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The Effects of *Viscum album* (Mistletoe) QuFrF Extract and Vincristine in Human Multiple Myeloma Cell Lines – A Comparative Experimental Study Using Several and Different Parameters

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1. Introduction

1.1 Multiple myeloma

Multiple myeloma (MM) is a haematological disorder of malignant plasma cells. B lymphocytes start in the bone marrow and move to the lymph nodes.

When they are activated to secrete antibodies, they are known as plasma cells, which are crucial part of the immune system. Due to the fundamental nature of the system affected, multiple myeloma manifests systemic symptoms that make it difficult to diagnose. Multiple myeloma is characterised by slow proliferation of the tumour cells, mainly in the bone marrow, by production of large amounts of immunoglobulins and osteolytic lesions. Multiple myeloma is a generally incurable disease at present, but remissions may be induced with stem cells transplants, steroids, chemotherapy and treatment with vincristine + doxorubicin + dexamethasone or thalidomide + dexamethasone or bortezomib based regimens or lenalidomide. The different therapeutic modalities have different “target location”.

1.2 Epidemiology of multiple myeloma

Multiple myeloma mainly affects older adults, but its causes and other risk factors are unknown. Yearly incidence is 3-6/100 000 worldwide, accounts for 1-2 % of all human cancer. Median survival is 50-55 months.

1.3 The role of cytokines in the growth, progression and dissemination of multiple myeloma

Cytokines are soluble proteins, peptides or glycoproteins that are released by cells. Cytokines can affect the cells via an autocrine and/or paracrine regulation mechanisms. In case of an autocrine regulation mechanism the endogenous produced cytokine affects the same type of cell. In case of a paracrine regulation mechanism the target cell is near to the

cytokine produced cell. Cytokine binds to a specific receptor and causes a change in function or in development (differentiation) of the target cell. In both cases, i.e. autocrine and paracrine regulation mechanisms the expression of the membrane receptor is altered.

1.3.1 Interleukins are a group of cytokines which are produced by a wide variety of body cells. The majority of interleukins are synthesized by helper CD4⁺ T lymphocytes, monocytes/macrophages and by endothelial cells. If the produced cytokine is released, then it is measurable in the supernatant, if not then the cytokine is measurable only intracellular.

Interleukin-6 (IL-6) originally defined as a B cell differentiation factor is produced by different cell types and certain tumour cells. Interleukin-6 acts as a pro-inflammatory and an anti-inflammatory cytokine. As a pro-inflammatory cytokine regulates inflammatory reactions either directly or indirectly. As an anti-inflammatory cytokine Interleukin-6 reduces the inflammatory reactions. IL-6 exists in three molecular weights, i.e. 21-30 kDa, 150-200 or 450 kDa. The biological activity of IL-6 depends on binding to its specific receptors. These membrane receptors composed the glycoprotein gp80 Interleukin-6 receptor alpha (IL-6R, also called CD126) and a signal-transducing component gp130 (also called CD 130). The complex IL-6+IL-6R+gp130 initiate a signal transduction cascade through JAKs (Janus kinases) and STATs (Signal Transducer-Activator of Transcription). The membrane receptors are released from the cells as soluble receptor proteins (sIL-6R and sgp130). As agonist, sIL-6R enhances the biological activity of IL-6 and sgp130 is an antagonist against the complex IL-6+sIL-6R.

Interleukin-6 is a major proliferative factor for the malignant plasma cells (multiple myeloma cells). This cytokine produces by the plasma cells and it affects the cells by an autocrine regulation mechanism with an additional paracrine signalling. IL-6 in a concentration of 2pg/ml can induce 50% proliferation in myeloma cells.

The multiple myeloma cells can be classified into three groups depending on exogenous IL-6: (a) both proliferation and survival of the cells are dependent on IL-6, (b) only proliferation of the myeloma cells is affected by IL-6, (c) the cells are dependent on IL-6 only for survival, but not for proliferation. However there are also some cell lines that are independent of IL-6 both for survival and proliferation. The serum values of IL-6 in 35% or in 97% or in 42% of multiple myeloma patients were significantly higher than in healthy persons (Nachbour et al., 1991; DuVillard et al., 1995; Wierzbowska et al., 1999).

Because about 70% of the secreted IL-6 forms a complex with sIL-6R (Gaillard et al., 1987), the amount of the free IL-6 in serum is low. Therefore the serum level of the sIL-6R is an important parameter in the evaluation and in the progression of multiple myeloma (Papadaki et al., 1997; Wierzbowska et al., 1999).

Interleukin-10 (IL-10) is known as a human cytokine synthesis inhibitory factor (CSIF). It produces by Thelper2 cells, monocytes/macrophages, by B lymphocytes and some tumour cells. Interleukin-10 has (1) immunosuppressive effect and (2) immunostimulatory effect. It down-regulates the expression of Thelper1 cytokines, MHC class II antigens and co-stimulatory molecules on macrophages. Interleukin-10 is a pleiotropic cytokine which increases Bcl-2 levels and protects cells from steroid or doxorubicin-induced apoptosis.

How might the presence of IL-10 contribute to a poor prognosis for some cancer? One possibility: Interleukin-10 is a growth factor for tumour cells. Second possibility: Interleukin-10 suppresses the anti-tumour immune responses.

Interleukin-10 enhances the survival and proliferation of B cells. IL-10 is a growth factor for myeloma cells (Kovacs, 2010a), enhances the proliferation of freshly explanted myeloma cells in a short-term bone marrow culture (Lu et al., 1995). Three out of seven human myeloma cell lines produce IL-10. Elevated IL-10 levels were detected in serum from about 50% of patients having multiple myeloma showing a relation to the clinical manifestation (Otsuki et al., 2000; 2002). Interleukin-6 leads to a marked production of Interleukin-10 in several human multiple myeloma cells. Interleukin-10 is an Interleukin-6 related growth factor for these tumour cells (Kovacs, 2010a).

1.4 Cytostatic effect and cytotoxic effect

Cytotoxic effect: It is known that there are two important pathways against tumours: To inhibit the tumour cell proliferation (**cytostatic effect**) and/or to induce the death of the tumour cells (**cytotoxic effect**). **Cytotoxic effect:** apoptosis or necrosis.

The apoptosis is a physiological process in the life of healthy cells, whereas necrosis is a pathological process for tumour cells. **Cytotoxicity** is the quality of being toxic to cells. There are a **direct** and an **indirect** (cell-mediated) **cytotoxicity**. In case of direct cytotoxicity the cells are treated with cytotoxic compounds leading to necrosis.

