Chapter 3
Lectins from Medicinal Plants: Bioeffectors with Diverse Activities

Alexander V. Timoshenko, Irina V. Gorudko and Hans-Joachim Gabius

Abstract Lectins represent a family of glycan-binding proteins that are differentially expressed in various plant tissues and organs. As a component of traditional herbs, some purified plant lectins are known to possess immunomodulatory, cytotoxic, and anticancer activities with a potential biomedical application. In this chapter, we summarize our procedures for lectin isolation from medicinal plants and methods for lectin screening and biotesting based on a variety of cellular responses (cell aggregation, generation of hydrogen peroxide, and secretion of vascular endothelial growth factor C). The importance of detecting and characterizing lectins in herbal preparations is discussed in the context of safety and efficacy of lectin-based phytotherapeutical approaches.

Keywords Cancer · Innate immunity · Lectins · Medicinal plants · Phytochemicals

3.1 Introduction

Cellular glycans are increasingly recognized as a versatile basis to store and transmit biological information [1]. Due to this appreciation of sugars as the third alphabet of life forming the sugar code [1, 2], the ways sugar-encoded information is translated into cellular effects have become an attractive study area. A key role in this process is played by lectins, glycan-binding proteins, which are neither glycoenzymes such as glycosyltransferases or glycosidases, nor glycan-specific antibodies, nor transporters for free glycans [2]. Starting with H. Stillmark's pioneering work on a respective activity in *Ricinus communis* in 1888, plants have proven to be a rich

A. V. Timoshenko (✉)
Department of Biology, Western University, London,Ontario N6G 5B7, Canada
e-mail: atimoshe@uwo.ca

I. V. Gorudko
Department of Biophysics, Belarusian State University,
220030 Minsk, Belarus

H.-J. Gabius
Institute of Physiological Chemistry, Faculty of Veterinary Medicine,
Ludwig-Maximilians-University, 80539 Munich, Germany

R. Jetter (ed.), *Phytochemicals—Biosynthesis, Function and Application,*
Recent Advances in Phytochemistry 44, DOI 10.1007/978-3-319-04045-5_3

Table 3.1 The list of medicinal plants specified in 2012 US Pharmacopeia that contain lectins based on a search of peer-reviewed sources on PubMed

Latin name of plants	PubMed hits[a] (all/lectin)	Lectin MW, kDa	Nominal glycan-binding specificity	References
Aesculus hippocastanum	368/2	132	O-glycan	[12]
Allium sativum	4,119/75	25, 24, 48	α(1→3)Man	[13–15]
Curcuma longa	1,664/11	19.6, 20.3, 404	Man	[16]
Glycine max	21,088/923	110	GalNAc	[17]
Trifolium pratense	1,192/5	ND	ND	[18][b]
Urtica dioica	332/49	8.3–9.5	(GlcNAc)n	[19, 20]
Vitis vinifera	1,469/2	11.9–13.2	p-nitrophenyl β-D-Gal/Glc	[21]
Withania somnifera	521/3	30	ND	[22]
Zingiber officinale	1,724/4	16.2	Man	[23]

[a] The search was performed using first Latin names of the plants as key words and then in their combination with "lectin" as second key word
[b] In silico analysis
ND not determined

source for lectins, then referred to as phytohaemagglutinins because of their capacity to agglutinate erythrocytes in a carbohydrate-dependent manner [3]. The highest concentration of lectins is usually detected in seeds, for example with 2.1 g/100 g in beans [3]. With purification yields in this range, it is evident why these glycan-binding proteins have become a popular tool for structural research [1–3].

The enormous toxicity of certain lectins, especially the AB toxins such as ricin, and also other activities on mammalian cells such as mitogenicity or the simple capacity to define aspects of the glycophenotype have made plant lectins study objects in biomedicine. Owing to their stability, they maintain their active form in vivo even after consuming a lectin-containing meal, e.g., with roasted peanuts [4]. Of interest in this context, purified plant lectins are known to affect aspects of functional activity of mammalian cells that are associated with immunomodulation (e.g., mitogenic stimulation of lymphocytes, activation of innate immunity, production of cytokines, and growth factors) [5–8], by virtue of binding to distinct glycans, e.g., α 2,3/6-sialylated N- and O-glycans. Detailed glycan mapping, with plant lectins as sensors, in combination with tissue lectins, has been instrumental to define, for instance, the master-regulator potency of a tumor suppressor which downregulates α 2,6-sialylation [9, 10]. Along this line, the significance and role of a lectin component in medicinal plants can deserve attention in phytopharmacology, despite the relatively low content of lectins in traditional pharmacopoeial materials, e.g., leaves and flowers. As a consequence, available information about lectins in medicinal plants that are listed in national pharmacopeias is rather limited [11]. In particular, a PubMed-based search (December 2012) on lectins (also using the synonymous term "agglutinin") in 43 herbs from Dietary Supplements Official Monographs of the 2012 US Pharmacopeia has revealed that information about lectins in medicinal plants is available only for ~ 21 % of the listed herbs (Table 3.1) [12–23].

