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Immunotherapy of C3H/HeJ mammary adenocarcinoma with interleukin-2, mistletoe lectin or their combination: effects on tumour growth, capillary leakage and nitric oxide (NO) production

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Abstract

Clinical application of interleukin (IL)-2-based immunotherapy of cancer has been limited by a major side-effect known as 'capillary leak syndrome', resulting from nitric oxide (NO) overproduction. A galactoside-specific lectin from *Viscum album* L. (VAA) has been reported to induce certain lymphokines and upregulate IL-2 receptors on lymphocytes. Present study was, therefore, designed to compare the effects of combination therapy with IL-2 (10^4 Cetus units/mouse, intraperitoneal (i.p). every 8 h, given as 5 day rounds per week, for one or two rounds) and VAA (1 ng/kg subcutaneous (s.c.), biweekly) with those of IL-2 or VAA therapy alone in C3H/HeJ female mice bearing s.c. transplants of a highly metastatic C3L5 mammary adenocarcinoma. IL-2 therapy alone reduced tumour growth and metastasis, but caused significant water retention indicative of capillary leakage in the kidneys after both rounds of therapy, whereas pleural effusion was only evident after the first round and not the second round. A sharp rise in the systemic NO levels after the first round, followed by a decline after the second round of IL-2 therapy suggested a causal relationship of increased NO levels to pleural effusion. A strong immunostaining for nitrotyrosine (a marker for the production of peroxynitrite) was noted in the renal tubules at the end of both rounds of therapy suggestive of a causal association of this toxic NO-metabolite with capillary leakage in the kidneys. Addition of VAA to IL-2 therapy had no effect on any of the above parameters. Unexpectedly, however, VAA therapy alone stimulated tumour growth as well as lung metastases. NO induction in the C3L5 cells by VAA was excluded as a possible reason for this stimulation. Present results suggest the need for exercising caution in the use of VAA as an immunoadjuvant in human cancer therapy.

Keywords: Alternative (complementary) medicine; Immunotherapy; Interleukin-2; Lectin; Mistletoe; Nitric oxide; Biological response modifier

1. Introduction

High dose interleukin (IL)-2 therapy has shown efficacy in animal [1–4] as well as human [5–7] cancer models. However, its application has been limited by a life-threatening side-effect called capillary leak syndrome which is characterised by a rapid fluid accumulation in the tissue spaces and severe hypotension [8]. Combination of chronic indomethacin therapy with IL-2 therapy was shown to improve antitumour and antimetastatic effects of IL-2 therapy because of the improved activation of lymphokine-activated killer (LAK) cells *in situ* [2,3], but have no effect on IL-2-induced capillary leakage [9]. We subsequently discovered that endothelial damage responsible for this leakage resulted primarily from an overproduction of nitric oxide (NO) due to iNOS induction *in vivo* and that IL-2-induced capillary leakage can be ameliorated by combined therapy with NOS inhibitors [10–12]. A possible beneficial role of other biological response modifiers in improving the therapeutic efficacy and reducing the toxicity of IL-2 therapy has not been explored.

In recent years, immunostimulating effects of certain lectins have evoked considerable interest for application in cancer biotherapy. The galactoside-specific lectin from *Viscum album* L. (VAA or formerly ML-1) is considered to be the key immunostimulating component of

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commercially available mistletoe extracts historically employed in phytotherapy [13–14]. *In vitro*, VAA has been reported to upregulate the gene expression and secretion of proinflammatory cytokines including IL-1-α, IL-1-β, IL-6, IL-12, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte macrophage-colony stimulating factor (GM-CSF) by human leucocytes [15–18]. Furthermore, VAA was shown to stimulate natural killer (NK) cell activity in rat splenocytes [18], as well as NK and LAK cell cytotoxicity in cultures of human peripheral blood lymphocytes [19]. Administration of VAA by parenteral routes was shown to stimulate the activity and incidence of NK cells in healthy rabbits, rats and human breast cancer patients [18,20] and upregulate the expression of IL-2 receptors (CD25) on lymphoid cells of breast cancer patients [21]. The therapeutic value of the administration of VAA and VAA-containing extracts remains a highly debated area because of reports indicating beneficial, as well as detrimental effects. VAA therapy was shown to reduce experimental metastasis formation by murine sarcoma [22] lymphoma [23] and melanoma [24] cells, reduce the growth of a rat glioma [25], and improve the survival of immunodeficient mice transplanted with a human ovarian carcinoma cell line [26]. However, VAA therapy stimulated the growth of chemically-induced rat bladder carcinomas [27,28]. Application of mistletoe therapy to human cancer has been confounded with the problems of inadequate rationale and therapeutic designs and the questionable anticancer potential of lectin-induced cytokines which can stimulate as well as inhibit tumour growth [13,29,30]. VAA-induced stimulation of the proliferation of human tumour cells in histotypic cultures [31], and apparent stimulation of tumour growth at the injection site in a patient with non-Hodgkin's lymphoma [32] have raised additional concerns against VAA therapy.

In view of the putative immunostimulating function of VAA, including the ability to upregulate IL-2 receptors on lymphocytes, we wanted to test whether inclusion of non-toxic, but immunostimulant, doses of VAA could improve the antitumour and antimetastatic effects of IL-2 therapy without an adverse influence on the IL-2, induced capillary leakage. The present study was, therefore, designed to compare the effects of the combined therapy with IL-2 and VAA with those of IL-2 and VAA therapy alone in a highly metastatic C3L5 mammary adenocarcinoma model in C3H/HeJ mice. The following parameters were measured: tumour growth and spontaneous metastases in the lungs, capillary leakage (pleural effusion and wet/dry weight of the lungs and the kidneys), NO production (nitrite + nitrate levels in the serum, pleural fluids, kidney and lung homogenates), and the level of immunostaining for nitrotyrosine, which is a marker for the production of peroxynitrite, a toxic NO-metabolite implicated in capillary leakage.

2. Materials and methods

2.1. Reagents

Biotinylated anti-rabbit IgG and Vectastain ABC kit (PK-4000) were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Immunoaffinity-purified polyclonal IgG antibody to nitrotyrosine produced in rabbits were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Lipopolysaccharide from *Escherichia coli* 026:B6 (LPS) and 3-nitro-L-tyrosine (antigen to neutralise nitrotyrosine antibody) were obtained from Sigma (Oakville, Canada). Nitrate/Nitrite Colorimetric Assay Kit was from Cayman Chemical (Ann Arbor, MI, USA). IFN-γ and all reagents to maintain cell culture were from GIBCO BRL (Burlington, Canada). Recombinant human IL-2 (Lot PA0522A) with activity of 18×10^6 International Units (World Health Organization (WHO)) or 3×10^6 Cetus Units/mg protein was kindly provided by the Chiron Corporation (Emeryville, CA, USA). Biochemically-pure and endotoxin-free VAA and its carbohydrate-binding B-chain were isolated from buffer extracts of *Viscum album* L. using lactose-Sepharose 4B as an affinity matrix in the chromatography [33].

