Antiproliferative Effects of Mistletoe (*Viscum album* L.) Extract in Urinary Bladder Carcinoma Cell Lines

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Abstract. *Background: The aim of the study was to evaluate the antiproliferative potency of Viscum album extract (VA-E) in human bladder carcinoma cell lines with regard to its possible use for intravesical therapy of superficial bladder cancer. Materials and Methods: Proliferation (MTT-test or ^{3}H-thymidine incorporation), necrotic disintegration (^{3}H-thymidine release of prelabelled cells) and portions of apoptotic and/or necrotic cells (Annexin-V binding, propidium iodide (PI) labelling and DNA-fluorescence profiles by flow cytometry) were measured in four different human bladder carcinoma cell lines (T24, TCCSUP, J82 and UM-UC3) cultured in vitro. Results: Antiproliferative effects of VA-E were observed in the four bladder carcinoma cell lines tested. Metabolic activity could also be completely abrogated by short-time contact of the cells with VA-E. Apoptosis and necrosis, as underlying mechanisms of action, were differentially expressed by the different cell lines. Conclusion: VA-E and cytotoxic proteins, i.e., mistletoe lectins (ML) and viscotoxins (VT), were able to block the growth of bladder carcinoma cells. Together with the immunomodulating properties of VA-E, the observed antiproliferative potency might give a rationale for the topical intravesical application of VA-E for the treatment of superficial bladder cancer.*

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Approximately 80% of all bladder cancer is confined to the epithelium and to the submucosa and is termed superficial. This group of tumors is heterogeneous with differing natural histories (1). Superficial bladder tumors have a strong tendency to recur, with reported 5-, 10- and 15-year recurrence rates of 65%, 81% and 88%, respectively (2). In the majority of cases, the recurrence is also superficial, but in 5% to 30% of patients, the recurrent tumor invades into the muscular layer of the bladder (*i.e.,* progression). Once the tumor has penetrated the muscle, there is an increased risk of tumor metastasis and death from bladder cancer. This has led to a strategy of vigilant surveillance and follow-up of superficial bladder tumors.

Therefore, different adjuvant instillation therapies with cytostatic and immunomodulating substances, such as adriamycin, thiotepa, mitomycin, epirubicin, doxorubicin and bacillus Calmette-Guérin (BCG), have been undertaken to prevent relapses (3, 4). Currently, the intravesical application of BCG has proved to be the therapy of choice for recurrent superficial transitional cell carcinoma of the bladder after surgical resection (5, 6). However, this therapy has been associated with local and systemic side-effects and even rare cases of death (7). Unfortunately, not all patients respond to these treatments so that new therapeutic approaches with better patient tolerance for the patients are needed.

Aqueous extracts of mistletoe (*Viscum album* L.) have been in use for the treatment of cancer patients in Europe for more than 80 years, with alleged antitumor effects (8). ML I, II, III and VT are regarded to be the main active components, exerting antiproliferative, cytotoxic and immunomodulatory effects (9). Immunomodulation with mistletoe extracts includes stimulation of the release of different cytokines, but also the activation of natural killer

cells, large granular lymphocytes and antibody-dependent cell-mediated cytotoxicity (9-13), which are believed to play crucial roles in therapy with BCG. The antiproliferative activity of mistletoe is based mainly on the apoptotic effect of ML and necrosis induced by VT (14-16).

These pharmacological features of mistletoe extracts might justify the attempt to evaluate the effectiveness of commercial mistletoe extracts for the topical therapy of superficial bladder cancer. In an animal model, intravesically-applied VA-E reduced the incidence of chemically-induced bladder tumors (17). The clinical observation of single cases of intravesical instillation therapy with VA-E resulted in favorable outcomes in the absence of any severe side-effects in all the patients observed (18). A study of the antiproliferative effect of VA-E on urinary bladder carcinoma cell lines is presented here to provide experimental data for the evaluation of intravesical VA-E therapy.

