ISSN 0025-7680

International Symposium
NEW DIRECTIONS IN CANCER MANAGEMENT
Academia Nacional de Medicina
Buenos Aires, 6-8 June 2007

MEDICINA (Buenos Aires) 2007; 67 (Supl. II): 97-106

GENE EXPRESSION SIGNATURES OF PATHWAY ALTERATIONS IN TUMOR CELLS CAUSED BY PLANT EXTRACTS

ISABELLE WAGSCHAL[a], JENNY EGGENSCHWILER[a], LEOPOLD von BALTHAZAR[a], ANDREA PATRIGNANI[b], HUBERT REHRAUER[b], RALPH SCHLAPBACH[b], MAC H. RAMOS F[c], ANGELIKA VIVIANI[a]

[a]*University of Applied Sciences Wädenswil;* [b]*Functional Genomics Center Zurich, ETHZ and University of Zurich;* [c]*Weleda AG, Arlesheim, Switzerland*

Abstract Extracts of *Viscum album* from different host trees (Iscador®Qu, M, P and A) and isolated mistletoe lectin showed distinct gene-expression profiles using cDNA microarrays. We were able to confirm that genes of apoptosis, the cell cycle and the immune system were affected by mistletoe preparations and we also found that signal transduction, cell communication and cancer related pathways were overregulated in the tested breast cancer (HCC-1937, MCF-7, KPL-1, Mfm-223) and lymphoma cell lines (WSZ-NHL). The effect of a specific mistletoe extract on a particular cell line was individual, so that the distinction between the reaction of the cell type and/or the influence of the mistletoe preparation could be used in targeted mistletoe therapy.

Key words: *Viscum album,* cancer cells lines

Resumen ***Perfiles de expresión génica en células tumorales inducidos por extractos de plantas.*** Extractos de *Viscum album* de diferentes arboles hospedadores (Iscador®Qu, M, P and A) y lectinas aisladas de muérdago demostraron diferentes patrones de expresión génica utilizando la técnica de microarray. Pudimos confirmar que genes involucrados en apoptosis, ciclo celular y el sistema inmune fueron afectados por preparaciones de muérdago. Por otra parte encontramos que vías de señalización, comunicación entre células y vías relacionadas con cáncer estaban sobreexpresadas en las líneas de cáncer de mama estudiadas (HCC-1937, MCF-7, KPL-1, Mfm-223) y en la línea de linfoma WSZ-NHL. El efecto de extracto específico de muérdago sobre una particular línea celular fue individual, de manera que la distinción entre la reacción del tipo celular y/o la influencia de la preparación, podrían usarse como blanco en la terapia con extractos de muérdago.

Palabras clave: *Viscum album,* líneas celulares neoplásicas

The cytotoxic effect of *Viscum album* extracts on human cancer cells is well documented, and it is known that cancer cells respond individually to specific mistletoe preparations, depending on the host tree and the manufacturing process[1-11]. The ways in which mistletoe extracts affect cancer cells have been described for some cellular mechanisms (apoptosis[12-19], cell cycle[15, 20], protein synthesis[21], immune modulation[21-25], However, the cell response seems to be very unique and has to be analysed individually for each cell type. Describing a cancer cell type according to its corresponding IC_{50} gives us the information on whether a cancer cell is sensitive or resistant to mistletoe extract, but the characteristic, unique properties of each cell are not taken into account. A method to characterize the common and individual cellular alterations is transcriptome analysis. We analyzed changes of mistletoe extract-treated cells compared to untreated ones within the 41,000 genes of the Whole Human Genome chip (Agilent) on the mRNA level. The obtained gene expression profiles allow us to distinguish the pathway deregulations caused by either a certain mistletoe extract or by a specific cancer cell type or subtype. The gene regulation studies were carried out with 4 different breast cancer and two lymphoma cell lines.

The aim of the project is to predict the sensitivity of a specific cancer type to the most efficient mistletoe preparation.

- - - -
e-mail: a.viviani@hsw.ch

Methods

Viscum album *extracts and isolated mistletoe lectin*

The mistletoe preparations Iscador® Qu, M, A and P were provided by Weleda AG, Arlesheim, Switzerland. Isolated mistletoe lectin (ML I) was a gift of Hiscia AG, Arlesheim, Switzerland.

Cancer cell lines

Four different breast cancer cell lines, MCF-7, KPL-1, HCC-1937 and MFM223, and two lymphoma cell lines, WSU-NHL and DOHH-2, were used.