1.5 Viscum album (VA) extract

Viscum album (VA) extract from **European mistletoe** plants has fermented and non-fermented preparations. Active components of VA extracts include mistletoe lectins (I, II, III) and viscotoxin, additionally aminoacids, polysaccharides and lipids. The fermented preparations are used either alone or in combination with chemo/radiotherapy in the treatment of tumour patients.

The Viscum album QuFrF (VAQuFrF) is an aqueous and unfermented extract of mistletoe plants growing in the oak tree. It contains 1 µg lectin and 6 µg viscotoxin in 5 mg/ml or 2 µg lectin and 10 µg viscotoxin in 10 mg/ml. The extract is an experimental drug that is not yet used in the treatment of tumour patients.

1.6 Vincristine

Vincristine is a vinca alkaloid. As a chemotherapeutic agent is used mainly in combination with other chemotherapeutic substances in the therapy of multiple myeloma. Vincristine inhibits the proliferation of these tumour cells and as a CCS blocker (El Alaoui, 1997; Lin et al., 1998; Mastberger et al., 2000) arrests the cell cycle phase G2/M by blocking the mitotic spindle formation (Harmsma et al., 2004).

2. Aim

2.1 Comparison of the effects of Viscum album QuFrF extract with those of Vincristine.

2.2 Mode of action of Viscum album QuFrF extract and Vincristine.

2.3 To assess the effective doses of Viscum album QuFrF extract and to transfer these doses to the in vivo situation.

3. Materials and methods

3.1 Test substances

Viscum album QuFrF (VAQuFrF) extract was obtained from the Hiscia Institute (Arlesheim, Switzerland). According to the manufacturer the aqueous unfermented solution of extract 10 mg/ml contains 2 µg lectin and 10 µg viscotoxin. Vincristine sulfate salt was obtained from Sigma GmbH (Schnelldorf Germany, No 8879). Recombinant human interleukin-6 (rh IL-6) was obtained from R & D Systems (No. 206-IL, United Kingdom) and reconstituted in phosphate-buffered saline with 0.18% bovine serum albumin.

3.2 Cells and culture condition

Human myeloma cell lines (MOLP-8, LP-1, RPMI-8226, OPM-2, U-266, COLO-677, KMS-12-BM, were obtained from DSMZ (Braunschweig, Germany). Five cell lines derived from blood, COLO-677 from lymph node, KMS-12-BM from bone marrow. The cells were cultivated in RPMI 1640 supplemented with 10-20% foetal calf serum, 2mM L-glutamine and 1 % gentamicin in a humidified atmosphere with 5% CO₂ at 37°C. The doubling times of tumour cell lines were between 35 and 96 hours. For the measurement of the parameters the cell cultures were used within 4-6 weeks after thawing.

3.3 Treatment of cells with VAQuFrF extract or vincristine

- a. To measure viability, cytokine production, membrane expression of IL-6 receptor, cell cycle phases and apoptosis /necrosis the cells were cultured at a density of 0.5-0.7x10⁶ cells/ml, except for COLO-677 (0.2x10⁶ cells/ml). After 24 hours the cells were incubated with VAQuFrF extract or Vincristine (doses: 0, 10, 50 or 100 µg/10⁶ cells/ml). The parameters were measured after 24, 48 and 72 hours.
- b. To measure proliferation the cells were cultured at a density of 0.5-0.7x10⁵ cells/100 µl, except for COLO-677 (0.2x10⁵ cells/100 µl). After 24 hours the cells were incubated with VAQuFrF extract or Vincristine (doses: 0, 1, 5, 10 µg/10⁵ cells/100µl). The parameter was measured after 24, 48 and 72 hours.

3.4 Treatment of cells with Interleukin-6

- a. To measure viability, cytokine production, membrane expression of IL-6 receptor, cell cycle phases and apoptosis /necrosis the cells were cultured at a density of 0.5-0.7x10⁶ cells/ml, except for COLO-677 (0.2x10⁶ cells/ml). After 24 hours the cells were incubated with IL-6 (dose: 5 ng/10⁶cells/ml). The parameters were measured after 24, 48 and 72 hours.
- b. To measure proliferation the cells were cultured at a density of 0.5-0.7x10⁵ cells/100 µl, except for COLO-677 (0.2x10⁵ cells/100 µl). After 24 hours the cells were incubated with IL-6 (dose: 0.5 ng/10⁵cells/100µl). The parameter was measured after 24, 48 and 72 hours.

3.5 Measurement of viability

The viabilities of the cultivated tumour cells were determined by 7-aminoactinomycin D (7-AAD), to exclude the non-viable cells in flow cytometric assays. The values are given in %.

3.6 Measurement of cytokine production

The IL-6 production or IL-10 production in the supernatant of the cultured cells was determined by chemiluminescent immunometric assay. The lowest detectable level was 2 pg/ml or 5 pg/ml.

3.7 Measurement of membrane expression of IL-6 receptor

For immunofluorescence staining 3×10^5 cells/100 μ l were incubated with 20 μ l phycoerythrin (PE) conjugated monoclonal antibody (CD 126, Immunotech, France) for 30 min at 4 °C. Then the cells were washed, sedimented and analysed in the FACSCalibur flow cytometer. For the expression of the membrane IL-6R (CD 126) the signal intensity (geometric mean of the fluorescence intensity \times counts) was used as parameter. The signal intensity of the treated samples was compared with that of untreated samples, which were taken as 100%.

3.8 Measurement of the cell cycle phases

The cell cycle phases G0/G1, S, G2/M were assessed using the cycle test plus DNA reagent kit on a flow cytometer (BD, BioSciences, San Jose, USA No 340242). Briefly: 5×10^5 cells were incubated at room temperature with trypsin buffer and additionally with trypsin inhibitor+RNase buffer. The values are expressed in percentage of total viable cell number (100%).

3.9 Measurement of apoptosis and necrosis

Apoptosis was measured using Annexin V-FITC (BD Biosciences Pharmingen, San Diego, USA No 556 570). Necrosis was measured using propidium iodide (PI). Briefly: 1×10^5 cells were incubated with Annexin V-FITC or PI at room temperature in the dark. Thereafter the samples were analysed in a flow cytometer. Apoptotic cells: Annexin V-FITC positive and PI negative. Necrotic cells: Annexin V-FITC positive and PI positive. The values are given in percent of total cell number.

