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VISCUM ALBUM EXTRACTS ISCADOR®P AND ISCADOR®M COUNTERACT THE GROWTH FACTOR INDUCED EFFECTS IN HUMAN FOLLICULAR B-NHL CELLS AND BREAST CANCER CELLS

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Abstract Mistletoe therapy has been implemented in cancer therapy in Germany for the last 30 years, whereby its application in the clinic ranges between 46 to 70% of patients with malignant diseases including follicular B Non-Hodgkin's Lymphoma (B-NHL) and breast cancer. In the majority of cases, mistletoe extracts (also named *Viscum album* (VA) extracts) are given as an adjuvant therapy, e.g. in combination with chemotherapy. The use of VA extracts in the treatment of follicular B-NHL is still subject to controversial discussions. On the one hand, various studies indicated that intravenous application caused elevated interleukin-6 (IL-6) serum levels in patients, which would be fatal for B-NHL patients since IL-6 is a proliferation factor for neoplastic B-cells. On the other hand, we and other groups have shown that the VA extract Iscador®P does not provoke an IL-6 deregulation in follicular B-NHL cell lines and that subcutaneous application of Iscador®P does not cause increased IL-6 serum levels. Here we investigated the influence of the VA extract Iscador®P on the IL-6 induced proliferation of two follicular B-NHL cell lines. Interestingly, Iscador®P had a more profound inhibitory effect on the proliferation of follicular B-NHL cell lines if these were stimulated with IL-6. Thereby, Iscador®P acts differently on the investigated cell lines. For instance, in the WSU-NHL B-NHL cell line, Iscador®P and IL-6 co-treatment caused a bax up-regulation, which correlated with an increased number of apoptotic cells. In contrast, an increased number of apoptotic cells was not detectable in Sc-1 cells, albeit the proliferation rate of Iscador®P and IL-6 co-treated cells was markedly decreased. The observation that VA extracts are more potent if applied with proliferatory stimulus was also observed for the human breast cancer cell line MDA-MB-468-HER2. Here, the VA extract Iscador®M efficiently counteracts the EGF induced proliferation and migration of these cells. In summary, our data provide new insights in the potency of VA extracts for cancer treatment.

Keywords: *Viscum album* extracts, follicular B-Non-Hodgkin's Lymphoma, breast cancer, growth factor, proliferation, cell movement

Resumen ***Los extractos de* Viscum album *Iscador®P e Iscador®M contrarrestan los efectos inducidos por factores de crecimiento sobre células humanas de B-NHL folicular y de cáncer de mama.*** La terapia con muérdago ha sido utilizada en Alemania desde hace 30 años, y su aplicación en la clínica varía entre un 46 y un 70% de pacientes con enfermedades malignas, incluyendo linfoma No-Hodgkin folicular B (B-NHL) y cáncer de mama. En la mayoría de los casos, los extractos de muérdago (también denominados extractos de *Viscum album* (VA)) son suministrados como terapia adyuvante, es decir, en combinación con quimioterapia. La utilización de extractos de VA en B-NHL es todavía controvertida. Por otra parte, varios estudios indicaron que la aplicación intravenosa llevó a altos niveles de interleuquina-6 (IL-6) en suero de pacientes, lo cual podría resultar fatal en pacientes con B-NHL dado que la IL-6 es un factor proliferativo para células B neoplásticas. Por otra parte, nosotros y otros grupos hemos demostrado que extractos Iscador®P de VA no provocan una desregulación de la IL-6 en líneas foliculares de B-NHL y que la aplicación subcutánea de Iscador®P no aumenta los niveles séricos de IL-6. En este trabajo investigamos la influencia del extracto Iscador®P de VA sobre la proliferación inducida por IL-6 de dos líneas foliculares de B-NHL. Se encontró que Iscador®P tuvo un profundo efecto inhibitorio de la proliferación estimulada por IL-6 en estas dos líneas celulares. Por lo tanto, Iscador®P actuaría en forma diferencial sobre distintas líneas celulares estudiadas. Por ejemplo, en la línea de B-NHL WSU-NHL el tratamiento simultáneo con Iscador®P y IL-6 causó un incremento de Bax, que correlacionó con un incremento del número de células apoptóticas. En contraste, no se detectó un incremento de células apoptóticas en células Sc-1, aunque sí se notó una disminución en la proliferación con el mismo tratamiento. La observación de que los extractos de VA son más potentes en células que son estimuladas a proliferar también es válida en la línea de cáncer de mama humano MDA-MB-468-HER2. En este caso el extracto de VA Iscador®M contrarresta eficientemente la proliferación y migración inducidas por EGF. En resumen, nuestros datos proveen nuevas observaciones sobre la potencialidad de los extractos de VA como tratamiento para el cáncer.