Of interest in this context, the market in Central and Eastern Europe offers a variety of so-called lectin-containing herbal teas that are based on medicinal plants such

as *Agastache rugosa* (wrinkled giant hyssop), *Calendula officinalis* (pot marigold), *Hypericum perforatum* (St. John's wort), *Salvia officinalis* (sage), *Melissa officinalis* (lemon balm), *Mentha piperita* (peppermint), *Nepeta cataria* (catmint), and *Zea mays* (corn silk). To the best of our knowledge, no peer-reviewed publications on lectin composition in the aqueous extracts of the above-mentioned plants, except for *Z. mays* seeds but not silk, are available in PubMed, and thus the significance of leaf lectins is unclear. In fact, recent studies have identified several leaf lectins with potential antimicrobial and antiproliferative properties in medicinal plants including *Morus* and *Schinus* species [24, 25]. To date, only one leaf lectin, a galactoside-binding lectin (agglutinin) from mistletoe leaves (*Viscus album* agglutinin, VAA), was examined rather rigorously as a drug. Based on a purely spiritual concept, mistletoe extracts had been suggested to be able to cure cancer by acting on the dysbalance between nonmaterial spheres within the patient's personality [26]. Biotesting of the purified galactose-binding lectin from clinically approved mistletoe extracts had shown that its application in ng quantities had diverse immunomodulatory effects both in vitro and in vivo, including the increase in secretion of cytokines such as interleukin-6, which has a potential to stimulate tumor growth [7, 27, 28]. When increasing the concentration, mistletoe lectins were shown to be cytotoxic for tumor and normal cells alike [29]. Overall, these studies indicate that many medicinal plants may contain biologically active lectins that warrant investigation. To this end, methods for lectin isolation and characterization must be established and optimized. The purpose of this chapter is to summarize our ongoing work in this field, first illustrating the procedure for lectin isolation (Sect. 3.2), then the assays used for biotesting of plant lectins in different cellular systems, e.g., isolated human blood cells (Sects. 3.3 and 3.4) and cancer cells (Sects. 3.5 and 3.6). Special aspects covered are applicability of lectins from medicinal plants and the specification of different levels of lectin-mediated modulation of selected cellular responses, such as cell aggregation (Sect. 3.3), hydrogen peroxide generation (Sect. 3.4), and vascular endothelial growth factor C (VEGF-C) secretion (Sect. 3.5). Based on this, it will be discussed whether treatment with a lectin immunomodulator can cause harmful effects in vivo (Sect. 3.6).

3.2 Isolation of Lectins from Medicinal Plants

A gold standard for lectin isolation is glycan-based affinity chromatography, which will need to be optimized for each protein studied. Such work is outlined in this section for the mistletoe lectin VAA, with an eye on yield [30]. To start with an important aspect of this technique, the proper matrix should be selected and substituted with covalently attached glycans using a spacer of sufficient length and flexibility to bind lectins. To isolate the galactoside-binding lectin from mistletoe leaves, we have employed Sepharose 4B with immobilized lactose. The preparation of this affinity matrix included activation (50 mL, rinsed with 1 L of distilled H_2O and then 0.5 M Na_2CO_3 buffer, pH 11) with 6 mL divinyl sulfone for 90 min. The divinyl sulfone-activated resin was then mixed with a solution of 20% lactose and left overnight

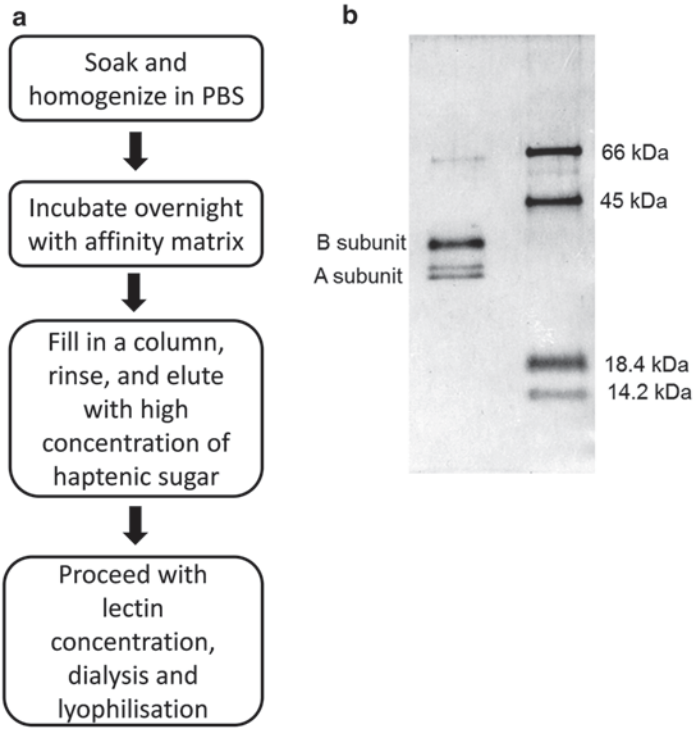

Fig. 3.1 Isolation and characteristics of Gal-binding lectin from mistletoe leaves. **a** Main steps of the lectin purification. **b** SDS-PAGE gel of purified VAA showing the presence of glycan-binding B subunit (34 kDa) and two closely related toxic subunits A1 (28.5 kDa) and A2 (29.5 kDa); protein standards were bovine serum albumin (66 kDa), egg albumin (45 kDa), β-lactoglobulin (18.4 kDa), and α-lactoglobulin (14.2 kDa)

under gentle rotation or stirring for lactose immobilization. Final steps of Sepharose 4B-lactose resin preparation required consecutive washes with distilled H_2O, 0.5 M $NaHCO_3$ buffer (pH 8.5), 2 h treatment with 4% β-mercaptoethanol to block any residual activated groups, and finally extensive washes with distilled H_2O and then 20 mM phosphate-buffered saline (PBS, pH 7.2) to completely remove any reagents.