3.10 Measurement of the proliferation

The proliferation was assessed using cell proliferation reagent WST-1 (Roche, Mannheim, Germany, No 1644 807). The colorimetric assay is based on the reduction of the tetrazolium salt WST-1 by viable cells. The reaction produces the soluble formazan salt. The quantity of the formazan dye is directly correlated to the number of the metabolically active cells. The proliferation rate was measured 1, 2 and 4 h after incubation with the reagents at time points 24, 48 and 72 h. The upper limit of the absorbance was 2.0–2.1. The intra-sample variance of the untreated cells was <10% (3–8%).

3.11 Statistical analysis

Three to four independent measurements were carried out. For the evaluation of the parameters the Mann-Whitney U-test was used. The limit of significance was taken as $P < 0.05$.

4. Results

4.1 Production of Interleukin-6 in supernatant of human multiple myeloma cells

Objectives: (a) spontaneous production, (b) production after treatment with VAQuFrF or Vincristine.

Table 1 presents the values of IL-6 in myeloma cell line MOLP-8, LP-1, RPMI-8226, OPM-2, COLO-677. None of the five multiple myeloma cell lines produced Interleukin-6 spontaneously. This means that all the investigated cell lines are IL-6 independent or have **autocrine/paracrine** regulation mechanisms. In case of an **autocrine** regulation mechanism the cytokine is produced endogenously and affects its membrane receptor directly. In case of **paracrine** regulation mechanism the exogenous cytokine also affects the membrane receptor. In two cell lines (RPMI-8226 and OPM-2) exogenous IL-6 led to a high expression of membrane IL-6R and enhanced levels of sIL-6R in the supernatant (Kovacs, 2003; and results are not shown) indicating a paracrine regulation mechanism.

Cell lines	Spontaneous	After treatment with	
		VAQuFrF	Vincristine
MOLP-8	ND	ND	ND
LP-1	ND	ND	ND
RPMI-8226	ND	ND	ND
OPM-2	ND	ND	ND
COLO-677	ND	ND	ND

Maeasurements at 24 and 48 hours after tretment with VAQufrF extractor Vincristine. Dose=50 µg/10⁶ cells in both cases.ND = not detectable.

Table 1. Production of Interleukin-6 in human multiple myeloma cell lines.

Treatment with VAQuFrF extract (dose: 50 µg/10⁶ cells) and with Vincristine (dose: 50 µg/10⁶ cells) also did not lead to IL-6 production in the five multiple myeloma cell lines. These results confirm the findings of previous studies (Kovacs et al., 2006; Kovacs, 2010b).

4.2 Production of interleukin-10 in supernatant of human multiple myeloma cells

Objectives: (a) spontaneous production, (b) production after treatment with IL-6 (dose: 5ng/10⁶ cells), (c) production after treatment with VAQuFrF or Vincristine (dose: 50 µg/10⁶ cells for both substances), (d) after treatment with IL-6+VAQuFrF or IL-6+Vincristine (doses: 5 ng/10⁶ cells +50 µg/10⁶ cells in each case). For the combined treatment IL-6 was added 2 hours before the test substances.

Table 2 presents the production of Interleukin-10 in five human multiple myeloma cell lines. Spontaneous IL-10 production was found in 4/5 cell lines: MOLP-8, LP-1, RPMI-8226, COLO-677, however the cell lines MOLP-8 and COLO-677 secreted IL-10 not every time confirming the findings of previous study (Kovacs, 2010a). IL-6 led to a marked increase of

IL-10 production (up to 946 pg/ml) in 5/5 cell lines. VAQuFrF extract and Vincristine reduced the spontaneous IL-10 production in MOLP-8, LP-1 and COLO-677 to non-detectable amounts.

With IL-6+VAQuFrF or IL-6+Vincristine the values were markedly lower after addition of IL-6 but higher than without IL-6 treatment. VAQuFrF and Vincristine reduced the induced IL-10 production to the same degree in cell lines RPMI-8226 and LP-1. In the cell lines OPM-2, MOLP-8, COLO-677 the extract of VAQuFrF inhibited the IL-10 production weaker than Vincristine.

Cell lines	No treatment	After treatment with			IL-6 + VAQuFF	IL-6 + Vincristine
		IL-6	VAQuFrF	Vincristine		
MOLP-8	ND-18	453-862	ND	ND	35-73	8-40
LP-1	10-22	34-95	ND	ND	25-53	31-44
RPMI-8226	32-124	510-946	36-14	15-27	290-334	370-404
OPM-2	ND	8-33	ND	ND	6-14	ND
COLO-677	ND-30	26-105	ND-11	ND	10-32	ND

Maesurements at 24 and 48 hors after tretment. IL-6:5ng/10⁶ cells.VAQuFrF and Vincristine: 50 µg/10⁶ cells. Range of 4 independent measurements. ND=not detectable

Table 2. Production of Interleukin-10 (pg/ml)in human multiple myeloma cell lines.

No treatment	After treatment with		
	IL-6	VAQuFrF	Vincristine
55-60	49-62	11-17	16-20
82-83	80-85	58-71	30-48
70-72	69-75	30-47	29-53
47-56	49-57	46-55	13-21
67-68	70-81	41-59	21-32

The values are presented in percentage. Range of four independent measurements.

Table 2.A. Viability of human multiple myeloma cells (cell lines see Table 2).

The IL-10 production was measured at 24 and 48 after incubation with IL-6. The results show that in tumour cell lines MOLP-8 and RPMI-8226 the IL-10 production was high during the two days. In the other three cell lines the production decreased slightly at 48 h.

Table 2/A. presents the range of cell viability without treatment and after treatment with the test substances: L-6, VAQuFrF extract and Vincristine. The viability of the untreated MM cells was different: LP-1>RPMI-8226>COLO-677>MOLP-8>OPM-2. The both test substances impaired the viability to different degrees.

IL-6 does not alter the viability, confirming the findings of previous investigations (Kovacs, 2006b, 2010a). It was reported that IL-6 enhances survival of the myeloma cells because it inhibits apoptosis of induction of the anti-Fas (Nordan & Potter, 1986; Hata et al., 1995).

Summarised: The results indicate that the effect of the both test substances on the IL-10 production is due to their apoptotic/necrotic effects. It is possible that VAQuFrF and Vincristine could also impair the membrane expression of IL-10 receptor. To explain this hypothesis further experiments are necessary.