2.2. Mice

C3H/HeJ female mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were acclimatised in the animal quarters of the University of Western Ontario for 3 weeks before starting experiments. Mice were randomly separated into five groups, each of 8–10 animals/plastic cage, and were kept on 12-h light/dark cycle with free access to standard mouse chow and water *ad libitum*. The mean weight of the mouse was 22 g at the onset of experiments. The guidelines set by the Canadian Council of Animal Care were strictly followed during all treatments of mice.

2.3. Tumour cell line, murine tumour transplantation and measurement of tumour growth

C3L5 murine mammary adenocarcinoma cell line maintained in this laboratory is an extensively characterized clonal derivative of a spontaneous C3H/HeJ mammary tumour [3]. It is strongly positive for endothelial (e) NOS, and NO production by the tumour cells has been shown to promote tumour progression by stimulating tumour cell migration, invasiveness [34] and angiogenesis [34,35]. These cells can also be induced in the present of IFN-γ and LPS to produce additional NO which stimulates their invasive function *in vitro* [36]. The cells were grown in monolayer in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented

with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator fed with 5% CO_2 at 37°C. Cells were harvested by brief exposure to 0.05% trypsin-phosphate-buffered saline-ethylene diamine tetra acetic acid (PBS-EDTA) and resuspended in RPMI-1640 medium at a concentration of 10^6 cells/ml. The cell viability was more than 96% according to a dye exclusion test with trypan blue. Tumour cell transplantation was done by subcutaneous (s.c.) injections of 5×10^5 viable cells in 0.5 ml cell suspension in the mammary line of the left axillary region of C3H/HeJ female mice. Starting at 1 week after tumour transplantation, the minimal and maximal diameters (a and b) of the tumours were measured on alternate days using digital calipers and the tumour volume was calculated according to the equation $V = 0.52a^2b$ [34].

2.4. Experimental protocol for treatment of mice with IL-2 and VAA

Two series of experiments were conducted utilising a total of 90 mice. In the first series, five experimental groups of mice ($n = 10$ animals/group) were employed, namely (1) healthy mice, (2) control tumour-bearing mice, (3) IL-2- treated tumour-bearing mice, (4) VAA-treated tumour-bearing mice, (5) combined IL-2 + VAA-treated tumour-bearing mice. The treatment of mice with both drugs was started on day 7 after tumour transplantation. The groups (3) and (5) received two rounds of therapy with IL-2 as intraperitoneal (i.p.) injections at a dose of 10^4 Cetus Units (i.e. 6×10^4 IU)/mouse every 8 h during 5 days, followed by a 4-day recess and then a similar 5-day round with IL-2. IL-2 was dissolved in RPMI-1640 medium at a concentration of 10^5 Cetus Units/ml, so that each injection was 100 μl of the solution. The treatment of mice with VAA was in accordance with the dosage and schedule established for immunomodulation, namely a non-toxic dose 1 ng/kg body weight biweekly [13,20,37]. This protocol is also in line with clinical recommendations by commercial distributors of standardised mistletoe extracts in Germany. The drug preparation was deliberately designed to match earlier animal studies [27,28]. In brief, lyophilised VAA was freshly dissolved in PBS solution containing 50 μg/ml of mouse albumin (MA) to prevent lectin adsorption on the tube surface, diluted up to a concentration of 0.22 ng/ml in the same vehicle and injected s.c. with 100 μl of the solution on days 7, 10, 13, 16, and 19 after tumour transplantation. Mice in control groups (1) and (2) received blank injections of RPMI-1640 and PBS-MA. On day 21 after tumour transplantation, and 2 h after the last injection of IL-2, mice were killed by i.p. injection of 100 μl of euthanol solution (135 mg/ml) and samples of blood were collected and the lungs and kidneys were removed for further analyses.

In a second series of experiments, five similar groups of animals were used as above except for the fact that 8 animals were used per group and the animals were killed at the end of one round of therapy (on day 5 after the initiation of therapy, that is, 12 days after tumour transplantation).

2.5. Measurements of lung metastases

Right lungs were inflated with Bouin's fixative and the visible metastatic colonies which stand out on the lung surface were counted under a dissecting microscope. Metastasis count was only possible in the first experimental series representing animals at 21 days following tumour transplantation, i.e. those receiving two rounds of therapy. No visible lung metastasis appeared in any of the animals in the second series, in which case animals were killed at 12 days after tumour transplantation.

2.6. Measurements of capillary leakage

Fluid accumulation in the pleural cavity is a major sign of the capillary leak syndrome. The volume of pleural effusion, if any, was measured by removal from the pleural cavities as reported earlier [12] before removing the lungs. Increased water retention in the organs is a good measure of capillary leakage. Left lungs and kidneys were weighed (wet weight) immediately after removing and placed into a freezer at −20°C. Shortly thereafter, the collected samples were freeze-dried for 48 h to a constant weight. The weight of the dried samples was measured (dry weight) and the wet/dry weight ratios of organs were calculated as an indicator of the water content in these organs to assess capillary leakage [10,11].

2.7. Measurements of NO in blood serum and tissue homogenates

The coagulated blood was centrifuged for 10 min at 15 000 rpm using the Biofuge 17 R (Baxter Scientific Products), the serum was collected into Eppendorf tubes and kept at −20°C. To analyse nitrite and nitrate levels in the tissues, the freeze-dried left lungs and kidneys were used. Each tissue sample was homogenised in 15 or 30 volumes of PBS, pH 7.35, depending on the initial wet weight of the lung or kidney, respectively, using Polytron PT-MR2100 from Kinematica AG, CH. The homogenates were centrifuged at 4°C for 30 min at 15 000 rpm and the supernatants were collected into Eppendorf tubes, which were kept on ice. Both blood serum and tissue homogenates were ultrafiltered through 10 kDa molecular weight cut-off Ultrafree filters from regenerated cellulose membrane (Sigma) to get protein-free samples prior to NO analyses. Nitrate/Nitrite

Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA) was applied to measure total nitrate + nitrite ($[NO_2^- + NO_3^-]$) concentration in deproteinised serum and supernatants of the tissue homogenates. This assay is based on the conversion of nitrate to nitrite by nitrate reductase followed by application of the Griess reagent. In the case of lung and kidney homogenates, the water content of these organs and the dilution factor in making homogenates were applied in computing the $[NO_2^- + NO_3^-]$ levels.