Cytotoxity assay

All cell lines were tested in regard to their responsiveness to mistletoe extracts. The IC_{50} was determined by exposing the corresponding cancer cells to the appropriate mistletoe extract in a concentration-dependent manner for 48 hours. The survival of the cells was analysed by determining the mitochondrial activity (MTT assay).

Microarray experiments

Cell lines were treated for 24 hours with 0.1 mg Iscador preparation. Then, the mRNA was isolated from cell lysates of $1\text{-}2\cdot10^6$ cells. Only those RNA samples with a 260 nm/280 nm ratio between 1.8 - 2.1 and a 28S/18S ratio within 1.5 - 2 were further processed.

RNA samples (2.5 µg) were reverse-transcribed into double-stranded cDNA, which was labeled by *in vitro* transcription incorporating Cyanine 3-CTP (samples) or Cyanine 5-CTP (reference). Each Cy-3 labeled sample (1 µg) was combined with the Cy-5 labeled control (1 µg) and hybridized onto Agilent Whole Human Genome 41k arrays for 17 h at 65 °C.

Hybridized arrays were scanned and the scanned images were quantified using the Agilent Feature Extraction Software 7.1. Data were excluded for genes that did not have an expression signal above 200 in either channel.

For each cell line, the cells incubated with the four different Iscador preparations were compared with untreated ones to obtain lists of mistletoe affected genes using Genespring. Then, the obtained gene lists were used to find the pathways where the regulated genes were significantly overrepresented. Overrepresentation was declared as significant by t-test if the p-value for overrepresentation was below 0.01 and within a pathway if the genelist vs pathway random overlap p-value was below 0.05.

Each experiment was repeated three times.

Results

Cytotoxicity of Iscador preparations and isolated mistletoe lectin on cancer cells

In order to achieve an overview of whether mistletoe extracts and their lectin content affect the chosen cancer cells and whether the effects were comparable or totally different, we determined the IC_{50} value for each cell line and mistletoe extract (Table 1). In addition to Iscador Qu (oak-mistletoe), Iscador M (apple tree-mistletoe), Iscador A (white fir-mistletoe) and Iscador P (pine-mistletoe) isolated mistletoe lectin, which is known to be the strongest cytotoxic component of mistletoe extracts, was tested. Iscador Qu and M are lectin-rich with approximately 25-50 ng lectin/mg extract, whereas the lectin-poor Iscador A and P contain only about 1 ng lectin/mg extract.

The lymphoma cells WSU-NHL were most sensitive to lectin, with an IC_{50} of 22 ng/ml, followed by Mfm-223 and DOHH-2. MCF-7 and HCC-1937 are more than 10 times less responsive to the isolated component. Nevertheless, the IC_{50} values of the whole extracts of Iscador Qu, M or A were all around 0.1 mg/ml for MCF-7 and for Mfm-223 whereas the cells of WSU-NHL, KPL-1 and HCC-1937 reacted similarly to the lectin-rich Iscador Qu and M and less strongly to the lectin-poor Iscador A (0.3 mg/ml). DOHH-2 showed the lowest sensitivity to Iscador preparations compared to the other cell lines. Iscador P did not cause strong cytotoxic effects on any of the cell lines.

We conclude that lectin is highly cytotoxic. However, its cytotoxic influence within the multi-component mistletoe extract was observed in regard to the responsiveness of cancer cell types only in special cases, but there is not yet enough evidence to predict these cases reliably

Pathway alterations in cancer cells after Iscador and lectin treatment

IC_{50} values quantitatively describe the efficacy of an extract on a certain cell line in general without referring to cellular processes in detail. An equal IC_{50} value does not correspond to consistently equal molecular responses in different cell lines. Therefore, cellular mechanisms were analysed using microarray data at the level of pathways to characterize cell behaviour in the presence of mistletoe components. From a total of 188 Keggs pathways in Genespring, 125 were found to be involved in the action of mistletoe extracts or mistletoe lectin, causing changes in the cancer cell mechanism from which the most important will be selected. In the following Tables (2-5)

TABLE 1.– *IC_{50} values of the mistletoe preparations Iscador Qu, M, A and P and isolated mistletoe lectin I. The lymphoma (WSU-NHL, DOHH-2) and breast cancer cells (MCF-7, KPL-1, HCC-1937, MFM223) were exposed for 48 hours to the corresponding test solutions*

