Direct and Eosinophil Dependent Mechanisms Underlying Mistletoe Therapy in the Treatment of Melanoma

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ABSTRACT

Mistletoe is one of the most widely used and studied complementary medicines for the treatment of cancer patients. It is one of the most prescribed drugs for cancer in German-speaking Central Europe and has been in use since 1920.

Among all the active ingredients of mistletoe extracts, mistletoe lectins (ML) and viscotoxin have shown antiproliferative or growth inhibition activity against a variety of cancer cell lines. Interestingly, this growth inhibitory property is not universal and different studies have shown that mistletoe extracts may or may not inhibit cell growth and may even induce cell proliferation depending on preparation, presence of multiple low molecular compounds of unknown activity and concentration of mistletoe extract, as well as the type of cell line used.

Clinically, mistletoe extract has been widely used for the treatment of gynaecological and breast cancer patients, administered via subcutaneous or intravenous injections as an adjuvant to conventional chemotherapy. A systematic review of clinical studies examining the effectiveness of mistletoe extract in these patients has found some positive effects. Incorporation of mistletoe extract (Viscum album, VA) as an adjuvant therapy in cancer treatment has been shown to improve the prognosis and quality of life of the patients by enhancing the antitumour response, strengthening the immune system, and reducing the side effects of mainstream chemotherapies. Patients treated with VAQ (mistletoe extract from the oak tree), had increased survival rates and improved quality of life, linked to leukocytosis and prolonged tissue-associated eosinophilia. Eosinophil infiltration at the tumour site is not associated with other mistletoe extracts, suggesting that eosinophils may play a role in the anti-cancer activity induced specifically by VAQ treatment.

Most studies on mistletoe have focussed on its effects as an adjuvant, but in isolated cases in humans and animal models, mistletoe extracts and ML have shown promising anticancer functions due to direct cytotoxic and apoptotic effects.

Malignant melanoma is one of the leading causes of death from cancer in Australia. There are only limited clinical studies looking into the efficacy of mistletoe extracts on malignant melanoma with confounding results. While one multicentre epidemiological cohort study involving 686 patients with intermediate to high-risk malignant melanoma who received long-term adjuvant treatment with mistletoe extract (VAL) showed significantly improved survival, another phase III trial involving 830 patients with high-risk primary melanoma and lymph node metastasis showed no difference between immunotherapy and mistletoe extracts. Preclinical studies in a mouse model have shown a better outcome. In one study, ML reduced melanoma growth and spread in a severe combined immunodeficiency (SCID) mouse xenograft model. In another study, triterpenoids from mistletoe had enhanced anti-tumour effects on murine B16F1 melanoma in C57/Bl6 mice. Interestingly, in the

SCID mouse model, a lower dose of ML was more effective in reducing tumour size and metastatic deposit compared to higher doses. *In-vitro* studies examining the effect of mistletoe extracts on melanoma cell lines have also yielded variable results. In one study, low dose lectin stimulated cell proliferation instead of inhibition in the malignant melanoma cell lines SK-MEL-28 and HT-144 but not in Malme-3M and C32.

Taken together with data from the Simson group, it is clear that VA and ML have high potential as complementary treatments. However, given some of the confounding data (described above) it is important to understand the cellular mechanisms that underlie the clinical symptoms in order to enable the most appropriate use of VA/ML.

The overall aim of this study was to investigate VA-mediated inflammation and immune modulation in-vivo with respect to tumour eradication, focusing on the role of eosinophils in these processes. The specific aims addressed in the study were to investigate the:

1. Role of VAQ and ML in reducing tumour growth, survival and metastasis.

VAQ and ML both significantly reduced transplantable melanoma (B16F1) growth as well as metastasis to the lungs in male Black6 mice (C57/Bl6). VAQ and ML not only reduced melanoma growth but also significantly improved survival rate of the mice. Reduced subcutaneous melanoma was associated with increased eosinophil accumulation at the tumour site compared to the untreated controls.

2. Molecular mechanism underlying the tumour reduction role of VAQ and ML

The study was further expanded to find out the possible molecular mechanism for melanoma reduction by investigating the effects of VAQ and ML on B16F1 growth proliferation *in-vitro*. Both VAQ and ML showed significant growth reduction of B16F1 *in-vitro* compared to untreated cells. These compounds also caused significant apoptosis of B16F1 cells and decreased CD47 expression by the B16F1 cells. This is the first study to look into the role of VAQ and ML on CD47 expression in the B16F1 cell-line. CD47 is a “marker of self” which prevents effective phagocytosis and also induces apoptosis. These *in-vitro* results indicate that effects of mistletoe extracts on tumour growth are multifaceted from induction of apoptosis to enhanced phagocytosis.

3. Role of eosinophils in the anti-tumour effects of VAQ and ML

One of the aims of the study was to investigate the role of eosinophils in solid tumours and possible effects of mistletoe extracts on eosinophils. B16F1 cell lines, when co-cultured with eosinophils treated with VAQ or ML, showed reduced cell growth compared to those co-cultured with untreated eosinophils. Ex vivo treated eosinophils also showed increased Major Basic protein (MBP) degranulation compared to untreated eosinophils. MBP and other eosinophil granules are toxic and have shown the ability to kill cancer cells in various studies. One of the ligands for CD47 in myeloid

cells, including eosinophils, is signal regulatory protein alpha (SIRP-α). SIRP-α is an inhibitory regulator of myeloid cells. Ligation of CD47 and SIRP-α has been implicated as one of many mechanisms by which tumours escape immune surveillance and clearance. This study showed for the first time that *in-vitro*, VAQ and ML reduce expression of SIRP-α by the eosinophils. These results suggest that VAQ and ML may enhance the eosinophil mediated anti-tumour response by reducing inhibitory signalling pathway response and by enhancing the secretion of toxic proteins.

