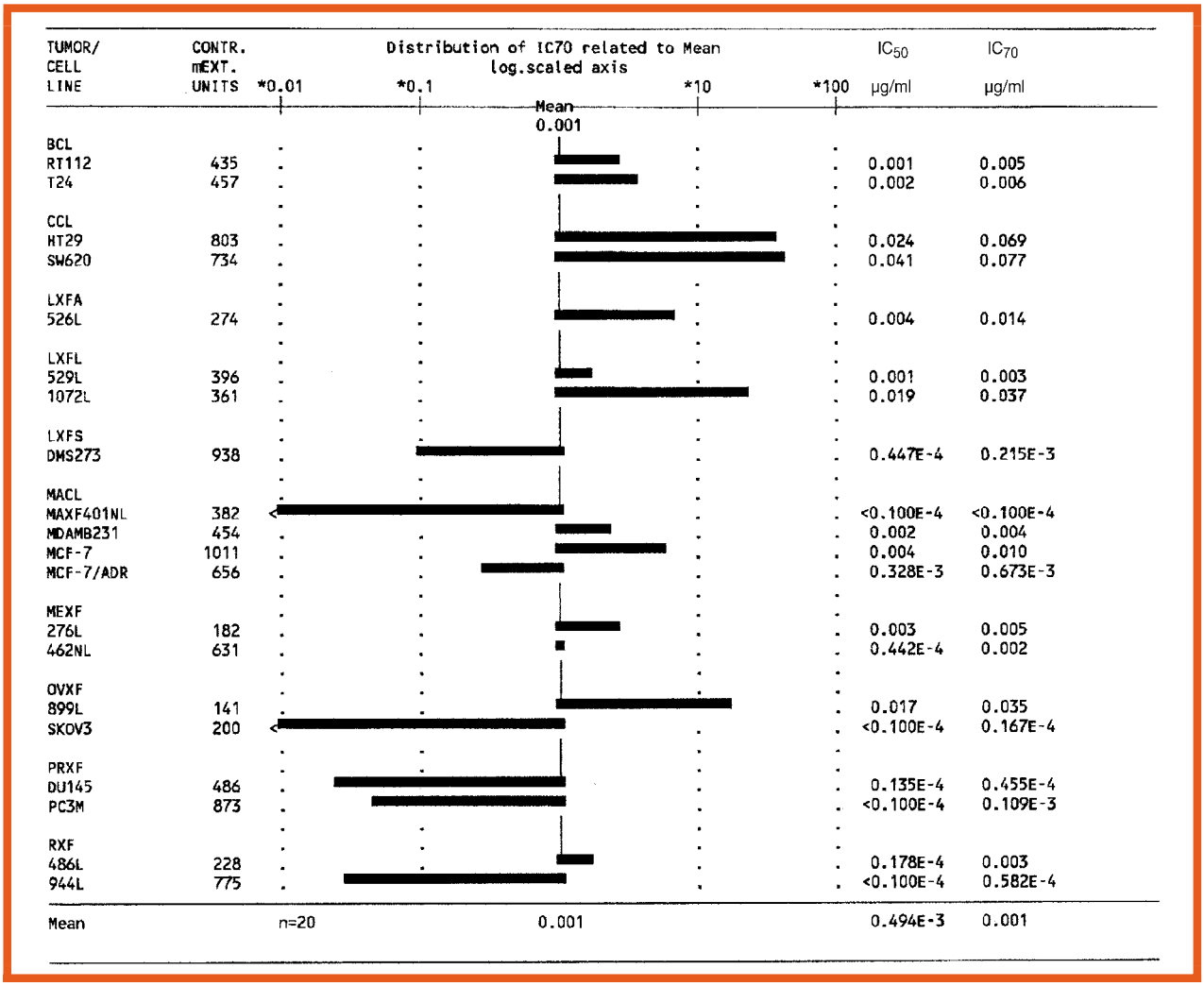

Fig. 3: IC_{70} plot of AME, SRB assay. Shown are the deviation of individual tumors tested from the mean IC_{70} value. Bars to the left present the more sensitive tumor types, bars to the right indicate the more resistant tumors. The tumor designation and histologies are listed in Table 1. Contr. m. ext. units = mean extinction units (optical density at 490 nm) of control wells; IC_{50} (IC_{70}) = inhibitory concentration 50 % (70 %).

