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MISTLETOE EXTRACTS (VISCUM ALBUM L.) ISCADOR® INTERACT WITH THE CELL CYCLE MACHINERY AND TARGET SURVIVAL MECHANISMS IN CANCER CELLS

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Abstract The molecular and cellular mechanisms by which mistletoe extracts exert their cytotoxic and immunomodulatory anti-tumoral effects are largely unknown. Active components in these preparations include lectins and viscotoxins. In this study we tested the hypothesis that Iscador preparations induce tumor regression by cell cycle inhibition and/or interference with apoptotic signaling pathways in tumor cells. Also a possible effect on angiogenesis, which is a prerequisite for tumor growth *in vivo*, is studied in endothelial cell cultures. Furthermore, we examined which apoptotic signaling route(s) is (are) activated by Iscador by studying specific pro-apoptotic proteins in cultured cells. To characterize these properties, human carcinoma cell lines of different origin, two lymphoma cell lines, one epidermis derived cell line, and two endothelial cell cultures were incubated with different concentrations of either Iscador® Quercus Spezial, Iscador® Malus Spezial, both containing defined concentrations of lectins, or Iscador® Pinus containing low amounts of lectin, but a relatively high concentration of viscotoxins. Cell cycle kinetic parameters were measured by bromodeoxyuridine (BrdU) pulse labeling and spindle microtubule staining. Apoptotic responses were detected by M30 CytoDeath or Annexin V/ propidium iodide assays. Characterization of the apoptotic pathway(s) was performed by staining cells for amongst others active caspase 3 and cytochrome C (mitochondrial pathway), as well as active caspase 8 (death receptor pathway). The sensitivity to Iscador treatment varies strongly between different cell lines and also amongst the different Iscador® preparations. In sensitive cell lines, including those derived from small cell lung cancer, adenocarcinoma of the lung and breast, B-cell lymphoma, as well as endothelial cell cultures, Iscador caused early cell cycle inhibition followed by apoptosis in a dose dependent manner. Amongst the low responders are cell lines derived from colorectal carcinoma. In general Iscador® Malus exerted a stronger response than Iscador® Quercus. Apoptosis was induced by activating the mitochondrial, but not the death receptor dependent pathway, at least in case of Iscador® Quercus. Iscador® Malus also seemed to induce apoptosis via the death receptor route, which may explain the higher sensitivity of cancer and endothelial cells to this preparation. Iscador® Pinus, tested in the lymphoma cell lines, was found to induce mainly necrosis, most probably due to the viscotoxins present in these preparations.

Key words: Iscador, carcinomas, lymphomas, apoptotic pathways, cell cycle, caspases, endothelial cells

Resumen ***Extractos de* muérdago (Viscum album *L.) Iscador® interfieren con el ciclo celular y afectan la sobrevida de las células malignas.*** Los mecanismos moleculares por los cuales los extractos de “mistletoe” (muerdago) llevan a cabo sus efectos citotóxicos e inmunomodulatorios anti-tumorales son mayormente desconocidos. Componentes activos de estas preparaciones incluyen lectinas y viscotoxinas. En este trabajo pusimos a prueba la hipótesis de que las preparaciones de Iscador inducen regresión tumoral por inhibir el ciclo celular y/o interferir con las vías de señalización apoptóticas. También un posible efecto sobre la angiogénesis, que es un prerrequisito para el crecimiento tumoral, fue evaluado en cultivo. Más aún, examinamos qué vías de señalización apoptóticas son activadas por Iscador analizando proteínas pro-apoptóticas específicas en cultivos celulares. Para realizar estas caracterizaciones, líneas de carcinoma humano de distintos orígenes (dos de linfoma, una derivada de epidermis y dos líneas endoteliales) fueron incubadas con diferentes concentraciones de Iscador® Quercus Spezial, Iscador® Malus Spezial, ambas conteniendo concentraciones definidas de lectinas, o con Iscador® Pinus, que contiene baja concentración de lectinas, pero altas de viscotoxinas. La cinética del ciclo celular fue evaluada por incorporación de bromodeoxiuridina (BrdU) y marcación de microtúbulos. La respuesta apoptótica fue detectada por M30 CytoDeath o tinción con Anexina V/ioduro de propidio. La caracterización de las vías apoptóticas fue realizada tiñendo células contra caspasa 3 activa y citocromo C (vía mitocondrial), así también contra caspasa 8 activa (vía de receptor de muerte). La sensibilidad a Iscador varía entre las líneas celulares y también con los distintos preparados de Iscador. En líneas sensibles, incluyendo aquellas derivadas de tumor de pulmón de células pequeñas, linfoma B y células endoteliales,