	Iscador Qu mg/ml	Iscador M mg/ml	Iscador A mg/ml	Iscador P mg/ml	Mistletoe lectin ng/ml
WSU-NHL	0.08 ± 0.01	0.11 ± 0.02	0.19 ± 0.02	1.1 ± 0.01	22 ± 5
DOHH-2	0.3 ± 0.04	0.46 ± 0.1	0.29 ± 0.07	2.7 ± 0.2	94 ± 17
Mfm-223	0.05 ± 0.02	0.12 ± 0.03	0.07 ± 0.03	1.4 ± 0.25	38 ± 3
KPL-1	0.1 ± 0.01	0.12 ± 0.022	0.31 ± 0.06	1.94 ± 0.3	141 ± 29
MCF-7	0.09 ± 0.02	0.12 ± 0.02	0.10 ± 0.02	1.61 ± 0.25	410 ± 59
HCC1937	0.11 ± 0.02	0.1 ± 0.02	0.31 ± 0.02	2.14 ± 1.0	320 ± 73

TABLE 2.– *The cellular metabolisms (carbohydrate metabolism, lipid metabolism, amino acid metabolism, metabolism of other amino acids, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins) were involved in alterations in pathways of the breast cancer (MCF-7, KPL-1, HCC-1937, MFM223) and lymphoma cell lines (WSU-NHL, DOHH-2) after treatment with 0.1 mg* Viscum album *preparation/ml or mistletoe lectin (at IC_{50}) for 24 hours.*

Carbohydrate metabolism							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Aminosugars metabolism (50)	Iscador A		+++				
	Iscador P		+++++				
Butanoate metabolism (66)	Lectin	++++		++++			
	Iscador Qu					+++	
Citrate cycle (49)	Lectin	+++	+++				
	Iscador Qu		+++				
	Iscador A		++				
Glyoxylate and dicarboxylate metabolism (23)	Iscador Qu					**+++++**	
	Iscador M					**+++++**	
Pentose phosphate pathway (43)	Lectin	++++		+++++	++++		
	Iscador Qu					+++	
	Iscador M					++	

Lipid metabolism							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Biosynthesis of steroids (25)	Lectin		**+++++**	**+++++**			
	Iscador Qu					**+++++**	
	Iscador M					**+++++**	
Fatty acid elongation in mitochondria (14)	Lectin	++++	++++	**+++++**			
Sphingolipid metabolism (61)	Lectin	**+++++**	++++	++++			
Synthesis and degradation of ketone bodies (13)	Iscador Qu		+++++			**+++++**	
	Iscador M					**+++++**	

Amino acid metabolism							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Glycine, serine and threonine metabolism (74)	Lectin	**+++++**					
	Iscador Qu	+++				++++	
	Iscador M					+++	
Methionine metabolism (37)	Lectin	++++					
	Iscador Qu					**+++++**	
	Iscador M					++++	
Tryptophan metabolism (140)	Lectin	++++		**+++++**			
	Iscador Qu				+		
	Iscador M		++				
	Iscador A		++				
	Iscador P		++				
Valine, leucine and isoleucine biosynthesis (14)	Iscador Qu	+++++	+++++			+++++	
	Iscador M					**+++++**	
	Iscador A		+++++				+++++

(Continuación Table 2)

Metabolism of other amino acids							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Glutathione metabolism (65)	Lectin	**+++++**	+++				
	Iscador Qu	+++					
	Iscador M						++*
	Iscador A			+			++
Selenoamino acid metabolism (64)	Lectin	+++++					
	Iscador Qu					**+++++**	
	Iscador M					+++++	

Glycan biosynthesis and metabolism							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Chondroitin sulfate biosynthesis (26)	Lectin		**+++++**	+++++			
	Iscador Qu	++++				+++	
Glycan structures - biosynthesis 1 (165)	Lectin		**++++**	**+++++**			
	Iscador A	++					
Glycosphingolipid biosynthesis - ganglioseries (26)	Lectin	+++++					
	Iscador Qu	**+++++**					
N-Glycan biosynthesis (65)	Lectin			+++++			
	Iscador A	++					
N-Glycan degradation (24)	Lectin			+++++			
	Iscador Qu	+++++					
O-Glycan biosynthesis (39)	Lectin		+++++	**+++++**			

Metabolism of cofactors and vitamins							
Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
Nicotinate and nicotinamide metabolism (57)	Lectin	+++++	**+++++**				
	Iscador Qu					++	
One carbon pool by folate (36)	Iscador M					++++	
	Iscador Qu					+++++	
	Iscador M					++++	
	Iscador A		++++				
Ubiquinone biosynthesis (13)	Iscador Qu					+++++	
	Iscador M					**+++++**	

+++++: ≥ 20% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++++: ≥ 15% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+++: ≥ 10% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++ ≥ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+: ≤ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
***+ in bold:** genelist vs pathway random overlap p-value below 0.001*
**: only 1 experiment was carried out*

the affected pathways were divided into functional groups such as metabolism, genetic information processing, environmental information processing, cellular processes and human diseases, which are further subdivided into clearly defined pathways.

