

Viscumins functionally modulate cell motility-associated gene expression

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Abstract. In Europe extracts from *Viscum album* L., the European white-berry mistletoe, are widely used as a complementary cancer therapy. Viscumins (mistletoe lectins, ML) have been scrutinized as important active components of mistletoe and exhibit a variety of anticancer effects such as stimulation of the immune system, induction of cytotoxicity, reduction of tumor cell motility as well as changes in the expression of genes associated with cancer development and progression. By microarray expression analysis, quantitative RT-PCR and RT-PCR based validation of microarray data we demonstrate for the *Viscum album* extract Iscador Qu and for the lectins Aviscumine and ML-1 that in glioma cells these drugs differentially modulate the expression of genes involved in the regulation of cell migration and invasion, including processes modulating cell architecture and cell adhesion. A variety of differentially expressed genes in ML treated cells are associated with the transforming growth factor (TGF)- β signaling pathway or are targets of TGF- β . ML treatment downregulated the expression of TGF- β itself, of the TGF- β receptor II (TGFBR2), of the TGF- β intracellular signal transducer protein SMAD2, and of matrix-metalloproteinases (MMP) MMP-2 and MMP-14. Even if the changes in gene expression differ between Aviscumine, Iscador Qu and ML-1, the overall regulation of motility associated gene expression by all drugs showed functional effects since tumor cell motility was reduced in a ML-dependent manner. Therefore, ML containing compounds might provide clinical benefit as adjuvant therapeutics in the treatment of patients with invasively growing tumors such as glioblastomas.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor in adults. The overall survival, even at best therapy and standard care, is normally less than 15 months, also long-term survival is rare (1). Surgical resection followed by irradiation and chemotherapy using temozolomide is currently still the standard treatment for patients with GBM. However, despite novel therapy approaches, the outcome remains poor. GBM are characterized by a diffuse and infiltrative growth into the surrounding healthy brain parenchyma (2). Even if GBM does not metastasize outside the brain, the expression of many proteins involved in metastatic processes like MMPs that destroy the extracellular matrix (ECM), cell adhesion molecules (CAM) such as integrins, cadherins or selectins that are necessary for cell-cell or cell-ECM interactions, members of the TGF- β signaling cascade which regulates cell migration and proteins involved in cell metabolism or transcription factors involved in the epithelial to mesenchymal transition (EMT) are dysregulated in GBM cells (3-8).

Today, *Viscum album* extracts are known under several trade names, such as Iscador, AbnobaVISCUM, Helixor and others which are mainly available in European countries. Dependent on the host tree the mistletoe is growing on, the time the plant is harvested as well as on the technique how the drug is prepared, the compounds in different *Viscum album* extracts vary. Important compounds of mistletoe extracts providing anticancer effects are ML-1, ML-2, ML-3 and viscotoxins (VT). VT are small peptides inducing cytotoxicity whereas MLs are glycosylated type II ribosomal inhibitory proteins (RIP II) of two subunits of which the α -chain serves as a toxic 28 S rRNA N-glycosidase. Other ingredients of *Viscum album* extracts with known effects on mammalian cells are oligo- and polysaccharides as well as flavonoids and triterpenes. ML-1 is known to be an important anticancer compound within the MLs (9,10).

We and others have shown that in cancer cells ML containing drugs not only block protein translation as predicted by the ML function such as RIPs, but also provide multimodal anticancer functions. At higher concentration these drugs induce cytotoxicity in cancer cells, potentiate cellular anticancer immune responses, alter the expression of cancer associated genes and

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reduce tumor growth in mice (11-15). Until today it is not completely understood how these plant extracts transmit their function as regulators of gene expression. Are VTs or other minor compounds like triterpenoids or flavonoids present in the extracts, beside the MLs, involved in the observed alteration in gene expression? Do MLs have to be glycosylated to be efficiently taken up by tumor cells and to transmit their function? To determine whether different ML containing drugs alter the expression of genes involved in cancer progression such as metastasis/motility associated genes and if the glycosylation of MLs, or minor additional compounds present in some preparations, are involved in gene regulation, we used three different ML containing drugs: Iscador Qu, a *Viscum album* extract, Aviscumine, a recombinant ML-1 and purified ML-1 from mistletoe plants growing on ash trees. We could demonstrate that these three drugs, even to a different level, modulate gene expression of motility associated genes and reduce glioma cell motility in a ML-dependent way.

Materials and methods

Mistletoe preparations. Iscador Qu, kindly provided by Iscador AG (Lörrach, Germany), is a fermented extract prepared from the mistletoe growing on oaks. It contains high amounts of MLs as well as VTs and other minor compounds such as oligo- and polysaccharides, triterpenes, flavonoids and others. ML and VT contents were as follows: Iscador Qu₂₀ (Charge 4080/3: total ML 1095 ng/ml as determined by the provider using an ELISA assay detecting ML1-3, VT 48 µg/ml). Aviscumine (ME-503), a recombinant, but non-glycosylated ML-1 produced in *E. coli* (16) and purified to GMP quality, was a kind gift of Hans Lentzen (Melema Pharma GmbH, Hamburg Germany). Purified glycosylated ML-1 isolated from mistletoes growing on ash trees was a kind gift of Christoph Heyder (Abnoba GmbH, Pforzheim, Germany) (17-21).

Cell culture. LNT-229 human malignant glioma cells, kindly provided by Nicolas de Tribolet (Lausanne, Switzerland), were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum (FCS; Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml) in a humidified atmosphere containing 5% CO₂. Cell viability was measured using crystal violet staining as previously described (15).