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Iscador causó una inhibición del ciclo celular y apoptosis en forma dosis dependiente. Entre las líneas poco respondedoras están aquellas derivadas de carcinoma colorrectal. En general Iscador® Malus ejerció un efecto más fuerte que Iscador® Quercus. La apoptosis se indujo por activación de la vía mitocondrial en el caso de Iscador® Quercus. Iscador® Malus, por otra parte, indujo apoptosis vía el receptor de muerte, lo cual podría explicar la mayor sensibilidad de células de carcinoma y endoteliales a esta preparación. Finalmente Iscador® Pinus, que fue evaluado en células de linfoma, indujo sobre todo necrosis, probablemente como consecuencia de la alta concentración de viscotoxinas en esta preparación.

Palabras claves: Iscador, linfoma, vías apoptóticas, ciclo celular, caspasas, células endoteliales

There are several ways by which anti-carcinogenic therapy can interfere with tumour growth. Tumour cells can be inhibited in the progression of the cell cycle or induced to undergo cell death. Anti-carcinogenic therapy can also interfere with the formation of new blood vessels (angiogenesis) by inhibition of cell growth or the killing of endothelial cells[1]. The inhibition of angiogenesis causes a limitation of the growth rate or even starvation of the tumour due to the lack of oxygen and nutrient supply and the accumulation of waste products at the site of the tumour.

Two major types of cell death can be distinguished, i.e. necrosis and apoptosis. Necrosis occurs when a cell is damaged in such a way that it looses its membrane integrity and releases its contents into the environment. This causes extensive inflammation. Apoptosis, also called programmed cell death, is a tightly regulated process in which the cell disassembles while maintaining its membrane integrity. Apoptotic cells show several characteristic features, among which the exposure of phosphatidylserine (a membrane lipid) at the outer leaflet of the membrane[2]. Because of this, they are recognized by phagocytic cells and removed from the circulation[3]. Apoptosis does not induce inflammatory responses.

There are several ways by which cells can be induced to undergo apoptosis. Two of the major pathways are the death receptor pathway and the mitochondrial pathway[4]. The death receptor pathway is activated by the interaction of ligands with death receptors at the cell surface. This leads to a series of events in which caspase-8 is activated, which in turn activates other caspases downstream in the apoptotic process. The mitochondrial pathway is characterized by loss of mitochondrial membrane potential and subsequent leakage of cytochrome C into the cytosol. Cytochrome C is released from mitochondria when the proapoptotic proteins Bax and Bak are translocated to the outer mitochondrial membrane and induces the activation of caspase 9. Both pathways converge in the activation of caspase-3, which ultimately elicits the morphological hallmarks of apoptosis.

Apart from inducing apoptosis, anti-carcinogenic agents can also inhibit tumour cells or endothelial cells in their cell cycle progression. This may ultimately lead to apoptosis, but can also result in cell cycle delay, senescence or in mitotic catastrophe[5]. The relationship between the mechanisms that induce cell cycle delay and those that induce apoptosis are still elusive. Although in first instance it may seem that inducing senescence is a proper way to block tumour growth, there are several indications that senescent cells can induce renewed tumour growth after a period of time, because senescent cells remain metabolically active and produce several secreted proteins, among which proteins with tumour promoting activities.

The major aim of our studies was to unravel the mode of action of Iscador® at a molecular and cellular level. Another goal is to explain why certain cancer types show a good response towards Iscador treatment, whereas others do not respond or to a lesser extent.

In the present study we examined the efficacy of Iscador® preparations in cell cycle inhibition and the induction of apoptosis in human cell lines derived from different tumor types. Furthermore, we identified the apoptotic route(s) involved in Iscador® induced apoptosis. Also the anti-angiogenic effect(s) of Iscador® was (were) determined by analyzing the apoptotic rate and cell cycle kinetics in endothelial cells. A comparison was made between these cytostatic and cytotoxic effects of Iscador® Malus Spezial (hereafter referred to as Iscador® M) and Iscador® Quercus Spezial (hereafter Iscador® Q), both extracts derived from Viscum album growing on apple and oak trees, respectively, and containing a defined amount of lectins. Iscador® Pinus (hereafter Iscador® P), an extract derived from Viscum album growing on pine trees, was only tested on lymphoma cells, and contains low concentrations of lectins.