Palabras clave: extractos de *Viscum album*, linfoma folicular B Non-Hodgkin, cancer de mama, factor de crecimiento, proliferación, migración celular

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To date, cancer is still the second most prevalent cause of death after cardiovascular diseases in the industrialized world. Thereby, the primary cause of cancer is not attributed to primary tumor formation, but rather to the growth of metastases at distant organ sites[1]. Among the hallmarks of cancer, the progression of the primary and secondary tumor towards unrestricted proliferation and cell migration has been reported to be crucial for the pathogenesis of cancer[2]. Proliferation and migration of tumor cells are chiefly directed by the interplay of hormones, chemokines, cytokines, and growth factors, whereby these factors act via autocrine and paracrine stimulatory loops.

Mistletoe therapy has been implemented in cancer therapy in Germany for the last 30 years, whereby its application in the clinic ranges between 46 to 70% of patients with a malignant disease[3]. In the majority of cases, *Viscum album* (VA) extracts are given as an adjuvant therapy, i.e. in combination with chemotherapy[4]. Clinically proved benefits for the patients are a measurable improvement of life quality, e.g. due to reduced side-effects[5]. Furthermore, VA extracts stimulate the immune system via activation of the Natural Killer Cells (NK-Cells), Monocytes/Macrophages, Granulocytes and T-cells[6, 7].

A previous study by our group clearly showed that treatment of follicular B-Non-Hodgkin's Lymphoma (B-NHL) cell lines with the VA extract Iscador®P did not cause changes in the expression levels of IL-6 and its receptor subunits IL-6 receptor (IL-6R) and gp130 indicating that the VA extract Iscador®P does not cause an IL-6 deregulation in B-NHL cells[4]. IL-6 is a proliferation factor for B-cells[8] and deregulation of IL-6 has been associated with B-cell neoplasia progression[9]. Furthermore, neoplastic B-NHL cells contain high amounts of IL-6, which may be responsible for elevated IL-6 serum levels in B-NHL patients[10]. However, since IL-6 could also act in a paracrine fashion on B-NHL cells, possibly released by macrophages due to inflammatory conditions, we investigated the influence of the VA extract Iscador®P on the IL-6 induced gene expression pattern of the apoptotic relevant proteins bax and bcl-2 as well as on the IL-6 induced proliferation of follicular B-NHL cells. Additionally, we also investigated the influence of the VA extract Iscador®M on the EGF induced proliferation and migration of the human MDA-MB-468-HER2 cell line, which has been referred to as a model cell line for malignant EGFR/c-erbB-2 double positive breast cancer[11]. The VA extract Iscador®M is commonly used as an adjuvant therapy in breast cancer and shows clinically relevant effect on breast cancer tumor progression as measured by overall survival as well as by the time of recurrences, lymphatic or distant metastases[12, 13].

Here we show that the VA extracts Iscador®P and Iscador®M effectively counteract the cytokine/ growth factor induced effects in follicular B-NHL cell lines and the breast cancer cell line MDA-MB-468-HER2. Thereby, the inhibitory effects of the applied VA extracts are likely attributed to different mechanisms suggesting that VA extracts inhibits cellular functions in a versatile fashion.

Materials and Methods

Cell culture and reagents

The human follicular B-Non-Hodgkin´s Lymphoma cell lines Sc-1 (European Collection of Cell Culture (ECACC), Salisbury, UK) and WSU-NHL (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany), and the human breast adenocarcinoma cell line MDA-MB-486 HER2[11] were maintained in RPMI 1640 (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (MDA-MB-486 HER2, DoHH-2, and Sc-1, PAA) or 10% fetal calf serum gold (WSU-NHL, PAA) at 37 °C in a humidified atmosphere with 5% CO_2. The Viscum album extracts Iscador®P (host plant: stone pine) and Iscador®M (host plant: apple tree) were kindly provided by the Verein für Krebsforschung, Arlesheim, Switzerland. The extracts contain standardized amounts of mistletoe lectin and viscotoxin.