Figure 3.1a shows the flow chart for isolation of VAA using the Sepharose 4B-lactose affinity matrix prepared as described above. All steps were performed at room temperature if not otherwise indicated. First, 100 g of dried leaves of *V. album* from a local drug store were macerated overnight in 400 mL of 20 mM PBS (pH 7.2), homogenized with additional 200 mL of PBS in a blender for 3 min, and filtered through a Buchner funnel. Second, the filtrate was centrifuged at 14,000 rpm for 20 min at 4 °C, in a Beckman J2-21 centrifuge (JA-14 rotor), and the supernatant was mixed with 30 mL of the Sepharose 4B-lactose matrix. The mixture was incubated overnight in a cold room (4 °C) with gentle shaking. Third, a glass column (2.5 × 7 cm) was filled with the gel suspension, washed with 600 mL PBS (flow rate of 150 mL/h), and, after washing thoroughly, VAA was eluted (rate of 50 mL/h) with 150 mL of

0.3 M lactose in PBS (pH 7.2). Fourth, the eluate was concentrated down to 10 mL in an Amicon ultrafiltration cell 8200 (YM5 membrane), dialyzed first against PBS (3 days with daily buffer exchange of 2 L) and then distilled H_2O (day 4), aliquoted at 500 μg, frozen and lyophilized. The entire isolation procedure lasted 1 week and can result in 4–6 mg/100 g d.w. of mistletoe leaves. The purity of the lectin attained was confirmed by SDS-PAGE as described by Laemmli [31] using vertical slab gels ($10 \times 7 \times 0.1$ cm) that combined a 4% stacking gel (length is 1.5 cm) and 10% separating gel (length is 8.5 cm). The gels were run at a constant current of 20 mA in a Mini-PROTEAN system and stained using $AgNO_3$ as described elsewhere [32]. This isolation method produced the heterodimer with molecular weight of 62 kDa that consists of a carbohydrate-binding B subunit (34 kDa) and a mixture of two slightly different toxic subunits A1 and A2 of 28.5 kDa and 29.5 kDa, respectively (Fig. 3.1b). The toxicity of A subunits is based on their ability to inactivate eukaryotic ribosomes by cleaving a single N-glycosidic bond at adenosine-4324 of 28S rRNA [33]. Further analysis of the lectin revealed a concentration-dependent equilibrium in the quaternary structure and preferential lectin activity to the so-called Tyr site in the dimer [34, 35].

3.3 Lectin-Induced Cell Aggregation Assay

Cell aggregation or agglutination activity is a 'natural' property of many lectins, because they can serve as molecular bridges between cell surface glycans of contacting cells, if harbouring more than one glycan-binding site as VAA does [36]. As such, the lectin-induced aggregation assay senses the accessibility of reactive glycans on the cell surface. To run this assay, lectins (usually 1–50 μg/mL) are added to the suspension of isolated human/animal cells ($1–5 \times 10^6$ cells/mL) in a balanced saline solution (PBS or HBSS). The formation of cell aggregates can be detected microscopically and spectrophotometrically by recording changes in the turbidity of cell suspensions [37]. Microscopic detection, although mostly qualitative, is however required to ascertain the formation of cell aggregates (Fig. 3.2a–c). Turbidimetric cell aggregation assay is quantitative and requires respective equipment, i.e., cell aggregometers, which are available from several suppliers such as SOLAR (Minsk, Belarus) or Chrono-Log (Havertown, PA), or any spectrophotometer with stirring and temperature-controlled cell.

Figure 3.2d, e (trace 1) show the courses of aggregation of human neutrophils (2×10^6 cells/mL) by two lectins with different glycan-binding properties, i.e., VAA (5 μg/mL) and wheat germ agglutinin (WGA) (50 μg/mL). Both lectins induced increase in light transmission of cell suspensions at 560 nm. This increase correlates well with microscopic observations of either individual cells or cell aggregates in the absence and presence of lectins, respectively (Fig. 3.2a, b). The glycan-binding specificity of lectins can also be measured in an aggregation assay by adding inhibitory (or haptenic) sugars prior or together with lectin(s). For example, as seen in Fig. 3.2d, e (trace 2), the aggregation response to VAA is inhibited by lactose

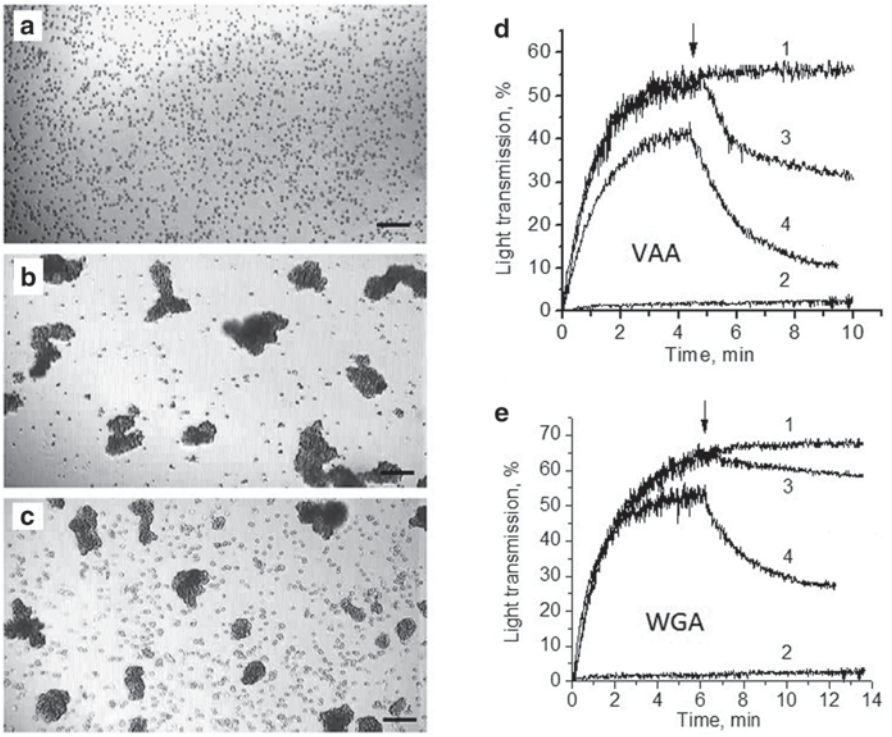

Fig. 3.2 Microscopical and turbidimetric detection of lectin-induced aggregation of human neutrophils. **a** Control suspension of neutrophils (2×10^6 cells/mL). **b** neutrophil aggregates formed in response to VAA (5 μg/mL) at 10–15 min corresponding to the plateau phase of the aggregation response. **c** VAA-induced neutrophil aggregates after addition of lactose to a concentration of 55 mM to cell aggregates at the plateau phase. **d** and **e** aggregation kinetics of human neutrophils recorded as changes in the light transmission of cell suspensions at 560 nm after the addition of VAA (5 μg/mL) and WGA (50 μg/mL), respectively: *1* a regular kinetics of lectin-induced cell aggregation, *2* inhibition of cell aggregation by haptenic sugars added before lectins (55 mM lactose to inhibit VAA and 100 mM GlcNAc to inhibit WGA), *3* formation of HSR contacts as proven by adding haptenic sugar to cell aggregates (indicated by *arrow*), *4* inhibition of HSR contacts in the presence of N-ethylmaleimide (50 μM) as revealed by cell aggregate dissociation induced by haptenic sugars (reproduced with permission from [37]). Bar = 100 μm (**a–c**)