Materials and Methods

Mistletoe extracts and toxins. The VA-E Iscador Qu spezial 200 mg (fermented extract from 200 mg plant material in 1 ml, standardized for the total amount of ML; lot number CH. B. 0135; Verein für Krebsforschung, Arlesheim, Switzerland) was used. The presence of 17.1 μg/ml ML and 505 μg/ml VT was measured in the extract by ELISA and HPLC, respectively (19, 20). The galactose-binding ML I and the N-acetylgalactosamine (galNAc)-binding ML III were kindly provided by Prof. U. Pfüller (University Witten/Herdecke, Germany). The RNA synthesis inhibitor actinomycin D (Act D), the DNA synthesis inhibitor mitomycin C (Mit C) and the protein kinase C inhibitor staurosporine (STPN) were purchased from Sigma (Deisenhofen, Germany).

Cell culture. The human urinary bladder carcinoma cell lines T24 (grade III carcinoma, p53 mutation), TCCSUP (grade IV carcinoma), J82 (transitional cell carcinoma, ras [H-ras] oncogene-positive) and UM-UC3 (transitional cell carcinoma) and lymphoblastic Jurkat T cells were used. T24, TCCSUP and Jurkat cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany and J82 and UM-UC3 from the American Type Culture collection. The jurkat cells were maintained in culture in RPMI 1640 supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS, heat-inactivated) and 1% penicillin-streptomycin. The T24 cell line was cultured in RPMI 1640 containing 25 mM Hepes, 2 mM glutamine, 10% FCS (heat-inactivated) and 1% penicillin-streptomycin. TCCSUP, J82 and UM-UC3 cells were cultured in MEM (Earle's modification) supplemented with 4 mM L-glutamine, 1 mM pyruvate, 25 mM Hepes, 1% penicillin-streptomycin and 10% FCS (heat-inactivated). The adherent urothelial bladder cancer cells were maintained in exponential growth and harvested by brief exposure to trypsin-EDTA solution (Sigma, Switzerland).

Cell proliferation, apoptosis, necrosis. Pre-evaluation of the test conditions showed confluence of the cells after 6 days (J82, T24). After 4 days, none of the four cell lines had reached full confluence. The incubation time was therefore limited to 4 days. At time 0, the confluence of the four cell lines used in the MTT-test was very low.

All assays were performed at least in triplicate with cells in exponential growth after adhesion to the bottom of the plates. Cell proliferation was measured indirectly by use of a colorimetric assay based on the reduction of the tetrazolium salt MTT (Cell Proliferation Kit I, Roche Diagnostics, Rotkreuz, Switzerland) in viable cells. The assay started with 10^3 cells per well seeded into a 96-well plate, incubated with different concentrations of VA-E and viability was measured at time 0, 48 h and 96 h. DNA synthesis directly correlating with the proliferative activity of the cells was quantified by measuring ^{3}H-thymidine incorporation into a insoluble fraction of the cells. Briefly, $2.5x10^3$ cells per well seeded into a 96-well plate were incubated in 200 μl medium with appropriate concentrations of VA-E. At times 0, 24 h and 48 h, 20 μl of (methyl-^{3}H)-thymidine (18.5 kBq, specific activity 4.62 GBq/nmol; Amersham Biosciences, Otelfingen, Switzerland) were added and the cells were incubated for 30 min at 37˚C before radioactivity retained on glass fiber filters in a cell harvester was measured, as described in (25).

For the assessment of necrotic disintegration by VA-E, $3x10^6$ cells in 75-cm^2 plates were labelled with ^{3}H-thymidine (1100 kBq in 5 ml culture medium) over 24 h and then washed thoroughly (21). These pre-labelled cells were used to measure the radioactivity released into the supernatant in VA-E dose-response experiments.

Flow cytometric analyses, of at least 5,000 events, were performed on an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany). The viability of the cultured cells was analyzed by binding of Annexin-V to phosphatidyl serine on cells undergoing cell death, as described previously (22). Briefly, after washing the cultured cells with PBS and resuspension in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), 100 μl of cells were stained with 5 μl FITC-labelled Annexin-V (PharMingen, San Diego, CA, USA) and propidium iodide (PI; 5 μg/ml; Sigma). To measure the sub-G1 peak of the cultured cells (which reflects the hypodiploid DNA content of apoptotic cells), the cells were pelleted and resuspended in hypotonic fluorochrome solution (PI, 50 μg/ml in 0.1 % sodium citrate and 0.1 % Triton X-100; Sigma) and kept at 4˚C in the dark overnight before analysis, as described (15). To investigate the cytotoxic effects of VA-E or the other cytotoxic compounds by flow cytometry, concentrations previously shown to be effective, *i.e.,* 10, 50 and/or 100 ng/ml of ML I and ML III, 1 μg/ml of Act D and STPN, 10 μg/ml of Mit C or 10, 100 and 500 μg/ml of VA-E were used (22, 23).