Metabolic pathways

If any cell is subjected to environmental changes by drugs, very specific pathways are affected; also the basic metabolism can be overregulated (Table 2). The

TABLE 3.– *The pathways of environmental information processing (membrane transport, signal transduction, signalling molecules and interaction) were overregulated by the breast cancer (MCF-7, KPL-1, HCC-1937, MFM223) and lymphoma cell lines (WSU-NHL, DOHH-2) after treatment with 0.1 mg* Viscum album *preparation/ml or mistletoe lectin (at IC_{50}) for 24 hours.*

Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
			Membrane transport				
ABC transporters (67)	Lectin		++++	+++++			
	Iscador A		+++	+++++			
			Signal transduction				
Calcium signalling	Iscador Qu						+*
pathway (292)	Iscador M			**+**			
	Iscador A			**+**			+*
	Iscador P			**+**			
Hedgehog signalling	Lectin		+++	+++++			
pathway (79)	Iscador Qu	+++					++*
	Iscador M					++	
	Iscador A						++*
MAPK signalling	Lectin	**+++++**	**++++**	**+++++**	**+**		
pathway (472)	Iscador M		++				
	Iscador A	++					
Notch signalling	Lectin			**+++++**			
pathway (93)	Iscador Qu		++				
	Iscador M		++				
TGF-beta signalling	Lectin	+++++		**+++++**	+		
pathway (137)	Iscador Qu	+++					
	Iscador M					+	
	Iscador A				++		
	Iscador P					**++**	
VEGF signalling	Lectin		+++				
pathway (129)	Iscador M		++				
	Iscador A	+++					
Wnt signalling	Lectin	+++	**++++**	**+++++**			
pathway (273)	Iscador Qu			**++**			
	Iscador M		++		+		++*
	Iscador A			+	++		++*
			Signalling molecules and interaction				
Cytokine-cytokine	Lectin	**+++++**	**+++**	+++++	**++**		
receptor	Iscador Qu			+	+	**++**	
interaction (349)	Iscador M			**+**	+	**+**	++*
	Iscador A			+	++		
	Iscador P			+		+	
ECM-receptor	Lectin			+++++	+		
interaction (146)	Iscador Qu	+++				+	
	Iscador M					+*	++*
	Iscador A					+*	++*
Regulation of actin	Iscador M			+			
cytoskeleton (394)	Iscador A		++			+	
	Iscador P			+			

+++++: ≥ 20% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++++: ≥ 15% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+++: ≥ 10% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++ ≥ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+: ≤ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
***+ in bold:** genelist vs pathway random overlap p-value below 0.001*
**: only 1 experiment was carried out*

TABLE 4.– *The pathways of cellular processes belong to cell growth and death, cell communication, endocrine system, immune system and behaviour and some are best known to be induced by the breast cancer (MCF-7, KPL-1, HCC-1937, MFM223) and lymphoma cell lines (WSU-NHL, DOHH-2) after treatment with 0.1 mg* Viscum album *preparation/ml or mistletoe lectin (at IC_{50}) for 24 hours.*

Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
			Cell growth and death				
Apoptosis (145)	Lectin	+++++	**+++++**	**+++++**			
	Iscador Qu				+		
	Iscador M				+		
	Iscador A				++		
	Iscador P				+		
Cell cycle (183)	Lectin			**+++++**			
	Iscador Qu					++	
	Iscador M					++	
			Cell communication				
Adherens junction (161)	Iscador M					++	
	Iscador A		++				**+**
Focal adhesion (363)	Iscador M		++		+		**+**
	Iscador A	++					+*
	Iscador P			+			
Gap junction (139)	Iscador Qu	+++					
	Iscador A	++					
Tight junction (229)	Iscador M						+*
	Iscador A	++					
	Iscador P	++					+*
			Endocrine system				
Adipocytokine signalling pathway (125)	Lectin		+++	++++		+	+
	Iscador Qu	+++					
GnRH signalling pathway (165)	Lectin	++++				+	+
	Iscador A	++					
Insulin signalling pathway (241)	Lectin		+++			+	+
PPAR signalling pathway (109)	Lectin	+++++				+	+
	Iscador Qu			++		++	
	Iscador M					++	
			Immune system				
Antigen processing and presentation (153)	Lectin		++++	**+++++**			
	Iscador Qu					+++	
	Iscador M					**++++**	
	Iscador A			+			
	Iscador P			+			
B cell receptor signalling pathway (106)	Lectin		**+++++**				
	Iscador Qu			++	+		
	Iscador M		++		++		
	Iscador P				+		
Complement and coagulation cascades (111)	Lectin	+++++	+++++			+	+
	Iscador Qu				++		+*
	Iscador M				++		**+++***
	Iscador A		++		++	+	**++***
	Iscador P			**++***			