(55 mM), whereas the aggregation response to GlcNAc-binding WGA is inhibited by GlcNAc (100 mM). Haptenic sugars can also be added in the course of the aggregation reaction, stopping cell aggregation or inducing dissociation of cell aggregates (Fig. 3.2d, e, trace 3). Remarkably, not all lectin-induced cell aggregates can be dissociated by haptenic sugars, indicating that additional intercellular contacts could be formed in response to lectins as demonstrated earlier [38–41]. Such lectin-inducible haptenic-sugar-resistant (HSR) contacts resulted from activation of transmembrane signaling in cells, because they can be inhibited by compounds [39, 40] that affect signaling pathways involved in cell adhesion [42]. For example, a very efficient inhibitor of HSR contacts is the sulfhydryl-blocking reagent N-ethylmaleimide, addition of which leads to dissociation of cell aggregates in the presence of

haptenic sugars (Fig. 3.2d, e, trace 4). It should be noted that lectin-induced increase in cell adhesion and expression of adhesion receptors was documented for human eosinophils and monocytes [43, 44]. Thus, analysis of cell aggregation and dissociation responses enables detecting both the presence of lectin-binding glycans on the cell surface and the ability of lectins to link counterreceptors, a process which can lead to stimulation of transmembrane signaling in cells. This approach is especially important for lectins from medicinal plants, because it provides a powerful and rapid method for screening lectins with potential immunomodulatory activity, for testing immune cells such as neutrophils and lymphocytes. To quantify the extent of cell aggregation and disaggregation, three parameters are suited, i.e., the maximal rate of cell aggregation based on the slope of the aggregation curve, the maximal extent of aggregation based on the light transmission plateau, and the stability of lectin-induced cell aggregates based on the response to haptenic sugars [37]. Interestingly, while there was a significant correlation between the aggregation rate and extent, the stability of lectin-induced aggregates, for obvious reasons, did not correlate in the majority of cases with those of the two conventional aggregation indices.

3.4 Lectin-Induced Generation of Hydrogen Peroxide by Human Neutrophils

Many plant lectins are able to activate plasma membrane NADPH oxidase, a pivotal enzyme complex of phagocytic cells (neutrophils, eosinophils, and macrophages) which generates superoxide anion radicals [45]. Superoxide radicals are dismutated spontaneously or enzymatically into H_2O_2, which is often used as an indicator of the phagocyte NADPH oxidase activity [6, 46]. The biological importance of this special oxidase is to make an effector in innate immunity available, because this reactive oxygen species contributes to the inactivation of pathogens taken up by phagocytosis [47]. The functionally active NADPH oxidase complex is assembled in cholesterol-enriched lipid rafts with plasma membrane flavocytochrome b558 (heterodimer of p22phox and gp91phox) and four cytoplasmic subunits (p40phox, p47phox, p67phox, and Rac) [48]. The only glycosylated component of this complex is gp91phox (NOX2), which is a key catalytic subunit and a founding member of NOX family of NADPH oxidases [48, 49]. We demonstrated that the sustainability of lectin-induced generation of H_2O_2 by human neutrophils depends on whether or not lectins interact with cytochrome b558, i.e., its glycosylated subunit gp91phox, and this interaction minimizes a requirement of presentation in lipid rafts [50]. This aspect of lectin activity can be disclosed by examining H_2O_2 generation by human neutrophils treated with drugs impairing the integrity of lipid rafts, e.g., with methyl-β-cyclodextrin which removes cholesterol from the cells without abolishing their viability [50].

A convenient assay of the phagocyte NADPH oxidase activity utilizes scopoletin, a fluorescent substrate of horseradish peroxidase (HRP) [6, 46]. To run this assay, neutrophils are used at a concentration of 1×10^6 cells/mL in PBS or HBSS (pH 7.2–7.3) containing 1 μM of scopoletin, 20 μg/ml of HRP, and 1 mM of NaN_3. Sodium azide is an optional component and included to inhibit endogenous myeloperoxidase and