2.8. Study of lectin effects on NO synthesis by C3L5 cells in vitro

To measure NO synthesis by C3L5 cells, the RPMI-1640 media was replaced by the Dulbecco's modified Eagle medium, which contains a negligible level of nitrate (2.5×10^{-7} M ferric nitrate). Aliquots (800 μl) of a cell suspension (2×10^5 cells/ml) were grown in 24-well plates in triplicate in standard conditions with or without the presence of VAA or its non-toxic carbohydrate-binding B-chain (1, 5, 10, 50, 100, 1000 ng/ml) as well as LPS (10 μg/ml) + IFN-γ (500 U/ml). The culture media were collected at 48 h and processed with the Cayman kit to measure the $[NO_2^- + NO_3^-]$ concentration.

2.9. Immunostaining for nitrotyrosine

Nitrotyrosine is produced as a result of nitration of tyrosine residues of cellular proteins (including tyrosine kinases) by peroxynitrite which is a reaction product of NO with superoxide. Thus immunostaining for nitrotyrosine is considered to be a reliable marker for peroxynitrite production *in vivo* [38]. We have strong evidence to suggest that NO-mediated capillary leakage induced by IL-2 therapy is due to the formation of peroxynitrite and that nitrotyrosine immunostaining of the kidneys provides a good reflection of IL-2 therapy-induced peroxynitrite formation (Lala, data not shown). The right kidneys were removed from mice and fixed in 10% buffered neutral formalin (VWR Scientific Products, West Chester, PA, USA). The paraffin-embedded samples were cut to get 4 μm-thick sections, which were then routinely processed for immunohistochemical staining. Briefly, the deparaffinised sections were incubated for 10 min at room temperature with 3% H_2O_2 in methanol to inhibit endogenous peroxidases, blocked for 1 h with 10% normal goat serum in 1% bovine serum albumin (BSA)-PBS solution, treated overnight with the primary rabbit polyclonal anti-tyrosine antibody (1:500 dilution) at 4°C, and then for 45 min at room temperature with the secondary antibody (biotinylated anti-rabbit IgG, 1:500 dilution). The ABC-kit from Vector was then applied for 45 min at room temperature. Specificity controls were obtained by combined application of the primary antibody with 0.01 M 3-nitro-L-tyrosine which neutralises the antibody. The visualisation of nitrotyrosine sites in the kidney sections was done using 3,3′-diaminobenzidine as a peroxidase substrate. The immunostained sections were finally counterstained lightly with Mayer's haematoxylin, dehydrated and mounted with Permount (Fisher Scientific, Fair Cawn, NJ, USA). They were analysed under an axiophot (Zeiss) microscope and pictures were recorded at 630× magnification with a digital image recorder.

2.10. Statistics

Each group of mice at the time of final data analysis contained 7–10 animals because of death of an occasional animal in the IL-2-treated groups prior to data collection. The mean values, standard deviations, *P* values from the Student's test, and Pearson correlation coefficients were calculated using Microsoft® Excel tools. For analysing data on pulmonary metastases, the Mann–Whitney rank sum test was applied, because of non-parametric distribution of the data. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Effects of therapies on subcutaneous tumour growth and pulmonary metastasis

Growth curves for C3L5 tumours in the various treated groups are presented in Fig. 1. Administration of IL-2 (10^4 Cetus Units/mouse, every 8 h) during two

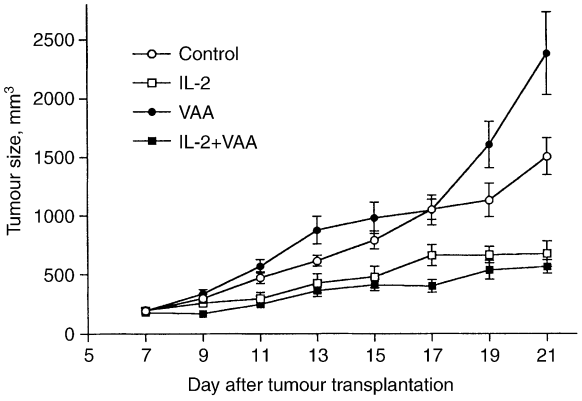

Fig. 1. Effects of intraperitoneal (i.p.) injection of interleukin-2 (IL-2) and subcutaneous (s.c.) injections of VAA, as well as their combination on tumour growth after s.c. inoculation of 5×10^5 C3L5 cells in C3H/HeJ female mice. Data indicate mean ± standard error (S.E.). First 3 data points (days 7, 9 and 11) are derived from both experimental series ($n = 18$), whereas subsequent data points are derived from experimental series I ($n = 9$–10), following the sacrifice of those in experimental series II on day 12. Significant ($P < 0.05$) reduction of tumour growth was noted in both IL-2 and IL-2 + VAA-treated mice from day 13 onwards, whereas a stimulation ($P < 0.05$) is noted in VAA-treated mice on day 19 or 21.

rounds of injections (days 7–11 and 16–21) significantly suppressed the tumour growth corroborating the previous data from this laboratory using different IL-2 treatment regimens and treatment periods [3,11]. However, the tumour growth was stimulated in the group of mice receiving biweekly s.c. injections of VAA alone at a dose of 1 ng/kg (Fig. 1) starting at day 7 after tumour cell transplantation. When VAA was combined with IL-2 therapy, the tumour growth was suppressed to the same extent as noted with the IL-2 therapy alone.

The rates of primary tumour growth were positively correlated with the number of lung metastases visible at 3 weeks after tumour transplantation in the various treated groups, as presented in Table 1. The incidence of lung metastasis was significantly increased by VAA therapy and reduced equally in both animal groups treated with IL-2 alone or combined IL-2 + VAA. Furthermore, the size of visible lung nodules was consistently much larger in the VAA-treated animals, compared with the other groups (data not shown). Histological examination of the lungs (data not shown) revealed more frequent and larger foci of intrapulmonary metastases in the VAA-treated group than in other groups confirming the macroscopic data.

3.2. IL-2-induced capillary leakage

The salient clinical feature of IL-2 therapy (but not VAA therapy)-induced capillary leakage in tumour-bearing mice was the development of pleural effusion during the first round of immunotherapy. The mean volumes of pleural effusions detected in mice after 5 days of IL-2 injections in the present protocol were 1.2 ± 0.2 ml ($n = 8$) and 1.1 ± 0.2 ml ($n = 8$) for IL-2 and IL-2 + VAA treated mice, respectively. These values were not significantly different from each other. Pleural effusion was the main cause of morbidity (six out of 36 mice in the two experimental series combined, receiving IL-2 or IL-2 + VAA, i.e. 17%) observed up to the end of the first round of immunotherapy. The volume of pleural fluid was more than 1.3 ml in these cases. No morbidity was noted in the other groups. Remarkably, mice surviving the first round of IL-2 therapy tolerated well the second round of IL-2 and no pleural effusion was observed in these animals at sacrifice. This finding confirms our earlier observations with a different IL-2 regimen [12].