MAXF 401NL and ovarian cancer SKOV3 followed by both prostate cancers DU145 and PC3M. ADR was most effective in the breast cancer cell lines MAXF 401NL and MCF-7. Interestingly, the ADR-resistant subline of MCF-7, namely MCF-7/ADR, was more sensitive to AME than the parental MCF-7 cells, indicating a possible independence of multidrug resistance mechanisms.

Endotoxin was inactive at the concentrations (0.05, 0.5 and 50 ng/ml) used in this assay.

3.3. Propidium iodide assay

The antiproliferative activity of AME was also evaluated in 5 human hematological tumor cell lines in concentrations ranging from 0.1 pg/ml to 1 µg/ml. AME showed a similar potency as in the clonogenic assay with IC_{50} and IC_{70} values of 0.05 and 0.12 ng/ml in these suspension cell lines. On a molar basis, AME was active in the pM range (0.8 pmol to 2 pmol). It was most active in the HL-60 and least active (6 times compared to IC_{70}) in the lymphoma U937 cell line (Fig. 5).

4. Discussion

The in vitro antiproliferative activity of AME was evaluated in panels of human tumor xenografts growing as colonies in soft agar, permanent human tumor cell lines derived from solid tumors and of hematological origin. Three in vitro proliferation assays, namely colony formation, the SRB and PI assays were used. In all systems, AME was shown to be a highly cytotoxic agent with mean IC_{70} values of 0.17 to 1 ng ML/ml (2.8 to 17 pmol). As shown from toxicological studies, such concentrations can be easily reached in the blood of dogs following i.v. treatment of systemically non-toxic doses of AME (unpublished data). A no effect level could not be defined in the present in vitro studies as several of the solid tumors used were sensitive even at the lowest tested concentration of 10 pg ML/ml. The results are in line with previous findings showing that the cytotoxicity of ML was high in human U937 promonocytes and breast cancer lines at concentrations between 30 to 100