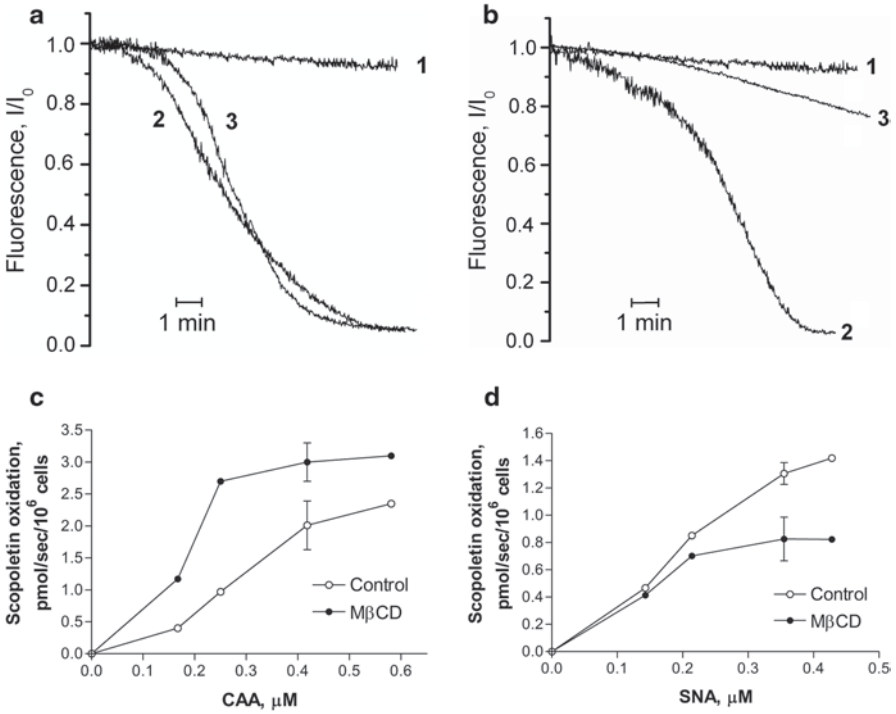

Fig. 3.3 Lectin-induced oxidation of scopoletin by human neutrophils as a measure of H_2O_2 generation. **a** and **b** Cell responses to GalNAc-binding lectin from *Caragana arborescens* (50 μg/mL) and NeuNAcα2,6Gal/GalNAc-binding lectin from *Sambucus nigra* (50 μg/mL): *1* basal oxidation, *2* lectin-induced oxidation, *3* lectin-induced oxidation in the presence of 10 mM methyl-β-cyclodextrin. **c** and **d** dose-dependent effects of lectins from *Caragana arborescens* (CAA) and *Sambucus nigra* (SNA) on scopoletin oxidation in the absence and presence of 10 mM methyl-β-cyclodextrin. Error bars were included for only one tested concentration on cells isolated from at least 3–5 donors. The reaction was performed in suspensions of human neutrophils in PBS, pH 7.2 (10^6 cells/mL) supplemented with 1 μM scopoletin, 20 μg/mL horseradish peroxidase, and 1 mM NaN_3 at 37 °C. (Reproduced with permission from [50])

catalase activity that provides better conditions for the detection of total H_2O_2 production. To start the reaction, a lectin is added (final concentration ranges from 1 to 50 μg/mL) to 1.5–2 mL of the stirred cell suspension at 37 °C, and the kinetics of scopoletin oxidation is recorded as a decrease in fluorescence intensity at 460 nm (excitation at 350 nm) (Fig. 3.3). The quantification of lectin-induced H_2O_2 generation yields two parameters; these are the maximal rate of scopoletin oxidation based on the slope of the kinetics and the lag-period, i.e., the initial time required to assemble NADPH oxidase complex. To infer whether lectins interact with gp91phox, the neutrophils are treated with methyl-β-cyclodextrin (extracting cholesterol from lipid microdomains; 10 mM) for 10 min at 37 °C and the regular response to lectins is measured. Methyl-β-cyclodextrin-induced inhibition of the lectin response suggests no direct interaction between lectin and flavocytochrome b558, while the sustained or increased H_2O_2 generation implies an interaction. Figure 3.3 shows the

corresponding kinetics and dose-dependent curves for two experiments with lectins from *Caragana arborescens* and *Sambucus nigra,* with opposite properties.

3.5 Lectin-Induced Secretion of VEGF-C by Cancer Cells

Binding of lectin with cells can induce the secretion of diverse types of immune modulators and cytokines having been noted above [27, 28]. This cellular response can be harmful in vivo as implied by an ongoing paradigmatic shift considering the tumor-promoting activity spectrum of some cytokines [51, 52]. To substantiate this concept, we present respective experimental data, first introducing a rather novel activity in this context. A dysregulated presence of growth factors required for angiogenesis and lymphangiogenesis is considered as a signature of cancer cells that require an efficient vascular system in tumors [53]. We showed that overproduction of VEGF-C, which is a major lymphangiogenic factor, is a feature of highly metastatic human breast cancer cell line MDA-MB-231 [54]. The level of VEGF-C in the cell culture medium may reach up to 10 ng/mL as can be readily measured by commercially available ELISA kits. Plant lectins with different glycan-binding properties were able to stimulate VEGF-C secretion by MDA-MB-231 cells, to a similar extent as human lectins (Fig. 3.4), what may also suggest a protumoral aspect of activity of these lectins. However, interpretation should be done cautiously for at least two reasons: First, there was evidence that other breast cancer cell lines (MCF7 and T-47D) showed differential responses to galectins and concanavalin A [55] and second, VEGF-C can mediate alternative functions in vivo affecting the permeability of blood vessels [56] and activating $\alpha^9\beta^1$ integrin and neuropilins that are involved in cell migration [57]. Thus, VEGF-C testing with cancer cells is an example of an assay for lectins from medicinal plants to gain insights on their biomedical potential. To decide on in vivo benefit or problems, animal models will provide salient information.

3.6 Tumor-Promoting Effects of Mistletoe Lectin In Vivo

In order to address the issue on potentially harmful effects of immunomodulation, a lectin can be tested at doses effective to increase cytokine levels. Such work has been done for the mistletoe lectin in two animal models. First, long-term VAA therapy was used to treat urinary bladder carcinomas in rats induced by N-methyl-N-nitrosourea or N-butyl-N-(4-hydroxybutyl)-nitrosamine [58, 59]. The standard regime of subcutaneous injections of VAA (1 ng/kg body weight, biweekly) was applied for 15 months. In both cases, no protection against chemically induced bladder carcinomas was noticed. In contrast, evidence for tumor stimulation was obtained. Second, short-term VAA therapy was applied to a breast cancer model in C3H/HeJ mice, which were transplanted with a metastatic C3L5 mammary adenocarcinoma cell line [52]. This model provides a convenient in vivo tool to monitor

