

Chapter 2

How Plants Can Contribute to the Supply of Anticancer Compounds

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Abstract Plants were the first sources of medicines used by humankind, with evidence of herbal remedies dating back at least 60,000 years. Many plants have been used medicinally because they produce secondary metabolites with pharmacological properties, including compounds such as paclitaxel (Taxol) that inhibit cell division and can therefore be used as a treatment for cancer. With the advent of recombinant DNA and molecular biotechnology in the 1970s, plants have also been modified genetically to produce more of their native pharmaceutically active substances, or even nonnative compounds. The scope of medicinal plants has also expanded beyond secondary metabolites to include pharmaceutical recombinant proteins, such as human antibodies. This chapter provides an overview of the anticancer compounds naturally produced in plants and how gene technology has been used to facilitate their production. It also considers how plant-based expression systems can help to supply modern healthcare systems with protein-based anticancer compounds such as monoclonal antibodies, lectins, and anticancer vaccines.

Keywords Lectins • Monoclonal antibodies • Plant molecular pharming • Plant secondary metabolites • Therapeutic anticancer vaccines

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCs	Antibody-drug conjugates
APIs	Active pharmaceutical ingredients
ATPS	Aqueous two-phase systems

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CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary
DoE	Design-of-experiments
EBA	Expanded-bed adsorption
EBV	<i>Epstein-Barr virus</i>
FDA	Food and Drug Administration
GMP	Good manufacturing practice
HBsAg	Hepatitis B-soluble antigen
HBV	<i>Hepatitis B virus</i>
HCPs	Host cell proteins
HPV	<i>Human papillomavirus</i>
mAbs	Monoclonal antibodies
ML1	Mistletoe lectin 1
NK	Natural killer
PAT	Process analytical technology
PEG	Polyethylene glycol
QbD	Quality-by-design
R&D	Research and development
RIP	Ribosome-inactivating protein
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
T _h 1	T-helper
T-DNA	Transfer DNA
VEGF	Vascular endothelial growth factor
VFUs	Vertical farming units
VLPs	Virus-like particles

Introduction

Cancer is one of the major challenges in modern medicine and healthcare systems (Yabroff et al. 2011) due to the severe physiological and psychological burden suffered by patients and their families (Faller et al. 2013; Linden and Girgis 2012). Cancer also has a negative impact on the economy in general, costing an estimated \$895.2 billion in healthcare-related payments and reduced productivity in 2008 (American Cancer Society 2010).

There were 6.2 million cancer-related deaths in 2003, equivalent to approximately 13% of all deaths worldwide (McGuire 2016). The cancer-related mortality rate is higher in developing countries than industrialized countries because of the socioeconomic conditions that restrict access to anticancer therapies (Sankaranarayanan 2014). More than 14 million new cancer cases are reported each year, and this is expected to increase by 26% over the next 35 years due to demographic changes and improved diagnostics (Pritzkeleit et al. 2010; Rottenberg et al. 2010).

Cancer is not a narrowly defined condition with a single cause, but the collective term for more than 100 different yet related diseases (American Cancer Society 2015). There are gender-specific differences in the incidence of certain cancers that may reflect developmental differences between the sexes; for example women are more frequently diagnosed with breast cancer than men, or lifestyle factors; for example men are more frequently diagnosed with lung cancer than women (American Cancer Society 2015; McGuire 2016). The latter is also the single most frequent cause of cancer-related deaths (1.59 million per year) (McGuire 2016). The common link between all cancer types is that a subset of cells acquires the ability to proliferate in a rapid and uncontrolled manner. In most tissues, cancer cells initially form a localized malignant tumor, but cells eventually break away from the primary tumor and spread through the blood and/or lymphatic systems to form secondary tumors, a process known as metastasis (Alberts et al. 2002). Some tumors remain benign and noninvasive, and these are not classified as cancers (Silverstein et al. 2006). A set of six characteristics has been proposed to define cancer, i.e., (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan and Weinberg 2000). An expansion of this set has been proposed more recently, including (7) deregulated metabolism, (8) evasion of the immune system, (9) genome instability, and (10) inflammation (Hanahan and Weinberg 2011). However, these definitions may focus too much on the cellular rather than the tissue level of the diseases (Sonnenschein and Soto 2013).

A predisposition to cancer can be inherited, but it often occurs spontaneously due to exposure to environmental risk factors such as smoking, high-energy radiation, or carcinogenic chemical substances. Cancer can also be caused by infections with certain bacteria, e.g., *Helicobacter pylori* (Hong et al. 2012). More often, cancer can be caused by viruses (Cummins and Tangney 2013; Weinberg 2006), including *Human papillomavirus* (HPV) (Chen et al. 2015), the major cause of cervical cancer, and presumably *Epstein-Barr virus* (EBV), which is linked to Burkitt's lymphoma (Brady et al. 2007). Ultimately, all triggers result in mutations and/or epigenetic changes in DNA structure that inactivate tumor-suppressor genes such as *TP53* (Bieging et al. 2014) or activate proto-oncogenes such as *HER-2* (Chial 2008). The trigger may in some cases be directly mutagenic, e.g., (1) the induction of point mutations by alkylating agents, nucleoside analogs, or intercalating chemicals; (2) the incorrect repair of DNA double-strand breaks induced predominantly by radiation; or (3) the integration of foreign DNA, disrupting the original genetic context and causing aberrant gene expression as observed for some viruses (Akagi et al. 2014). The effects can also be indirect, e.g., the induction of chronic inflammation or infections that promote the proliferation of a subset of cells, e.g., B-lymphocytes, increasing the likelihood of uncontrolled growth as assumed for EBV in Burkitt's lymphoma.

Due to the heterogeneous nature of cancer there is no universal treatment. Four different general approaches are available, which can be followed alone or in combination (Sudhakar 2009). Surgery involves the physical removal of malignant tumor tissue. This can in theory effect a complete cure in a single procedure, but a

small number of cancer cells may remain at the excision site eventually leading to the formation of a new tumor. Therefore, healthy tissue adjacent to the tumor is often removed to create a safety margin, which is an undesirable side effect and does not necessarily increase the survival rate (Hernandez et al. 2009; Kubota 2011). Furthermore, in advanced cancers, small secondary tumors may be difficult to locate and/or remove. Therefore, surgery is often combined with chemotherapy or radiotherapy to increase the likelihood of a cure (Salama and Chmura 2014).