Statistics. The data are presented as means±SD. A comparison of results was performed by a non-parametric two-sample Wilcoxon signed rank test. A value of $p<0.05$ was considered to indicate significant differences between the groups.

Results

The effect of VA-E was measured in four urinary bladder carcinoma cell lines (J82, T24, UM-UC3, TCCSUP). Cell growth was assessed by measuring metabolic activity, which reflects viability in the MTT-test in the course of 96 h of continued exposure to the substances tested. Dose-response experiments showed antiproliferative effects of VA-E (Figure 1); 100 μg/ml VA-E almost completely abrogated the metabolic activity of the four cell lines. The sensitivity was highest in UM-UC3 and lowest in T24. Mit C,

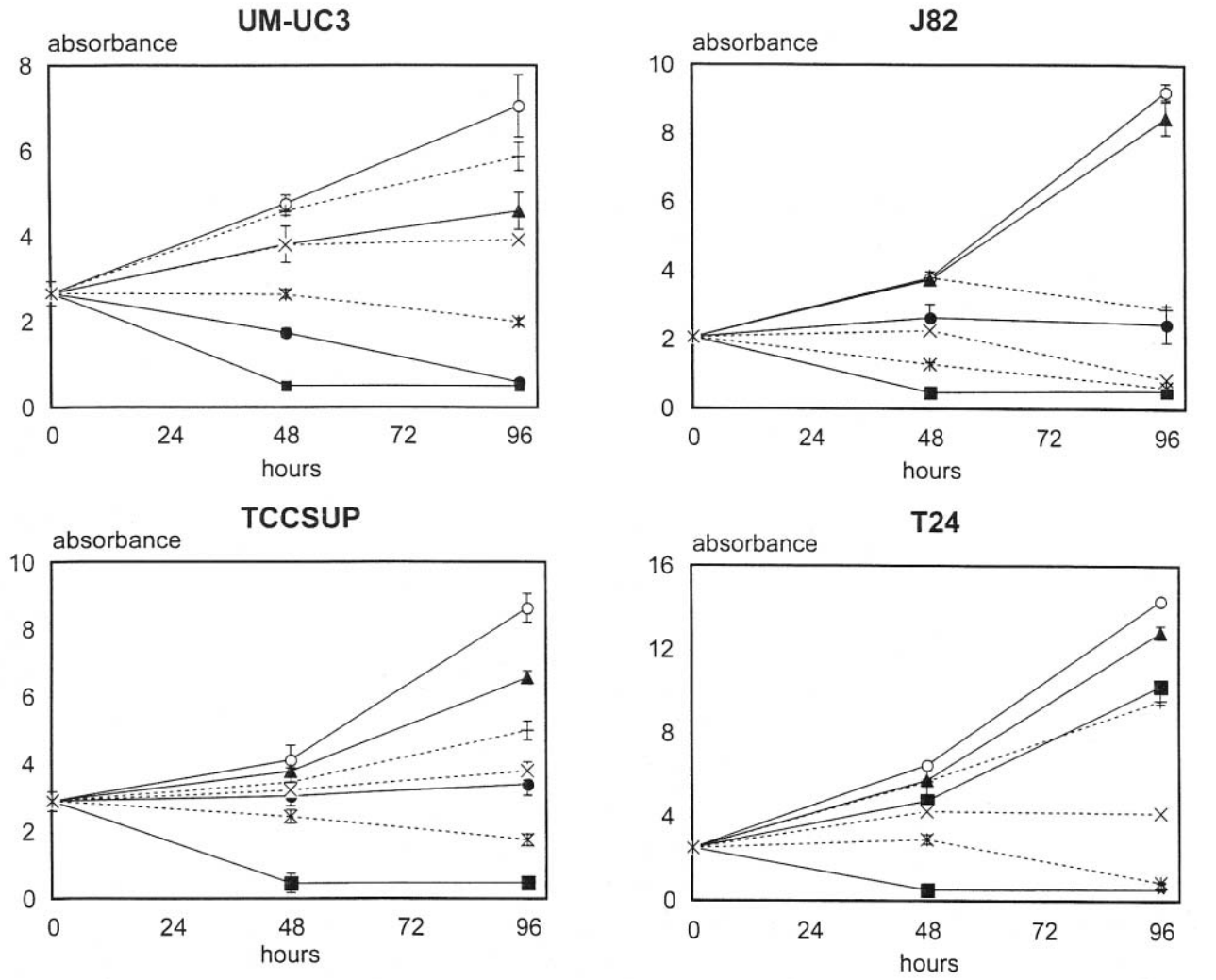

Figure 1. *Growth of urinary bladder carcinoma cell lines J82, UM-UC3, T24 and TCCSUP in the presence of VA-E (solid line; ○: control, ▲: 10 μg/ml, ●: 100 μg/ml, ■: 1000 μg/ml) and mytomycin (hatched line; +: 0.01 μg/ml, x: 0.1 μg/ml, *: 1 μg/ml) in the course of 4 days (see Materials and Methods).*