Proliferation assay

Follicular B-Non-Hodgkin´s Lymphoma cells were seeded in dublicates (DoHH-2 0.6 $\times 10^6$/ml, WSU-NHL 1.2 $\times$ 10^6/ml, Sc-1 1.6 $\times$ 10^6/ml) in a 24-well-plate (0.5ml/well) and were incubated with IL-6 (1ng IL-6/0.1 $\times$ 10^6 cells; R&D Systems, Wiesbaden, Germany), clinically relevant does of Iscador®P (0.15, 1.5, 15 µg/ml /0.2 $\times$ 10^6 cells) and a combination of both for up to six days. Cell number was counted were counted in the lag-, log-, and plateau-phase. IL-6 and Iscador® P were added according to the increase of the cell density. Three independent experiments were carried, whereby untreated cell served as a control.

The proliferation of the MDA-MB-468-HER2 cell line was assessed by the XTT-assay as described previously[1, 14]. In brief, MDA-MB-468-HER2 cells were seeded in a 96-well-plate (5000 cells/well). After 24h incubation at 37 °C in a humidified atmosphere with 5% CO_2 the medium was replaced with 300 µl RPMI 1640 including EGF (0.1ng/ml; Sigma, Deisenhofen, Germany), Iscador® M (0.5, 1µg/ml/0.2 $\times$ 10^6 cells), and a combination of both, and cells were cultivated for additional 24 h. Subsequently, the medium was aspirated and replaced by 250 µl colorless RPMI 1640 (PAA, Cölbe, Germany) and 50 µl XTT-solution (25 µl 5mM Phenazine Methosulfate + 5ml XTT-stocksolution (1mg/ml)). Cells were incubated for 4h at 37 °C. The absorbance was quantified with a Microplate Reader (Model 550, BIORAD, Hercules, CA) at 450nm. Three independent experiments were performed, whereby each experiment was carried out in quadruplicates. Untreated MDA-MB-468-HER2 cells served as a control.

Cell cycle analysis

Follicular B-Non-Hodgkin´s Lymphoma cells were seeded in dublicates (DoHH-2 0.6 $\times$ 10^6/ml, WSU-NHL 1.2 $\times$ 10^6/ml, Sc-1 1.6 $\times$ 10^6/ml) in a 24-well-plate (0.5ml/well) and were incubated with IL-6 (1ng IL-6/0.1 $\times$ 10^6 cells; R&D Systems, Wiesbaden, Germany), clinically relevant does of Iscador®P (0.15, 1.5, 15 µg/ml /0.2 $\times$ 10^6 cells) and a combination of both for 48h. For cell cylce analysis, cells were collected, washed once in PBS and were resuspended in a hypotonic lysis buffer (0.1% Triton X100, 3.8 mM sodium citrate, 0.05 mg/ml propi-

TABLE 1.– *Summary of the primer pairs and TaqMan® probes used in this study*

Gene	Primer	Sequence (5´ to 3´)
ubiquitin	Forward primer	ATTTGGGTCGCGGTTCTTG
(UBC)	Reverse primer	TGCCTTGACATTCTCGATGGT
	TaqMan®-probe	TCAAGTGACGATCACAGCGATCCACAA
β-glucoroni-	Forward primer	CTCATTTGGAATTTTGCCGATT
dase (GUS)	Reverse primer	CCGAGTGAAGATCCCCTTTTTA
	TaqMan®-probe	TGAACAGTCACCGACGAGAGTGCTGG
bcl-2	Forward primer	GGCCTTCTTTGAGTTCGGT
	Reverse primer	ATCCACAGGGCGATGTTGT
	TaqMan®-probe	TCATGTGTGTGGAGAGCGTCAACCG
bax	Forward primer	AGGCACCCGAGCTG
	Reverse primer	TCAGCTTCTTGGTGGACG
	TaqMan®-probe	TGGACCCGGTGCCTCAGGATG

dium iodide; all chemicals were obtained from Sigma). Cells were incubated for 30min at 4 °C, filtered once (pore size 40-70 µm), and analyzed by flow cytometry (FACScalibur; Becton Dickenson, Heidelberg, Germany).

RealTime-PCR

Gene expression of bax and bcl-2 of Sc-1 and WSU-NHL cells was quantified by RealTime-RT-PCR as described previously[4] using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Cells were incubated with Iscador® P as described above. Total RNA of 2×10^6 cells was isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Reverse transcription was performed using the RevertAid™ First Strand cDNA Synthesis Lit (Fermentas, St. Leon-Rot, Germany) as described in the manufacturers' guidelines. RealTime-PCR was performed using the qPCR Core Kit (Eurogentec, Köln, Germany) according to the manufacturers' protocol under the following cycler conditions: 95 °C: 10min; 40 cycles [95 °C: 9sec; 60 °C: 60 sec]. Quantitative analysis of cDNA was accessed by the TaqMan®-technology. The used primer pairs and TaqMan®-probes for bax, bcl-2, and housekeeping genes (beta-glucoronidase and ubiquitin) are listed in Table 1. All TaqMan®-probes were labelled with FAM (6-Carboxy-Fluorescein) except ubiquitin, which was labelled with JOE (2,7-Dimethhoxy-4,5-dichloro-6-carboxyfluorescein). Both housekeeping genes were measured in one well via multiplex reaction. The obtained ΔC_T values for bax and bcl-2 in treated cells were calculated relative to the ΔC_T values for bax and bcl-2 of untreated cells, which were set to 1.