Radiotherapy uses X-ray or gamma radiation to damage the DNA of rapidly dividing cancer cells beyond repair, causing the cells to undergo apoptosis. Even though healthy cells typically have more efficient DNA repair mechanisms than cancer cells and can thus better withstand the radiation (Bernstein and Bernstein 2015; Gajecka et al. 2005), they can be affected by radiotherapy as well which can give rise to severe side effects such as the depletion of hematopoietic precursor cells and (in the longer term) even the induction of other forms of cancer (Berrington de Gonzalez et al. 2011; Mauch et al. 1995). The efficacy of high-energy radiation is dependent on the oxygen concentration in the tumor tissue, because the free radicals formed when oxygen interacts with the radiation cause further damage to the DNA of the cancer cells. However, hypoxia is often observed in large solid tumors, which means that radiotherapy is less effective against larger tumors (Harrison et al. 2002).

Chemotherapy is the treatment of cancer with drugs. This approach is advantageous because it can kill residual cancer cells and small, undetectable secondary tumors (Polireddy and Chen 2016). Chemotherapy can also be combined with radiotherapy to increase the therapeutic efficacy (Shahid 2016). One drawback is that the efficacy of chemotherapy depends on the way drugs are distributed in tissues, and poor results are often observed with larger solid tumors due to the limited vascularization, which prevents effective tumor penetration (Minchinton and Tannock 2006). The active pharmaceutical ingredients (APIs) used for chemotherapy are often small molecules, such as paclitaxel (Chabner and Roberts 2005). Such molecules can circulate relatively freely and reach the tumor site(s) even if their precise location is unknown. The first generation of chemotherapeutics were developed to disrupt the metabolism and/or mitotic activity of rapidly dividing cells, whereas the second generation instead targeted signaling components, such as protein kinases or growth factor receptors (Chabner and Roberts 2005). For example, paclitaxel is a first-generation drug that disrupts mitosis by preventing tubulin depolymerization, whereas gefitinib is a second-generation drug that inhibits signaling via the epidermal growth factor receptor (Chabner and Roberts 2005; Wani and Horwitz 2014). Whereas some cancer drugs have a simple structure suitable for total chemical synthesis (Neidle and Thurston 2005), most are complex molecules that must be produced using biotechnology (Baldi et al. 2008; Howat et al. 2014). Paclitaxel provides a useful example of the latter scenario. This compound was originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) (Wani and Horwitz 2014), but is now produced in transgenic plant cell suspension cultures at the 75,000-L scale (Zhong 2002). Some cancer drugs demonstrate limited selectivity but most also affect rapidly dividing healthy cells, such as hair follicle cells and B-lymphocytes, resulting in the common side effects of chemotherapy: hair loss and a compromised immune system (Sfikakis et al. 2005; Trueb 2010).

Immunotherapy harnesses the immune system against cancer, and is the most selective treatment approach and therefore the treatment associated with the least severe side effects (Caspi 2008; Schuster et al. 2006). Immunotherapy can take several forms, including the use of vaccines to prevent cancer, as seen with the vaccine against HPV to prevent cervical cancer (De Vincenzo et al. 2013; Poljak 2012), introduction of cytokines to manipulate the immune response, or antibody therapy to target cancer cells in the same way that antibodies normally target pathogens (Schuster et al. 2006). In the latter case, monoclonal antibodies (mAbs) are directed against cancer-specific cell surface structures including receptors and other surface proteins that are overexpressed in tumors, or glycan structures that are more common in cancer cells—these tumor-selective targets are collectively described as tumor markers (Christiansen et al. 2014; Chung and Christianson 2014; Duffy et al. 2014; Weiner et al. 2012). After binding to cancer cells, the mAbs can elicit antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) through their constant domains, causing natural killer (NK) cells to force the cancer cells into apoptosis (Mellstedt 2003). Alternatively, the antibodies may block the binding of growth factors (Gong et al. 2004) or carry a toxic conjugate such as monomethyl auristatin E (Polakis 2016), which is taken up into the tumors. Such antibody-drug conjugates (ADCs) can combine the ADCC of regular antibodies with the additional toxic effect of a conjugated cytotoxic effector (Peters and Brown 2015; Scott et al. 2012). Despite these benefits and several dozen approved products (Scott et al. 2012), antibody therapy requires large doses of expensive and highly pure mAb, e.g., 3 mg per kg body mass (Ben-Kasus et al. 2007) or ~750 mg per square meter of body surface area (Cheson and Leonard 2008), sometimes meaning the equivalent of 6–12 g per patient (Chames et al. 2009) (Table 2.1). This is why immunotherapy can cost several thousand euros per year per patient. The situation can be even worse if the antibody has a short serum half-life or induces an immune response in the patient, because this reduces the effective concentration of the protein and increases the required doses (Glassman and Balthasar 2014; Senter 2009). Furthermore, the comparably large size of an antibody (~150 kDa) can inhibit tumor penetration and reduce the effectiveness of the treatment against large solid tumors (Beckman et al. 2011).

These drawbacks can be circumvented by anticancer vaccines. The benefit of such vaccines is that, in theory, they require only a single low-dose treatment for each patient (e.g., 15 µg), which significantly reduces the costs of production and administration (Table 2.1). Anticancer vaccines exploit the ability of the human adaptive immune system to raise neutralizing antibodies against foreign epitopes (Murphy et al. 2008), in this case epitopes unique to or more abundant on cancer cells or the pathogens that cause cancer. For example, GSK and Merck & Co. have developed vaccines that form viruslike particles (VLPs) based on the L1 surface protein of the most virulent HPV strains, to reduce the risk of infection and the resulting HPV-related cervical cancer (De Vincenzo et al. 2013; Poljak 2012). These vaccines act in a preventive manner, whereas the HPV E6 and E7 proteins are being developed as therapeutic vaccines, i.e., vaccines that prevent the onset of cervical cancer even when an HPV infection has been established (Buyel et al. 2012; Massa et al. 2007; Venuti et al. 2009). Immunotherapy can also be combined with chemotherapy (Bang et al. 2010).