Microarray analysis and quantitative RT-PCR. For microarray analysis RNA of treated and untreated cells was isolated using the RNeasy lysis kit (Qiagen, Crawley, UK) and transcribed into cDNA using SuperScript II (Invitrogen, Karlsruhe, Germany). For each sample 10 µg reverse transcribed total RNA in a volume of 1080 µl in DNase-free water was mixed with 1080 µl TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), dispensed in 20 µl/well into the TaqMan Array Human Tumor Metastasis 96-well plate (cat. no. 4414098; Thermo Fisher Scientific) and run on an ABI 7500 system.

For quantitative reverse transcribed polymerase chain reaction (RT-qPCR) cDNA was prepared as for the microarray analysis. Quantitative PCR was determined using SYBR-Green Master Mix (Thermo Fisher Scientific) on a Roche

LightCycler 480. Relative mRNA expression was quantified ($[E^{ACT}(\text{gene of interest})/E^{ACT}(\text{housekeeping gene})]$). The following primers were used: EPHB2 forward, 5'-CCACTCATCATCGGCTCCTC-3' and EPHB2 reverse, 5'-GCTCAAACCCCGTCTGTTA-3'; GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and GAPDH reverse, 5'-GGCATGGACTGTGGTCATGAG-3'; IL1B forward, 5'-GCTCGCCAGTGAATGATGG-3' and IL1B reverse, 5'-GGTGGTCGGAGATTCGTAGC-3'; MMP-2 forward, 5'-CCAGAGACAGTGGATGATGCC-3' and MMP-2 reverse, 5'-GGAGTCCGTCCTTACCGTCAA-3'; MMP-14 forward, 5'-CGGCCCTTTCCAGCCTCTG-3' and MMP-14 reverse, 5'-GAGGTCTGAGGGTCTGCC-3'; MTA1 forward, 5'-CCAGTAGGGGTCTGGCAA-3' and MTA1 reverse, 5'-GGTAGGACTTCCCGTTGAGC-3'; NME forward, 5'-AGCCGGAGTTCAAACCTAAGC-3' and NME reverse, 5'-TTTGTGTGTCTGCCTCCCCT-3'; PTGS2 forward, 5'-GTTCCACCCGCAGTACAGAA-3' and PTGS2 reverse, 5'-AGGGCTTCAGCATAAAGCGT-3'; SERPINB5 forward, 5'-CATCCAGGTCTTTGTGCTCCT-3' and SERPINB5 reverse, 5'-GGGCCTGGAGTCACAGTTATC-3'; TGFB1 forward, 5'-GCCCTGGACACCAACTATTG-3' and TGFB1 reverse, 5'-CGTGTCCAGGCTCCAAAAG-3'; TGFB2 forward, 5'-CAAAAGCCAGAGTGCCTGAA-3' and TGFB2 reverse, 5'-CAGTTACATCGAAGGAGAGC-3'; TGFB2 forward, 5'-GGAGTTTCTGTTTCCCCG-3' and TGFB2 reverse, 5'-AGGGAAGCTGCACAGGAGTC-3'; TIMP-2 forward, 5'-GTTTATCTACACGGCCCCCT-3' and TIMP-2 reverse, 5'-TCGGCCTTTCTGCAATGAG-3'. RT-qPCR cycling conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec, 60°C for 1 min and 72°C for 20 sec.

Immunoblot analysis. The general procedure has been previously described (22). Supernatants were generated by culturing cells in serum deprived medium for 48 h, followed by a centrifugation step to avoid contamination. Protein contents were analyzed according to Bradford (BCA; Thermo Fisher Scientific). Following antibodies were used: anti-MMP-2 (Merck Millipore, Darmstadt, Germany), anti-MMP-14 (Epitomics, Burlingame, CA, USA), anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Smad2/3 (Cell Signaling Technology, Danvers, MA, USA) and anti-TIMP-2 (R&D Systems GmbH, Wiesbaden, Germany). Protein contents were quantified using the ChemiDoc MP system and ImageLab software (Bio-Rad Laboratories GmbH, Munich, Germany).

Immunofluorescence. LNT-229 cells (1×10^5) were seeded on poly-L-lysine coated glass coverslips in 12-well plates and treated after attachment for different time-points with 8 ng/ml of ML Iscador Qu, Aviscumine, ML-1 or were left untreated. The cells were fixed in 4% paraformaldehyde for 10 min, washed three times with phosphate-buffered saline (PBS) and blocked in 10% goat serum with 0.3% Triton X-100 in PBS for 1 h followed by overnight incubation with 2 µg/ml anti-ML antibody or isotype rabbit IgG (Santa Cruz Biotechnology) at 4°C. The polyclonal rabbit anti-ML antibody was a kind gift of the Iscador AG. To visualize the bound antibody the cells were stained with a goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fisher Scientific) for 1 h, mounted with Vectashield HardSet Mounting medium with DAPI (Vector Laboratories

Table I. ML preparations used in the present study.

Preparation	Manufacturer	Host tree	Characteristics
Iscador Qu	Iscador AG	Oak	Fermented extract, high ML-content, contains glycosylated MLs, and minor compounds such as viscotoxins, flavonoids and triterpenes, oligo- and polysaccharides
Aviscumine	Melema Pharma GmbH	-	Recombinant, unglycosylated ML-1 produced in <i>E. coli</i>
ML-1	Abnoba AG	Ash	Purified ML-1, glycosylated protein

Inc., Burlingame, CA, USA) and examined by confocal microscopy using a Zeiss LSM 510. Images were analyzed with the software ImageJ.

TGF- β ELISA. LNT-229 cells were seeded and treated with Iscador Qu, Aviscumine, ML-1 (8 ng/ml of ML) for 24 h or were left untreated. To block the effect of the ML treatment, the polyclonal rabbit anti-ML antibody (4.8 μ g/ml) was added to ML containing medium 30 min before adding the drugs to LNT-229 cells. After addition of MLs, cell supernatants were generated and concentrated using 4 kDa Amicon centrifugal filters (Merck Millipore). Protein concentrations for normalization were determined using the Bradford array. The ELISA was performed according to instruction of the manufacturer (RayBiotech, Norcross, GA, USA).