Cell migration analysis

The locomotory activity of MDA-MB-468-HER2 cells within three-dimensional collagen lattices was recorded by time-lapse videomicroscopy and analysed by computer-assisted cell tracking as described previously[1, 11, 15]. In brief, 8×10^4 tumor cells were mixed with 150µl carbonate-buffered liquid collagen (Flow, Mc Lean, VA) containing Eagle´s MEM (Minimal Essential Medium, Sigma-Aldrich, Deisenhofen, Germany). Depend on the experimental setting, EGF (100ng/ml), Iscador® M (0.5, 1 µg/ml/0.2×10^6 cells), and a combination of both, was added. The cell-collagen mixtures were filled into self-constructed migration-chambers and allowed to polymerize for 30 minutes at 37 °C in a humidified atmosphere with 5% CO_2. The migration behavior in the 3D collagen lattice was recorded for at least 15 h at 37 °C. For data analysis, 30 cells of each sample were randomly selected and two-dimensional projections of the paths were digitized as x/y coordinates in 20 min intervals.

Results

Iscador®P efficiently counteracts the IL-6 induced proliferation in follicular B-NHL cell lines

Two low malignant follicular B-NHL cell lines were used in this study. The WSU-NHL cell line is a nodular histiocytic lymphoma cell line, whereas the Sc-1 cell lines is derived from the small-cleaved type. Both cell lines were positive for IL-6R and gp130 expression[4] and were cultivated with IL-6 (1ng/ml), Iscador®P (15 µg/ml), and a combination of both until cells have reached the plateau phase. Cells were counted in the lag-, log, and plateau-phase, whereby the relative cell number was calculated in relation to the cell number of control cells, which was set to 1.

In contrast to the Sc-1 cell line, which showed a markedly increased IL-6 dependent proliferation rate (Fig. 1A), the IL-6 effect on WSU-NHL was rather moderate (Fig. 1B). Compared with untreated Sc-1 cells, the relative cell number of IL-6 treated Sc-1 cells was increased of about 14% (log-phase) and up to 27% (plateau phase), respectively (Fig. 1A). On the contrary, the cell number of IL-6 stimulated WSU-NHL cells was solely enhanced of about 10% both in the log- and plateau-phase (Fig. 1B). Treatment of Sc-1 and WSU-NHL with Iscador®P alone had no effect on the proliferation rate as compared with control cells (data not shown). However, Iscador®P in combination with IL-6 showed a markedly decreased proliferation rate of both Sc-1 and WSU-NHL cells (Fig. 1). Compared to IL-6 stimulation, the relative cell number of IL-6 and Iscador®P co-treated Sc-1 cells was decreased to 95% in