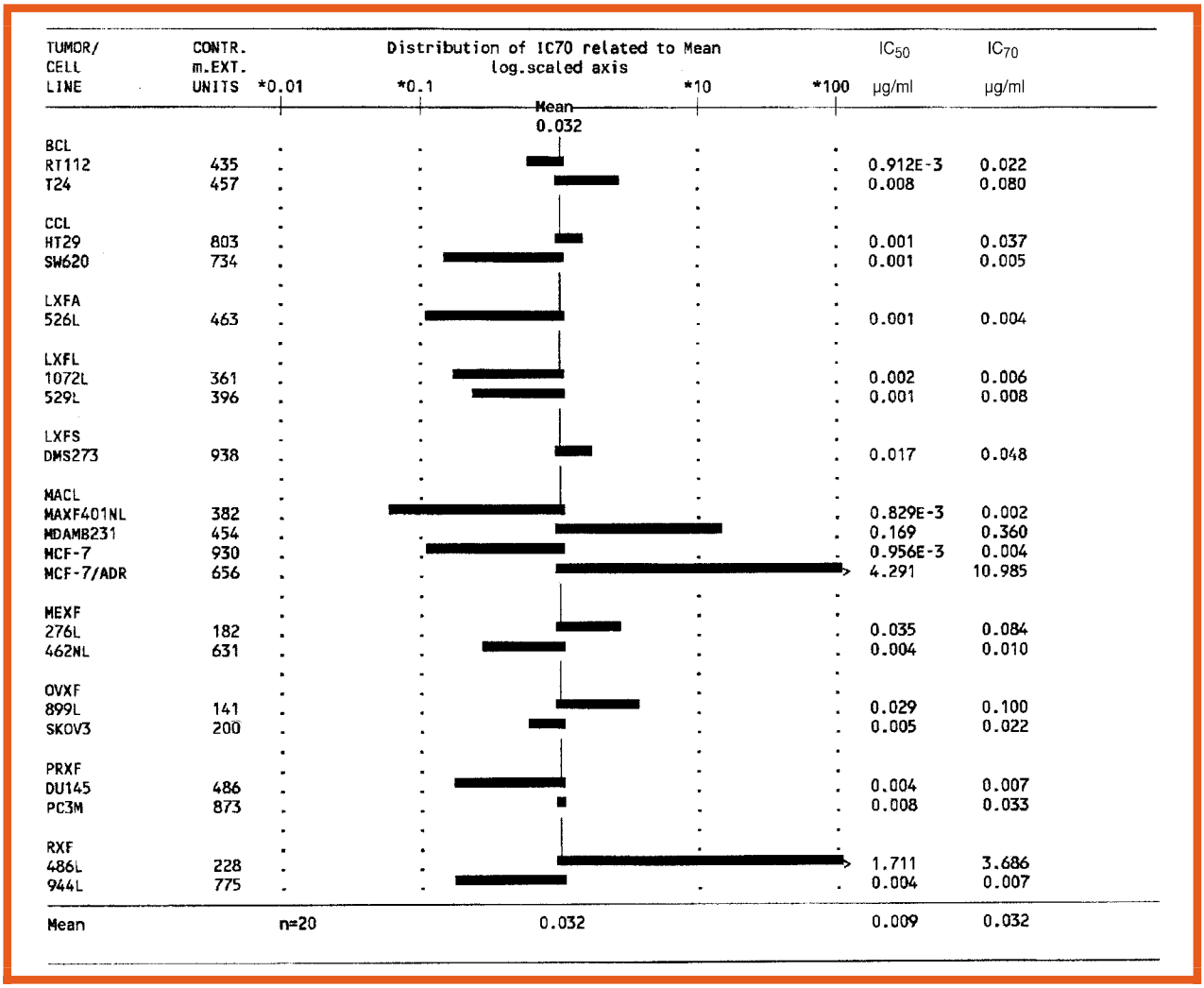

Fig. 4: IC_{70} plot of adriamycin, SRB assay. Shown are the deviation of individual tumors tested from the mean IC_{70} value. Bars to the left present the more sensitive tumor types, bars to the right indicate the more resistant tumors. The tumor designation and histologies are listed in Table 1. Contr. m. ext. units = mean extinction units (optical density at 490 nm) of control wells; IC_{50} (IC_{70}) = inhibitory concentration 50 % (70 %)

ng/ml [24, 25], taken into account that the drug exposure time in these studies was considerably shorter (72 h) than in our experiments (4 to 15 days). In a similar concentration range, immunostimulatory effects were shown for a mistletoe extract as well as for the isolated natural mistletoe lectin and its recombinant form. After incubation of cultured mononuclear blood cells from human donors, an increased secretion of several cytokines was observed at concentrations of 1 and 10 ng ML/ml. For IL-1-β, increased levels were even found at 0.1 ng ML/ml [15].

The activity of AME was compared to the standard anticancer agent ADR in the clonogenic and SRB assay. AME was, on a molar basis 10^3–10^4 times more potent than ADR in either assay. Both compounds displayed an interesting pattern of differential cytotoxicity. AME showed selective activity against prostate (2/2) and breast cancer (2/4), whilst ADR was most active in non-