Materials and Methods

Apoptosis assays

The bivariate Annexin V/ propidium iodide apoptosis assay is based on the facts that during the early stages of apoptosis:

- phosphatidylserine (PS) is exposed at the outer surface of the cell membrane to which Annexin V strongly binds[2].
- plasma membrane integrity is maintained and thus the dye propidium iodide (PI) is excluded from the cell.

For this assay cells were incubated for several hours in culture medium containing various concentrations of Iscador. Subsequently, cells were harvested and resuspended in binding buffer containing Annexin V-FITC and propidium iodide (PI). The percentage of Annexin V positive/PI negative cells is indicative of the percentage of apoptotic cells. The percentage of Annexin V positive/PI positive cells reflects the (secondary) necrotic cell fraction.

The M30-CytoDeath assay is based on the fact that cleavage of cytokeratin-18 is an early apoptotic event in cells containing this protein. Cleaved cytokeratin-18 can be detected specifically using the M30-CytoDeath antibody. In this assay apoptosis is detected and quantified as described previously[6].

To determine which apoptotic route(s) is (are) activated upon treatment with Iscador®, cells are stained for several pro-apoptotic proteins. A positive staining for caspase-8 is indicative for activation of the death receptor pathway, whereas a positive staining for Bax indicates activation of the mitochondrial pathway. Activation of the mitochondrial pathway is also accompanied by leakage of cytochrome C into the cytoplasm. Therefore staining for cytochrome C results in a diffuse staining pattern when the mitochondrial pathway is activated, whereas a dot-like staining pattern will be observed in cells with intact mitochondria. Finally, a staining for activated caspase-3 is included to detect activation of apoptotic route(s) in general. The antibody reagents used for these tests have been described earlier[7].

Cell cycle assays

The cell cycle can be subdivided into G1-, S-, G2- and M-phase. The relative frequency of cells in the different phases can be measured based on the differences in DNA content. G2- and M-phase cells have twice the amount of DNA as compared to cells in the G1-phase of the cell cycle, while S-phase cells show intermediate values varying from near G1-phase to near G2-/M-phase values. When cells are stained with a DNA-specific fluorescent dye and the amount of fluorescence is measured on a per cell basis, a DNA histogram can be obtained using flow cytometry. This technique provides, however, a static measurement. No information is obtained about the dynamics of cell cycle progression, i.e. no information can be obtained about the duration of the different phases of the cell cycle. The circumvent this problem a distinct cohort of cells should be marked and followed as they progress through the cell cycle. This can be achieved by incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the DNA of cells during the S-phase of the cell cycle. BrdU labeled cells can subsequently be visualized by immunocytochemistry using anti-BrdU-specific antibodies. When an exponentially growing population of cells is briefly exposed to BrdU and subsequently cultured in medium without BrdU, the labeled cohort of cells can be followed through the cell cycle. Directly after pulse labeling cells in the S-phase of the cell cycle will incorporate BrdU and this cohort of labeled cells will progress towards the G2- and M-phase. After cell division a BrdU-labeled cell will give rise to two labeled daughter cells in the G1-phase of the cell cycle (Fig. 1A and B). The progression of a labeled cohort of cells at a time point when part of the labeled cohort has moved through cell division is estimated using special algorithms that are able to calculate the number and kinetics of cells in the different phases of the cell cycle[7, 8, 9].

Iscador® preparations

Fresh working solutions were prepared for each assay by dissolving the desired amount of Iscador® Quercus 5mg Spezial (375 ng lectins/ml; 0-1 mg Isc Q/ml culture sup), Iscador® Malus 5 mg Spezial (containing 250 ng lectins/ml; 0-1.5 mg Isc M/ml culture sup), or Iscador® Pinus (10 mg/ml; 0-1.5 mg Isc P/ml culture sup) in the appropriate culture medium.

Cell lines and culturing conditions

To investigate the effect of Iscador® preparations on cell cycle progression and apoptosis, the following cell lines were used:

HaCat	squamous epithelium skin
MR65	squamous cell carcinoma lung
NCI-H125	adenocarcinoma lung
NCI-H82	small cell carcinoma lung
NCI-H69	small cell carcinoma lung
CaC02	adenocarcinoma colon
HT29	adenocarcinoma colon
MCF7	adenocarcinoma breast
Jurkat	myeloid cells
HUVEC	umbilical vein endothelial cells
HMEC	dermal microvascular endothelial cells (SV40 transformed)
DoHH-2	human B cell lymphoma
WSU-NHL	human B cell lymphoma