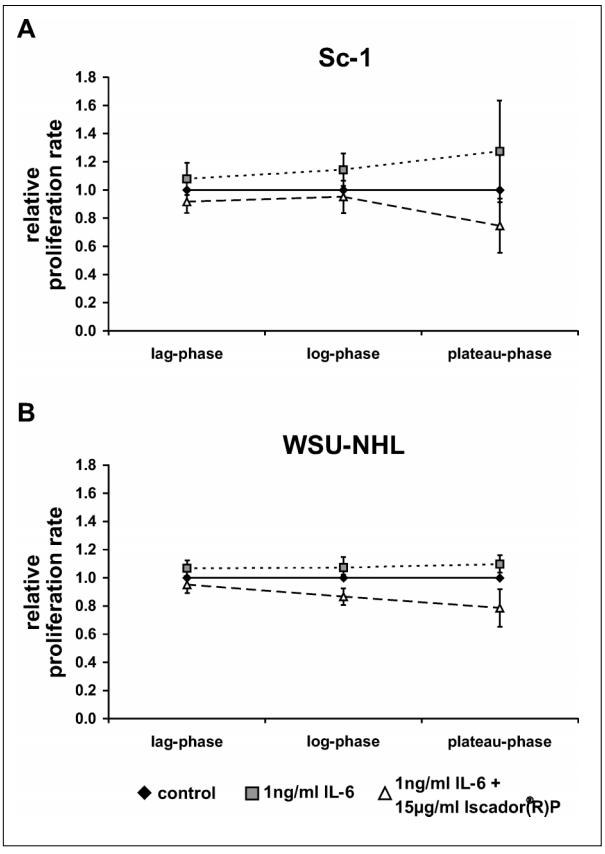

Fig 1. – Iscador®P efficiently counteracts the IL-6 induced proliferation of follicular B-NHL cell lines. A: relative proliferation rate of the follicular B-NHL cell line Sc-1; B: relative proliferation rate of the follicular B-NHL cell line WSU-NHL. Cells were grown in duplicates and counted in the lag-, log-, and plateau-phase. Cell numbers were calculated in relation to the number of control cells, which were set to 1.

the log-phase and to 75% in the plateau phase (Fig. 1A). Similar results were achieved for the WSU-NHL cell line. Here, the relative cell number of IL-6 and Iscador®P co-treated cells was decreased to 87% (log-phase) and 79% (plateau-phase), respectively (Fig. 1B). In summary, these results indicate that the VA extract Iscador®P is a potent inhibitor of the IL-6 induced proliferation of follicular B-NHL cell lines.

Iscador®P acts differently on Sc-1 and WSU-NHL cells

To investigate the mode of action how Iscador®P blocks the IL-6 induced proliferation in folicullar B-NHL cell lines, we investigated the proportion of the G_0/G_1-, S-, and M-phases as well as analyzed the changes in the gene expression profile of bax and bcl-2. Our data show that the inhibitory effect of Iscador®P on the IL-6 induced proliferation must be attributed to different mechanisms in the investigated cell lines.

Cell cycle analysis of Sc-1 and WSU-NHL cells revealed no significant differences between the single cell cycle phases of IL-6 and IL-6 plus Iscador®P treated cells as compared with their appropriate controls (Table 2). However, realtime-PCR analysis indicated the up-regulation of the pro-apoptotic protein bax in IL-6 plus Iscador®P treated WSU-NHL cells (Fig. 2B), which was accompanied by an increased number of apoptotic cells (data not shown). Interestingly, the up-regulation of the pro-apoptotic protein bax was not observable in WSU-NHL cells solely treated with IL-6 or Iscador®P, respectively (Fig. 2B). By contrast, the anti-apoptotic protein bcl-2 was up-regulated two-fold in IL-6 treated Sc-1 cells (Fig. 2A). This IL-6 dependent bcl-2 up-regulation was slightly counterbalanced by co-treatment with Iscador®P (Fig. 2A). However, co-treatment of Sc-1 cells with IL-6 plus Iscador®P also resulted in a slightly decreased bax expression level (Fig. 2A). In accordance with that, we did not observed an increased number of apoptotic Sc-1 cells that have been co-treated with IL-6 plus Iscador®P (data not shown). These results indicate that the Iscador®P blocks the IL-6 induced proliferation of WSU-NHL cells by inducing apoptosis. On the contrary, the Iscador®P mediated inhibition of the IL-6 induced proliferation of Sc-1 cells must be attributed to a different mechanism.

TABLE 2.– *Cell cycle analysis of follicular B-NHL cell lines treated*

	cell cycle phase	control %	IL-6 %	IL-6 + Iscador®P %
Sc-1	G_0/G_1	36.7	31.9	30.9
	S	51.2	56.4	60.1
	M	12.1	11.7	9.1
WSU-NHL	G_0/G_1	42.1	42.3	48.2
	S	33.9	35.6	29.0
	M	24.0	22.2	22.8

Iscador®M efficiently counteracts the EGF induced proliferation of MDA-MB-468-HER2 cells

Our data show that Iscador®P is a potent inhibitor of the IL-6 induced proliferation of follicular B-NHL cell lines. In order to investigate whether VA extracts do also inhibit the growth factor induced proliferation of solid cancer cells we analyzed the influence of the VA extract Iscador®M on the epidermal growth factor (EGF) induced proliferation of MDA-MB-468-HER2 cells. Iscador®M was chosen since this VA extract is commonly used in breast cancer treatment[12, 13]. MDA-MB-468-HER2 cells represent a valid model for EGFR/c-erbB-2 double positive breast cancer[11].