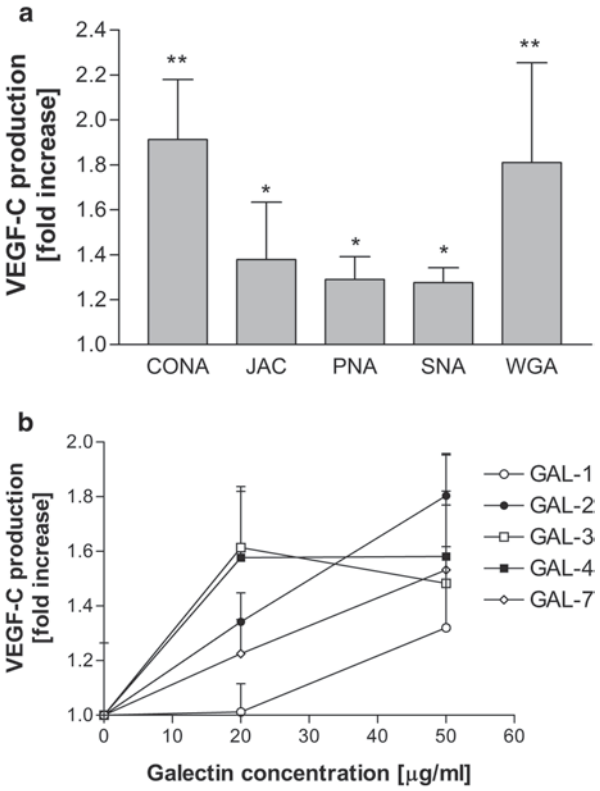

Fig. 3.4 Effect of plant lectins and human galectins on VEGF-C production by MDA-MB-231 cells. **a** Accumulation of VEGF-C in the cell culture medium (DMEM) of 80–90 % confluent culture in response to plant lectins (5 μg/ml) at 24 h (ConA concanavalin A, *JAC* jacalin, PNA peanut agglutinin, SNA *Sambucus nigra* agglutinin, WGA wheat germ agglutinin). **b** concentration-dependent secretion of VEGF-C by the cells in response to human galectins (GAL) at 24 h. The basal production of VEGF-C by untreated cells was 1127 ± 343 pg/ml ($n = 4$). *$p < 0.05$, **$p < 0.001$. (Reproduced with permission from [55])

tumor growth over a period of few weeks. While tumor growth was inhibited by the routine intraperitoneal administration of interleukin-2, subcutaneous injections of VAA (1 ng/kg body weight biweekly) promoted tumor growth and the incidence of lung metastasis (Fig. 3.5). The C3L5 cell line, which was used in this study, is characterized by a high metastatic activity resulting most likely from high cyclooxygenase-2, eNOS, and VEGF-C/VEGF-D expression [52, 60] that may make it sensitive to lectin-induced cytokines. Potential of growth stimulation in human histocultures [29] further adds to the notion that the actual physiological context determines the outcome of lectin application. It is therefore mandatory to rigorously exclude any risk potential of an immunomodulatory application (*primum non nocere*).

3.7 Conclusions

Medicinal plants in many cases contain lectins. They can readily be isolated using affinity chromatography, as demonstrated for a galactoside-binding lectin from mistletoe leaves. As a part of aqueous extracts, these lectins may contribute to the

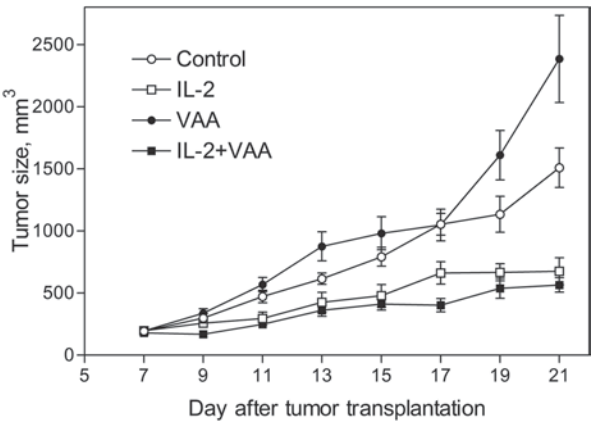

Fig. 3.5 Effects of intraperitoneal injection of interleukin-2 (IL-2, 6×10^4 IU every 8 h during 5 days, followed by a 4-day recess and then a similar 5-day round) and subcutaneous (s.c.) injections of VAA (1 ng/kg body weight, biweekly), as well as their combination on tumor growth after s.c. inoculation of 5×10^5 C3L5 cells in C3H/HeJ female mice. Starting at 1 week after tumor transplantation, the minimal and maximal diameters (*a* and *b*) of the tumors were measured on alternate days using digital calipers and the tumor volume was calculated according to the equation $V = 0.52a^2b$. Data indicate mean ± standard error ($n = 6$–10). (Reproduced with permission from [52])

activity profile of herbal medications, which needs to be thoroughly assessed. As instructive examples, different features of activity can be examined, e.g., using the aggregation assay in conjunction with inhibitory sugars and assays measuring the production of regulatory molecules such as reactive oxygen species and growth factors. In vivo work, as shown, is essential to identify and exclude harmful consequences that may arise from an administration of lectin-containing extracts. The set of methods that we have described in this chapter can thus be applied for screening and characterizing lectins in medicinal plants, with the aim of lectin-based assessment and standardization of herbal extracts, required to ensure optimal efficacy and herbal medicine safety.