4.3 The effect of Interleukin-6, VAQuFrF and IL-6+VAQuFrF on the membrane expression of Interleukin-6 receptor in human multiple myeloma cells

Objectives: (a) in untreated cells, (b) after treatment with IL-6 (dose: 5 ng/10⁶cells), (c) after treatment with VAQuFrF (dose: 50 µg/10⁶ cells), (d) after treatment with IL-6+VAQuFrF(dose: 5 ng/10⁶ cells+50 µg/10⁶ cells). For the combined treatment IL-6 was added 2 hours before the test substance. For the expression of the membrane IL-6R the signal intensity (geometric mean of the fluorescence intensity x counts) was used as parameter. This parameter was measured at 24 and 48 hours after incubation. The signal intensity of the treated samples, expressed in percentage was compared with that of untreated samples, which were taken as 100%.

Table 3 presents the mean values of the membrane expression of Interleukin -6 receptor in the cell lines LP-1, RPMI-8226 and OPM-2.

Cell lines	No treatment	Treatment with		
		IL-6	VAQuFrF	IL-6 + VAQuFrF
LP-1	100	128 ^a	28 ^a	77 ^{ab}
RPMI-8226	100	191 ^a	61 ^a	81 ^b
OPM-2	100	148 ^a	94	116 ^b

No treatment	Treatment with		
	IL-6	VAQuFrF	IL-6 + VAQuFrF
100	168 ^a	34 ^a	81 ^b
100	134 ^a	42 ^a	67 ^{ab}
100	104	92	85

Measurements at 24 h after treatment.

Measurements at 48 h after treatment.

The mean values of three independent measurementa are expressed in percentage of untreated samples (100). a=p<0.05 vs.untreated samples, b=p<0.05 vs. with IL-6 treated samples.

Table 3. Membrane expression of Interleukin-6 receptor in human multiple myeloma cell lines.

The surface expressions of IL-6R in untreated cells of all three cell lines were in the similar range (results are not shown). Exogenous IL-6 increased the membrane expression its receptor significantly ($P<0.05$). VAQuFrF reduced the membrane expression markedly in LP-1 and RPMI-8226 ($P<0.05$), it had no effect in OPM-2. With IL-6+VAQuFrF the values were lower than with IL-6 ($P<0.05$), but higher than after treatment with VAQuFrF.

In cell lines MOLP-8, COLO-677 and KMS-12-BM exogenous IL-6 led to down-regulation of its receptor, signalling the possible process of endocytosis (results are not shown). It is interesting that all three cell lines in which IL-6 upregulated its membrane receptor sourced from blood. To investigate of the membrane expression of IL-6 receptor in the cell lines MOLP-8, COLO-677 and KMS-12-BM additional experiments are planned.

4.4 Inhibition of proliferation of multiple myeloma cells (cytostatic effect). Induction of apoptosis and necrosis in multiple myeloma cells (cytotoxic effect)

Figure 1 and **Figure 2** present the mean values of the proliferation and those of apoptosis/necrosis in six human multiple myeloma cell lines treated with IL-6 or VAQuFrF or Vincristine. The cell lines MOLP-8, LP-1, RPMI-8226, OPM-2 sourced from blood, COLO-677 which is a derivative of RPMI-8226 from lymph node, KMS-12-BM from bone marrow.

To measure the proliferation the following doses were applied (1) IL-6: 0.5 ng/ 10^5 cells, (2) VAQuFrF or Vincristine: 1, 5, 10 $\mu\text{g}/10^5$ cells. To measure apoptosis/ necrosis (1) IL-6: 5 ng/ 10^6 cells, (2) VAQuFrF or Vincristine; 10, 50, 100 $\mu\text{g}/10^6$ cells. The parameters were measured at 24, 48 and 72 hours after incubation with the test substances.

Proliferation: The values of the treated samples are expressed as percentages of the untreated samples and are the average of four independent experiments. Significance was assessed versus untreated samples (100%).

Apoptosis/necrosis: The values are expressed as percentage of total cell numbers and are the average of four independent experiments. In the untreated samples the percentage of apoptotic cells lay in the range of 5-38%, that of necrotic cells 10-35% during 72 hours. There were big differences between the tumour cell lines.

4.4.1 MOLP-8

Proliferation: IL-6 increased the proliferation on average up to 130-155%. Comparison of VAQuFrF with Vincristine: 24 and 48 hours after incubation VAQuFrF at the dose of 5 and 10 $\mu\text{g}/10^5$ cells was more effective than Vincristine. 72 hours after there was no difference between the substances in any dose.

Apoptosis/necrosis: In the untreated tumour cells the values of apoptosis lay either in the range of necrosis or above them. IL-6 treatment did not impair either the apoptosis or necrosis. To measure the effects of the two substances on the apoptosis/necrosis we applied ten times less doses. VAQuFrF increased the apoptosis and necrosis at 5 and 10 $\mu\text{g}/10^6$ cells ($P<0.05$ and $P<0.01$). Vincristine had the same effect as VAQuFrF.