(Continuación Tabla 4)

Fc epsilon RI signalling	Lectin	+++++			
pathway (126)	Iscador M				+
	Iscador A				+
Hematopoietic	Lectin	+++++			
cell lineage (137)	Iscador Qu				**++**
	Iscador M				+
	Iscador A		++	+	
	Iscador P		++	+	+
Natural killer cell mediated	Iscador A		++		
cytotoxicity (205)	Iscador P	++			
T cell receptor signalling	Lectin	**+++++**	**++++**	+++++	
pathway (158)	Iscador Qu			++	**+**
	Iscador M				**++**
	Iscador A				++
	Iscador P				**++**
Toll-like receptor	Lectin	+++++	++++		+
signalling pathway (126)	Iscador Qu			+	+
	Iscador M				+
	Iscador A			+	
			Behaviour		
Circadian rhythm (23)	Lectin	**++++++**	+++++	+++++	
	Iscador Qu			++++	

+++++: ≥ 20% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++++: ≥ 15% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+++: ≥ 10% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++ ≥ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+: ≤ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
***+ in bold:** genelist vs pathway random overlap p-value below 0.001*
**: only 1 experiment was carried out*

metabolic pathways of Mfm-223 and DOHH-2 cells were not induced by mistletoe components whereas the lipid and amino acid metabolism, as well as the glycan biosynthesis of HCC-1937, MCF-7 and WSU-NHL were overregulated in the presence of isolated mistletoe lectin. In contrast, the whole mistletoe extracts did not produce remarkable alterations in the metabolic pathways of all tested cell lines.

The only exception was WSU-NHL, on which Iscador Qu and M activated the glyoxylate and dicarboxylate metabolism, the biosynthesis of steroids, the synthesis and degradation of ketone bodies, the glycine, serine and threonine metabolism, the methionine metabolism, the valine, leucine and isoleucine biosynthesis, the seleno-amino acid metabolism, one carbon pool by folate metabolism and ubiquinone biosynthesis very strongly and independently from the other cell lines tested, so that a selective geneinduction by these two extracts can be postulated for this cell line.

Pathways of environmental information processing

If a cell is exposed to a drug, internal cell processes will be altered to remove the foreign agent and to reconstitute the cellular order immediately. Depending on the character of the cell, different signalling pathways will be induced to inform the cellular environment and to induce the immune-response by releasing or activating signal substances. It has to be considered that the pathways, calcium signalling, regulation of actin cytoskeleton, MAPK signalling, Wnt signalling and cytokine-cytokine receptor interaction, contain from 273 to 472 genes, many more than the metabolic pathways, and that 20% of the overregulated genes in these pathways comprise 55 to 95 genes.

In the presence of isolated mistletoe lectin, 9 of the 11 pathways (ABC transporter, Hedgehog signalling pathway, MAPK signalling pathway, Notch signalling pathway, TGF-beta signalling pathway, VEGF signalling pathway, Wnt signalling pathway, Cytokine-cytokine receptor interaction, ECM-receptor interaction) were mostly strongly overre-

TABLE 5.– *The pathways of human diseases are affected by the breast cancer (MCF-7, KPL-1, HCC-1937, MFM223) and lymphoma cell lines (WSU-NHL, DOHH-2) after treatment with 0.1 mg* Viscum album *preparation/ml or mistletoe lectin (at IC_{50}) for 24 hours.*