Boyden chamber migration assay. LNT-229 cells were seeded in 12-well plates, were allowed to attach and treated with Iscador Qu, Aviscumine, ML-1 (8 ng/ml ML) or were left untreated. To block the effect of ML, a pan-specific polyclonal rabbit anti-ML antibody (Iscador AG) neutralizing the effects of ML-1, -2 and -3 was added 30 min prior to the addition of ML containing drugs to glioma cells. After 24 h of ML treatment, the cells were washed to remove residual ML and 2×10^4 cells were seeded in doublets in membrane inserts of transwell migration chambers (8 μ m pores; Corning Incorp., Corning, NY, USA). Migrated cells were fixed 24 h later with methanol, stained with hematoxylin and counted as previously described (15). To avoid errors in the quantification of cell migration derived from differences in cell proliferation between ML treated and untreated cells, in parallel 1×10^4 of the cells were seeded in triplets in microwell plates, stained with crystal violet as described 24 h later (22) and were used for normalization of cell migration.

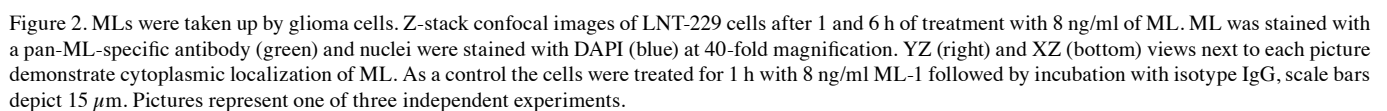
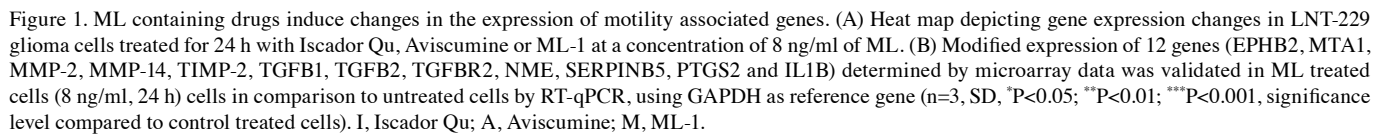
Statistical analysis. If not mentioned otherwise, the figures show the mean or one representative experiment out of at least three independent experiments as indicated. Quantitative data were assessed for significance by unpaired Student's t-test ($P < 0.05$; $P < 0.01$; $P < 0.001$).

Results

Different effects of Iscador Qu, Aviscumine and ML-1 on the expression of cell motility associated genes in LNT-229 glioma cells. In our previous experiments we have observed that ML-rich Iscador Qu, but not ML-poor Iscador P, reduces

the expression of genes involved in tumor development and progression such as genes regulating proliferation, cell survival or immune surveillance. Genes found to be upregulated in glioma were mainly downregulated by Iscador Qu whereas genes found to be downregulated in GBM specimen were upregulated (15 and data not shown). To analyze the influence of different ML preparations on the expression of motility associated genes and to evaluate whether the glycosylation of MLs influences gene expression, we used three different ML containing drugs (Table I). To avoid cytotoxicity-related changes in gene expression we first determined the EC₅₀ values (24 h) for all preparations in LNT-229 cells and found them to be 37 ng/ml of ML (Iscador Qu), 95 ng/ml (Aviscumine) and >240 ng/ml (ML-1). For further experiments we used ML concentrations that were far below EC₅₀ values (up to 8 ng/ml ML for Iscador Qu, up to 8 ng/ml Aviscumine and up to 16 ng/ml ML-1). To analyze differences in gene expression induced by the three preparations we treated LNT-229 glioma cells for 24 h with 8 ng/ml of ML (Iscador Qu). This concentration induces <15% of cell number reduction in LNT-229 (15). A concentration of 8 ng/ml of ML in Iscador Qu counts for 0.35 μ g/ml of VT, a concentration at which we have never observed cytotoxicity if treating glioma cells with a comparable VT concentration present in ML poor Iscador P (15). For Aviscumine or ML-1, we used concentrations up to 16 ng/ml ML, which is also far below the determined EC₅₀ value. All concentrations used in the experiments are below those that are necessary to kill non-tumor brain cells (15 and data not shown).

Using a PCR based microarray measuring the expression of 92 metastasis associated genes, quantitative RT-PCR of well-known genes involved in the regulation of cell motility, as well as RT-PCR based validation of differentially expressed genes identified by the microarray analysis, we identified 33 cell motility/invasion modulating genes that were regulated by at least one ML containing drug in LNT-229 glioma cells. The levels of 18 mRNAs decreased, whereas the levels of 15 mRNAs rose upon treatment. For Iscador Qu, 15 mRNAs were downregulated and 7 upregulated. For Aviscumine, 8 mRNAs were downregulated and 14 upregulated. For ML-1, 8 mRNAs were downregulated and 10 upregulated suggesting that minor compounds present in the mistletoe extract Iscador Qu might enhance downregulation of mRNA expression (Fig. 1 and Table I). Besides this, we detected differences in mRNA expression after the treatment of LNT-229 cells with either non-glycosylated Aviscumine or glycosylated ML-1, especially in the group of upregulated mRNAs (Fig. 1). In the



