Resistance to Targeted Anti-Cancer Therapeutics 6
Series Editor: Benjamin Bonavida

Rama Shanker Verma *Editor*

Resistance to Immunotoxins in Cancer Therapy

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Resistance to Targeted Anti-Cancer Therapeutics

Volume 6

Series Editor
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For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor-specific and exhibited severe toxicities in many cases. But during the last several years, targeted cancer therapies have been developed. Targeted cancer therapies are drugs or other agents (e.g. antibodies) that block the growth and spread of cancer by interfering with specific gene products that regulate tumor cell growth and progression. Targeted cancer therapies are also sometimes called "molecularly targeted drugs." We have witnessed in the last decade a significant explosion in the development of targeted cancer therapies developed against various specific cancers. These include drugs/antibodies that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells and to deliver toxic drugs to cancer cells. One of the major problems that arises following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to therapies remains a major problem and several strategies are being considered to reverse the resistance to various manipulations.

Resistance to Targeted Anti-Cancer Therapeutics will focus on the basic and translational research behind the molecular mechanisms of resistance found in many kinds of anti-cancer therapeutics.

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Preface

Cancer includes a large class of diseases of abnormal cell growth and is the most life-threatening disease mankind has ever seen; it accounted for an astonishing 14.6% of all human deaths globally, according to the world cancer report in 2014. In the United States, 18,860 projected new cases and 10,460 deaths were reported in 2014 for acute myeloid leukemia—the most common form of leukemia. Although most of the global research is focused on developing therapeutics for cancers and cancer-related diseases, the successful treatment of cancer has been limited—due primarily to the emergence of resistance that leads to recurrence of a more aggressive form of the disease. As a result, there is an urgent need to reconsider current strategies of treatment and investigate the mechanisms of resistance.

This volume focuses on mechanisms of resistance and strategies to improve targeted therapy using immunotoxins and related therapies. The development of recombinant immunotoxins, using toxins as killing moieties conjugated with antibodies or ligands against cancer cell surface proteins, met the criteria of improving specificity and the cytotoxicity. Immunotoxin therapy, due to its specificity, is one of the major strategies used in targeted therapy that has shown promise in clinical trials. The toxins used are usually derived from bacterial or plant toxins, which are highly immunogenic. To reduce immunogenicity, genetically engineered versions were made by either silencing immunogenic epitopes or developing humanized versions of immunotoxins. However, the emergence of resistance and failure of the immunotoxins in many clinical trials have raised concerns about their utility, although immunotoxin is still the most promising approach used so far. The targeted therapeutic approach has been an attractive alternative in contrast to conventional treatment modalities. The development of monoclonal antibodies to specific surface targets on cancer cells led to the exponential growth of targeted therapy for better efficacy; this is evident from the fact that several monoclonal antibody-based therapies have been approved by the FDA. (These antibodies were later modified into single chain and recombinant versions.) Further, to minimize immunogenicity, humanized monoclonal antibodies were developed and, to improve activity, antibodies were conjugated with drugs or protein toxins. However, the problems of immunogenicity, specificity, and toxicity have yet to be completely eliminated.

Chapter 1 describes the history, construction, and types of immunotoxins developed to date. Chapter 2 presents a comprehensive view of resistance to cancers, different mechanisms involved in immunotoxin resistance, the role of cancer stem cells in resistance, and underlying mechanisms; it also presents future perspectives about strategies that could be used to target resistant cancer stem cells. Chapter 3 describes the factors associated with sensitivity and potential resistance of cancer cells to pseudomonas exotoxin-derived immunotoxins. Chapter 4 provides an overview of signaling pathways involved in resistance. Chapter 5 focuses on the treatment of hematologic neoplasms using antibody-drug conjugates, and immunotoxins. Chapter 6 discusses the challenges involved in pseudomonas exotoxin-based immunotoxins. Chapter 7 deals with resistance to the antibody-drug conjugate gentuzumab ozogamicin, while Chapter 8 discusses overcoming resistance to apoptosis using engineered human Granzyme B and Angiogenin. Chapter 9 presents immune responses in cancer and their therapeutic impact.

Resistance to Immunotoxins in Cancer Therapy, which includes reports about and reviews of cancer resistance, molecular and cellular mechanisms involved in resistance to targeted therapy, and methods to overcome resistance, is a comprehensive guide to the complete understanding of resistance to immunotoxins.

Rama Shanker Verma, Ph.D.

About the Editor

Rama Shanker Verma is Professor, Indian Institute of Technology, Department of Biotechnology, Chennai, India. He is also Visiting Professor, Pondicherry University, Department of Biochemistry and Molecular Biology, and Visiting Professor, University of Madras. His awards and fellowships include a UNESCO fellowship and the Genomic Pioneer Recognition Award from HUGO. Professor Verma was recently elected Fellow, AMI, and is Fellow, Indian Academy of Science, India.

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Chapter 1
Targeted Cancer Therapy: History and Development of Immunotoxins

Sudarshan Gadadhar and Anjali A. Karande

Abstract Though a number of new drugs have been and are being formulated to treat cancer, newer therapeutic approaches are needed due to increased instances of drug resistance and toxic side effects. One relatively new approach for treatment is immunotherapy, using antibodies or ligands to cell surface molecules as vehicles to deliver toxins to specific cells, thus increasing the efficacy of the treatment by several folds. Such conjugates, of antibodies and toxins termed 'immunotoxins' are generated either as chemical conjugates using hetero-bifunctional cross-linkers that link the antibody to the toxin, or as 'fusion proteins', wherein, the gene for the antibody and the gene for the toxin are cloned together as one construct into bacterial expression systems and expressed as recombinant proteins. Several toxins, mainly the inhibitors of translation, are being explored for preparing immunotoxins. This chapter provides an overview of the treatment modalities adopted to date for the cancer management and introduces the field of immunotherapy. The chapter also focuses on the different toxins used in generating immunotoxins and on those that have made it to clinical trials.

Keywords Immunotherapy · Immunotoxins · Antibodies · Targeted therapy · Ribosome · Inactivating proteins · Toxins

Abbreviations

5-FU	5-fluorouracil
ADCC	Antibody-dependent cellular cytotoxicity
bsAb	Bispecific antibody
BiTE	Bispecific T cell engager
CT	Cancer-testis
CEA	Carcinoembryonic antigen
CLL	Chronic lymphocytic leukemia

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CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CDR	Complementarity determining region
CTCL	Cutaneous T-cell lymphoma
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DT	Diphtheria toxin
ER	Endoplasmic reticulum
EPHA3	Ephrin receptor A3
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
ERAD	ER-associated degradation
eEF2	Eukaryotic elongation factor 2
FATE-1	Fetal and adult testis-expressed transcript 1
FAP	Fibroblast activation protein
GBM	Glioblastomamultiformes
GM-CSF	Granulocyte-macrophage colony stimulating factor receptor
HCL	Hairy cell leukemia
HGFR	Hepatocyte growth factor receptor
HER2	Human epidermal growth factor receptor 2
Ig	Immunoglobulin
IT	Immunotoxin
ITAM	Immunotyrosine-activating motif
IGF1R	Insulin-like growth factor 1 receptor
IL-10	Interleukin 10
IL-2R	Interleukin-2 receptor
MHC	Major histocompatibility complex
MART-1	Melanocyte antigen recognized by T-cells 1
MAC	Membrane-attack complex
MMP	Mitochondrial membrane potential
mAb	Monoclonal antibody
NK	Natural killer
NHS	N-hydroxysuccinimide
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate
SMPT	Succinimidyloxycarbonyl-C-methyl-C-(2-pyridyldithio) toluene
PARP	Poly-ADP ribose polymerase
PE	Pseudomonas exotoxin
ROS	Reactive oxygen species
RANKL	Receptor activator of nuclear factor-xB ligand
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RIP	Ribosome inactivating protein
SAGE-1	Sarcoma antigen 1
ST	Shiga toxin

scFv	Single chain Fv
SAPK	Stress Activated Protein Kinase
$(scFv)^2$	Tandem scFv
TRAIL1	TNF-related apoptosis inducing ligand 1
TGF	Transforming growth factor
TEM	Triethylenemaleamine
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VAMP	Vincristine, Amethopterin, 6-mercaptopurine, Prednisone.

1.1 Introduction

Cancer, in basic medical terminology, is termed as a disease of uncontrolled proliferation of cells in an organism, which has deleterious consequences. Malignant cells have the propensity to multiply indefinitely, and also have a high metabolic rate. Another important aspect, attributed to these cells, is the rapid rate of mutations they undergo, thus making it difficult to treat the disease. Irrespective of the type, treatment of cancer is to arrest the growth of tumor cells. Treatment of cancer has been a challenge for ages, especially with respect to the selection destruction of cancer cells, leaving the normal ones unharmed. Towards this goal, the more recently developed immunotoxins appear promising. The following review is a discussion on the development of these molecules as cell targeted therapeutic agents for the treatment of cancer.

1.2 Chemotherapy

Mankind knows Cancer since 460 BC, when Hippocrates gave the name ‘karkinos’ to this disease. Celsus and Leonides, of the Roman period, mentioned the procedure of mastectomy [1]. From the first century AD, a number of medicinal herbs were in use for topical application for the treatment of tumors. During the 1880s, the first few surgeries were performed to remove tumors from patients [2]. Ehrlich, in the early part of 1900s, coined the term chemotherapy, for the treatment of diseases with chemicals [3]. The discovery of Roentgen ray, and the subsequent discovery of radium, in 1896, were a shot in the arm as radiation therapy came into use for the treatment of cancer [2]. Another important milestone was the discovery that hormones could play a key role in suppressing tumor growth [3] and that in animals receiving prolonged treatment of estrogens there was the development of breast tumors [4]. A number of steroid hormones coupled with surgical procedures like oophorectomy were used to control blood cancers and breast cancer [5]. Nutritional research identified that folic acid was important for the proper functioning of the bone marrow and folic acid antagonists e.g., methotrexate [6] and the purine antagonist, 6-mercaptopurine [7, 8] were shown to have anti-leukemic activity [9].

In the mid-1950s, an analogue of the pyrimidine, uracil, called 5-fluorouracil (5-FU), was targeted towards cancer of the liver, based on the observation that rat hepatoma had a higher uptake and use of uracil, as compared to the normal tissue. This was the first account of 'targeted therapy', wherein the biochemical pathway of uptake and utilization of uracil was targeted [10] and was found to be effective for the management of several solid tumors [11]. The next generation of anti-cancer drugs was antibiotics, like actinomycin, mitomycin, doxorubicin and bleomycin [12, 13]. In the early 1960s, alkaloids from a plant, *Vincarosea*, called vincristins and vinblastine, were reported to be mitotic inhibitors [2] and had significant effects on hematological tumors [14]. In the later part of the twentieth century, the first inorganic drug called cisplatin was reported [15] and clinically tried as an anti-cancer agent [2].

Several drugs were tested for their anti-tumor efficacy but as most of them failed to cure, combination chemotherapy with a number of combination drugs like VAMP (vincristine, amethopterin, 6-mercaptopurine and prednisone) were used [3]. Each of these had a different mechanism of action and owing to the combination of different drugs the concentration of each of these was brought down drastically. The results showed that remissions lasted for long periods [3].

Research was later focused on understanding the signaling pathways involved in tumorigenesis [16]. Pioneering work in this regard was the development of the Bcr-Abl tyrosine kinase inhibitor, Imatinib [17], for the treatment of chronic myelocytic leukemia. Imatinib fits into the ATP-binding pocket of the aberrant kinase leading to its inhibition. The success of imatinib led to the development of small molecule drugs to target specific proteins, like kinases and eight such agents have been approved by the U.S. Food and Drug Administration (FDA), for the treatment of diseases that were effectively resistant to chemotherapy [18].

Another promising field of research is the use of cancer vaccines, with the ultimate goal of eliciting a potent immune response that can eradicate tumor and also provide a long-term anti-tumor memory to the immune system.

1.3 Cancer Immunotherapy

The advent of monoclonal antibodies, in 1975, enhanced the effects of chemotherapy and were proven to be useful for clinical use in the mid-1990 [3]. The ability of antibodies to target specific antigens on the tumor cells makes them more specific than conventional chemotherapeutic drugs. Many drugs like trastuzumab, cetuximab and bevacizumab have antibodies as an integral part. The use of antibodies in cancer therapy is discussed below.

1.3.1 Antibodies in Cancer Therapy

Although tumor cells are distinct from normal cells in a number of aspects, the immune system recognizes them as self, owing to a number of similar proteins

they express and hence, any immune response that has been induced against them is not enough to eradicate them [19]. Several key factors could also play a role in the failure of our immune system from eliciting an effective response against a tumor. However, the belief in tumor immunology was resurrected, with the advent of hybridoma technology in 1975, by George Köhler and César Milstein [20]. They described an elegant method of obtaining pure antibodies, of pre-determined specificities, on a large scale, which can be used for various purposes in Biology and Medicine. During this time, a number of tumor antigens were being discovered, paving the way for the use of antibodies for therapy of cancer and leading to a scientific revolution in the field of tumor immunotherapy.

1.3.2 Tumor Antigens

The selection of the antigen, for the synthesis of antibodies, was the first hurdle in the use of antibodies for immunotherapy. A tumor-associated antigen should be abundant and accessible to the antibody, as well as be expressed homogenously, consistently and exclusively on cancer cells. [21]. For antibody-based therapy, the secretion of a chosen antigen should be minimum, so that the effective concentration of antibodies binding to the surface molecule is not reduced by the circulating soluble antigen [21].

Research on understanding the antigens expressed by tumors led to the discovery of a number of tumor-associated antigens that are potential targets for antibody-therapy. Some of the well-known ones are: *Haematopoetic differentiation antigens*: for e.g., CD19, CD20, CD30, CD33 and CD52 [22]; *Growth and differentiation antigens*: for e.g., receptor tyrosine protein kinase (ERBB3), the human epidermal growth factor receptor 2 (HER2,) etc., *Antigens in angiogenesis*: e.g., vascular endothelial growth factor (VEGF), integrin CV03 and integrin C501[23], and many others.

1.3.3 Antibodies for the Clinic

Identification of tumor antigens paved the way to generate a number of antibodies that would target these antigens. The first treatment of cancer with monoclonal antibody was by Nadler, in 1980, where he treated a patient with non-Hodgkin's lymphoma with an antibody designated AB 89. The treatment failed to rescue the patient but it indicated the possibility of an antibody capable of arresting the growth of a tumor [24]. Following this, there was a revolution in the field of monoclonal antibodies used for cancer therapeutics but met with failures. The major reason being that most of the antibodies were murine in origin, which elicited an immune reaction, leading to adverse effects [24]. Murine antibodies were then 'humanized'. The first such modified antibody to be successfully assayed for its ability to arrest tumor growth was the chimeric antibody targeting CD20 in non-Hodgkin's lymphoma patients, called Rituximab or Rituxan, in treating lymphomas [24], leukemia

and non-Hodgkin's lymphoma [25]. Cetuximab, a chimeric EGFR specific IgG1, functions by preventing ligand binding and receptor dimerization. Cetuximab is also used in combination chemotherapy along with folinic acid, 5-fluorouracil and irinotecan, collectively called FOLFIRI chemotherapy. This treatment has been shown to prolong the survival of patients with metastatic colon cancer, especially of those who harbor the wild type *KRAS* gene [26]. One of the most exciting targets for antibody-based cancer therapy is HER2. This proto-oncogene is expressed in 20–30% of breast and other carcinomas [27]. In contrast to EGFR, HER2 has no known ligand binding to it. The first antibody targeting HER2 was Trastuzumab, also called Herceptin, used for the treatment of invasive breast cancer that over-expresses *HER2.* Monotherapy with trastuzumab exhibited 35% response by those metastatic breast cancers that were not exposed to chemotherapy [28]. It acts by preventing receptor dimerization, endocytic destruction of the receptor and also, immune activation [29]. Another antibody, called pertuzumab, also targets HER2, but its epitope is different from that of Trastuzumab. It affects the dimerization of the receptor and has shown considerable success in breast cancer [30]. Both of these antibodies targeting HER2 are used in combination with chemotherapy.

Vascular endothelial growth factors (VEGFs) are essential for stimulation of angiogenesis and tumor vasculature. Humanized monoclonal antibodies, namely Bevacizumab (35) and Ramucirumab (36), which block the binding of VEGF to its receptor, VEGFR, on the vascular endothelium have shown therapeutic benefit in pre-clinical and clinical studies [31].

CD52 is over-expressed in patients with chronic lymphocytic leukemia and Alemtuzumab, a humanized anti-CD52 antibody is proving to be successful for use in therapy of patients with chronic lymphocytic leukemia [24].

Table 1.1 summarizes the different antibodies developed to target the immune cells, and their effects on these cells. It is pertinent to mention here that since 1997, twelve antibodies, summarized in Table 1.2, have received approval from the U.S. FDA for the treatment of various solid tumors and hematological malignancies.

1.3.4 Antibodies as Carriers

Antibodies were considered to be 'magic bullets' for treating cancer, but their inability to effectively curb the disease led scientists to consider the possibility of using them as conjugated agents, instead of unconjugated mAbs. One major consideration was to conjugate antibodies with radionuclides, a technique called radioimmunotherapy (RIT) [32]. The cytotoxic ability of radioisotopes like iodine-125, iodine-131, yttrium-80 and bismuth-213 was coupled with antibodies to target specific sites. One successful isotope is radioiodine, ^{131}I, which is a beta-emitter and can induce cell death in any tissue it is administered to. Owing to its high potential to undergo nuclear fission, releasing free radicals, and its stability compared to other radioisotopes of iodine, it has been used to generate a number of radiolabeled antibodies for cancer therapy.^{131}I-Tositumomab, which targets CD20 on lymphomas,

Table 1.1 Antibodies targeting molecules expressed on immune cells: Adapted from [26]

Generic name (Trade name)	Target antigen	Target expression	Antigen function	Effect of antibody on tumor cells
Dacetuzumab (SGN-40)	CD40	DCs, B cells, monocytes and macrophages	DC maturation germinal center formation, Ig-isotype switching and affinity maturation	Apoptosis in some tumors and increased tumor specific CD8+T cells
Tremelimumab (CP-675,206)	CTLA4	Activated T cells	Inhibition of T cell proliferation	Tumor rejection, protection from re-challenge, enhanced tumor specific T-cell response
CT-011	PD1	Activated lymphocytes	Negative regulator of lymphocyte proliferation and cytokine production	Maintenance and expansion of tumor specific memory T cells and NK cell activation
BMS-663513	CD137	Activated T cells, Treg cells, NK cells, NKT cells, DCs, neutrophils and monocytes	Expansion of T cells,CD8+ T cell survival, NK cell proliferation and IFNγ production	Regression of established tumors and expansion of CD8+T cells
Daclizumab (Zenapax)	CD25	Activated T cells	Promotes T cell proliferation. Expression is high in Tregcells	Transient depletion of CD25+ Treg cells Enhanced tumor regression Increase number of effector T cells

has been approved by the FDA for clinical therapy. Another isotope that has seen considerable success in tumor radioimmunotherapy is Yttrium-90 (^{90}Y). It is also a β-emitter and compared to ^{131}I, which yields a dose rate of 5 rad/h, ^{90}Y yields 15 rad/h. In the case of Hodgkin's lymphoma patients, who had failed to respond to chemotherapy, when administered with a single dose of 30 millicurie (mCi) ^{90}Y-antiferretin, complete remission of the lymphoma was seen [33]. The current FDA approved radiolabeled antibody for clinical therapy of cancer is ^{90}Y-labeled Ibritumomab which targets CD20 and has shown considerable success in the treatment of non-Hodgkin's lymphoma [34]. Another isotope, studied extensively in the production of radiolabeled antibodies, is Bismuth-213 (^{213}B) which is an α-particle emitter and has been tagged to various antibodies [32].

Despite their high cytotoxic ability, radioisotopes are not considered potent therapeutic agents for cancer therapy, as they have severe side effects, with some of them being potential carcinogens themselves. Also, the non-tumor bound isotopes accumulate in the liver and spleen, resulting in hepatotoxicity as well as increased

Table 1.2 Therapeutic antibodies approved by FDA for use in cancer immunotherapy: Adapted from [26]

Generic name	Trade name	Target antigen	Antibody format	Target cancer
Rituximab	Rituxan	CD20	Chimeric IgG1	Non-Hodgkin lymphoma
Trastuzumab	Herceptin	HER2	Humanized IgG1	Breast cancer
Alemtuzumab	Campath	CD52	Humanized IgG1	Chronic lymphocytic leukemia
Cetuximab	Erbitux	EGFR	Chimeric IgG1	Colorectal cancer
Bevacizumab	Avastin	VEGFA	Humanized IgG1	Colorectal, breast and lung cancer
Panitumumab	Vectibix	EGFR	Human IgG2	Colorectal cancer
Ofatumumab	Arzerra	CD20	Human IgG1	Chronic lymphocytic leukemia

hematologic toxicity [33]. Another major setback for this mode of treatment is the rapid de-halogenation of labeled antibodies, in vivo, within 24 h of treatment, which leads to a lack of effective concentration of the radioisotope at the tumor site.

Owing to several drawbacks of radioimmunotherapy, other potential agents were explored for use in targeted cancer therapy, the most potent ones being, toxins of bacteria and plants. This led to the development of a new field of cancer therapeutics, called 'Immunotoxins' where antibodies act as carriers for toxins that inhibit important cellular processes like protein synthesis, leading to cell death. The next section focuses on the development of immunotoxins and their applications in targeted cancer therapy.

1.4 Immunotoxins in Cancer Therapy

The ineffectiveness of antibodies alone as therapeutic agents led to their use as targeting moieties for the delivery of radioisotopes or chemical drugs used widely for the treatment of cancer. Since anti-cancer drugs act stoichiometrically, the number of anti-cancer drug molecules delivered to the tumor cells might be lower than the threshold levels required [35]. The use of toxins or subunits of toxins then emerged. Owing to their catalytic efficiency, toxins would be much more effective and would have lower threshold levels than the conventional anti-cancer drugs [35]. A new approach in the field of cancer therapeutics, referred to as 'IMMUNOTOXINS' was described.

Immunotoxins (ITs) consist predominantly of two components: (1) catalytically active, highly toxic proteins that enzymatically inhibit protein synthesis, either by inactivating the eukaryotic elongation factor 2 (eEF2), or by destabilizing the 60S ribosomal subunit, leading to the loss of binding of the eEF2 to the elongation site on the ribosome [36] and (2) antibodies to receptors on tumor cells or cytokines or, hormones targeting their cognate receptors, which act as vehicles to deliver these

potent toxins to the tumor. Immunotoxins are more potent than other antibody-drug conjugates as they act catalytically (reacting repeatedly with multiple targets) rather than stoichiometrically (reacting once with a single intracellular target) [37].

1.4.1 Targeting Moiety

The targeting moiety of the ITs is the vehicle that delivers the toxin to the tumor. Targeting agents used currently are monoclonal antibodies, growth factors or cytokines. The antibodies used are those that recognize differentiating antigens on the surface of tumor cells or tumor associated antigens, as tumor-specific antigens are few [38]. Although the targeted tumor-associated antigens are expressed on normal cells of the body as well, the expression is generally many-fold higher on tumor cells, than normal cells, resulting in a preferential killing [39]. Some of the antigens targeted extensively by mAb-based ITs include the receptors CD3, CD5, CD7, CD19, CD20, CD22, CD33, on hematological cells [37] and carcinoembryonic antigen (CEA), EGFR, epithelial cell adhesion molecule (EpCAM) and HER2 which are expressed on solid tumors [40]. Even certain carbohydrate antigens like the gangliosides GM2, GD2 and GD3, $Lewis^Y$ and globo-H, which are neutral glycolipids and glycoproteins like Tn, Tissue factor (TF, also called platelet tissue factor) and sialylatedTn (sTn) are over-expressed in a variety of cancers and are potential targets for cancer therapy [41]. Of the different classes of antibodies present, the preferred choice for the construction of an immunotoxin generally is the IgG. Although IgM has many more antigen binding sites, it tends to aggregate and it also has a poorer tissue penetration [42]. Not only whole antibodies, but the Fab fragment is also used for the production of immunotoxins. The Fab region, obtained by limited proteolysis of an antibody by pepsin, consists of bivalent $(Fab')^2$ fragments, which upon reduction yields two free Fab' regions, with a free -SH group that is used for the construction of the IT. Currently, antibodies are engineered in a number of different ways to enable efficient internalization and greater retention within the body. Figure 1.1 depicts the structures of the different antibody carriers that are being used for the generation of ITs.

Another potential targeting molecule for the construction of immunotoxins is the cytokine. But since antibody is the predominant vehicle used, the conjugates of toxins with cytokines and growth factors are also placed under the same family of proteins, immunotoxins [38].Cytokines are effective targeting agents as their affinity to their ligands is many fold higher than that of typical antibodies [43]. Once bound to their receptors, the cytokine-based IT is effectively internalized via receptor-mediated endocytosis. Further, cytokine receptors are often well modulated during growth and differentiation and enable targeting a specific population of cells effectively [44]. Some of the cytokine receptors that have been targeted by cytokine-based ITs include receptors for interleukin-2 (IL-2R), interleukin-4 (IL-4R) and interleukin-6 (IL-6R) [45]. Apart from cytokines, certain growth factors like epidermal growth factor (EGF), transforming growth factor-α (TGF-α) can also be used to generate immunotoxins, taking care that the growth factor does not retain its agonistic effect, which would lead to proliferation of the target cells [39].

1.4.2 Toxins in Cancer Immunotherapy

The construction of an immunotoxin requires two basic building blocks: a targeting moiety and a toxic moiety. The toxic moieties, used currently, belong to the class of proteins, called protein synthesis inhibitors. These are further divided into inhibitors of translation and ribosome inactivating proteins (RIPs). Though knowledge of these toxins dates back to 1887, interest in these toxins was revived only in the 1960s when Lin et al. showed that tumor cells were more susceptible to the toxicity of these proteins than normal cells [46]. This was followed by extensive research to identify and understand new toxins derived from both, plants and microbial sources.

Translation inhibitors are predominantly bacterial toxins, like the exotoxin from *Pseudomonas aeruginosa* and the toxin from *Corynebacterium diphtheriae*. These are single chain toxins, with different binding, translocation and catalytic domains [42] (Fig. 1.2a).

Ribosome inactivating proteins are proteins mostly from various tissues of angiospermic plants. Apart from plants like *Ricinuscommunis* (ricin) and *Abrusprecatorius* (abrin), certain bacteria like *Pseudomonas aeruginosa*,

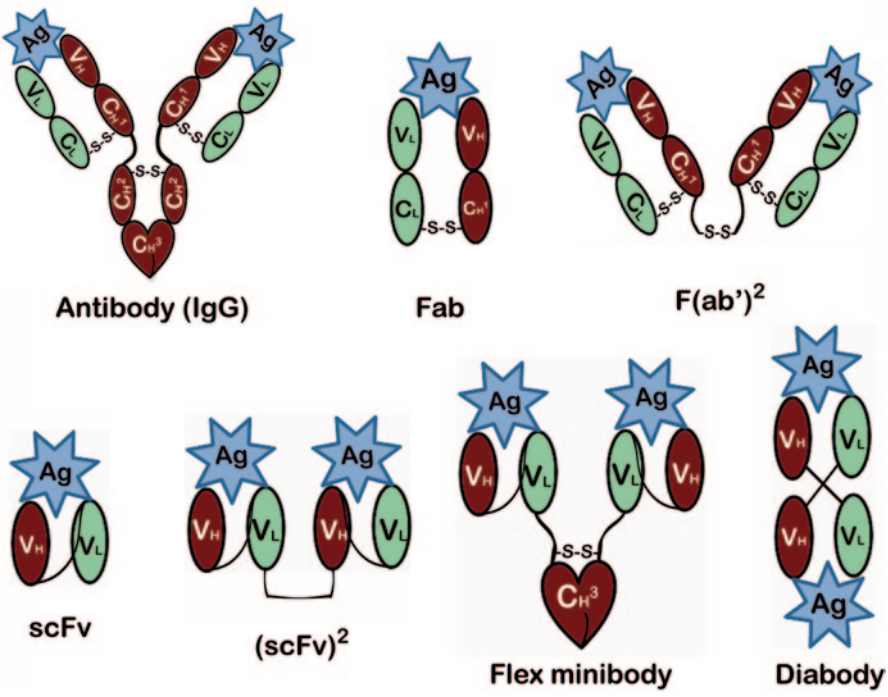

Fig. 1.1 Antibody-based targeting proteins: Different antibody-based molecules, produced by enzymatic digestion of intact antibodies, or using molecular engineering techniques, as recombinant proteins, are used to enhance the penetration of antibodies into solid tumors. IgG: Intact immunoglobulin; scFv and $(scFv)_2$: single chain Fv region of both heavy and light chains, linked by a linker, obtained as recombinant protein; Fab and $F(ab')_2$: Proteolytically cleaved antibody

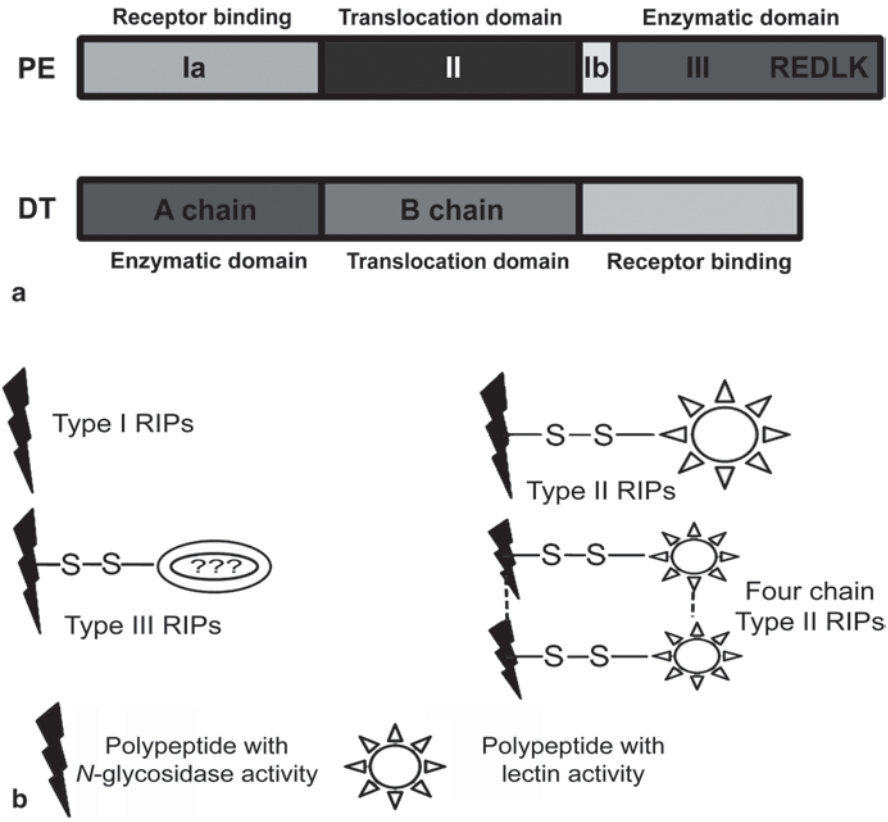

Fig. 1.2 Different types of toxins: **a** Bacterial toxins: The schematic representation depicting the different domains of the bacterial toxins, *Pseudomonas* enterotoxin and diphtheria toxin. **b** Different classes of RIPs: Diagrammatic representation of the different classes of ribosome inactivating proteins

Corynebacteriumdiphtheriae, *Shigelladysentriae* and certain fungi like *Aspergillusgiganteus*, *Aspergillusrestrictus* also produce protein synthesis inhibitors, which are as potent as the plant toxins [38]. Most of the toxins produced by plants are glycoproteins that inhibit translation in eukaryotic cells. Ribosome inactivating proteins are a large family of proteins classified into different types based on their polypeptide organization. They are broadly classified into three types [47] (Fig. 1.2b):

I. **Type I RIPs:** Proteins belonging to this class consist of a single subunit with RNA-N-glycosidase activity. Examples include saporin (*Saponariaofficinalis*), momordin (*Momordicacharantia*), pokeweed antiviral protein (PAP) (*Phytolaccaamericana*), and maize RIP (*Zea mays*) among others. Fungal toxins like α-sarcin (*Aspergillusgiganteus*) and restrictocin (*Aspergillusrestrictus*) also belong to type I RIPs.

II. **Type II RIPs:** Proteins of this group consist of an enzymatic A (Active) chain with RNA-*N*-glycosidase activity, and a B (Binding) chain with lectin activity specific to galactose. The two subunits are linked by a disulfide bond. Examples of type II RIPs include ricin (*Ricinuscommunis*), abrin (*Abrusprecatorius*), mistletoe lectin I (*Viscum album*), modeccin (*Adeniadigitata*), and volkensin (*Adeniavolkensii*) from plant sources. Bacterial toxins like Shiga toxin from *Shigelladysentriae*also belongs to this group of RIPs. Though most of the Type II RIPs are dimers, with one A and one B chain, some RIPs are tetramers with 2 dimers of the heterodimers linked by either non-covalent interactions or by disulfide bonds. Hemagglutinins like *Ricinuscommunis* agglutinin and *Abrus-precatorius* agglutinin also belong to the same family.

III. **Type III RIPs:** Reinbothe et al [48] demonstrated that some RIPs like the JIP60, obtained from barley (*Hordeumvulgare*), had an A chain similar to the Type I RIPs but bound to a C-terminal domain of a protein of unknown function. Proteins like JIP60 were thus classified as type III RIPs.

Among the different classes of RIPs, the type II RIPs are the more potent owing to the lectin-binding B chain, which binds to galactose on the cell surface glycoproteins and glycolipids, leading to the internalization of the protein. The rest of the discussion will focus mainly on the type II RIPs.

Synthesis and Storage of Type II RIPs Most RIPs are encoded by small multi-gene families devoid of any introns. Translation of the exons leads to the synthesis of a pre-pro form that is cleaved by a protease to yield the active form. In plants, RIPs are synthesized as the inactive pre-proform with the N-terminal signal sequence and a linker peptide between the A chain and the B chain [49]. The signal sequence mediates the co-translational translocation of the polypeptide into the endoplasmic reticulum (ER), where the signal sequence is cleaved. Then, the pro-RIPs are glycosylated and the intra-and inter-chain disulfide bonds are established. Between the A and B chains, in the pro-RIPs, there exists a linker peptide of 12–25 amino acids that targets these pro-RIPs into vacuoles (Fig. 1.3), also called protein bodies, where the mature, but inactive form of RIPs is stored.

Only when the toxins reach the target tissue are the two chains separated and the active form of RIP is released into the cytosol to carry out its activity [36, 50]. Different plants store RIPs in different organelles [36]. Table 1.3 lists out some of the RIPs and their organelles of storage.

Cytotoxic Pathway of RIPs The cytotoxic pathway of RIPs is well established. Type I RIPs are endocytosed predominantly by pinocytosis as they lack the cell-surface binding polypeptide in them. Bacterial toxins like *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT), have a separate binding domain in their polypeptide structure [51] which enables their binding and internalization into the cells.

Type II RIPs bind to the cell surface by the lectin activity of the B chain. The B chain has specificity to the terminal galactose and, hence, can bind to any cell surface receptor-bearing terminal galactose residues. Once bound, the protein is internalized by receptor-mediated endocytosis [36, 52, 53]. Endosomes carrying

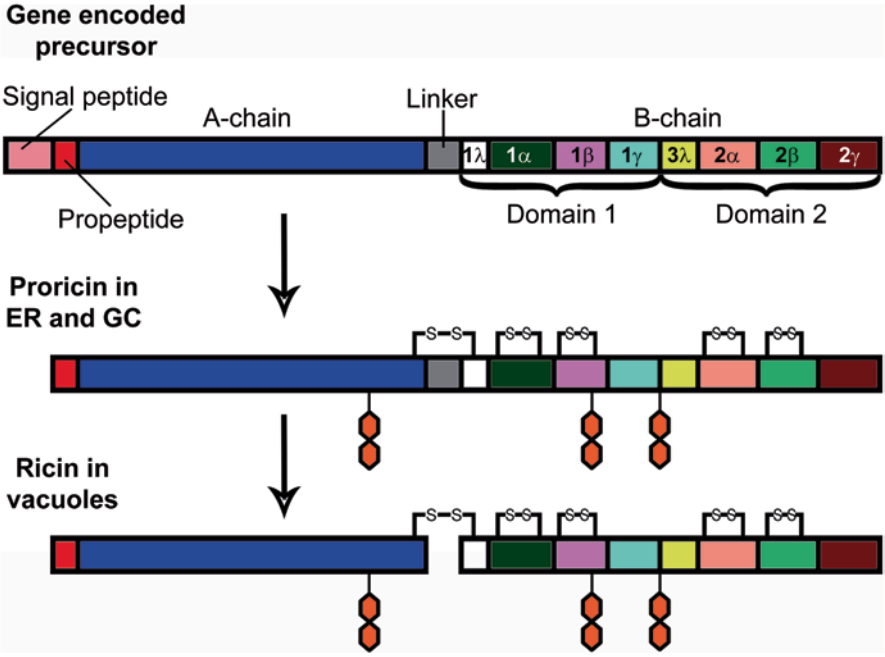

Fig. 1.3 Synthesis of Ricin: Ricin, a type II RIP, is synthesized and stored in the seeds of the plant *Ricinus communis*. The signal peptide directs co-translational translocation of the protein in the ER. This is cleaved in the ER to give a pre-pro form of ricin, which is targeted to the Golgi, where both the A and the B chains are glycosylated. Once glycosylation and the intra-and inter-chain disulfide bonds are developed, the pro-ricin is targeted to the storage vesicles by the linker peptide, of 12–25 amino acids, bearing the sequence LIRP. In the storage vesicles, this linker is cleaved and the mature ricin is stored

the toxins are targeted to either the lysosomes or the *trans*-Golgi network (TGN) [52]. Thereafter, the proteins are targeted to the endoplasmic reticulum (ER) via the COP I vesicles, a process referred to as the retrograde transport pathway. The mechanism of the retrograde transport differs for different RIPs [52, 53]. Translation inhibitors like *Pseudomonas* exotoxin, cholera toxin and *E.coli* heat labile toxin bear a C-terminal KDEL or related sequence, which targets them to the ER [52]. Toxins like ricin, Shiga toxin and absin the do not harbor the C-terminal KDEL or a related peptide translocate to the ER by binding to a recycling glycoprotein or other ER-targeted proteins like calreticulin, which bears the KDEL sequence, via the galactose specificity of the B chain [53]. Once the toxin reaches the ER, the protein disulfide isomerase (PDI) family of proteins reduces the disulfide bond between the two polypeptides, the A and the B chains. The A chain is then released into the cytosol through the ER-associated degradation (ERAD) pathway, where the A chain reaches the 60S ribosomal subunit and cleaves the *N*-glycosidic bond between the adenine and its sugar, leading to inhibition of translation [36].

Table 1.3 Storage of RIPs in plants

Plant genus	RIP	Storage
Type I RIPs		
Asparagus officinalis	Asparin 1 and 2	Seeds
Saponariaofficinalis	Saporin -L1, L2 Saporin -R1, R2, R3 Saporin – S5, S6, S8, S9	Leaves Roots Seeds
Momordicacharantia	Momordin I and II	Seeds
Geloniummultifi orum	Gelonin	Seeds
Phytolaccaamericana	PAP, PAP II PAP–S PAP-C	Leaves Seeds Culture
Type II RIPs		
Ricinuscommunis	Ricin D, E and Ricinuscommunisagglutinin (RCA)	Seeds
Abrusprecatorius	Abrin a, Abrin b, Abrin c, Abrin d and Abrusprecatoriusagglutinin (APA)	Seeds
Adeniadigitata	Modeccin, Modeccin 6B	Roots
Sambucusebulus	Ebulin I	Leaves
Viscum album	Viscumin	Leaves

Biological Activity of RIPs Protein synthesis inhibitors are broadly classified into two groups, based on their mode of action: inhibitors of translation and in activators of ribosomes [38]. Of all the different types of RIPs classified, the type II family of RIPs are the most potent toxins known to date; one molecule is capable of depurinating about 1500 ribosomes per minute [36, 50]. Table 1.4 below indicates the toxins which have been studied extensively.