Table 1
Effects of therapy with IL-2, VAA, and their combination on the number of visible metastatic colonies on the lung surface in mice sacrificed at 21 days after tumour transplantation in the experimental series I

Experimental group (number of mice)	Number of lung surface metastases	
	Range	Mean ± S.D.
1. Control ($n = 10$)	1–21	7.5 ± 7.1
2. IL-2 treatment ($n = 7$)	1–6	2.3 ± 1.9*
3. VAA treatment ($n = 10$)	5–61	27.6 ± 22.3*
4. Combined IL-2 + VAA treatment ($n = 9$)	0–9	2.3 ± 2.8*

VAA, a galactoside-specific lectin from *Viscum album* L.; IL-2, interleukin-2; S.D., standard deviation.

*$P < 0.028$ compared with the control group. Mice were killed on day 21 after subcutaneous (s.c.) transplantation of C3L5 cells (5×10^5 cells/mouse) into C3H/HeJ mice.

An increase in the water content of organs (wet/dry weight ratio) is a good indicator of capillary leakage induced by IL-2 therapy [10,11]. Table 2 documents the data for the lungs and the kidneys after one and two rounds of therapy. Significant capillary leakage was identified in the lungs only in the IL-2 + VAA-treated group after one round of therapy, when water contents were compared with those in the control tumour-bearing mice. However, significant leakage was observed in the kidneys after both rounds of therapy in the IL-2- as well as IL-2 + VAA-treated groups. No difference in the water contents of the kidneys was observed between these two groups. Thus, in summary, the addition of VAA to IL-2 therapy neither reduced nor increased the extent of pleural effusion and fluid leakage in the kidneys.

3.3. NO levels in the serum, pleural fluid and different tissues of IL-2- and VAA-treated mice

The level of $[NO_2^- + NO_3^-]$ in the serum, pleural fluid, kidneys and lungs of control and experimental mice was measured at two time points corresponding to the end of the first round of immunotherapy (day 12 after tumour transplantation) and the second round (day 21 after tumour transplantation). A significantly elevated level of $[NO_2^- + NO_3^-]$ ($P < 0.03$) was detected in the blood serum, kidneys and lungs after the administration of IL-2 during the first round (Table 3). Additionally, a high level of $[NO_2^- + NO_3^-]$ was also observed in the pleural fluids of IL-2- and IL-2 + VAA-treated tumour-bearing mice, namely 152.2 ± 59.5 μM ($n = 8$) and 108.9 ± 67.5 μM ($n = 8$), respectively, very similar to the respective levels in the serum of the same animals. The correlation coefficients (Pearson) were 0.9824 and 0.9832 for the IL-2- and IL-2 + VAA-treated groups, respectively. Similarly, increased levels of $[NO_2^- + NO_3^-]$ were also observed in kidneys and lungs of IL-2- as well as IL-2 + VAA-treated mice (Table 3). In essence, $[NO_2^- + NO_3^-]$ levels in the various tissues appeared to reflect the common NO-status of the individual animal group, as defined by the activity of different NOS enzymes including those induced by IL-2. Furthermore, administration of VAA alone had no significant effect on the levels of $[NO_2^- + NO_3^-]$ in control or IL-2-treated mice (Table 3).

NO levels in the different groups after the second round of therapies are presented in Table 4. Unexpectedly, the levels of $[NO_2^- + NO_3^-]$ in all tested tissues were significantly reduced ($P < 0.03$) in IL-2 ($\pm$VAA)-treated animal groups when compared with control tumour-bearing or healthy mice. The reduced levels of $[NO_2^- + NO_3^-]$ correlated with the absence of any pleural exudations in these mice.

3.4. Nitrotyrosine expression in the kidneys

NO is a free radical which can rapidly combine with available oxygen free radicals. Peroxynitrite is such a reactive intermediate which may mediate the endothelial damage responsible for capillary leakage. This molecule is toxic, unstable and reacts with tyrosine residues in proteins to form stable 3-nitrotyrosine [38], which appears to be a reliable footprint of peroxynitrite formation. We had earlier established that normal lungs exhibit significant nitrotyrosine immunostaining because of high endogenous levels of NO in this organ (Lala, data not shown). However, kidneys were found to serve as a better indicator for IL-2-induced nitrotyrosine formation. The right kidneys of all the mice were processed for nitrotyrosine immunostaining. Kidney tissues from healthy and control tumour-bearing mice, as well as from VAA-treated mice demonstrated the absence of immunostaining or sparse, patchy, very light to light, immunostaining in both the cortex and medulla (Fig. 2a–d). However, IL-2 treatment of the tumour-bearing mice, alone or in combination with VAA, led to marked enhancement of immunostaining of the renal tubules in both the cortex and the medulla (Fig. 2, e and f). In addition, macrophages within the stroma (renal capsule) were strongly positive in these mice (data not shown).

The negative control samples (antibodies pretreated with 3-nitro-L-tyrosine) in all groups were devoid of immunostaining, indicating the specificity of the staining (Fig. 2g and h). Furthermore, no nitrotyrosine staining was seen in the glomerul, adipose tissue (perirenal fat) and adrenal glands in treated as well as control groups, serving as intrinsic negative controls in these tissue sections. Remarkably, the strong expression of nitrotyrosine in the renal tubules was observed both after the first and the second rounds of IL-2 therapy. This was in contrast to the reduced level of $[NO_2^- + NO_3^-]$ in the different tissues including the kidney after the second round of IL-2 therapy, indicating that enhanced expression of nitrotyrosine persists in this organ probably resulting from the high NO levels during the first round of IL-2 therapy. These results reinforce the notion that the persistent peroxynitrite-mediated injury to the kidneys may be responsible for the capillary leakage, even in the absence of a high local NO level after the second round of IL-2 therapy.

Table 2
Water content in the left lung and kidney of treated mice, expressed as the wet/dry weight ratio of the organs (experimental series I and II)

Mice group	Wet/dry weight ratio, mean±S.D.			
	Lung		Kidney	
	1 round	2 rounds	1 round	2 rounds
1. Healthy mice	5.63±0.42 (8)	4.97±0.67 (10)	3.67±0.20 (8)	3.66±0.20 (10)
2. Control tumour-bearing mice	5.45±0.52 (8)	5.31±0.51 (10)	3.53±0.40 (8)	3.68±0.19 (10)
3. Treatment with IL-2	5.91±0.81 (8)	5.02±0.42 (7)	4.16±0.48 (8)**	4.07±0.15 (7)***
4. Treatment with VAA	5.63±0.58 (8)	4.94±0.44 (10)	3.63±0.21 (8)	3.86±0.29 (10)
5. Combined treatment with IL-2 and VAA	5.97±0.32 (8)*	5.05±0.60 (9)	4.23±0.35 (8)**	4.11±0.17 (9)***

IL-2, interleukin-2; VAA, a galactoside-specific lectin from *Viscum album* L.; S.D., standard deviation.
*$P < 0.02$, **$P < 0.01$, ***$P < 0.001$ compared with control tumour-bearing mice. Number in parentheses indicates the number of animals.