Table 2.1 Drug types used for cancer therapy with the corresponding amounts of API required per treatment

Drug type	API	Disease	Treatment duration [weeks]/ number of doses [-] (for vaccines)	ø API per patient [mg]	Reference
Small molecule	Paclitaxel	Breast cancer	25	5994	Committee for Medicinal Products for Human Use (CHMP (2015a))
Vaccine	Inactivated pertussis toxoid	Acellular pertussis	3	11	Thierry-Carstensen et al. (2013)
	HBsAg ^a	Hepatitis B	4	440	Aziz et al. (2006)
	HBsAg	Hepatitis B	3	6	Baldy et al. (2003)
	HPV L1 protein	HPV infection	3	180	McCormack (2014)
	HPV L1 protein	HPV infection	3	30	Committee for Medicinal Products for Human Use (CHMP (2016))
	Hemagglutinin	Influenza	1	9	Kenney et al. (2004)
	Hemagglutinin	Influenza	1	10	Chi et al. (2010)
	Hemagglutinin	Influenza	1	12	Beran et al. (2009)
	Pf ^b epitope fusion to HBV ^c core particles	Malaria	3	35	Nardin et al. (2004)
mAb	Trastuzumab	Breast cancer	36–52	6569	Ben-Kasus et al. (2007)
	Bevacizumab	Colon carcinoma	240	67,140	Committee for Medicinal Products for Human Use (CHMP (2015b))
	Cetuximab	Colon carcinoma	240	134,940	Committee for Medicinal Products for Human Use (CHMP (2004))
	Aflibercept ^d	Colorectal cancer	6	2238	Committee for Medicinal Products for Human Use (CHMP (2012))
	Panitumumab	Colorectal cancer	6	1902	Committee for Medicinal Products for Human Use (CHMP (2007))
	Rituximab	Lymphoma	4	1306	Cheson and Leonard (2008)
	Rituximab	Lymphoma	8–16	103,800	Chames et al. (2009)
	Pembrolizumab	Metastatic melanoma	20	995	Committee for Medicinal Products for Human Use (CHMP (2015c))

^aHBsAg hepatitis B-soluble antigen^bPf *Plasmodium falciparum*^cHBV *Hepatitis B virus*^dThis is a fusion protein used as a decoy receptor for vascular endothelial growth factor (VEGF)

Lectins are another class of molecules that can be used for immunotherapy or chemotherapy (Jiang et al. 2015). These plant-derived proteins bind to various carbohydrate structures on the cell surface and can induce immunomodulatory effects or apoptosis (Souza et al. 2013). For example, viscumin, also known as mistletoe lectin 1 (ML1) because it is the most abundant lectin in mistletoe (*Viscum album*), is a type II ribosome-inactivating protein (RIP) (Olsnes et al. 1982) that can be used to treat solid tumors (Zwierzina et al. 2011).

Ultimately, the choice of treatment depends on the type and grade of the cancer, its histology and location in the body, the stage of the disease, as well as the geographical region and healthcare options available to the patient (Manegold 2014; Merrett 2014). The difficult task is to identify a treatment that will effectively clear malignant tumors from the body with minimal side effects, e.g., the number of cycles of chemotherapy required (Heydarnejad et al. 2011). In the future, these issues could be reduced by individualized cancer treatments for each patient. The concept of individualized medicine has attracted increasing interest over the last decade because implementation is more feasible given the recent improvements in cancer diagnostics, molecular biology, high-throughput screening, donor cell cultivation, process scale-down, and single-use production systems (Klutzn et al. 2015; Schilsky 2010). Personalized medicine requires a precise diagnosis for each patient, followed by the identification of patient-specific tumor markers based on blood or biopsy samples and then the production, selection, or even de novo development of highly selective small-molecule anticancer compounds, mAbs, or ADCs and their subsequent application with constant monitoring for therapeutic progress (Millner and Strotman 2016). Personalized therapy would require not only a paradigm change in the way clinical trials are designed today (Schork 2015), but also a platform that can manufacture complex APIs in comparably large amounts (gram range) in a short time, which will be difficult to achieve with the current, mostly cell culture-based expression systems.

Even without considering the specific challenges of personalized medicine, there remain major challenges for the economically feasible production of anticancer drugs with sufficient purity and at the necessary volume (Siddiqui and Rajkumar 2012). Chemical synthesis is often impractical for small-molecule drugs due to the complexity of anticancer compounds and not economical for entire proteins. Prokaryotic expression systems are generally unsuitable for mAbs and other therapeutic proteins due to the lack of some posttranslational modifications (e.g., *N*-linked glycosylation, which is often required for therapeutic proteins to function properly) and the inefficiency of others (e.g., disulfide bond formation, which is necessary for proteins to fold correctly). The inefficient formation of disulfide bonds causes insoluble proteins to accumulate as inclusion bodies that need to be solubilized and refolded in vitro, which reduces the yield and adds to the production costs (Eiberle and Jungbauer 2010). On the other hand, expression platforms based on mammalian cells, such as Chinese hamster ovary (CHO) cells, may be incompatible with the production of anticancer small-molecule drugs and proteins that target cell division.

The following sections of the chapter focus on plants as either natural sources of anticancer compounds or as hosts for the production of recombinant anticancer

biopharmaceutical proteins. Some additional benefits of plants for the production of anticancer therapeutics, such as their scalability and sustainability, are discussed in concert with their potential merits as a platform for the manufacture of personalized medicines.

Plant Secondary Metabolites as Anticancer Drugs

Natural Sources of Plant Metabolites with Anticancer Activity

Plants have been used as medicines for at least 60,000 years (Fabricant and Farnsworth 2001) reflecting their ability to produce cocktails of secondary metabolites with a broad range of pharmacological properties, including anticancer activity (Kuttan et al. 1997; Zarkovic et al. 1998). Phenolic compounds, such as flavonoids, are the most promising plant-derived secondary metabolites for the treatment of cancer (Asensi et al. 2011; Wahl et al. 2011). Historically, plant-derived medicines were administered orally, either by the direct consumption of plant tissues or by the preparation of crude extracts, which is advantageous because these methods are both simple and inexpensive. Furthermore, the administration process is painless for the patient and safe because no syringe-based injection or other invasive procedures are required. The number of publications reporting the anticancer activity of plant extracts is expanding rapidly: a literature search in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) on August 16th, 2016, resulted in 4122 hits including 462 review articles when using the search term “plant extract anti cancer” and a massive increase in publication activity can be observed in the field since 2000 (Fig. 2.1a). It is beyond the scope of this chapter to review all the plant-derived substances that have potential anticancer activity, their mode of action, and efficacy, and it is unlikely that all these extracts will be able to replicate their anticancer effects in larger scale tests (Fritz et al. 2013; Ioannidis 2005; Nuzzo 2014). Furthermore, even if the anticancer effects are genuine (Kwon et al. 2016), the crude mixtures will need to be tested for safety and efficacy because they may contain, as well as the API, additional metabolites with undesirable effects, and the relative concentrations of the API and other ingredients can be difficult to control. For example, a raw extract of poppy (*Papaver somniferum*) seeds not only contains sanguinarine (an alkaloid with anticancer activity) (Selvi et al. 2009) but also hallucinogenic and addictive opiates.