Table 1.4 Classification of toxins based on their mode of action

Toxin	Source	Poylpeptide
RNA N-glycosidase of 28S rRNA		
Abrin	Plant	Two chain
Ricin	Plant	Two chain
Modeccin	Plant	Two chain
Saporin	Plant	Single chain
Momordin	Plant	Single chain
α-sarcin	Fungi	Single chain
Restrictocin	Fungi	Single chain
Shiga toxin	Bacteria	Two chain
ADP ribosylation of eEF-2		
Pseudomonas exotoxin	Bacteria	Single chain
Diphtheria toxin	Bacteria	Single chain

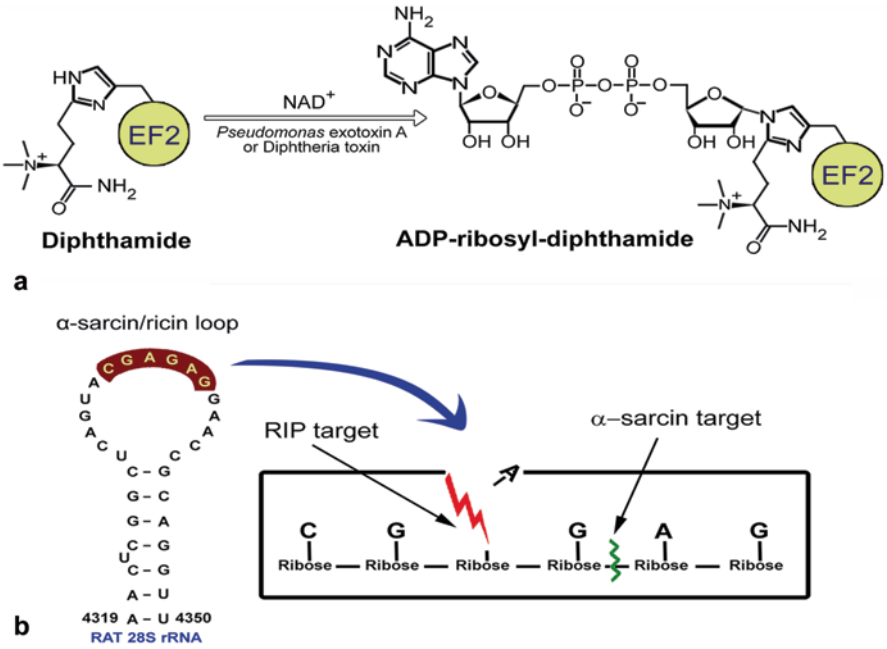

Fig. 1.4 Mechanism of action of proteins that inhibit translation **a** Inhibitors of translation: like PE and DT bring about their cytotoxic effect by ADP ribosylation of the modified diphthamide residue of eEF2. They use the cellular store of $NAD^+$for the substrate ADP that is required. This ADP-ribosyl-diphthamide will become incapable of binding to the elongation site of the ribosome, thus stalling protein synthesis. **b** Ribosome inactivating proteins: like ricin and saporin inhibit protein synthesis by cleaving the glycosidic bond between an adenine at position 4324, on the α-sarcin/ricin loop, and its ribose sugar, on the 28S rRNA of the 60S ribosomal subunit. Proteins like α-sarcin, on the other hand, cleave the link between the ribose sugar bound to guanine 4325 and its subsequent sugar. This destabilizes the 60S ribosomal subunit and prevents its binding to the eEF2, shutting down the protein synthesis machinery irreversibly

Diphtheria toxin and *Pseudomonas* exotoxin inhibit translation by ADP-ribosylation of the eukaryotic elongation factor-2 (eEF2). This blocks the binding of eEF2 to the ribosome, thereby stalling protein synthesis [51, 54]. Ribosome inactivating proteins, on the other hand, bind to the 28S rRNA of the 60S ribosomal subunit [36, 50, 55, 56] that results in the cleavage of the *N*-glycosidic bond between an adenine at position 4324 and its ribose sugar in the α-sarcin/ricin loop of the RNA [57]. This leads to destabilization of the RNA and, in turn, the ribosomal subunit which disables the binding of the 60S subunit to eEF2 and, thus, inhibition of translation [50, 58]. Both the irreversible mechanisms are unique as can be seen from Fig. 1.4.

Most of the RIPs are inactive on prokaryotic ribosomes, whereas they are very potent on eukaryotic ribosomes. This might be because of the complexity of the interaction between the RIP and the ribosomes, which is a much more intricate mechanism than just the recognition of a primary RNA structure. It is reported that the

toxicity of the RIPs is aided by their interaction with the ribosomal proteins. The ribosomal proteins may directly interact with the RIPs or maintain the conformation of the 28S rRNA such that it is easily accessible to the RIP, leading to the cleavage of the specific adenine residue [55]. During the late 1980s, it was observed that the A chain of most RIPs was able to depurinate other rRNAs as well when they were free from the ribosomes, though the concentration of protein required for this is much higher than the concentration required to inhibit protein synthesis in eukaryotic cells.

One of the major effects of inhibition of protein synthesis in any cell is the stress leading to cell death. It was initially believed that inhibition of protein synthesis by RIPs led to necrotic cell death because of loss of protein synthesis. But on analysis of the cell morphology, it was observed that cells undergo apoptosis [59]. It has been well documented that most of the RIPs like Shiga toxin 1, ricin, abrin, mistletoe lectin, saporin etc., trigger apoptosis in various cells and cell lines [60, 61]. RIPs induce apoptosis in eukaryotic cells via the intrinsic pathway, involving the activation of caspase-9 by the release of cytochrome c by the mitochondria [62]. RIPs cause apoptosis in cells mainly by inducing ribotoxic stress that triggers a cascade of signals [63]. The cascades include:

- *Ribotoxic stress response mediated cell death*: RIPs like Ricin and α-sarcin were tested on cell lines inducing apoptosis through Stress Activated Protein Kinase (SAPK/JNK)-mediated response [63]. Toxins like Shiga toxin are reported to activate p38 MAP kinase and JNK leading to apoptosis [64].
- *Stress induced mitochondrial pathway*: RIPs like abrin were shown to induce apoptosis in cells by bringing about perturbation of their mitochondrial membrane potential (MMP), which leads to the release of cytochrome c, thereby leading to apoptosis mediated via caspase 9 [65]. Toxins like ST induce stress, which might lead to an increase in the intracellular calcium levels, thus increase in ROS, leading to cell death [66].
- *Regulation of anti-apoptotic/pro-apoptotic factors*: RIPs like Shiga toxin can induce apoptosis by inhibiting the synthesis of anti-apoptotic factors like Mcl1 [67] and also by upregulation of pro-apoptotic proteins like Bax [68].
- *Apoptosis induction due to NAD + and ATP downregulation*: RIPs not only induce apoptosis by inhibiting protein synthesis, but also by decreasing the levels of critical components of the cell metabolism. Two major components controlled are NAD + and ATP. On treating monocytic cells with ricin, poly-ADP ribose polymerase (PARP), a substrate of caspase-3, was upregulated, leading to decreased levels of NAD^+ and thus ATP. PARP is implicated in DNA strand break repair, for which it utilizes NAD^+as its substrate, finally leading to cell apoptosis [69].
- *ER stress and mitochondrial pathway*: Proteins like abrin inhibit translation leading to accumulation of unfolded proteins in the ER. This leads to ER stress, which in turn activates stress kinases like p38 MAP kinases. These in turn can activate caspase-2 and caspase-8-mediated perturbation of the mitochondrial membrane potential, leading to apoptosis [70].

Thus, the signaling pathway activated by individual RIPs might differ in the context of the type of cell and hence, it is cumbersome to delineate the link between protein

synthesis inhibition and apoptosis. It has also been reported that in some cases, rRNA depurination might not be the cause of induction of apoptosis as non-toxic mutants induce apoptosis though they do not depurinate the ribosomal rRNA [71]. Understanding the biological activity of RIPs is important in the context of using these as immunotherapeutic agents, especially in the treatment of cancer, as RIPs are more effective against tumor cells than conventional chemotherapeutic drugs and radioisotopes.

1.5 Construction of an Immunotoxin

The first immunotoxin to be described was by Sponberg et al. in the 1970s [72]. Ever since, a number of researchers across the world have tried and tested various immunotoxins for different cancers, both hematologic and solid tumors. The initial phase of the production of an immunotoxin was focused on the chemical conjugation of an antibody to the toxin using heterobifunctional cross-linkers (Fig. 1.5a) [73]. But the major limitation of using this method was the inability of the large molecule to reach the interiors of a tumor. Hence, most of the conventional chemical conjugates failed in clinical trials. To overcome the limitation, recombinant immunotoxins were generated, wherein the gene for the toxin was cloned with the gene for the Fv region of the antibody, and expressed in *E. coli* as fusion proteins (Fig. 1.5b) Their drawback however, was the decreased half-life of these proteins. Despite their drawbacks, some of the ITs, either as chemical conjugates or as fusion proteins, have had some success in clinical trials and in the future, they may act as potential drugs for targeted cancer therapy.

1.6 Internalization and Cytotoxic Activity of Immunotoxins

Like most of the other molecules, ITs bound to their cognate receptors are internalized by receptor-mediated endocytosis [74] and then targeted to the lysosomes, where they are degraded. However, a few molecules of the ITs escape degradation to reach the cytosol [75], a mechanism that is still not well understood. A recent report on an abrin immunotoxin from our laboratory sheds light on the possible mechanism of trafficking of the immunotoxin, as opposed to its parent toxin [76] (Fig. 1.6).

The active moiety, the toxin, must be released from the conjugate, to be able to exert its cytotoxic effect. The mechanism by which the A chain is released from the conjugate is also not well established, although recent reports, from our group and other groups, have described a role for the thioredoxin system in the cytosol in releasing the A chain from the conjugate by cleaving the disulfide bond between

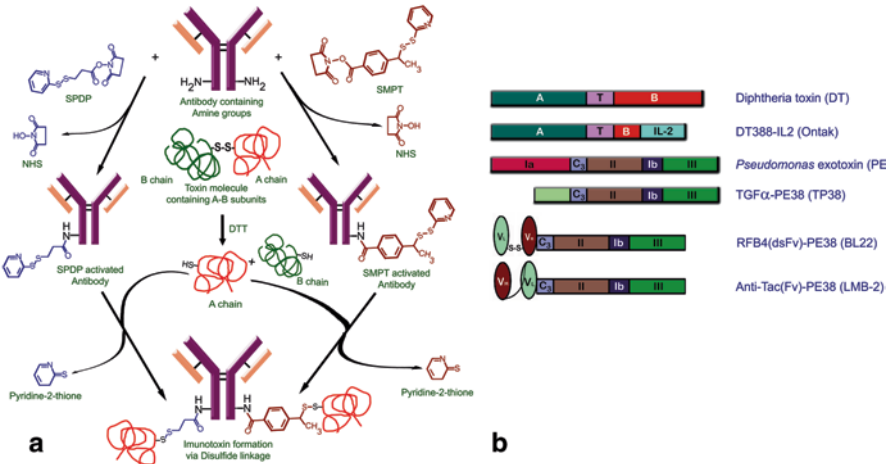

Fig. 1.5 **a** Construction of an immunotoxin by chemical conjugation: Heterobifunctional cross-linkers like SPDP and SMPT are used in the chemical conjugation for an immunotoxin preparation. They have an NHS-ester group at one end and a disulfide group at the other. The antibody used for IT preparation is first activated with the cross-linker, where it binds to the ε-NH_2 group of lysine residues through the NHS ester. The antibody-cross-linker complex is then treated with the A chain, of type II RIP, or single chain RIPs, wherein the toxin binds to the cross-linker via the disulfide linkage, releasing a pyridine-2-thione group to form the immunotoxin. **b** Recombinant immunotoxins: Owing to the ease of production of bacterial toxins, they are used in the construction of recombinant immunotoxins. The coding sequence for the receptor-binding domain of these bacterial toxins is replaced by the genes for antibody scFv or dsFv regions, or the gene for cytokines and hormones like IL-2 and TGF-α. These are then cloned into expression vectors, bacterial cells transformed with these vectors, and expressed as fusion proteins

the cross-linker and the A chain [76, 77]. The cytotoxic activity of ITs is generally much lower than the corresponding native protein. One major factor responsible for this is the effective binding of ITs to the cell surface. Native type II RIPs bind to any glycoprotein or glycolipid that bears a terminal galactose residue, through their B chain. ITs, on the other hand, bind only to those receptors against which the antibodies are raised. Thus, the concentration of ITs binding to the cells is much lower than the native protein. Another factor reducing the activity of ITs is the intracellular degradation of proteins [75]. Thus, for an IT to be successfully used for cancer therapy, it should be cytotoxic but exhibiting less adverse effects.

1.7 Immunotoxins in Clinical Study

Though Sponberg and his group generated the first IT in the early part of the 1970s, it was not until the late 1980s and the 1990s that the technique was accepted and taken up in a large scale by research groups across the world. One of the major reasons for this was the ability to humanize the antibodies. Many groups tried and

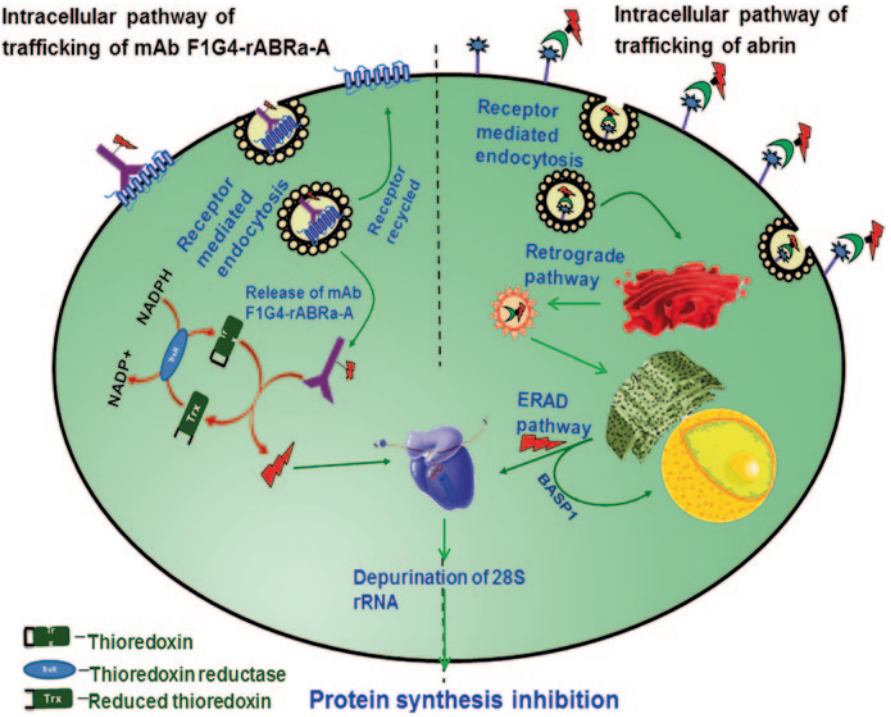

Fig. 1.6 Proposed model for the internalization and cytotoxicity of ITs: The IT binds to the receptor via the antibody. Once bound, it is internalized via receptor-mediated endocytosis through clathrin coated pits. The protein is then released from the vesicles into the cytosol. In the cytosol, the S–S bond between rABRa-A and the cross-linker SMPT is cleaved by thioredoxin, giving rise to free recombinant A chain. The thioredoxin, on the other hand, gets oxidized. This oxidized thioredoxin is reduced back by the enzyme, thioredoxin reductase, using protons donated by cytosolic NADPH, which gets oxidized to NADP+. This overall pathway is different from that observed for abrin, shown in the right half of the figure, wherein the protein, once internalized, follows the retrograde pathway to reach the ER. In the ER, the disulfide bond is cleaved, releasing the A chain to the cytosol through the ERAD pathway. In the cytosol, irrespective of the pathway followed, the A chain binds to the 60S ribosomal subunit, depurinating the 28S rRNA, thus inhibiting translation

tested various antibody-toxin combinations as potential ITs, but many failed to reach clinical trials. This was either due to the lack of proper uptake of the ITs, or due to the increased side effects observed in mice. Most of the immunotoxins that have reached clinical trials for cancer therapy are recombinant immunotoxins, although a few conventional chemical conjugates have also been successful. In the next couple of sections, we discuss some of the key immunotoxins that have successfully reached clinical trials, for the treatment of either hematological malignancies or solid tumors.

Immunotoxins Against Hematologic Malignancies Hematologic malignancies are best suited for treatment with immunotoxins as these cells are in circulation and,

therefore, easily accessible to the intravenously administered drug [51]. In spite of this advantage, only a handful of immunotoxins have been successful in treating these malignancies.

One of the most sought-after antigens for treating hematologic malignancies is the interleukin-2 receptor (IL-2R) and one of the earliest immunotoxins targeting IL-2R was generated using an antibody against its α-subunit, also called CD25, using the deglycosylated ricin A chain. The immunotoxin, RFT5-dgA was marginally successful in the remission of Hodgkin's lymphoma in clinical trials [51]. Later, a recombinant IT using IL-2 fused with a truncated form of DT, DAB486 was constructed, which showed significant effect, in clinical trials, on patients with hematologic malignancies and had significant reduction in the transaminase elevation, which is a hallmark of this disease. To improve the efficacy of DAB486IL-2, a new fusion toxin, wherein, amino acids 389–486 were removed from the DT to generate the toxin, DAB389IL-2, also called denileukindiftitox (or Ontak). DAB389IL-2 had a significantly improved half-life, cytotoxicity and tolerance in animals and was the most effective IT in clinical trials, for the treatment of hematologic malignancies. In the critical Phase III trial, 30% of the patients with advanced cutaneous T-cell lymphoma (CTCL) showed significant remission of the disease, thus being approved by the FDA for the treatment of CTCL [51, 78].

The potency of Ontak is limited because of the low expression of the high-affinity IL-2R on malignant cells. Researchers targeted one of the subunits, CD25, to generate immunotoxins as its levels are significantly elevated in many hematological malignancies. [51, 78]. CD25 is a lymphoid activation marker with high expression in Hodgkin's lymphoma and other hematological malignancies. One IT that seems promising to treat lymphomas with high CD25 expression, is LMB-2 (Anti-Tac(Fv)-PE38KDEL), wherein the Fv region of the mAb to Tac is fused with the truncated form of PE [79], wherein the binding domain of the toxin is removed. The major modification of the toxin here is that the C-terminal REDL sequence of the toxin is replaced with KDEL, to enhance the trafficking of the IT into the ER. Patients with chronic lymphocytic leukemia (CLL) were the most sensitive to LMB-2, in comparison to those with hairy cell leukemia (HCL). The toxin is currently in clinical trials, but results seem to be offset, owing to increased non-specificity due to the KDEL sequence in the toxin.

CD22 expressed on a number of B-cell malignancies is a potential target for ITs. Of the few ITs targeting it, RFB4-dgA was the more successful one in clinical trials [51]. To improve the IT, the Fv regions were connected by a disulfide bond, instead of a linker, to form a dsFv, with the VH being fused to PE38, leading to the formation of the IT, BL22. BL22 is the first completely recombinant immunotoxin in which the disulfide bond forms naturally during renaturation *in vitro* [51]. BL22 is currently in phase II clinical trials, and it is the first agent, since the advent of purine analogs, reported to induce complete remission in patients with HCL [51]. Recently, an IT with an improved mAb portion called HA22 [80] has sidelined clinical trials of BL22. The IT, Moxetumomabpasudotoxis is able to bind CD22 owing to the 3 mutations added in the complementarity-determining regions (CDRs) of the antibody. This enhances its efficacy towards HCL and B-CLL by 50-folds [81].

The IT, currently in Phase II clinical trials, showed complete remission in 46% of the patients with HCL under Phase I of clinical trials, making it a much more potent IT than BL22 [82].

A key component of the T-cell receptor, CD3ε, is overexpressed in a number of T cell NHLs and, hence, is a potent molecule for targeted therapy. One of the most potent DT-based ITs, for treating hematological malignancies, is the bivalent anti-human T cell immunotoxin, A-dmDT390-bisFv (UCHT1) [83, 84]. The IT, a single chain fusion protein consists of the catalytic and translocation domains of DT fused to two tandem scFv molecules targeting CD3. The translocation domain comprises of two point mutations, eliminating its glycosylation sites, making it more specific to CD3. The IT had minimal pharmacological and toxicological effects in pre-clinical trials in rats and monkeys, with mild leukocytosis, minimal subacute hepatic inflammation and mild renal multifocal mineralization [84]. The IT is currently in Phase I clinical trials and appears to be a promising tool for treating NHLs.

CD22 and CD19, as mentioned earlier, are two of the vital targets for therapy when it comes to hematological malignancies as both are surface antigens expressed highly in malignant B cells. To obtain a better therapeutic index, with reduced side effects, groups have started generating a combination of different ITs. One such attempt is the generation of 'Combotox', a combination of two separate immunotoxins, RFB4-dgA, targeting CD22, and HD37-dgA, targeting CD19. This is an equimolar mixture of the two individual immunotoxins, which renders increased antitumor specificity with minimal residual disease [85]. Currently, in Phase I clinical trials on patients with relapsed or refractory acute lymphoblastic leukemia (ALL), combotox showed a higher maximum tolerated dose (MTD) than individual ITs and 2 of the 17 patients tested so far developed a grade 3 elevation in liver function tests. Further trials are underway to ascertain the potency of combotox over individual ITs.

Another surface marker that is markedly expressed in myeloid leukemia is CD33. Michael G. Rosenblum and his group, at the MD Anderson Cancer Centre, USA, generated a humanized version of the anti CD33 mAb, M195, and fused it with the recombinant version of the plant RIP, Gelonin [86]. The IT, HuM195/rGel successfully repressed AML. The advantage of this IT over ricin-based anti-CD33 ITs was the fact that rGel was less toxic, but also showed lesser vascular leak, which is associated with ricin and abrin. Currently, the IT is in Phase I clinical trials of patients with refractory or relapsed leukemia, with moderate clinical activity.

Though a number of similar ITs have been generated, targeting molecules like granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) [78], (DTGM); chemical conjugates of anti B4-blocked ricintargeting CD19; anti-CD30 mAb, Ki, conjugated to dgA (Ki-dgA) etc, most of them were not successful in clinical trials because of limited activity, possibly due to limited tumor penetration or lower half-life in vivo. Table 1.5 is a list of most of the immunotoxins generated, targeting hematologic tumors that are in clinical trials at the time of writing this review.

Table 1.5 : Immunotoxins targeting hematologic tumors: Hematologic cancers targeted by immunotoxins include non-Hodgkin's lymphoma (NHL) of both B-cells (B-NHL) and T-cells (T-NHL), chronic lymphocytic leukemia (CLL), Acute lymphoblastic leukemia (ALL), cutaneous T-cell lymphoma (CTCL), hairy cell leukemia (HCL) and acute myelogenous leukemia (AML). Toxins used to generate these immunotoxins include pokeweed antivirus protein (PAP), ricin, deglycosylated ricin A chain (dgA), truncated Pseudomonas exotoxin (PE38) and truncated diphtheria toxin (DAB389 and DAB 388)

Immunotoxin	Antigen	Targeting moiety	Toxin	Disease
Chemical conjugates				
RFT5-dgA	CD25	mAb RFT5	Ricin dgA	Hodgkin's disease
RFB4-dgA	CD22	mAb RFB4	Ricin dgA	B-NHL, CLL
RFB4-Fab'-dgA	CD22	Fab' of mAb RFB4	Ricin dgA	B-NHL
HD37-dgA	CD19	mAb HD37	Ricin dgA	B-NHL
Anti CD7-dgA	CD7	mAb	Ricin dgA	T-NHL
Ki-4.dgA	CD30	mAb Ki-4	Ricin dgA	Hodgkin's disease
B43-PAP	CD19	mAb B43	PAP	ALL
Anti-B4-bRicin	CD19	mAb anti-B4	blocked Ricin	B-NHL
Ber-H2-Sap6	CD30	mAb Ber-H2	Saporin S6	Hodgkin's disease
Recombinant toxins				
Ontak	IL-2R	IL-2	DAB389	CTCL, CLL,NHL
BL22	CD22	mAb RFB4 (dsFv)	PE38	HCL, CLL, NHL
LMB-2	CD25	anti-Tac (scFv)	PE38	NHL, leukemias
DT388-GM-CSF	GM-CSFR	GM-CSF	DT388	AML
HA22	CD22	Anti CD22 (dsFv)	PE38	HCL,ALL,NHL,CLL
Moxetumomabpasudodotox	CD22	Anti CD22 (dsFv)	PE38	HCL, B-CLL, NHL
UCHT1	CD3ε	Anti CD3ε bisFv	DT390	T-cell lymphoma/ leukemia
DT388-IL3	IL-3R	IL-3	DT388	AML,MDS
RFT5-dgA	CD25	mAb RFT5	Ricin dgA	CTCL, NHL, melanoma
Combotox (RFB4-dgA + HD37-dgA)	CD19 / CD22	Anti CD22 + Anti CD19 mAbs	Ricin dgA	ALL
HuM195/rGel	CD33	Humanized Anti CD33	r-Gelonin	Leukemia

Immunotoxins Targeting Solid Tumors Targeting immunotoxins to solid tumors is a more challenging task, as compared to hematologic malignancies. Not only are the cellular junctions tighter, and the tumor cells more tightly packed, but the patients are also less immuno-suppressed and more likely to generate anti-IT antibodies that can neutralize the immunotoxins [51]. None-the-less, a number of immunotoxins have been generated, by conventional conjugation methods and as

fusion toxins, to target various antigens on solid tumors. Some of these have made their way into clinical trials.

One of the first ITs generated was to the epidermal growth factor receptor (EGFR). The targeting moiety was either the anti-EGFR antibody, or the ligands, epidermal growth factor (EGF) or transforming growth factor-α (TGFα). All of these were linked to the toxin PE, to generate the IT [87]. Most of these, however, were not tolerated in patients as liver cells express EGFR, and, thus, the IT led to hepatotoxicity. Fusion toxins targeting the EGFR have made significant progress in this field and a couple of them are in clinical trials presently. One such IT is DAB389EGF, which has the truncated DT as its toxin. A fusion toxin, TP38, which is a fusion between TGFC and PE38 was constructed to target glioblastoma multiformes (GBMs). Phase I clinical trials with TP38 in patients with recurrent primary or metastatic malignant brain tumor showed encouraging results, some showing partial remission of the tumor [78].

To overcome the drawback of chemical conjugates with respect to their ability to penetrate solid tumor tissue, a number of recombinant immunotoxins were generated, which would have lesser vascular retention time. These included B3(Fv)-PE38 (LMB-7), B3(dsFv)-PE38 (LMB-9), B1(dsFv)PE-33 and BR96 sFv-PE40 (SGN-10). However, none of these was able to show any significant tumor regression. However, among some of those that made it to pre-clinical and clinical trials, one immunotoxin, erb38 is noticeable. Erb38 is a fusion of dsFv of anti-erbB2, mAb e23, and PE38[88]. Erb38 was the first dsFv-PE38 based IT to enter clinical trials [89]Erb38 was much more potent in pre-clinical studies carried out in mice, than its parent IT, e23 dsFv-PE38. It had a better retention time in mice owing to its larger size, and also had better anti-tumor activity with an approximately 13-fold increase in activity than the monovalent IT [88]. But the IT failed in Phase I clinical trials as it did not have any preventive advantage over the monovalent IT and patients administered with the IT had severe hepatotoxicity. An improved IT, targeting the ErbB2 receptor, was generated more recently, wherein the scFv of mAb FRP5, which targets the extracellular domain of ErbB2, was fused with the truncated form of PE, that lacked the cell-binding domain. In Phase I clinical trials, scFv(FRP5)-ETA, scored over erb38 in that it had reduced the dosage of other chemotherapeutic drugs administered to the patients suffering from metastatic breast, prostate, head and neck and non-small cell lung cancers [90]. Also, a much higher dosage of the IT could be administered, with minimal hepatotoxicity. Further analyses are underway with scFv(FRP5)-ETA, to understand how the dosage of the IT can be enhanced, but at the same time, keeping hepatotoxicity at check.

One of the recent patents from NIH claims that an IT that is of much interest in the field of cancer therapeutics, is the one targeting mesothelin, which is expressed on solid tumors of the mesothelia, ovary, pancreas and the lungs. The IT, SS1P, is a fusion between anti-mesothelinFv and PE38. Although pre-clinical trials showed much promise for the IT, it has been undergoing a struggle in phase I clinical trials [91–93]. One of the key reasons for the failure of the IT is its high immunogenicity, wherein 75% of the patients administered with the IT developed anti-IT antibodies. The other reason for the failure of the IT in clinical trials is the fact that free

mesothelin in circulation competes with membrane-associated mesothelin, for the IT. Hence, there is a sequestration of the IT by the circulating mesothelin, which reduces the effective concentration of the IT available to attack the tumors [94].

The epithelial cell adhesion molecule (EpCAM) is a key component of the cell cytoskeleton and its expression is enhanced in almost all solid tumors. This makes it one of the favorable molecules for immunotoxin based therapies. One promising immunotoxin to have been generated in early 2000s [95] is VB4-845, or commercially called Oportuzumabmonatox. The IT is a fusion between anti-EpCAMscFv and *Pseudomonas* exotoxin A. Currently in Phase II clinical trials, the IT has been promising as recurrent intratumoral injection of the IT has not resulted in any immunogenicity and it has been well tolerated as well. The IT has a favorable safety profile, with an instillation of 30 mg of VB4-845 once a week for six consecutive weeks showed a response rate of 27% complete recovery and 16% in a disease-free status for more than a year [96].

Another class of ITs was generated to target receptors of IL-4. Phase I immunotoxins generated by fusing IL-4 with PE showed limited binding as the toxin interfered with the binding of IL-4 to IL-4R. To overcome this problem, a circularly permuted mutant of IL-4 was used, which was fused with PE [51, 75], resulting in enhanced cytotoxicity. The IT, IL4(38-37)-PE38KDEL, showed severe hepatotoxicity even at low doses and hence, intravenous injection of this IT was ruled out. Since intra-tumoral injections of the IT were encouraging, Phase II trials are underway to determine the efficacy of this IT as a cancer therapeutic.

Brain tumor is one of the forerunners when it comes to developing a drug to cure a disease. The scientific community is directing considerable amount of effort and money to find a cure for glioblastoma multiforme, which is the most prevalent primary brain tumor. Among the molecules that can be targeted, one important protein is the receptor for interleukin-13 (IL13-R). Cintredekinbesudotox or IL13-PE38QQR is a chimeric toxin, wherein human IL13 is fused to PE38, which has its lysines at positions 590 and 606 replaced by glutamines (Qs) and the lysine at 613 by arginine (R)[83]. The IT has reached Phase III clinical studies, with great efficacy demonstrated in Phase I and II of the trials. The IT is the first to be used for a state-of-the-art drug administration technique called Convection-enhanced delivery (CED), wherein the drug is administered loco-regionally—relying on a continuous pressure gradient—into the interstitial space of the brain. The interval of administration is from a few hours to a few days which enables the drug to bypass the blood-brain barrier, increasing its concentration at the target tissue [97]. Although patients with GBM tolerated the drug, survival was not enhanced, when compared to conventional therapies using Gliadel wafers [97]. Groups using IL13-PE38QQR are now trying to fine-tune several parameters to try and understand how the IT can be made more efficacious to the treatment of GBM.

Transferrin (Tf) receptor is a key component of cells as they are involved in the uptake of iron. The expression of the receptor tends to increase in cells when they are rapidly dividing and hence, it is a key molecule for targeted therapy of cancer as many cancers do overexpress the receptor. After considerable efforts, one IT was generated, that targeted the Tf receptor, especially in GBMs, called transferrin-

CRM107 (Tf-CRM107) wherein CRM107 is the diphtheria toxin bearing a point mutation [98]. The IT showed a lot of promise in pre-clinical and Phase I clinical trials. But in Phase II trials, the response rate at maximal tolerated dose was at 35% and so, further trials were not approved as it was unlikely that the IT improved the overall patient survivability compared to the current modalities of treatment.

Thus, although a number of immunotoxins have been generated targeting various antigens on the surface of solid tumors, only a few have made it to clinical testing due to limitations in dose-response rate, renal tubular acidosis, hepatotoxicity and immunogenicity [35, 51]. Table 1.6 is a summary of the immunotoxins generated against antigens on solid tumors, which have progressed to the initial stages of clinical trials.

1.8 Conclusions

The foregoing discussion makes it evident that cancer therapeutics have undergone a change, from the times of using herbs to control tumor growth, to the generation of immunotoxins using recombinant DNA technologies. As more and more cancer specific antigens expressed on the cancer cell surface are getting identified, these antigens become potential targets for delivering the RIP toxins by means of specific antibodies, or antibody fragments. It is important to generate an immunotoxin with high potency, and also understand the trafficking pathway of the immunotoxin in vivo. In spite of so many immunotoxins being generated, not many have been successful. Of all the ITs, only one, Ontak (denileukindiftitox) has been approved by the FDA, for the treatment of hematologic malignancies. The drawback with ITs has to do with poor binding or poor penetration of the antibodies into solid tumors or low specificity towards hematologic tumors. In case of recombinant toxins, one of the major drawbacks is the short half-life of these proteins, of about 30–40 min, in comparison to 5–10 h of the intact antibody conjugate. Also, low tumor localization, rapid hepatic uptake and liver toxicity are some of the major problems associated with the intravenous injections of ITs. These can be overcome by using intra-tumoral injections [35]. Another major drawback of this therapy is the toxin itself. Many toxins, like ricin, are extensively glycosylated, which would require them to be deglycosylated before use. If bacterial toxins are used, then there are possibilities that the patients are pre-exposed to these toxins, leading to prevalence of anti-toxin antibodies in them. In this context, a toxin like abrin, which is a type II RIP, and whose A chain is devoid of glycosylation, can be considered as a potential tool for immunotherapy.

Conflict of Interest No potential conflicts of interest were disclosed.

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Table 1.6 Immunotoxins targeting solid tumors: Toxins used in immunotoxins constructed to target solid tumors include recombinant ricin A chain (rRA), truncated Pseudomonas exotoxins (PE38, PE40, PE38KDEL and PE38QQR), mutated diphtheria toxin (CRM107) and truncated diphtheria toxin (DAB389)

Immunotoxin	Antigen	Targeting moiety	Toxin	Disease
Chemical conjugates				
LMB-1	Ley	Anti-Ley	Lys-PE38	Carcinoma
TF-CRM107	TFR	Transferrin	CRM107	Glioma
454A12-rRA	TFR	Anti-TFR	rRA	Cerebrospinal fluid cancer
N901-bR	CD56	Anti-CD56	Blocked Ricin	Small cell lung cancer
OvB3-PE		Antibody	PE	Ovarian cancer
Recombinant toxins				
B3(Fv)-PE38	Ley	mAb B3 (scFv)	PE38	Carcinoma
B3 (dsFv)-PE38	Ley	mAb B3 (dsFv)	PE38	Carcinoma
TP40	EGFR	TGF-c	PE40	Bladder cancer
TP38	EGFR	TGF-c	PE38	Glioblastoma
BR96 (scFv)-PE40	Ley	mAb BR96 (scFv)	PE40	Carcinoma
erb38	erbB2	Anti-erbB2 (dsFv)	PE38	Breast cancer
NBI-3001	IL-4R	IL-4 (38-37)	PE38KDEL	Glioma
Cintredekinbesu-dotox	IL-13R	IL-13	PE38QQR	Brain cancer, Renal cell carcinoma
SS1P	Mesothelin	mAb SS1 (dsFv)	PE38	Mesothelioma
DAB389EGF	EGFR	EGF	DAB389	Carcinoma
scFv(FRP5)-ETA	erbB2	Anti-erbB2 (FRP5) dsFv	PE38	Solid tumors
Oportuzumab-monatox	EpCAM	Anti-EpCAM (scFv)	PE40	Carcinomas
Naptumomabe-stafenatox	5T4	Anti-5T4 (Fab)	SEA	Renal cell carcinoma
MR1-1	EGFRv-III	Anti-EGFRv-III (scFv)	PE	Brain cancer
TP-38	EGFR	TGFα	PE38	Glioblastoma

References

1. Papac RJ. Origins of cancer therapy. Yale J Biol Med. 2001;74(6):391–8.
2. Wright JC. Cancer chemotherapy: past, present, and future–part I. J Natl Med Assoc. 1984;76(8):773–84.
3. DeVita JVT, Chu E. A history of cancer chemotherapy. Cancer Res. 2008;68(21):8643–53.
4. Wright JC. Clinical cancer chemotherapy. N Y State J Med. 1961;61:249–80.
5. Wright JC. Cancer chemotherapy: past, present, and future–part II. J Natl Med Assoc. 1984;76(9):865–76.

6. Farber S, Diamond LK. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. N Engl J Med. 1948;238(23):787–93.
7. Elion GB, Singer S, Hitchings GH. Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. J Biol Chem. 1954;208(2):477–88.
8. Hitchings GH, Elion GB. The chemistry and biochemistry of purine analogs. Ann N Y Acad Sci. 1954;60(2):195–99.
9. Burchenal JH, Murphy ML, Ellison RR, Sykes MP, Tan TC, Leone LA, Karnovsky DA, Craver LF, Dargeon HW, Rhoads CP. Clinical evaluation of a new antimetabolite, 6-mercaptopurine, in the treatment of leukemia and allied diseases. Blood. 1953;8(11):965–99.
10. Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D, Greisbach L, Duschinsky R, Schnitzer RJ, Pleven E, Scheiner J. Fluorinated pyrimidines, new class of tumour-inhibitory compounds. Nature. 1957;179(4561):663–66.
11. Heidelberger C, Ansfield FJ. Experimental and clinical use of fluorinated pyrimidines in cancer chemotherapy. Cancer Res. 1963;23:1226–43.
12. Wright JC, Medrek TJ, Walker DG, Lyons MM. The current status of chemotherapy and hormone therapy for cancer. Prog Clin Cancer. 1965;10:264–307.
13. Bonadonna G, Monfardini S, De Lena M, Fossati-Bellani F. Clinical evaluation of adriamycin, a new antitumour antibiotic. Br Med J. 1969;3(5669):503–6.
14. Dyke RW, Nelson RL. Phase I anti-cancer agents: vindesine (desacetyl vinblastine amide sulfate). Cancer Treat Rev. 1977;4(2):135–42.
15. Rosenberg B, Vancamp L, Krigas T. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature. 1965;205:698–99.
16. Fischinger PJ, DeVita JVT. Governance of science at the National Cancer Institute: perceptions and opportunities in oncogene research. Cancer Res. 1984;44(10):4693–6.
17. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 1996;2(5):561–6.
18. Sparreboom A, Verweij J. Advances in cancer therapeutics. Clin Pharmacol Ther. 2009;85(2):113–7.
19. Davis ID. An overview of cancer immunotherapy. Immunol Cell Biol. 2000;78(3):179–95.
20. Llewelyn MB, Hawkins RE, Russell SJ. Discovery of antibodies. BMJ. 1992;305(6864):1269–72.
21. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12(4):278–87.
22. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol. 2010;10(5):301–16.
23. Schliemann C, Neri D. Antibody-based vascular tumor targeting. Recent Results Cancer Res. 2010;180:201–16.
24. Oldham RK, Dillman RO. Monoclonal antibodies in cancer therapy: 25 years of progress. J Clin Oncol. 2008;26(11):1774–7.
25. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, Morel P, Van Den Neste E, Salles G, Gaulard P, Reyes F, Lederlin P, Gisselbrecht C. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235–42.
26. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol. 2010;10(5):317–27.
27. Eccles SA. Monoclonal antibodies targeting cancer: 'magic bullets' or just the trigger? Breast Cancer Res. 2001;3(2):86–90.
28. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20(3):719–26.
29. Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med. 2007;357(1):39–51.

30. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5(4):317–28.
31. Mackey J, Gelmon K, Martin M, McCarthy N, Pinter T, Rupin M, Youssoufian H. TRIO-012: a multicenter, multinational, randomized, double-blind phase III study of IMC-1121B plus docetaxel versus placebo plus docetaxel in previously untreated patients with HER2-negative, unresectable, locally recurrent or metastatic breast cancer. Clin Breast Cancer. 2009;9(4):258–61.
32. Weiner LM, Adams GP. New approaches to antibody therapy. Oncogene. 2000;19(53):6144–51.
33. Order SE, Sleeper AM, Stillwagon GB, Klein JL, Leichner PK. Radiolabeled antibodies: results and potential in cancer therapy. Cancer Res. 1990;50(3 Suppl):1011s–3s.
34. Weiner LM, Murray JC, Shuptrine CW. Antibody-based immunotherapy of cancer. Cell. 2012;148(6):1081–84.
35. Pirker R. Immunotoxins against solid tumors. J Cancer Res Clin Oncol. 1988;114(4):385–93.
36. Barbieri L, Battelli MG, Stirpe F. Ribosome-inactivating proteins from plants. Biochim Biophys Acta. 1993;1154(3–4):237–82.
37. Hertler AA, Frankel AE. Immunotoxins: a clinical review of their use in the treatment of malignancies. J Clin Oncol. 1989;7(12):1932–42.
38. Thrush GR, Lark LR, Clinchy BC, Vitetta ES. Immunotoxins: an update. Annu Rev Immunol. 1996;14:49–71.
39. Baselga J, Mendelsohn J. Receptor blockade with monoclonal antibodies as anti-cancer therapy. Pharmacol Ther. 1994;64(1):127–54.
40. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov. 2006;5(2):147–59.
41. Ragupathi G. Carbohydrate antigens as targets for active specific immunotherapy. Cancer Immunol Immunother. 1996;43(3):152–57.
42. Brinkmann U, Pastan I. Immunotoxins against cancer. Biochim Biophys Acta. 1994;1198(1):27–45.
43. Gadina M, Newton DL, Rybak SM, Wu YN, Youle RJ. Humanized immunotoxins. Ther Immunol. 1994;1(1):59–64.
44. Wen ZL, Tao X, Lakkis F, Kiyokawa T, Murphy JR. Diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion toxin. Internal in-frame deletion from Thr387 to His485 results in the formation of a highly potent fusion toxin which is resistant to proteolytic degradation. J Biol Chem. 1991;266(19):12289–93.
45. Walz G, Zanker B, Brand K, Waters C, Genbauffe F, Zeldis JB, Murphy JR, Strom TB. Sequential effects of interleukin 2-diphtheria toxin fusion protein on T-cell activation. Proc Natl Acad Sci U S A. 1989;86(23):9485–8.
46. Lin JY, Tserng KY, Chen CC, Lin LT, Tung TC. Abrin and ricin: new anti-tumour substances. Nature. 1970;227(5255):292–3.
47. Peumans WJ, Hao Q, Van Damme EJ. Ribosome-inactivating proteins from plants: more than RNA N-glycosidases? FASEB J. 2001;15(9):1493–1506.
48. Reinbothe S, Reinbothe C, Lehmann J, Becker W, Apel K, Parthier B. JIP60, a methyl jasmonate-induced ribosome-inactivating protein involved in plant stress reactions. Proc Natl Acad Sci U S A. 1994;91(15):7012–6.
49. Kozlov IV, Sudarkina OI, Kurmanova AG. Ribosome-inactivating lectins from plants. Mol Biol (Mosk). 2006;40(4):711–23.
50. Stirpe F, Battelli MG. Ribosome-inactivating proteins: progress and problems. Cell Mol Life Sci. 2006;63(16):1850–66.
51. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J. 2006;8(3):E532–551
52. Lord JM, Roberts LM. Toxin entry: retrograde transport through the secretory pathway. J Cell Biol. 1998;140(4):733–6.
53. Day PJ, Owens SR, Wesche J, Olsnes S, Roberts LM, Lord JM. An interaction between ricin and calreticulin that may have implications for toxin trafficking. J Biol Chem. 2001;276(10):7202–8.

54. Zhang Y, Zhu X, Torelli AT, Lee M, Dzikovski B, Koralewski RM, Wang E, Freed J, Krebs C, Ealick SE, Lin H. Diphthamide biosynthesis requires an organic radical generated by an iron-sulphur enzyme. Nature. 2010;465(7300):891–6.
55. Endo Y, Tsurugi K. The RNA N-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA. J Biol Chem. 1988;263(18):8735–9.
56. Endo Y, Mitsui K, Motizuki M, Tsurugi K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. J Biol Chem. 1987;262(12):5908–12.
57. Endo Y, Wool IG. The site of action of alpha-sarcin on eukaryotic ribosomes. The sequence at the alpha-sarcin cleavage site in 28 S ribosomal ribonucleic acid. J Biol Chem. 1982;257(15):9054–60.
58. Stirpe F. Ribosome-inactivating proteins. Toxicon. 2004;44(4):371–83.
59. Griffiths GD, Leek MD, Gee DJ. The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissues and intestine. J Pathol. 1987;151(3):221–9.
60. Vervecken W, Kleff S, Pfuller U, Bussing A. Induction of apoptosis by mistletoe lectin I and its subunits. No evidence for cytotoxic effects caused by isolated A- and B-chains. Int J Biochem Cell Biol. 2000;32(3):317–26.
61. Kojio S, Zhang H, Ohmura M, Gondaira F, Kobayashi N, Yamamoto T. Caspase-3 activation and apoptosis induction coupled with the retrograde transport of Shiga toxin: inhibition by brefeldin A. FEMS Immunol Med Microbiol. 2000;29(4):275–81.
62. Narayanan S, Surendranath K, Bora N, Surolia A, Karande AA. Ribosome inactivating proteins and apoptosis. FEBS Lett. 2005;579(6):1324–31.
63. Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Chen SL, Magun BE. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol. 1997;17(6):3373–81.
64. Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, Thorpe CM. Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect Immun. 2003;71(3):1497–1504.
65. Narayanan S, Surolia A, Karande AA. Ribosome-inactivating protein and apoptosis: abrin causes cell death via mitochondrial pathway in Jurkat cells. Biochem J. 2004;377(Pt 1):233–40.
66. Fujii J, Matsui T, Heatherly DP, Schlegel KH, Lobo PI, Yutsudo T, Ciraolo GM, Morris RE, Obrig T. Rapid apoptosis induced by Shiga toxin in HeLa cells. Infect Immun. 2003;71(5):2724–35.
67. Erwert RD, Eiting KT, Tupper JC, Winn RK, Harlan JM, Bannerman DD. Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. Microb Pathog. 2003;35(2):87–93.
68. Jones NL, Islur A, Haq R, Mascarenhas M, Karmali MA, Perdue MH, Zanke BW, Sherman PM. Escherichia coli Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. Am J Physiol Gastrointest Liver Physiol. 2000;278(5):G811–9.
69. Komatsu N, Nakagawa M, Oda T, Muramatsu T. Depletion of intracellular NAD(+) and ATP levels during ricin-induced apoptosis through the specific ribosomal inactivation results in the cytolysis of U937 cells. J Biochem. 2000;128(3):463–70.
70. Mishra R, Karande AA. Endoplasmic reticulum stress-mediated activation of p38 MAPK, Caspase-2 and Caspase-8 leads to abrin-induced apoptosis. PLoS ONE. 2014;9(3):e92586. doi:10.1371/journal.pone.0092586.
71. Li XP, Baricevic M, Saidasan H, Tumer NE. Ribosome depurination is not sufficient for ricin-mediated cell death in Saccharomyces cerevisiae. Infect Immun. 2007;75(1):417–28.
72. Moolten FL, Cooperband SR. Selective destruction of target cells by diphtheria toxin conjugated to antibody directed against antigens on the cells. Science. 1970;169(3940):68–70.
73. FitzGerald DJ, Kreitman R, Wilson W, Squires D, Pastan I. Recombinant immunotoxins for treating cancer. Int J Med Microbiol. 2004;293(7–8):577–82.
74. Olsnes S, Sandvig K, Madshus IH, Sundan A. Entry mechanisms of protein toxins and picornaviruses. Biochem Soc Symp. 1985;50:171–91.

75. Pastan I, Willingham MC, FitzGerald DJ. Immunotoxins. Cell. 1986;47(5):641–8.
76. Gadadhar S, Karande AA. Abrin immunotoxin: targeted cytotoxicity and intracellular trafficking pathway. PLoS ONE. 2013;8(3):e58304. doi:10.1371/journal.pone.0058304.
77. Bellisola G, Fracasso G, Ippoliti R, Menestrina G, Rosén A, SoldÃ S, Udali S, Tomazzolli R, Tridente G, Colombatti M. Reductive activation of ricin and ricin A-chain immunotoxins by protein disulfide isomerase and thioredoxin reductase. Biochem Pharmacol. 2004;67(9):1721–31.
78. Choudhary S, Mathew M, Verma RS. Therapeutic potential of anticancer immunotoxins. Drug Discov Today. 2011;16(11–12):495–503.
79. Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, Waldmann TA, Pastan I. Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol. 2000;18(8):1622–36
80. Alderson RF, Kreitman RJ, Chen T, Yeung P, Herbst R, Fox JA, Pastan I. CAT-8015: a second-generation pseudomonas exotoxin A-based immunotherapy targeting CD22-expressing hematologic malignancies. Clin Cancer Res. 2009;15(3):832–9. doi:10.1158/1078-0432.CCR-08-1456.
81. Salvatore G, Beers R, Margulies I, Kreitman RJ, Pastan I. Improved cytotoxic activity toward cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. Clin Cancer Res. 2002;8(4):995–1002.
82. Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler-Stevenson M, Fitzgerald DJ, Lechleider R, Pastan I. Phase I trial of anti-CD22 recombinant immunotoxin moxetumomab pasudotox (CAT-8015 or HA22) in patients with hairy cell leukemia. J Clin Oncol. 2012;30(15):1822–28. doi:10.1200/JCO.2011.38.1756.
83. Dosio F, Stella B, Cerioni S, Gastaldi D, Arpicco S. Advances in anticancer antibody-drug conjugates and immunotoxins. Recent Pat Anti-cancer Drug Discov. 2014;9(1):35–65.
84. Woo JH, Bour SH, Dang T, Lee YJ, Park SK, Andreas E, Kang SH, Liu JS, Neville DM, Jr., Frankel AE. Preclinical studies in rats and squirrel monkeys for safety evaluation of the bivalent anti-human T cell immunotoxin, A-dmDT390-bisFv(UCHT1). Cancer Immunol Immunother. 2008;57(8):1225–39. doi:10.1007/s00262-008-0457-x.
85. Schindler J, Gajavelli S, Ravandi F, Shen Y, Parekh S, Braunchweig I, Barta S, Ghetie V, Vitetta E, Verma A. A phase I study of a combination of anti-CD19 and anti-CD22 immunotoxins (Combotox) in adult patients with refractory B-lineage acute lymphoblastic leukaemia. Br J Haematol. 2011;154(4):471–6. doi:10.1111/j.1365-2141.2011.08762.x.
86. Pagliaro LC, Liu B, Munker R, Andreeff M, Freireich EJ, Scheinberg DA, Rosenblum MG. Humanized M195 monoclonal antibody conjugated to recombinant gelonin: an anti-CD33 immunotoxin with antileukemic activity. Clin Cancer Res. 1998;4(8):1971–6.
87. FitzGerald DJ, Trowbridge IS, Pastan I, Willingham MC. Enhancement of toxicity of anti-transferrin receptor antibody-Pseudomonas exotoxin conjugates by adenovirus. Proc Natl Acad Sci U S A. 1983;80(13):4134–8.
88. Bera TK, Viner J, Brinkmann E, Pastan I. Pharmacokinetics and antitumor activity of a bivalent disulfide-stabilized Fv immunotoxin with improved antigen binding to erbB2. Cancer Res. 1999;59(16):4018–22.
89. Pai-Scherf LH, Villa J, Pearson D, Watson T, Liu E, Willingham MC, Pastan I. Hepatotoxicity in cancer patients receiving erb-38, a recombinant immunotoxin that targets the erbB2 receptor. Clin Cancer Res. 1999;5(9):2311–5.
90. von Minckwitz G, Harder S, Hovelmann S, Jager E, Al-Batran SE, Loibl S, Atmaca A, Cimpoiasu C, Neumann A, Abera A, Knuth A, Kaufmann M, Jager D, Maurer AB, Wels WS. Phase I clinical study of the recombinant antibody toxin scFv(FRP5)-ETA specific for the ErbB2/HER2 receptor in patients with advanced solid malignomas. Breast Cancer Res. 2007;7(5):R617–26. doi:10.1186/bcr1264.
91. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. Clin Cancer Res. 2007;13(17):5144–9. doi:10.1158/1078-0432.CCR-07-0869.

92. Hassan R, Ho M. Mesothelin targeted cancer immunotherapy. Eur J Cancer. 2008;44(1):46–53. doi:10.1016/j.ejca.2007.08.028.
93. Kreitman RJ, Hassan R, Fitzgerald DJ, Pastan I. Phase I trial of continuous infusion anti-mesothelin recombinant immunotoxin SS1P. Clin Cancer Res. 2009;15(16):5274–9. doi:10.1158/1078-0432.CCR-09-0062.
94. Pak Y, Zhang Y, Pastan I, Lee B. Antigen shedding may improve efficiencies for delivery of antibody-based anticancer agents in solid tumors. Cancer Res. 2012;72(13):3143–52. doi:10.1158/0008-5472.CAN-11-3925.
95. Di Paolo C, Willuda J, Kubetzko S, Lauffer I, Tschudi D, Waibel R, Pluckthun A, Stahel RA, Zangemeister-Wittke U. A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. Clin Cancer Res. 2003;9(7):2837–48.
96. Kowalski M, Guindon J, Brazas L, Moore C, Entwistle J, Cizeau J, Jewett MA, MacDonald GC. A phase II study of oportuzumab monatox: an immunotoxin therapy for patients with noninvasive urothelial carcinoma in situ previously treated with bacillus Calmette-Guerin. J Urol. 2012;188 (5):1712–8. doi:10.1016/j.juro.2012.07.020.
97. Kunwar S, Chang S, Westphal M, Vogelbaum M, Sampson J, Barnett G, Shaffrey M, Ram Z, Piepmeier J, Prados M, Croteau D, Pedain C, Leland P, Husain SR, Joshi BH, Puri RK. Phase III randomized trial of CED of IL13-PE38QQR vs Gliadel wafers for recurrent glioblastoma. Neuro Oncol. 2010;12(8):871–81. doi:10.1093/neuonc/nop054.
98. Weaver M, Laske DW. Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. J Neurooncol. 2003;65(1):3–13

Chapter 2
Immunotoxins, Resistance and Cancer Stem Cells: Future Perspective

Sithambaram Devilakshmi, Jayaprakasam Madhumathi and Rama Shanker Verma

Abstract Cancer relapse or recurrence has been the greatest challenge in the treatment of this life threatening disease, which occurs due to resistance of cancer cells to drug or radiation therapy. Most often this resistance is developed during treatment, which makes it even more complicated, leading to the failure of chemo or radiation therapy in the majority of cases. To circumvent these problems associated with conventional therapies, newer strategies were adopted like targeted therapy using monoclonal antibodies, immunotoxins and antibody-drug conjugates. However, targeted therapy also showed failure in many *in vitro* and *in vivo* studies that was again attributed to the emergence of resistant cells. Here, we discuss the various factors and cellular mechanisms responsible for resistance against conventional therapies and targeted approaches like recombinant immunotoxins. Cancer stem cells (CSC's) were identified as the major reason for resistance and their role in cancer relapse has been proved convincingly in recent studies. Hence, resistance mechanisms involved in CSC's have been elaborated. We also summarize the strategies being adopted currently to overcome resistance and different means of targeting resistant cancer stem cells that could be used in the future.

Keywords Immunotoxins · Cancer resistance · Cancer stem cells · Drug efflux · Survival pathway · Chemotherapy

Abbreviations

ABC	ATP-Binding Cassette
AML	Acute myeloid leukemia
BCRP	Breast cancer resistance protein
BTK	Bruton tyrosine kinase
CSC	Cancer stem cells

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DAMP	Damage-associated molecular patterns
DT	Diphtheria toxin
EMT	Epithelial Mesenchymal transition
GCS	GlcCer synthase
GO	Gemtuzumab ozogamicin
GSIs	Gamma-secretase inhibitors
HIF	Hypoxia inducible factor
IT	Immunotoxin
MCL	Mantle cell lymphoma
MDR	Multi Drug Resistance
MRP1	Multidrug resistance protein 1
OV	Oncolytic viruses
P-gp	P-glycoprotein
SCLC	Small Cell Lung Cancer
SCNP	Single cell network profiling
T-ALL	T cell acute lymphoblastic leukemia
TMM	Telomere maintenance mechanisms
Tnfaip3	Tumor necrosis factor alpha induced protein 3
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor

2.1 Introduction

Cancer is the most devastating disease that confers threat to human health. The widely used conventional treatment for all types of cancers is chemo- and radiation therapies. However, the emerging resistance towards various chemotherapeutic drugs remains a great challenge in cancer treatment. Transcriptional misregulation of genes and accumulation of mutations commonly result in gaining resistance to the panel of chemotherapeutic drugs referred as Multi Drug Resistance (MDR). MDR can be defined as "a state of resilience against structurally and mechanistically unrelated drugs". MDR is the principal mechanism by which many cancers develop resistance to chemotherapeutic drugs, leading to the failure of treatment in patients with a variety of blood cancers and solid tumors, including breast, ovarian, lung, and lower gastrointestinal tract cancers [1–3].

To combat chemotherapy resistance newer strategies were developed like targeted approaches which include immunotoxins, monoclonal antibodies, antibody fragments (ScFv, Fab), antibody-drug conjugates etc. However, even targeted therapy faces challenges due to the emergence of resistance by more complex mechanisms. Here, we describe the various factors involved in cancer resistance with a focus on resistance against immunotoxins, the role of cancer stem cells in resistance and strategies to overcome resistance.

2.2 Factors Responsible for Cancer Resistance

The hallmark of a cancer cell is the increased genomic instability and higher mutation rates. Drug resistant tumour is most often acquired with characteristic features like gene mutations, gene amplification, or epigenetic changes that influence the drug metabolism, or export of drugs from cells. The daughter cells from cancer cells acquire changes (mutations) at a high rate. Acquiring gene mutation is a common characteristic feature of cancer cells. Normal cells can be targeted by drug treatment. However, a mutated cell with a modified gene may have a function, which can no longer be the drug target [4]. Mutations in any of the genes involved in metabolic pathways that are essential for cell growth, survival, maintenance of cellular homeostasis and cell division may result in resistance.

Other than mutations, the expression of drug transport proteins and the tumor niche play a major role in resistance. The tumor niche is a microenvironment which consists of diverse populations of malignant cells, with variable degree of resistance, few of them being highly resistant [5]. Chemotherapy kills drug-sensitive cells, but a small population called "side population" survives, which then drives the cancer relapse. As the tumor begins to grow again, chemotherapy may fail to eliminate resistant populations that culminate into a poor prognostic recurrence of disease or even death. These side populations survive, proliferate at a higher rate and finally rebuild the tumor microenvironment.

2.2.1 Gene Mutations in Signalling Pathways

A20 is a protein encoded by the gene tumor necrosis factor alpha-induced protein 3 (TNFAIP3). It regulates the canonical NF-κB activation and also acts as the cell's autocrine inhibitory molecule. It interacts with NF-κB upstream signalling components which keeps the pathway activation under control [6]. Recent reports suggested that NF-κB-dependent A20 exerts cell-type specific anti- or pro-apoptotic functions. Increased A20 expression in few solid human tumors likely contributes to both carcinogenesis and response to chemotherapy. However, a current approach of analysing a unique molecular signature of each tumor holds promise for a personalized chemotherapeutic regimen comprising specific A20-targeting agents i.e., both inhibitors and enhancers [7].

Ibrutinib, an FDA approved drug, targets and inhibits the Bruton tyrosine kinase (BTK), which is found in increased levels in several types of B-cell malignancies, including mantle cell lymphoma (MCL) [8]. However, about one-third of these patients do not respond to the treatment and those that respond also become resistant to the drug [9–11]. It was identified later that there was a patient-relapse–specific mutation, C481S, in patients who initially had a durable response to ibrutinib but then showed disease progression. The mutation resulted in increased BTK activation which leads to AKT activation, further driven by the cell-cycle regulator CDK4 [12, 13].

FLT3 is a cytokine receptor belonging to the receptor tyrosine kinase class III family. Mutations in the FLT3 gene linked to a poor prognosis in acute myeloid leukemia (AML). Early trials of FLT3 inhibitors gained resistance to treatment and the development of a new FLT3 inhibitor AC220 showed promising activity in patients with highly resistant leukemia [14].

Glucocorticoid resistance is a major cause of therapeutic failure in T cell acute lymphoblastic leukemia (T-ALL). AKT1 impairs glucocorticoid-induced gene expression by direct phosphorylation of NR3C1 at position S134 and blocking glucocorticoid-induced NR3C1 translocation to the nucleus. Conversely, pharmacologic inhibition of AKT with MK2206 effectively restores glucocorticoid-induced NR3C1 translocation to the nucleus, increases the response of T-ALL cells to glucocorticoid therapy and effectively reverses glucocorticoid resistance *in vitro* and *in vivo* [10].

2.2.2 Drug Transporters

ATP-binding cassette (ABC) transporters play a major role in drug resistance. Chemotherapeutic drugs are rapidly effluxed out by this family of transporters which are localized in the plasma membrane of resistant cells. Three well studied transporters involved in multi-drug resistance are—P-glycoprotein (P-gp, MDR1, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2).

P-glycoprotein had shown strong evidence in support of its role in pleiotropic drug resistance in 1982, when it was shown that DNA from resistant cell lines transformed in non-resistant cells conferred resistance which also correlated with protein expression [15]. The gene for P-glycoprotein, called MDR-1, was cloned in 1985, and the protein's putative function was postulated on the basis of sequence homologies with bacterial hemolysin transport protein as an energy-dependent pump that expels small molecules from inside the cells [16, 17]. Recent work suggests that in non-small cell lung cancer cells: there is a correlation between MRP and mutant p53 expression, suggesting that it can be used as a prognostic marker.

ABCG2 is the mitoxantrone resistance gene also known as breast cancer resistance protein (BCRP), or ABC transporter in placenta (ABC-P). Mutant ABCG2 protein is an ideal candidate for human stem cell protection and for use as a selectable marker in gene therapy [18].

2.2.3 Tumor Microenvironment and Accessibility

Hypoxia and accumulation of HIF-1 alpha in solid tumor tissues are associated with resistance to chemotherapy, radiotherapy and immunotherapy [19]. Activated HIF-1 induces the expression of vascular endothelial growth factor (VEGF) in cancer. Increase in VEGF levels promotes tumor metastasis by angiogenesis [20]. Anti-

angiogenic therapy using humanized VEGF antibody and VEGF receptor tyrosine kinase inhibitors have been shown to have promising effect in solid cancer therapy.

Another important, but little studied, cause of drug resistance is the accessibility of drugs to tumor tissue. Since the diluted concentration of drug that reaches the target site, which is much less than the potential lethal concentration, it may trigger resistance in cancer cells [21].

2.3 Resistance to Immunotoxins

Targeting cancer cells by inducing apoptosis is one of the earliest approaches to control cancer. However, cancer cells bypass apoptosis by activating alternate survival pathways and/or by blocking/inactivating apoptotic pathways. Another strategy by which resistant cells evade apoptosis is through the efflux of drugs by membrane transporters by expressing multi-drug resistant genes.

Immunotoxins bind to specific cell surface receptor and are internalized into endocytic vesicles. After priming, it is then translocated to the cytosol, and inhibiting protein synthesis by acting upon ADP-ribosylation of elongation factor 2 in the cytosol. Resistance can be induced by interference at any of these steps like down regulation of receptor or poor binding of immunotoxins, degradation of internalized toxins and failure of ADP-ribosylation of EF-2 by an escape mechanism in resistant cells (Table 2.1).

Gemtuzumab ozogamicin (GO) is a conjugate of monoclonal antibody targeting CD33 and the toxic drug calicheamicin. The poor expression of CD33 or the uptake of GO was correlated with resistance in AML cell lines [22]. Similarly, higher expression of HER2 was associated with response to T-DMI, which is a conjugate of anti-HER2 antibody, trastuzumab and toxic drug moiety DMI, a derivative of maytansine, in cancer cells [23].

2.3.1 Dysfunctional Apoptotic Pathways

Anti-apoptotic factors downstream of DNA damage play a major role in gaining resistance against GO in AML. The AML cell line KG1a displayed resistance to GO due to defect in activation of the pro-apoptotic proteins Bak and Bax [24]. Activation of caspase-3 signaling was observed in HL60 and NB4 AML cells but not in GO-exposed KG1a AML cells. Bcl-2 family of anti-apoptotic proteins were reported to be involved in GO resistance in few studies. HL-60 cells with stable overexpression of Bcl-2 or Bcl-XL were reported to be resistant to GO [25]. Despite inhibition of protein synthesis by PE-based immunotoxins, cell death was not significant due to apoptosis via pro-survival proteins like Bcl-2 family. ABT-737, a BH3-only mimetic that inhibits Bcl-2 protein could restore sensitivity in resistant cell lines like DLD1 by neutralizing Bcl-2, Bcl-xl, and Bcl-w [26].

Table 2.1 Resistance to immunotoxins

Factors involved in resistance		Immunotoxin	Target molecule	Cells	Preventive strategy	Reference
Lower receptor expression/poor internalization	Lower CD33 expression	GO (Anti CD33-calicheamicin)	CD33	AML		Walter et al. [22]
	Lower expression and internalization of Her2	T-DMI (Anti HER2 DMI)	HER2	Breast cancer cells		Barok et al. [23]
	Lower CD33 expression	GO	CD33	HL60		Cianfriglia et al. [27]
Apoptotic Pathway	Bak and Bax	GO	CD33	KG1a		Haag et al. [24]
	Bcl2	GO	CD33	HL60		Linenberger et al. [25]
	Bcl2	HB21-PE40 SS1P	TfR Mesothelin	DLD1	ABT-737	Traini et al. [26]
ABC transporters	MDR1-Pgp	GO	CD33	HL60		Cianfriglia et al. [27]
	Pgp, MRP1, and MRP2	GO	CD33	NB4, HL-60, TF1 cells, AML samples	Cyclosporine MK-571	Walter et al. [28]
	MRP1 and MRP2	GO	CD33	AML samples		Linenberger et al. [25]
	P-gp	HuM195-gelonin	CD33	MDR RV+ HL60 and K562	Bafilomycin A	McGrath et al. [29]
Lysosomal degradation		B3(dsFv)-PE38	CD22	CA46, Daudi, Raji, and Ramos		Weldon et al. [30]
		Gelonin based IT's	CD33	MDR RV+ HL60 and K562		McGrath et al. [29]
		Ricin A-chain IT (p67-7.dgA)	CD33	HL60 cells		Engert et al. [32]

Table 2.1 (continued)

Factors involved in resistance		Immunotoxin	Target molecule	Cells	Preventive strategy	Reference
Other factors	Methylation of CpG in DPH1 promoter	HA22 (Anti-CD22-PE IT)	CD22	KOPN-8 cells		Hu et al. [33]
	Methylation of CpG in DPH4 promoter	HA22	CD22	ALL	5-azacytidine	Wei et al. [34]
	Modification of diphthamide in EF2	PE based IT	CD22	Lymphoma cell line		Wei et al. [35]
	PI3K/AKT signalling	GO		AML cell lines	AKT inhibitor MK-2206	Rosen et al. [36]

2.3.2 ABC Transporters

Multidrug resistance protein1 (MRP1 or ABCC1), is overexpressed in 7–30 % AML cases and was associated with resistance to GO. Expression of P-gp in AML blast cells were correlated with resistance by treatment with GO in phase II clinical trials. MDR1-Pgp and MRP1 efflux systems were reported to be engaged by CalCγ1 in the resistant HL60 cell line but only MDR1-Pgp over-expression could abrogate drug cytotoxicity in MDR cells [27].

The expression of multi drug resistance proteins P-gp, MRP1 and MRP2 was characterized in CD33 + cell lines and AML samples. The MRP inhibitor MK-571 showed cytotoxicity to GO in MRP-positive NB4 and HL-60 cells and the Pgp inhibitor cyclosporine (CSA) increased susceptibility to GO in P-gp-positive/MRP-positive TF1 cells [28]. In a case study, it was reported that MRP activity in all patient samples and 17 out of 23 patients showed Pgp activity and further 12 pgp positive samples were found sensitive after treatment with CSA. This proves the important role of multi-drug resistance genes in gaining cancer resistance.

Gelonin is a RIP from the seeds of *Gelonium multiflorum* used in the immunotoxin. The IT HuM195-gelonin that consists of a humanized mAb specific for CD33 conjugated to a recombinant gelonin toxin. P-gp was reported to be involved in resistance to HuM195-gelonin immunotoxin [25]. Resistance to HuM195-gelonin and to free rGelonin was also reported in The human leukemic cell lines HL60 and K562 by mediating multi-drug resistance through over expression of the P-glycoprotein (P-gp). Inhibiting the function of P-gp was shown to reverse resistance to IT. However, they showed that the same cells were sensitive to other protein synthesis inhibitors like cycloheximide, saponin, and *Pseudomonas* exotoxin A [29].

2.3.3 Lysosomal Degradation

Lysosomes are involved in degrading internalized exogenous macromolecules which include active immunotoxin in the cytosol. Weldon et al. [30] observed that PE-based immunotoxin B3(dsFv)-PE38 targeting CD22 was susceptible to the lysosomal degradation pathway in CD22-positive human Burkitt lymphoma cell lines. Resistance to gelonin-based IT was suggested to be mediated by increased lysosomal degradation [29]. Misfolded proteins are cleared by the ER and translocated to the proteasome in the cytosol for degradation by retrotranslocation via a pathway known as ER associated degradation (ERAD). IT is known to exert its activity by translocation from the ER to the cytosol. AB toxins were suggested to act by mimicking misfolded proteins and entering the ERAD pathway to the cytosol [31]. However, this will also result in the majority of proteins being degraded through this pathway which is one of the reasons for resistance.

Resistance of myeloid cells to ricin A-chain IT CD33, p67-7·dgA, was attributed to fast and efficient lysosomal degradation. The IT was found to bind to HL60 cells but was not capable of killing whereas anti-TfR immunotoxin could kill the cells [32].

2.3.4 Other Factors

In a recent study, Moxetumomab pasudotox (HA22), an anti-CD22 Fv fused to *Pseudomonas* exotoxin A, showed resistance in KOPN-8 cells due to methylation of the CpG island in the DPH1 promoter that was overcome by the methylation inhibitor 5-Azacytidine [33]. Earlier Wei et al. [34] showed that HA22 resistant cell lines had low levels of DPH4 expression which in turn prevents diphthamide biosynthesis in ALL. The CpG island in the promoter region of *DPH4* gene was hypermethylated in resistant cells which was reversed when incubated with 5-azacytidine.

The PE conjugated IT targeting CD22 was found to be effective in drug resistant hairy cell leukemia but not in acute lymphoblastic leukemia. In a recent work, Wei et al. [35] claimed that resistance was due to the failure of the immunotoxin to ADP-ribosylate and inactivate EF2 in the HA22-resistant lymphoma cell line. They showed that this was, in turn, owing to the deletion of the diphthamide synthesis gene *WDR85,* which results in the modification of diphthamide in EF2 and, thus, cannot be inactivated by the immunotoxin.

The PI3K/AKT signaling pathway is actively involved in cell growth, survival and apoptosis mediated by AKT phosphorylation. The activation of the AKT signaling pathway has been correlated with failure to therapy in AML. Recently, Rosen et al., [36] noted the association of AKT signaling with GO resistance *in vitro* using the single cell network profiling (SCNP) assays with the AKT inhibitor, MK-2206, in AML samples.

2.4 Cancer Stem Cells and Resistance

The involvement of cancer stem cells (CSC's) in tumor recurrence and invasion has been a long debated concept. Several studies have proved the presence of cancer stem cells in drug resistant cancers convincingly (Fig. 2.1). In a recent report by Ding et al., [37], CSC's have been proven to be involved in trastuzumab resistance in mammary carcinoma cells. Cojoc et al., [38] have extensively reviewed the mechanisms of resistance in cancer stem cells.

2.4.1 Drug Efflux

Cells that efflux drugs termed as side population (SP) are identified in resistant cancer cells. In cancer stem cells these MDR proteins are up-regulated and, thus, show resistance towards many chemotherapeutic drugs. The ABCG2 transporter has been reported to be involved in resistance to various drugs like methotrexate, doxorubicin, imatinib, daunorubicin, topotecan, mitoxantrone etc [39]. The ABC family of transporters was also associated with resistance in targeted therapies such as the ty-

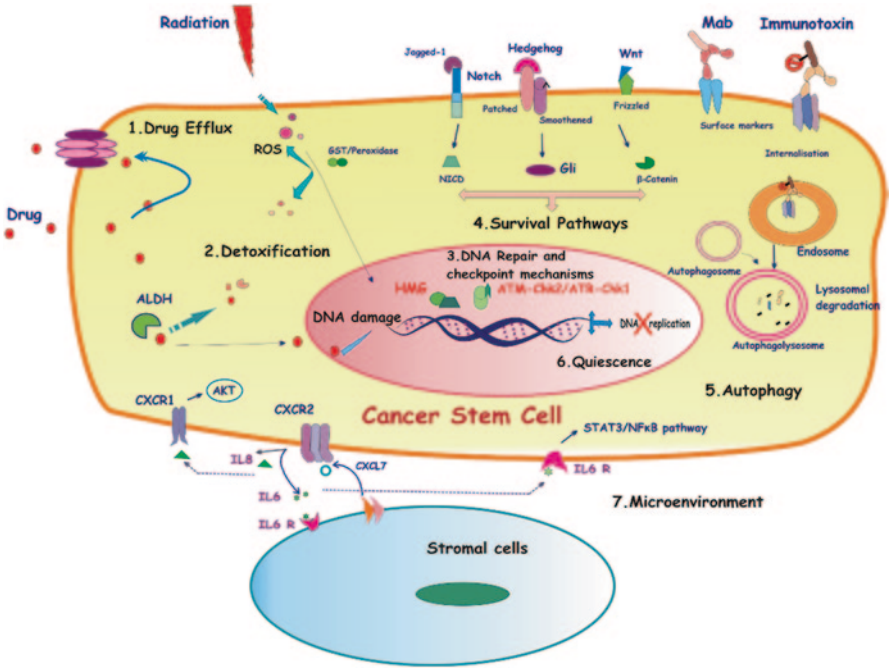

Fig. 2.1 Mechanisms involved in cancer stem cell resistance. *1* Small molecule drugs are effluxed by the membrane transporters P-gp/MDR1, MRP1 and BCRP/ABCG2. *2* Some drugs are detoxified by the enzyme ALDH. ROS generated during radiation therapy are scavenged by enzymes like Glutathione S transferase, Peroxidase and Catalase. *3* DNA damage induced by radiation therapy or drugs is repaired by HMG proteins and the cell cycle checkpoint mechanisms ATM-Chk2 and ATR-Chk1 are activated. *4* Anti-apoptotic and developmental signaling pathways needed for survival like Notch, Wnt and Hedgehog pathways are activated. *5* Intracellular digestion by autophagy is activated by fusion of autophagosomes with endosomes and lysosomes. Targeted therapy using monoclonal antibodies and immunotoxins lead to specific binding to cell surface markers but are internalized and could be degraded by lysosomal enzymes. *6* CSC's adopt quiescence by shutting down replication and remaining in a dormant state with no metabolic activity. *7* The cancer microenvironment protects CSC's from therapeutics or other stress. The CSC niche includes stromal fibroblasts, immune cells, mesenchymal stem cells, extra cellular matrix, growth factors and cytokines released by these cells and also physiologic factors like hypoxia and pH

rosine kinase inhibitors Sorafenib, Imatinib, Nilotinib, Gefitinib and Erlotinib [38]. Cancer stem cells use drug efflux mechanisms actively to prevent the drug from acting and this property is used to even sort CSC populations based on their ability to efflux dyes like Hoechst 33342.

2.4.2 Detoxification and Cellular Repair

Resistance to radiation therapy has been associated with increased involvement of ROS scavenging mechanisms which enhance cell survival by eliminating ROS

generated during therapy. The level of glutathione, one of the ROS scavengers has been correlated in gastrointestinal cancer cells and HNSCC. Other genes involved in ROS scavenging like *superoxide dismutase*, *glutathione peroxidase* and *catalase* were reportedly upregulated in CD44+CD24−breast CSCs [38]. CSC's were consistently found to display high ALDH activity which is involved in detoxification and are associated with drug resistance. Levels of ALDH1 have been frequently used as a marker for identification and sorting of CSC's. Administration of ALDH1 inhibitors could reduce tumor growth and resistance [40].

Damage-associated molecular patterns (DAMP's) are molecules released by damaged cells that initiate repair and survival mechanisms in cells. These molecules are recognized by pattern recognition receptors (PRR). Hombach-Klonisch et al. [41], suggest that DAMP signaling via several PRR may be one of the major tumor survival response associated with cell proliferation, inflammatory and autophagy responses in cancer stem cells.

2.4.3 DNA Repair and Modification

Cancer stem cells often display highly efficient DNA repair systems. Higher expression of DNA repair genes have been reported to be involved in chemo-resistance. Cells with higher expression of High Mobility Group (HMG) proteins show enhanced DNA repair mechanisms and thus evade killing by drugs. Cojoc et al. [38] has reviewed several studies which showed that DNA repair mechanisms are activated in cancer stem cells in glioma, nasopharyngeal carcinoma, lung, breast and mouse mammary tumors. Also, the checkpoint mechanisms have been induced in cancer stem cells via the kinase signaling pathways ATM-Chk2 and ATR-Chk1.

In another study genes involved in chromatin modification, such as *KDM5A/Jarid1A*, a *histone H3K demethylase* and *histone deacetylases (HDACs)* were over expressed in drug resistant cells in Non-small cell lung cancer cell lines [42]. Thus, chromatin modifications and epigenetic changes can be partly responsible for resistance [43]. The unlimited replicative potential is one of the hallmarks of cancer and it requires activation of telomere maintenance mechanisms (TMMs). Two TMMs are currently known in human cancer, namely, telomerase activity and the alternative lengthening of telomere (ALT) mechanisms. Although both TMMs appear to be equivalent in their ability to support immortalization, their contribution to tumor growth and survival and consequently patients' prognosis may differ [44].

Telomeres are specialized DNA-protein structures located at the end of eukaryotic chromosomes. They are essential for continued cell proliferation. Indeed, telomere attrition, which occurs within each cell division, represents a molecular clock that counts the number of times a cell can divide and determines its entry into senescence [45]. Other than acting as a mitotic clock, telomeres play an important role in the maintenance of genomic integrity. As suggested by Feijoo et al. [46], telomere erosion in a context of impaired cell cycle checkpoint may constitute an important mechanism during tumoriogenesis.

2.4.4 Survival Pathways

Different studies have proven the involvement of developmental pathways, wingless-type MMTV integration site family (WNT), Notch signaling and Hedgehog pathways in cancer stem cells and resistance. These pathways are involved in self-renewal of normal stem cells. Activators of Wnt, Notch and Hedgehog signaling pathways could induce proliferation of HSC's. Inhibition of β-catenin of the Wnt pathway by axin was found to reduce the self-renewal capacity [47]. Cojoc et al. [38] in his review compiled the studies that have shown over expression of genes involved in these pathways in cancer stem cells. Inhibitors of these pathways like gamma-secretase inhibitors (GSIs) and cyclopamine rendered the cells susceptible to treatment.

2.4.5 Autophagy and EMT

Autophagy is a lysosomal degradation pathway which is involved in the degradation of intracellular materials and the removal of damaged organelles, protein aggregates or microbes. It plays a major role in cell survival in metabolic stress and preventing apoptosis in cancer cells. The epithelial Mesenchymal transition (EMT) which has also been associated with resistance to cancer therapy is known to be related to autophagy and stemness. Cancer stem cells utilize alternative mechanisms of survival to manage environmental stress, autophagy being one of them. Cojoc et al. [38], have shown increased resistance in prostate and pancreatic cancer cell lines by the induction of autophagy by Neurophilin-2.

2.4.6 Quiescence

One of the important features of CSC's is their dormancy or quiescence. Dormant CSC's are extremely slow cycling with an arrest in the GO phase and, hence, have a minimum energy requirement. They have been reported to show highest capacity for self-renewal. Quiescence was considered as a major factor responsible for the ability of CSC's to survive harsh conditions and anti-cancer therapy. Drug resistance was suggested to be due to the fact that most drugs target DNA replication and proliferation of cells or metabolic pathways which are greatly reduced in these cells. Their dormancy could be broken by the addition of cytokines involved in activating dormant CSC's during injury like G-CSF and IFNα, which induced proliferation. This has been used as a strategy to eliminate CSC's [48].

2.4.7 Microenvironment

CSC's are often hidden in the hypoxic core of cancer tissue in a unique niche that contributes to its survival. This niche called the microenvironment is vital for their

existence as they are surrounded by stromal fibroblasts and an extra cellular matrix (ECM) that release cytokines and signaling factors. Tumor and stromal-derived factors have been shown to play a key role in CSC maintenance and therapy resistance. CXCL12/CXCR4 signaling and TGF-β/SMAD signaling are major pathways induced by these growth factors. Growth factors released in the tumor niche like PDGF, IL1β, TNF, TGF-β, chemokine CXCL12 and MMPs were involved in the development and regulation of CSC's [38].

CSC's are protected from the environmental stress and attack by therapeutic agents in the microenvironment. One of the major reasons for radio resistance was found to be hypoxia in the CSC niche since oxygen is required for radiation-induced killing. Also, the hypoxia-inducible factor (HIF) signaling is activated in hypoxic condition which in turn activates survival pathways.

2.5 Strategies Used to Overcome Resistance

2.5.1 Inhibitors of Anti-apoptotic Proteins

Blocking anti-apoptotic proteins Bcl-2, Bcl-xl, and Bcl-w is an important strategy used by many investigators to combat resistance. Oblimersen, was used to target Bcl-2, showed improvement in GO treatment in 25 % of AML patients in a phase II clinical trial [49]. IL-2/granzyme A fusion protein improved doxorubicin sensitivity of the MDR + lm1-mdr cell line by inducing caspase-independent apoptosis [50].