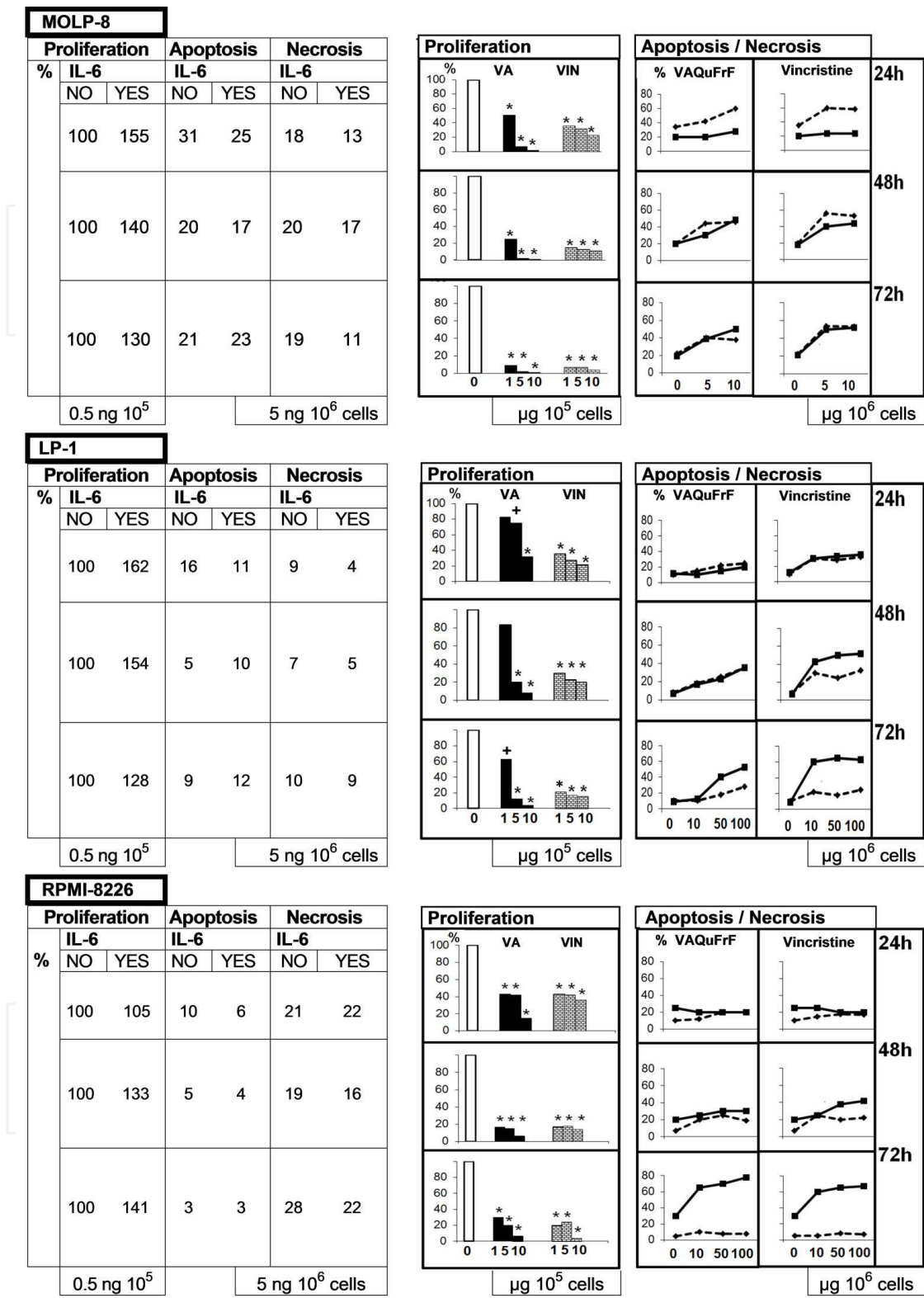


Fig. 1. The effects of IL-6, VAQuFrF extract and Vincristine on the proliferation and on apoptosis/necrosis in human multiple myeloma cell lines MOLP-8, LP-1 and RPMI-8226. The mean values of four independent experiments are expressed as percentage of untreated samples (100%). Proliferation=105 cells. Apoptosis/necrosis=106 cells. - - -● apoptotic cells, - - -● necrotic cells. +P<0.05, *P<0.01 compared with untreated samples (Mann-Whitney U-test).

4.4.2 LP-1

Proliferation: With IL-6 the proliferation rate lay on average between 128-162% during 72 hours. VAQuFrF at the dose of 10 $\mu\text{g}/10^5$ cells inhibited the proliferation more effectively ($P<0.01$) than Vincristine.

Apoptosis/necrosis: In the untreated cell the values of apoptosis lay in the range of necrosis. There was no difference between the values of untreated and with IL-6 treated cells. VAQuFrF did not greatly alter the apoptosis during the investigation time. There was a necrotic effect with a dose dependence from 50 up to 100 $\mu\text{g}/10^6$ cells ($P<0.05$). Vincristine increased the number of apoptotic cells and that of necrotic cells ($P<0.05$), however without dose dependence. The number of necrotic cells was higher than that of apoptotic cells at each dose after 48 and 72 hours ($P<0.05$ and $P<0.01$).

4.4.3 RPMI-8226

Proliferation: IL-6 increased the proliferation on average up to 105-141%.

The test substances inhibited the proliferation: After 24 and 48 hours VAQuFrF in dose of 10 $\mu\text{g}/10^5$ cells was more effective than Vincristine ($P<0.01$). In lower doses (5 $\mu\text{g}/10^5$ cells and 1 $\mu\text{g}/10^5$ cells) VAQuFrF had the same effect as Vincristine.

Apoptosis/necrosis: The values of apoptosis in untreated cells lay below the necrosis. There were no differences between the values of cells treated with IL-6 and that those of untreated cells.

VAQuFrF and Vincristine did not alter the apoptosis. At 72 hours after treatment with both substances the numbers of necrotic cells was higher than those of apoptotic cells at each dose ($P<0.05$ and $P<0.01$).

4.4.4 OPM-2

Proliferation: IL-6 increased the proliferation on average between 110-130%. Comparison of VAQuFrF with Vincristine: The inhibitory effect of VAQuFrF was weaker than that of Vincristine at each dose and at investigated time point. Additional investigation indicate that higher doses increase the effect of VAQuFrF (results are not presented).

Apoptosis/necrosis: In the untreated cells the values of apoptosis lay in the range of necrosis. IL-6 did not impair either the apoptosis or necrosis of the cells. None of the test substances altered the apoptosis. Vincristine increased markedly the number of necrotic cells between 10 and 100 $\mu\text{g}/10^6$ cells without dose dependence after 72 hours of treatment. VAQuFrF was ineffective.

4.4.5 COLO-677

Proliferation: With IL-6 the values of proliferation lay on average between 110-115%.

The inhibitory effect of VAQuFrF was weaker than that of Vincristine in doses of 1 and 5 $\mu\text{g}/10^5$ cells. At the dose of 10 $\mu\text{g}/10^5$ cells the anti-proliferative effects of VAQuFrF and Vincristine was the same at each investigated time point.