In the light of the risks presented by crude extracts, APIs with anticancer activity are usually isolated from plants allowing them to be tested for a specific mode of action at defined concentrations without side effects caused by other metabolites. The most prominent example is paclitaxel, a taxane found in the bark of the Pacific yew tree (Camidge 2001; Wani and Horwitz 2014). Paclitaxel is used to treat ovarian, breast, and pancreatic cancers among others (Committee for Medicinal Products for Human Use (CHMP) 2015a; Wani and Horwitz 2014), and was approved by the US Food and Drug Administration (FDA) in 1992. It was shown that in combination with gemcitabine, paclitaxel increased the median survival time of patients suffering

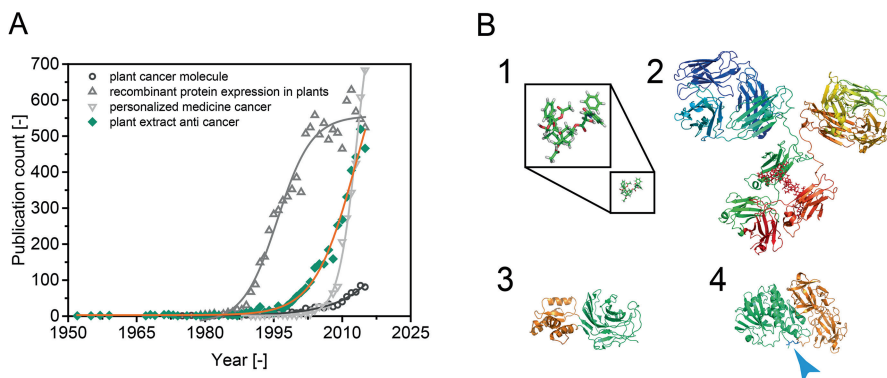


Fig. 2.1 Publication activity and anticancer compounds. **(a)** Publication history for four search queries related to anticancer compounds derived from or produced in plants. The most relevant query is shown as *green diamonds*. A Boltzmann function was fitted to each data series to illustrate the general trends in publication frequency. **(b)** Schematic illustration of the structures of molecules typically used for the treatment of cancer, all shown at the same size scale. (1) The small-molecule paclitaxel (sdf file: TA1_ideal_taxol; rcsb database; <http://www.rcsb.org>) with a zoomed-in box. (2) IgG2a monoclonal antibody from mice (pdb file: 1IGT) (Harris et al. 1997). The F_c part is shown in *red* and *green*, and the F_1 regions are shown in *blue* and *orange*. (3) Therapeutic HPV vaccine candidate LicKM-E7-GGG (“3Djigsaw” (<https://bmm.crick.ac.uk/~3djigsaw/>) homology model using automated template selection). The HPV-E7 fusion part is shown in *orange*, and the lichenase part is shown in *green* (Buyel et al. 2012). (4) Mistletoe lectin viscumin (pdb file: 1M2T) (Krauspenhaar et al. 2002). The toxic A-chain is shown in *green*, the lectin-like B-chain is shown in *orange*, and the linking disulfide bond is highlighted by a *blue arrow*

from pancreatic cancer by >25% compared to treatment with gemcitabine alone (Von Hoff et al. 2013). Paclitaxel is an inhibitor of tubulin depolymerization, thereby stabilizing the microtubule cytoskeleton and blocking mitosis in a concentration-dependent manner (Wani and Horwitz 2014).

Other small-molecule anticancer compounds naturally found in plants include genistein (an isoflavone angiogenesis inhibitor), lycopene (a carotenoid found in many fruits), and resveratrol (a stilbene found in grape berries and therefore also in wines) which are currently undergoing clinical trials to test their efficacy against breast and oral cancer (Bosviel et al. 2012; King-Batoon et al. 2008; Schneckeburger et al. 2014; Zlotogorski et al. 2013). Additional examples of plant-derived metabolites that have shown anticancer activity *in vitro* include several pregnane glycosides (e.g., desmiflavaside D) and alkaloids (e.g., mahanine) which selectively inhibit the growth of breast and prostate cancer cell lines, respectively (Jagadeesh et al. 2007; Raees et al. 2016). Antitumor activity has also been reported for non-psychoactive cannabinoids (McAllister et al. 2015). The number of known plant secondary metabolites with potential anticancer activity is steadily increasing (Schneckeburger et al. 2014) and diverse ecosystems such as tropical rainforests and coral reefs are thought to harbor a plethora of yet unknown molecules with similar or even improved efficacy as well as completely new modes of action (Mukherjee et al. 2001; Pereira et al. 2012).

Despite the potency of such compounds, large amounts of plant biomass must be harvested due to the high doses required, e.g., ~3 g of paclitaxel per patient (Small and Catling 1999), the miniscule amounts produced in native plants (Kelsey and Vance 1992), and the typically poor recovery of such compounds after extraction, which can be as low as 0.004% (Wani and Horwitz 2014). The naturally available production capacity for such anticancer compounds therefore tends to be insufficient to meet demands, and the source species may be driven close to extinction, as observed for the Pacific yew, which was initially the only source of paclitaxel (Wani and Horwitz 2014).

Production of Small-Molecule Anticancer Compounds in Plants

The unsustainable depletion of natural plant populations can be avoided by using genetic engineering to extend an existing metabolic pathway or to introduce an entirely new metabolic pathway in a more suitable host plant (Wilson and Roberts 2012). This allows the selection of hosts with better growth rates, shorter generation times, greater biomass productivity, or more suitability for biomass processing. For example, the extraction of paclitaxel from Pacific yew trees (Wani and Horwitz 2014) requires a large cultivation area to grow a sufficient number of trees over a period of up to 200 years. The trees must be logged and stripped of bark to extract paclitaxel, killing the trees in the process. The size of the trees and the rigidity of the bark mean that expensive large-scale equipment is required. Even with optimal cultivation and processing infrastructure, the area-wise productivity is low. Assuming that a 100-year-old yew tree is covered with ~3 kg of bark containing paclitaxel at a concentration of 0.14 g kg⁻¹ (Small and Catling 1999) and that such a tree requires 30 m² for cultivation, the productivity is only 0.14 mg a⁻¹ m⁻². In contrast, tobacco (*Nicotiana tabacum*) produces biomass at the rate of 10 kg a⁻¹ m⁻² (Stoger et al. 2002). Even if genetic engineering in tobacco achieves 10% of the yields in yew trees (0.014 g kg⁻¹ biomass), the productivity is still 140 mg a⁻¹ m⁻² and thus 1000-fold higher than the natural source. The production of secondary metabolites can also be increased by external factors, e.g., the lighting conditions (Buyel et al. 2015a).