Pathway (number of genes/pathway)	Treatment	HCC-1937	MCF-7	KPL-1	Mfm-223	WSU-NHL	DOHH-2
			Neurodegenerative disorders				
Neurodegenerative	Iscador Qu		++				**+++++***
disorders (66)	Iscador A				++		**+++++***
			Infectious disease				
Epithelial cell signaling	Iscador Qu			++	**+**		
in Helicobacter pylori	Iscador M				**+**		
infection (106)	Iscador P			+			
Pathogenic Escherichia	Iscador Qu	+++					
coli infection (94)	Iscador M					++	
	Iscador A	+++					
	Iscador P	++					
			Cancers				
Chronic myeloid	Lectin			+++++			
leukemia (142)	Iscador Qu				+		
	Iscador A	++					
Colorectal cancer (153)	Lectin		+++	+++++			
	Iscador Qu		++				
	Iscador M		++				
	Iscador A		++		++		
Glioma (112)	Lectin			++++	+		
	Iscador A	++	++				
Huntington's	Lectin				++		
disease (57)	Iscador Qu					+++	
Pancreatic cancer (134)	Lectin			++++			
	Iscador A	++					

+++++: ≥ 20% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++++: ≥ 15% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+++: ≥ 10% genes overregulated, genelist vs pathway random overlap p-value below 0.1
++ ≥ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
+: ≥ 5% genes overregulated, genelist vs pathway random overlap p-value below 0.1
***+ in bold:** genelist vs pathway random overlap p-value below 0.001*
**: only 1 experiment was carried out*

gulated in the breast cancer cell lines HCC-1937, MCF-7 and KPL-1. The calcium signalling pathway and regulation of actin cytoskeleton seem to be lectin-independent. The calcium signalling pathway was induced by Iscador M, A and P on KPL-1, and Iscador Qu and A on DOHH-2. The regulation of actin cytoskeleton was affected by Iscador M on KPL-1, Iscador A on MCF-7 and WSU-NHL and Iscador P on KPL-1. The most sensitive cell line in all the environmental information processes was KPL-1. Mfm-223 and WSU-NHL are well represented under signalling molecules and interaction so that we conclude that they were activated on different receptor sides by Iscador Qu, M or A. Genes of the signal transduction pathways were targets favoured by Iscador Qu and M.

Cellular processes

Pathways in this category contain up to 100 genes, except circadian rhythm with only 23.

It is well documented that apoptosis and cell cycle are affected by mistletoe extracts. Under the given experimental conditions (0.1mg extract/ml, 24 hours incubation time) apoptosis was induced by mistletoe lectin only on HCC-1937, MCF-7 and KPL-1. Mfm-223 cells became apoptotic under the influence of Iscador Qu, M, A and P, but not isolated lectin. Cell cycle deregulation was found in WSU-NHL in the presence of Iscador Qu and M, and in KPL-1 in the presence of isolated lectin.

The immunomodulatory effects of mistletoe extracts are well known. Interestingly, even cancer cells react with strong alterations in immune response-related genes, especially all breast cancer cells tested. Lectin affected all immune system pathways in HCC-1937, MCF-7 and/or KPL-1; the Mfm-223 cells were also immune-responsive, but only to the Iscador preparations, not to lectin. Whereas the breast cancer cell lines showed overregulations in the innate and adaptive immune system, the lymphoma cell lines were affected only in pathways of the innate immune system, namely in antigen processing and presentation and complement and coagulation cascades. It can be concluded that immunomodulation is induced in all cell lines by mistletoe components or extracts; however the involvement of a specific pathway is dependent on the individual cancer cell type.

Alterations in pathways of cell communication, the endocrine system and circadian rhythm are new aspects involved in describing the action of *Viscum album* extracts on cancer cells. Cell communication did not depend on lectin, which could not overregulate any of the pathways involved, but was influenced mainly by Iscador A in breast cancer cell lines and by Iscador A and M in lymphomas. In contrast, the endocrine system was lectin-dependent and was induced by lymphoma and breast cancer cells. Finally, 22-40% of the genes in the circadian rhythm pathway were changed by isolated mistletoe lectin in HCC-1937, MCF-7 and KPL-1, so that this aspect has to be pursued in future studies *in vitro* as well *in vivo*.

Human Diseases

Progress in molecular genomics allows the analysis of disease-related genes which can be influenced by pharmaceutical agents. Iscador Qu and A activate genes of neurodegenerative disorders in MCF-7, Mfm-223 moderately and in DOHH-2 extremely. Infectious disease genes were overregulated in HCC-1937 at the level of pathogenic *Escherichia coli* infection and KPL-1 at that of epithelial cell signalling in *Helicobacter pylori* infection. Cancer-related genes were affected by isolated lectin in KPL-1 only, whereas Iscador Qu, M and A were overregulated in the other three breast cancer cell lines. Iscador A was the best represented within all cancer pathways (chronic myeloid leukemia, colorectal cancer, glioma, Huntington's disease and pancreatic cancer) and breast cancer cell lines.