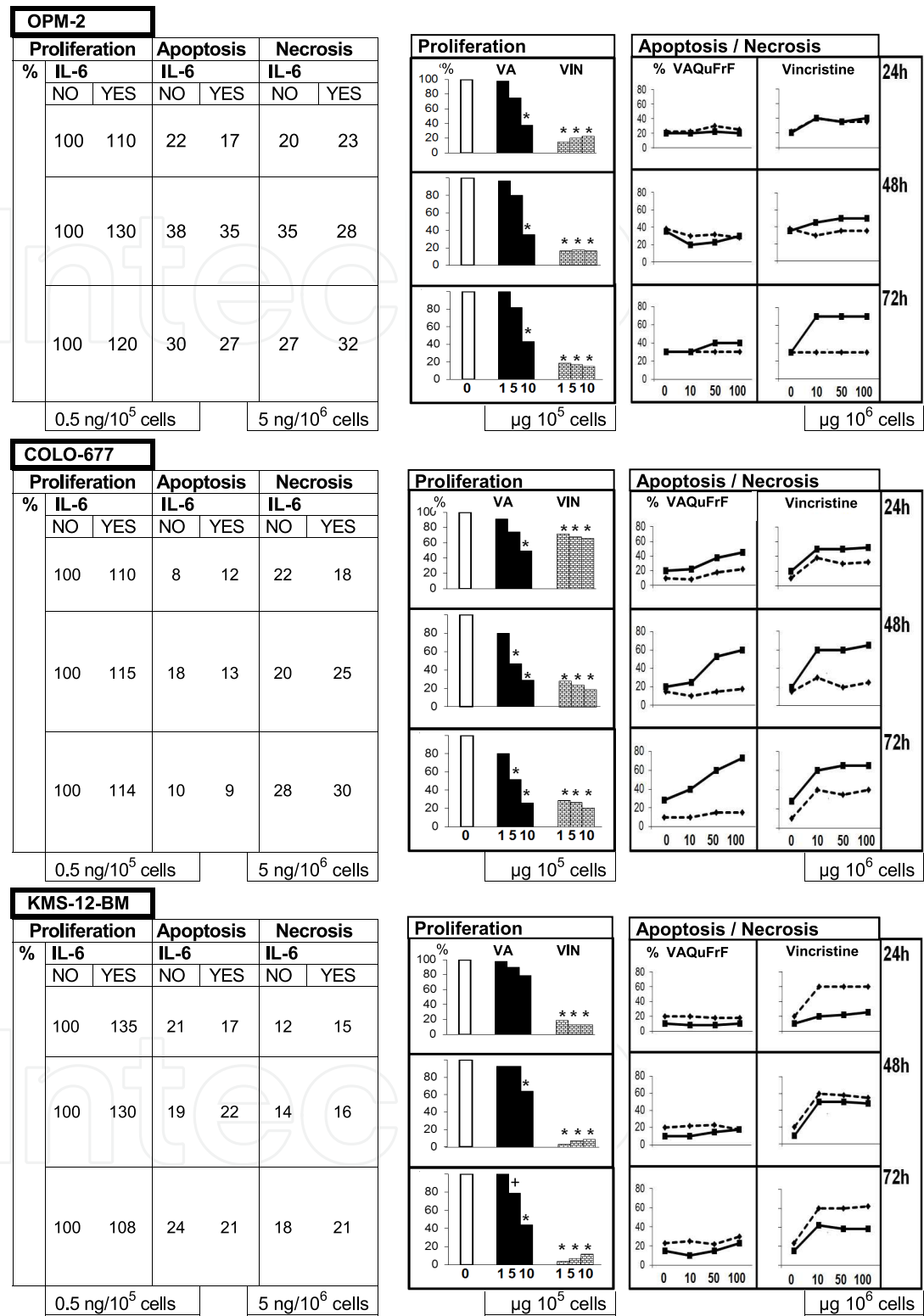


Fig. 2. The effects of IL-6, VAQuFrF extract and Vincristine on the proliferation and on the apoptosis/necrosis in human multiple myeloma cell lines OPM-2, COLO-677 and KMS-12-BM. The mean values of four independent experiments are expressed as percentage of untreated samples (100%). Proliferation=105 cells. Apoptosis/necrosis=106 cells. ●- - -● apoptotic cells, ●- - -● necrotic cells. +P<0.05, *P<0.01 compared with untreated samples (Mann-Whitney U-test).

Apoptosis/necrosis: In the untreated tumour cells the values of apoptosis lay either in the range of necrosis or below them. There was no alteration after treatment with IL-6. VAQuFrF did not alter the apoptosis. There was a necrotic effect with a dose dependence (from 50 up to 100 $\mu\text{g}/10^6$ cells) ($P<0.05$). Vincristine increased the number of apoptotic cells ($P<0.05$). The number of necrotic cells was higher than that of apoptotic at each dose and at each time point ($P<0.05$ and $P<0.01$). The apoptotic /necrotic effects of Vincristine were not dose-dependent.

4.4.6 KMS-12-BM

Proliferation: IL-6 increased the proliferation on average between 108 and 135%. VAQuFrF inhibited the proliferation only in dose of 10 $\mu\text{g}/10^5$ cells after 48 and 72 hours. Vincristine inhibited the proliferation markedly however without dose dependence at each dose and at each investigated time point.

Apoptosis/necrosis: The values of apoptosis in untreated cells lay above the values of necrosis. IL-6 did not alter the apoptosis and necrosis. VAQuFrF did not impair either the apoptosis or the necrosis in this cell line. Vincristine was effective in KMS-12-BM: The number of apoptotic/necrotic cells was significantly higher ($P<0.01$) at each time point, but without dose dependence.

It was reported that that inhibition of cell proliferation is a stronger prognostic indicator than the apoptosis (Stokke et al., 1998). There is a quantitative correlation between the inhibition of proliferation and apoptosis in lymphoma cells (Leoncini, et al., 1993).

Chemotherapeutic agents influence apoptosis through a mitochondrial pathway (Oancea, et al., 2004). Multiple myeloma cells overexpress Bcl-2, a mitochondrial membrane protein which suppresses apoptosis (Chanen-Khan, 2004; Tsujimoto & Shimizu, 2007). VAQuFrF decreases the levels of Bcl-2 in B and T lymphocytes (Duong Van Huyen et al., 2001).

Summarised: In this study the apoptotic/necrotic effect of Vincristine was more marked than its proliferative effect in all cell lines. There was no dose dependence between 10, 50 or 100 $\mu\text{g}/10^6$ cells/ml in both parameters. It is possible that Vincristine impairs the proliferation and apoptosis/necrosis with dose dependence only in a lower dose range.

VAQuFrF first inhibits the proliferation and then the cells die by apoptosis and/or necrosis in the MM cell lines LP-1, RPMI-8226 and COLO-677, confirming the findings with RPMI-8226 presented in a previous study (Kovacs et al., 2006a). The inhibitory effect of VAQuFrF was markedly weaker than that of Vincristine in cell lines OPM-2 and KMS-12-BM at each dose and at investigated time point.