Table 3
The level of nitrate + nitrite (μM) in mice after one round of therapy (experimental series II)

Animal group	Blood serum	Pleural effusion	Left kidney	Left lung
1. Healthy mice	42.2±5.9 (8)	–	33.3±8.5 (8)	49.2±22.6 (8)
2. Control tumour-bearing mice	36.0±11.8 (8)	–	31.3±11.0 (8)	41.4±25.3 (8)
3. IL-2 treatment	145.6±51.9 (6)*	152.2±59.5 (8)	94.3±34.9 (8)***	160.2±59.1 (8)*****
4. VAA treatment	41.3±8.9 (8)	–	23.2±10.7 (8)	79.8±60.6 (8)
5. Combined IL-2 and VAA treatment	101.4±72.6 (7)**	108.9±67.5 (8)	69.8±35.9 (8)****	101.3±35.4 (8)******

IL-2, interleukin-2; VAA, a galactoside-specific lectin from *Viscum album* L.
*$P = 0.0016$, **$P = 0.0274$, ***$P = 0.000541$, ****$P = 0.009622$, *****$P = 0.00023$, ******$P = 0.000971$ in comparison with control tumour-bearing mice. The levels in the kidneys and lungs were recalculated on the basis of water content in these organs. Numbers in parentheses indicate the number of animals.

3.5. Effect of VAA on NO synthesis in culture of C3L5 cells

Endogenous production of NO by C3L5 cells because of eNOS expression was shown to stimulate tumour cell migration, invasiveness and angiogenesis [34,35]. To evaluate whether tumour growth and the metastasis-promoting activity of VAA resulted from additional NO induction by VAA in the C3L5 cells, we tested the effects of the lectin on $[NO_2^- + NO_3^-]$ accumulation in C3L5 cell cultures.

The basal level of $[NO_2^- + NO_3^-]$ in the cell media after 48 h of cultivation was 3.8 ± 1.0 μM ($n = 3$). The level significantly increased to 59.9 ± 0.8 μM ($n = 3$) in the presence of 10 μg/ml LPS and 500 U/ml IFN-γ, indicating an induction of iNOS in these cells, as previously reported by us [36]. The cells incubated for 48 h with VAA at concentrations of 1, 5, 10, 50, 100 or 1000 ng/ml generated $[NO_2^- + NO_3^-]$ ranging between 0.9 and 1.7 μM, which represent unchanged or reduced ($P < 0.05$) levels. To test whether the minor reduction could be due to the toxicity of VAA, its carbohydrate-binding non-toxic B-subunit was added at the same concentrations to the C3L5 cells. This treatment also led to unchanged or decreased ($P < 0.05$) levels ranging between 0.4–7.1 μM. Thus, additions of VAA and its B-subunit at immunomodulatory and higher concentrations failed to activate iNOS in the C3L5 cells.

4. Discussion

The principal objective of the present study was to test whether a biochemically purified plant lectin VAA given to mice at non-toxic immunomodulatory dosage provided additional therapeutic benefits in combination with IL-2 therapy. We utilised a well-characterised, eNOS expressing C3L5 murine mammary carcinoma model in C3H/HeJ mice to measure the effects of VAA and IL-2 therapy alone or in combination, on primary tumour growth, spontaneous lung metastasis, capillary leakage, NO production *in vivo*, and additionally the formation of the toxic NO-metabolite peroxynitrite, as indicated by the presence of immunoreactive nitrotyrosine in the kidney. Results of the VAA therapy alone were intriguing. They revealed that VAA given as biweekly injections for two weeks promoted the growth of primary tumours and stimulated the development as well as growth of spontaneous lung metastases. This occurred in spite of the inability of VAA to induce additional NO production by C3L5 cells *in vitro*. IL-2 therapy alone suppressed tumour growth and metastasis, but caused capillary leakage as evidenced by pleural effusion after the first round of therapy and fluid retention in the kidneys following both rounds of therapy. This was associated with sharp rises in NO levels in the serum (and pleural fluid) and organs (kidneys and lungs) after the first round, but a decline in the levels after the second round of therapy. Formation of peroxynitrite was evidenced by strong staining for nitrotyrosine in the renal tubules after both rounds of IL-2 therapy. Addition of VAA to IL-2 therapy provided no additional benefit nor detriment to IL-2 therapy as indicated by an absence of change in any of the above parameters in this tumour model.

Table 4
The level of nitrate + nitrite (μM) in mice after two rounds of therapy (experimental series I)

Animal group	Blood serum	Left kidney
1. Healthy mice	66.8 ± 19.3 (10)	61.5 ± 16.4 (10)
2. Control tumour-bearing mice	47.2 ± 12.6 (10)*	52.9 ± 30.1 (10)
3. IL-2 treatment	21.4 ± 5.5 (7)**	29.7 ± 12.2 (7)****
4. VAA treatment	47.0 ± 6.6 (10)	41.6 ± 13.4 (10)
5. Combined IL-2 and VAA treatment	17.0 ± 6.1 (9)***	28.8 ± 11.0 (9)*****

IL-2, interleukin-2; VAA, a galactoside-specific lectin from *Viscum album* L.

*$P = 0.008308$, **$P = 0.0000342$, ***$P = 0.00000641$, ****$P = 0.023962$, *****$P = 0.018511$. The levels in the kidneys was re-calculated on the basis of water content in this organ. Numbers in parentheses indicate number of animals.

Present findings of antitumour and antimetastatic effects of IL-2 therapy alone are a confirmation of our earlier studies with this tumour model utilising a different IL-2 regimen [3,11]. We have also confirmed the phenomenon of IL-2-induced capillary leakage in this tumour model, including the observation that mice surviving the pleural effusion after the first round do not develop pleural effusion after the second round of the IL-2 therapy [10–12]. In the present study, we noted that NO levels in the IL-2-treated mice rise sharply after the first round, but decline after the second round. We propose that pleural vessels can repair NO-mediated injury after withdrawal of high local NO levels. Thus a decline in the systemic and organ NO levels after the second round of IL-2 may explain the absence of pleural effusion. This decline is due likely to a negative feedback loop by which a sharp rise in NO levels can downregulate NOS expression, as well as NOS activity, as a protective mechanism against NO-mediated injury. This view is supported by a number of studies showing that high NO levels could inhibit both constitutive and inducible NOS activity. Feedback NOS inactivation appeared to be due to competition with oxygen (O_2) binding of NO to the haem iron prosthetic group [39] in association with a conserved tryptophan 409 residue [40]. Inhibitory action of NO on iNOS activity were demonstrated in macrophage cell lines J774 [41] and RAW 264.7 [42] using a set of NO donors, e.g. sodium nitroprusside and S-nitro-acetyl-penicillamine. Similarly, NO donors downregulated NO synthesis by the