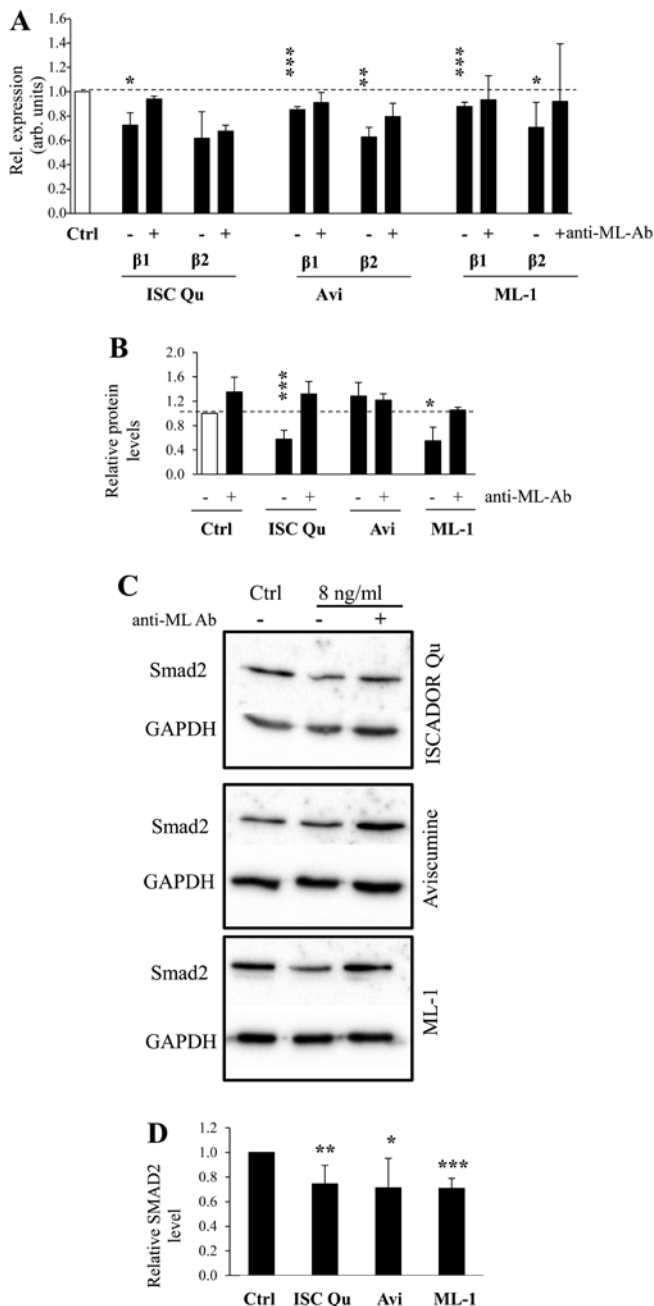


Figure 3. ML containing drugs modulate the expression of TGF- β and its intracellular transducer SMAD2. (A) Downregulated expression of TGF- β 1 and TGF- β 2 in Iscador Qu, Aviscumine or ML-1 treated LNT-229 cells (8 ng/ml ML, 24 h) in comparison to untreated cells by RT-qPCR, using GAPDH as reference. Coincubation of ML drugs with a ML-neutralizing antibody (4.8 μ g/ml, 30 min) was used to block ML specific effects. (B) Protein levels of TGF- β 1 in LNT-229 supernatants were quantified by ELISA. The cells were treated as in A, and afterwards cultivated in serum-free medium for 48 h to generate supernatants. (C) SMAD2 protein expression in ML treated LNT-229 cells. The cells were treated as in A and protein analysis was done by immunoblot, one of three independent experiments is shown. (D) Quantification of SMAD2 protein was prepared from three independent experiments. (A/B/D, n=3, SD, *P<0.05; **P<0.01; ***P<0.001, significance level compared to control treated cells).

group of the 18 downregulated mRNAs, 16 mRNAs code for genes harboring pro-migratory or pro-invasive function, the function of two genes (RBL1 and KISS1R) is controversially discussed. In the group of 15 upregulated mRNAs, 4 mRNAs

are known to provide anti-motility effects (BRMS1, FGF2, NME and SERPINB5), 7 are described to be pro-migratory (FAT1, IL-18, KRAS, MMP1, PTGS2, S100A4 and SERPIN1) and 4 mRNAs provide either pro- or anti-migratory effects dependent on the micro-milieu in which they are expressed (HGF, IL-1B, MET and MYC) (Fig. 1).

Differential expression of motility associated genes is independent from variations in the lectin uptake by glioma cells. To analyze whether the differences in gene expression induced by Iscador Qu, Aviscumine or ML-1 might be caused by differences in the uptake of MLs present in these drugs and might also depend on the glycosylation of MLs, this influencing the uptake, we treated the cells with a concentration that counts for a subtoxic concentration of 8 ng/ml of ML and performed immunofluorescence experiments using a pan-specific ML antibody. As demonstrated in Fig. 2, MLs from all three preparations were incorporated by the cells 1 h after treatment. At this time-point, the intensity of ML staining was approximately equal for Iscador Qu and Aviscumine, whereas ML-1 uptake was enhanced. At a later time-point (6 h), MLs were taken up equally independent of their glycosylation or from additional compounds being present in Iscador Qu.

ML containing drugs influence the expression of proteins involved in the TGF- β signaling pathway in glioma cells. TGF- β is one of the most important tumor promoting cytokines in GBM since in glioma cells TGF- β provides immunosuppressive function and induces a more migratory phenotype (23). We have demonstrated previously that TGF- β was downregulated by Iscador Qu (15). We now demonstrate that both TGF- β 1 and - β 2 mRNAs were also downregulated by Aviscumine and ML-1, and co-incubation of the preparations with a ML-specific antibody prior to the treatment of glioma cells reversed this effect (Fig. 3A). In addition to TGF- β , also TGF- β receptor type II (TGFR2) mRNA is reduced (Fig. 1B). The downregulation of TGF- β mRNA in ML treated LNT-229 glioma cells also leads to a downregulation of the TGF- β 1 protein by at least Iscador Qu and ML-1 (Fig. 3B). Notably, even if SMAD2, a prominent intracellular transducer protein of the TGF- β signaling, was not modulated on its mRNA level (data not shown), SMAD2 protein is reduced in ML treated glioma cells ML-dependently (Fig. 3C and D) suggesting that this effect might be induced by the function of MLs as RIPs.