introduced into the test series as a positive control, showed the lowest activity in UM-UC3, high activity in J82 and TCCSUP and intermediate strength in T24. The sensitivities of the cells to VA-E did not correspond to their sensitivities towards Mit C.

Intravesical therapy of the bladder brings VA-E in direct contact with the urothelium only for a limited time. To imitate this situation *in vitro,* the layers of adherent bladder carcinoma cells were exposed to VA-E for only 2 h. By this treatment, the proliferative potency (DNA synthesis) of the four urinary bladder carcinoma cell lines was suppressed in a dose-dependent manner (Figure 2). This short-time effect, induced by concentrations of VA-E between 0.1 to 16 mg/ml, was most pronounced in the UM-UC3 cells (IC_{50}=0.38±0.03 mg/ml). The corresponding IC_{50} of VA-E in the T24, J82 and TCCSUP cells were 1.64±0.27, 1.88±0.17 and 1.92±0.17 mg/ml, respectively.

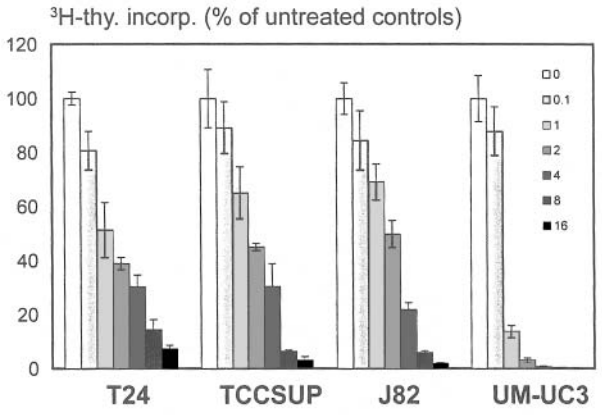

Figure 2. *Proliferation (^{3}H-thymidine incorporation, % of untreated controls) of T24, TCCSUP, J82, UM-UC3 urinary bladder carcinoma cells after 2 h of treatment with different concentrations of VA-E (0 [control], 0.1, 1, 2, 4, 8, 16 mg/ml).*