Small-molecule anticancer compounds can also be produced in cultured plant cells (Tabata 2004). Despite the higher investment costs for fermentation infrastructure and the need for a sterile environment, cell cultures can be advantageous because it is possible to derive suspension cells directly from the native species or a close relative, benefiting from the intrinsic metabolic capability. Genetic engineering can then be used to increase yields if necessary. Paclitaxel provides a key example of this approach. Phyton Biotech (Delta BC, Canada) has implemented a 75,000-L-scale process compliant with good manufacturing practice (GMP) using *Taxus* sp. cells to produce the API for Bristol-Myers Squibb (New York, USA) (Zhong 2002). The product has been marketed since 1992 with annual sales of \$1.592 billion in 2000 (Exposito et al. 2009). Since then, several approaches have been pursued to increase the paclitaxel titers during cultivation, e.g., using elicitors such as methyl jasmonate

(Cusido et al. 2014). Other approved compounds with anticancer activity include berberine and shikonin, which are manufactured in *Coptis japonica* and *Lithospermum erythrorhizon* cells, respectively (Fujita 2007). The typical scale of plant cell cultures is ~1000 L and above (Georgiev and Weber 2014).

Protein-Based Anticancer Compounds from Plants

The use of small-molecule anticancer compounds is limited by their poor selectivity, leading to side effects caused by nonspecific effects on the enzymes, metabolism, and cell cycle components of healthy cells. Proteins are more complex molecules that offer alternative mechanisms of action and can also be used to target cancer cells, thus reducing side effects. Small-molecule drugs and anticancer proteins are compared in Fig. 2.1b.

Anticancer Activity of Lectins

Lectins are a heterogeneous group of glycoproteins produced by many different plant species and have probably evolved as part of the molecular defense repertoire against pests and herbivores (Lannoo and Van Damme 2014; Van Damme 2014; Vandenberghe et al. 2011). Lectins may comprise several polypeptide chains, forming, e.g., homotetramers like ArtinM from jackfruit (*Artocarpus heterophyllus*) (Souza et al. 2013) or heterodimers like the mistletoe lectin viscumin (Kourmanova et al. 2004). The common feature of lectins is their affinity towards carbohydrate structures, with different lectins favoring different oligosaccharides. This selectivity allows some lectins to bind more or less specifically to carbohydrates displayed on tumor cells, resulting in immunomodulatory or anticancer activity (Souza et al. 2013).

Viscumin is the most prominent example of a plant lectin with potential anticancer applications (Zwierzina et al. 2011). Viscumin is synthesized as a single polypeptide precursor which is activated by proteolytically removing a central amino acid linker sequence. The active form of the protein comprises an A-chain (former N-terminus) with N-glycosidase activity and a B-chain (former C-terminus) which binds to carbohydrates on the cell surface (Walsh et al. 2013). The two chains are covalently linked by a disulfide bond (Olsnes et al. 1982). The A-chain features β -sheet secondary structures but is dominated by α -helices (Fig. 2.1b) (Krauspenhaar et al. 2002). The mode of action of the A-chain involves the cleavage of the N-glycosidic bond at position A4324 in the 28S rRNA of the large subunit of eukaryotic ribosomes, hence the classification of viscumin as a type II RIP (Endo et al. 1988). The toxicity of purified viscumin (intravenous LD₅₀ in mice) is 2.4 $\mu\text{m kg}^{-1}$ (Olsnes et al. 1982). In comparison, the structurally related ricin toxin from the seeds of *Ricinus communis* (castor bean) has a toxicity of 30 $\mu\text{m kg}^{-1}$ (Audi et al. 2005).

In contrast to the A-chain, the carbohydrate-binding B-chain of viscumin is mostly composed of β -sheets (Fig. 2.1b) and has three intra-chain disulfide bonds. Viscumin was initially shown to bind to β -galactosides, especially terminal galactose in the oligosaccharides of glycoproteins (Gabijs et al. 1992; Lee et al. 1994; Olsnes et al. 1982) but it also binds selectively to terminal sialic acids, e.g., IV^{Neu5Ac}-nLc4Cer residues in a mammalian context (Muthing et al. 2004), especially on glycoproteins and gangliosides (Zwierzina et al. 2011). The ganglioside CD75s carries an IV^{Neu5Ac}-nLc4Cer residue and is associated with several types of solid tumor, including pancreatic cancer (Distler et al. 2008). The antitumor activity of viscumin therefore appears to reflect the ability of the B-chain to bind selectively to tumor cells allowing the toxic A-chain to be internalized and block protein synthesis. Previous studies have shown that viscumin also stimulates T-helper (T_h1) cells, thereby inducing the cellular component of the adaptive immune system to attack cancer cells (Zwierzina et al. 2011).

N-glycans are added to the viscumin B-chain as it passes through the secretory pathway to the apoplast (Niwa et al. 2003). These posttranslational modifications are not required for its antitumor activity, because aglycosylated recombinant viscumin purified from *Escherichia coli* inclusion bodies was also efficacious in phase I clinical trials (Zwierzina et al. 2011). However, authentic N-linked glycosylation may increase the potency of viscumin. Furthermore, the resolubilization and refolding of protein from inclusion bodies are laborious and inefficient (Eiberle and Jungbauer 2010). The expression of recombinant lectins in plants may be therefore ideal to achieve high yields, straightforward purification, and more potent APIs.