To further establish the role of eosinophils in solid tumours, B16F1 cells were implanted in IL 5 transgenic (C57/BL6-IL5$^{+/+}$) male mice which have 30-40% eosinophils in contrast to wild type Black 6 (C57/BL6) mice which have only 3-5% eosinophils. C57/BL6-IL5$^{+/+}$ mice showed significantly reduced melanoma growth compared to the C57/BL6 mice. Immunohistochemistry (IHC) staining for MBP showed increased degranulation in C57/BL6-IL5$^{+/+}$ mice compared to wild type mice.

In conclusion, results from this study suggest that mistletoe extracts have the ability to reduce tumour growth and improve survival through complex, multifaceted mechanisms including direct cytotoxicity as well as the promotion of apoptosis and phagocytosis. Findings of this study also suggest that eosinophils are capable of exerting anti-tumour responses and VAQ and ML may improve immune surveillance by eosinophils by increasing degranulation and reducing the inhibitory signalling pathway response through reduction of CD47and SIRP-α expression.

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List of Commonly Used Abbreviations

Akt	Protein Kinas B
AML	Acute myeloid leukaemia
ANXA1	Annexin A1
APC	Antigen presenting cell
Apaf	Apoptotic activation factor
APAF	apoptotic protease activating factor 1
B cell	B lymphocytes
BAI1	Brain-specific angiogenesis inhibitor 1
BCs	Body condition score
BM	Bone Marrow
BSA	Bovine serum albumin
CAD	Caspase associated DNase
CAF	Cancer-associated fibroblasts
CCL11/24/26	Eotaxin
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
CPM	Count per minute
CRT	Calreticulin
CSC	Cancer stem cells
CSF-1	Colony stimulating factor 1
CTL	Cytotoxic T lymphocytes
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
E2F	Elongation factors 2
ECM	Extracellular matrix
ECP	Eosinophil cationic proteins
EDN	Eosinophil derived Neurotoxin
EDTA	Ethelenediaminetetraacetic acid
EGF	Epidermal growth factor
EM	European Mistletoe
EM	Electron microscope
EMT	Epithelial-mesenchymal transition
EPO	Eosinophil peroxidase
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence Activated Cell sorting
FBS/FCS	Fetal bovine serum/ calf serum
FGF	Fibroblast growth factor
GMCSF	Granulocyte macrophage-colony stimulating factor
HBSS	Hank's balanced salt solution
H&E	Haematoxylin and Eosin
HLA	Human leukocyte antigen

HMGB	High mobility group box protein-1
HSC	Hematopoietic stem cell
IAP	Integrin-associated protein
IC	Inhibitory Concentration
IHC	Immunohistochemistry
IL	Interleukin
IFN	Interferon γ
IP	Intraperitoneal
IP 10	Interferon gamma inducing protein 10
IRAK1	Interleukin-1 receptor-associated kinase-1
IT	Intra tumoral
IV	Intravenous
JNK	Jun N-terminal kinases
KMC/VAC	Korean mistletoe
LAIR	Leukocytes –associated immunoglobulin like receptor
LIAR	Local immunity and repair/remodelling
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP1	monocytes chemoattractant protein 1
MDM2	Murine double minute protein
ME	mistletoe extracts
MIP-1α	Macrophage inflammatory protein
ML	mistletoe lectin
MMP	Matrix metalloproteinase
mTOR	Mechanistic target of rapamycin
NBCS	Newborn calf serum
NBF	Neutral buffer formalin
NF-kB	Nuclear factor kB
NK cell	Natural Killer cells
NKG2-A/B	Natural Killer cells receptor 2
NMRI	Naval medical research institute
OA	Oleanic Acid (Ingredient of VA)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
PI3	Phosphatidylinositol 3 Phosphate
PMD	Piecemeal degranulation
PRR	pattern recognition receptors
QoL	Quality of Life
RAGE	Receptor for advanced glycation end product
RANTES	Regulated on activation, normal T cell expressed and secreted
RB	Retinoblastoma
RIP	Ribosome inactivating protein
rML	Recombinant mistletoe lectin
RNA	Ribo nucleic acid
RNAase	Ribonuclease

RPMI	Roswell Park Memorial Institute media
RTK	Receptor tyrosine kinase
SC	Subcutaneous
SCID	Severe combined immune deficient
SD	Standard deviation
SEM	Standard error of mean
SHP	Src homology-2 domain containing protein tyrosine phosphatases
Siglec	Sialic acid binding Ig-like lectins
SIRP	Signal regulatory protein alpha
T cell	T lymphocytes
TABE	Tumour-associated blood eosinophilia
TAM	Tumour-associated macrophages
TATE	Tumour-associated tissue eosinophilia
TBS	TRIS Buffer Solution
TGF β	Transforming growth factor β
Th	T helper lymphocytes
THP1	Monocytic leukaemia monocytes
TIL	Tumour infiltrating leukocytes
TIM	T-cell immunoglobulin and mucin domain-containing molecule
TLR4	Toll-like receptor 4
TNF	Tumour Necrosis Factor
TSP-1	Thrombospondin 1
UC	University of Canberra
UCAEC	University of Canberra Animal Ethics Committee
VA	Viscum Album
VAA	Viscum Album from fir tree or Abies
VAE	Viscum Album extract
VAM	Viscum Album from apple tree or Malus
VAP	Viscum Album from Pine Tree
VAQ	Viscum Album Quercus
VEGF-A	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
VT	Viscotoxin
MDSC	Myeloid-derived suppressor cells
T reg	Regulatory T cells
TAM	Tumour-associated macrophage

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