Mistletoe extract-specific genes of the breast cancer cell lines

From the pathway studies we conclude that the cancer cell type responds individually to a certain mistletoe extract which always includes several pathways. However, the possibility of cellular interactions is of great diversity, so that common pathways for all treatments and cell lines were difficult to define within the small cell type population tested.

It is easier to evaluate the specific genes which were affected by treatment with only one mistletoe extract in all the breast cancer cell lines HCC-1937, MCF-7, KPL-1 and Mfm-223. We found 1387 specific genes when the cells were treated with isolated lectin, 177 for Iscador Qu, 194 for Iscador M, 7 for Iscador A and 28 for Iscador P. Further studies will be undertaken to explain the function of these mistletoe extract-specific genes.

Conclusion

In summary, mistletoe extracts produced individual responses in the different cancer cell lines tested. However, the wide variety in the characteristics of the different cancer cell types makes classification difficult with regard to predictable responsiveness to a specific mistletoe extract. Therefore more cell lines and primary cancer cells have to be tested.

Isolated mistletoe lectin and the lectin-rich preparations Iscador Qu and M affected pathways in the breast cancer cell lines HCC-1937, MCF-7 and KPL-1, and produced comparable gene-expression profiles in signal transduction, the immune system, cancer and some metabolic pathways. On the other hand, WSU-NHL and Mfm-223 cells exhibited the lowest IC_{50} with lectin; nevertheless only a few pathways were overregulated. Therefore, it cannot be concluded directly that an activation of many pathways by one mistletoe extract correlates with stronger sensitivity. Quite similar is the situation in which a higher number of overregulated genes represent a better efficacy of this extract compared to the others. This is absolutely not true for Iscador A.

All our results are based on only one experimental condition (0.1 mg mistletoe extract/ml, 24 hour-incubation) and on the strong statistical restriction in gene analysis.

However, it can be concluded that transcriptome analyses of mistletoe extracts resulted in clear distinction of the gene expression profiles between isolated lectin, the lectin-rich mistletoe extracts (Iscador Qu and M) and the lectin-poor ones (Iscador A and P). These results can lead to targeted mistletoe therapies with a selection of specific, mistletoe-related genes on a microarray chip or as a set of oligos for qRT-PCR if the different cancer cell types can be assigned to specific clusters.

References

1. Knopfl-Sidler F, Viviani A, Rist L, Hensel, A. Human cancer cells exhibit in vitro individual receptiveness towards different mistletoe extracts. *Pharmazie* 2005; 60: 448-54.