HaCaT cells[10] were cultured in EMEM (minimal essential medium with Earle's salt; Gibco) supplemented with 1% L-glutamine, 10% heat-inactivated fetal calf serum, 1% non

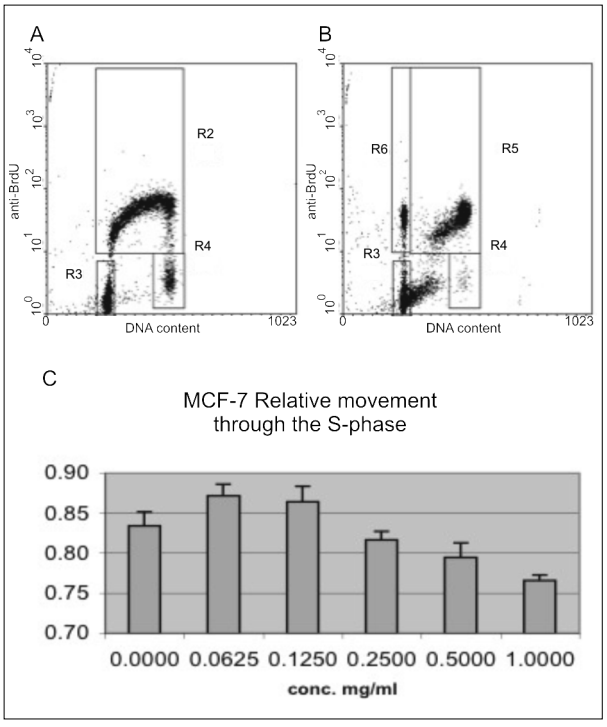

Fig. 1.– Using bivariate BrdU/DNA analysis by flow cytometry, progression of a labeled cohort of cells through the cell cycle can be visualized when cells are double stained for their DNA content and for incorporated BrdU. Fig. 1A depicts a dotplot of double stained cells directly after pulse labeling with BrdU, while Fig. 1B shows the pattern observed after a chase period of 7 hours. The amount of DNA per cell is shown on the x-axis and the amount of incorporated BrdU on the y-axis. In the dotplot regions can be set to identify BrdU-positive S-phase cells (region R2), BrdU-negative G1- (region R3) and G2-/M-phase cells (region R4). Fig. 1C illustrates the decreasing relative movement of MCF7 breast cancer cells through S-phase upon treatment with Iscador® Q, which was added in increasing concentrations to the cell culture.

essential amino acids and 0,1% gentamycin. HUVEC were obtained as primary cultures after trypsinisation of human umbilical vein. HUVEC and HMEC were cultured in RPMI (Gibco) supplemented with 20% human pool serum (HUVEC; Sanquin, Amsterdam, The Netherlands) or 10% fetal calf serum (HMEC), 1% L-glutamine and 0,1% gentamycin in tissue culture flasks that were pretreated with 0.1% gelatin for at least 1 hour at 37 °C. The gelatin solution was aspired before cells were added. HUVEC were used at passage 2 to at most 4. DoHH-2 (human B cell lymphoma) and WSU-NHL (human B cell lymphoma) were grown in RPMI 1640 (Gibco) containing 1% L-glutamine, 10% FCS and 0.1% gentamycin and split approximately 1:4 every 3-4 days. The other cell lines were grown as described before[11]. Cells were kept at 37 °C and 5% CO_2 in a humidified incubator.

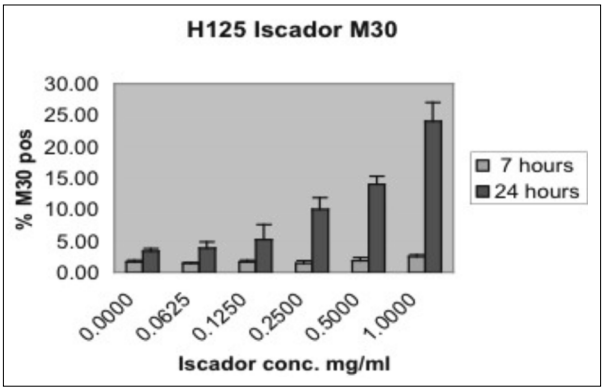

Fig. 2.– Apoptosis induction by Iscador® Q in NCI-H125 lung adenocarcinoma cell culture in a concentration- and time-dependent manner.