Recombinant Protein Expression in Plants

Benefits and Challenges

In addition to the production of native proteins with anticancer activity by some plant species, many species of plants (or the cells and tissues derived from them) have been used as expression systems for recombinant therapeutic proteins. The use of plants for the production of recombinant proteins is known as “molecular farming,” and to emphasize the medical relevance when these are therapeutic proteins the alternative spelling “molecular pharming” is also used (Fischer et al. 1999, 2013; Ma et al. 2005; Menkhaus et al. 2004; Wilken and Nikolov 2012). In the context of biopharmaceutical manufacturing, plants are beneficial because they can synthesize complex proteins with authentic posttranslational modifications (e.g., glycosylation, disulfide bond formation), combined with low-cost upstream production, inherent process safety based on the inability of human pathogens to replicate in plants (Commandeur et al. 2003), and potential for flexible and very-large-scale production (Buyel et al. 2016c). The last two aspects are particularly important when comparing plants to mammalian cells because disastrous contamination with human pathogens is unlikely (Bethencourt 2009; Zimran et al. 2011) and the manufacturing capacity can be rapidly adapted to market demands. Two general types of expression strategies are available for plants: (1) transient expression using viral or bacterial

vectors or combinations thereof and (2) expression in transgenic plants or plant cells (Fischer and Schillberg 2006; Paul and Ma 2011; Twyman et al. 2003).

In contrast to the production of proteins in microbes and animal cells, where a limited number of host systems have been standardized by the biomanufacturing industry (The CMC Biotech Working Group 2009), a plethora of plant species and expression strategies have been considered by academic and industrial development teams. In terms of intact plants, tobacco and its close relative *N. benthamiana* are now emerging as standard platforms for the production of recombinant proteins by stable transformation and transient expression, respectively, and cereals are regarded as promising hosts for stable expression because the recombinant proteins can be stored in seeds (Spiegel et al. 2016). Standardized platforms are also emerging based on tobacco, rice, and carrot cell suspension cultures (Santos et al. 2016; Schillberg et al. 2013). Scalable unit operations and more structured purification schemes are now being implemented for plant-derived products (Buyel and Fischer 2014c, e; Buyel et al. 2015c). The initial extraction method for plant-derived APIs depends on the tissue and subcellular localization used for expression. Products secreted into hydroponic medium (intact plants) or the fermentation broth (cell culture) can be recovered directly, similar to other cell culture-based processes (Drake et al. 2009). Recombinant proteins expressed in leaf or seed tissues are typically extracted by blade-based homogenizers/presses or mills, respectively (Bals and Dale 2011; Buyel and Fischer 2014d, e; Farinas et al. 2005; Hassan et al. 2014; Hassan et al. 2008; Kim et al. 2013). Aqueous buffers within the pH range of 7.0–8.0 are typically used for extraction (Buyel et al. 2015c).

Product Recovery and Purification

During downstream processing, the process schemes rely on the same methods used in cell culture-based systems, e.g., filtration and solid-liquid chromatography, but must be adapted to suit the characteristics of plants, e.g., a high particle burden (Buyel et al. 2014b) and a high concentration of host cell proteins (HCPs) such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) if the proteins are not secreted into the medium (Buyel and Fischer 2014c), and product concentrations below 0.5 g L^{-1} in the raw extracts (Buyel 2015) in contrast to $5\text{--}10 \text{ g L}^{-1}$ achieved in cultured mammalian cells (Li et al. 2010; Shukla and Thommes 2010), which can be challenging during initial product recovery (Winkelkemper and Schembecker 2010). These modifications have been major cost drivers for plant-derived APIs in the past, accounting for up to 80% of the process costs (Buyel et al. 2015c; Wilken and Nikolov 2012). In particular, the clarification of the raw plant extracts has been characterized by low filter capacities ($<100 \text{ L m}^{-2}$) increasing the consumable costs of production. However, flocculants and other filter aids have recently been shown to improve particle retention on low-cost bag filters and can increase the downstream filter capacity to $>1000 \text{ L m}^{-2}$ (Buyel 2016; Buyel and Fischer 2014b; Buyel et al. 2014b, 2015b).

A number of genetic modifications have been used to facilitate the purification of proteins expressed in plants from the clarified extract. These modifications include oleosins as direct (Kapchie et al. 2011; Markley et al. 2006; Napier et al. 1996) or indirect fusions (McLean et al. 2012), zein (Geli et al. 1994; Torrent et al. 2009),

elastin-like polypeptides (Conley et al. 2009; Tian and Sun 2011; Urry 1988), soybean agglutinin (Tremblay et al. 2011), or the frequently used hexa-histidine (His₆) tag (Buyel et al. 2012). All of these modifications confer specific properties that facilitate product purification, e.g., by two-phase extraction or affinity chromatography, but may not be suitable for anticancer compounds that are used for long-term treatment because the same tags may be immunogenic, thus reducing therapeutic efficacy (Fischer et al. 2012; Li 2011), or their commercial use may be restricted (Conley et al. 2011).

Process-based solutions for the challenging purification of plant-derived APIs have therefore been developed that are compatible with the requirements of biopharmaceutical proteins. Heat precipitation at ~70 °C has proven useful to remove >90% of all HCPs from extracts containing heat-stable products at a very early process stage, reducing the likelihood of product oxidation or proteolytic degradation (Buyel et al. 2014a, 2016b; Menzel et al. 2016). The low-pH (<5.0) precipitation of HCPs is an alternative step for heat-sensitive products (Azzoni et al. 2002; Buyel and Fischer 2014c). Additionally, abundant HCPs such as RuBisCO can be precipitated with polyethylene glycol (PEG) (Arfi et al. 2016). The subsequent purification and polishing steps to achieve the required final purity use chromatography modes such as affinity chromatography (e.g., protein A chromatography for the purification of mAbs) followed by orthogonal combinations of anion- and cation-exchange chromatography, hydrophobic interaction, and mixed-mode chromatography or size-exclusion chromatography (Nfor et al. 2011). For example, a two-step process consisting of protein A and ceramic hydroxyapatite chromatography was used to isolate an mAb from clarified tobacco extracts for phase I clinical studies (Ma et al. 2015). Because the product concentration can fall below 0.05 g L⁻¹ (Menkhaus and Roseland 2008), the use of membrane absorbers instead of packed-bed columns can be advantageous, also reducing the process times due to the higher flow rates (Orr et al. 2013). Alternatively, ultrafiltration/diafiltration setups can be used to reduce the process volume (Lightfoot and Moscariello 2004; Lightfoot et al. 2008). Integrated approaches such as expanded-bed adsorption (EBA) chromatography and aqueous two-phase systems (ATPS) have been tested for the purification of recombinant proteins from plants but were either more expensive than conventional processes (Menkhaus and Glatz 2005) or attracted regulatory scrutiny due to the presence of fusion tags (Reuter et al. 2014).