In a detailed study by Traini et al. [26], ABT-737 and PE immunotoxin could inhibit apoptosis in combination. ABT-737 could bind to the hydrophobic core of Bcl-2 proteins Bcl-2, Bcl-xl, and Bcl-w while the immunotoxin degraded Mcl-1 protein, thus releasing the inhibition of the apoptotic pathway. ABT-263 as well as ABT-737 have been reported to show synergistic killing with PE immunotoxin targeting transferring receptor in Small Cell Lung Cancer (SCLC) cell lines that were otherwise resistant to the immunotoxin. Killing was observed in 6 h with loss of Mcl-1. The same effect was observed *in vivo* also when the immunotoxin was administered in combination with ABT-737 in nude mice with H69AR tumor [51].

TNF-related apoptosis-inducing ligand (TRAIL) reportedly induces apoptosis independent of major pathways controlling chemotherapy resistance [52]. The MDR + subline MDR-U2OS was shown to be TRAIL sensitive due to reduced AKT activation [53]. Apoptosis could be induced in resistant cells with low Bak using mesothelin conjugated with anti-TRAIL receptor 2 [54]. Anti-sense oligonucleotides have been employed in clinical studies to sensitize cancer cells to apoptotic triggers [55, 56]. However, there is emerging evidence to support the novel mechanism of death inhibition by Bcl-2 involving its ability to modulate cellular redox status and mitochondrial metabolism.

2.5.2 Blocking Membrane Drug Transporters

Hamada and Tsuruo [57] developed two monoclonal antibodies (MRK16 and MRK17) against the membrane transporter P-glycoprotein. Fitzgerald et al. [58], reported the use of MRK16 coupled with PE toxin in killing multi-drug resistant KB cell lines. The anti-P-glycoprotein monoclonal antibody MRK16 could overcome bone marrow resistance against daunomycin, doxorubicin, vincristine, vinblastine, etoposide, and taxol in multi-drug resistant transgenic mice. The MRK16-PE conjugate was also successfully shown to kill bone marrow cells in a dose-dependant manner [59]. MRK16 was used along with Saponin immunotoxin that could eliminate 99% of MDR cells [60]. A recombinant single-chain Fv fragment against P-gp was developed by Niv et al. [61].

It was proven that the combination of antibody conjugates with chemosensitisers (cyclosporin A, D, G) that block P-gp transporters restored the sensitivity of MDR cell lines [62]. Two inhibitors of ABCG2 and ABCB1 transporters, GF120918 and tariquidar, have been approved for clinical studies. Although CD33 is expressed in 90% of AML patients, more than 50% of patients show remission due to GO resistance mediated via the membrane transporters [63]. Addition of U0126, a MEK1/2 inhibitor, was reported to prevent GO resistance induced in HL-60/GO resistant cells. Combination of the MDR modifiers PSC833 or MS209 with Gemtuzumab ozogamicin (CMA-676) was observed to reverse resistance in CD33+AML with P-gp-related MDR [64] by inhibiting the efflux of therapeutic agents.

2.5.3 Delivery and Intracellular Trafficking

HPMA hydrogels were used successfully to prolong the delivery of antibody-drug conjugates with different targeting moieties (anti-CD71, anti-thymocyte globulin, anti-CD4, transferrin) tested on human multidrug resistance (MDR) cell lines [62]. The trafficking route *via* specific organelles was found to play a major role in the case of LMB2, an IT comprising PE38 and an Fv against IL2 receptor [65].

ABT-737 showed 20-fold enhanced killing of resistant cell lines by PE IT's by increasing the delivery of IT from the ER to the cytosol by a mechanism poorly understood. However, it was hypothesized that ABT-737 induces ER stress and facilitates its transport to the cytosol [26]. Recently, IT named RG7787, a PE-based toxin targeting mesothelin was reported to be efficient due to resistance to lysosomal degradation in breast and gastric cancers [66].

2.5.4 Inhibition of DNA Repair and Telomerase Activity

Inhibition of DNA repair and DNA damage checkpoint mechanisms like the kinase pathways ATM-Chk2 and ATR-Chk1 were utilized by some workers. The chk1 inhibitor AZD7762, debromohymenial-disine (DBH) that inhibits both Chk1

and Chk2 kinases and the ATM inhibitor KU55933 were effective against resistant populations along with chemo or radiotherapy [67]. Santambrogio et al. [68] used microRNAs to impair telomerase activity or to affect telomere functions in cancer cells. Crees et al. [69], Romaniuk et al. [70] and Uziel and Lahav [71] described the approaches developed during the last decades to inhibit telomerase, aimed to interfere with the enzyme's catalytic activity. Overall, accumulating evidence from preclinical studies on the effects of telomerase inhibition in human cancer has provided persuasive arguments to indicate that the enzyme is a well-validated cancer target and an ideal tumor-associated antigen [45].

2.5.5 Combination Therapy

Two treatment strategies were devised based on cancer cell genetic findings. It involves the serial use of two anti-cancer drugs, the first to weaken or "prime" the cancer cells, and the second to deliver an added impact. To prime the cancer cells, researchers used Palbociclib (which selectively inhibits two cell-cycle promoting proteins, CDK4 and CDK6) to slow down the cancer's growth and sensitize cells being targeted by the second drug. Previous clinical studies have shown that palbociclib itself can significantly inhibit the growth of mantle cell lymphoma. In the cells with a mutated BTK, palbociclib was administered first, and then the second drug idelalisib. In lymphoma cells lacking the BTK mutation, the investigators also started with palbociclib, followed by ibrutinib, since both drugs are well tolerated by the patients.

7-hydroxystaurosporine (UCN-01) is a novel protein kinase inhibitor that increases chemotherapy-induced apoptosis *in vitro* and is in early phases of clinical development [72]. *In vitro*, UCN-01 is synergistic with multiple cytotoxic agents and increases fludarabine-induced apoptosis in a human breast cell line. These results suggest that UCN-01 sensitized the lymphoma to the cytotoxic effects of EPOCH, possibly by modulating the "threshold" for apoptosis, and may illustrate a new paradigm for reversal of drug resistance.

Immunotoxins were also used in combination with other drugs. Anti-CD138 IT B-B4-SO6 with doxorubicin were used as a combination therapy for the drug-resistant multiple myeloma (MM)-derived cell line RPMI8226. The authors conclude that combination of IT and chemotherapy could prevent drug resistance that arises due to exposure to chemotherapy alone [73]. In another early study, the combination of ricin conjugated IT targeting CD19 (anti-B4 blocked ricin) combined with drugs like cisplatin, cyclophosphamide and etoposide showed long term cure *in vivo* in SCID mice with disseminated tumors of the multidrug-resistant human B-cell lymphoma Namalwal/mdr-1 [74].

IT containing anti-melanoma antibody ZME-018 recognizing a 240-kDa surface glycoprotein (gp 240) and the plant toxin gelonin was tested in resistant human melanoma cells (A375-M). Combination with cisplatin, IFN-γ, IFN-α, and etoposide were observed to enhance the cytotoxic effects of ZME-gelonin against resistant cells [75]. Other combinations with GO include G-CSF that induced AML cells

to enter G2/M and hypodiploid phase and Valproic acid, a histone deacetylase inhibitor [63]. As mentioned earlier, the combination of ABT-737 with immunotoxin could enhance killing by 20-fold in resistant cell lines by neutralizing anti-apoptotic proteins and by increasing the delivery of the immunotoxin from the ER to the cytosol [26].

2.5.6 Nanotechnology

Nanotechnology holds great promise in establishing efficacious, innovative strategies to overcome chemoresistance and may facilitate complementary treatment methods and cancer diagnostics. Various nanomedical devices are being introduced and evaluated, demonstrating encouraging results. While stealth liposomes serve as a benchmark, astonishing progress is witnessed in polymeric nanovehicles. It can be also combined with low molecular weight surfactants, inhibiting drug resistance in addition to solubilizing drugs. A nanocrystalline silver particle (8 nm) modified with TAT (AgNP-TAT) was developed for MDR cancer cell treatment. The antitumor activity was reported in both MDR cells and non- resistant cells [76]. AgNP-TAT showed significant enhancement in tumor cell killing, up to 24-fold higher cytotoxic effect compared to its counter-part lacking the TAT conjugation. AgNP-TAT NPs were able to effectively inhibit tumor growth in mice bearing malignant melanoma at a dose of 1 nmol/kg (compared with 4.3 μmol/kg of DOX), and showed significantly reduced adverse toxicity *in vivo* [77]. Various nanoparticle-based approaches have been investigated to overcome efflux-mediated resistance. These include the use of formulation excipients that inhibit transporter activity and co-delivery of the anticancer drug with a specific inhibitor of transporter function or expression [78].

2.5.7 Other Novel Strategies

Oncolytic viruses (OV) are promising anti-cancer agents, capable of selectively targeting replication in tumor cells. Genetically modified oncolytic viruses (OVs) kill tumor cells via completely unique mechanisms compared to small molecule chemotherapeutics typically used in lung cancer treatment and can also be used to deliver specific toxic, therapeutic or immunomodulatory genes to tumor cells. Recent pre-clinical and clinical studies with oncolytic vaccine approaches have revealed promising combination strategies that enhance oncolysis of tumor cells and circumvent tumor resistance mechanisms [79]. Synergistic effects of therapy based on combining OV and various cytostatics are in preclinical studies and have shown promising results.

Over-expression of recombinant GlcCer synthase (GCS) confers resistance to adriamycin and to ceramide in GlcCer synthase-transfected human breast cancer cells, suggesting that drug resistance is related to stimulation of glycosylation of

ceramide and the resultant inhibition of drug induced apoptotic signalling. Blocking glycosylation of ceramide has been shown to increase cancer cell sensitivity to cytotoxic drugs. Drug combinations that enhance ceramide generation and limit glycosylation have been shown to enhance effectiveness of chemotherapy by inducing apoptosis in cancer cell models

Targeting intracellular compartments is another challenging approach. A particularly interesting direction which shows promise for targeted anticancer nanomedicine is the use of viral components against drug resistant cancer cells. Hence, newly discovered anticancer- and antimetastatic drugs may be combined with a broad spectrum of molecules which includes small-molecule inhibitors, interfering RNA molecules, microRNA, oncolytic viruses, and also naturally occurring substances. This combination with anti-inflammatory and adjuvant therapies seems to be a very promising treatment approach [80].

2.6 Targeting Cancer Stem Cells

It is clear from various studies that cancer stem cells play a major role in resistance against all kinds of therapy. Almost all the factors listed as responsible for resistance in IT therapy are found to overlap in CSC's. It is also quite evident that CSC's are not only responsible for resistance against chemo and radiotherapy but also against IT therapy since they can use any of the following ways to handle IT's conveniently:

i) They can be protected from exposure to IT in their microenvironment
ii) Efflux IT's using membrane transporters,
iii) Utilize autophagy to degrade and get rid of recombinant IT's,
iv) Use detoxification and repair pathways to circumvent the damage,
v) Recruit anti-apoptotic proteins to prevent apoptosis,
vi) Use alternative survival pathways to escape cell death and
vii) Remain quiescent with inactive cellular machinery.

Although the strategies mentioned earlier have been successful to some extent in avoiding resistance, it is highly unlikely that these strategies alone would be completely effective in dealing resistance since the major contributor to resistance remains hidden and active. Hence, currently several groups are studying the possibility of targeting CSC's to destroy cancer permanently. The targets include proteins involved in signaling pathways in CSC's like WNT, NOTCH and Hedgehog pathways. drug transporters, CSC specific surface markers, ALDH, quiescence factors, anti-apoptotic proteins and factors involved in the CSC niche [81, 82].

2.6.1 Targeting Signaling Pathways in CSC's

Various modes of therapies are being investigated to kill CSC's which that have been summarized in the review by Han et al. [81]. Inhibition of the Hedgehog path-

way with drugs like GDC-0449, LDE225 and GSIs like RO4929097 and MK-0752 have been used along with chemotherapy with paclitaxel, carboplatin, capecitabine, cinblastine, gemcitabine and temozolo-mide [38]. The steroid-like compound, cyclopamine, was used to target SMO of hedgehog signaling that could eliminate prostate cancer cells in mice xenograft tumors *in vivo* and was shown to be effective in killing CSC's in glioma sphere cells. Arsenic trioxide (As2O3) inhibits the glioma-associated oncogene homolog (Gli) and has been used in combination with the SMO inhibitors cyclopamine and GDC-0449. (-)-epigallocatechin-3-gallate (EGCG) with quercetin could inhibit self- renewal capacity in CSC's by inhibiting the sonic hedgehog (SHh) pathway.

Inhibition of the Notch signaling using the γ-secretase inhibitor GSI-18 could eliminate CD133 + medulloblastoma cells while MRK-003 was effective in killing CSC's in breast cancer. The Wnt signaling pathway has also been widely targeted like cAMP response-element binding protein (CBP)/b-catenin antagonist ICG-001, used to target leukemic stem cells [81]. Targeting mTOR involved in PI3/AKT using rapamycin could deplete leukemic stem cells [82]. The Notch signaling pathway has been inhibited by several other groups using GSI, siRNA or antibody against the Notch ligand, delta-like 4 ligand (DLL4), which either reduced the CSC population or rendered the CSC's susceptible to drug therapy [38]. Recent study have reported that c-Met silencing could inhibit CSC's in head and neck squamous carcinoma by down regulation of the Wnt/β-catenin signaling [83].

NF-κB is activated during lymphoid development and is used as a target in few studies. Inhibition of NF-κB activation using the proteasome inhibitors, bortezomib or MG-132, and inhibition of IκB kinase (IKK) by Parthenolide were used to target AML stem cells. However, CML stem cells were resistant to ABL kinase inhibitors imatinib and its derivative nilotinib [47].

2.6.2 Targeting Apoptosis and Cellular Repair Mechanisms in CSC's

Inducing apoptosis by MSC's expressing TNF-related apoptosis-inducing factor (TRAIL) along with mitoxantrone was effective in putative CSC's. In our laboratory, Madhumathi et al., (unpublished data) have successfully used TRAIL-based immunotoxins to induce apoptosis in CSC's isolated from leukemic cell lines by culturing cells in the presence of methotrexate. The Methotrexate resistant side population was found to be enriched in the CSC population. Inducing apoptosis selectively in CSC's using IT's conjugated with TRAIL, targeting different surface markers of CSC's has been a promising strategy used in our laboratory for all cancers.

Inhibition of ALDH activity using all-trans retinoic acid (ATRA), synthetic retinoids, disulfiram, 4- diethylaminobenzaldehyde (DEAB) or ALDH1A1 shRNA were used in combination with chemotherapeutic drugs in various studies. Targeting ROS scavengers by buthionine sulfoximine (BSO) reduced radioresistance in CSC's by inhibiting glutamate-cysteine ligase [38]. Sorafenib and sulforaphane could be used to inhibit ALDH1 activity and thus was postulated as potential drugs for CSC's [40].

2.6.3 Targeting Autophagy and Microenvironment in CSC's

Autophagy has been inhibited in another strategy of killing CSC's using lysosomotropic anti-malaria drug chloroquine/hydroxychloroquine. Targeting tumor microenvironment or hypoxic niche by improving tumor oxygenation has also been tested along with radio and chemotherapy. Inhibition of cytokine and chemokine receptors like IL-8 receptor CXCR1 by antibody or by repertaxin was successful in reducing breast CSC's. Inhibition of TGF-β/SMAD pathway also showed reduction in CSC's. Mab against VEGF, bevacizumab in mice glioma cell xenografts could decrease CD133+ cancer stem cells by anti-angiogenesis while treatment with IFN-α alone could kill side population in ovarian cancer [82]. Quiescence of CSC's has been inhibited using Arsenic trioxide, G-CSF or IFN α as an alternative strategy [48].

2.6.4 Targeting Membrane Transporters and CSC Surface Markers

ABC transporters have been inhibited by drugs like phosphodiesterase-5 inhibitors and fumitremorgin-type indolyl diketopiperazine, dofequidar fumarate, Ko143, ABCG2 siRNA, or ABCG2 inhibitor YHO-13351, in different types of cancers which could be used to target CSC's since they over-express these transporters [38]. The monoclonal antibody (Mab) H90 targeting CD44 could bind and kill leukemic stem cells in AML *in vivo*. Since GO targets the CD33 receptors which are highly expressed in CSC's, it was presumed that the activity of GO could be due to killing of CD33 + AML stem cells. Micro-RNAs have also been shown to be involved in inhibiting CSC's [84, 85]. Lentiviral-mediated shRNA was used to target the neuronal cell surface adhesion molecule LiCAM in CD133 + glioma stem cells [86].

Antibodies against other cell surface molecules like VLA-4 and CLL-1 (C-type lectin-like molecule-1) are being evaluated as potential targets [47]. Immunotoxins targeting CSC's have been recently developed using ligands or antibodies that specifically bind CSC's. IL3 conjugated with diphtheria toxin (DT), targeting CD123 receptor that is over-expressed in leukemic stem cells, has been used for AML [87].

2.7 Conclusion

It is evident from the factors involved in resistance, that CSC's are the major contributors of therapy resistance for all kinds of treatments—either conventional or targeted therapies. Many mechanisms observed in immunotoxin resistance were also identified as a major feature of CSC's like drug efflux, anti-apoptotic pathways, lysosomal degradation, etc. Thus, it could be concluded that CSC's are responsible for resistance against all treatment modalities since they have innumerable ways to

handle all kinds of stress. They have mechanisms to evade any attack in order to survive in adverse conditions. Targeting two or three of these key survival strategies together by means of combination therapies would be ideal in managing cancer in the future, instead of targeting only one factor. Targeted therapy using immunotoxins should be combined with blocking other alternative survival pathways for maximum efficacy in treatment.

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References

1. Harris AL, Hochhauser D Mechanisms of multidrug resistance in cancer treatment. Acta Oncol. 1992;31(2):205–13.
2. Jamroziak K, Robak T. Pharmacogenomics of MDR1/ABCB1 gene: the influence on risk and clinical outcome of haematological malignancies. Hematology. 2004;9(2):91–105.
3. Leighton JC Jr, Goldstein LJ. P-glycoprotein in adult solid tumors. Expression and prognostic significance. Hematol Oncol Clin North Am. 1995;9(2):251–73.
4. Hanahan D, Weinberg RA. Hallmarks of Cancer: the next generation. Cell. 2011;144(5):646–74.
5. Giaccia AJ, Schipani E. Role of carcinoma-associated fibroblasts and hypoxia in tumor progression. Curr Top Microbiol Immunol. 2010;345:31–45.
6. da Silva CG, Minussi DC, Ferran C, Bredel M. A20 expressing tumors and anticancer drug resistance. Adv Exp Med Biol. 2014;809:65–81.
7. Pujari R, Hunte R, Khan WN, Shembade N. A20-mediated negative regulation of canonical NF-κB signaling pathway. Immunol Res. 2013;57(1–3):166–71.
8. Perez-Galan P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. Blood. 2011;117:26–38.
9. Liang DC, Shih LY, Hung IJ, Yang CP, Chen SH, Jaing TH, Liu HC, Wang LY, Chang WH. FLT3-TKD mutation in childhood acute myeloid leukemia. Leukemia. 2003;17(5):883–6.
10. Piovan E, Yu J, Tosello V, Herranz D, Ambesi-Impiombato A, Silva AC D, Sanchez-Martin M, Perez-Garcia A, Rigo I, Castillo M, Indraccolo S, Cross JR, de Stanchina E, Paietta E, Racevskis J, Rowe JM, Tallman MS, Basso G, Meijerink JP, Cordon-Cardo C, Califano A, Ferrando AA. Direct reversal of glucocorticoid resistance by AKT inhibition in acute lymphoblastic leukemia. Cancer Cell. 2013;24(6):766–76.
11. Small S. Ibrutinib approved for the treatment of mantle cell lymphoma. Clin Adv Hematol Oncol. 2013;11(12):808.
12. Wilson WH, Sorbara L, Figg WD, Mont EK, Sausville E, Warren KE, Balis FM, Bauer K, Raffeld M, Senderowicz AM, Monks A. Modulation of clinical drug resistance in a B cell lymphoma patient by the protein kinase inhibitor 7-hydroxystaurosporine: presentation of a novel therapeutic paradigm. Clin Cancer Res. 2000;6(2):415–21.
13. Chiron D, Liberto M D, Martin P, Huang X, Sharman J, Blecua P, Mathew S, Vijay P, Eng K, Ali S, Johnson A, Chang B, Ely S, Elemento O, Mason CE, Leonard JP, Chen-Kiang S. Cell-cycle reprogramming for PI3K inhibition overrides a relapse-specific C481S BTK mutation revealed by longitudinal functional genomics in mantle cell lymphoma. Cancer Discov. 2014;4(9):1022–35.
14. Williams AB, Nguyen B, Li L, Brown P, Levis M, Leahy D, Small D. Mutations of FLT3/ITD confer resistance to multiple tyrosine kinase inhibitors. Leukemia. 2013;27(1):48–55.

15. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell. 1986;47(3):381–9.
16. Coley HM. Overcoming multidrug resistance in cancer: clinical studies of p-glycoprotein inhibitors. Methods Mol Biol. 2010;596:341–58.
17. Pietro A D, Dayan G, Conseil G, Steinfels E, Krell T, Trompier D, Baubichon-Cortay H, Jault J. P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure-function relationships. Braz J Med Biol Res. 1999;32(8):925–39.
18. Sarkadi B, Ozvegy-Laczka C, Nemet K, Varadi A. ABCG2- a transporter for all seasons. FEBS Lett. 2004;567:116–20.
19. Yasuda H. Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. Nitric Oxide. 2008;19(2):205–16.
20. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev. 2007;26(2):225–39.
21. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 1994;269(38):23757–63.
22. Walter RB, Raden BW, Kamikura DM, Cooper JA, Bernstein ID. Influence of CD33 expression levels and ITIM-dependent internalization on gemtuzumab ozogamicin-induced cytotoxicity. Blood. 2005;105:1295–302.
23. Barok M, Joensuu H, Isola J. Trastuzumab emtansine: mechanisms of action and drug resistance. Breast Cancer Res. 2014;16(2):209.
24. Haag P, Viktorsson K, Lindberg ML, Kanter L, Lewensohn R, Stenke L. Deficient activation of Bak and Bax confers resistance to gemtuzumab ozogamicin-induced apoptotic cell death in AML. Exp Hematol. 2009;37(6):755–66.
25. Linenberger ML. CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. Leukemia. 2005;19(2):176–82.
26. Traini R, Ben-Josef G, Pastrana DV, Moskatel E, Sharma AK, Antignani A, Fitzgerald DJ. ABT-737 overcomes resistance to immunotoxin-mediated exotoxin-based proteins to the cell cytosol apoptosis and enhances the delivery of pseudomonas. Mol Cancer Ther. 2010;9(7):2007–15.
27. Cianfriglia M, Mallano A, Ascione A, Dupuis ML. Multidrug transporter proteins and cellular factors involved in free and mAb linked calicheamicin-Á1 (gentuzumab ozogamicin, GO) resistance and in the selection of GO resistant variants of the HL60 AML cell line. Int J Oncol. 2010;36(6):1513–20.
28. Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID, Linenberger ML. Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. Blood. 2003;102(4):1466–73.
29. McGrath MS, Rosenblum MG, Philips MR, Scheinberg DA. Immunotoxin resistance in multidrug resistant cells. Cancer Res. 2003;63(1):72–9.
30. Weldon JE, Xiang L, Chertov O, Margulies I, Kreitman RJ, FitzGerald DJ, Pastan I. A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity. Blood. 2009;113(16):3792–800.
31. Hazes B, Read RJ. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. BioChemistry. 1997;36(37):11051–4.
32. Engert A, Brown A, Thorpe P. Resistance of myeloid leukaemia cell lines to ricin A-chain immunotoxins. Leuk Res. 1991;15(11):1079–86.
33. Hu X, Wei H, Xiang L, Chertov O, Wayne AS, Bera TK, Pastan I. Methylation of the DPH1 promoter causes immunotoxin resistance in acute lymphoblastic leukemia cell line KOPN-8. Leuk Res. 2013;37(11):1551–6.

34. Wei H, Xiang L, Wayne AS, Chertov O, FitzGerald DJ, Bera TK, Pastan I. Immunotoxin resistance via reversible methylation of the DPH4 promoter is a unique survival strategy. Proc Natl Acad Sci U S A. 2012;109(18):6898–903.
35. Wei H, Bera TK, Wayne AS, Xiang L, Colantonio S, Chertov O, Pastan IA. Modified form of diphthamide causes immunotoxin resistance in a lymphoma cell line with a deletion of the WDR85 gene. J Biol Chem. 2013;288(17):12305–12.
36. Rosen DB, Harrington KH, Cordeiro JA, Leung LY, Putta S, Lacayo N, Laszlo GS, Gudgeon CJ, Hogge DE, Hawtin RE, Cesano A, Walter RB. AKT signalling as a novel factor associated with in vitro resistance of human AML to gemtuzumab ozogamicin. PLoS ONE. 2013;8(1):e53518.
37. Ding K, Banerjee A, Tan S, Zhao J, Zhuang Q, Li R, Qian P, Liu S, Wu ZS, Lobie PE, Zhu T. Artemin, a member of the glial cell line-derived neurotrophic factor family of ligands, is HER2-regulated and mediates acquired trastuzumab resistance by promoting cancer stem cell-like behavior in mammary carcinoma cells. J Biol Chem. 2014;289(23):16057–71.
38. Cojoc M, Mäbert K, Muders MH, Dubrovska A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. Semin Cancer Biol. 2014;31:16–27.
39. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275–84.
40. Vinogradov S, Wei X Cancer stem cells and drug resistance: the potential of nanomedicine. Nanomedicine (Lond). 2012;7(4):597–615.
41. Hombach-Klonisch S, Natarajan S, Thanasupawat T, Medapati M, Pathak A, Ghavami S, Klonisch T. Mechanisms of therapeutic resistance in cancer (stem) cells with emphasis on thyroid cancer cells. Front Endocrinol (Lausanne). 2014;5:37.
42. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong KK, Brandstetter K, Wittner B, Ramaswamy S, Classon M, Settleman J. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell. 2010;141(1):69–80.
43. Borst P. Cancer drug pan-resistance: pumps, cancer stem cells, quiescence, epithelial to mesenchymal transition, blocked cell death pathways, persisters or what? Open Biol. 2012;2(5):120066.
44. Folini M Editorial: targeting telomere maintenance mechanisms in cancer therapy. Curr Pharm Des. 2014;20(41):6359–60.
45. Reddel RR. Telomere maintenance mechanisms in human cancer: clinical implications. Curr Pharm Des. 2014;20(41):6361–74.
46. Feijoo P, Dominguez D, Tusell L, Genesca A. Telomere-dependent genomic integrity: evolution of the fusion-bridge-breakage cycle concept. Curr Pharm Des. 2014;20(41):6375–85.
47. Krause DS, Van Etten RA. Right on target: eradicating leukemic stem cells. Trends Mol Med. 2007;13(11):470–81.
48. Essers MAG, Trumpp A. Targeting leukemic stem cells by breaking their dormancy. Mol Oncol. 2010;4:443 -450.
49. Moore J, Seiter K, Kolitz J, Stock W, Giles F, Kalaycio M, Zenk D, Marcucci G. A phase II study of Bcl-2 antisense (oblimersen sodium) combined with gemtuzumab ozogamicin in older patients with acute myeloid leukemia in first relapse. Leuk Res. 2006;30:777–83.
50. Grodzovski I, Lichtenstein M, Galski H, Lorberboum-Galski H. IL-2-granzyme A chimeric protein overcomes multidrug resistance (MDR) through a caspase 3-independent apoptotic pathway. Int J Cancer. 2011;128(8):1966–80.
51. Mattoo AR, FitzGerald DJ. Combination treatments with ABT-263 and an immunotoxin produce synergistic killing of ABT-263-resistant small cell lung cancer cell lines. Int J Cancer. 2013;132(4):978–87.
52. Secchiero P, Vaccarezza M, Gonelli A, Zauli G. TNF-related apoptosis-inducing ligand (TRAIL): a potential candidate for combined treatment of hematological malignancies. Curr Pharm Des. 2004;10(29):3673–81.

53. Cenni V, Maraldi NM, Ruggeri A, Secchiero P, Del Coco R, De Pol A, Cocco L, Marmiroli S. Sensitization of multidrug resistant human ostesarcoma cells to Apo2 Ligand/TRAIL-induced apoptosis by inhibition of the Akt/PKB kinase. Int J Oncol. 2004;25(6):1599–608.
54. Du X, Xiang L, Mackall C, Pastan I. Killing of resistant cancer cells with low Bak by a combination of an antimesothelin immunotoxin and a TRAIL receptor 2 agonist antibody. Clin Cancer Res. 2011;17:5926–34.
55. Yamanaka K, Rocchi P, Miyake H, Fazli LA, So A, Zangemeister-Wittke U, Gleave ME. Induction of apoptosis and enhancement of chemosensitivity in human prostate cancer LNCaP cells using bispecific antisense oligonucleotide targeting Bcl-2 and Bcl-xL genes. BJU Int. 2006;97:1300–8.
56. Indran IR, Tufo G, Pervaiz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim Biophys Acta. 2011;1807:735–45.
57. Hamada H, Tsuruo T Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. Proc Natl Acad Sci U S A. 1986;83(20):7785–9.
58. FitzGerald DJ, Willingham MC, Cardarelli CO, Hamada H, Tsuruo T, Gottesman MM, Pastan I. A monoclonal antibody-Pseudomonas toxin conjugate that specifically kills multidrug-resistant cells. Proc Natl Acad Sci U S A. 1987;84:4288–92.
59. Mickisch GH, Pai LH, Gottesman MM, Pastan I. Monoclonal antibody MRK16 reverses the multidrug resistance of multi-drug resistant transgenic mice. Cancer Res. 1992;52(16):4427–32.
60. Dinota A, Tazzari PL, Michieli M, Visani G, Gobbi, M, Bontadini A, Tassi C, Fanin R, Damiani D, Grandi M, Pileri S, Bolognesi A, Stirpe F, Baccarani M, Tsuruo T, Tura S. In vitro bone marrow purging of multidrug-resistant cells with a mouse monoclonal antibody directed against Mr 170,000 glycoprotein and Saporin-conjugated anti-mouse antibody. Cancer Res. 1990;50:4291–4.
61. Niv R, Assaraf YG, Segal D, Pirak E, Reiter Y. Targeting multidrug resistant tumor cells with a recombinant single-chain FV fragment directed to P-glycoprotein. Int J Cancer. 2001;94(6):864–72.
62. St'astný M, Strohalm J, Plocová D, Ulbrich K, Ríhová B. A possibility to overcome P-glycoprotein (PGP)-mediated multidrug resistance by antibody-targeted drugs conjugated to N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier. Eur J Cancer. 1999;35(3):459–66.
63. Takeshita A. Efficacy and resistance of gemtuzumabozogamicin for acute myeloid leukemia. Int J Hematol. 2013;97(6):703–16.
64. Matsui H, Takeshita A, Naito K, Shinjo K, Shigeno K, Maekawa M, Yamakawa Y, Tanimoto M, Kobayashi M, Ohnishi K, Ohno R. Reduced effect of gemtuzumabozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers. Leukemia. 2002;16(5):813–9.
65. Tortorella LL, Pipalia NH, Mukherjee S, Pastan I, Fitzgerald D, Maxfield FR. Efficiency of immunotoxin cytotoxicity is modulated by the intracellular itinerary. PLoS ONE. 2012;7:e47320.
66. Alewine C, Xiang L, Yamori T, Niederfellner G, Bosslet K, Pastan I. Efficacy of RG7787, a next generation mesothelin-targeted immunotoxin, against triple-negative breast and gastric cancers. Mol Cancer Ther. 2014;13(11):2653–61.
67. Folini M Editorial: targeting telomere maintenance mechanisms in cancer therapy. Curr Pharm Des. 2014;20(41):6359–60.
68. Santambrogio F, Gandellini P, Cimino-Reale G, Zaffaroni N, Folini M. MicroRNA-dependent regulation of telomere maintenance mechanisms: a field as much unexplored as potentially promising. Curr Pharm Des. 2014;20(41):6404–21.
69. Crees Z, Girard J, Rios Z, Botting GM, Harrington K, Shearrow C, Wojdyla L, Stone AL, Uppada SB, Devito JT, Puri N. Oligonucleotides and G-quadruplex stabilizers: targeting telomeres and telomerase in cancer therapy. Curr Pharm Des. 2014;20(41):6422–37.

70. Romaniuk A, Kopczy_ski P, Ksi_ek K, Rubi B. Telomerase as a target for anticancer therapies. Curr Pharm Des. 2014;20(41):6438–51.
71. Uziel O, Lahav M. Conventional anticancer therapeutics and telomere maintenance mechanisms. Curr Pharm Des. 2014;20(41):6452–65.
72. Jabbour E, Ottmann OG, Deininger M, Hochhaus A. Targeting the phosphoinositide 3-kinase pathway in hematologic malignancies. Haematologica. 2014;99(1):7–18.
73. Post J, Vooijs WC, Bast BJ, De Gast GC. Efficacy of an anti-CD138 immunotoxin and doxorubicin on drug-resistant and drug-sensitive myeloma cells. Int J Cancer. 1999;83(4):571–6.
74. Liu C, Lambert JM, Teicher BA, Blättler WA, O'Connor R. Cure of multidrug-resistant human B-Cell lymphoma xenografts by combinations of anti-B4-blocked ricin and chemotherapeutic drugs. Blood. 1996;87(9):3892–8.
75. Rosenblum MG, Cheung L, Kim SK, Mujoo K, Donato NJ, Murray JL. Cellular resistance to the antimelanoma immunotoxin ZME-gelonin and strategies to target resistant cells. Cancer Immunol Immunother. 1996;42(2):115–21.
76. Wagstaff KM, Jans DA. Protein transduction: cell penetrating peptides and their therapeutic applications. Curr Med Chem. 2006;13(12):1371–87.
77. Liu J, Zhao Y, Guo Q, Wang Z, Wang H, Yang Y, Huang Y. TAT-modified nanosilver for combating multidrug-resistant cancer. Biomaterials. 2012;33(26):6155–61.
78. Kirtane AR, Kalscheuer SM, Panyam J. Exploiting nanotechnology to overcome tumor drug resistance: challenges and opportunities. Adv Drug Deliv Rev. 2013;65(13–14):1731–47.
79. Beljanski V, Hiscott J. The use of oncolytic viruses to overcome lung cancer drug resistance. Curr Opin Virol. 2012;2(5):629–35.
80. Pavelic J. Editorial: combined cancer therapy. Curr Pharm Des. 2014;20(42):6511–2.
81. Han L, Shi S, Gong T, Zhang Z, Sun X. Cancer stem cells: therapeutic implications and perspectives in cancer therapy. Acta Pharmaceutica Sinica B. 2013;3(2):65–75.
82. Ischenko I, Seeliger H, Schaffer M, Jauch KW, Bruns CJ. Cancer stem cells: how can we target them? Curr Med Chem. 2008;15:3171–84.
83. Sun S, Liu S, Duan SZ, Zhang L, Zhou H, Hu Y, Zhou X, Shi C, Zhou R, Zhang Z. Targeting the c-Met/FZD8 signaling axis eliminates patient-derived cancer stem-like cells in head and neck squamous carcinomas. Cancer Res. 2014;74(24):7546–59.
84. Huang H, Hu M, Li P, Lu C, Li M. Mir-152 inhibits cell proliferation and colony formation of CD133 + liver cancer stem cells by targeting KIT. Tumour Biol. 2014;36(2):921–8.
85. Bao B, Azmi AS, Ali S, Zaiem F, Sarkar FH. Metformin may function as anti-cancer agent via targeting cancer stem cells: the potential biological significance of tumor associated miRNAs in breast and pancreatic cancers. Ann Transl Med. 2014;2(6):59.
86. Bao S, Wu Q, Li Z, Sathornsumetee S, Wang H, McLendon RE, Hjelmeland AB, Rich JN. Targeting cancer stem cells through L1CAM suppresses glioma growth. Cancer Res. 2008;68(15):6043–8.
87. Hogge DE, Feuring-Buske M, Gerhard B, Frankel AE. The efficacy of diphtheria-growth factor fusion proteins is enhanced by co-administration of cytosine arabinoside in an immunodeficient mouse model of human acute myeloid leukemia. Leuk Res. 2004;28(11):1221–6.

Chapter 3
Factors that Determine Sensitivity and Resistances of Tumor Cells Towards Antibody-Targeted Protein Toxins

Sebastian Stahl, Fabian Mueller, Ira Pastan and Ulrich Brinkmann

Abstract Recombinant immunotoxins are composed of antibody-derived targeting entities fused to truncated toxins. Pseudomonas toxins inactivate eEF2 by ADP-ribosylation and are potent antitumoral agents in clinical development. The sensitivity of tumor cells towards such fusion proteins, and hence their therapeutic efficacy, is influenced by multiple factors: (i) access to tumor cells, (ii) target antigen binding and internalization, (iii) entry into the cytosol, (iv) enzymatic modification of the intracellular target eEF2, and (v) induction of apoptosis. Parameters that affect these steps and hence modulate sensitivity include: (i) protein stability and immunogenicity, (ii) presence, density and internalization of target antigen, (iii) cellular factors involved in processing, routing or translocation of the toxin, (iv) factors involved in diphthamide synthesis on eEF2, and (v) factors that influence cellular susceptibility towards apoptosis. This chapter describes sensitivity or resistance factors for Pseudomonas exotoxin -derived immunotoxins that were identified experimentally and/or observed in clinical studies.

Keywords Recombinant immunotoxin · Pseudomonas exotoxin · Diphtheria toxin · Diphthamide · ADP-ribosylation · Immunogenicity · Biodistribution

Abbreviations

ADC	Antibody drug conjugates
ADPR	ADP-ribosylation
DT	Diphtheria toxin
eEF2	Eukaryotic translation elongation factor 2
ER	Endoplasmic reticulum
Fab	Fragment antigen-binding
Fv	Fragment crystallizable

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mAb	Monoclonal antibody
PARP	Poly-ADP-ribose polymerase
PE	Pseudomonas exotoxin A
PK	Pharmacokinetics
RIT	Recombinant immunotoxin
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
SAM	S-adenosyl-methionine
TGN	Trans-Golgi network
TNFα	Tumor necrosis factor alpha

3.1 Introduction

In cancer therapy, a paradigm shift is taking place from 'classical' approaches like chemotherapeutic drugs, radiation and hormonal therapies (with rather nonspecific activities and associated side effects) to highly specific targeted therapies. One promising targeted therapy approach is the application of antibody drug conjugates (ADCs) and ADC-like entities. ADCs combine antibody-mediated tumor specificity with the potency of cytotoxic compounds [1]. They are composed of three components: the monoclonal antibody (mAb), the pharmacologically active drug, and the linker in-between. Ideally, the mAb specifically binds to an internalizable antigen that is abundantly presented on tumor cells, but not or only scarcely expressed on healthy tissue. Upon binding to their target on the surface of tumor cells, ADCs become internalized (e.g. via receptor mediated endocytosis) and deliver their cytotoxic payload into the cell. Subsequently, the cytotoxic compound is released from the antibody (e.g. in lysosomes) [2], exits vesicular compartments, and thereby enters the cytoplasm or nucleus of cells to unfold cytotoxic activity [3].

In 'classical' ADCs, very potent small molecule toxins such as auristatins, maytansines, amanitines, pyrrolobenzamidines, calicheamicins [4–6] and others are conjugated to antibodies. These compounds are chemically connected to the antibody at lysine or cysteine residues via a linker structure which is of sufficient stability to prevent premature drug release in the circulation. Linker stability limits or avoids non target cytotoxic side effects of free cytotoxics and shall also ensure a long circulating half-life of the ADC to provide extended time of exposure post injection. On the other hand, the linker must also enable release of the cytotoxic compounds from the large antibodies once the ADC has entered the cells. Examples for these types of ADCs that are already applied in cancer therapy are Kadcyla (T-DM1), Emtansine coupled to Trastuzumab, targeting Her2 on tumor cells [7], and Adcetris (Brentuximab vedotin), a CD30 binding ADC which targets auristatin E to CD30 positive Hodgkin's Lymphoma cells [8].