2. Sidler F, Wermelinger T, Rist L, Hensel A, Viviani A. Influence of mistletoe extracts and its components on in vitro physiology of cancer cells. In: Animal cell technology meets genomics. Proceeding of the 18th ESACT meeting, Granada, Spain, 2005; 11-14, 2003.
3. Zuzak T, Rist L, Viviani A, et al. Das Mistelpräparat Iscucin®-Herstellung, Analytik, Wirkung in vitro. *Merkurstab* 2004; 6: 467-73.
4. Zuzak TJ, Rist L, Eggenschwiler J, Grotzer MA, Viviani A. Paediatric Medulloblastoma cells are susceptible to *Viscum album* (mistletoe) preparations. *Anticancer Research* 2006; 26: 3485-92.
5. Ulrich W, Mechelke F. Reactions of in vitro cultures of human fibroblasts, of HeLa-cells and of murine L-cells on application of a drug from *Viscum album* L. *Arzneianibamittelforschung* 1980; 30: 1722-5.
6. Hülsen H, Doser C, Mechelke F. Differences in the in vitro effectiveness of preparations produced from mistletoes of various host trees. *Arzneimittelforschung* 1986; 36: 433-6.
7. Lichtenstein A, Kahle J. Anti-tumor effect of inflammatory neutrophils: characteristics of in vivo generation and in vitro tumor cell lysis. *Int J Cancer* 1985; 35:121-7.
8. Doser C, Doser M, Hülsen H, Mechelke F. Influence of carbohydrates on the cytotoxicity of an aqueous mistletoe drug and of purified mistletoe lectins tested on human T-leukemia cells. *Arzneimittelforschung* 1989; 39: 647-51.
9. Lieberman MD, Shou J, Sigal RK, Yu J, Goldfine J, Daly JM. Transfusion-induced immunosuppression results in diminished host survival in a murine neuroblastoma model. *J Surg Res* 1990; 48: 498-503.
10. Lieubeau B, Heymann MF, Henry F, Barbieux I, Meflah K, Gregoire M. Immunomodulatory effects of tumor-associated fibroblasts in colorectal-tumor development. *Int J Cancer* 1999; 81: 629-36.
11. Li R, Sonik A, Stindl R, Rasnick D, Duesberg P. Aneuploidy vs. gene mutation hypothesis of cancer: recent study claims mutation but is found to support aneuploidy. *PNAS* 2000; 97: 3236-41.
12. Van Huyen JP, Delignat SS, Bloch MF, Kazatchine MD, Kaveri SV. Variable sensitivity of lymphoblastoid cells to apoptosis induced by Viscum album Qu FrF, a therapeutic preparation of mistletoe lectin. *Exp Chemotherapy* 2001; 47: 366-76.
13. Urech K, Scher JM, Hostanska K, Becker H. Apoptosis inducing activity of viscin, a lipophilic extract from Viscum album L. *Pharmaceutical Pharmacology* 2005; 57:101-9.
14. Lavastre V, Pelletier M, Salleer R, Hostanska K, Girard D. Mechanisms involved in spontaneous and Viscum album agglutinin-I- induced human neutrophil apoptosis: Viscum album agglutinin-I accelerates the loss of antiapoptotic Mcl-1 expression and the degradation of cytosceletal paxillin and vimentin proteins via caspases. *J immunol* 2002; 168: 1419-27.
15. Harmsma M, Gromme M, Ummelen M, Dignef W, Tusenius KJ, Ramaekers FC. Differential effects of Viscum album extract IscadorQu on cell cycle progression and apoptosis in cancer cells. *Int J Oncol* 2004; 25: 1521-9.
16. Thies A,· Nugel D, Pfüller U, Ingrid Moll I,Schumacher U. Influence of mistletoe lectins and cytokines induced by them on cell proliferation of human melanoma cells in vitro. *Toxicology* 2005; 207: 105-16.
17. Choi SH, Lyu SY, Park WB. Mistletoe lectin induces apoptosis and telomerase inhibition in human A253 cancer cells through dephosphorylation of Akt. *Arch Pharm Res* 2004; 27: 68-76.
18. Hostanska K, Vuong V, Rocha S, et al. Recombinant mistletoe lectin induces p53-independent apoptosis in tumour cells and cooperates with ionising radiation. *BJC* 2003; 88: 1785-92.
19. Savoie A, Lavastre V, Pelletier M, Hajto T, Hostanska K, Girard D. Activation of human neutrophils by the plant lectin Viscum album agglutinin-I: modulation of de novo protein synthesis and evidence that caspases are involved in induction of apoptosis. *J Leuk Biol* 2000; 68: 845-53.
20. Harmsma M, Gromme M, Ummelen M, Dignef W, Tusenius KJ, Ramaekers FC. Effects of mistletoe (Viscum album L.) extracts Iscador on cell cycle and survival of tumor cells. *Arzneimittelforschung/Drug Research* 2006; 56: 474-82.
21. Kienle GS and Kiene H. Die Mistel in der Onkologie. Fakten und konzeptionelle Grundlagen. Stuttgart Schattauer Verlag, 2003.
22. Lichtenstein A, Kahle J. Anti-tumor effect of inflammatory neutrophils: characteristics of in vivo generation and in vitro tumor cell lysis. *Int J Cancer* 1985; 35: 121-7.
23. Thies A,· Nugel D, Pfüller U, Ingrid Moll I,Schumacher U. Influence of mistletoe lectins and cytokines induced by them on cell proliferation of human melanoma cells in vitro. *Toxicology* 2005; 207:105-16.
24. Elluru S, Duong Van Huyen JP, Delignat S, et al. Molecular Mechanisms Underlying the Immunomodulatory Effects of Mistletoe (Viscum album L.) Extracts Iscador. *Arzneim Forsch Drug Res* 2006; 56: 461-6.
25. Eggenschwiler J, Patrignani A, Wagner U, et al. Gene expression profile compared with responsiveness of different breast cancer cells to fermented preparations of Viscum album from oak (Quercus), pine (Pinus), white fir (Abies) and apple tree (Malus) in vitro. *Arzneim Forsch Drug Res* 2006; 56: 483-96.

- - - -

La succession de chercheurs est comparable à un seul homme qui apprend indéfiniment.

La sucesión de investigadores es comparable a un solo hombre que aprende indefinidamente.

Blaise Pascal (1623-1662)