Results

Cell cycle inhibition by Iscador®

A clear effect of Iscador® Q on cell cycle progression was observed for HaCaT, NCI-H125, MCF7 (Fig. 1) and HUVEC cells. Progression through S-phase was delayed for all four cell lines. At the highest dose (1.0 mg/ml) an almost complete inhibition of S-phase progression (i.e. relative movement of 0.5) was seen in HaCaT and MCF7 cells. Also a slight but significant accumulation of BrdU positive MCF7, HaCaT and NCI-H125 cells in S+G2M phase occurred, accompanied by a reduction of BrdU positive MCF7 and HaCaT cells in G1 phase. NCI-H125 showed a temporary increase in BrdU positive G1 cells at 0.125 and 0.250 mg Iscador® Q/ml, followed by a decrease at 1.0 mg Iscador® Q/ml. The cytostatic effect was concentration dependent and observed already after a 7 hours incubation. No or only small effects of Iscador® Q on cell cycle progression were observed in HT-29, CaCo-2 and MR65. A slight decrease in BrdU positive G1 cells was visible for CaCo-2 and HT-29. Relative movement through S-phase was slightly impaired at higher Iscador® Q concentrations in HT-29 and MR65 cells. However, at low concentration (0.0625 mg/ml Iscador® Q), MR65 cells showed a slight increase in relative movement through S-phase.

When the influence of Iscador® Q on cell cycle kinetics is compared to that of Iscador® M , the relative movement is more strongly impaired upon Iscador® Q treatment as compared to Iscador® M treatment. In the BrdU positive cells a decrease is seen in G1 phase for both Iscador® types. However, this decrease is only temporary and non-significant in Iscador® M treated cells. For BrdU positive cells in late S + G2M phase, a temporary increase followed by a decrease is seen after incubation with both Iscador® preparations at comparable lectin concentrations. Both the increase and the decrease are most pronounced in the Iscador® Q treated cells, whereas in Iscador® M treated cells these changes are not significant. Furthermore, HUVEC cells responded to both Iscador® preparations with a decrease of BrdU negative cells in G2M phase, whereas they did not have a clear influence on the percentage of BrdU negative cells in G1 phase. This probably reflects loss of cells from G2M phase due to apoptosis (mitotic catastrophe).

A concentration dependent effect of Iscador® Q and Iscador® P was seen on cell cycle inhibition in the DoHH-2 lymphoma cells, while Iscador® M has a less obvious effect on the cell cycle of these cells. Strong fluctuations and no obvious concentration dependent effect was seen on the cell cycle of WSU-NHL lymphoma cells with all three Iscador® preparations, with the possible exception of Iscador® Q, which has some effect at its highest concentration.

Iscador® Q does not induce microtubule dysfunction

To detect whether Iscador® Q causes cell cycle inhibition or apoptosis by directly targeting the microtubules and thereby causing a mitotic block, cells were stained for α- and β-tubulin. Iscador® Q treated versus untreated HaCaT or MR65 cells show similar tubulin staining patterns[7, 11]. Therefore Iscador® Q treatment does not seem to exert its cytostatic effect by affecting the microtubular network, as for example seen for alkaloids.

Iscador® induces apoptosis in several tumor cell lines and endothelial cells

The concentration ranges used for Iscador® M and Iscador® Q in the apoptosis studies were comparable with regard to their lectin content. Maximal apoptotic responses were obtained at concentrations of 1 mg Iscador® Q/ml or 1.5 mg Iscador® M/ml.

The proportion of cells that could be induced to undergo apoptosis in the different cell lines upon stimulation with the different Iscador® Q or M preparations is

highly variable. The lung cancer cell line NCI-H82 showed 80% cell death after already 7 hours with 21% of the cells being apoptotic and 59% necrotic. It has to be kept in mind, however, that a considerable fraction of NCI-H82 becomes apoptotic without Iscador® treatment. MR65, HT-29 and CaCo-2 showed only a minor or no apoptotic response after 24 hours. Medium to high apoptotic responses were found in HaCaT, NCI-H125 (Fig. 2) and MCF7 cell lines. In all cell lines, except NCI-H82, execution of apoptosis due to Iscador® is a rather late event, with the effect being maximal after 24 hours (Fig. 2).The percentage of cells undergoing apoptosis was shown to be dose dependent.

When tested on the B-cell lymphoma cell lines DOHH-2 and WSU-NHL, all three Iscador® Q, M and P preparations induce a strong cell death response in a concentration dependent manner. Iscador® P induces necrosis rather than apoptosis as seen for Iscador® Q, and M, propably through viscotoxins, and is less effective in inducing cell death. Long term effects (3 days) with low concentrations of the drugs are, however, most prominent for Iscador® P.