Other types of targeted toxins which possess similar functionalities as ADCs are recombinant fusion proteins composed of antibody derivatives and bacterial or plant-based protein toxins [9]. These recombinant immunotoxins share many

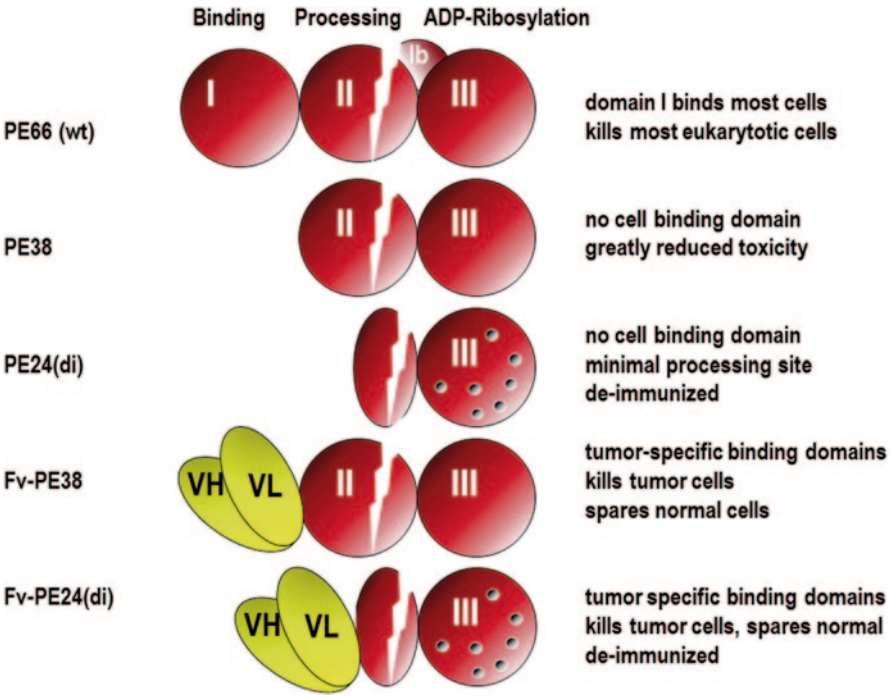

Fig. 3.1 Pseudomonas exotoxin A-and PE-derived immunotoxins. Removal of the non-specific cell binding domain I from Pseudomonas toxin (PE66, MW = 66 kDa) results in truncated derivatives PE38 (38 kDa) or PE24 (24 kDa) which can be de-immunized by removing B- and T cell epitopes [16, 17]. These toxin derivatives are 'de-toxified' because their access to cells is greatly diminished [18]. Replacement of the N-terminal cell binding domain by specific binding domains such as disulfide-stabilized Fvs [19] generates entities that specifically bind to and kill antigen-expressing cells

features with ADCs: high potency of their toxin payload, covalent linkage of payload to an antibody moiety which targets the toxin to the surface of cancer cells, internalization into vesicular compartments, processing to release the toxin from the targeting vehicle, and finally entry of the toxin into the cytoplasm of tumor cells.

Recombinant immunotoxins, e.g. those that contain truncated pseudomonas exotoxin as toxic moiety (Fig. 3.1) have unique properties. Since an enzymatic active molecule can modify many targets over time, only a few molecules inside a cell are sufficient to efficiently cause cell death [10]. Hereby the mode of action can alter depending on the immunotoxin. For example, diphtheria toxin (DT) and Pseudomonas exotoxin A(PE) induce cell death by inactivation of protein synthesis via ADP-ribosylation of the translation elongation factor 2 (eEF2), whereas ricin, gelonin, and other RIPs such as debouganin, sarcin and saporin lead to modification of rRNA and, thereby, inactivate ribosomes. While inhibition of protein synthesis can be sufficient to kill cells, in many cases toxin-mediated protein synthesis arrest also triggers induction of apoptosis of tumor cells [11, 12].

Recombinant fusion proteins can not only kill proliferating cells but also the resting slow growing cells. These cells are often not susceptible to toxins that interfere predominantly with dividing cells such as those having tubulin or DNA as target. Nevertheless, these cells can be killed by immunotoxins due to their dependency on an intact protein synthesis. Examples for recombinant immunotoxin enzymes that block protein synthesis that are applied in the clinic or in experimental cancer therapy are DT-IL2,HA22, an anti-CD22 Fv fused to a portion of Pseudomonas exotoxin A which is used to treat CD22 expressing B-cell malignancies, and SS1PE, an anti-mesothelin Fv genetically fused to a truncated form of the Pseudomonas exotoxin derivative, PE38 [13–15].

This chapter (which is complemented by the chapter 'Challenges for Therapeutic Application of Pseudomonas Exotoxin-Based Immunotoxins' by V Dergachev and I Benhar) describes the factors that influence the sensitivity or potential resistances of cancer cells towards recombinant immunotoxins which contain truncated and/or mutated derivatives of Pseudomonas exotoxin as cytotoxic payloads.

3.2 Intoxication Pathways Define Determinants for Sensitivity and/or Resistances of Tumor Cells Towards Immunotoxins

Complex series of events are necessary for a toxin to kill cells. Defining the steps that are essential for toxicity is critical for understanding resistances or factors that modulate sensitivity. Figure 3.2 summarizes the sequence of events all of which need to be fulfilled for successful cell killing. Five major steps that are important for toxin function are described below.

Step 1
Delivery to target cells: Following application to patients, sufficient amounts of intact immunotoxin molecules must access target receptors on cell surfaces. Similar to ADC requirement of linker stability preventing release of payload from an antibody, immunotoxins need to be of sufficient stability to 'survive' in the circulation until encountering the target cell. In addition to molecular stability, bacterial toxins tend to be immunogenic and patients develop neutralizing antibodies. Therefore, a limiting factor for sufficient delivery of therapeutic immunotoxins is rapid clearance of the toxin by neutralizing antibodies. These lead to degradation of the immunotoxins and thereby preventing delivery of sufficient amounts to target cells.

Step 2
Binding to the target antigen and internalization: Once the immunotoxin encounters the tumor cell, it binds to the cell surface antigen that is recognized by the antibody moiety. This results in internalization of the immunotoxin together with the target antigen. The presence of the target antigen in sufficient density, further processing, and a functional internalization machinery are therefore an absolute requirement

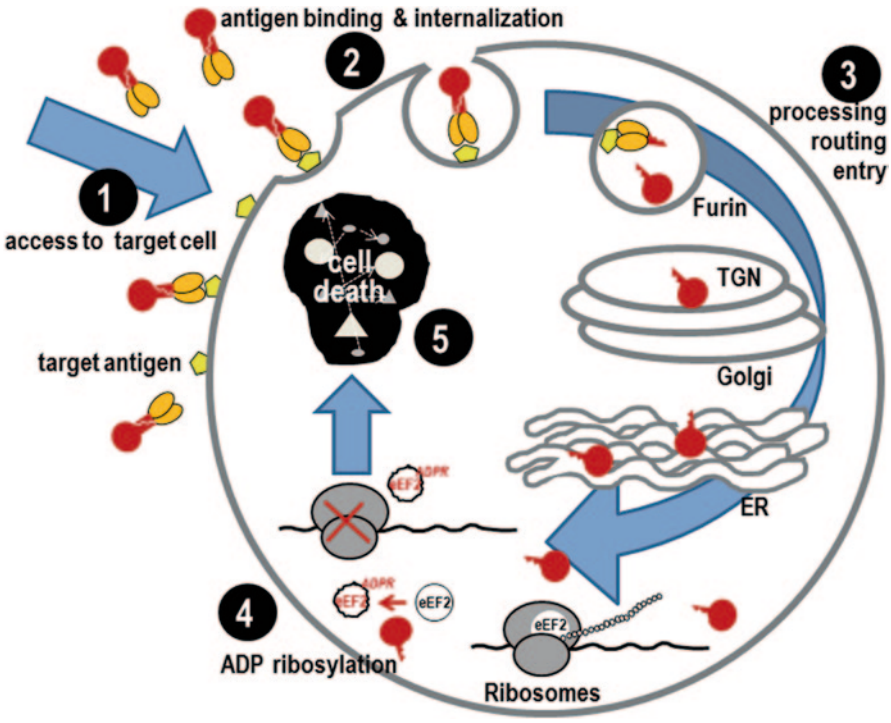

Fig. 3.2 **'Toxin pathway'**. After binding of the immunotoxin to the target antigen and internalization, the toxin fragment is released from the cell-targeting antibody and routed to the ER. Then, the toxin enters the cytoplasm where it ADP-ribosylates eEF2 which results in the inhibition of protein translation and induction of apoptosis

for cytotoxic activity of immunotoxins. One feasible resistance mechanism is the loss of target antigen, which prevents specific binding of the immunotoxin to tumor cells.

Step 3

Processing, routing and translocation into the cytoplasm: Following internalization, the toxin portion must be released from the antibody moiety. The antibody moiety bound to the receptor gets degraded in the lysosome whereas the toxin portion is routed to the ER. PE-derived immunotoxins contain a recognition site for processing by the protease furin which is present in vesicular compartments of most mammalian cells including tumor cells. Furin cleavage releases the enzymatically active toxin fragment from the cell targeting antibody. The toxin fragment contains a C-terminal routing motif that binds the KDEL receptor and directs the toxin fragment to the endoplasmic reticulum (ER). There, the toxin utilizes the pathway for secreted proteins in a retrograde manner to enter the cytoplasm.

Step 4
Inactivation of eEF2 by ADP-ribosylation: Once in the cytoplasm, the C-terminal domain of PE modifies the diphthamide of eukaryotic translation elongation factor 2 (eEF2) on position 715. Diphthamide is a posttranslational modification of His 715 by seven different cellular enzymes. PE induces an ADP-ribosylation at this position leading to an arrest of protein synthesis due to inhibition of the elongation step of translation. The ADP-ribosylation reaction requires NAD as substrate and is identical to the eEF2 inactivation by diphtheria toxin. Because eEF2 ADP-ribosylation occurs only on diphthamide modified eEF2, loss of the diphthamide makes cells resistant to the inhibition of protein synthesis caused by ADP-ribosylating toxins like PE and DT.

Step 5
Arrest of protein synthesis and induction of apoptosis: ADP-ribosylated eEF2 is non-functional and hence cannot support the elongation step of ribosomal protein translation. As a direct consequence, cells can die due to their inability to produce new proteins. In addition to that, stalled protein synthesis that cannot be resolved over time frequently is accompanied with the induction of apoptosis. The balance of pro- and anti-apoptotic proteins in cells is strictly regulated but alterations in the apoptosis machinery that are very common in cancer cells. Thus, apoptosis and susceptibility of cells towards apoptosis can be additional factors determining sensitivity of cells to protein toxins.

The sensitivity or resistance of tumor cells can be influenced by factors that are associated with the various steps of intoxication. Examples and observations of sensitivity and resistance mechanisms that are associated with them are described in more detail below.

3.3 Step 1—Access to Target Cells: Immunogenicity can be a Relevant Factor for Immunotoxin Therapy

After injecting immunotoxins into the blood stream of patients, sufficient amounts of intact immunotoxin have to reach target cells. Therefore, immunotoxins need to have the appropriate stability to ‘survive’in the circulation until they encounter their target cell. One factor that might influence immunotoxin levels and thereby reduce potency could be proteolysis of immunotoxins. In preclinical analyses and clinical trials performed with PE derived immunotoxins, rather short serum half-lives (some hours) were observed [20–22]. However, this short half-life might be determined by the size and lack of the antibody Fc regions rather than by proteolysis of the toxin or the antibody Fv fragments. Early work on protease sensitivity of whole PE toxin showed that removal of protease sensitive sites can extend the serum half-life of toxins (without an attached antibody) in animals [23]. Recent work by J Weldon et al. have demonstrated that within the context of a recombinant immunotoxin-removal of large parts of domain II eliminates protease sensitive sites and thereby stabilizes the toxin (against endosomal proteases) [24].

One major factor that still limits the therapeutic efficacy of recombinant immunotoxins at the initial 'access step' is immunogenicity. The toxin moiety is of bacterial origin and, hence, recognized as foreign by the human immune system. Therefore, in patients with normal immune systems it elicits rapid immune responses characterized by the generation of neutralizing antibodies which inactivate immunotoxins before they can bind to and attack cancer cells. Interestingly, immunogenicity in leukemia and lymphomas patients whose immune system is damaged by the disease is less of a limitation than in applications that target solid tumors. Examples for immunotoxins that target such diseases are immunotoxins that target the IL2 receptor [25], or the CD22 antigen. In solid tumor applications, initial trials showed some promising results but neutralizing immune responses prevented repeated dosing and, hence, limited therapeutic success [26]. Subsequent trials with co-administration of immunosuppressants enabled repeated dosing and increased the therapeutic potency [27]. Thus, for solid tumor treatment with recombinant immunotoxins, immunogenicity is a relevant resistance factor which may be overcome by immunosuppressive co-therapy. In addition, approaches to humanize the bacterial toxin by elimination of T- and/or B-cell epitopes have been developed [28, 29].

3.4 Step 2—Target Cell Binding: Loss or Reduction of Target Antigens Reduce Sensitivity of Tumor Cells Towards Targeted Toxins

After the antibody moiety binds to the antigen on the tumor cell, the immunotoxin together with the target antigen are internalized. (Rates vary from 10–20 % per hour (mesothelin) to 100 % per hour (CD22)). Therefore, if the levels of the target antigen on the surface of the tumor cell are reduced, fewer amounts of immunotoxins will bind and be internalized. It was shown that the response to an anti-CD22 immunotoxin on cells with high CD22 expression levels was significantly better than on those with lower levels [30]. Kreitman et al. also reported that the efficacy of the recombinant immunotoxin RFB4(dsFv)-PE38(BL22) to kill tumor cells of patients with B-cell leukemia highly depends on the presence and the absolute number of target molecules on tumor cells *in vitro* [31, 32].

These observations indicate that the presence of the target antigen in sufficient density is a very important factor for therapeutic potency. Tumor cells can become resistant to targeted toxins if they reduce or lose the expression of the antigen that is recognized by the immunotoxin. This mode of resistance is not specific for targeted protein toxins or inhibitors of protein synthesis, but is common for all types of ADCs and ADC-like molecules. One way to ameliorate or overcome this limitation may be the generation of ADCs or immunotoxins which recognize and bind more than one cell surface antigen. This reduces the chance of resistance due to the lesser probability of a cancer cell losing both target antigens simultaneously.

3.5 Step 3—Entry of Toxins into Cells: Loss of Processing Enzymes and Modulation of Vesicular Compartments Reduce Toxin Activity in Cultured Cells

Once the recombinant fusion protein has been internalized it must be cleaved from the antibody moiety and transferred to the ER where it is processed and finally released to the cytoplasm. PE-derived immunotoxins contain a recognition site for processing by the protease furin, which is present in vesicular compartments of most mammalian cells including tumor cells. To release the enzymatically active toxin fragment from the cell targeting antibody, cleavage by furin is essential.

Cells which do not possess furin are compromised in this processing step. For example, LoVo cells, which do not express functional furin, are quite resistant to PE [33]. Other proteases within vesicular compartments may be able to partially compensate for lack of furin, albeit to a much lesser degree. As a consequence, cells with reduced furin levels or cells without furin have a greatly diminished sensitivity to PE (as well as to DT). KDEL-receptor mediated routing of the furin-processed C-terminal toxin fragment is another important step in the intoxication process. In cell culture, it was shown that interference with vesicular routing or binding to the KDEL receptor interferes with toxin activity and causes resistance. For example, S Seetharam et al. have shown that interference with vesicular routing causes toxin resistances [34], and VK Chaudhary et al. showed that the C-terminal sequence that binds to the KDEL receptor is a requirement for toxin activity [35]. Thus, cellular alterations that modulate or interfere with these routing mechanisms will also affect the sensitivity of tumor cells to recombinant toxins. The importance of routing and translocation for immunotoxin activity may be further supported by the recent finding that ABT-737, a BH3 mimetic that can synergize with immunotoxin activity, can promote the entry of the toxin from the lumen of the ER into the cytosol [16].

In a genome-wide RNAi screen that identified genes required for ricin and PE intoxications, D Moreau et al. reported that genes that encode proteins involved in trafficking and acidification of vehicles can have a significant influence on toxin sensitivity [36]. Down-regulation of these genes in tumor cells could, thereby, also lead to resistance towards immunotoxins. The relevance of these potential resistance mechanisms in cancer therapy has not yet been shown. All experiments so far have been performed in cell culture. Also, the capability to process precursor proteins (furin), and the capability for vesicular transport and routing are important to sustain optimal metabolism and growth of cells, including tumor cells. Defective processing and routing pathways in most cases reduce cell growth, most likely also those of tumor cells. Therefore, it is questionable if cancer cells will acquire resistances to immunotoxins to a significant degree which are based on interference with these mechanisms in a clinically relevant setting.

3.6 Step 4—ADP-Ribosylation of eEF2: Reduced or Altered Expression of Diphthamide Synthesis Genes is Associated with Immunotoxin Resistances

Once the toxin reaches the cytosol, the C-terminal domain of PE modifies the eukaryotic translation elongation factor 2 at His 715 by catalyzing ADP-ribosylation of the diphthamide modification on eEF2 using NAD as ADP-ribosyl donor. This arrests protein translation leading to cell death [37]. ADP-ribosylation of eEF2 occurs only when His715 of eEF2 contains a diphthamide. Diphthamide is generated by a pathway including seven diphthamide genes called *DPH1-7* [38]. A pathway model has been described for yeast by Liu et al. and Lin et al. [39, 40]. This biosynthesis process for cells with intact synthesis pathway (i.e. with all enzyme functionalities present) is summarized in Fig. 3.3.

Because eEF2 ADP-ribosylation occurs exclusively on diphthamide modified eEF2, loss of the diphthamide renders eEF2 resistance to the ADP-ribosylating toxins PE and DT. Interestingly, diphthamide modification, even though highly

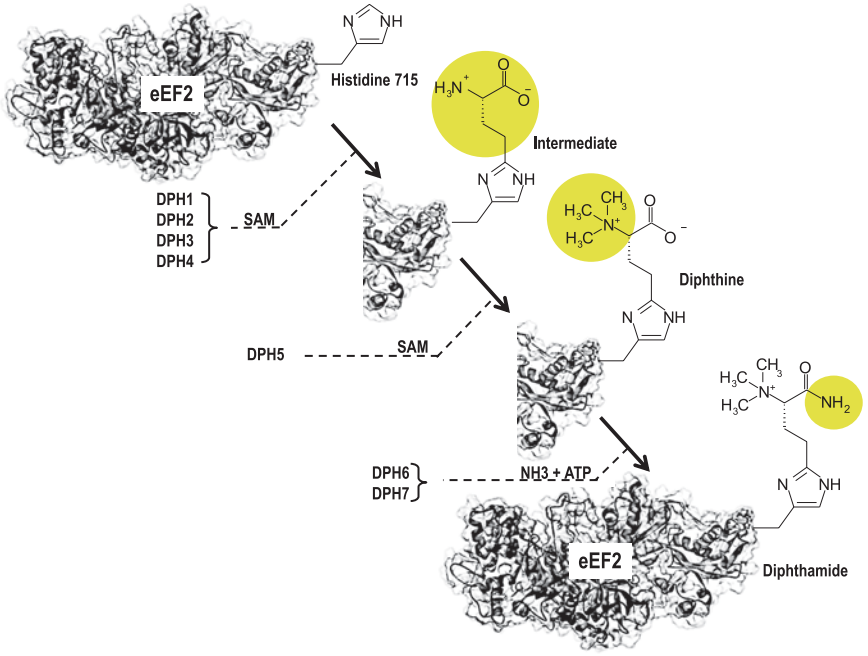

Fig. 3.3 Diphthamide synthesis. The diphthamide pathway was adapted from the model by Liu et al. and Lin et al. [39, 40] for yeast. *SAM* S-adenosyl-methionine. The role of DPH5 and/or alternative pathways and products in cells that lack one or more enzymes may need further clarification. [41]

conserved in all eukaryotic cells as well as archaebacteria, appears not to be essential for the viability of cultured cells, albeit it is necessary for the development of mice [42–44]. Therefore, cancer cells are able to modify or deregulate genes and products in the diphthamide synthesis pathway.

One way of acquiring immunotoxin resistance by cancer cells is via a reversible methylation of their DPH4 promoter, which has been described by H. Wei et al. [45]. HAL-01 cells isolated from acute lymphoblastic leukemia (ALL) patients only showed poor response to HA22, a recombinant immunotoxin composed of an anti-CD22 Fv fused to a portion of Pseudomonas exotoxin A. In resistant cells no ADP-ribosylation and inactivation of eEF2 were detected due to low levels of DPH4 mRNA and protein, which prevent diphthamide biosynthesis. Furthermore, it was shown that low expression levels of DPH4 could be explained by specific methylation of a CpG island in the DPH4 promoter. Interestingly, resistance to HA22 was not permanent but reversible. ALL cells cultivated without HA22 reverted to HA22 sensitivity in 4 months. Also, treatment of sensitive cells with the DNA methylation inhibitor 5-azacytidine prevented the emergence of resistant cells [45]. These results indicate that combinational treatments might be worth to consider in the future.

The HAL-01 cell line was not the only one found to be resistant against HA22. KOPN-8, a human pre-B leukemia cell line, was also resistant to HA22 treatment due to a hyper-methylation of a DPH promoter. The difference was that in KOPN-8 CpG islands of the DPH1 promoter were highly methylated, whereas in HAL-01 it was DPH4. Both genes encode for proteins needed for diphthamide synthesis. Another difference is that in the HAL-01 cell line resistance to HA22 was reversible, whereas in the KOPN-8 cells resistance was quite stable [46].

Not only hyper-methylation of a DPH promoter can promote the loss of toxin sensitivity. Wei et al. also showed that deletion of the *DPH7* gene (also known as *WDR85*) can cause toxin resistance [41]. In an HA22 resistant lymphoma cell line the *DPH7* gene was deleted leading to inability of HA22 to ADP-ribosylate and inactivate eEF2. By inactivation of DPH7 a novel form of diphthamide with an additional methyl group catalyzed by DPH5 was generated, which prevented ADP-ribosylation [41].

3.7 Step 5—Signaling and Apoptosis: Protective Factors and Pathways can Reduce Toxin Sensitivity

ADP-ribosylated eEF2 is non-functional and, hence, disrupts the elongation step of ribosomal protein translation. As a consequence, protein synthesis is stalled and cells subsequently die due to their inability to generate and/or replace essential proteins. In addition, arrested protein synthesis that cannot be resolved over time frequently leads to the induction of apoptosis. Thus, apoptosis, and susceptibility of cells towards apoptosis can be an additional factor that determines the sensitivity of cells to protein toxins, or that influence the time that is necessary for the cell to die.

One example that demonstrates that apoptosis factors influence the sensitivity of tumor cells to PE and immunotoxins isan *in vitro* cell culture experiment that described sensitivity in the presence of Bcl-2. B-cell lymphoma 2 (Bcl-2) belongs to the Bcl-2 family which regulates cell death, by either inducing or inhibiting apoptosis. Bcl-2 is specifically considered as an important anti-apoptotic protein [47, 48]. In this regard, it was shown that overexpression of Bcl-2 in MCF-7 breast cancer cells, which were stably transfected with a Bcl-2 expression plasmid, became less sensitive to immunotoxins [49]. However, overexpression of Bcl-2 leads only to a limited degree of resistance to immunotoxins PE, DT, and ricin whereas the cells were almost completely resistant to tumor necrosis factor alpha (TNFα, 1000-fold) [49]. In accordance, several leukemia cell lines were found to be sensitive to a PE containing immunotoxin even though they showed increased levels of Bcl-2 expression. These results indicate that overexpression of Bcl-2 can affect the sensitivity of cancer cells to immunotoxins, but that anti-apoptotic factors such as Bcl-2 do not confer 'absolute' drug resistance [50]. Another influence of apoptosis proteins on toxin sensitivity is supported by experiments investigating toxin sensitivity in the presence of caspase inhibitors. Induction of caspases upon application of immunotoxin shows that apoptosis is induced rapidly after toxin exposure [51]. In accordance with the caspase activation, these experiments on MCF-7 breast cancer cells with B3(Fv)-PE38 showed cleavage of poly-ADP-ribose polymerase (PARP). This cleavage could be prevented by treatment of the cells with caspase inhibitors as well as by overexpression of Bcl-2. This provides evidence for the involvement of caspases in toxin-induced cell killing. Further evidence for the contribution of apoptosis-related proteins to the activity of PE-derived immunotoxins is the observations by Du et al. [52] who analyzed the activity of PE in mouse embryo fibroblasts that were deficient in Bak or Bax. The results of these analyses indicated that PE-mediated apoptosis is associated with MCL-1 degradation and dependent on Bak activation.

An alternative approach to determine cellular factors that influence sensitivity or resistance of cells to immunotoxins has been undertaken by an expression cloning approach [53, 54]. In an attempt to identify toxin sensitivity or resistance factors, they transfected a cDNA expression library containing human cDNAs into MCF-7 cells and subsequently selected cDNAs that conferred resistance of MCF-7 breast cancer cells to immunotoxins. One set of these resistance-mediating plasmids contained antisense cDNA fragments homologous to the yeast chromosome segregation gene *CSE1*. The *CSE1L/CAS* gene is involved in multiple cellular processes [55]. It plays a role in cell division, in mitosis [56–58], in nuclear transport (export factor for importin alpha [59], as well as in apoptosis [55, 60]. The latter functionality (apoptosis) most likely contributes to toxin sensitivity. Antisense-mediated reduction of the human CSE1 homologue CAS protein generated a resistance against the ADP-ribosylating toxins PE and DT, as well as to tumor necrosis factor -α and β. Cells stably transfected with the antisense plasmid revealed reduced apoptosis compared to controls. CAS antisense did not affect cell death induced by staurosporine, cycloheximide, or etoposide indicating that CAS may play a role in selected but not all pathways of apoptosis. Moreover, it was shown that neither ADP-ribosylation

of the eukaryotic elongation factor 2 by PE, DT nor TNF binding was prevented by depletion of the CSE1L/CAS protein. Nevertheless, transfectants could recover after removal of the toxin whereas normal MCF-7 cells died after exposure to the toxin [60]. The mechanism by which the reduction of CAS protein leads to resistance against PE, DT and TNF remains elusive. However, it was shown that CAS not only plays a role in the regulation of apoptosis but also of proliferation [55]. In several tumors such as colon and breast cancer and in lymphoid neoplasms, CAS expression (mRNA and protein) is upregulated [61, 62]. Therefore, it is possible that CAS, like MYC, P53 or Bcl-2, is involved in the regulation of apoptosis as well as proliferation [63–65], and, hence, influences toxin sensitivity of the apoptotic level.

Other described intracellular signaling pathways that affect the sensitivity of cells towards targeted toxins include cell surface receptors (receptor tyrosine kinases, RTKs).One of these, the insulin receptor (INSR), promotes cell growth and protects against loss of viability and apoptosis. It is activated by insulin and insulin-like growth factor I (IGF-I), which activate pathways involved in cell growth and survival processes [66–68]. SiRNA-mediated knockdown of the insulin receptor enhanced the cytotoxic activity of SS1P (a recombinant immunotoxin fragment of PE attached to the Fv portion of a mAb targeting mesothelin) in several human cancer cell lines. The knockdown of insulin receptor also increased the cleavage of SS1P by furin, potentially liberating more toxins from the antibody, to reach the cytosol and inactivate the elongation factor 2 [69].

Other tyrosine kinases which are similar to INSR include HCK, SRC, PDGFR-α and BMX. SiRNA-mediated reduction of the mRNA levels of these RTKs also increased the cytotoxic potency of SS1P. Especially, HCK knockdown substantially enhanced SS1P efficacy. Similar to siRNA knockdown of INSR, decreased HCK levels promoted cleavage of SS1P by furin. The same effect could be achieved by treating tumor cells with Src inhibitors (SU6656 and SKI-606) leading to enhanced killing of PE-derived recombinant immunotoxins [70].

3.8 Conclusions and Outlook

This chapter has provided evidence that sensitivity and resistance of tumor cells towards PE-derived targeted toxins can be rationally associated with different steps in the intoxication pathways. A concluding summary of these factors, which have either been described in vitro or have been observed in animals, or in clinical trials is listed in Table 3.1.

The amounting knowledge about pathways and factors that are required for modulating immunotoxin sensitivity enables us not only to understand resistances, but also to devise treatment options to improve immunotoxin therapy, and to enhance antitumor efficacy. These include the development of deimmunized entities to circumvent neutralization before the toxins can even bind to their target cells (ongoing), the use of bispecific targeting modules to reduce the effects of potential

Table 3.1 Factors that determine sensitivity or resistances to PE-derived immunotoxins

Intoxication step	Relevant factor(s)	Effects of immunotoxin therapy	Demonstrated in	Reference(s)
Access to target cells	Protease sensitivity	Stability & PK	Cells, animals	[23, 24, 27–29]
	Immunogenicity	Neutralizes RITs	Patients	[26]
Binding to target cells	Loss of antigen	Potency	Cells	[30, 32]
			Patients	[31]
Processing, routing, and cell entry	Loss of furin, interference with vesicular trafficking	Potency	Cells	[33–35]
Inactivation of eEF2	DPH gene products	Potency	Cells	[37, 41, 46]
			Patients	[45]
Signaling & apoptosis	Apoptosis factors RTK pathways	Potency	Cells	[49, 51]
				[69, 70]

PK pharmacokinetics, *rIT* recombinant immunotoxin, *RTK* receptor tyrosine kinase

reduction or loss of target antigen, or co-administration of compounds that reduce immunogenic responses or that sensitize tumors and tumor cells to immunotoxins.

Disclosure of Potential Conflicts of Interest S.S. and U.B. are employed by Roche. Roche has an interest in the development of antibody targeted protein toxins.

I.P. is an inventor on several patents on immunotoxins that have all been assigned to NIH and has a Cooperative Research and Development Agreement with Roche Pharmaceuticals.

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References

1. Alley SC, Okeley NM, Senter PD. Antibody-drug conjugates: targeted drug delivery for cancer. Curr Opin Chem Biol. 2010;14(4):529–37.
2. Sutherland MS, Sanderson RJ, Gordon KA, Andreyka J, Cerveny CG, Yu C, Lewis TS, Meyer DL, Zabinski RF, Doronina SO, Senter PD, Law CL, Wahl AF. Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates. J Biol Chem. 2006;281(15):10540–7.
3. Carter PJ, Senter PD. Antibody-drug conjugates for cancer therapy. Cancer J. 2008;14(3):154–69.
4. Doronina SO, Toki BE, Torgov MY, Mendelsohn BA, Cerveny CG, Chace DF, DeBlanc RL, Gearing RP, Bovee TD, Siegall CB, Francisco JA, Wahl AF, Meyer DL, Senter PD. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat Biotechnol. 2003;21(7):778–84.

5. Law CL, Cerveny CG, Gordon KA, Klussman K, Mixan BJ, Chace DF, Meyer DL, Doronina SO, Siegall CB, Francisco JA, Senter PD, Wahl AF. Efficient elimination of B-lineage lymphomas by anti-CD20-auristatin conjugates. Clin Cancer Res. 2004;10(23):7842–51.
6. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug Chem. 2002;13(1):47–58.
7. Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W, Girish S, Tibbitts J, Yi JH, Sliwkowski MX, Jacobson F, Lutzker SG, Burris HA. Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. J Clin Oncol. 2010;28(16):2698–704.
8. Younes A, Gopal AK, Smith SE, Ansell SM, Rosenblatt JD, Savage KJ, Ramchandren R, Bartlett NL, Cheson BD, de Vos S, Forero-Torres A, Moskowitz CH, Connors JM, Engert A, Larsen EK, Kennedy DA, Sievers EL, Chen R. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. J Clin Oncol. 2012;30(18):2183–9.
9. Weidle UH, Tiefenthaler G, Schiller C, Weiss EH, Georges G, Brinkmann U. Prospects of bacterial and plant protein-based immunotoxins for treatment of cancer. Cancer Genomics Proteomics. 2014;11(1):25–38.
10. Shapira A, Benhar I. Toxin-based therapeutic approaches. Toxins. 2010;2(11):2519–83.
11. Chang MP, Bramhall J, Graves S, Bonavida B, Wisnieski BJ. Internucleosomal DNA cleavage precedes diphtheria toxin-induced cytolysis. Evidence that cell lysis is not a simple consequence of translation inhibition. J Biol Chem. 1989;264(26):15261–7.
12. Allam M, Bertrand R, Zhang-Sun G, Pappas J, Viallet J. Cholera toxin triggers apoptosis in human lung cancer cell lines. Cancer Res. 1997;57(13):2615–8.
13. Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler-Stevenson M, Fitzgerald DJ, Lechleider R, Pastan I. Phase I trial of anti-CD22 recombinant immunotoxin moxetumomab pasudotox (CAT-8015 or HA22) in patients with hairy cell leukemia. J Clin Oncol. 2012;30(15):1822–8.
14. Kelly RJ, Sharon E, Pastan I, Hassan R. Mesothelin-targeted agents in clinical trials and in preclinical development. Mol Cancer Ther. 2012;11(3):517–25.
15. Potala S, Verma RS. Modified DT-IL2 fusion toxin targeting uniquely IL2Ralpha expressing leukemia cell lines-Construction and characterization. J Biotechnol. 2010;148(2–3):147–55.
16. Antignani A, Sarnovsky R, FitzGerald DJ. ABT-737 promotes the dislocation of ER luminal proteins to the cytosol, including pseudomonas exotoxin. Mol Cancer Ther. 2014;13(6):1655–63.
17. King C, Garza EN, Mazor R, Linehan JL, Pastan I, Pepper M, Baker D. Removing T-cell epitopes with computational protein design. Proc Natl Acad Sci U S A. 2014;111(23):8577–82.
18. Weldon JE, Pastan I. A guide to taming a toxin–recombinant immunotoxins constructed from Pseudomonas exotoxin A for the treatment of cancer. FEBS J. 2011;278(23):4683–700.
19. Reiter Y, Brinkmann U, Jung SH, Pastan I, Lee B. Disulfide stabilization of antibody Fv: computer predictions and experimental evaluation. Protein Eng. 1995;8(12):1323–31.
20. Wayne AS, Kreitman RJ, Findley HW, Lew G, Delbrook C, Steinberg SM, Stetler-Stevenson M, Fitzgerald DJ, Pastan I. Anti-CD22 immunotoxin RFB4(dsFv)-PE38 (BL22) for CD22-positive hematologic malignancies of childhood: preclinical studies and phase I clinical trial. Clin Cancer Res. 2010;16(6):1894–903.
21. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. Clin Cancer Res. 2007;13(17):5144–9.
22. Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, Waldmann TA, Pastan I. Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol. 2000;18(8):1622–36.
23. Brinkmann U, Pai LH, FitzGerald DJ, Pastan I. Alteration of a protease-sensitive region of Pseudomonas exotoxin prolongs its survival in the circulation of mice. Proc Natl Acad Sci U S A. 1992;89(7):3065–9.

24. Weldon JE, Xiang L, Chertov O, Margulies I, Kreitman RJ, FitzGerald DJ, Pastan I. A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity. Blood. 2009;113(16):3792–800.
25. Robbins DH, Margulies I, Stetler-Stevenson M, Kreitman RJ. Hairy cell leukemia, a B-cell neoplasm that is particularly sensitive to the cytotoxic effect of anti-Tac(Fv)-PE38 (LMB-2). Clin Cancer Res. 2000;6(2):693–700.
26. Kreitman RJ, Hassan R, Fitzgerald DJ, Pastan I. Phase I trial of continuous infusion anti-mesothelin recombinant immunotoxin SS1P. Clin Cancer Res. 2009;15(16):5274–9.
27. Gelber EE, Vitetta ES. Effect of immunosuppressive agents on the immunogenicity and efficacy of an immunotoxin in mice. Clin Cancer Res. 1998;4(5):1297–304.
28. Mazor R, Vassall AN, Eberle JA, Beers R, Weldon JE, Venzon DJ, Tsang KY, Benhar I, Pastan I. Identification and elimination of an immunodominant T-cell epitope in recombinant immunotoxins based on Pseudomonas exotoxin A. Proc Natl Acad Sci U S A. 2012;109(51):E3597–603.
29. Liu W, Onda M, Lee B, Kreitman RJ, Hassan R, Xiang L, Pastan I. Recombinant immunotoxin engineered for low immunogenicity and antigenicity by identifying and silencing human B-cell epitopes. Proc Natl Acad Sci U S A. 2012;109(29):11782–7.
30. Tembhare PR, Marti G, Wiestner A, Degheidy H, Farooqui M, Kreitman RJ, Jasper GA, Yuan CM, Liewehr D, Venzon D, Stetler-Stevenson M. Quantification of expression of antigens targeted by antibody-based therapy in chronic lymphocytic leukemia. Am J Clin Pathol. 2013;140(6):813–8.
31. Kreitman RJ, Squires DR, Stetler-Stevenson M, Noel P, FitzGerald DJ, Wilson WH, Pastan I. Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. J Clin Oncol. 2005;23(27):6719–29.
32. Kreitman RJ, Margulies I, Stetler-Stevenson M, Wang QC, FitzGerald DJ, Pastan I. Cytotoxic activity of disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) toward fresh malignant cells from patients with B-cell leukemias. Clin Cancer Res. 2000;6(4):1476–87.
33. Inocencio NM, Moehring JM, Moehring TJ. Furin activates Pseudomonas exotoxin A by specific cleavage in vivo and in vitro. J Biol Chem. 1994;269(50):31831–5.
34. Seetharam S, Chaudhary VK, FitzGerald D, Pastan I. Increased cytotoxic activity of Pseudomonas exotoxin and two chimeric toxins ending in KDEL. J Biol Chem. 1991;266(26):17376–81.
35. Chaudhary VK, Jinno Y, FitzGerald D, Pastan I. Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. Proc Natl Acad Sci U S A. 1990;87(1):308–12.
36. Moreau D, Kumar P, Wang SC, Chaumet A, Chew SY, Chevalley H, Bard F. Genome-wide RNAi screens identify genes required for Ricin and PE intoxications. Dev Cell. 2011;21(2):231–44.
37. Zhang Y, Liu S, Lajoie G, Merrill AR. The role of the diphthamide-containing loop within eukaryotic elongation factor 2 in ADP-ribosylation by *Pseudomonas aeruginosa* exotoxin A. Biochem J. 2008;413(1):163–74.
38. Su X, Lin Z, Lin H. The biosynthesis and biological function of diphthamide. Crit Rev Biochem Mol Biol. 2013;48(6):515–21.
39. Lin Z, Su X, Chen W, Ci B, Zhang S, Lin H. Dph7 catalyzes a previously unknown demethylation step in diphthamide biosynthesis. J Am Chem Soc. 2014;136(17):6179–82.
40. Liu S, Milne GT, Kuremsky JG, Fink GR, Leppla SH. Identification of the proteins required for biosynthesis of diphthamide, the target of bacterial ADP-ribosylating toxins on translation elongation factor 2. Mol Cell Biol. 2004;24(21):9487–97.
41. Wei H, Bera TK, Wayne AS, Xiang L, Colantonio S, Chertov O, Pastan I. A modified form of diphthamide causes immunotoxin resistance in a lymphoma cell line with a deletion of the WDR85 gene. J Biol Chem. 2013;288(17):12305–12.
42. Chen CM, Behringer RR. Ovca1 regulates cell proliferation, embryonic development, and tumorigenesis. Genes Dev. 2004;18(3):320–32.

43. Liu S, Wiggins JF, Sreenath T, Kulkarni AB, Ward JM, Leppla SH. Dph3, a small protein required for diphthamide biosynthesis, is essential in mouse development. Mol Cell Biol. 2006;26(10):3835–41.
44. Webb TR, Cross SH, McKie L, Edgar R, Vizor L, Harrison J, Peters J, Jackson IJ. Diphthamide modification of eEF2 requires a J-domain protein and is essential for normal development. J Cell Sci. 2008;121(Pt 19):3140–5.
45. Wei H, Xiang L, Wayne AS, Chertov O, FitzGerald DJ, Bera TK, Pastan I. Immunotoxin resistance via reversible methylation of the DPH4 promoter is a unique survival strategy. Proc Natl Acad Sci U S A. 2012;109(18):6898–903.
46. Hu X, Wei H, Xiang L, Chertov O, Wayne AS, Bera TK, Pastan I. Methylation of the DPH1 promoter causes immunotoxin resistance in acute lymphoblastic leukemia cell line KOPN-8. Leuk Res. 2013;37(11):1551–6.
47. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. Ann Rev Immunol. 1998;16:395–419.
48. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol. 2014;15(1):49–63.
49. Brinkmann U, Mansfield E, Pastan I. Effects of BCL-2 overexpression on the sensitivity of MCF-7 breast cancer cells to ricin, diphtheria and Pseudomonas toxin and immunotoxins. Apoptosis. 1997;2(2):192–8.
50. Gottesman MM. Mechanisms of cancer drug resistance. Ann Rev Med. 2002;53:615–27.
51. Keppler-Hafkemeyer A, Brinkmann U, Pastan I. Role of caspases in immunotoxin-induced apoptosis of cancer cells. Biochemistry. 1998;37(48):16934–42.
52. Du X, Youle RJ, FitzGerald DJ, Pastan I. Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Mol Cell Biol. 2010;30(14):3444–52.
53. Brinkmann U, Brinkmann E, Gallo M, Pastan I. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. Proc Natl Acad Sci U S A. 1995;92(22):10427–31.
54. Brinkmann U, Brinkmann E, Pastan I. Expression cloning of cDNAs that render cancer cells resistant to Pseudomonas and diphtheria toxin and immunotoxins. Mol Med. 1995;1(2):206–16.
55. Brinkmann U. CAS, the human homologue of the yeast chromosome-segregation gene CSE1, in proliferation, apoptosis, and cancer. Am J Hum Genet. 1998;62(3):509–13.
56. Xiao Z, McGrew JT, Schroeder AJ, Fitzgerald-Hayes M. CSE1 and CSE2, two new genes required for accurate mitotic chromosome segregation in *Saccharomyces cerevisiae*. Mol Cell Biol. 1993;3(8):4691–702.
57. Irniger S, Piatti S, Michaelis C, Nasmyth K. Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell. 1995;81(2):269–78.
58. Ogryzko VV, Brinkmann E, Howard BH, Pastan I, Brinkmann U. Antisense inhibition of CAS, the human homologue of the yeast chromosome segregation gene CSE1, interferes with mitosis in HeLa cells. Biochemistry. 1997;36(31):9493–500.
59. Kutay U, Bischoff FR, Kostka S, Kraft R, Gorlich D. Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell. 1997;90(6):1061–71.
60. Brinkmann U, Brinkmann E, Gallo M, Scherf U, Pastan I. Role of CAS, a human homologue to the yeast chromosome segregation gene CSE1, in toxin and tumor necrosis factor mediated apoptosis. Biochemistry. 1996;35(21):6891–9.
61. Ried T, Knutzen R, Steinbeck R, Blegen H, Schrock E, Heselmeyer K, du Manoir S, Auer G. Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. Genes, Chromosomes Cancer. 1996;15(4):234–45.
62. Wellmann A, Krenacs L, Fest T, Scherf U, Pastan I, Raffeld M, Brinkmann U. Localization of the cell proliferation and apoptosis-associated CAS protein in lymphoid neoplasms. Am J Pathol. 1997;150(1):25–30.
63. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994;266(5192):1821–8.
64. Hermeking, H., Eick D. Mediation of c-Myc-induced apoptosis by p53. Science. 1994; 265(5181):2091–3.