4.5 The effect of VAQuFrF on the proliferation of cells with high proliferation rate

The effect of VAQuFrF with doses of 5 and 10 $\mu\text{g}/10^5$ cells was investigated in cell line RPMI-8226 with high proliferation rate, which remained unaltered during 2-3 days. VAQuFrF was more effective in cells having high proliferation rates than in those with low proliferation rates (Kovacs et al., 2006a). Recently the same findings were observed in cell lines LP-1 and OPM-2 (results are not shown).

4.7 Investigation of cell cycle phases in human multiple myeloma

Cell division consists of mitosis (M) and interphase, which divides into phases G1, G2, and S. Non dividing cells are in the stable resting phase, called the G0 phase. The blockade in the cell division leads to an arrest in the different cycle phases. This arrest appears as an accumulation of the tumour cells.

The following cell lines: were investigated: MOLP-8, LP-1, RPMI-8226, OPM-2, U-266, COLO-677, KMS-12-BM. The myeloma cells were treated (a) with IL-6 (dose: 5 ng/10⁶ cells) (b) with VAQuFrF and with Vincristine (doses: 10, 50, 100 µg/10⁶ cells). The investigation was carried out 24, 48 and 72 hours after treatment.

Table 4 presents the range of the values of untreated cells in different cell cycle phases in percentage in the total viable cell number (100%) 24 hours after treatment. **Table 5** presents the mean values resp. the accumulation of treated cells 24 hours after treatment. For the cell lines RPMI-8226, OPM-2 and U-266 the presented values signalize the effects 48 and 72 hours after treatment with VAQuFrF or Vincristine. For a significant increase or decrease, the percentage of the cell number of treated samples was compared with those of untreated samples.

CELL LINES	G0/ G1	S	G2/ M
MOLP-8	41 - 52	26 - 44	17 - 23
LP-1	50 - 63	27 - 37	8 - 12
RPMI-8226	60 - 78	13 - 28	8 - 15
OPM-2	51 - 62	20 - 38	10 - 19
U-266	48 - 59	17 - 25	9 - 21
COLO-677	36 - 49	42 - 50	10 - 21
KMS-12-BM	45 - 53	39 - 48	3 - 10

Measurements at 24 hour after incubation. Values are expressed in percentage of total viable cell number (100%). Range of four independent experiments.

Table 4. Values of untreated human multiple myeloma cells in different cell cycle phases.

Phases GO/G1: IL-6 did not affect this cell cycle phase. VAQuFrF led to an accumulation of cells in cell line MOLP-8, LP-1 and in KMS-12-BM ($p < 0.05$ and $p < 0.01$). Vincristine had effect in the cell lines MOLP-8 and LP-1 ($p < 0.05$). The both substances were effective at each time point.

Phase S: With IL-6 the number of cells was increased markedly in all cell lines during 72 hours except with OPM-2 and KMS-12-BM. In these cell lines there was no effect at 72 hours. VAQuFrF increased the cell number ($p < 0.01$) in RPMI-8226 and U-266 ($p < 0.05$), Vincristine in RPMI-8226, OPM-2 and U-266 ($p < 0.01$ and $p < 0.05$). Both substances were effective only at 48 and 72 hours after incubation.

CELL	G0/G1						S						G2/M								
	IL-6	VAQuFrF			Vincristine			IL-6	VAQuFrF			Vincristine			IL-6	VAQuFrF			Vincristine		
	LINES	5 ng 10 ⁶ / cells	10	50	100	10	50	100	5 ng 10 ⁶ / cells	10	50	100	10	50	100	5 ng 10 ⁶ / cells	10	50	100	10	50
MOLP-8	no effect	↑ 46	↑ 65	↑ 51	↑ 59	↑ 64		↑ 55	no effect			no effect			no effect	no effect			no effect		
LP-1	no effect	↑ 68	↑ 72		↑ 78	↑ 70		↑ 40	no effect			no effect			no effect	no effect			no effect		
RPMI-8226	no effect	no effect			no effect			↑ 37	↑ 25	↑ 33		↑ 20	↑ 42	↑ 45	no effect	no effect			↑ 49	↑ 52	↑ 50
OPM-2	no effect	no effect			no effect			↑ 45	no effect			↑ 30	↑ 31	↑ 39	no effect	no effect			no effect		
U-266	no effect	no effect			no effect			↑ 39	↑ 22	↑ 31		↑ 23	↑ 23	↑ 28	no effect	no effect			no effect		
COLO-677	no effect	no effect			no effect			↑ 58	no effect			no effect			no effect	↑ 18	↑ 22		↑ 54	↑ 62	↑ 68
KMS-12-BM	no effect	↑ 54			no effect			↑ 53	no effect			no effect			no effect	no effect			↑ 44	↑ 56	↑ 70

Treatment with IL-6(5ng/106 cells) or VAQuFrF or Vincristine(10,50,100 µg/106 cells); The investigation was carried out 24, 48 and 72 hours after treatment. Evaluation of three or four independent experiments ↑=accumulation. The numbers present the mean values in percentage.

Table 5. Accumulation of human multiple myeloma cells in different cell cycle phases.

Phases G2/M: IL-6 treatment led to accumulation of cells in LP-1, RPMI-8226 and OPM-2 after 48 and 72 hours. VAQuFrF was effective in COLO-677 (p < 0.05). Vincristine led to marked increase of the cell numbers in cell lines RPMI-8226, COLO-677 and KMS-12-BM (p < 0.01).

With IL-6 the cell number of each cell line was enhanced in the S phase and in some cell lines in the G2/M phase too. This means that either the DNA synthesis of the cells is increased or the cells are arrested in these cell cycle phases. In this investigation IL-6 led to high proliferation in all cell lines indicating an increased DNA synthesis. This could lead with to arrest in the cycle phase G2/M. In fact we found the accumulation of cells 48 and 72 hours after treatment in some cell lines.