Matrix metalloproteinases are major players in the destruction of the ECM, a process necessary for invasive tumor cell growth. It is well known that TGF- β modulates the expression and activity of MMPs and also of MMP inhibitors and activators in invasive cancer cells and also in gliomas (3,24). We have also shown in a recent publication that Iscador Qu downregulates MMP expression (15). By microarray, qPCR and immunoblot analyses we now demonstrate that this, but to a different extent, is also true for the other ML containing drugs. In LNT-229 cells, the treatment with Iscador Qu significantly reduced the expression of MMP-2, of the tissue inhibitor of metalloproteinase (TIMP)-2 and of MT-1MMP/ MMP-14 mRNA at a concentration of 8 ng/ml of ML, whereas this effect was minor for ML-1 and not detectable for Aviscumine. For MMP-14, a significant downregulation

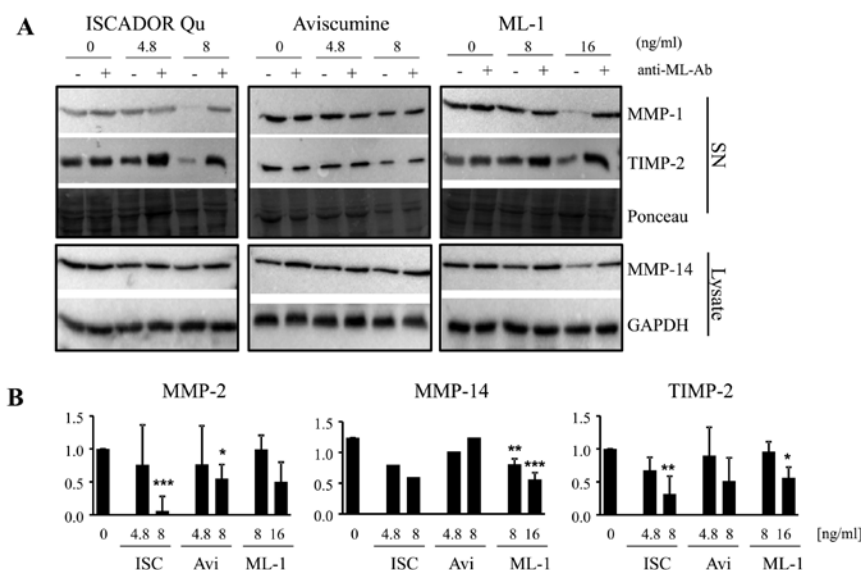


Figure 4. Iscador Qu, Aviscumine and ML-1 reduce MMP-2, MT1-MMP/MMP-14 and TIMP-2 expression. (A) LNT-229 cells were treated with increasing concentrations of Iscador Qu (ISC), Aviscumine (Avi) or ML-1. Coincubation of ML containing drugs with a ML neutralizing antibody (4.8 µg/ml, 30 min) prior to treatment was used to block ML specific effects. Supernatants and protein lysates were prepared as described in Materials and methods. (B) Quantification of MMP-14, MMP-2 and TIMP-2 protein levels from three independent experiments. For normalization of specific protein contents in supernatants, ponceau S stained lanes were used, in lysates GAPDH serves as housekeeping gene (n=3, SD).

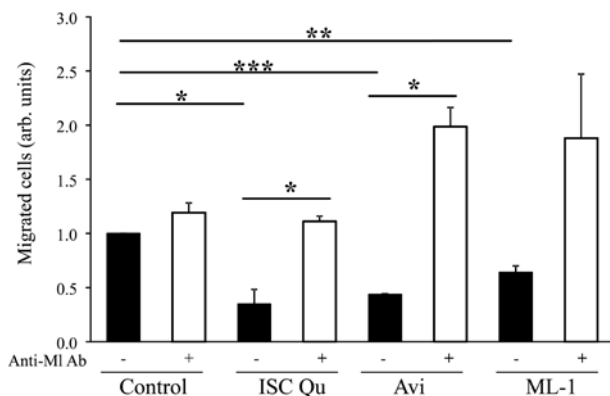


Figure 5. Iscador Qu, Aviscumine and ML-1 mitigate the migration of LNT-229 cells. LNT-229 cells were treated with Iscador Qu (ISC), Aviscumine (Avi), ML-1 (8 ng/ml ML, 24 h) or left untreated (control). A pan-specific ML antibody (4.8 µg/ml) was used to reverse the effect of the ML treatment as described before. For migration, 20,000 treated cells were seeded in Transwell migration chambers. After 24 h migrated cells were fixed, stained with haematoxylin and counted. (n=3, SD, *P<0.05; **P<0.01; ***P<0.001).

of MMP-14 mRNA was observed exclusively in Iscador Qu treated cells whereas TIMP-2 mRNA was reduced by both Iscador Qu and Aviscumine (Fig. 1B). Notably, the differences in MMP-2, MMP-14 and TIMP-2 mRNA downregulation by the different preparations are not directly translated into protein levels. Whereas Iscador Qu downregulates MMP-2 and TIMP-2 at a concentration of 8 ng/ml of ML in glioma cell supernatants, at this ML concentration lesser effects were detectable for Aviscumine and not detectable for ML-1. MT1-MMP/MMP-14, that converts inactive pro-MMP-2 into its active form, was slightly reduced by Iscador Qu and ML-1, but not by Aviscumine at 8 ng/ml. A concentration of 16 ng/ml of ML-1 has to be used to achieve the same effect as determined for Iscador Qu. In contrast to changes in the mRNA

expression observed by ML treatment, the downregulation in MMP and TIMP protein levels was dependent on the presence of MLs since addition of a ML-specific antibody reversed the observed effect almost completely (Fig. 4).