65. Nishioka WK, Welsh RM. Susceptibility to cytotoxic T lymphocyte-induced apoptosis is a function of the proliferative status of the target. J Exp Med. 1994;179(2):769–74.
66. Dupont J, LeRoith D. Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. Horm Res. 2001;55(Suppl 2):22–6.
67. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7(2):85–96.
68. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, Vigneri R. The role of insulin receptors and IGF-I receptors in cancer and other diseases. Arch Physiol Biochem. 2008;114(1):23–37.
69. Liu XF, FitzGerald DJ, Pastan I. The insulin receptor negatively regulates the action of Pseudomonas toxin-based immunotoxins and native Pseudomonas toxin. Cancer Res. 2013;73(7):2281–8.
70. Liu XF, Xiang L, FitzGerald DJ, Pastan I. Antitumor effects of immunotoxins are enhanced by lowering HCK or treatment with SRC kinase inhibitors. Mol Cancer Ther. 2014;13(1):82–9.

Chapter 4
Cell Signaling and Resistance to Immunotoxins

Roland B. Walter

Abstract The use of toxic plant or microbial proteins or polypeptides as immunotoxins has been a long-pursued strategy to increase the efficacy of targeted anticancer therapeutics. However, although these toxins can be highly potent, resistance has repeatedly been observed. Resistance to immunotoxin scan occur because of neutralizing antibodies or limited tumor cell access but also because of protective cellular signaling events in cancer cells. An increasing number of preclinical studies indicate that the latter form of resistance can be caused by a variety of mechanisms that either pre-exist because of genetic or epigenetic alterations or are induced by the immunotoxin itself, including modulation of cell surface expression of target antigens, altered trafficking or cleavage of toxin molecules, reduced synthesis of modified amino acid residues that are required for the toxin's inhibition of protein synthesis, inhibited caspase activation or activation of other pro-survival pathways, and perhaps activation of drug transporter proteins. While the clinical relevance of these potential resistance mechanisms remains to be demonstrated in future studies, they provide a conceptual framework for cellular resistance to immunotoxins, and may form the basis for the development of rational strategies aimed at improving immunotoxin-based cancer therapy.

Keywords Antibody · Cancer · Cellular · Immunotherapy · Immunotoxin · Resistance · Targeted Therapeutic · Toxin

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Abbreviations

Bcl-2	B-cell lymphoma-2
cAMP	Cyclic AMP
CSEL1/CAS	Cellular apoptosis susceptibility gene
DT	Diphtheria toxin
EF-1	Elongation factor-1
EF-2	Elongation factor-2
IAP	Inhibitor of apoptosis protein
IFNγ	Interferon gamma
IGF	Insulin like growth factor
IL-1α	Interleukin:1 alpha
IL-3	Interleukin-3
IL-4	Interleukin-4
JNK	C-Jun NH2-terminal kinase
NAD	Nicotinamide adenine dinucleotide
NRG1-β1	Neuregulin:1 beta1
PARP	Poly (ADP) ribose polymerase
PKA	Protein kinase A
PKC	Protein kinase C
PE	*Pseudomonas* exotoxin A
PI3K	Phosphatidylinositol 3-kinase
rRNA	Ribosomal RNA
TNFα	Tumor necrosis factor alpha
TRAIL	TNF related apoptosis-inducing ligand
TWEAK	TNF like weak inducer of apoptosis
XIAP	X-linked inhibitor of apoptosis protein

4.1 Introduction

The use of toxic plant or microbial proteins or polypeptides as immunotoxins has been a long-pursued strategy to increase the efficacy of therapeutic antibodies and cell-binding ligands [1, 2]. Although other purposes, such as the modulation of immune responses or the treatment of viral or parasitic infections have been envisioned, the majority of immunotoxins has been generated for the treatment of human cancers [3, 4]. With the availability of recombinant technologies, progress in the development of anti-tumor immunotoxins is rapidly accelerating [5]. Still, the vast majority of such agents comprise toxic moieties that are based on *Pseudomonas* exotoxin A (PE), Diphtheria toxin (DT), cholera toxin, the deglycosylated ricin A chain, recombinant gelonin, or saporin [3, 4, 6–10]. These toxins are exquisitely potent: in some instances, very few molecules can kill a eukaryotic cell [11]. Nonetheless, during the development of immunotoxins, less-than-desired efficacy has repeatedly been observed, highlighting the need to understand the mechanisms underlying clinically relevant resistance of cancer cells to these targeted agents.

Relative resistance to immunotoxins can occur through a variety of ways, ranging from the development of neutralizing antibodies—mostly directed at the toxic portion of the immunotoxin—to limited tumor cell access or binding to cellular mechanisms that limit the cytotoxic effects of toxin molecules. This chapter will review the latter form of resistance to immunotoxins, with particular emphasis on the resistance conferred by signaling events in target cells.

4.2 Conceptual Considerations on Cellular Immunotoxin Resistance

The commonly used toxins each have an enzyme domain that must reach the cytosol of the target cell in order to exert their cytotoxic activity [3, 4, 6–10]. This requires a complex chain of events that includes target cell recognition and binding, internalization of the receptor/immunotoxin complex, intracellular trafficking of processed toxin, cytosolic translocation of the catalytic domain, and effective induction of a signaling cascade that ultimately leads to cell death. Each of these steps, if suboptimal, could lead to relative toxin resistance, offering the target cell ample opportunity to interfere with the cytotoxic activity of immunotoxins. Detailed insight into this multistep process is, therefore, key in understanding how cellular resistance could arise and, conversely, how immunotoxins could be rendered more efficacious.

Some but not all of the elements of the journey of the immunotoxin from the initial docking onto the targeted cell surface antigen to the cytosol have been elucidated mechanistically. For DT, the immunotoxin is internalized after ligand binding via receptor-mediated endocytosis and clathrin-coated pits into an endosome, where the DT catalytic and translocation domains undergo furin-mediated cleavage. Upon endosome acidification, the translocation domain changes its conformation, is inserted into the endosomal membrane, and forms a channel through which the catalytic domain translocates into the cytosol. APE-immunotoxin is similarly internalized via receptor-mediated endocytosis, after which PE undergoes a conformational change and the catalytic and translocation domains are cleaved by furin-mediated proteolysis in endosomes at low pH. Following reduction of the single disulfide bond holding the proteolytic fragments together, the catalytic domain is routed to the trans-Golgi network, where the C-terminal exposed KDEL-like sequence binds the KDEL intracellular sorting receptor. This allows transportation to the endoplasmic reticulum, from where the catalytic domain translocates into the cytoplasm. Like PE, ricin travels backward from the Golgi to the endoplasmic reticulum, where its disulfide-linked chains are separated by protein disulfideisomerase, with subsequent translocation of the catalytic domain into the cytosol. On the other hand, saporin may not rely on Golgi-mediated retrograde transport but translocates to the cytoplasm from the endosomes [3, 4, 6–10].

Relative to this complex cellular uptake, processing, and trafficking process, the enzymatic action that initiates the cell death process is quite simple and well understood: for example, the catalytic domains of DT and PE transfer the adenosine 5'-diphosphate-ribosyl moiety of nicotinamide adenine dinucleotide (NAD) to a modified

histidine residue (diphthamide) in elongation factor-2 (EF-2); this ADP ribosylation inactivates EF-2 and blocks the elongation step of polypeptide assembly, inhibiting protein synthesis. Ricin A, gelonin, and saporin are N-glycosidases that inactivate ribosomes by depurinating a critical adenine residue in 28 S ribosomal RNA (rRNA), thereby preventing the association of elongationfactor-1 (EF-1) and EF-2 with the 60 s ribosomal subunit and inhibiting protein translation. In contrast, cholera toxin acts by ADP ribosylation of the Gs-a subunit of G proteins leading to an increased cyclic AMP (cAMP) level and pore formation in the cell membrane [3, 4, 6–10].

While other mechanisms are involved [12–14], one pathway—well studied for PE, DT, and ricin—involves the loss of the short-lived myeloid cell leukemia sequence 1 (Mcl-1; a pro-survival Bcl-2 family protein) and X-linked inhibitor of apoptosis protein (XIAP), induction of Bak, caspase activation, cleavage of poly(ADP)-ribose polymerase (PARP), DNA fragmentation, and subsequent induction of apoptotic cell death [12, 13, 15–23]. At least for a DT-immunotoxin, some experimental evidence also exists to suggest that the toxin activates the caspase cascade through a Fas-associated death domain protein (FADD)-dependent mechanism that, however, does not involve the death receptors Fas, TFNR1, and TRAIL receptors DR4 and DR5 [24]. Similar to PE, DT, and ricin, cholera toxin also activates caspases leading to cleavage of PARP, triggering of DNA fragmentation, and apoptosis [25–29], and also has shown to inhibit the c-Raf/Mek/Erk signaling pathway [30].

In the following sections, some of the mechanisms by which cancer cells escape immunotoxins will be summarized. Of note, resistance mechanisms have generally been studied in cell line models of human cancer, and although such studies provide a conceptual framework, their clinical relevance remains to be demonstrated. Many of these mechanisms may be pre-existent, that is genetic or epigenetic aberrations have led to alterations of cellular signaling and expression of resistance factors such anti-apoptotic Bcl-2 family proteins. Thus, the immunotoxin meets an already resistant cell. However, while less explored, some studies indicate that the immunotoxin itself can activate cellular signaling events that, ultimately, may lead to relative immunotoxin resistance. For example, Andersson et al. have provided experimental evidence that a PE-immunotoxin can induce an initial stress response that leads to activation of survival pathways with activation of the c-Jun NH2-terminal kinase (JNK) and the AMP-activated protein kinase, effects that could partially block PE-immunotoxin-induced apoptosis [31]. On the other hand, cholera toxin exposure may lead to cAMP-mediated induction of members of the inhibitor of apoptosis protein (IAP) family (BIRC3 and BIRC7) [32], similarly causing relative protection from apoptosis, again indicating that the toxin itself can cause signals that could inhibit the effective induction of cell death.

4.2.1 Alteration of Immunotoxin Resistance via Modulation of Caspase Activation Pathways

Caspase-mediated apoptosis can be initiated intrinsically via diverse stimuli that provoke cell stress or damage and then typically activate one or more members

of the BH3-only protein family, which overcome the inhibitory effect of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family members and promote the assembly of Bak–Bax oligomers within mitochondrial outer membranes or extrinsically via extracellular ligands such as tumor necrosis factor-alpha (TNFα) or Fas ligand that then activate transmembrane death receptors [33]. While tightly regulated in normal cells, defects in apoptosis signaling pathways are a hallmark of many human cancers [34–37]. Given the central role of caspase activation in the cell death of immunotoxin-targeted tumor cells, it is not surprising that modulation of both the mitochondrial or death receptor pathway affects the sensitivity of cancer cells to poisons such as PE, DT, ricin, and cholera toxin. Best studied in this regard are the effects of pro- and anti-apoptotic Bcl-2 family members. For example, overexpression of the pro-apoptotic protein, Bak, can increase the sensitivity to PE-based immunotoxins, whereas silencing of Bak expression causes relative resistance [38]. Conversely, upon treatment with PE, DT, ricin, or cholera toxin, toxin-induced PARP cleavage and apoptotic cell death is inhibited in cell lines forced to overexpress Bcl-2, leading to a modest relative cellular resistance [12, 39, 40]. Similarly, PE-immunotoxin-mediated apoptosis can be reduced by overexpression of the anti-apoptotic proteins, Mcl-1 or Bcl-xL [22]. Because of the well-recognized importance of Bcl-2 family proteins in the resistance of cancer cells to conventional chemotherapeutics and targeted immunotherapies, there is a long-standing interest in the use of pharmacological approaches that lower the threshold to mitochondrial apoptosis. For immunotoxins, the validity of this strategy is suggested by preclinical data with ABT-263 and ABT-737– BH3-only mimetics that bind to and neutralize Bcl-2, Bcl-xL, and Bcl-w [41, 42]—which yield synergistic killing when combined with a PE-based immunotoxin in tumor cell lines.Intriguingly, this sensitization may at least partly be due to an increase in endoplasmic reticulum permeability and promotion of the dislocation of PE from the endoplasmic reticulum to the cytosol, resulting in enhanced inhibition of protein synthesis [43–47].

Signaling events through cell death receptors have similarly been identified as modulators of immunotoxin resistance. For example, tumor necrosis factor-alpha (TNFα) sensitizes to DT- and PE-based but not ricin-based immunotoxins and can overcome immunotoxin resistance in targeted cell lines *in vitro* [16, 17, 48]. Likewise, treatment with an anti-Fas antibody that can mimic TNFα in mediating cytotoxicity against certain target cell lines enhanced DT-immunotoxin-mediated apoptosis; pretreatment with interferon-gamma (IFNγ) upregulated Fas antigen expression and further augmented the cytotoxic activity of the combination of anti-Fas antibody and DT-based immunotoxin [49]. Furthermore, the use of TNF-related apoptosis-inducing ligand (TRAIL) or the anti-TRAIL receptor 2 agonist antibody, lexatumomab (HGS-ETR2), which activate the extrinsic pathway of apoptosis, together with a PE-based immunotoxin targeting mesothelin synergistically triggered caspase-8 recruitment and activation, Bid cleavage and Bax activation and led to subsequent cell death in pancreatic cancer cell lines, even in the absence of functional Bak protein [38]. Consistent with this, the addition of TRAIL also sensitized acute myeloid leukemia cells to the cytotoxic activity of a DT-based immunotoxin targeting interleukin-3 (IL-3) in some cell models [50].

4.2.2 Resistance Mediated by the Cellular Apoptosis Susceptibility Gene

The cellular apoptosis susceptibility gene (*CSEL1/CAS*) has been found highly expressed in a variety of human cancers, and expression levels have been positively correlated with high tumor state, high tumor grade, and worse clinical outcome [51]. Perhaps somewhat paradoxically, CSEL1/CAS was initially identified in breast cancer cells that were resistant to PE, PE-immunotoxin, diphtheria toxin, and TNFα. In these cells, toxin resistance was associated with reduction of CSEL1/CAS protein levels [52, 53], indicating that this protein facilitates apoptosis induced by PE or diphtheria toxin.

4.2.3 Resistance Mediated by Insulin-Like Growth Factor Signaling

The insulin-like growth factor (IGF) signaling system is a key physiologic regulator of tissue growth, development, and energy metabolism, and is exploited by cancers and underlying cancer stem cells for their proliferation, survival, invasion, and metastasis [54–59]. Recent data suggest that the IGF signaling axis also interferes with the intracellular activation of immunotoxins and can mediate relative resistance to this therapeutic strategy. Specifically, siRNA-mediated knockdown of the insulin receptor in several human mesothelin-expressing cell lines enhanced to cytotoxicity of both PE and a PE-based immunotoxin targeting either mesothelin or the transferrin receptor but did not increase cytotoxicity of DT. Mechanistically, this effect of insulin receptors on PE-mediated cell death was attributed to an increased cleavage of the toxin by furin, resulting in greater inhibition of protein synthesis [60].

4.2.4 Immunotoxin Resistance via Interference with Diphthamide Synthesis

As reviewed above, DT and PE ADP ribosylate diphthamide, a uniquely modified histidine residue, in EF-2 to inhibit protein synthesis and initiate cell death. Post-translational modification of this histidine residue is under the control of several proteins, including Dph1-5 [61]. Several studies suggest that interference with the activity of the proteins governing diphthamide synthesis is an effective strategy of cancer cells to counteract the effects of immunotoxins. For example, reversible silencing of either the *DPH1* or the *DPH4* gene via promoter methylation has been demonstrated to lead to relative resistance of acute lymphoblastic leukemia cells to a PE-immunotoxin targeting CD22 [62, 63]. The observations that a deletion of the *WDR85* gene, which has recently been shown to be involved in diphthamide synthesis, and a dominant-negative mutant of Dph2 similarly lead to resistance to

DT- and/or PE-immunotoxins [64, 65] suggest that other proteins could be the target for survival signaling strategies of cancer cells.

4.2.5 Modulation of Immunotoxin Resistance via PI3K/AKT Signaling

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway, a key physiologic regulator of transcription, translation, cell cycle progression, differentiation, metabolism, and apoptosis is often dysregulated in human cancers and has been implicated in their competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance [66–69]. Limited data suggest that the PI3K/Akt pathway also modulates the cellular resistance to immunotoxin-based therapies. Using a PE-immunotoxin targeting the prostate-specific membrane antigen, Baiz et al. showed that treatment of prostate cancer cells with a pan-PI3K inhibitor significantly increased immunotoxin-mediated cytotoxicity [70]. Likewise, studies by Davol et al. indicated that the PI3K inhibitor, wortmannin, could enhance the cytotoxicity of several immunotoxins, in particular those containing saporin or gelonin. However, the same effects were not observed with another PI3K inhibitor (LY294002), raising the suspicion that the effect observed with wortmannin may have occurred through pathways not involving PI3K [71].

4.2.6 Immunotoxin Resistance via Drug Transporter Activity

One of the most common mechanisms of cancer cell resistance to chemotherapy is the expression of ATP-binding cassette (ABC) transporter proteins [72–74]. As is well established for one family member, ABCB1 (MDR1, P-glycoprotein), drug resistance via these transporter proteins is influenced by several signaling pathways and transcription factors, including PI3K/Akt and camp/protein kinase A (PKA), among others [75, 76]. Only a limited number of studies have examined the ability of ABC transporter proteins to cause cellular resistance to immunotoxins, and partly mixed results were reported. Specifically, de Jong et al. found that drug-selected tumor cells that either overexpressed ABCB1 or ABCG1 (breast cancer resistance protein [BCRP]) or were transfected with cDNA for ABCC2-5 (multidrug resistance protein [MRP] 2–5) remained sensitive to a PE-containing immunotoxin targeting interleukin-4 (IL-4), whereas tumor cells overexpressing ABCC1 (MRP1)were relatively resistant to the PE-immunotoxin but not PE itself [77]. Consistent with these findings, McGrath et al. found no evidence that ABCB1 overexpression reduced the sensitivity to PE in myeloid leukemia cells. Similarly unaffected was the cellular sensitivity to ricin, whereas ABCB1 overexpression was associated with resistance to gelonin and a gelonin-based immunotoxin targeting CD33 as well as a very weak resistance to DT; mechanistic studies suggested that increased accumulation and degradation of the immunotoxin within the acidified lysosomal compartment

may underlie this ABCB1-associated resistance to gelonin-immunotoxins [78]. In contrast, no evidence of resistance to a gelonin-immunotoxin targeting the TNF-like weak inducer of apoptosis (TWEAK; TNFSF12) receptor Fn14 (TNFRSF12A) was found in human melanoma cells engineered to overexpress ABCB1 and drug-selected human ovarian cancer cells [79].

4.2.7 Modulation of Immunotoxin Resistance via Cytokine Signaling

Several lines of evidence suggest that cytokine signaling can modulate the sensitivity of tumor cells to the cytotoxic effects of immunotoxins in different ways. As mentioned above, some cytokines such as TNFα can activate cell death receptors and the extrinsic pathway of apoptosis, and augment the cytotoxic effects of some immunotoxins. Other cytokines can activate signaling cascades that, ultimately, impact immunotoxin sensitivity via modulation of target antigens on the cell surface of cancer cells and/or alterations of trafficking/processing of the immunotoxin after internalization, as for example shown for interferon-gamma (IFNγ), interleukin-1 alpha (IL-1α), and neuregulin-1 (NRG1)-β1 [79–81].

4.2.8 Modulation of Immunotoxin Resistance via Protein Kinase C Signaling?

The protein kinase C (PKC) isozymes transduce a myriad of signals that, in many human cancers, are linked to tumor cell proliferation, survival, multidrug resistance, invasion, metastasis, and tumor angiogenesis [82, 83]. Given these properties, it is conceivable to hypothesize that PKC signaling could mediate immunotoxin resistance. This idea would be supported by the observation that PKC inhibition with chelerythrine modestly increased the cytotoxic activity of cholera toxin in lung cancer cell lines [27]. This idea would further be supported by findings with the PKC inhibitor, enzastaurin, which enhanced the cytotoxic effects of a PE-immunotoxin targeting mesothelin at least partly through loss of anti-apoptotic Bcl-2 family proteins and activation of caspases [84]. However, 2 other PKC inhibitors (Go6976 and sotrastaurin) did not sensitize the cancer cell lines to the PE-immunotoxin, suggesting that the mechanism underlying enzastaurin's impact on PE-immunotoxin-mediated cytotoxicity may have included effects on non-PKC signaling pathways [84]. Moreover, activation of PKC signaling via bryostatin 1 sensitized chronic lymphocytic leukemia cells to the cytotoxic activity of a PE-immunoconjugate targeting CD22, perhaps at least in part via upregulation of CD22 expression on the leukemia cells [85].

4.3 Conclusion

The available studies suggest that cancer cells dispose of numerous possibilities to counteract and avoid the cytotoxic effects of immunotoxins. Given the complex uptake and processing that is required for toxins to reach their site of action, it is likely that many more mechanisms exist that have yet to be discovered. Still, the available preclinical data should be useful in informing hypothesis-driven correlative studies that can be conducted during the clinical testing of immunotoxins so that the clinically relevant mechanisms of resistance can be determined. The latter should then provide the ultimate impetus to develop rational combination strategies aimed at overcoming cellular resistance to immunotoxins and improving immunotoxin-based cancer therapies.

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References

1. Thorpe PE, Ross WC, Cumber AJ, Hinson CA, Edwards DC, Davies AJ. Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin. Nature. 1978;271:752–5.
2. Blythman HE, Casellas P, Gros O, Gros P, Jansen FK, Paolucci F, Pau B, Vidal H. Immunotoxins: hybrid molecules of monoclonal antibodies and a toxin subunit specifically kill tumour cells. Nature. 1981;290:145–6.
3. Shapira A, Benhar I. Toxin-based therapeutic approaches. Toxins (Basel). 2010;2:2519–83.
4. Antignani A, Fitzgerald D. Immunotoxins: the role of the toxin. Toxins (Basel). 2013;5:1486–502.
5. Dosio F, Stella B, Cerioni S, Gastaldi D, Arpicco S. Advances in anticancer antibody-drug conjugates and immunotoxins. Recent Pat Anticancer Drug Discov. 2014;9:35–65.
6. Kreitman RJ. Immunotoxins in cancer therapy. Curr Opin Immunol. 1999;11:570–8.
7. Deng Q, Barbieri JT. Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. Annu Rev Microbiol. 2008;62:271–88.
8. Choudhary S, Mathew M, Verma RS. Therapeutic potential of anticancer immunotoxins. Drug Discov Today. 2011;16:495–503.
9. Madhumathi J, Verma RS. Therapeutic targets and recent advances in protein immunotoxins. Curr Opin Microbiol. 2012;15:300–9.
10. Polito L, Bortolotti M, Mercatelli D, Battelli MG, Bolognesi A. Saporin-S6: a useful tool in cancer therapy. Toxins (Basel). 2013;5:1698–722.
11. Yamaizumi M, Mekada E, Uchida T, Okada Y. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell. 1978;15:245–50.
12. Keppler-Hafkemeyer A, Brinkmann U, Pastan I. Role of caspases in immunotoxin-induced apoptosis of cancer cells. Biochemistry. 1998;37:16934–42.
13. Keppler-Hafkemeyer A, Kreitman RJ, Pastan I. Apoptosis induced by immunotoxins used in the treatment of hematologic malignancies. Int J Cancer. 2000;87:86–94.
14. Risberg K, Fodstad O, Andersson Y. Anti-melanoma activity of the 9.2.27PE immunotoxin in dacarbazine resistant cells. J Immunother. 2010;33:272–8.

15. Chang MP, Bramhall J, Graves S, Bonavida B, Wisnieski BJ. Internucleosomal DNA cleavage precedes diphtheria toxin-induced cytolysis. Evidence that cell lysis is not a simple consequence of translation inhibition. J Biol Chem. 1989;264:15261–7.
16. Morimoto H, Bonavida B. Diphtheria toxin- and Pseudomonas A toxin-mediated apoptosis. ADP ribosylation of elongation factor-2 is required for DNA fragmentation and cell lysis and synergy with tumor necrosis factor-alpha. J Immunol. 1992;149:2089–94.
17. Morimoto H, Bonavida B. Ricin-mediated cell-lysis and apoptosis of drug sensitive and resistant tumor-cells. Int J Oncol. 1993;2:363–71.
18. Kochi SK, Collier RJ. DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. Exp Cell Res. 1993;208:296–302.
19. Decker T, Oelsner M, Kreitman RJ, Salvatore G, Wang QC, Pastan I, Peschel C, Licht T. Induction of caspase-dependent programmed cell death in B-cell chronic lymphocytic leukemia by anti-CD22 immunotoxins. Blood. 2004;103:2718–26.
20. Andersson Y, Juell S, Fodstad O. Downregulation of the antiapoptotic MCL-1 protein and apoptosis in MA-11 breast cancer cells induced by an anti-epidermal growth factor receptor-Pseudomonas exotoxin a immunotoxin. Int J Cancer. 2004;112:475–83.
21. Jenkins CE, Swiatoniowski A, Issekutz AC, Lin TJ. Pseudomonas aeruginosa exotoxin A induces human mast cell apoptosis by a caspase-8 and—3-dependent mechanism. J Biol Chem. 2004;279:37201–7.
22. Du X, Youle RJ, Fitzgerald DJ, Pastan I. Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Mol Cell Biol. 2010;30:3444–52.
23. Liu Z, Feng Z, Zhu X, Xu W, Zhu J, Zhang X, Fan Z, Ji G. Construction, expression, and characterization of an anti-tumor immunotoxin containing the human anti-c-Met single-chain antibody and PE38KDEL. Immunol Lett. 2013;149:30–40.
24. Thorburn J, Frankel AE, Thorburn A. Apoptosis by leukemia cell-targeted diphtheria toxin occurs via receptor-independent activation of Fas-associated death domain protein. Clin Cancer Res. 2003;9:861–5.
25. Lanotte M, Riviere JB, Hermouet S, Houge G, Vintermyr OK, Gjertsen BT, Doskeland SO. Programmed cell death (apoptosis) is induced rapidly and with positive cooperativity by activation of cyclic adenosine monophosphate-kinase I in a myeloid leukemia cell line. J Cell Physiol. 1991;146:73–80.
26. Yankelevich B, Soldatenkov VA, Hodgson J, Polotsky AJ, Creswell K, Mazumder A. Differential induction of programmed cell death in CD8 + and CD4 + T cells by the B subunit of cholera toxin. Cell Immunol. 1996;168:229–34.
27. Allam M, Bertrand R, Zhang-Sun G, Pappas J, Viallet J. Cholera toxin triggers apoptosis in human lung cancer cell lines. Cancer Res. 1997;57:2615–8.
28. Yan L, Herrmann V, Hofer JK, Insel PA. beta-adrenergic receptor/cAMP-mediated signaling and apoptosis of S49 lymphoma cells. Am J Physiol Cell Physiol. 2000;279:C1665–74.
29. Pessina A, Croera C, Savalli N, Bonomi A, Cavicchini L, Turlizzi E, Guizzardi F, Guido L, Daprai L, Neri MG. Bcl-2 down modulation in WEHI-3B/CTRES cells resistant to Cholera Toxin (CT)-induced apoptosis. Cell Res. 2006;16:306–12.
30. Zheng X, Ou Y, Shu M, Wang Y, Zhou Y, Su X, Zhu W, Yin W, Li S, Qiu P, Yan G, Zhang J, Hu J, Xu D. Cholera toxin, a typical protein kinase A activator, induces G1 phase growth arrest in human bladder transitional cell carcinoma cells via inhibiting the c-Raf/MEK/ERK signaling pathway. Mol Med Rep. 2014;9:1773–9.
31. Andersson Y, Le H, Juell S, Fodstad O. AMP-activated protein kinase protects against anti-epidermal growth factor receptor-Pseudomonas exotoxin A immunotoxin-induced MA11 breast cancer cell death. Mol Cancer Ther. 2006;5:1050–9.
32. Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L. Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. Proc Natl Acad Sci U S A. 2003;100:8921–6.
33. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008;9:231–41.

34. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001;411:342–8.
35. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004;305:626–9.
36. Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer. 2005;5:876–85.
37. Plati J, Bucur O, Khosravi-Far R. Dysregulation of apoptotic signaling in cancer: molecular mechanisms and therapeutic opportunities. J Cell Biochem. 2008;104:1124–49.
38. Du X, Xiang L, Mackall C, Pastan I. Killing of resistant cancer cells with low Bak by a combination of an antimesothelin immunotoxin and a TRAIL Receptor 2 agonist antibody. Clin Cancer Res. 2011;17:5926–34.
39. Brinkmann U, Mansfield E, Pastan I. Effects of BCL-2 overexpression on the sensitivity of MCF-7 breast cancer cells to ricin, diphtheria and Pseudomonas toxin and immunotoxins. Apoptosis. 1997;2:192–8.
40. Bogner C, Dechow T, Ringshausen I, Wagner M, Oelsner M, Lutzny G, Licht T, Peschel C, Pastan I, Kreitman RJ, Decker T. Immunotoxin BL22 induces apoptosis in mantle cell lymphoma (MCL) cells dependent on Bcl-2 expression. Br J Haematol. 2010;148:99–109.
41. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435:677–81.
42. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, Johnson EF, Marsh KC, Mitten MJ, Nimmer P, Roberts L, Tahir SK, Xiao Y, Yang X, Zhang H, Fesik S, Rosenberg SH, Elmore SW. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res. 2008;68:3421–8.
43. Traini R, Ben-Josef G, Pastrana DV, Moskatel E, Sharma AK, Antignani A, Fitzgerald DJ. ABT-737 overcomes resistance to immunotoxin-mediated apoptosis and enhances the delivery of pseudomonas exotoxin-based proteins to the cell cytosol. Mol Cancer Ther. 2010;9:2007–15.
44. Fitzgerald DJ, Moskatel E, Ben-Josef G, Traini R, Tendler T, Sharma A, Antignani A, Mussai F, Wayne A, Kreitman RJ, Pastan I. Enhancing immunotoxin cell-killing activity via combination therapy with ABT-737. Leuk Lymphoma. 2011;52(Suppl 2):79–81.
45. Mattoo AR, Fitzgerald DJ. Combination treatments with ABT-263 and an immunotoxin produce synergistic killing of ABT-263-resistant small cell lung cancer cell lines. Int J Cancer. 2013;132:978–87.
46. Antignani A, Sarnovsky R, Fitzgerald DJ. ABT-737 promotes the dislocation of ER luminal proteins to the cytosol, including pseudomonas exotoxin. Mol Cancer Ther. 2014;13:1655–63.
47. Hollevoet K, Antignani A, Fitzgerald DJ, Pastan I. Combining the antimesothelin immunotoxin SS1P with the BH3-mimetic ABT-737 induces cell death in SS1P-resistant pancreatic cancer cells. J Immunother. 2014;37:8–15.
48. Morimoto H, Safrit JT, Bonavida B. Synergistic effect of tumor necrosis factor-alpha- and diphtheria toxin-mediated cytotoxicity in sensitive and resistant human ovarian tumor cell lines. J Immunol. 1991;147:2609–16.
49. Morimoto H, Yonehara S, Bonavida B. Overcoming tumor necrosis factor and drug resistance of human tumor cell lines by combination treatment with anti-Fas antibody and drugs or toxins. Cancer Res. 1993;53:2591–6.
50. Horita H, Frankel AE, Thorburn A. Acute-myeloid-leukemia-targeted toxins kill tumor cells by cell-type-specific mechanisms and synergize with TRAIL to allow manipulation of the extent and mechanism of tumor cell death. Leukemia. 2008;22:652–5.
51. Tai CJ, Hsu CH, Shen SC, Lee WR, Jiang MC. Cellular apoptosis susceptibility (CSE1 L/CAS) protein in cancer metastasis and chemotherapeutic drug-induced apoptosis. J Exp Clin Cancer Res. 2010;29:110.

52. Brinkmann U, Brinkmann E, Gallo M, Pastan I. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. Proc Natl Acad Sci U S A. 1995;92:10427–31.
53. Brinkmann U, Brinkmann E, Gallo M, Scherf U, Pastan I. Role of CAS, a human homologue to the yeast chromosome segregation gene CSE1, in toxin and tumor necrosis factor mediated apoptosis. Biochemistry. 1996;35:6891–9.
54. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer. 2004;4:505–18.
55. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer. 2008;8:915–28.
56. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nat Rev Cancer. 2012;12:159–69.
57. Weroha SJ, Haluska P. The insulin-like growth factor system in cancer. Endocrinol Metab Clin North Am. 2012;41:335–50, vi.
58. Singh P, Alex JM, Bast F. Insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) signaling systems: novel treatment strategies for cancer. Med Oncol. 2014;31:805.
59. Malaguarnera R, Belfiore A. The emerging role of insulin and insulin-like growth factor signaling in cancer stem cells. Front Endocrinol (Lausanne). 2014;5:10.
60. Liu XF, Fitzgerald DJ, Pastan I. The insulin receptor negatively regulates the action of Pseudomonas toxin-based immunotoxins and native Pseudomonas toxin. Cancer Res. 2013;73:2281–8.
61. Greganova E, Altmann M, Butikofer P. Unique modifications of translation elongation factors. FEBS J. 2011;278:2613–24.
62. Wei H, Xiang L, Wayne AS, Chertov O, Fitzgerald DJ, Bera TK, Pastan I. Immunotoxin resistance via reversible methylation of the DPH4 promoter is a unique survival strategy. Proc Natl Acad Sci U S A. 2012;109:6898–903.
63. Hu X, Wei H, Xiang L, Chertov O, Wayne AS, Bera TK, Pastan I. Methylation of the DPH1 promoter causes immunotoxin resistance in acute lymphoblastic leukemia cell line KOPN-8. Leuk Res. 2013;37:1551–6.
64. Roy V, Ghani K, Caruso M. A dominant-negative approach that prevents diphthamide formation confers resistance to Pseudomonas exotoxin A and diphtheria toxin. PLoS ONE. 2010;5:e15753.
65. Wei H, Bera TK, Wayne AS, Xiang L, Colantonio S, Chertov O, Pastan I. A modified form of diphthamide causes immunotoxin resistance in a lymphoma cell line with a deletion of the WDR85 gene. J Biol Chem. 2013;288:12305–12.
66. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296:1655–7.
67. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov. 2005;4:988–1004.
68. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261–74.
69. Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR signaling in cancer. Front Oncol. 2014;4:64.
70. Baiz D, Hassan S, Choi YA, Flores A, Karpova Y, Yancey D, Pullikuth A, Sui G, Sadelain M, Debinski W, Kulik G. Combination of the PI3K inhibitor ZSTK474 with a PSMA-targeted immunotoxin accelerates apoptosis and regression of prostate cancer. Neoplasia. 2013;15:1172–83.
71. Davol PA, Bizuneh A, Frackelton AR, Jr. Wortmannin. a phosphoinositide 3-kinase inhibitor, selectively enhances cytotoxicity of receptor-directed-toxin chimeras in vitro and in vivo. Anticancer Res. 1999;19:1705–13.
72. Wu CP, Calcagno AM, Ambudkar SV. Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. Curr Mol Pharmacol. 2008;1:93–105.
73. Tiwari AK, Sodani K, Dai CL, Ashby CR, Jr., Chen ZS. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. Curr Pharm Biotechnol. 2011;12:570–94.

74. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer. 2013;13:714–26.
75. Chen KG, Sikic BI. Molecular pathways: regulation and therapeutic implications of multidrug resistance. Clin Cancer Res. 2012;18:1863–9.
76. Sui H, Fan ZZ, Li Q. Signal transduction pathways and transcriptional mechanisms of ABCB1/Pgp-mediated multiple drug resistance in human cancer cells. J Int Med Res. 2012;40:426–35.
77. De Jong MC, Scheffer GL, Broxterman HJ, Hooijberg JH, Slootstra JW, Meloen RH, Kreitman RJ, Husain SR, Joshi BH, Puri RK, Scheper RJ. Multidrug-resistant tumor cells remain sensitive to a recombinant interleukin-4-Pseudomonas exotoxin, except when overexpressing the multidrug resistance protein MRP1. Clin Cancer Res. 2003;9:5009–17.
78. Mcgrath MS, Rosenblum MG, Philips MR, Scheinberg DA. Immunotoxin resistance in multidrug resistant cells. Cancer Res. 2003;63:72–9.
79. Zhou H, Hittelman WN, Yagita H, Cheung LH, Martin SS, Winkles JA, Rosenblum MG. Antitumor activity of a humanized, bivalent immunotoxin targeting fn14-positive solid tumors. Cancer Res. 2013;73:4439–50.
80. Francisco JA, Kiener PA, Moran-Davis P, Ledbetter JA, Siegall CB. Cytokine activation sensitizes human monocytic and endothelial cells to the cytotoxic effects of an anti-CD40 immunotoxin. J Immunol. 1996;157:1652–8.
81. Salard D, Kuzel TM, Samuelson E, Rosen S, Bakouche O. Interleukin-1 alpha increases the preferential cytotoxicity of an interleukin-2-diphtheria toxin fusion protein against neoplastic lymphocytes from patients with the Sezary syndrome compared to normal lymphocytes. J Clin Immunol. 1998;18:223–34.
82. Podar K, Raab MS, Chauhan D, Anderson KC. The therapeutic role of targeting protein kinase C in solid and hematologic malignancies. Expert Opin Investig Drugs. 2007;16:1693–707.
83. Newton AC. Protein kinase C: poised to signal. Am J Physiol Endocrinol Metab. 2010;298:E395–402.
84. Mattoo AR, Pastan I, Fitzgerald D. Combination treatments with the PKC inhibitor, enzastaurin, enhance the cytotoxicity of the anti-mesothelin immunotoxin, SS1P. PLoS ONE. 2013;8:e75576.
85. Biberacher V, Decker T, Oelsner M, Wagner M, Bogner C, Schmidt B, Kreitman RJ, Peschel C, Pastan I, Meyer Zum Buschenfelde C, Ringshausen I. The cytotoxicity of anti-CD22 immunotoxin is enhanced by bryostatin 1 in B-cell lymphomas through CD22 upregulation and PKC-betaII depletion. Haematologica. 2012;97:771–9.

Chapter 5
Antibody-Drug Conjugates and Immunotoxins for the Treatment of Hematologic Neoplasms

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Abstract The antibody-drug conjugate (ADC) is a unique combination of potent cytotoxic drugs covalently linked to monoclonal antibodies (mAb) through a stable specialized chemical linker. Recombinant immunotoxins, fusion proteins which contain the cytotoxic portion of a protein toxin fused to the Fv portion of an antibody, represent the most promising group of ADCs. Antibody-drug conjugates maximize drug delivery to tumor cells without increasing toxicity to normal cells. There are a number of ADCs in preclinical and clinical developments in haematological malignancies that target CD19, CD22, CD25, CD30, CD33, CD37, CD74, and CD79b. One of them, brentuximab vedotin, is approved for use in Hodgkin's lymphoma and systemic anaplastic large cell lymphoma. This chapter focuses on the use of mAbs or fragments of mAbs attached to cytotoxic agents produced by bacteria or plants, including high-molecular-weight protein toxins and low-molecular-weight chemical entities such as calicheamicin, mytansinoids or auristatin, in the treatment of acute myeloid leukemia, B- and T-cell lymphoid malignancies, Hodgkin lymphoma and multiple myeloma.