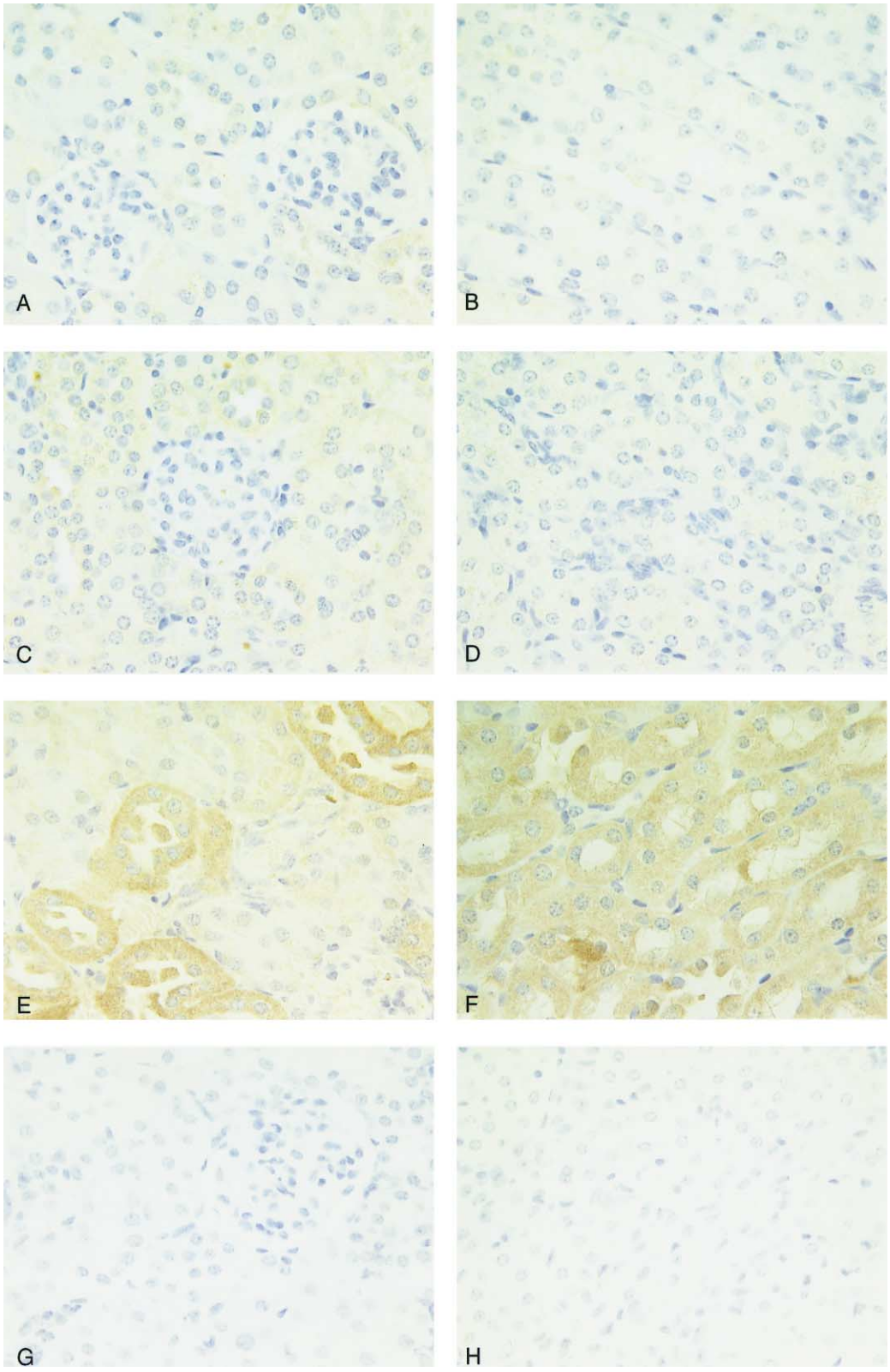

Fig. 2. Immunoperoxidase staining for nitrotyrosine in formalin-fixed paraffin-embedded kidney sections (×630) from C3L5 mammary adenocarcinoma-bearing C3H/HeJ female mice after two rounds of immunotherapy: (a, b) control tumour-bearing mice; (c, d) VAA-treated mice; (e, f) IL-2-treated mice; (g, h) negative control of IL-2-treated-mice (immunostaining with primary antibody was performed in the presence of 3-nitro-L-tyrosine). Left side (a, c, e, g) illustrates the cortical and the right side (b, d, f, h) the medullary regions of the kidneys. Kidney sections from VAA + IL-2-treated mice are not shown since their staining patterns were identical to those from IL-2-treated mice. Five sections from the right kidney of each animal ($n = 8$–10 mice/group) in the various groups were processed, and the pictures are representative for each group.

rat gastric myenteric plexus [43]. In other studies, NO has been shown to inhibit reversibly the activity of purified eNOS [44], as well as eNOS gene expression, in cultures of human coronary artery endothelial cells via a cyclic guanosine monophosphate (cGMP)-mediated mechanism [45]. In line with these observations, our data demonstrate for the first time the feedback regulation of NO production *in vivo* in the tumour-bearing mice treated with IL-2. Our results reveal that in spite of a decline in systematic NO levels after the second round of IL-2 therapy, kidneys exhibit significant capillary leakage, as opposed to the absence of pleural effusion. Thus pleural and renal capillaries may behave differently. It is possible that the latter is more sensitive to the injury resulting from peroxynitrite formation. Indeed, we have shown that in spite of a drop in renal NO levels, nitrotyrosine, a marker for peroxynitrite, was abundantly present in the kidneys after both rounds of IL-2 therapy.

In view of an increasing appreciation of the role of glycan chains of cellular glycoconjugates in cell signalling via interaction with endogenous lectins, research on plant lectins as diagnostic and therapeutic tools has attracted considerable interest [46,47]. Unfortunately, however, in recent times, popular alternative (complementary) modalities of human cancer therapy have frequently ascribed antitumour functions to mistletoe extracts, in spite of the lack of any clinical validation of such claims and documented opposing actions of lectin-induced cytokines *in vivo* [29,30]. The recent introduction of lectin-standardised extracts instead of preparations with variable lectin contents in the market is an attempted improvement to mimic the immunomodulatory capacity of the lectin. So far, case reports with extract application in the human remain discouraging, including some cases of stimulation of tumour growth [32,48]. As reviewed earlier, data derived from models of animal cancer remains conflicting, indicating beneficial [22–26] as well as detrimental [27,28] effects.