Iscador Qu, Aviscumine and ML-1 reduce glioma cell migration. Since a variety of genes associated with glioma cell invasion and migration are differentially regulated by ML treatment and even by different ML containing drugs, we analyzed whether the regulation of motility associated gene expression induced by ML functionally affects glioma cell motility. As demonstrated by Podlech *et al* (15) for Iscador Qu and now validated for Iscador Qu, but also shown for Aviscumine and ML-1, ML containing drugs significantly reduce glioma cell migration. The reduction of cell migration is dependent on the presence of MLs since addition of a ML-binding antibody completely abrogates the migration inhibitory effect (Fig. 5).

Discussion

Until today no effective therapy regimens are available for malignant GBM. One important characteristic issue for the treatment failure in glioma is its highly invasive growth that makes a complete resection impossible. A therapeutic drug that inhibits GBM cell motility, does not provide severe side-effects and that can be used as an adjuvant during radiation and chemotherapy might therefore further improve the prognosis of GBM patients. *Viscum album* extracts or ML-1 containing drugs show, at least *in vitro* and in cancer mouse models, therapeutic impact such as induction of cell death, inhibition of tumor cell proliferation and stimulation of the anticancer immune response (21,25-31). Besides this, several clinical trials have demonstrated benefits of this therapy for cancer patients such as enhancement of the quality of life, lesser side-effects of chemotherapy and, for certain cancer entities,

also slower tumor growth and a prolongation of survival (9,32-35). We have previously shown that Iscador Qu, a ML rich extract, also mitigates the motility of glioma cells (15). The focus of this project was to identify motility associated genes regulated by ML containing drugs to determine if different preparations provide comparable effects in gene regulation and whether changes in motility associated gene expression by ML containing drugs also lead to the inhibition of glioma cell motility.

Working concentrations of the different ML preparations were chosen for three reasons: i) Since no further information was available about the concentration of ML-1 in Iscador Qu, we chose a concentration that counts for maximal 8 ng/ml of total ML and a non-toxic concentration of 0.35 ng/ml VT. These concentrations do not induce cell death or caspase activity in LNT-229 cells and provide <15% reduction in cell growth (15). ii) There is a direct comparison possible regarding the effects of Aviscumine and ML-1 since these agents only differ in their glycosylation status: ML-1 being naturally glycosylated and Aviscumine not. iii) Due to the lower toxic effect of ML-1 ($EC_{50} > 240$ ng/ml) higher concentrations of ML-1 might be used during cancer therapy. For this we also tested the effect of 16 ng/ml of ML-1 on gene expression in glioma cells. On the level of reduced expression in treated glioma cells, all tested ML containing drugs downregulate the pro-migratory genes ephrin B2 (EPHB2), metastasis-associated protein 1 (MTA1), TGFBR2, its ligands TGF- β 1 and - β 2 and the TGF- β intracellular transducer protein SMAD2, however to different levels (Figs. 1 and 3). TGF- β is one of the most important pro-tumorigenic cytokines in glioma. Besides its immunosuppressive function, it enhances glioma cell invasion and migration via triggering the activation of MMPs and therefore, destruction of the ECM (3,23). SMAD2 is a central transducer protein in the TGF- β signaling cascade and its activity is regulated by phosphorylation (36). However, if the ML-induced reduction of TGF- β alone is sufficient for the reduction of the intracellular TGF- β signaling and the downregulation of TGF- β target genes, or if the downregulation of TGFBR2 and SMAD2 further enhances these effects, remains unclear. Overexpression of the TGF- β target EPHB2 in glioma cells reduces cell adhesion and induces cell migration and invasion *in vivo* and *in vitro*, whereas silencing of EPHB2 decreases tumor cell migration and is connected to the proto-oncogene Ras (37,38), which we have also found to be downregulated by Iscador Qu and ML-1. MTA1, also a target of TGF- β , has been described to promote motility and invasiveness of pancreas carcinoma cells and is amplified in highly invasively growing cells developed from recurrent GBM (39). Whether the downregulation of EPHB2 mRNA or other TGF- β target genes like MMP-2 is a direct effect of MLs or whether it is mediated by reducing the intracellular TGF- β signal has not been examined so far. Iscador Qu, that besides MLs, also contains VTs and minor ingredients such as triterpenes, flavonoids and others, provides superior effects in mRNA downregulation compared to Aviscumine and ML-1. In Iscador Qu treated glioma cells, 15 mRNAs were downregulated, whereas only 8 mRNAs were downregulated by Aviscumine or ML-1, respectively. The differences in the mRNA regulatory effects do not seem to be dependent on the uptake of MLs since after 6 h, intracellular ML was equally detectable for Iscador Qu, Aviscumine and

ML-1 (Fig. 2). Even if mRNA downregulation was dependent on at least ML-1 present in all preparations as demonstrated by the addition of the ML-specific antibody (Figs. 3 and 4), the glycosylation of ML-1 does not seem to be important for its inhibitory function since nearly the same profile of downregulated genes were identified in Aviscumine and ML-1 treated glioma cells (Fig. 1). The superior effects of Iscador Qu are potentially caused by additional compounds in this extract. It has been recently published that triterpenes as well as flavonoids provide antitumor activity, can influence mRNA expression involving the expression, secretion and activation of MMP-2, a mRNA we solely found to be significantly downregulated in Iscador Qu treated LNT-229 cells (Fig. 1) (40,41). In the panel of mRNAs that were only downregulated by Iscador Qu we found genes with prominent pro-migratory function. In glioma cells, a reduction of caspase-8 (CASP8) below basal levels results in a reduction of cell migration (42), CEACAM1 is an adhesion molecule that promotes migration and invasion of several cancers (43,44) and the netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating CDC42 and Rac1 (45). The TGF- β responsive genes MMP-2, MMP-14 and TIMP-2, the latter also serving as an activator for MMP-2, are important proteins of glioma cell motility (46) and the nuclear proto-oncogene SET1 stimulates cell migration in a Rac1-dependent manner, its knockdown inhibits cell migration and invasion of breast cancer cells (47). The function of the KISS1 receptor (KISS1R) and the retinoblastoma like protein (RBL1) are controversially discussed. KISS1R can enhance tumor cell spreading but has been also described to stimulate invadopodia formation and cancer cell invasion (48). In GBM and metastatic colon cancer cells, RBL1/p107 showed enhanced expression compared to normal tissue, but its level falls during invasion (49).