The endothelial cell lines HUVEC and HMEC were highly responsive to Iscador® treatment, although a considerable proportion of HUVEC cells underwent spontaneous apoptosis. At concentrations inducing the maximal apoptotic response, Iscador® M induces stronger apoptotic and necrotic responses in HUVEC cells than Iscador® Q. Compared to the control incubation the apoptotic response increases 7.3-fold and the necrotic response 5.1-fold upon incubation with 1.5 mg/ml Iscador® M, whereas it increases only 2.1-fold and 3-fold, respectively, upon incubation with Iscador® Q.

Apoptotic pathways induced by Iscador®

To detect which apoptotic pathways are activated by Iscador® Q and M, HaCaT cells were immunostained for several apoptosis related components. Caspase cleaved cytokeratin 18 was used to detect apoptotic cells in general. The proteolytic cytokeratin 18 fragment is indeed present in apoptotic cells indicating activation of caspase 3, 7 and/or 9. To further dissect the apoptotic pathways, cells were stained for activated caspase 3, activated caspase 8 as indicator of the death receptor pathway, and cytochrome C as indicator of the mitochondrial pathway. CMXRos was used to detect disruption of mitochondrial transmembrane potential, since this dye accumulates in polarized, but not in depolarized mitochondria. Activated caspase 3 could be detected in apoptotic cells while activated caspase 8, could not be detected in Iscador® Q treated apoptotic cells. Caspase 8 activation was however detectable in cells induced to undergo apoptosis by treatment with other apoptosis inducing componist[7, 11], suggesting that the death receptor pathway was not activated by Iscador® Q treatment.

Cytochrome C and CMXRos staining showed the typical dot like staining pattern in untreated cells, whereas several cells in the culture treated with Iscador® Q showed a more diffuse staining pattern, indicating leakage of cytochrome C and CMXRos from the mitochondria and therefore activation of the mitochondrial apoptotic pathway as well as disruption of transmembrane potential.

Iscador® M treated cells stained positive for cleaved cytokeratin-18 and activated caspase-3, which are indicators of activation of apoptotic routes in general. A fraction of the cells were, however, found to be positive for activated caspase-8, which is indicative for activation of the death receptor pathway, and for Bax, which shows involvement of the mitochondrial pathway. Also a diffuse staining for cytochrome C was observed in apoptotic cells after Iscador® M treatment, indicating leakage of cytochrome C from the mitochondria, demonstrating involvement of the mitochondrial pathway.

Conclusions

Extracts from European Mistletoe or *Viscum album L.* have been reported to exert cytotoxic and immunomodulatory effects *in vitro* and *in vivo*. The mechanism of this anti-tumoral activity is however, largely unknown. In this report we tested the hypothesis that Iscador® Q, M and P preparations, aqueous fermented extracts from the European mistletoe grown on oaks, apple tree and pine tree, respectively, induce tumor regression by cell cycle inhibition and/or interference with apoptotic signaling pathways in cancer cells. Also a possible effect on angiogenesis, which is a prerequisite for tumor growth in vivo, was studied in endothelial cell cultures. Furthermore, we examined which apoptotic signaling routes are activated by staining cells for specific pro-apoptotic proteins. In conclusion we can state that Iscador® may inhibit tumor and endothelial cell growth by delaying cell cycle progression and by causing apoptotic cell death. However, sensitivity to these Iscador® preparations varies strongly between different tumor cell lines. Immunostaining for pro-apoptotic proteins in Iscador® Q treated HaCaT cells only revealed involvement of the mitochondrial pathway, whereas in Iscador® M treated cells the death receptor pathway as well as the mitochondrial pathway seem to be involved.

Our data suggest that Iscador® affects tumors by two different mechanisms, i.e. a direct cytostatic and apoptosis inducing effect on tumor cells, and an indirect effect via endothelial cells leading to inhibition of vasculature formation and subsequent starvation of the tumor.

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Tumor immunologists have attempted to harness and boost the immune system's power in all sorts of ways. Their hopes have often been raised, but never fulfilled. Perversely, their string of failures seems to have immunized them against despair. They are ready to try again.

Los inmunólogos tumorales han tratado de frenar o estimular el sistema inmune de mil maneras. Sus esperanzas fueron despertadas pero nunca satisfechas. Perversamente, esas cadenas de fracasos parecen haberlos inmunizados contra la desesperanza. Están listos para empezar de nuevo.

The Economist, 1994