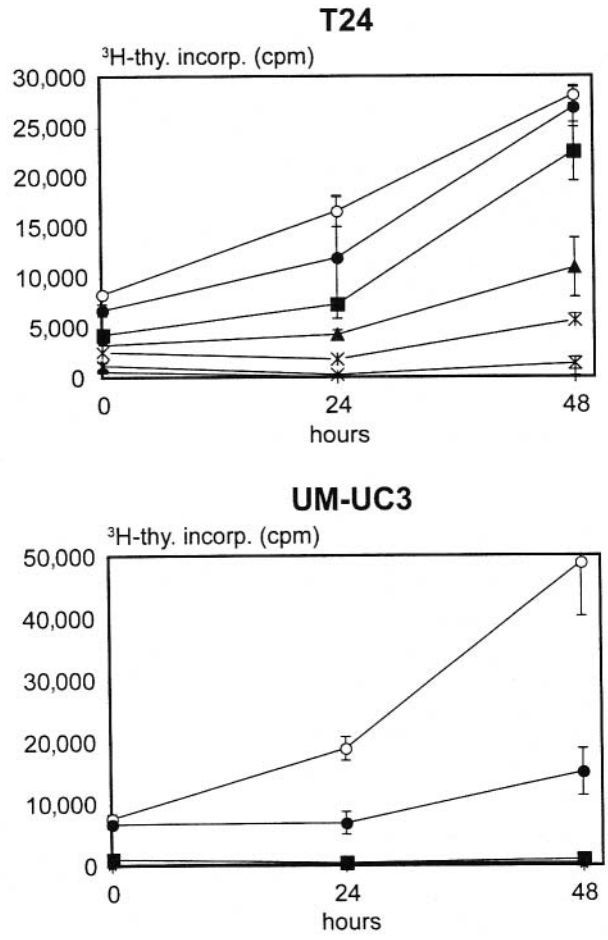

Figure 3. *Proliferation (^{3}H-thymidine incorporation) of the urinary bladder carcinoma cell lines T24 and UM-UC3 pretreated by VA-E (○: untreated control; ●: 0.1 mg/ml; ■: 1 mg/ml; ▲: 2 mg/ml; *: 4 mg/ml;, x: 8 mg/ml; +: 16 mg/ml). After 2 h of treatment with VA-E, the cells were washed and proliferation was measured directly afterwards (0 h) and after 24 and 48 h.*

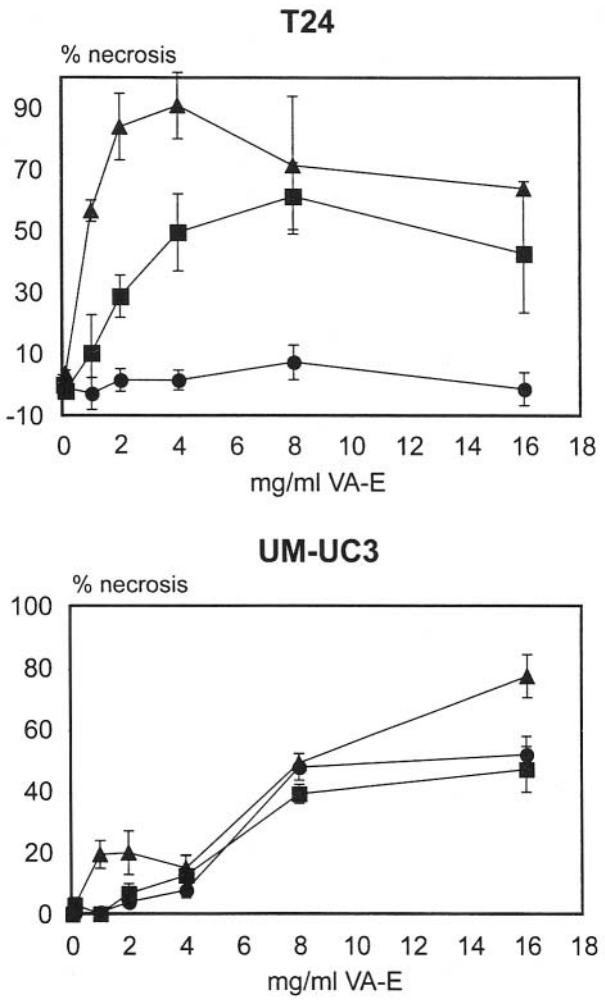

Figure 4. *Induction of necrotic disintegration of UM-UC3 and T24 urinary bladder carcinoma cells after 2-h (●), 8-h (■) and 24-h (▲) exposure to different concentrations of VA-E (100% necrosis corresponds to the effect of 1% TritonX100).*

Further growth of the 2-h-treated cells was followed in fresh medium for 48 h after elimination of VA-E. The UM-UC3 cells heavily impaired by 2-h exposure to 1 to 16 mg/ml VA-E did not recover after 48 h in fresh medium (Figure 3). Only the lowest concentration of 0.1 mg/ml VA-E allowed for a slow recovery of cell growth. In T24, the two highest concentrations of VA-E (8 and 16 mg/ml) appeared to have irreversibly damaged the cell population. The lower concentrations allowed the cells to recuperate growth gradually, however starting at reduced levels. Almost the same kinetics observed in T24 could also be observed in the J82 and TCCSUP cells (results not shown).