In brief, Iscador®M potently blocked the EGF (1ng/ml) induced proliferation of MDA-MB-468-HER2 cells (1ng/ml EGF: 116.0% vs. 1ng/ml EGF + 0.5µg/ml Iscador®M:

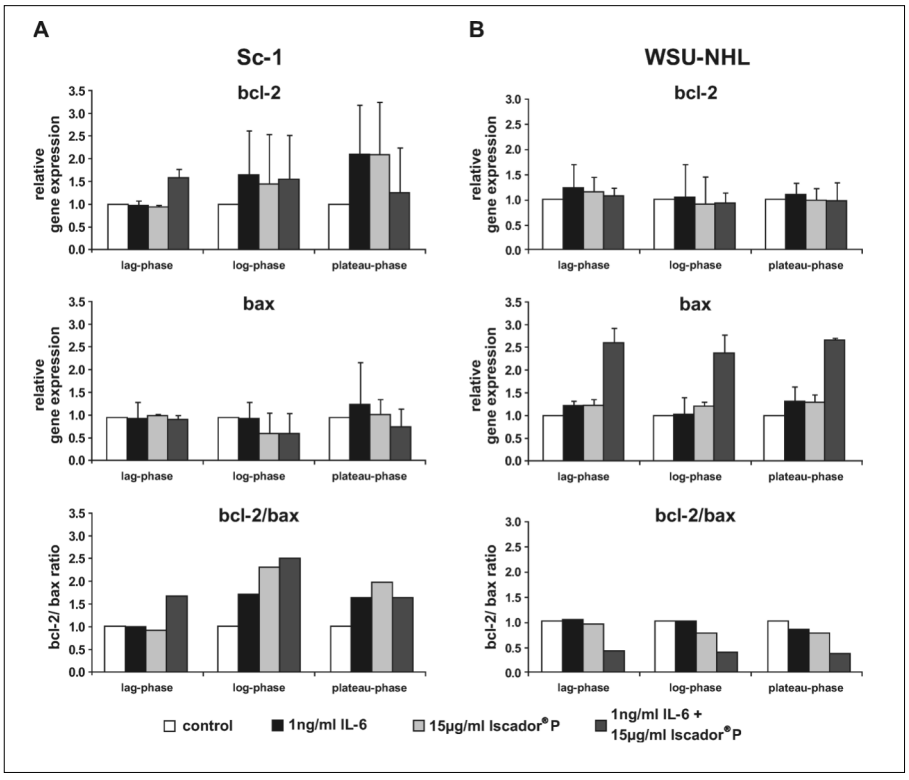

Fig. 2. – RealTime-PCR analysis of the bax and bcl-2 expression levels in Sc-1 and WSU-NHL cells. A: relative gene expression levels of bax and bcl-2 in Sc-1 cells; B: relative gene expression levels of bax and bcl-2 in WSU-NHL cells. IL-6 and Iscador®P caused bcl-2 up-regulation in Sc-1 cells, which is decreased if cells were treated with a combination of Iscador®P plus IL-6. By contrast, solely Iscador®P plus IL-6 co-treatment caused a bax up-regulation in WSU-NHL cells, which correlated with an increased number of apoptotic cells.

89.7% vs. 1ng/ml EGF + 1µg/ml Iscador®M: 91.2%; Fig. 3A). However, in contrast to follicular B-NHL cells we also observed a slightly reduced proliferation rate of solely Iscador®M treated MDA-MB-468-HER2 cells. Compared to control cells, the proliferation rate of Iscador®M treated cells was reduced to 92.9% (0.5µg/ml Iscador®M) and 97.3% (1µg/ml Iscador®M), respectively (Fig. 3A). Nonetheless, the decrease in the proliferation rate of MDA-MB-468-HER2 was more profound when cells were co-treated with a combination of EGF and Iscador®M.

Iscador®M efficiently blocks the EGF induced migration of MDA-MB-468-HER2 cells

It is well recognized that co-expression of EGFR and c-erbB-2 of breast cancer cells determines a motogenic phenotype due to constitutive EGFR/ c-erbB-2 heterodimerization[16], which modulates the kinetics of the adaptor protein phospholipase C-γ1 (PLC-γ1)[11].