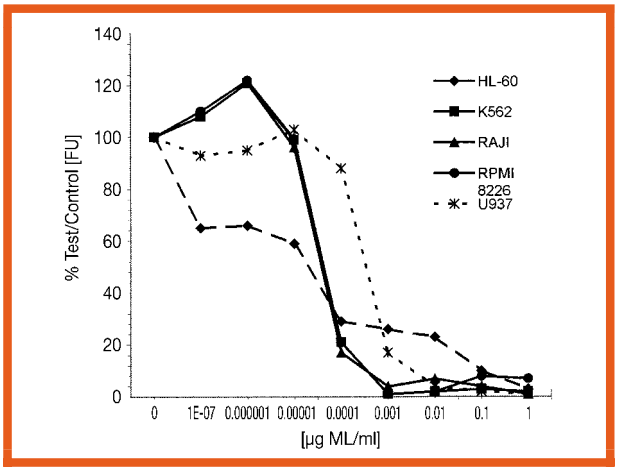

Fig. 5: Antiproliferative activity of AME in human hematological tumor cell lines. Growth curves are shown as % test/control of measured fluoresecence units (FU) plotted against ML concentration. Mean FU for 100 % K562 = 1869; U937 = 1811; RAJI = 1916; RPMI 8226 = 1199; HL-60 = 2326.

small cell (2/2) and breast (2/4) tumors in the SRB assay. This pattern was confirmed and extended in the clonogenic assay with human tumor explants grown s.c. in nude mice. In this assay, AME was particularly active against small cell lung (2/2), non-small cell lung (4/5), breast (4/6), prostate (3/3) and renal cell tumors (4/5). ADR was differentially active against small cell lung (2/2), breast (3/5) and gastric (3/3) tumors (Fig. 1 to 4). Since it has been shown in several studies in the past that the clonogenic assay is predictive for clinical response [19, 23], it would be worthwhile to investigate the in vivo antitumor activity of AME in breast, lung, prostate and renal tumors and, on the basis of the results in the PI assay, also in leukemia, lymphoma and myeloma (Fig. 5).

Generally, breast and small cell lung tumors respond to treatment with standard chemotherapeutic agents. The renal cell cancers, disseminated prostate tumors and adenocarcinomas of the lung, however, are intrinsically resistant to therapy [23]. In such cases, AME might provide an alternative to treat these refractory tumors. Moreover, another indication that AME might be valuable in treating resistant tumors is the fact that the MCF-7/ADR-resistant cell line was more sensitive to AME than the parental MCF-7 cell line. Thus, it appears as if AME might not be susceptible to development of multidrug resistance mechanisms. This aspect of AME-activity, however, needs to be studied in more detail.

We have tested AME at concentrations as low as 0.1 pg ML/ml to address the question whether growth stimulation could occur at such dose levels. This has been discussed for agents which are used in cancer therapy and for several cytokines [26, 27]. In the different tumor types examined, we did not see any tumor growth enhancement in vitro which could be considered biologically significant. In rare cases, such as in K562, RAJI, and RPMI 8226 cells, slight increases compared to controls in the order of 10 to maximal 20 % of control at concentrations of 1 pg/ml were observed (Fig. 5). Such small peaks, however, which have been reported as a certain risk potential by another group [28] can be interpreted as a result of biological variability and do not seem to be a relevant biological effect. This phenomenon is common and has been reported for a variety of cytotoxic agents before [29–31].

The differences in IC_{70} profiles of both drugs indicate that they have different modes of action. ADR is a DNA intercalating and topoisomerase II inhibiting agent. The mechanism of action of ML is not completely understood. ML belongs to the ribosome inhibiting proteins (RIP) type II, which consists of an A and B subunit. After internalization into the cell via the B subunit, the disulfide chain is separated and the A subunit exerts its RIP activity. This leads to an activation of cellular stress kinases and probably via this pathway to apoptosis of the target cell [32–34].

It has recently been shown that the recombinant form of ML exhibits the same cytotoxic pattern under identical experimental conditions [35]. This strongly supports the view that bioactive ML is the active ingredient of the used aqueous mistletoe extract (AME).

In conclusion, the present in vitro findings obtained from human tumor cell lines and xenografts indicate that AME has a cytotoxic/antiproliferative potential and thus it might be useful in adjuvant chemotherapy. Further studies are warranted which will be aimed to examine whether the in vitro cytotoxic potential can be translated into in vivo antitumor efficacy of AME.

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