Necrotic disintegration of cells was evaluated by measuring the release of ^{3}H-thymidine from prelabelled cells (Figure 4). Incubation of T24 cells for 2 h did not damage the cells over the whole dose range of VA-E from 1 to 16 mg/ml. Only after 8- and 24-h contact with VA-E did a dose- and time-dependent necrotic disintegration of the cells take place. The UM-UC3 cells, however, were disintegrated dose-dependently even after 2 h of treatment with VA-E. Incubation for 8 h and 24 h did not result in a considerable increase in the portion of lysed UM-UC3 cells.

To further examine the underlying mechanisms of the antiproliferative effect of VA-E, the basic mechanisms of cell death were analyzed by flow cytometry. After 48-h incubation with 500 μg/ml VA-E, elevated portions of early (Annexin-V^+/PI^-) and late (Annexin-V^+/PI^+) apoptotic cells could be observed in the four carcinoma cell lines tested (Table I). The T24 and J82 cells reacted most

Table I. *Cell death in urinary bladder carcinoma cells T24, TCCSUP, J82, UM-UC3 (n=3) and lymphoblastic Jurkat cells after 48-h exposure to VA-E and ML I.*

Parameter	Additive	% of total cells				
		T24	TCCSUP	J82	UM-UC3	Jurkat
AV^+PI^+	Medium control	9.7±4.7	7.3±2.1	11.7±10.7	3.0±1.0	18.7
	VA-E 10 μg/ml	9.7±2.9	7.3±3.2	4.5±0.7	6.7±6.4	23.9
	VA-E 100 μg/ml	18.7±4.0*	10.7±4.7	18.3±15.3	6.0±2.6	81.2
	VA-E 500 μg/ml	40.0±20.1*	17.0±6.0*	49.0±17.5*	18.0±5.6*	85.7
	ML I 10 ng/ml	7.0±2.6	8.7±3.5	10.0±5.2	7.7±9.0	66.8
	ML I 50 ng/ml	17.7±6.0*	9.7±4.0	37.0±23.4*	9.3±4.0	82.6
AV^+PI^-	Medium control	1.7±1.2	3.7±1.5	2.3±1.2	2.0±1.0	6.3
	VA-E 10 μg/ml	1.2±1.1	3.1±1.0	2.0±1.4	2.0±1.7	12.6
	VA-E 100 μg/ml	9.2±6.2*	4.3±2.1	3.7±2.1	3.0±1.0	12.5
	VA-E 500 μg/ml	21.3±21.9*	11.0±8.5*	17.0±15.1*	8.3±3.5*	8.4
	ML I 10 ng/ml	2.0±1.7	4.7±2.3	1.7±1.2	2.0±1.7	18.7
	ML I 50 ng/ml	6.6±7.4*	5.0±2.0	10.3±8.5*	3.0±2.6	10.3
Sub-G1-peak	Medium control	1.0±0	4.0±1.0	6	2	6
	VA-E 10 μg/ml	1.3±0.6	4.7±1.5	8	–	30
	VA-E 100 μg/ml	24.0±3.6*	4.3±1.2	23	4	89
	VA-E 500 μg/ml	67.3±5.5*	6.7±0.6*	50	15	89
	ML I 10 ng/ml	5.3±4.0	4.3±1.2	11	3	10
	ML I 50 ng/ml	54.0±4.4*	4.7±0.6	46	–	91

AV^+PI^- : cells binding Annexin-V (AV) to phosphatidylserine on the outer leaflet of the cell membrane and excluding PI (early apoptosis). AV^+P^+ : cells binding AV and taking up PI (late apoptosis and/or primary necrosis). Sub-G1-peak: cells containing hypodiploid DNA (apoptosis). *Significant difference from control ($p<0.05$).

strongly, whereas the number of dead TCCSUP and UM-UC3 cells increased only slightly. Using ML I at 50 ng/ml, the J82 and, to a lesser extent, T24 cells underwent cell death, while the TCCSUP and UM-UC3 cells showed no, or only weak, reactions. A strong induction of cell death occurred in the Jurkat cells even at a concentration of 100 μg/ml VA-E and 10 ng/ml ML I, which did not, or only slightly, affect the carcinoma cell lines. The results were confirmed by determination of the DNA profile in the cultured cells. The greatest number of cells with a hypodiploid (apoptotic) DNA content were observed in the Jurkat, J82 and T24 cells incubated with VA-E at 100 and 500 μg/ml and ML I at 50 ng/ml.