References

1. Gabius HJ (ed) (2009) The sugar code. Fundamentals of glycosciences. Wiley-VCH, Germany
2. Gabius HJ, André S, Jiménez-Barbero J, Romero A, Solís D (2011) From lectin structure to functional glycomics: principles of the sugar code. Trends Biochem Sci 36:298–313
3. Rüdiger H, Gabius HJ (2001) Plant lectins: occurrence, biochemistry, functions and applications. Glycoconj J 18:589–613
4. Wang Q, Yu LG, Campbell BJ, Milton JD, Rhodes JM (1998) Identification of intact peanut lectin in peripheral venous blood. Lancet 352:1831–1832

5. Kilpatrick DC (1998) Mechanisms and assessment of mitogenesis: an overview. Methods Mol Med 9:365–378
6. Timoshenko AV, Kayser K, Gabius HJ (1998) Lectin-triggered superoxide/H_2O_2 and granule enzyme release from cells. Methods Mol Med 9:441–451
7. Gabius HJ (2001) Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie 83:659–666
8. Shanmugham LN, Castellani ML, Salini V, Falasca K, Vecchiet J, Conti P, Petrarca C (2006) Relevance of plant lectins in human cell biology and immunology. Riv Biol 99:227–249
9. André S, Sanchez-Ruderisch H, Nakagawa H, Buchholz M, Kopitz J, Forberich P, Kemmner W, Böck C, Deguchi K, Detjen KM, Wiedenmann B, von Knebel DM, Gress TM, Nishimura S, Rosewicz S, Gabius HJ (2007) Tumor suppressor $p16^{INK4a}$: modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J 274:3233–3256
10. Amano M, Eriksson H, Manning JC, Detjen KM, André S, Nishimura S, Lehtiö J, Gabius HJ (2012) Tumour suppressor $p16^{INK4a}$: anoikis-favouring decrease in N/O-glycan/cell surface sialylation by down-regulation of enzymes in sialic acid biosynthesis in tandem in a pancreatic carcinoma model. FEBS J 279:4062–4080
11. Timoshenko AV (2003) Lectins in medicinal plants. Proc Natl Acad Sci Belarus Biol N2:104–113
12. Antoniuk VO (1992) Isolation of lectin from horse chestnut (*Aesculus hippocastanum* L.) seeds and study of its interaction with carbohydrates and glycoproteins. Ukr Biokhim Zh 64:47–52
13. Kaku H, Goldstein IJ, van Damme EJ, Peumans WJ (1992) New mannose-specific lectins from garlic (*Allium sativum*) and ramsons (*Allium ursinum*) bulbs. Carbohydr Res 229:347–353
14. van Damme EJ, Smeets K, Torrekens S, van Leuven F, Goldstein IJ, Peumans WJ (1992) The closely related homomeric and heterodimeric mannose-binding lectins from garlic are encoded by one-domain and two-domain lectin genes, respectively. Eur J Biochem 206:413–420
15. Dam TK, Bachhawat K, Rani PG, Surolia A (1998) Garlic (*Allium sativum*) lectins bind to high mannose oligosaccharide chains. J Biol Chem 273:5528–5535
16. Boonmee A, Srisomsap C, Chokchaichamnankit D, Karnchanatat A, Sangvanich P (2011) A proteomic analysis of *Curcuma comosa* Roxb. rhizomes. Proteome Sci 9:43. doi:10.1186/1477–5956-9–43
17. Lis H, Sharon N (1972) Soybean (*Glycine max*) agglutinin. Methods Enzymol 28:360–365
18. Gubaidullin II, Baimiev AK, Baimiev AK, Chemeris AV (2007) The carbohydrate-binding sequences in lectins of the clovers *Trifolium repens, T. pratense,* and *T. trichocephalum*. Russ J Genet 43:376–380
19. Shibuya N, Goldstein IJ, Shafer JA, Peumans WJ, Broekaert WF (1986) Carbohydrate binding properties of the stinging nettle (*Urtica dioica*) rhizome lectin. Arch Biochem Biophys 249:215–224
20. Does MP, Ng DK, Dekker HL, Peumans WJ, Houterman PM, Van Damme EJ, Cornelissen BJ (1999) Characterization of *Urtica dioica* agglutinin isolectins and the encoding gene family. Plant Mol Biol 39:335–347
21. Berthier L, Marchal R, Debray H, Bonnet E, Jeandet P, Maujean A (1999) Isolation of isolectins from *Vitis vinifera* L. Cv. Chardonnay grape berries. J Agric Food Chem 47:2193–2197
22. Ghosh M (2009) Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. Fitoterapia 80:91–95
23. Chen Z, Kai G, Liu X, Lin J, Sun X, Tang K (2005) cDNA cloning and characterization of a mannose-binding lectin from *Zingiber officinale* Roscoe (ginger) rhizomes. J Biosci 30:213–220
24. Deepa M, Priya S (2012) Purification and characterization of a novel anti-proliferative lectin from *Morus alba* L. leaves. Protein Pept Lett 19:839–845
25. Gomes FS, Procópio TF, Napoleão TH, Coelho LC, Paiva PM (2013) Antimicrobial lectin from *Schinus terebinthifolius* leaf. J Appl Microbiol 114:672–679
26. Burkhard B (2000) Anthroposophische Arzneimittel. Eine kritische Betrachtung. GOVI-Verlag, Eschborn