Vincristine blocks the mitotic process by binding to tubulin leading to an arrest of the cycle phase in G2/M (El Alaaoui et al., 1997; Lin et al., 1998). In this study Vincristine led to an accumulation of the cells in cycle phase G2/M in only three out of seven multiple myeloma cell lines. It was effective in S and in G0/G1 phases of five cell lines, indicating that Vincristine also affects these cycle phases. It is interesting that it was effective both in the S and in G2/M cycle phases of the cell line RPMI-8226. VAQuFrF extract had the same effects as Vincristine in five out of seven tumour cell lines; however in a higher dose range. We postulated that different tumour cell lines from the same disorder (multiple myeloma) show a different sensitivity to Vincristine or VAQuFrF.

The inhibition of the G0/G1 phases in different malignancies correlates with anti-proliferative substances (El-Sherbiny et al., 2000; Pellizaro et al., 2008). In fact VAQuFrF blocked the cells in the G0/G1 phases in cell lines MOLP-8 and LP-1 and also inhibited the cell proliferation.

5. Summary and conclusion

In this experimental study we compared **Viscum album (Mistletoe) extract** and **Vincristine** in several human multiple myeloma cell lines using the parameters: (a) the IL-6 production, (b) the IL-10 production, (c) the expression of membrane IL-6 receptor, (d) the proliferation, (e) the apoptosis/necrosis, (f) the cell cycle phases. The following parameters were measured in a “package” i.e. they measured simultaneously: (a) the IL-6 production, (b) the IL-10 production, (c) the proliferation, (d) the apoptosis/necrosis.

The parameters were measured at different times (24, 48 and 72 hours) after incubation with VAQuFrF and Vincristine. Interleukin-6 is a major proliferative factor for the malignant plasma cells (multiple myeloma cells). Therefore this cytokines was measured parallel to the test substances.

Viscum album QuFrF (VAQuFrF) is an experimental drug that is not yet used in the treatment of tumour patients. For this reason it was necessary and important to compare with a well-known clinic-substance. Vincristine is used mainly in combination with other chemotherapeutic substances in the therapy of multiple myeloma.

5.1 Key results

- a. Interleukin-6 leads to a markedly increased IL-10 production. Interleukin-6 upregulates markedly the expression of its membrane receptor (IL-6R). IL-6 increases the proliferation and it is effective in the S cell cycle phase. IL-6 does not affect the apoptosis/ necrosis.
- b. Neither VAQuFrF nor Vincristine produce IL-6 or lead to an enhanced IL-10 production in any cell line. VAQuFrF and Vincristine inhibits the spontaneous IL-10 production. Both substances **counteract** the increased IL-10 production induced by IL-6. The effects of the two substances are comparable. The results indicate that the effect of the both test substances on the IL-10 production is due to their apoptotic/necrotic effects.
- c. VAQuFrF **inhibits** the membrane expression of IL-6 receptor. VAQuFrF **counteracts** the enhanced membrane expression of this receptor induced by IL-6.
- d. The **cytotoxic effect** of Vincristine is more marked than its **cytostatic** effect in all cell lines.
The effect of VAQuFrF focuses on the inhibition of proliferation (**cytostatic effect**). VAQuFrF first inhibits the proliferation and then the cells die by apoptosis and/or necrosis.
- e. VAQuFrF inhibits the proliferation in cells with **high proliferation rate more effectively** than in those with **low growth** rate.
- f. **Cell cycle phases:** VAQuFrF extract has the same effect as Vincristine in five out of seven tumour cell lines, however in a higher dose range.

The findings indicate that VAQuFrF extract could be a novel drug in the therapy of multiple myeloma.

To assess the effective doses of *Viscum album* QuFrF extract and to transfer these doses to the in vivo situation.

The *Viscum album* QuFrF (VAQuFrF) is an aqueous and unfermented extract of mistletoe plants growing in the oak tree. It contains 2 µg lectin and 10 µg viscotoxin in 10 mg/ml. It

was tested in dose range of 10, 50, 100 $\mu\text{g}/10^6$ cells. 10 μg extract contains 0.002 μg lectin + 0.01 μg viscotoxin, 50 μg extract contains 0.01 μg lectin + 0.05 μg viscotoxin, 100 μg extract contains 0.02 μg lectin + 0.1 μg viscotoxin.

Dosage of Vincristine in the therapy for multiple myeloma: In combination with other chemotherapeutic agents as a part of the VAD regimen 0.4 mg/day intravenously (400 $\mu\text{g}/\text{day}$). In these experimental studies Vincristine was applied in dose range of 10, 50, 100 $\mu\text{g}/10^6$ cells. The effects of Vincristine on the proliferation and the apoptosis/necrosis were in each cell line without dose dependence. This means that these doses lay in a saturation range. It is planned to investigate the effects of Vincristine in a lower dose range.

The efficient dose range for VAQuFrF lies between 50 and 100 $\mu\text{g}/10^6$ cells (0.01 μg lectin + 0.05 μg viscotoxin and 0.02 μg lectin + 0.1 μg viscotoxin). These data concern the cell lines LP-1, RPMI-8226 and COLO-677. For cell lines OPM-2, KMS-12-BM the dose range lies higher. In MOLP-8 cell line VAQuFrF inhibits the proliferation more effectively than Vincristine.

Our findings suggest that the in vivo effective (active) dose for VAQuFrF will be about 10-20 times higher than that of Vincristine.

6. Future research

1. **Viscum album** (VA) QuFrF extract contains two active components: mistletoe lectins (I, II, III) and viscotoxins.
Question: Which component is responsible for the effects of this extract? Is this the **lectin(s)** or **viscotoxin** or **both**? Further study is planned to clarify this **question**.
The non-fermented preparation from VAQuFrF extract contains 2 μg lectin and 10 μg viscotoxin in 10 mg/ml (**ratio between lectin and viscotoxin: 0.2**).
The **ratio between lectin and viscotoxin from the fermented preparations is 0.06** respectively **0.08**. The fermented preparations are used either alone or in combination with chemo /radiotherapy in the treatment of tumour patients. The extract presented in this study is an experimental drug that is not yet used in the treatment of tumour patients.
2. The results presented in this study indicated that VAQuFrF could effect the membrane expression of IL-6 receptor by antagonism. **Question:** Is this substance a competitive- or a non-competitive antagonist? Additional experiments will give answer to this (important) **question**.
3. To investigate more human multiple myeloma cell lines.
To investigate **tumour cells isolated from bone marrow** of patients with multiple myeloma.
4. To investigate the anti-proliferative and apoptotic/necrotic effects of Vincristine in a lower dose range, respectively the anti-proliferative and apoptotic/necrotic effects of VAQuFrF extract in a higher dose range.

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