The TCCSUP and UM-UC3 cells were less sensitive to the cytotoxic potential of mistletoe, as compared to the Jurkat, J82 and T24 cells. As shown in Table II, the galNAc-binding ML III was also ineffective in inducing cell death in the TCCSUP cells. Using other toxins, such as Act D, Mit C and STPN, this ML-insensitive cell line underwent cell death, while the ML-sensitive T24 cells, in contrast, did not respond to Act D and responded weakly to Mit C as compared to the TCC-SUP cells.

Table II. *Cell death in urinary bladder carcinoma cells T24 and TCCSUP after 48-h incubation with ML III from V. album (100 ng/ml), Act D (1 μg/ml), Mit C (10 μg/ml) and STPN (1 μg/ml).*

Parameter	Additive	% of total cells	
		T24	TCCSUP
AV^+PI^+	Medium control	22.2±4.3	10.4±2.9
	ML III	57.0±18.2*	14.7±2.9
	Act D	25.3±4.6	64.7±3.0*
	Mit C	40.5±4.5*	65.1±3.8*
	STPN	78.4±11.3*	71.9±2.2*

AV^+P^+: AV and PI binding cells (late apoptosis and/or primary necrosis). *Significant difference from control ($p<0.05$).

Discussion

VA-E suppressed growth in the four bladder carcinoma cell lines tested. The induction of apoptosis could be detected in response to VA-E treatment. Differences in the sensitivity of the four cell lines to the antiproliferative

effects of VA-E, however, did not correspond to the differences in sensitivity towards the apoptosis-inducing potency of VA-E. Growth was distinctly inhibited by VA-E in the two cell lines, UM-UC3 and TCCSUP, but these lines underwent apoptosis only to a minor degree.

Necrosis was detected in UM-UC3 cells only at higher concentrations of VA-E. The low level of necrosis in the flow-cytometric detection of the membrane permeability of these cells after 48-h treatment might be explained by the detachment and dissolution of necrotic cells. Complete resistance to the primary necrotic action of VA-E was registered in the T24 cells, which highly expressed apoptotic signals. Reduction of cell growth reflects either a decreased proliferation rate or enhanced cell death by necrosis or apoptosis. It cannot be excluded that the cell division cycles were slowed down.

The VA-E employed in the present experiments contained 85.5 μg ML per g extracted plant and 2.5 mg/g VT. The ED_{50} values of antiproliferative ML are reported to range from 10 pg/ml to 12.8 μg/ml, depending on the isoform of ML and on the cell line used (24, 25). The ED_{50} values of VT range from 0.2 to 4.6 μg/ml (20, 26). The concentrations of ML and VT present in the assays with the bladder carcinoma cells presented here ranged from 0.86 to 1.370 ng/ml and from 0.025 to 40 μg/ml, respectively, and thus easily reached the cytotoxic range known from the literature. Cytotoxicity was assumed to be responsible for the antitumoral activity observed with topical treatment of chemically-induced bladder tumors in a murine system (17). The present results on bladder carcinoma cells showed that cytotoxic effects might be expected at the concentrations and exposure times of VA-E commonly used for instillation therapy of superficial bladder cancer.

Our comparative study on the induction of cell death by VA-E, ML I, ML III and three common chemotherapeutic agents points to cell-specific characteristic patterns of sensitivity to the different cytotoxic agents. In some cell lines less sensitive to distinct chemotherapeutic drugs, VA-E might be effective on the basis of the different mechanisms of action associated with mistletoe compounds. Three different isoforms of ML (ML I, ML II, ML III) and four VT (A1, A2, A3, B) were reported to be present in the VA-E used here (20, 27), each isoform having its specific and characteristic pharmacological properties (10, 20, 24, 26, 28, 29). In the topical treatment of superficial bladder cancer, the presence of various cytotoxic and immuno-modulating agents with different mechanisms of action might be beneficial. Evidence from preclinical experimental results and from first observational data from clinical applications (18) suggests that VA-E may represent a potential intravesical therapeutical option for patients with superficial bladder cancer and should be evaluated in phase II clinical trials.

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