27. Hajto T, Hostanska K, Gabius HJ (1989) 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 49:4803–4808
28. Hajto T, Hostanska K, Frei K, Rordorf C, Gabius HJ (1990) 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 50:3322–3326
29. Gabius HJ, Darro F, Remmelink M, André S, Kopitz J, Danguy A, Gabius S, Salmon I, Kiss R (2001) 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 19:114–126
30. Gabius HJ (1990) Influence of type of linkage and spacer on the interaction of β-galactoside-binding proteins with immobilized affinity ligands. Anal Biochem 189:91–94
31. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
32. Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99
33. Endo Y, Tsurugi K, Franz H (1988) The site of action of the A-chain of mistletoe lectin I on eukaryotic ribosomes: the RNA N-glycosidase activity of the protein. FEBS Lett 231:378–380
34. Jiménez M, Sáiz JL, André S, Gabius HJ, Solís D (2005) Monomer/dimer equilibrium of the AB-type lectin from mistletoe enables combination of toxin/agglutinin activities in one protein: analysis of native and citraconylated proteins by ultracentrifugation/gel filtration and cell biological consequences of dimer destabilization. Glycobiology 15:1386–1395
35. Jiménez M, André S, Siebert HC, Gabius HJ, Solís D (2006) AB-type lectin (toxin/agglutinin) from mistletoe: differences in affinity of the two galactoside-binding Trp/Tyr-sites and regulation of their functionality by monomer/dimer equilibrium. Glycobiology 16:926–937
36. Mikeska R, Wacker R, Arni R, Singh TP, Mikhailov A, Gabdoulkhakov A, Voelter W, Betzel C (2005) Mistletoe lectin I in complex with galactose and lactose reveals distinct sugar-binding properties. Acta Crystallogr F61:17–25
37. Gorudko IV, Buko IV, Cherenkevich SN, Polonetsky LZ, Timoshenko AV (2008) Lectin-induced aggregates of blood cells from patients with acute coronary syndromes. Arch Med Res 39:674–681
38. Timoshenko AV, Gorudko IV, Kaltner H, Cherenkevich SN, Gabius HJ (1997) Metabolic inhibitors as tools to delineate participation of distinct intracellular pathways in enhancement of lactose-induced dissociation of neutrophil and thymocyte aggregates formed by a plant lectin. Biochem Mol Biol Int 43:477–487
39. Timoshenko AV, Gorudko IV, Cherenkevich SN, Gabius HJ (1999) Differential potency of two crosslinking plant lectins to induce formation of haptenic-sugar-resistant aggregates of rat thymocytes by post-binding signaling. FEBS Lett 449:75–78
40. Timoshenko AV, Gorudko IV, Kaltner H, Gabius HJ (1999) Dissection of the impact of various intracellular signaling pathways on stable cell aggregate formation of rat thymocytes after initial lectin-dependent cell association of using a plant lectin as model and target-selective inhibitors. Mol Cell Biochem 197:137–145
41. Timoshenko AV, Gorudko IV, André S, Gabius HJ (2000) Cell-type dependence of stability modulation of lectin-initiated contacts by impairment of multivalent carbohydrate binding and intracellular signaling. Biosci Rep 20:199–209
42. Ruoslahti E, Obrink B (1996) Common principles in cell adhesion. Exp Cell Res 227:1–11
43. Fattah D, Page KR, Bezbaruah S et al (1996) A rapid activation assay for human eosinophils based on adhesion to immobilized ICAM-1, VCAM-1 and IgG. Cytokine 8:248–259
44. Tahara K, Nishiya K, Hisakawa N, Wang H, Hashimoto K (2003) Suppressive effect of iron on concanavalin A-induced multinucleated giant cell formation by human monocytes. Immunol Invest 32:229–243

45. Timoshenko AV, Gabius HJ (1993) Efficient induction of superoxide release from human neutrophils by the galactoside-specific lectin from *Viscum album*. Biol Chem Hoppe Seyler 374:237–243
46. Jones OTG, Hancock JT (1994) Assays of plasma membrane NADPH oxidase. Methods Enzymol 233:222–229
47. Segal BH, Grimm MJ, Khan AN, Han W, Blackwell TS (2012) Regulation of innate immunity by NADPH oxidase. Free Radic Biol Med 53:72–80
48. Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4:181–189
49. Taylor RM, Baniulis D, Burritt JB, Gripentrog JM, Lord CI, Riesselman MH, Maaty WS, Bothner BP, Angel TE, Dratz EA, Linton GF, Malech HL, Jesaitis AJ (2006) Analysis of human phagocyte flavocytochrome b_{558} by mass spectrometry. J Biol Chem 281:3745–3756
50. Gorudko IV, Mukhortava AV, Caraher B, Ren M, Cherenkevich SN, Kelly GM, Timoshenko AV (2011) Lectin-induced activation of plasma membrane NADPH oxidase in cholesterol-depleted human neutrophils. Arch Biochem Biophys 516:173–181
51. Gabius HJ, Gabius S (1999) Immunomodulierende Misteltherapie durch Lektinstandardisierung: Ein zweischneidiges Schwert? Versicherungsmedizin 51:128–136
52. Timoshenko AV, Lan Y, Gabius HJ, Lala PK (2001) Immunotherapy of C3H/HeJ mammary adenocarcinoma with interleukin-2, mistletoe lectin or their combination: effects on tumor growth, capillary leakage and nitric oxide (NO) production. Eur J Cancer 37:1910–1920
53. Holopainen T, Bry M, Alitalo K, Saaristo A (2011) Perspectives on lymphangiogenesis and angiogenesis in cancer. J Surg Oncol 103:484–488
54. Timoshenko AV, Chakraborty C, Wagner GF, Lala PK (2006) COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. Br J Cancer 94:1154–1163
55. Timoshenko AV, Kaltner H, André S, Gabius HJ, Lala PK (2010) Differential stimulation of VEGF-C production by adhesion/growth-regulatory galectins and plant lectins in human breast cancer cells. Anticancer Res 30:4829–4833
56. Brkovic A, Sirois MG (2007) Vascular permeability induced by VEGF family members in vivo: role of endogenous PAF and NO synthesis. J Cell Biochem 100:727–737
57. Timoshenko AV, Rastogi S, Lala PK (2007) Migration-promoting role of VEGF-C and VEGF-C binding receptors in human breast cancer cells. Br J Cancer 97:1090–1098
58. Kunze E, Schulz H, Gabius HJ (1998) Inability of galactoside-specific mistletoe lectin to inhibit *N*-methyl-*N*-nitrosourea-induced tumor development in the urinary bladder of rats and to mediate a local cellular immune response after long-term administration. J Cancer Res Clin Oncol 124:73–87
59. Kunze E, Schulz H, Adamek M, Gabius HJ (2000) 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 126:125–138
60. Xin X, Majumder M, Girish GV, Mohindra V, Maruyama T, Lala PK (2012) Targeting COX-2 and EP4 to control tumor growth, angiogenesis, lymphangiogenesis and metastasis to the lungs and lymph nodes in a breast cancer model. Lab Invest 92:1115–1128