In the group of 15 upregulated mRNAs, 4 mRNAs are known to provide anti-motility or anti-invasive function (BRMS1, FGF2, NME and SERPINB5), 7 are described to be pro-migratory (FAT1, IL-18, KRAS, MMP1, PTGS2, S100A4 and SERPIN1) and 4 mRNAs provide either pro- or anti-migratory effects dependent on the circumstances under which they are expressed in tumor cells (HGF, IL1B, MET and MYC; Table II). In the panel of upregulated anti-migratory/anti-invasive mRNAs, the breast-cancer metastasis suppressor 1 (BRMS1) has been described to suppress glioma progression by regulating invasion, migration and adhesion (50). Higher expression of fibroblast growth factor 2 (FGF2) was observed in the lesser invasive proneural GBM subtype compared to invasively growing mesenchymal subtype GBMs. Besides this, GBM patients with a lower expression of the FGF2-dependent PDGF receptor A (PDGFRA) have a better prognosis than patients with high amounts of PDGFRA (51,52). Nucleoside diphosphate kinase A (NME) has been suggested to play an important role in the suppression of glioma invasion and migration (53,54) and the serpin peptidase inhibitor, member 5 (SERPIN5B/MASPIN) which is often silenced in GBM by promoter hypermethylation, effectively suppresses migration and invasiveness of malignant cancer cells (55,56). However, some genes upregulated by mistletoe treatment harbor pro-migratory or pro-invasive function, but this does not lead to functional consequences (Fig. 5). Even if the expression of

Table II. List of cell motility regulating genes differentially expressed by ML containing drugs.

Gene	Protein	Pro-/ anti-migratory	Array			RT-qPCR			Function	Connected to TGF-β signaling	Ref.
			I	A	M	I	A	M			
Downregulated genes											
EPHB2	Ephrin type-B receptor 2	Pro-migratory	↓	↓	↓	↓	↓	↓	EPHB2 is a TGF-β target important for TGF-β-mediated invasion and migration in breast cancer. EphB2 expression stimulates glioma neurosphere cell migration and invasion. Its overexpression in glioma cells results in reduced cell adhesion and increased cell invasion.	Yes	(34,38,57)
CASP8	Caspase-8	Pro-migratory	↓					n.d.	Downregulation of caspase-8 results in an inhibition of the migratory potential of glioma cells.	Yes	(42)
CEACAM1	Carcinoembryonic antigen related cell adhesion molecule 1	Pro-migratory	↓					n.d.	CEACAM1 inhibits cell-matrix adhesion and promotes cell migration and promotes invasiveness of thyroid cancer.	Yes	(43,44)
DCC	Deleted in colon cancer	Pro-migratory	↓					n.d.	The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1.	No	(45)
KISS1R	KISS-1 receptor	Controversial	↓					n.d.	KISS1R signaling promotes invadopodia formation in human breast cancer.	Yes	(48)
MMP-2	Matrix metalloproteinase-2	Pro-migratory	No change				↓		MMP-2 expression is elevated by the degree of malignancy of glioma. MMP-2 and -9 together play an important role in the invasiveness of gliomas by modulating the degradation of the ECM.	Yes	(58)
MMP-14/ MT1-MMP	Matrix metallo- proteinase-14	Pro-migratory	↓	↓	↓	↓	↓		MMP14 promotes glioma invasion, proliferation and angiogenesis. Coexpression of MMP-14 and MMP-19 predicts poor survival in human glioma.	Yes	(59,60)
MMP-9	Matrix metalloproteinase-9	Pro-migratory	↓	↓	↓		Very low expression		MMP-9 expression strongly associates with poor prognosis in p53+ GBM. Epigenetic regulation of miRNA-211 by MMP-9 governs glioma cell malignant features.	Yes	(61,62)
MTA1	Metastasis- associated protein 1	Pro-migratory	↓				↓	↓	MTA1 is upregulated in glioma tissue and amplified in aggressive stem cells during glioma recurrence. Its expression promotes motility and invasiveness of pancreatic carcinoma cells.	Yes	(39,63)
MTA2	Metastasis associated protein 2	Pro-migratory		↓				n.d.	MTA2 knockdown suppresses proliferation and invasion of human glioma cells.	Yes	(64)
RBL1	Retinoblastoma- like 1	Controversial	↓					n.d.	RBL-1 expression is enhanced in glioma compared to normal tissue In colon cancer, RBL-1 expression rises towards carcinogenesis and falls during invasion.	?	(49)

Table II. Continued.