Keywords A-dmDT390-bisFv · Brentuximab vedotin · Combotox · DCDTS4501A · DCDT2980S · Denileukin diftitox · Gemtuzumab ozogamicin · Inotuzumab ozogamicin · Indatuximab ravtansine · Lorvotuzumab mertansine · Moxetumomab pasudotox · Polatuzumab vedotin · SAR-3419

Abbreviations

ADC	Antibody drug conjugate
ADCC	Antibody-dependent cell-mediated cytotoxicity
AE	Adverse event
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia

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ALCL	Anaplastic large cell lymphoma
Ara C	Cytarabine
ASCT	Autologous stem cell transplantation
BCR	B-cell receptor
BV	Brentuximab vedotin
CDC	Complement mediated cytotoxicity
CHOP	Cyclophosphamide adriamycin-vincristine-prednison
CLC	Capillary leak syndrome
CLL	Chronic lymphocytic leukemia
CR	Complete response
CRp	CR with no platelet recover
CTCL	Cutaneous T-cell lymphomas
DFS	Disease-free survival
DLBCL	Diffused large B-cell lymphoma; dose limiting toxicity
DM4	N^2'-deacetyl-N^2'-(4-mercapto-4-methyl-1-oxopentyl) maytansine
DLT	Dose-limiting toxicity
FL	Follicular lymphoma
FDA	Food and Drug Administration
GO	Gemtuzumab ozogamicin
HCL	Hairy cell leukemia
HL	Hodgkin lymphoma
IO	Inotuzumab ozogamicin
IR	Indatuximab ravtansine
MM	Multiple myeloma
mAb	Monoclonal antibody
MCL	Mantle cell lymphoma
MM	Multiple myeloma
MRD	Minimal residual disease
LM	Lorvotuzumab mertansine
LBL	Lymphoblastic lymphoma
MTD	Maximum tolerated dose
MMAE	Monomethyl auristatin E
MMAF	Monomethyl auristatin F
NHL	Non hodgkin lymphoma
OR	Overall response
PFS	Progression free survival
PK	Pharmacokinetics
PV	Polatuzumab vedotin
PR	Partial response
PTCL	Peripheral T-cell lymphoma
RFS	Relapse free survival
SD	Stable disease
VLS	Vascular leak syndrome

5.1 Introduction

Over the last few years, several antibody-drug conjugates (ADCs) have been investigated in clinical trials in patients with hematologic malignancies, and some of them have important clinical value. The binding domains of monoclonal antibodies (mAbs) and antigen binding fragments (Fabs) can be used to deliver cytotoxic substances to cells which express cognate antigens on their surface [1, 2]. Recombinant immunotoxins are fusion proteins which contain the cytotoxic portion of a protein, toxins produced by bacteria or plants fused to the Fv portion of an antibody [3]. Protein toxins are highly potent enzymes, and only a small number of molecules need to be delivered to the site of action, the cell cytosol. Most immunotoxins administered in the clinic are derived from *Pseudomonas* exotoxin-A, diphtheria toxin or ricin. These agents mediate their cytotoxicity by inhibiting protein synthesis. In addition, mAbs, or fragments of mAbs, can be attached to low-molecular-weight cytotoxic agents such as calicheamicin, mytansinoids or auristatin. The antibody binds to an antigen on the target cell and the highly toxic protein is internalized by the target cells. The enzymatic fragment of the toxin then translocates to the cytosol, whereupon the cell is killed by the enzymatic inhibition of protein synthesis and initiation of the apoptotic cascade [4]. Toxic proteins can be attached directly to antibodies via peptide bonds, and they can be modified easily with engineered modifications of toxin genes. Immunotoxins maximize drug delivery to tumor cells without increasing toxicity to normal cells. These drugs use a different mechanism to induce cell death than traditional cytotoxic agents, thus potentially circumventing chemoresistance.

There are a number of antibody–drug conjugates (ADCs) in preclinical and clinical development that target CD19, CD22, CD25, CD 30, CD33, CD37, CD74, and CD79b (Table 5.1). Antibodies against these targets are good choices as carriers of drugs for the treatment of leukemias and lymphomas, because they are internalizing. Here we discuss on ADCs which are attached to cytotoxic agents produced by bacteria or plants, including high-molecular-weight protein toxins and low-molecular-weight chemical entities, such as calicheamicin, mytansinoids, and auristatin, in the treatment of acute myeloid leukemia (AML), B- and T-cell lymphoid malignancies, Hodgkin lymphoma (HL) and multiple myeloma (MM).

5.2 Anti-CD33 Immunotoxins for Acute Myeloid Leukemia

Acute myeloid leukemia is a clonal disease characterized by proliferation and accumulation of myeloid progenitor cells in the bone marrow, leading ultimately to hematopoietic failure. This is the most common type of leukemia in adults, yet continues to have the lowest survival rate [5]. In the US, 18,860 projected new cases of AML and 10,460 deaths were reported in 2014 [6]. Combinations of cytarabine

Table 5.1 Antibody drug coniugates and immunotoxins active in hematologic neoplasms

Agent	Target	Characteristics
Gemtuzumab ozogamicin (Mylotarg™, Pfizer Inc.)	CD33	Anti-CD33 mAb conjugated to the cytotoxic antitumor antibiotic, calicheamicin
AVE9633 (ImmunoGen Inc/Sanofi-Aventis)	CD33	Anti-CD33 humanized mAb huMy9-6, linked to the maytansine derivative DM4
HUM-195/rGel (M.D. Anderson Cancer Center)	CD33	Anti-CD33 humanized mAb M195 conjugated to recombinant gelonin
SGN-CD33A (Seattle Genetics, Inc.)	CD33	Humanized anti-CD33 mAb with engineered cysteines conjugated to a highly potent, synthetic DNA cross-linking pyrrolobenzodiazepine dimer
Inotuzumab ozogamicin (CMC-544, Wyet/Pfizer)	CD22	Anti-CD22 IgG4 mAb linked to calicheamicin
BL22 (CAT-3888; National Cancer Institute)	CD22	Anti-CD22 disulfide-linked Fv antibody fragment linked to *Pseudomonas* exotoxin PE38
Moxetumomab pasudotox (CAT-8015, AstraZeneca)	CD22	Fv portion of the anti-CD22 mAb covalently fused to a 38 KDa fragment of *Pseudomonas* exotoxin-A
Pinatuzumab vedotin (DCDT2980S, RG7593, Genentech Inc)	CD22	Anti-CD22 mAb conjugated to monomethyl auristatin E
LMB-2 (National Cancer Institute)	CD25	Recombinant immunotoxin containing variable domains of anti-Tac antibody and truncated *Pseudomonas* exotoxin.
SAR-3419 (Sanofi-Adventis, NJ, USA)	CD19	Humanized mAb huB4 conjugated to the maytansine derivative DM4
Combotox (National Cancer Institute/Montefiore Medical Center)	CD19/CD22	Mixture of two immunotoxins prepared from deglycosylated ricin A chain conjugated to mAbs against CD22 and CD19
DT2219ARL (Scott and White Hospital and Clinic)	CD19/CD22	Bispecific immunotoxin composed of two scFv antibodies recognizing CD19 and CD22 and truncated form of diphtheria toxin
SGN-CD19A (Seattle genetics)	CD19	Humanized anti-CD19 mAb conjugated with monomethyl auristatin F
MDX-1203 (Bristol-Myers Squibb)	CD70	Anti-CD70 antibody conjugated to CC-1065 (rachelmycin) analogue MED-2460
SGN-75 (Seattle Genetics)	CD70	Anti-CD70 mAb h1F6 conjugated to the duocarmycin-based toxin monomethyl auristatin F
Polatuzumab vedotin (DCDTS4501A, Genentech)	CD79b	Anti-CD79b mAb conjugated to monomethyl auristatin E

Table 5.1 (continued)

Agent	Target	Characteristics
IMGN529 (ImmunoGen, Inc.)	CD37	Anti-CD37 mAb K7153A conjugated to the maytansinoid DM1
Brentuximab vedotin (Adcetris®, SGN-35; Seattle Genetics)	CD30	Anti-CD30 mAb cAC10 covalently attached to monomethyl auristatin E
Denileukin diftitox (DAB 486-IL2; Ontak®, Eisai Inc., Seragen, Inc.)	IL-2 receptor	Recombinant fusion product of diphtheria toxin fused to amino acid residues of human IL-2 binding to IL-2 receptors
A-dmDT390-bisFv(UCHT1) (Angimmune LLC)	CD3ε	Anti-T cell immunotoxin consisted of 1-390 amino acid residues of chain A diphtheria toxin joined to the Fv fragment of UCHT1 connected to a second UCHT1 Fv fragment
Milatuzumab-doxorubicin (Immunomedics Inc.)	CD74	Humanized anti-CD74 mAb conjugated to doxorubicin
Indatuximab ravtansine (BT062, Biotest AG)	CD138	Conjugate of anti-CD138 chimerized mAb nBT062 and the maytansinoid DM4
Lorvotuzumab mertansine (IMGN901, ImmunoGen)	CD56	Anti-CD56 mAb, lorvotuzumab, linked to the tubulin-binding maytansinoid DM1

mAb monoclonal antibody

(Ara-C) and anthracyclines are still the mainstay of induction therapy, and the use of high-dose Ara-C is now a standard consolidation therapy in AML patients aged < 60 years. Although several new agents have shown promise in treating AML, it is unlikely that these agents will be curative when administered as monotherapy. It is more likely that they will be used in combination with other new agents or with conventional therapy. CD33 represents an attractive target for antibody-based therapy in patients with AML. It is a transmembrane cell surface glycoprotein receptor that is specific for myeloid cells [7]. The CD33 antigen is present on approximately 90% of AML myeloblasts, including leukaemic clonogenic precursors as well as normal myeloid precursor cells, but not on $CD34^+$ pluripotent hematopoietic stem cells or in non-haematopoietic tissues [8]. However, CD33 is expressed in more committed myeloid precursors and is not present in AML stem cells [9].

5.2.1 Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin (GO, Mylotarg™, Wyeth/Pfizer Inc) is an immunotoxin composed of a humanized IgG4 kappa anti-CD33 antibody conjugated to the cytotoxic antitumor antibiotic, calicheamicin, isolated from the products of fermentation by the bacterium *Micromonospora echinospora. Subsp.* The antibody portion of GO binds specifically to the CD33 antigen on the surface of immature normal cells of myelomonocytic lineage including myeloid blasts, but not on normal hematopoietic stem cells.

The results of early nonrandomized studies showed GO to be effective in patients over 60 years old with relapsed AML, for whom intensive treatment was not suitable [10]. The results of phase II studies revealed an overall response (OR) rate of 30%, including 23 patients with complete response (CR) and 19 with CR with incomplete platelet recovery (CRp), among 142 patients with recurrent AML treated with two doses of GO [11, 12]. These results led to the accelerated approval of GO by the US Food and Drug Administration (FDA) in May 2000 for treatment of patients aged 60 years and older with recurrent AML who were not considered candidates for other chemotherapy. Unfortunately, the trial on simultaneous administration of GO with intensive chemotherapy in induction and consolidation in younger patients with AML was stopped prematurely when no improvement in clinical benefit was observed, and after a higher number of deaths was observed in patients who received GO. In June 2010, the FDA withdrew GO and it is no longer commercially available [13, 14].

Subsequent randomized clinical trials have shown a survival benefit to be associated with GO in subgroups of patients with AML [Table 5.2; 15–18]. More recently, other randomized studies have been completed that support the efficacy of GO in combination with chemotherapy, in specific subsets of patients with newly diagnosed AML with acceptable toxicity. In particular, the results from the LRF AML14 and NCRI AML16 indicate that the addition of GO to low-dose Ara-C doubled the remission rate but did not improve the overall survival in older patients

Table 5.2 Clinical trials evaluating antibody drug coniugates and immunotoxins in acute myeloid leukemia

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Larson et al. 2002 [10]	GO 9 mg/m^2 for two doses with 14 days between doses	First relapse, >60 yrs	101	OR—28%, CR—13%,OS—5.4 m for all patients and 14.5 m for patients achieving CR	Grade 3/4 neutropenia—99%, thrombocytopenia—99%; bilirubin elevations—4% and hepatic transaminases elevations—15%
Sievers et al. 2001 [11]	GO 9 mg/m^2 in 2-h i.v. infusion at 2-week intervals for two doses	AML in first relapse, median age 61 yrs	142	OR—30%; CR 16–30%; median relapse-free survival—6.8 m	Grade 3/4 myelosuppression—23%, hyperbilirubinemia—23%, elevated hepatic transaminases—17%
Burnett et al. 2012 [17]	GO 3 mg/m^2 d 1 of course 1 + daunorubicin/Ara-C or daunorubicin/clofarabine, vs chemotherapy alone	Untreated AML or high-risk MDS, >50 yrs, median age 67 yrs	559 vs 556	OR 69%, CR 60%; no difference between GO (70%) and no GO (68%) arms; 3-year cumulative relapse with GO 68% vs without GO 76% ($P = 0.007$)	Addition of GO in course 1 slightly increased grades of nausea, oral toxicity, and bilirubin, and requirement for more units of platelet and antibiotics
Burnett et al. 2013 [16]	LDAC+/- GO 5 mg on day 1 of each course	Untreated AML in older pts	495	OR (30% vs 17%, $P = 0.006$); OS (25% vs 27%). 3-year OS 25% v 20%, $P = 0.05$)	No difference in 30-or 60-day mortality and no major increase in toxicity with GO
Burnett et al. 2011 [15]	Single dose of GO (3 mg/m^2) on d 1 of induction course 1 with daunorubicin and Ara-C; Ara-C, daunorubicin, and etoposide; or fludarabine, Ara-C, G-CSF; and idarubicin	Untreated, <60 yrs	1113	Addition of GO improved survival in pts with favorable cytogenetics	Lack of increased toxicity in combination with chemotherapy
Petersdorf et al. 2013 [18]	Daunorubicin (45 mg/m^2 ds 1–3 + Ara-C (100 mg/m^2 days 1–7 + GO (6 mg/m^2 on day 4 vs daunorubicin (60 mg/m^2 + Ara-C). Pts in CR after consolidation randomized to three doses of GO (5 mg/m^2 every 28 days or no GO)	Untreated AML pts aged 18–60 yrs	637	CR 69% for DA + GO and 70% for DA ($P = 0.59$); 5-year relapse-free survival rate—43% DA + GO vs 42% DA ($P = 0.40$); 5-year OS 46% DA + GO vs 50% in the DA group ($P = 0.85$; DFS not improved with postconsolidation GO	Grade 4 or fatal nonhematologic induction toxicityin 21% DA + GO vs 12% DA; $P = 0.0054$); no fatal maintenance GO toxicities

Table 5.2 (continued)

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Lapusan et al. 2012 [20]	Phase I study of AVE9633 infusion on day 1 of a 21-day cycle, day 1 and 8 and day 1, 4 and 7 of a 28-day cycle	Relapsed/refractory AML	47	For D1–D7 DLT—150 mg/m^2 (and the MTD 130 mg/m^2; 1 CRp, 1 PR, biological activity in 5 pts	Allergic reaction during infusion, 1 keratitis, 1 liver toxicity
Borthakur et al. 2013 [25]	Phase I study of HUM-195/rGEL, 4 dose levels (12, 18, 28 and 40 mg/m^2 per course) in a "3 + 3" study design	Relapsed or refractory AML, RAEB, CMML, accelerated or myeloid blastic phase of CML	28	MTD—28 mg/m^2 total dose; reduction in peripheral blood blasts of at least 50% in 4 pts	Dose-limiting toxicity—infusion-related allergic reaction including hypoxia and hypotension

AML acute myeloid leukemia, *Ara-C* cytarabine, *CML* chronic myelogenous leukemia, *CMML* chronic myelomonocytic leukemia, *DA* Daunorubicin + Ara-C, *DFS* disease-free survival, *G-CSF* granulocyte colony-stimulating factor, *GO* gemtuzumab ozogamicin, *Ara-C* cytarabine, *CR* complete, *CY* cyclophosphamide, *LDAC* low-dose Ara-C, *MDS* myelodysplastic syndrome, *MTD* maximum tolerated dose, *NR*-not reported, *OR* overall response, *OS* overall survival, *PR* partial response, *RAEB* refractory anemia with excess blasts

[16]. However, a significant survival benefit was observed in the large randomized MRC AML15 trial in AML patients who demonstrated favorable cytogenetics [15]. In this study, 113 untreated younger patients were randomly assigned to receive a single dose of GO (3 mg/m^2) on day 1 of an induction course consisting of either daunorubicin and Ara-C; Ara-C, daunorubicin, and etoposide; or fludarabine, Ara-C, granulocyte colony-stimulating factor (G-CSF) and idarubicin. The addition of GO was well tolerated and no increase of toxicity was observed in the GO arm. The overall response (OR) and the overall survival (OS) were similar. However, although a significant survival benefit was noted for patients with favorable cytogenetics and a beneficial trend was seen in intermediate-risk patients, no such benefit was observed for patients with a poor-risk disease [18]. This randomized phase III clinical trial evaluated the potential benefit of the addition of GO to standard induction and postconsolidation therapy regime in patients with AML. Patients were randomly assigned to receive either a standard DA induction therapy regime comprising daunorubicin (60 mg/m^2 per day on days 1, 2, and 3) and Ara-C alone, or a DA + GO combination of daunorubicin (45 mg/m^2 per day on days 1, 2, and 3), Ara-C (100 mg/m^2 per day by continuous infusion on days 1–7) and GO (6 mg/m^2 on day 4). Patients who achieved CR received three courses of high-dose cytarabine. Those remaining in CR after consolidation were randomly assigned to receive either no additional therapy or three doses of GO (5 mg/m^2 every 28 days). From August 2004 until August 2009, 637 patients were registered for induction. The CR rate was 69 % for DA + GO and 70 % for DA ($P = 0.59$). Among those who achieved a CR, the 5-year relapse-free survival rate was 43 % in the DA + GO group and 42 % in the DA group ($P = 0.40$). The 5-year overall survival rate was 46 % in the DA + GO group and 50 % in the DA group ($P = 0.85$). One hundred and seventy-four patients in CR after consolidation underwent postconsolidation randomization. Disease-free survival (DFS) was not improved with postconsolidation GO (HR, 1.48; $P = 0.97$). In this study, the addition of GO to induction or postconsolidation therapy failed to show improvement in the CR rate, disease-free survival, or overall survival. The results of this trial failed to demonstrate improvement in the CR rate, relapse free survival (RFS), or OS survival when GO was added to either the induction therapy or the postconsolidation therapy.

5.2.2 AVE9633

AVE9633 (ImmunoGen Inc/Sanofi-Aventis) is a new immunoconjugate comprising of an anti-CD33 humanized mAb, huMy9-6, linked through a disulfide bond to the maytansine derivative DM4 (N^2'-deacetyl-N^2'-(4-methyl-4(oxobutyldithio)-1-oxopentyl)-maytansine), a potent tubulin inhibitor [19]. In a phase I study, AVE9633 was administered to 54 patients with refractory/relapsed AML, as drug infusion on day 1 of a 21-day cycle, day 1 and 8 and day 1, 4 and 7 of a 28-day cycle (NCT00543972) [Table 5.2; 20]. The primary objective of the study was to determine the maximum tolerated dose (MTD) of AVE9633 and to characterize the

dose limiting toxicity. Allergic reactions during infusion, particularly the presence of bronchospasms, was the main toxicity. Dose limiting toxicity (DLT) was reached for the three day schedule at 150 mg/m^2, with the MTD being 130 mg/m^2. The DLT was not reached for either the one or two-day schedules. Two responses were obtained in this trial, including one CR and one partial response (PR), and biological activity was seen in five other patients. However, the development of AVE9633 was discontinued after the completion of the treatment of study patients in this phase 1 trial.

5.2.3 HUM-195/rGEL

HUM-195/rGel (M.D. Anderson Cancer Center) is an immunotoxin containing an M195 anti-CD33 humanized mAb conjugated to recombinant gelonin via an N-succinimidyl-3-(2-pyridyl-dithio)-propionate linkage [21]. M195 is an IgG2a mAb derived from a mouse immunized with live human leukemic myeloblasts. Its reactivity is restricted to myeloid blasts and myeloid progenitors and is not present in mature myeloid cells [22]. The HUM-195 recombinant antibody has higher avidity for binding CD33 and better induces antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) than M195 [23]. Recombinant gelonin (rGel) is a single-chain recombinant version of the gelonin toxin originally isolated from the seeds of *Gelonium multi-florum*. It inactivates the ribosomal 60 S subunit by cleaving the RNA adenine N-glycoside bonds in a sequence-specific fashion and inhibits protein synthesis [24].

The HUM-195/Rge was investigated in a phase I study in patients with relapsed or refractory myeloid leukemias and the results were recently published [25]. The drug was administered to 28 patients intravenously at four dose levels (12, 18, 28 and 40 mg/m^2 per course). The MTD was established as 28 mg/m^2. The DLT was infusion-related allergic reaction including hypoxia and hypotension. Three patients showed a 38–50 % reduction in bone marrow blasts and four patients developed a reduction in peripheral blood blasts of at least 50 %. In addition, normalization of platelets was observed in one patient treated with 40 mg/m^2.

5.2.4 SGN-CD33A

SGN-CD33A (Seattle Genetics, Inc.) is a novel CD33-directed ADC which demonstrates preclinical antitumor activity against multi-drug resistant human AML. It is a humanized anti-CD33 antibody linked to pyrrolobenzodiazepine (PBD) dimers, highly potent cytotoxic DNA-crosslinking agents, by engineered cysteines (EC-mAb) using proprietary site-specific conjugation technology. In vitro studies with SGN-CD33A revealed approximately 3-fold higher activity than GO against primary AML cells [26, 27]. A phase I study examining the safety profile of SGN-CD33A administered as a single agent in patients with AML is ongoing (NCT01902329).

5.3 Antibody-Drug Conjugates for B-Cell Lymphoid Malignancies

Lymphoid malignancies comprise a heterogeneous group of disorders originating from clonal proliferation of B or T lymphocytes. B-cell lymphoid malignancies are more common than T-cell neoplasms, accounting for approximately 85–90% of all non-Hodgkin's lymphomas (NHL) [28]. The incidence of NHL has been increasing steadily over the past several decades. At present, it is the fifth most common cancer in the US, with an estimated 79,990 new cases and 20,170 deaths [29]. The most common subtypes of B-cell NHL are diffused large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). DLBCL accounts for approximately 30% of all new diagnosed cases and more than 80% of aggressive lymphomas. FL is the second most common lymphoma, representing approximately 70% of all indolent lymphomas and 22% of all lymphomas. In addition, mantle cell lymphoma (MCL) is a distinct disease entity in the WHO classification of malignant neoplasms, and comprises 7% of NHL [30]. Currently, ADC targeting the antigens CD22, CD19, CD25, CD30, CD37, CD70, CD79b and CD180 are in preclinical studies or clinical trials for B-cell malignancies (Table 5.3).

5.3.1 Anti-CD22 Immunotoxins

CD22 is a 135 kDa B-cell-specific transmembrane sialoglycoprotein whose expression is limited to B-cells. It is expressed in about 70% of B cell lymphomas and leukemias but is not expressed on plasma cells, memory B cells, stem cells, monocytes or T-cells [31]. CD22 is a signaling molecule that plays a role in cellular adhesion, regulation of B-cell homing, and modulation of B-cell activation. It regulates B-cell function, both as a lectin-like adhesion receptor and as a component of the B-cell activation complex. The function of CD22 through the B-cell receptor (BCR) complex is due to phosphorylation of three tyrosine-based inhibitory motifs on its intracellular tail upon BCR stimulation [32, 33]. CD22 is internalized into the cell when bound by an antibody. At least six different anti-CD22 immunotoxins are currently under clinical investigation, including inotuzumab ozogamicin, BL22, moxetumomab pasudotox, Anti-CD22-MCC-DM1, pinatuzumab vedotin and combotox [Table 5.3; 34].

5.3.1.1 Inotuzumab Zogamycin

Inotuzumab ozogamicin (IO, CMC-544, Wyet/Pfizer) is an immunotoxin, a close relative of gemtuzumab ozogamicin, comprising a hinge-stabilized humanized anti-CD22 IgG4 mAb covalently linked to calicheamicin, a toxic natural product of *Micromonospora echinospora* via an acid-labile acetylphenoxy butanoic linker [35]. The mAb is joined to calicheamicin via the acid hydrolysable 4-(4'-acteylphenoxy)

Table 5.3 Clinical trials evaluating antibody drug coniugates and immunotoxins in B-cell ALL

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Kantarjian et al. 2012 [38]	IO 1·8 mg/m^2 i.v. over 1 h every 3–4 weeks	Adults and children with refractory and relapsed ALL	49	OR—57% CR—18%; marrow CR—39%	Fever (gr ½–20 pts, gr 3/4–9 pts. Hypotension (gr 1/2–12 pts, grade 3–1pt Bilirubin: gr 1/2–12 pts, gr3–2 pts raised aminotransferases: gr ½–27 pts, gr 3–1pt
Kantarjian et al. 2013 [39]	IO 1.3–1.8 mg/m^2 every 3 to 4 wks—49 pts IO 0.8 mg/m^2 d y 1 and at a dose of 0.5 mg/m^2 on ds 8 and 15, every 3–4 wks—41 pts	Refractory-relapsed ALL	90	OR—58%. CR—19% CRp 30 PFS—7 m OS—6.2 months	Reversible bilirubin elevation, fever, hypotension
Jain et al. 2013 [40]	IO 1.3 to 1.8 mg/m^2 + mini-hyper-CVD (cyclophosphamide and dexamethasone at 50% dose reduction, methotrexate at 75% dose reduction, Ara-C 0.5 g/m^2 × 4 doses) + IO day 3	Pts ≥ 60 yrs (median age 69 yrs with newly-diagnosed B-cell ALL	15	CR/CRp—13 pts (93%), All pts in CR MRD negative	Grade 3 elevation in liver function test; VLS—2 pts Thrombocytopenia—6 pts
Herrera et al. 2009 [68]	Combotox 2 mg/m^2, 4 mg/m^2, 5 mg/m^2, and 6 mg/m^2	Pediatric patients with refractory B-lineage ALL	17	CR—3 pts Decrease of peripheral blood blasts >95%—6 pts	Elevated liver function tests
Schindler et al. 2011 [69]	Combotox 3, 5, 6, 7 and 8 mg/m^2 per dose, three doses every other day	Refractory B-lineage ALL	17	PR—1 SD—2	Dose-limiting toxicity—vascular leak syndrome, abnormality in liver function tests

ALL acute lymphocytic leukaemia, *Ara-C* cytarabine, *CR* complete response, *CRp*, CR with no platelet recovery, *IO* inotuzumab ozogamicin, *i.v.* intravenous, *NR* not reported, *OR* overall response, *PR* partial response, *VLS* vascular leak syndrome

butanoic acid (AcBut) linker. This linker is more effective than a more stable amide linker in both *in vitro* cytotoxicity and *in vivo* anti-tumor assays. Inotuzumab ozogamicin has shown promising single-agent activity in patients with relapsed/refractory B-lineage ALL and in CD22 positive indolent and aggressive NHL [36, 37].

In a study by Kantarjian et al., 49 adults and children with refractory and relapsed ALL were treated with single-dose, intravenous IO in a phase II study [Table 5.3; 38]. Patients were given 1.8 mg/m^2 IO intravenously over 1 h every 3–4 weeks. The OR rate was 57%, including 9 (18%) CR and 19 (39%) marrow CR. The most frequent adverse events (AE) were fever, hypotension and liver-related toxic effects. A subsequent study examined a group of 90 patients [39], 49 of whom were treated with single-dose, intravenous IO 1.3–1.8 mg/mg/m^2 every 3–4 weeks, while the remaining 41 received inotuzumab weekly at a dose of 0.8 mg/m^2 on day 1 and at a dose of 0.5 mg/mg/m^2 on days 8 and 15, every 3–4 weeks. In this study, the OR rate was found to be 58% including CR in 19%, CRp in 30% and a bone marrow CR (no recovery of counts) in 9%. The median remission duration was 7 months and the median OS was 6.2 months.

A combination of inotuzumab ozogamicin with low-intensity hyper-CVD (Mini-hyper-CVD) as frontline therapy has been also investigated in older patients with ALL [40]. Treatment was safe and showed very encouraging results. Of the 14 patients evaluable for response, 13 (93%) responded, including 12 CR and one CRp, all with flow-cytometric minimal residual disease (MRD) negative status. One-year disease-free survival (DFS) was 83% and OS 93%. No DLT was observed. Inotuzumab ozogamicin also demonstrated promising activity in pediatric patients with relapsed and refractory ALL [41].

The drug has demonstrated good tolerability and significant efficacy against CD22 positive B-cell NHL [42, 43]. In a phase I study, IO was administered intravenously as a single agent once every 3 or 4 weeks at doses ranging from 0.4 to 2.4 mg/m^2 to 79 patients with relapsed/refractory FL and DLBCL [44]. The OR rate was 39% and median PFS was 10 months for patients treated at the MTD. Median PFS was 10.4 months. In patients with FL treated at the MTD, the OR rate was 68% and median PFS was approximately 10.4 months. Corresponding values for patients with DLBCL were 15% and 49 days [45].

The safety and activity of IO and rituximab combination therapy was evaluated in a phase I/II study in 118 patients with relapsed/refractory CD20 and/CD22 positive NHL. The objective response rate was found to be 87% for patients with FL and 74% for patients with DLBCL, and the 2-year PFS rates were 68% and 42%, respectively. The median duration of response was 17.7 months for relapsed DLBCL and was not reached for patients with FL after a median follow-up of 40 months. Most common grade 3–4 AEs were thrombocytopenia, noted in 31% of patients, and neutropenia, noted in 22%. Studies exploring the combination of IO alone, and in combination with rituximab and other chemotherapeutic regimens are ongoing in a phase III trial (NCT00562965).

5.3.1.2 BL22

BL22 (RFB4(dsFv)-PE38, CAT-3888; National Cancer Institute) is an anti-CD22 immunotoxin fusion protein between a murine anti-CD22 disulfide-linked Fv (dsFv) antibody fragment and *Pseudomonas* exotoxin PE38. BL22 is cytotoxic to several CD22-positive cell lines, being 1.5–6.7-times more cytotoxic than the single-chain recombinant immunotoxin [46]. BL22 was investigated in chemotherapy-resistant hairy cell leukemia (HCL) [Table 5.4; 47]. All patients included into the study had circulating hairy cells that expressed CD22. BL22 was administered at doses between 0.2 and 4.0 mg as a 30 min intravenous infusion every other day to a total of three doses. After a partial response, patients could receive a total of 16 cycles of BL22, and patients who had a CR could receive two additional cycles. Of 16 patients treated with BL22, 11 (69%) had a CR and two had a PR. During a median follow-up of 16 months, 3 of the 11 patients who had a CR were retreated with BL22 because of relapse, and all of them had a second CR. A common toxic effect was transient hypoalbumina and an elevated aminotransferase level. Two patients developed reversible hemolytic uremic syndrome. BL22 demonstrated a <20% response rate in chronic lymphocytic leukemia (CLL) and ALL, diseases in which the leukemic cells contain much lower numbers of CD22 target sites.

Table 5.4 Clinical trials evaluating antibody drug coniugates and immunotoxins in hairy cell leukemia

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Kreitman et al. 2001 [46]	BL22, 0.2–4.0 mg i.v. every other day for a total of three doses	Classic HCL—13 pts, HCL variant—3 pts, all pretreated with median three courses of purine analogues	16	CR—11pts (3 pts relapsed after 8, 12, and 7 months, PR—2 pts	Reversible hemolytic-uremic syndrome—2 pts; common toxic effects—transient hypoalbuminemia and elevated aminotransferase levels
Kreitman et al. 2012 [49]	Moxetumomab pasudotox 5–50 μg/kg every other day for three doses (QOD × 3), with up to 16 cycles repeating at ≥4-week intervals	Classic HCL—26 pts, HCL variant—2 pts, 1–7 prior courses of purine analogs (median two courses)	28	OR—86%, CR—46%, 10 (80%) of 13 patients remaining in CR for a median of 29 months	Transient grade 2 hemolytic uremic syndrome—2 pts, hypoalbuminemia, aminotransferase elevations, edema, headache, hypotension, nausea, and fatigue
Kreitman et al. 2000 [57]	LMB-2 2–63 μg/kg i.v. over 30 min on alternate days for three doses (QOD × 3)	HCL pts previously treated with purine analogs	4	CR lasting 6 manths—1 pt, PR >44 days—3 pts	Transaminase elevations, fever, nausea, vomiting, diarrhea

CR complete response, *HCL* hairy cell leukemia, *OR* overall response, *PR* partial response

5.3.1.3 Moxetumomab Pasudotox

Moxetumomab pasudotox (CAT-8015, HA22, Astra Zeneca) is a new generation of CD22-specific targeted immunotoxins. It comprises a disulfide-stabilized, variable mouse CD22 fragment fused to a *Pseudomonas aeruginosa* exotoxin A PE38 fragment via a 7-mer linker. [48]. The drug was mutated from BL22 by changing SSY to THW at positions 100, 100a, and 100b of VH. Moxetumomab pasudotox is internalized upon binding to CD22, inhibiting protein translation and promoting apoptosis. This agent may have a role in the treatment of HCL, especially in patients where conventional therapies produce limited responses or treatment failure [49]. Moxetumomab pasudotox at doses up to 50 μg/kg QOD × 3 has activity in relapsed/refractory HCL and has a safety profile that supports further clinical development for treatment of this disease. The drug is up to 50-times more active on lymphoma cell lines and leukemic cells from patients with CLL and HCL than BL22. No DLT has been established in a phase I trial in 28 patients with refractory/relapsed HCL and MTD has not been reached [Table 5.4; 50]. Drug-related toxicities included grade 1–2 hypoalbuminemia, aminotransferase elevations, edema, headache, hypotension, nausea, and fatigue. Nineteen of 26 patients (73.1 %) responded with a CR rate of 34.6 % and a PR rate of 38.5 %. Moxetumomab pasudotox has been also clinically tested and showed antitumor activity in other B-cell malignancies including CLL, B-cell NHL, and ALL. (NCT01030536, NCT00659425, NCT00586924). The clinical activity and safety profile of this drug support further clinical development for the treatment of HCL and other CD22 positive lymphoid neoplasms.

5.3.1.4 Pinatuzumab Vedotin

Pinatuzumab vedotin (PV, DCDT2980S, RG7593, Genentech Inc) is an anti-CD22 monoclonal antibody conjugated to monomethyl auristatin E (MMAE), which is the same toxin incorporated in brentuximab vedotin [51, 52]. MMAE is a synthetic derivative of dolastatin 10, a potent anti-microtubule inhibitor, originally isolated from the marine shell-less mollusk *Dorabella auricularia*.

Pinatuzumab vedotin has therapeutic activity and acceptable toxicity in patients with relapsed or refractory B-cell NHL when used at doses of 1.8 and 2.4 mg/kg [53, 54]. MTD was defined as 2.4 mg/kg either as a single agent or in combination with rituximab at a dose of 375 mg/m^2 every 21 days [55]. The drug alone and in combination with rituximab were generally well-tolerated. The most frequent adverse events included neutropenia (26 %), hyperglycemia (10 %), peripheral sensory neuropathy (10 %), fatigue (5 %), and diarrhea (5 %). Peripheral sensory neuropathy was reversible in some patients with dose delays and reductions. The overall objective responses were observed in 41 % patients treated with PV and 31 % patients receiving PV in combination with rituximab. These results may indicate that the addition of rituximab does not appear to enhance the efficacy of PV. The combination of rituximab with PV is undergoing further evaluation in an ongoing randomized phase II study in patients with refractory/relapsed DLBCL and FL (NCT01691898).

5.3.2 Anti-CD25 Immunotoxins

CD25 is the interleukin 2 receptor (IL-2R) which is expressed on activated normal T and B cells and macrophages. CD25 is also present on the malignant cells of patients with a variety of hematologic malignancies, including acute T-cell leukemia (ATL), CLL, anaplastic large-cell lymphomas (ALCL), B-cell NHL, peripheral T-cell lymphomas (PTCL), cutaneous T-cell lymphomas (CTCL) and HCL. LMB-2 (Anti-Tac(Fv)-PE38, National cancer Institute) is an anti-CD25 recombinant immunotoxin containing variable domains of MAb anti-Tac and truncated *Pseudomonas* exotoxin [56]. LMB-2 was well tolerated and was found to have clinical activity in CD25 positive hematologic malignancies. In a phase I study, patients with $CD25^+$ hematologic malignancies refractory to standard and salvage therapies were treated with LMB-2 at doses which ranged from 2 to 63 μg/kg administered intravenously over 30 min on alternate days for three doses [57]. Dose-limiting toxicity (DLT) at the 63 μg/kg level included transaminase elevations, diarrhea and cardiomyopathy. LMB-2 induced one CR and seven PRs in 34 assessable patients. One HCL patient achieved a CR, which was ongoing at 20 months. In addition, seven PRs were noted in CTCL, HCL, CLL, HL and adult T-cell leukemia (ATL). Importantly, all four HCL patients responded to LMB-2 therapy. The phase II trial of LMB-2 in HCL is ongoing (NCT00321555).

5.3.3 Anti-CD19 Immunotoxins

CD19 is a 95-kDa type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily with expression restricted to B cells [58]. High expression levels of CD19 are observed on the vast majority of NHL, acute lymphoblastic leukemia (ALL) and CLL cells. However, it is not found on hematopoietic stem cells, plasma cells, or other healthy tissues. Since most B-cell lymphoid malignancies retain CD19 expression, it represents an excellent target for immunotherapy of these disorders. This molecule acts as a co-receptor, enhancing signaling and antigen processing by the B-cell receptor complex in response to antigen stimulation [59]. CD19 has a broader expression profile than that of CD20 and a more efficient internalization and it is thought that CD19 is a better target for immunotoxins.

5.3.3.1 SAR-3419

SAR-3419 (Sanofi-Adventis, NJ, USA) is an anti-CD19 composed of the humanized antibody huB4 conjugated to the maytansine derivative DM4 ($N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl) maytansine), via a cleavable disulfide linker N-succinimidyl-4-(2-pyridyldithio)butyrate (SPDB linker). DM4 is a thiol-containing maytansinoid, originally isolated from the bark of the African shrub *Maytenus ovatus* [60]. It is a highly potent tubulin inhibitor which inhibits tubulin

polymerization and enhances microtubule destabilization. In consequence, DM4 suppresses microtubule dynamics, resulting in a mitotic block and apoptotic cell death. DM4 binds to tubulin and inhibits the microtubule assembly in a similar manner to *Vinca* alkaloids, but is 100–1000-fold more potent [61]. In preclinical studies, SAR3419 was found to be more effective than rituximab and a standard cytotoxic chemotherapy regimen (cyclophosphamide-Adriamycin-vincristine-prednisone, CHOP) against CD19+ tumor cells in diffuse large B-cell lymphoma (DLBCL) and follicular small cleaved cell lymphoma models [62].

During evaluation in two phase I trials in refractory/relapsed patients with NHL, SAR3419 was found to possess significant activity and low hematological toxicity (Table 5.5) [63, 64]. In an every-3-weeks dosing trial, objective responses were observed in 6 (17%) out of 35 patients who received doses in the range of 10–270 mg/m^2. In a dose-escalation study, SAR3419 was administered alone by intravenous infusion in patients with refractory/relapsed NHL [64].

Patients were treated with escalating doses of SAR3419 repeated qw for eight to 12 doses. Forty-four patients were treated on seven dose levels ranging from 5 to 70 mg/m². The qw/q2w schedule allowed accumulation to be limited, resulting in a decrease in the SAR3419 plasma trough and average concentrations of around 1.4-times of those identified with the qw schedule. Around 30% of patients with either indolent or aggressive NHL responded to therapy. In these phase I studies, clinical responses were noted mainly in patients with FL and DLBCL. The qw/q2w schedule at 55 mg/m² showed an improved safety profile compared with the qw schedule. When administered weekly, SAR3419 was well tolerated and active. The qw/q2w was selected for clinical phase II studies. The dose-limiting toxicities were reversible and included severe blurred vision associated with microcystic epithelial corneal changes and neuropathy.

A phase II study evaluated a combination of SAR3419 and rituximab therapy in patients with relapsed/refractory DLBCL [65]**.** Fifty-three patients received 375 mg/m^2 of rituximab i.v. and 55 mg/m² of SAR3419 on days 1, 8, 15 and 22, followed by bi-weekly rituximab and SAR3419 at the same doses for two additional 28-day cycles. An OR rate of 58.3% was achieved for patients with relapsed DLBCL, while 42.9% was noted for refractory patients. Asthenia, nausea, cough, diarrhea and vomiting were the most common non-hematologic AEs. SAR3419 holds promise as a novel and well-tolerated therapy in B-cell lymphoid malignancies.