Process Design and Monitoring

Non-platform processes designed to accommodate the properties of individual products require time and investment, and therefore contribute to the steadily increasing R&D costs in the biopharmaceutical industry (PhRMA 2014). Rational protein design (Boes et al. 2011) and characterization of HCPs (Buyel et al. 2013b) using open-source (UniProt-Consortium 2012) and/or commercial software (Chemical Computing Group, Montreal, Canada) can be used to establish more standardized processes for plant-derived APIs. Furthermore, the concept of quality-by-design (QbD) and its associated tools, namely design-of-experiments (DoE) and

process analytical technology (PAT) (De Beer et al. 2008; Landgrebe et al. 2010; Rathore 2009; Rathore et al. 2008, 2010; Read et al. 2010a; b), are being integrated at the stage of process development but must be adapted to the large number of individual plants per batch in contrast to the smaller number of fermenters in conventional processes (D'Este et al. 2012; Juca et al. 2011; Sainz et al. 2013). For example, new PAT monitoring has been implemented for plant cell suspension cultures during upstream production (Buyel et al. 2016a; Holland et al. 2013) and the complex mechanisms of protein expression (Buyel and Fischer 2014a) and flocculation (Buyel 2016) have been described using DoE.

Single-use technologies, such as filters, sensors, fermenters, and chromatography columns, are becoming increasingly popular in the biopharmaceutical industry (Allison and Richards 2014; Eibl and Eibl 2011; Laukel et al. 2011; O'Brien et al. 2012; Shukla and Gottschalk 2013; Whitford 2010) and are likely to become an integral part of future manufacturing plants (Klutcz et al. 2015) because they facilitate process validation, accelerate change-over tasks, and avoid the need to clean equipment that comes into contact with the product (Fischer et al. 2012). Single-use technologies are also typically implemented in plant-based production processes (Ma et al. 2015) and the plants themselves can be regarded as single-use, biodegradable bioreactors.

Today, protein-based APIs derived from plants are still exotic products in the pharmaceutical industry. The only product of this class currently approved as a pharmaceutical for general human use is taliglucerase alfa (marketed as Elelyso) which was developed by Protalix Biotherapeutics and is produced in carrot cell suspension cultures (Mor 2015; Pastores et al. 2014). However, more than ten further product candidates are already in the clinical pipeline with still more in pre-clinical development (Gleba et al. 2014). These industry-derived products combined with further APIs developed in publically funded projects (Paul et al. 2013; Sparrow et al. 2007) have encouraged the regulatory authorities in the USA and EU to release draft guidelines for the production of plant-derived biopharmaceuticals (CPMP 2002; FDA 2002; Spok 2007), filling the legal vacuum that has thus far discouraged the big players in the pharmaceutical industry from making substantial investments into this sector (Das et al. 2008; Fischer et al. 2012; Ma et al. 2005), thus resulting in the focus on plant cell cultures instead of intact plants (Pastores et al. 2014). Plant-based production systems are now entering the biopharmaceutical arena as a competitive platform for the manufacture of biopharmaceutical proteins, including anticancer antibodies and lectins.

Transgenic Plants and the Potential Large-Scale Production of Anticancer Compounds

Scalability and Product Quality

In contrast to microorganisms and animal/plant cell cultures, transgenic plants can be cultivated as intact, multicellular organisms. One advantage is that sterile cultivation conditions and/or antibiotics are not required for upstream production, and

another is that the pharmaceutical crops can be grown on the same agricultural scale as food and feed crops, thus allowing the production of multiple tons of recombinant proteins to fulfil high-demand markets (Buyel et al. 2016c; Fischer and Schillberg 2006). For example, 179.7 million hectares of genetically modified crops were grown in 2015, which is a 100-fold increase compared to 1996 (James 2015). Yet another advantage of transgenic plants is that the modification is much more stable than the genes carried by engineered microbes or mammalian cell lines (Baneyx and Mujacic 2004; Gerngross 2004; Hellwig et al. 2004; Wurm 2004). This ensures a defined and stable genetic status and facilitates the generation of well-defined master and working seed banks (Fischer et al. 2012). Transgenic lines are typically established by transformation using *Agrobacterium tumefaciens* or physical methods such as particle bombardment to introduce the gene(s) of interest into the plant genome, before regeneration into intact plants (Lorence and Verpoorte 2004; Newell 2000; Rivera et al. 2012). The initial transformants are typically subjected to 3–8 breeding cycles resulting in homozygous transgenic plant lines. The transformation and breeding process can take 8–24 months depending on the plant species and genetic construct, and can require extra time to prepare the seed banks required for large-scale production (Twyman et al. 2003). Although the advent of genome editing tools such as CRISPR/Cas9 can reduce this time (Bortesi and Fischer 2015; Puchta and Fauser 2014), transgenic plants are probably inadequate for urgent demands, as would be required for personalized cancer therapy. In contrast, transgenic plants function better as a bulk production system for proteins that are required for standard therapies in a large number of patients, e.g., anticancer vaccines or mAbs such as rituximab.

One matter of concern when considering plants for the production of vaccines and antibodies is that the glycans added to proteins by plants are structurally different to those produced in humans, which means that injected APIs can be immunogenic. Although antibodies against plant glycans have been detected in human serum, no adverse responses against plant-derived recombinant therapeutic proteins have been reported (Bardor et al. 2003; Chargelegue et al. 2000; Koprivova et al. 2004). In some cases, the nonhuman glycan profiles produced by plants can even be advantageous, e.g., modulating the effector functions of some plant-derived mAbs due to the carbohydrates attached to the Fc region, and simplifying the production of the enzyme glucocerebrosidase which is used to treat Gaucher's disease. In the latter case, the enzyme produced in carrot cells (Elelyso, see above) is devoid of terminal sialic acid residues, allowing efficient uptake by mannose receptors on circulating macrophages. The same protein produced in mammalian cells contains terminal sialic acid residues that must be cleaved off *in vitro*, thus increasing production costs (Grabowski et al. 2014). Hence, plants can be regarded not only as an alternative production system but also as a potential source of “biobetters.” Where authentic human-type glycosylation is necessary for therapeutic efficacy, plants can be modified by mutating endogenous genes to remove plant-specific residues such as core α 1,3-fucose and β 1,2-xylose, and by introducing human genes needed for the synthesis of multi-antennary glycans, core α 1,6-fucose, β 1,4-galactose, and terminal sialic acids (Bakker et al. 2006; Strasser 2013, 2016; Strasser et al. 2008). These glyco-engineered lines can then be used as a platform for the production of recombinant proteins with authentic glycans.