Cell migration of MDA-MB-468-HER2 cells was determined by using the 3D-collagen matrix assay, which allows for a detailed analysis of cell migration on even a single cell level[1]. Stimulation of MDA-MB-468-HER2 cells with EGF (100ng/ml) resulted in an increased locomotory activity of up to 142% (Fig. 3B), which is in accordance with previous studies[11, 15, 17]. Treatment of MDA-MB-468-HER2 cells with Iscador®M alone did not alter the locomotory activity of the cells (0.5µg/ml Iscador®M: 104%, 1µg/ml Iscador®M: 101%). By contrast, Iscador®M potently blocked the EGF induced migration of MDA-MB-468-HER2 cells (Fig. 3B). The locomotory activity of 100ng/ml EGF plus 0.5µg/ml Iscador®M and 100ng/ml EGF plus 1µg/ml Iscador®M, respectively treated MDA-MB-468-HER2 cells was reduced to 116% and 105%, respectively (Fig. 3B), which also indicates a slight dose-dependent effect. Interestingly, solely Iscador®M as well as EGF plus Iscador®M MDA-MB-468-HER2 cells were still viable indicating that Iscador®M rather inhibits the EGF induced migration by interfering with the migratory molecular machinery instead of killing the cells.

Discussion

In the present study we investigated the influence of the VA extracts Iscador®P and Iscador®M on the cytokines/ growth factor induced proliferation of follicular B-NHL cell

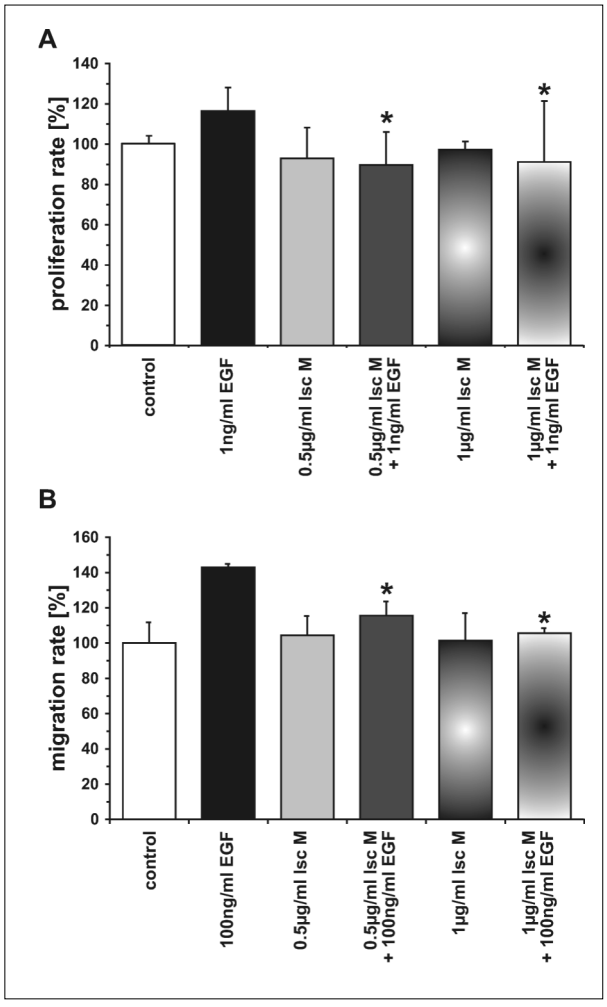

Fig. 3. - Iscador®M efficiently counteracts the EGF induced proliferation and migration of MDA-MB-468-HER2 cells. A: proliferation rate of MDA-MB-468-HER2 cells treated with EGF and Iscador®M; B: locomotory activity of MDA-MB-468-HER2 cells treated with EGF and Iscador®M. Control values were set to 100%. Statistical significance was calculated by an unpaired student's *t*-test (* = $P < 0.05$).

lines Sc-1 and WSU-NHL, and the breast cancer cell line MDA-MB-468-HER2.

IL-6 is a proliferation factor for B-cells and is used as a prognostic factor in various malignant B-cell neoplasias such as B-NHL and multiple myeloma, since elevated IL-6 serum levels are associated with a poor prognosis of the afflicted patients[9]. Studies of Kato et al. suggested an active role of neoplastic B-NHL cells by modulating the general status of B-NHL patients since these cells contained elevated intracellular concentrations of IL-6[10]. Previous results of our group clearly showed that the VA extract Iscador®P did not alter the gene expression of IL-6 and its receptor components IL-6R and gp130 in follicular B-NHL cell lines[4]. These findings are in agreement with data of Kovacs and Kuehn showing that elevated IL-6 serum levels were not detectable in Iscador®P treated follicular B-NHL patients[18]. In fact, IL-6 serum levels of long-term patients were significantly lower than those of controls.