Gene	Protein	Pro-/anti-migratory	Array			RT-qPCR			Function	Connected to TGF- β signaling	Ref.
			I	A	M	I	A	M			
SET1	SET nuclear proto-oncogene	Pro-migratory	↑	↑			n.d.		SET stimulates cell migration in a Rac1-dependent manner, its knock-down inhibits cell migration and invasion in breast carcinoma cells.	No	(65,66)
TIMP1	Tissue inhibitor of metalloproteinase 1	Anti-migratory	↓					↓	TIMP-1 is capable of inhibiting the activities of all known MMPs.	Yes	(67)
TIMP2	Tissue inhibitor of metalloproteinase 2	Controversial	No change			↓	↓	↓	Upregulation TIMP-2 promotes MMP-2 activation and cell invasion in a human GBM cell line.	No	(68)
TGFB1	Transforming growth factor β 1	Pro-migratory	↓	↓	↓	↓	↓	↓	Pro-migratory cytokine in GBM.		(7)
TGFB2	Transforming growth factor β 2	Pro-migratory	Not present			↓	↓	↓	Pro-migratory cytokine in GBM.		(7)
TGFBR2	Transforming growth factor β receptor type 2	Pro-migratory	↓	↓	↓	↓	↓	↓	Receptor for the pro-migratory cytokine TGF- β .	Yes	(7)
TWIST1	Twist-related protein 1	Pro-migratory	↓				n.d.		TWIST1 promotes invasion of human GBM cells through mesenchymal change.	Yes	(69)
Upregulated genes											
BRMS1	Breast cancer metastasis suppressor 1	Anti-migratory	↑				n.d.		BRMS1 suppresses glioma progression by regulating invasion, migration and adhesion of glioma cells.	Yes	(50)
FAT1	FAT atypical cadherin 1	Pro-migratory	↑				n.d.		FAT1 knockdown results in decreased migration and invasion in glioma cells.	?	(71)
FGF2	Fibroblast growth factor 2	Anti-migratory	↑	↑	↑		n.d.		Enhanced expression of FGF2 in the lesser aggressive and invasive proneural subtype of GBM compared to invasively growing mesenchymal subtype of GBM. Better prognosis of glioma patients expressing the FGF2-dependent PDGF receptor A.	Yes	(51,52)
HGF	Hepatocyte growth factor	Controversial	↑				n.d.		HGF is the ligand of the pro-invasive receptor MET. MET can also bind glioma secreted VEGF instead of HGF, this leading to lesser glioma cell invasion.	Yes	(72)
IL-18	Interleukin 18	Pro-migratory	↑				n.d.		IL-18 is a driver of GBM cell migration.	Yes	(73)
IL-1B	Interleukin 1 β	Controversial	↑	↑	↑	↑	↑	↑	Expression of IL-1 β in the tumor micro-environment significantly increases migration and invasion of glioma cells. Non-toxic concentrations of ML induce the release of pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-8 and IFN γ .	Yes	(74,75)

Table II. Continued.

Gene	Protein	Pro-/ anti-migratory	Array			RT-qPCR			Function	Connected to TGF- β signaling	Ref.
			I	A	M	I	A	M			
K-Ras	Kirsten Ras protooncogene	Pro-migratory	↑					n.d.	KRAS-induced interleukin-8 overexpression promotes cell growth and migration in non-small cell lung cancer.	Yes	(76)
MET	Hepatocyte growth factor receptor	Controversial	↑	↑				n.d.	MET is a pro-invasive receptor, it can also bind glioma secreted VEGF instead of its natural ligand HGF, this leading to lesser glioma cell invasion.	?	(72)
MMP-1	Matrix metalloproteinase 1	Pro-migratory	↑	↑				n.d.	EGF induces MMP-1 expression and invasion in glioma cell lines.	Yes	(77,78)
MYC	c-Myc	Controversial	↑	↑	↑			n.d.	Myc is a pro-proliferative proto-oncogene. Increased c-myc activity was found in migration-restricted proliferative glioma cells. Besides this, myc suppresses the activation of TGF β -induced genes.	Yes	(79)
NME1/ NM23/	Nucleoside diphosphate kinase A	Anti-migratory	↑	↑	↑	↑	↑	↑	NME1/NM23 is described as a metastasis suppressor and plays an important role in the suppression of glioma invasion and migration.	Yes	(53,80)
PTGS2	Prostaglandin-endoperoxide synthase 2	Pro-migratory	↑	↑	↑	↑	↑	↑	PTGS2 is associated with recurrence and metastatic disease in multiple cancers. In GBM cells with COX-2 or ID1 overexpression, PTGS2 demonstrates greater migration/invasive potential.	Yes	(81)
S100A4	S100 Calcium binding protein A4	Pro-migratory	↑					n.d.	Extracellular S100A4 stimulates the migration rate of astrocytic tumor cells.	Yes	(82)
SERPINE1	Serpine 1/plasminogen activator inhibitor-1 (PAI1)	Pro-migratory	↑	↑	↑			n.d.	SERPINE 1 is described as a marker protein for metastatic melanoma, it is highly expressed in GBM.	Yes	(83)
SERPINB5/ MASPIN	Serpine peptidase inhibitor, member 5/Maspin	Anti-migratory	↑	↑	↑	↑	↑	↑	Overexpression of SERPIN B5 effectively suppresses the invasiveness and motility of malignant cancer cells. SERPIN 5 is silenced in glioma cells promoter hypermethylation. Its overexpression inhibits cell growth of glioma cells.	Yes	(55)

n.d., not determined; ↑, upregulated, ↓, downregulated; I, Iscador Qu; A, Aviscumine; M, ML-1. Shown are the genes and their regulation by ISCADOR Qu, Aviscumine and ML-1 as determined by PCR microarray and RT-qPCR as well as their function and connection to TGF- β signaling in regard to cell motility.

genes involved in metastatic, pro-migratory or pro-invasive processes differs between the three ML preparations we tested, the anti-migratory effect of all substances was nearly equal. This might be a result not only by changes in gene expression induced by MLs, but also an effect transmitted by the function of MLs as inhibitors of ribosomal translation, or a secondary effect by modulating the transcription or translation of certain transcription or transcriptional cofactors. Nevertheless, ML containing compounds like Iscador Qu, Aviscumine or even ML-I might provide clinical benefit as adjuvant therapeutics in the treatment of patients with invasively growing tumors.

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