Monoclonal Antibodies

Monoclonal antibodies can be used as highly specific and efficacious APIs for cancer therapy (Chiarella 2011; Gaughan 2015; Scott et al. 2012). In 2013, a total of almost 10 tonnes of mAbs was manufactured with an estimated market value of \$US 75 billion (Ecker et al. 2015). Based on the current forecast of a 10% annual growth rate (Research 2013), this market will cross the \$US 200 billion mark before 2025. The demand for some major single products such as rituximab and etanercept is already about 1 tonne per year (Kelley 2007). This amount is likely to increase massively if the supply of these products can be expanded to developing countries, which currently have limited access to such high-quality cancer therapeutics due to the manufacturing costs. For example, assuming that only every second person among the 14.1 million new cancer patients every year (Pritzkeleit et al. 2010; Rottenberg et al. 2010) requires mAb therapy, with a typical dose of ~10 g of protein pre patient (Chames et al. 2009), the annual demand for mAbs related to cancer therapy will increase to 70.5 tonnes. This calculation does not take into account the increasing incidence of cancer expected in the future due to demographic changes and improved diagnostics, and also ignores the likelihood that per-patient mAb consumption may increase due to improved survival rates.

Multi-tonne production scales for mAbs are difficult to achieve using conventional expression systems alone, even though mAb titers of 5–10 g L⁻¹ are now possible on a regular basis (Li et al. 2010; Shukla and Thommes 2010). For example, despite such high titers, the investment costs for bioreactors would be enormous because even very-large-scale fermenters with volumes of 10,000–25,000 L (Kelley 2007; Li et al. 2010) would only produce ~5.0 tonnes of mAb per year, assuming a duration of 12 days per batch (Kelley 2007), and would thus cover less than 10% of the anticipated demand. Furthermore, the many benefits of single-use technologies are lost at such massive production scales because multiple disposable production trains would need to operate simultaneously, and at this point a fixed stainless-steel facility costs less to operate even when the up-front costs are included (Eibl et al. 2010; Shukla and Gottschalk 2013).

In contrast, plants provide their own in-built single-use bioreactors and the cost does not increase significantly regardless of the production scale, because plants can be numbered up as required and the only cost factor is the additional land or greenhouse space. The per se high biomass yield of some crops, e.g., 10,000 tonnes a⁻¹ km⁻² for tobacco (Stoger et al. 2002), can be increased to 91,000 tonnes a⁻¹ km⁻² if so-called vertical farming units (VFUs) are used for production instead of open-field cultivation (Buyel et al. 2016c; Holtz et al. 2015). Combining these biomass yields with antibody expression levels of 2 g kg⁻¹ (Zischewski et al. 2015) and a typical mAb recovery of 70% during purification from transgenic plants (Ma et al. 2015) implies that ~0.55 km⁻² of VFU area will be sufficient to produce 70 tonnes of purified mAb.

Additional benefits of VFUs include the improved containment and more tightly controlled growth conditions, which can be advantageous for GMP production processes (Fischer et al. 2012), as well as the potential for fully automated handling, even in a GMP environment (Wirz et al. 2012). To achieve the same output by

fermentation, a bioreactor volume of >350,000 L would be required. On top of the investment costs, such a setup would carry an immense monetary risk given the frequency of batch failures, e.g., due to contamination (Lolas 2013): assuming CHO cell media costs of \$US 55–90 L⁻¹, each failed batch would generate losses corresponding to \$US 19–32 million only for the media. A more detailed discussion of the benefits of plants for the very-large-scale production of mAbs can be found elsewhere (Buyel et al. 2016c), but independent from the upstream production step, the downstream processing and scale-up considerations would be similar to those for cell culture-based systems (Ma et al. 2015; The CMC Biotech Working Group 2009), e.g., continuous manufacturing and a preference for single-use technologies (Angarita et al. 2015; Baur et al. 2015; Klutz et al. 2015). Plant-based systems may also benefit from the experiences gathered in the food processing industry (Goody 1997). In summary, plants as a production platform could tap into the full potential of mAbs as anticancer agents for a broad range of patients in industrialized as well as developing countries.

Lectins

Lectins such as viscumin could potentially be developed as anticancer drug candidates and a GMP-compliant process for the production of viscumin has already been established using a standard *E. coli* expression system, allowing viscumin to be tested in clinical trials (Zwierzina et al. 2011). However, the A- and B-chains were produced by two different bacterial strains in separate fermentations and both polypeptides formed inclusion bodies, so laborious resolubilization and refolding were necessary leading to poor recoveries of ~5% based on the amount of unfolded polypeptide educts, which is typical for such refolding processes (Eiberle and Jungbauer 2010). Additionally, the product was not glycosylated, which may affect its stability and efficacy as discussed above (Li et al. 2012). Other plant lectins have been expressed in the yeast *Pichia pastoris* at levels of 6–20 mg L⁻¹ (Lannoo et al. 2007; Oliveira et al. 2008) but glycoproteins produced by yeast often bear predominantly high-mannose rather than complex-type glycans (Strasser 2016). Mammalian cells are unlikely to express viscumin at high titers because the lectin would be toxic to the host cells (Endo et al. 1988). Such issues can be overcome by using plants as expression hosts because lectins are native to plants and heterologous lectins are generally not toxic in plant systems. In the case of viscumin, the Fraunhofer Institute for Molecular Biology and Applied Ecology IME (IME) has started to develop a plant-based expression approach which has thus far achieved yields of ~5 mg kg⁻¹ (our unpublished data). This is comparable to the 1.5 mg mL⁻¹ achieved in *E. coli* after refolding, but the refolding step is not required. Purifying viscumin from the more complex plant matrix is unlikely to pose a major challenge because lactosyl-Sepharose can be used as an affinity resin, and the purification of proteins from plant extracts by affinity chromatography does not seem to be inhibited by the larger quantity of released HCPs compared to products secreted into the fermentation

Biotechnology and Production of Anti-Cancer
Compounds

Malik, S. (Ed.)

2017, XV, 328 p. 53 illus., 36 illus. in color., Hardcover

ISBN: 978-3-319-53879-2