In this context, our results provide new insights by showing that IL-6 might be useful in the Iscador®P-based therapy of follicular B-NHL. Cell proliferation studies clearly show that Iscador®P only blocks the proliferation of follicular B-NHL cell lines if Iscador®P was applied in combination with IL-6 (Fig. 1). Treatment of follicular B-NHL cell lines with Iscador®P alone had no effect on the proliferation rate of these cells (data not shown). Similar results were obtained for IL-6 dependent multiple myeloma cell lines (data not shown) indicating that the inhibitory effect of Iscador®P depends on IL-6. The observation that the combination of Iscador®P and IL-6 has a more profound inhibitory effect on the proliferation of follicular B-NHL cells is further substantiated by RealTime-PCR data concerning bax and bcl-2 expression levels. For instance, in WSU-NHL cells a bax up-regulation was solely detectable in cells co-treated with Iscador®P plus IL-6 (Fig. 2B) that was additionally correlated to an increased a number of apopotic cells (data not shown). Additionally, Iscador®P in combination with IL-6 counteracted that IL-6 induced bcl-2 up-regulation in Sc-1 cells (Fig. 2A). Interestingly, we did not observe an increased number of apoptotic Sc-1 co-treated with Iscador®P plus IL-6 (data not shown) albeit Iscador® plus IL-6 co-treated cells showed a markedly decreased proliferation rate. This indicates that the VA extract Iscador®P likely inhibits cell proliferation by a different yet unknown mechanism.

The finding that the inhibitory effect of VA extracts was more effective in the presence of a proliferatory stimulus was also observed for treatment of the breast cancer cell line MDA-MB-468-HER2 with Iscador®M. Here, both the EGF induced proliferation and cell migration was efficiently counteracted by the VA extract. However, we also noticed a slight reduced proliferation rate for MDA-MB-468-HER2 cells solely treated with Iscador®M. This effect might be attributed to the different composition of the used VA extracts. Iscador®P is derived from mistletoes growing on stone pine and contains low amounts of mistletoe lectins (10ng/ml), but high viscotoxin (12µg/ml) concentrations. By contrast, Iscador®M (harvested from apple tree) contains high concentrations of mistletoe lectins (488ng/ml), but low amounts of viscotoxins (6µg/ml). Nonetheless, compared to MDA-MB-468-HER2 cells solely treated with Iscador®M the proliferation of EGF plus Iscador®M co-treated cells was slightly much more decreased.

Iscador®M solely inhibits the EGF induced, but not the spontaneous migration of MDA-MB-468-HER2 cells. Cell migration is a complex process and is directed by the interplay of several signal transduction pathways initiated

by various ligands such as cytokine, chemokines, growth factors, and extracellular matrix components that activate growth factor receptors, chemokine receptors, and integrins[1]. The finding that Iscador®M solely inhibits the EGF induced migration indicates that this VA extract likely interferes with the EGF signal transduction cascade. Otherwise we should have been observed a decreased locomotory activity of Iscador®M treated MDA-MB-468-HER2 cells. We have recently demonstrated that cell migration is induced in EGFR/c-erbB-2 double positive breast cancer cells due to the c-erbB-2 modulated kinetics of the adaptor protein PLC-γ1[11]. The importance of this signal transduction pathway was further demonstrated by showing that recombinant PLC-γ1-$(SH2)_2$ proteins, which binds to the activated c-erbB-2 receptor, thereby preventing naïve PLC-γ1 docking, efficiently blocked the EGF induced migration of MDA-MB-468-HER2 cells[15]. Thus we assume that Iscador®M likely interferes with the EGF induced PLC-γ1 signal transduction cascade, thereby inhibiting cell migration.

Our data indicate that VA extracts are able to influence important tumor cell functions including proliferation and migration. Moreover, our results provide new insights in the IL-6 problem of B-cell neoplasia treatment by VA extracts. Here we show for the first time that the B-cell proliferation factor IL-6 might be helpful in the Iscador®P-based therapy of follicular B-NHL (and possibly multiple myeloma) since only Iscador®P plus IL-6, but not solely Iscador®P treated follicular B-NHL cells showed a reduced proliferation rate. This is in agreement with a well-known phenomenon in cancer therapy that mostly fast dividing cells are susceptible to therapy, whereas low proliferating cells are not affected by e.g. chemotherapy. Thus, the increase in IL-6 serum levels caused by VA extract application would be a positive side-effect allowing the VA extracts to fulfill their anti-cancer properties in B-cell neoplasia therapy.

In summary, we conclude from our data that VA extracts are suitable anti-cancer drugs for follicular B-NHL and breast cancer treatment. However, further studies are recommended to evaluate the necessity of proliferatory stimuli on the anti-cancer efficiency of VA extracts.

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