Our data demonstrate that the purified VAA promotes tumour growth and lung metastases in mice in the C3L5 mammary adenocarcinoma model. An important feature of this cell line is the constitutive expression of eNOS and induction of iNOS after stimulation with LPS and IFN-γ [36]. We had earlier shown that endogenous NO promoted tumour progression and metastasis in this tumour model by multiple mechanisms including stimulation of tumour cell migration, invasiveness and NO-mediated angiogenesis [34,35]. We have further shown that additional induction of iNOS in the presence of LPS and IFN-γ promotes invasiveness of these cells by an upregulation of matrix metalloprotease (MMP)-2 [36]. However, VAA and its carbohydrate-binding B-chain failed to activate iNOS in the C3L5 tumour cells in contrast to the action of LPS + IFN-γ. Thus VAA-induced progression of tumour growth and metastasis in this cancer model cannot be attributed to iNOS induction with VAA. In contrast to the present finding with VAA, certain lectins from *Canavalia ensiformis* (Con A), *Canavalia brasiliensis*, *Pisum arvense, Dioclea grandiflora* were found to induce a significant accumulation of nitrite in the culture of unfractionated peritoneal cells from female BALB/c mice [49]. Furthermore, i.p. administration of two lectins from *Tricholoma mongolicum* to normal and sarcoma-bearing C57BL/6 mice primed peritoneal macrophages to produce nitrite and inhibited tumour growth most likely due to NO-mediated tumour cell cytotoxicity [50]. However, in many human tumours, in particular, cancers of the breast, oropharynx, lungs and the pancreas, increased NOS activity and expression in tumours is positively associated with tumour progression [51] indicating the risks of using NO-inducing agents in the clinic. In the present case, VAA stimulation of C3L5 tumour growth may be due to a direct stimulation of cell proliferation [31] or indirect stimulation by VAA-induced cytokines *in vivo* [30]. These possibilities remain to be investigated. While animal studies may not always predict the clinical outcome in the human, our results suggest that the use of VAA for cancer therapy in the clinic should be considered with caution. They also demonstrate that there is no advantage in using VAA as an immuno-adjuvant for IL-2-based immunotherapy.

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References

1. Rosenberg SA, Mule JJ, Spiess PJ, Reichart CM, Schwarz SL. Regression of established pulmonary metastasis and subcutaneous tumor mediated by systemic administration of high dose recombinant interleukin 2. *J Exp Med* 1985, **161**, 1169–1188.
2. Lala PK, Parhar RS. Cure of B16F10 melanoma lung metastasis in mice by chronic indomethacin therapy combined with repeated rounds of interleukin 2: characteristics of killer cells generated in situ. *Cancer Res* 1988, **48**, 1072–1079.
3. Lala PK, Parhar RS. Eradication of spontaneous and experimental adenocarcinoma metastases with chronic indomethacin and intermittent IL-2 therapy. *Int J Cancer* 1993, **54**, 677–684.
4. Lala PK, Elkashab M, Kerbel RS, Parhar RS. Cure of human melanoma lung metastases in nude mice with chronic indomethacin therapy combined with multiple rounds of IL-2: characteristics of killer cells generated in situ. *Int Immunol* 1990, **2**, 1149–1158.
5. Rosenberg SA, Lotze MT, Muul LM, *et al*. A progress report on the treatment of 157 patients with advanced cancer using lym-

phokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 1987, **316**, 889–897.
6. Mertens WC, Bramwell VH, Banerjee D, Gwadry-Sridhar F, Lala PK. Sustained indomethacin and ranitidine with intermittent continuous infusion interleukin-2 in advanced malignant melanoma: a phase II study. *Clin Oncol* 1993, **5**, 107–113.
7. Mertens WC, Bramwell VH, Banerjee D, *et al*. Sustained oral indomethacin and ranitidine with intermittent continuous infusion interleukin-2 in advanced renal cell carcinoma. *Cancer Biother* 1993, **8**, 229–233.
8. Rosenstein M, Ettinghausen SE, Rosenberg SA. Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin 2. *J Immunol* 1986, **137**, 1735–1742.
9. Orucevic A, Lala PK. Effects of NG-methyl-L-arginine and indomethacin on IL-2 induced capillary leakage in tumor-bearing mice. *Proc Amer Assoc Cancer Res* 1993, **34**, 459.
10. Orucevic A, Lala PK. N^G-Nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthesis, ameliorates interleukin-2-induced capillary leak syndrome in healthy mice. *J Immunotherapy* 1996, **18**, 210–220.
11. Orucevic A, Lala PK. N^G-Nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthesis, ameliorates interleukin-2-induced capillary leakage and reduces tumour growth in adenocarcinoma-bearing mice. *Br J Cancer* 1996, **73**, 189–196.
12. Orucevic A, Hearn S, Lala PK. The role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome resulting from interleukin-2 therapy in mice. *Lab Invest* 1997, **76**, 53–65.
13. Gabius S, Joshi SS, Kayser K, Gabius H-J. The galactoside-specific lectin from mistletoe as biological response modifier. *Int J Oncol* 1992, **1**, 705–708.
14. Bocci V. Mistletoe (*Viscum album*) lectins as cytokine inducers and immunoadjuvant in tumor therapy. A review. *J Biol Regul Homeost Agents* 1993, **7**, 1–6.
15. Hajto T, Hostanska K, Frei K, Rordorf C, Gabius H-J. Increased secretion of tumor necrosis factor-α, interleukin-1, and interleukin-6 by human mononuclear cells exposed to the β-galactoside-specific lectin from clinically applied mistletoe extract. *Cancer Res* 1990, **50**, 3322–3326.
16. Hostanska K, Hajto T, Spagnoli GC, Fischer J, Lentzen H, Herrmann R. A plant lectin derived from Viscum album induces cytokine gene expression and protein production in cultures of human peripheral blood mononuclear cells. *Nat Immun* 1995, **14**, 295–304.
17. Ribereau-Gayon G, Dumont S, Muller C, Jung ML, Poindron P, Anton R. Mistletoe lectins I, II and III induce the production of cytokines by cultured human monocytes. *Cancer Lett* 1996, **109**, 33–38.
18. Hajto T, Hostanska K, Weber K, *et al*. Effect of a recombinant lectin, Viscum album agglutinin on the secretion of interleukin-12 in cultured human peripheral blood mononuclear cells and on NK-cell-mediated cytotoxicity of rat splenocytes in vitro and in vivo. *Nat Immun* 1998, **16**, 34–46.
19. Baxevanis CN, Voutsas IF, Soler MH, *et al*. Mistletoe lectin I-induced effects on human cytotoxic lymphocytes. I. Synergism with IL-2 in the induction of enhanced LAK cytotoxicity. *Immunopharmacol Immunotoxicol* 1998, **20**, 355–372.
20. Hajto T, Hostanska K, Gabius H-J. Modulatory potency of the β-galactoside-specific lectin from mistletoe extract (Iscador) on the host defense system *in vivo* in rabbits and patients. *Cancer Res* 1989, **49**, 4803–4808.
21. Beuth J, Ko HL, Gabius H-J, Burrichter H, Oette K, Pulverer G. Behavior of lymphocyte subsets and expression of activation markers in response to immunotherapy with galactoside-specific lectin from mistletoe in breast cancer patients. *Clin Investig* 1992, **70**, 658–661.
22. Beuth J, Ko HL, Gabius H-J, Pulverer G. Influence of treatment with the immunomodulatory effective dose of the β-galactoside-specific lectin from mistletoe on tumor colonization in BALB/c-mice for two experimental model systems. *In Vivo* 1991, **5**, 29–32.
23. Joshi SS, Komanduri KC, Gabius S, Gabius HJ. Immunotherapeutic effects of purified mistletoe lectin on murine large cell lymphoma. In: Gabius HJ, Gabius S, eds. *Lectins and Cancer*. Berlin/New York, Springer Publ. Co, 1991, 207–216.
24. Weber K, Mengs U, Schwarz T, *et al*. Effects of a standardized mistletoe preparation on metastatic B16 melanoma colonization in murine lungs. *Arznaimittelforschung/Drug Res* 1998, **48**, 497–502.
25. Lenartz D, Andermahr J, Plum G, Menzel J, Beuth J. Efficiency of treatment with galactoside-specific lectin from mistletoe against rat glioma. *Anticancer Res* 1998, **18**, 1011–1014.
26. Schumacher U, Feldhaus S, Mengs U. Recombinant mistletoe lectin (rML) is successful in treating human ovarian cancer cells transplanted into severe combined immunodeficient (SCID) mice. *Cancer Lett* 2000, **150**, 171–175.
27. Kunze E, Schulz H, Gabius H-J. Inability of galactoside-specific mistletoe lectin to inhibit N-methyl-N-nitrosourea-initiated tumor development in the urinary bladder of rats and to induce a local cellular immune response after long-term administration. *J Cancer Res Clin Oncol* 1998, **124**, 73–87.
28. Kunze E, Schulz H, Adamek M, Gabius H-J. Long-term administration of galactoside-specific mistletoe lectin in an animal model: no protection against N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder carcinogenesis in rats and no induction of a relevant local cellular immune response. *J Cancer Res Clin Oncol* 2000, **126**, 125–138.
29. Hauser SP. Unproven methods in cancer treatment. *Current Opinion Oncol* 1993, **5**, 646–654.
30. Gabius H-J, Gabius S. Immunomodulierende Misteltherapie durch Lektinstandardisierung: Ein zweischneidiges Schwert? *Versicherungsmedizin* 1999, **51**, 128–136.
31. Gabius H-J, Darro F, Remmelink M, *et al*. Evidence for stimulation of tumor proliferation in cell lines and histotypic cultures by clinically relevant low doses of the galactoside-binding mistletoe lectin, a component of proprietary extracts. *Cancer Invest* 2001, **19**, 114–126.
32. Hagenah W, Dorges I, Gafumbegete E, Wagner T. Subcutaneous manifestations of a centrocytic non-Hodgkin lymphoma at the injection site of a mistletoe preparation. *Dtsch Med Wochenschr* 1998, **123**, 1001–1004.
33. Gabius H-J. Influence of type of linkage and spacer on the interaction of β-galactoside-binding proteins with immobilized affinity ligands. *Anal Biochem* 1990, **189**, 91–94.
34. Jadeski L, Hum KO, Chakraborty C, Lala PK. Nitric oxide promotes murine mammary tumor growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis. *Int J Cancer* 2000, **86**, 30–39.
35. Jadeski L, Lala PK. Nitric oxide synthase inhibition by N^G-nitro-L-arginine methyl ester inhibits tumor-induced angiogenesis in mammary tumors. *Am J Pathol* 1999, **155**, 1381–1390.
36. Orucevic A, Bechberger J, Green AM, Shapiro RA, Billiar TR, Lala PK. Nitric oxide production by murine mammary adenocarcinoma cells promotes tumor-cell invasiveness. *Int J Cancer* 1999, **81**, 889–896.
37. Beuth J, Ko HL, Tunggal L, *et al*. Immunoactive action of mistletoe lectin-1 in relation to dose. *Arzneimittelforschung* 1994, **44**, 1255–1258.
38. Vander Vliet A, Eiserish JP, Kaur H, Cross CE, Halliwell B. Nitrotyrosine as biomarker for reactive nitrogen species. *Meth Enzymol* 1996, **269**, 175–184.
39. Griscavage JM, Hobbs AJ, Ignarro LJ. Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. *Adv Pharmacol* 1995, **34**, 215–234.
40. Adak S, Crooks C, Wang Q, *et al*. Tryptophan 409 controls the activity of neuronal nitric-oxide synthase by regulating nitric oxide feedback inhibition. *J Biol Chem* 1999, **274**, 6907–6911.

41. Assreuy J, Cunha FQ, Liew FY, Moncada S. Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br J Pharmacol* 1993, **108**, 833–837.
42. Hinz B, Brune K, Andreas P. Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages. *Biochem Biophys Res Comm* 2000, **271**, 353–357.
43. Hosoda K, Takahashi T, Fujino MA, Owyang C. Inhibitory effects of nitric oxide donors on nitric oxide synthesis in rat gastric myenteric plexus. *J Pharmacol Exp Ther* 1998, **286**, 1222–1230.
44. Ravichandran LV, Johns RA, Rengasamy A. Direct and reversible inhibition of endothelial nitric oxide synthase by nitric oxide. *Am J Physiol* 1995, **268**, H2216–H2223.
45. Vaziri ND, Wang XQ. cGMP-mediated negative-feedback regulation of endothelial nitric oxide synthase expression by nitric oxide. *Hypertension* 1999, **34**, 1237–1241.
46. Mody R, Joshi S, William C. Use of lectins as diagnostic and therapeutic tools for cancer. *J Pharmacol Toxicol Meth* 1995, **33**, 1–10.
47. Gabius H-J. Biological information transfer beyond the genetic code: the sugar code. *Naturwissenschaften* 2000, **87**, 108–121.
48. Heimpel H. Letter to the editor. *Tumordiagn u Ther* 1995, **16**, 205.
49. Andrade JL, Arruda S, Barbosa T, Paim L, Ramos MV, Cavada BS, Barral-Netto M. Lectin-induced nitric oxide production. *Cell Immunol* 1999, **194**, 98–102.
50. Wang HX, Ng TB, Ooi VEC, Liu WK, Chang ST. Actions of lectins from the mushroom *Tricholoma mongolicum* on macrophages, splenocytes and life-span in sarcoma-bearing mice. *Anticancer Res* 1997, **17**, 415–420.
51. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumor progression. *Lancet Oncology* 2001, **3**, 149–156.