5.3.3.2 Combotox

Combotox (National Cancer Institute/Montefiore Medical Center) is a 1:1 mixture of two immunotoxins prepared from deglycosylated ricin A chain (dgRTA) conjugated to mAbs directed against CD22 (RFB4-dgA) and CD19 (HD37-dgRTA [66, 67]. Both anti-CD19 (HD37) and anti-CD22 (RFB4) are murine IgG_1 mAbs. Preclinical studies have demonstrated that combotox has cytotoxic activity against pre-B-ALL cell lines and cells from patients with pre-B ALL. Combotox can be safely administered to children with refractory leukemia and has antileukemic activity in

Table 5.5 Clinical trials evaluating antibody drug coniugates and immunotoxins in B-cell NHL

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Advani et al. 2010 [37]	IO 0.4–2.4 mg/m(2) every 3 or 4 weeks	B-cell NHL pts that-progressed after ≥2 therapies	79	OR—39% for all pts, FL: OR—68%, PFS—317 ds DLBCL: OR—15%, PFS 49 ds	Thrombocytopenia (90%), asthenia (67%), nausea (51%), neutropenia (51%)
Fayad et al. 2013 [44]	IO doses 0.8 mg/m^2, 1.3 mg/m^2, and 1.8 mg/m^2 + Rituximab 375 mg/m^2	Relapsed FL—42 pts; Relapsed DLBCL—47 pts; Refractory NHL—30 pts	118	FL: OR 87%,CR 62%, 2-year PFS 68%; DLBCL: OR 74%, CR50%, 2-year PFS 42%; Refractory NHL: OR 20%, 2-year PFS rate of 10%	Thrombocytopenia—56%, nausea—57%, fatigue—53%, increased AST—41%, neutropenia—33%, increased alkaline phosphatase—30%, vomiting—28%
Advani et al. 2012 [54]	PV 0.1–3.2 mg/kg i.v. every 21 days until disease progression or unacceptable toxicity	Heavily pre-treated pts: DLBCL 18 pts, FL, 11 pts, transformed FL—4 pts, SLL—2 pts	35	DLBCL: 2 pts >75% reduction SPD and negative PET scans,2pts >50% reduction in tumor SPD; FL:1 PR	AEin 20% of pts: diarrhea (34%), fatigue (34%), nausea (31%), neutropenia (26%), decreased appetite (23%), vomiting (23%), peripheral edema (20%)
Advani et al. 2013 [55]	PV 1.8 mg/kg—2.4 mg/kg q21d +/-RTX 375 mg/m^2	Relapsed/refractory DLBCL or indolent NHL	46	OR: 19/46 (41%) DCDT and 5/16 (31%) DCDT+RTX pts; Median PFS for DLBCL—115 days and for indolent—227 days	Grade ≥3 Aes in ≥5% of pts: neutropenia (26%), hyperglycemia (10%), peripheral sensory neuropathy (10%), fatigue (5%), and diarrhea (5%)
Ogura et al. 2010 [43]	IO 1.3 and 1.8 mg//m^2 every 28 days	Patients with FL pre-treated with rituximab-based therapy	13	CR—54% PR—31% SD—15%	Thrombocytopenia (100%), leukopenia (92%), lymphopenia (85%), neutropenia (85%), elevated AST (85%), anorexia (85%), and nausea (77%)
Ogura et al. 2012 [42]	Rituximab 375 mg/m^2 i.v. on day 1+IO 1.8 mg/m2 i.v. on day 2 every 28 days for up to eight cycles	Relapsed/refractory B-cell NHL (FL—6, MCL—2, DLBCL—1, MALT—1)	10	OR−80% CR—7 PR—1	Thrombocytopenia (70%), neutropenia (50%), leukopenia (30%), lymphopenia (30%)

Table 5.5 (continued)

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Younes et al. 2012 [63]	SAR3419 10 mg/m^2 i.v	Relapsed/refractory B-cell NHL (FL—17, MCL—5, DLBCL—5, SLL—10, MZL—2)	39	OR—23.5% pts treated with MTD OR for FL—30%	Fatigue—17pts, Eye disorders—18 pts, Peripheral neuropaty 17 pts, Diarrhea 15 Pts, Dyspnea 6 pts
Ribrag et al. 2014 [64]	SAR3419 5–70 mg/m^2 i.v	Relapsed/refractory B-cell NHL (FL—19, DLBCL—16, other 9)	44	OR: weekly schedule—30% (2 CR and 4 Cru), optimized schedule—28% (1 CR and 3 Cru)	Gastrointestinal disorders (27%), , eye disorders (23%), asthenia/ fatigue (23%) patients, reversible paresthesias (11%)
Palanca-Wessels et al. 2013 [83]	DCDS4501A 1.8 mg/kg—2.4 mg/kg mg/kg. i.v.every 21 days or DCDS4501A 2.4 mg/kg+RTX until disease progression or unacceptable toxicity	Relapsed/refractory B-cell NHL (FL-14, DLBCL—11, MCL—4, MZL—2, transformed FL—1, SLL—1)	60	OR for DCDS4501A—53% OR for DCDS4501A + RTX—78%, PFS for DLBCL—149 days, PFS for indolent NHL—241 days.	Neutropenia (50%), diarrhea (45%), nausea (40%), pyrexia (38%), peripheral neuropathy (25%), peripheral sensory neuropathy (20%), hypokalemia (20%)
Bartlett et al. 2013 [85]	BV 1.8 mg/kg every 3 weeks by i.v. infusion	Relapsed/refractory B-cell lymphoma patients with CD30 expression (44 DLBCL and 18 other B-cell neoplasms)	62	DLBCL: OR—40%(CR—7. PR—10, PFS —36 weeks), Other B-cell neoplasms:OR—22%, CR-2, PR—2)	AEs occurring in >20% of patients: fatigue (40%), nausea(37%), neutropenia (37%, pyrexia (32%), diarrhea (31%), peripheral sensory neuropathy (26%), vomiting (23%), anemia (21%), constipation (21%)

AE adverse event, *BR* Brentuximab vedotin, *CRu* unconfirmed complete remission, *PV* Pinatuzumab vedotin, *DLBCL* diffuse large B-cell lymphoma, *FL* follicular lymphoma, *IO* Inotuzumab ozogamicin, *MCL* mantle cell lymphoma, *MALT* mucosa-associated lymphoid tissue lymphoma, *MZL* Marginal zone lymphoma, *NHL* non-Hodgkin's lymphoma, OR overall response, PR partial response, *RTX* rituximab, *SLL* small lymphocytic lymphoma, *SD* stable disease, *SPD* sum of perpendicular dimensions

this patient population. A phase I dose-escalation study evaluated the use of combotox in 17 children with refractory or relapsed B-lineage-ALL [Table 5.3; 68]. Combotox was administered at levels of 2 mg/m^2, 4 mg/m^2, 5 mg/m^2, and 6 mg/m^2: Of these, 5 mg/m^2 was established as the MTD and recommended for future studies. Complete remission was obtained in three patients, and in six additional patients, a decrease of percentage of peripheral blood blast counts > 95 % was observed.

In the adult ALL phase I study, all patients with peripheral blasts experienced decreased blast counts following the administration of combotox, and one out of 17 patients with refractory or relapsed B-lineage-ALL achieved a partial response [69]. Blast count reductions were rapid and were restricted to only the blast population, demonstrating specific cytotoxicity. Vascular leak syndrome was the dose-limiting toxicity and the MTD was 7 mg/m^2. The combination of combotox with Ara-C resulted in significantly longer median survival in a murine xenograft model of advanced ALL [70]. In this study, sequential administration of Ara-C and combotox was superior to concurrent administration. These results have led to a phase I clinical trial exploring this combination in adults with relapsed or refractory B-lineage ALL (NCT01408160).

5.3.3.3 DT2219ARL

DT2219ARL (Scott and White Hospital and Clinic) is a bispecific immunotoxin targeted against CD19 and CD22. Bispecific ligand directed toxins are single-chain biologicals produced by linking a truncated toxin to two well-established targeting ligands. DT2219ARL is composed of two scFv antibodies and a truncated form of a diphtheria toxin [71]. Catalytic DT390 was genetically modified by reverse orienting VH-VL domains and adding aggregation reducing/stabilizing linkers for superior in vivo anti-leukemia activity. These modifications resulted in increased long-term tumor-free survival in two highly aggressive human B cell malignancy models in SCID mice: a bioluminescent xenograft imaging model of Raji Burkitt's lymphoma and a Daudi model. A phase I dose escalation study of DT2219ARL for the treatment of relapsed or refractory B-lineage leukemia and lymphoma is ongoing (NCT00889408).

5.3.3.4 SGN-CD19A

SGN-CD19A (Seattle Genetics) is an immunotoxin designed for the treatment of B-cell lymphoid malignancies directed against CD19. It is composed of a humanized anti-CD19 monoclonal antibody conjugated with monomethyl auristatin F (MMAF) with a protease-sensitive peptide-based linker [72]. Preclinical data indicates that SGN-CD19A effectively binds to target cells, internalizes and induces durable tumor regressions in multiple preclinical cancer models. A phase 1 trial evaluating SGN-CD19A has demonstrated antitumor activity and an acceptable safety profile in heavily pretreated patients with ALL and aggressive types of NHL

including B-cell lymphoblastic lymphoma (B-LBL) and Burkitt lymphoma [73]. SGN-CD19A was administered on days 1 and 8 of 21-day cycles at up to seven cohort-specific doses (0.3–2.3 mg/kg) to nine patients with B-ALL, three patients with B-LBL and one patient with Burkitt lymphoma. Antitumor activity has been observed, including CR in one heavily pretreated B-ALL patient and stable disease in two patients with lymphoma. The most common drug-related AEs included pyrexia, nausea, chills, fatigue, headache, pain, blurred vision, cough and diarrhea. In the second trial, SGN-CD19A is given every 3 weeks to patients with aggressive B-cell NHL (NCT01786135).

5.3.4 Anti- CD70 Immunotoxins

CD70 is a type II integral membrane protein and the ligand for CD27, which belongs to the tumor necrosis factor receptor superfamily [74]. This antigen is expressed by DLBCL and FL and also by the malignant cells of HL, Waldenström macroglobulinemia, and multiple myeloma (MM) [75]. It is also aberrantly expressed on HTLV-1-associated T-cell lymphoma/leukemia, and EBV-associated malignancies [76]. However, this molecule has highly restricted expression in normal tissue. CD70 has been regarded as a novel potential therapeutic target for lymphoid malignancies. Two ADCs targeting CD70, SGN-75 and MDX-1203, are currently being investigated in phase I studies (NCT01015911, NCT00944905).

5.3.4.1 MDX-1203

MDX-1203 **(**Bristol-Myers Squibb) is a fully human anti-CD70 antibody conjugated to MED-2460 (duocarmycin), a CC-1065 (rachelmycin) analogue, via a cleavable peptide-based linker [77, 78]. MED-2460 is an inactive prodrug. Upon internalization, MED-2460 is released and binds to double-stranded B-DNA within the minor groove, thereby alkylating the—3 position of adenine, which may inhibit cellular proliferation of tumor cells. The cytotoxic activity of MDX-1203 is dependent on cleavage of the linker by lysosomal proteases. Study of MDX-1203 in patients with advanced/recurrent clear cell renal cell carcinoma and relapsed/refractory B-cell NHL has been completed (NCT00944905).

5.3.4.2 SGN-75

SGN-75 (Seattle Genetics, Inc.) consists of anti-CD70 the antibody h1F6 conjugated to the duocarmycin-based toxin MMAF through a plasma-stable linker [79, 80]. Upon binding to CD70, SGN-75 internalizes and releases cysmc MMAF which binds tubulin and induces G2/M arrest and apoptosis. A phase I trial in patients with renal cell carcinoma and NHL has been completed (NCT01015911) [81]. This dose-escalation, multicenter study investigated the safety, tolerability,

PK, pharmacodynamic effects, and antitumor activity of SGN-75 monotherapy in patients with CD70-positive metastatic renal cell carcinoma or relapsed/refractory NHL. SGN-75 has generally been well tolerated: the MTD has not been reached in either dosing schedule and doses up to 3 mg/kg q3wk and 0.6 mg/kg q1wk have been tolerated. The most common AEs were found to be fatigue, nausea, dyspnea, peripheral edema and thrombocytopenia. Best responses in NHL group were 1 CR and 6 stable disease (SD).

5.3.5 Anti-CD79: Polatuzumab Vedotin

Polatuzumab vedotin (PV, DCDTS4501A, RG7596, Genentech Inc.) is an anti-CD79b mAb conjugated to monomethyl auristatin E (MMAE) designed to target B-cells. CD79b is a component of the B-cell receptor (BCR) expressed on the surface of B-cells in the majority of B-cell lymphoid malignancies. Upon internalization and proteolytic cleavage, MMAE binds to tubulin and inhibits its polymerization, causes G2/M phase arrest and induces apoptosis. In phase I study, PV demonstrated anti-tumor activity and an acceptable toxicity profile in heavily pre-treated patients with relapsed/refractory B-cell NHL including FL, DLBCL and mantle cell lymphoma (MCL) [Table 5.5; 82]. Polatuzumab vedotin was generally well tolerated. The most common grade 3 AEs were neutropenia occurring in 39 % of the patients and leukopenia in 12 %. The observed toxicities, neutropenia and peripheral sensory neuropathy, were consistent with the known mechanism of action of the study drug and were manageable. A recommended dose of 2.4 mg/kg every 21 days (q21d) was established for phase II clinical trials. Among 17 patients treated at clinically relevant doses ≥ 1.8 mg/kg, 8 displayed an objective response to polatuzumab vedotin monotherapy with evidence of durable responses.

Recently, updated results were published regarding the treatment of a group of patients with refractory/relapsed DLBCL and indolent NHL with 1.8 mg/kg polatuzumab vedotin, with or without 375 mg/m^2 q21d rituximab, as well as an 2.4 mg/kg expansion cohort [83]. Sixty patients received polatuzumab vedotin at doses of 1.8 mg/kg–2.4 mg/kg or polatuzumab vedotin + rituximab. The PV + rituximab safety profile was similar to PV monotherapy. DCDTS4501A and the DCDTS4501A + rituximab combination were generally well-tolerated. Treatment-related AEs included neutropenia, diarrhea, nausea, pyrexia, peripheral neuropathy, peripheral sensory neuropathy and hypokalemia. The median PFS for DLBCL patients treated with PV or PV+ rituximab was 149 days and the median PFS for indolent NHL was 241 days. Additional studies of PV combined with immunochemotherapy are being planned.

5.3.6 Anti-CD30: Brentuximab Vedotin

Brentuximab vedotin (BV, Adcetris®**,** SGN-35; Seattle Genetics) is a next-generation ADC comprising the antibody cAC10, specific for human CD30, covalently

attached to the microtubule-disrupting agent monomethyl auristatin E (MMAE) via a protease-cleavable linker [84]**.** CD30 is a transmembrane glycoprotein receptor and is a member of the tumor necrosis factor receptor superfamily. After binding with CD30 on the surface of malignant cells, the BV-CD30 complex is internalized into a lysosome where enzymatic linker cleavage releases MMAE. Subsequently, MMAE disrupts the microtubules and spindle and causes G2/M cell cycle arrest and apoptosis. A Phase II clinical trial to evaluate the efficacy and safety of BV as a single agent in relapsed or refractory patients with CD30-positive NHL is ongoing (NCT01421667). This study also evaluates the safety and efficacy of BV in combination with rituximab in patients with relapsed or refractory DLBCL. Interim results in patients with DLBCL and primary mediastinal B-cell lymphoma (PMBL) has been recently reported [Table 5.5; 85]. In an interim analysis of 62 patients, compelling antitumor activity has been observed with BV. Of the 43 evaluable DLBCL patients, 40 % achieved an objective response, including 7 CR and 10 PR. The median PFS duration was 36 weeks. No correlation between CD30 expression and response rate was observed. The most frequently occurred AEs included fatigue, nausea and neutropenia, pyrexia, diarrhea, peripheral sensory neuropathy, vomiting, anemia and constipation

5.3.7 Anti-CD37: IMGN529

CD37 is a member of the tetraspanin superfamily of cell surface antigens, which are considered as a target for B cell malignancies, and is expressed by most B-cell malignancies [86]. It has been found to be significantly expressed on neoplastic cells of patients with CLL, HCL and NHL. In B-cell NHL, CD37 expression is observed in aggressive NHL, like Burkitt lymphoma, mantle cell lymphoma (MCL), FL and small lymphocytic lymphoma (SLL) [87]. However, it is absent or minimally expressed on CD10+ precursor B- cells in the bone marrow, terminally differentiated plasma cells, normal T cells, NK cells and myeloid cells—monocytes and granulocytes. CD37 is selectively expressed on normal mature B-cells and by most B-cell malignancies; these all demonstrate higher levels of CD37 expression than CD20. CD37-directed antibody-drug conjugates have been recently explored by Deckert et al. [88].

IMGN529 is a novel anti-CD37 K7153A antibody conjugated to the maytansinoid, DM1, a potent anti-microtubule agent, via the thioether linker, SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate). IMGN529 combines the intrinsic proapoptotic and immune effector activities of its anti-CD37 antibody component with the cytotoxic potency of its DM1 maytansinoid payload through apoptosis induction, ADCC, and CDC. IMGN529 was found to exhibit enhanced cytotoxicity against lymphoma cell lines, and induces G2/M cell cycle arrest following internalization and lysosomal processing to lysine-Nε-SMCC-DM1, the sole intracellular maytansinoid metabolite.

Beckwith et al. developed a transgenic mouse model over-expressing human CD37 exclusively in B cells [89]. IMGN529 was evaluated in double transgenic

mice characterized by the development of a CLL-like disease with hCD37+ leukemic cells in the peripheral blood and lymphoid organs. In this model, IMGN529 rapidly eliminated peripheral blood leukemia and improved overall survival. IMGN529 is directly cytotoxic to human CLL *in vitro*, depletes B-cells in the whole blood of the patient, and promotes their destruction by macrophages and NK cells. These results demonstrate the value of the novel human CD37 transgenic mouse model for evaluating anti-human CD37 therapeutics, and highlight the potential of IMGN529 for the treatment of CLL and other CD37-positive B-cell malignancies. IMGN529 was highly active against subcutaneous B-cell tumor xenografts in SCID mice with comparable or better activity than rituximab, CVP or bendamustine. In human blood cells, IMGN529 application resulted in potent and specific depletion of normal and CLL B cells and leukemic cells from CLL patients. IMGN529 appears to be a useful agent for the treatment of CD37-positive B-cell malignancies. A phase I study of IMGN529 in patients with relapsed or refractory NHL is ongoing (NCT01534715).

5.4 Immunotoxins for T-Cell Lymphoid Malignancies

T-cell lymphoid malignancies are a heterogeneous group of T-cell neoplasms arising from post-thymic mature T-cells. Compared with B-cell lymphomas, T-cell lymphomas are uncommon and account for 10–15 % of all NHL. In addition, there is a marked geographical variation in incidence of these disorders. Different T-cell lymphoma subtypes require different treatment approaches. Recently, novel treatments for T-cell lymphomas including new cytotoxic agents, monoclonal antibodies, epigenetic modifier and immunotoxins represent available treatment options [Table 5.6; 90].

5.4.1 Brentuximab Vedotin

Brentuximab vedotin is well tolerated and highly active in patients with refractory anaplastic large-cell lymphoma (ALCL). Systemic ALCL is a mature T cell lymphoma with characteristic morphological, immunophenotypic and cytogenetic features and constitutes approximately 2–3 % of all lymphoid neoplasms. The current WHO classification includes anaplastic lymphoma kinase (ALK)-positive and ALK-negative variants. The activity of BV was evaluated in immunologically deficient mice with human ALCL xenografts [91]. This agent was active at 1–3 mg/kg, which was lower than the maximum tolerated dose of approximately 100 mg/kg. Systemic ALCL is a CD30-positive aggressive subtype of mature T-cell lymphoma. For patients with relapsed ALCL, outcomes are poor, with a median OS of 7.0 months [92]. Brentuximab vedotin was evaluated in a phase II multicenter trial in 58 patients with relapsed or refractory systemic ALCL after at least one prior therapy (Table 5.6) (NCT00866047) [93, 94]. Patients received BV at a dose of

Table 5.6 Clinical trials evaluating antibody drug coniugates in T-cell lymphoid malignancies and Hodgkin lymphoma

Study	Treatment regimens	Patients characteristics	N	Efficacy	Adverse events
Pro et al. 2012, 2013 [93, 94]	BV 1.8 mg/kg i.v. every 3 weeks over 30 min up to 16 cycles.	Recurrent systemic ALCL after at least one prior therapy	58	OR—86%, CR—57%, PR—29% median PFS—14.6 m	Grade 3 or 4 AEs: neutropenia (21%), thrombocytopenia (14%), peripheral sensory neuropathy (12%)
Duvic et al. 2013 [96]	BV 1.8 mg/kg every 21 days.	CD30+ cutaneous T-Cell lymphomas and lympho proliferative disorders	56	OR—71% CR—35% Median PFS—1.68 yrs	AEs—peripheral neuropathy (60%):drug rashes (27%), diarrhea (24%), fatigue (30%), alopecia (16%), myalgias (16%), nausea (14%)
Younes et al. 2012[108], Gopal et al. 2013[109]	BV 1.8 mg/kg every 3 wks for up to 16 cycles (median of 9)	Relapsed or refractory HL	102	OR—75% CR—33% Median OS—40.5 m 36-m survival—54%	Most common (≥15%) Aes: peripheral sensory neuropathy, nausea, fatigue, neutropenia, and diarrhea
Olsen et al. 2001	DT—9 or 18 μg/kg/d for 5 days every 3 weeks for up to eight cycles	Previously treated CTCL	71	OR—30%, CR—10%, PR—20% Response −6.9 m	AEs: flu-like symptoms, acute infusion-related events, vascular leak syndrome, transient elevations of hepatic transaminase levels (61% with 17% grade 3 or 4)
Prince et al. 2010 [100]	DT 9 μg/kg/d or 18 μg/kg/d or placebo	Previously treated CD25—positive CTCL	144	OR—44% (CR—10%, PR—34%), PFS—124 ds	Capillary leak syndrome—10%, rigors—12%, pyrexia—11%, fatigue—12%, artralgia—5%
Foss et al. 2013 [103]	DT 18 μg/kg/d (days 1, 2) with CHOP (day 3) every 21 days for ≤6–8 cycles	Untreated PTCL	49	OR—65%, median duration of response—30 months, PFS—12 m	AEs ≥grade 3: lymphopenia (24.5%), neutropenia (20.4%), leukopenia (18.4%), fatigue, nausea

AE adverse event, *ALCL* anaplastic large-cell lymphoma, *BV* brentuximab vedotin, *CHOP* cyclophosphamide + doxorubicin + vincristine + prednisone, *CTCL* cutaneous T-cell lymphoma, *DT* Denileukin diftitox, *HL* Hodgkin lymphoma, *m* month, *OR* overall response, *PR* partial response, *PTCL* peripheral T-cell lymphoma

1.8 mg/kg intravenously every 3 weeks over 30 min. Of 58 patients treated in the study, the OR rate was 86 %, and CR was noted in 59 % of the patients. The median duration of OR was 12.6 months while CR was 13.2 months. Grade 3 or 4 AEs were neutropenia (21 %), thrombocytopenia (14 %), and peripheral sensory neuropathy (12 %). The updated results of the study have been presented recently [Table 5.6; 94]. At the time of the analysis, the median observation time from the first dose of BV was 33.4 months. The median duration of response was 13.2 months for all patients and 26.3 months for patients who obtained a CR. The median PFS for all patients was 14.6 months and the estimated 3-year OS rate was 63 %. A randomized phase III study has been initiated to evaluate the effectiveness of BV in combination with cyclophosphamide, doxorubicin, and prednisone for frontline treatment of CD30-positive mature T-cell lymphomas (NCT01777152).

The safety and antitumor activity of BV administered as a retreatment option for patients who had previously achieved an objective response with prior BV treatment have been recently reported [95]. Eight patients with systemic ALCL were retreated and three patients were retreated twice. The objective response rate in systemic ALCL patients who received BV retreatment was 88 %, including 63 % CR. The estimated median duration of response for ALCL patients was 12.3 months and the median PFS 12.9 months. Three systemic ALCL patients were retreated twice. One patient achieved a CR and two patients achieved a PR with both retreatment experiences. In August 2011, BV received accelerated approval for the treatment of systemic ALCL.

The phase II open-label clinical trial found that BV is active against mycosis fungoides (MF) and other CD30+ cutaneous T-cell lymphomas [96]. In this study, 48 patients with lymphomatoid papulosis (LyP) and primary cutaneous ALCL (pc-ALCL) or CD30+ MF were included. The criteria for inclusion comprised the presence of skin lesion expression of CD30, more than 10 LyP lesions, one or more tumors, and the need for systemic therapy. Brentuximab vedotin was given i.v. at 1.8 mg/kg every 21 days. Patients achieving a CR received two additional doses, while those achieving PR after eight cycles could receive up to 16 doses. Overall response was achieved in 34 (71 %) patients, including CR in 17 (35 %). The OR rate for 28 patients with MF was 50 %. Median PFS was 1.68 years from first dose. The most common AE of any grade was peripheral neuropathy, observed in 29 of 48 (60 %) evaluated patients.

5.4.2 Denileukin Diftitox

Denileukin diftitox (DD, DAB 486-IL2; Ontak®, Eisai Inc., Seragen, Inc.) is a recombinant fusion product of an amino-terminal methionine: the first 386 amino acid residues of mature diphtheria toxin fused to amino acid residues 1-133 of human IL-2 [97, 98]. Denileukin diftitox selectively binds to high-affinity interleukin-2 (IL-2) receptors (IL-2Rs) that are expressed on activated normal and malignant lymphocytes. Upon internalization and acidification, denileukin diftitox is

proteolytically cleaved within the endosomes to liberate the enzymatically-active portion of the diphtheria toxin. The fragment of diphtheria toxin is translocated across the endosomal membrane into the cytosol, where it inhibits protein synthesis via ADP-ribosylation of elongation factor-2, ultimately resulting in cell death. CD25 antigen, an interleukin-2 (IL-2) receptor alpha-chain, is expressed on the surface of CLL cells in 30–50 % of patients and at a higher density than on normal B cells.

Denileukin diftitox has been shown to be a useful agent in the treatment of patients with persistent or recurrent cutaneous T-cell lymphoma (CTCL) despite other therapeutic interventions. In these patients, the response rates ranged from 30 to 50 % [Table 5.6; 99, 100]. Denileukin diftitox showed meaningful and durable effects on the response rate. Patients receiving DD 9 μg/kg/day had OR rates between 23 % and 38 %. Patients receiving 18 μg/kg/day demonstrated higher response rates ranging between 36 % and 49 %. The median duration of response lasted between 2.7 and 46.1 months.

Recently, Duvic et al. found the duration of response in CTCL patients treated with denileukin diftitox who experienced PR or CR in 3 phase III studies to be 277 days vs. 81 days using placebo [101]. The duration of response was longer in patients who developed capillary leak syndrome (CLC) than in the patients without this complication (619 vs. 267 days, respectively) but the difference was not significant ($P = 0.28$). Nausea, pyrexia, fatigue, CLS, and rigors were the most frequent adverse events. Importantly, denileukin diftitox may bestow a clinically meaningful benefit in patients with CTCL who relapsed after an initial response to the drug [102]. In a phase II study, the safety and efficacy of a combination of DD and cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) was determined in 49 patients with newly diagnosed peripheral T-cell lymphoma (PTCL) [Table 5.6; 103]. The patients received DD 18 μg/kg/day on days 1 and 2 with CHOP on day 3, every 21 days for 6–8 cycles. The OR rate was 65 % with the median duration of response 30 months and median PFS 12 months The most frequent treatment-related grade three or higher AEs were lymphopenia (24.5 %) and neutropenia (20.4 %).

5.4.3 A-dmDT390-bisFv (UCHT1)

A-dmDT390-bisFv(UCHT1) is a bivalent anti-T cell immunotoxin consisting of 1-390 amino acid residues of the chain A diphtheria toxin (DT) joined via a spacer to the Fv fragment of UCHT1, which is connected to a second UCHT1 Fv fragment via a disulfide bond. This immunotoxin selectively kills CD3ε-positive T cells. The addition of the second Fv fragment overcomes the steric hindrance of immunotoxin binding due to the large N-terminal DT domain [104]. The maximum tolerated dose, pharmacokinetics and immunogenicity of A-dmDT390-bisFv(UCHT1) have been evaluated in studies based on rat and squirrel monkey models [105]. Both animal species received 0, 2.5, 25 or 56.25 μg/kg of A-dmDT390-bisFv(UCHT1) intravenously twice daily for four consecutive days. Transient elevation of liver

transaminases, transient lethargy, inappetence and weight loss were noted in the groups receiving high doses. The MTD of 200 μg/kg was found to be a dose sufficient for anti-tumor activity *in vitro* and in a rodent model. Phase I/II study in patients with surface CD3+ malignant T cell diseases is ongoing (NCT00611208).

5.5 Hodgkin Lymphoma

Hodgkin lymphoma (HL) arises from the germinal center and the post-germinal center B-cells, with tumors composed of a minority of neoplastic (Reed-Sternberg) cells in an inflammatory background of a variable number of lymphocytes, eosinophils, neutrophils, macrophages/histiocytes, plasma cells, fibroblasts, and collagen fibers. The crude incidence of HL in the European Union is 2.3 and the mortality is 0.4 cases/100 000/year. In the USA, 9190 new cases and 6250 deaths from HL were estimated in 2014 [6]. From an immunophenotypic perspective, virtually all classical Hodgkin lymphoma Reed-Sternberg cells express CD30, a marker that is very useful for the diagnosis of HL [106]. Classical HL is a highly curable cancer with long-term survival exceeding 80 % [107]. However, depending on the initial stage and on various prognostic features, up to 30–40 % of patients can relapse after frontline therapy.

Brentuximab vedotin represents an option in patients failing autologous stem cell transplantation (ASCT). A pivotal phase II study included 102 HL patients who had failed ASCT, 71 % of whom had relapsed within 1 year of ASCT [Table 5.6; [108, 109]. All received 1.8 mg/kg BV every 3 weeks as a 30-min outpatient i.v. infusion for a maximum of 16 cycles. The patients received a median of 9 cycles of BV for up to 16 cycles, resulting in an overall response rate of 75 % with a 34 % CR. The median duration of response was 6.7 months for responders and 20.5 months for complete responders. Brentuximab vedotin has a modest toxicity profile, with peripheral sensory neuropathy being one of the most clinically significant side effects, and this is largely reversible. Brentuximab vedotin-induced peripheral neuropathy was primarily sensory, although motor neuropathy has also been observed. The most common adverse events apart from peripheral neuropathy, were nausea, fatigue, neutropenia, and diarrhea. The findings of approximately 3 years of follow up study from this ongoing trial were recently presented, including characterization of patients who experienced long-term remissions [109]: the median observation time from first dose of BV was found to be 32.7 months. Fifty one (50 %) of the patients were alive and the median OS was 40.5 months. Fourteen patients remained in remission and were not treated with a new anti-cancer therapy, excluding five patients who received consolidative allo-SCT.

Retreatment with BV monotherapy was evaluated in patients with HL who relapsed after achieving CR or PR with initial therapy in a previous study [110]. Twenty-one patients with HL were retreated with 1.8 mg/kg BV intravenously approximately every 3 weeks over 30 min as an outpatient infusion. The objective response rate was 60 %, including 30 % CR, while the estimated median duration of

response was 9.5 months. Retreatment with BV was associated with a higher rate of peripheral motor neuropathy, than seen in previous trials. Recently, a randomized phase III study has been initiated to evaluate BV in combination with AVD (doxorubicin, vinblastine, and dacarbazine) versus ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) for frontline treatment of HL (NCT01712490). Brentuximab vedotin has received accelerated approval from the FDA for the treatment of classical HL that has relapsed either after ASCT or after two lines of combination chemotherapy in patients ineligible for ASCT [111].

5.6 Immunoconjugates for Multiple Myeloma

Multiple myeloma is an incurable neoplastic disease characterized by clonal proliferation of plasma cells in the bone marrow. In 2014, a total of 24,050 new cases and 11,090 deaths from multiple myeloma were estimated in the USA [6]. However, the incidence of MM is known to vary according to ethnicity. The use of novel targeted agents alone, or in combination with novel or high-dose conventional therapies, has improved the clinical outcome of MM patients. Despite the evolution of effective frontline treatment strategies, patients who enter clinical remission eventually relapse and become refractory to treatment. The established success of mAbs in the treatment of several neoplastic diseases has promoted interest in developing antibody-based therapies for MM, including antibodies conjugated to potent cytotoxic moieties [112]. Three such immunoconjugates which target cell surface proteins found on MM cells are currently in clinical development (Table 5.7). Milatuzumab-doxorubicin (MD) is an anti-myeloma ADC consisting of milatuzumab, a humanized anti-CD74 mAb, conjugated to the anthracycline antibiotic doxorubicin. Indatuximab ravtansine (IR) is an anti-CD138 antibody-maytansinoid conjugate. The third anti-MM immunoconjugate is lorvotuzumab mertansine (LM), which targets the neural cell adhesion molecule, CD56, expressed on the majority of myeloma cells [113].

5.6.1 Milatuzumab-Doxorubicin Antibody-Drug Conjugate

CD74 is an integral membrane protein and tumorassociated antigen (TAA). This molecule is an attractive target for ADC because it internalizes and recycles after antibody binding [114]. Milatuzumab-doxorubicin **(**hLL1-DOX, Immunomedics Inc.) is an immunoconjugate consisting of milatuzumab, a humanized anti-CD74 mAb conjugated to the anthracycline antibiotic doxorubicin. It selectively binds to CD74 on MM cell surfaces. After internalization, the doxorubicin moiety is released, where it intercalates between base pairs in the DNA helix and inhibits topoisomerase II. In consequence, doxorubicin prevents DNA replication and increases double-strand breakage, thus inhibiting the proliferation of cells which overexpress

Table 5.7 Clinical trials evaluating antibody drug coniugates and immunotoxins in multiple myeloma

Study	Treatment regimens	Patients character-istics	N	Efficacy	Adverse events
Heffner et al. 2012 [119]	BT062 40 mg/m^2–160 mg/m^2 days 1, 8, and 15, every 4 weeks	Relapsed or relapsed/ refractory MM	23	PR—1 pt SD—11pts PFS—112 (90–245) days	The most frequently reported AEs: anemia, diarrhea, fatigue
Kelly et al. 2013 [121]	BT062 80 mg/m^2–120 mg/m^2 (days 1, 8, and 15, every 4 weeks) + Lenalidomide (25 mg, daily on days 1–21) + Dexamethasone (40 mg on days 1, 8, 15, and 22)	Relapsed/ refractory MM	15	OR—78 % (CR 1 pt, very good PR 1pt, PR 5 pts, SD 2pts)	The most common AEs: fatigue, hypokalemia, diarrhea
Chanan-Khan et al. 2010 [125]	IMGN901 40–140 mg/m^2/ week	CD56+ relapsed/ refractory MM	37	OR—5 pts (2 partial responses and 3 minimal responses)	Fatigue, renal failure, weakness
Berdeja et al. 2012 [126]	IMGN901 (Days 1, 8, 15 every 28 days) + Lenalido-mide (25 mg) days 1 to 21 every 28 days + dexamethasone (40 mg) (Days 1, 8, 15, and 22 every 28 days)	CD56+ relapsed/ refractory MM	44	OR—59 CR—1pt VGPR—8 pts PR—9 pts	Peripheral neuropathy in most cases in cycles > 3, neutropenia,hyperuricemia, tumor lysis syndrome,neutropenia, thrombocytopenia, anemia, hemolytic anemia, and LDH increase

AE adverse event, *NHL* non Hodgkin's lymphoma, *NR* not reported, *OR* overall response, *PFS* progression free survival, *PR* partial response, *SD* stable disease, *VGPR* very good partial remission

CD74. Milatuzumab-doxorubicin is the first anti-CD74 immunoconjugate to enter clinical trials. A phase I/II study of MD for safety and tolerability in patients with MM is ongoing (NCT01101594).

5.6.2 Indatuximab Ravtansine

CD138 (Syndecan-1) is expressed on normal plasma cells, with no expression on other hematopoietic stem cells [115]. In addition, CD138 represents one of the most

specific target antigens for identification of MM cells. Its expression on MM cells is significantly higher than on normal plasma cells. Moreover, CD138 is highly overexpressed on various solid tumors including ovarian carcinoma, subtypes of lymphoma and leukemia, and neuroendocrine tumors such as Merkel cell carcinoma. Anti-CD138 immunoconjugates significantly inhibit growth of MM cell lines and primary tumor cells from MM patients.

Indatuximab ravtansine (BT062, Biotest AG Dreieich, Germany) is an anti-CD138 antibody-maytansinoid conjugate comprising the anti-CD138 chimerized mAb nBT062 and the maytansinoid DM4 as cytotoxic agent, joined through a disulfide linker [116]. It was developed by Biotest AG as a primary diagnostic marker for multiple myeloma [117]. After the binding of IR to CD138, the conjugate is internalized and releases DM4, leading to the death of a target cell [118]. As previous preclinical and early clinical studies of IR have shown this agent to have significant anti-myeloma activity, particularly after combination with lenalidomide and dexamethasone, a prospective, open label, dose-escalation phase I/IIa study was initiated to evaluate the safety and efficacy of IR in combination with lenadomide and dexamethasone (Table 5.7) (NCT00723359) [119–122]. Treatment cycles were repeated until progression of the underlying disease or occurrence of unacceptable toxicities. The overall response rate was 78 % among nine patients evaluated for efficacy, including one patient with CR receiving a dose of 120 mg/m^2. The MTD has been established as 100 mg/m^2. The most common AEs were diarrhea, fatigue and hypokalemia. The available data indicate that IR is well tolerated, both when administered as a single agent and as part of a combination regimen, at doses of up to 100 mg/m^2.

5.6.3 Lorvotuzumab Mertansine

CD56 is a member of the immunoglobulin superfamily of cell surface adhesion glycoproteins, originally identified by its role in neural cell adhesion and migration. It is expressed in up to 78 % of multiple myelomas [122, 123].

Lorvotuzumab mertansine (LM, IMGN901, ImmunoGen) is an antibody-drug conjugate composed of a humanized IgG1 version of the N901 (huN901) anti-CD56 antibody, lorvotuzumab, linked via a cleavable disulfide linker to the tubulin-binding maytansinoid DM1 [124]. DM1 is a synthetic derivative of maytansine, an *ansa* macrolide, that binds to tubulin at the vinca alkaloid-binding site, leading to inhibition of microtubule assembly and cell proliferation, and then to cell death. This agent displays antitumor activity in preclinical models of multiple myeloma. Lorvotuzumab mertansine can be also potentially useful in such other malignancies as small-cell lung cancer, Merkel cell carcinoma, and other cancers of neuroendocrine origin.

Lorvotuzumab mertansine has demonstrated single agent clinical activity and an acceptable safety profile in relapsed/refractory multiple myeloma patients (Table 5.7). In a phase I study of MM, the MTD of single-agent LM was 112 mg/

m² [125]. The results from a phase I study demonstrated that combination therapy incorporating LM with revlimid and dexamethasone is effective for relapsed and refractory MM patients [126]. Lorvotuzumab mertansine was administered intravenously at doses of 75 mg/m^2–112 mg/m^2 once per week for 3 weeks in a 4-week treatment cycle. In addition, all patients received 25 mg revlimid orally on days 1–21, as well as 40 mg dexamethasone orally once per week. Patients were treated until disease progression or the development of unacceptable side effects. Of the 44 patients enrolled, 41 were evaluable for safety. Peripheral neuropathy was the most common AE and occurred in 100 % of the patients receiving the drug at doses of 90 and 112 mg/m^2. The OR rate was 59 %, including one patient with CR. The MTD was established as 75 mg/m^2 when administered daily for 3 consecutive days every 3 weeks.

5.7 Conclusions

Immunoconjugates are composed of an antibody, or an antibody fragment, and a toxin portion. Major advances in the efficacy and safety of immunotoxins have been achieved by incorporating highly potent drugs and using stable linkers to better exploit the half-life of the mAb component of these drugs. Recent clinical data has clearly demonstrated that new generations of ADCs and immunotoxins have improved clinical activity and safety.

A major advance in the development of immunoconjugates resulted from the incorporation of highly potent cytotoxins such as calicheamicins, maytansinoids, auristatins, and duocarmycins into new agents. In addition, recombinant immunotoxins containing only the Fv fragment of the antibodies fused to a truncated toxin have been developed. This new approach will probably have a major impact on the treatment of lymphoid and myeloid malignancies. Responses have been observed in many patients, and additional studies are now required to define the optimal dose, schedule, and proper combinations for particular diseases.

However, the clinical development of immunoconjugates has been hindered by a variety of problems, including poor antigen specificity, nonspecific toxicities, immunogenicity and induction of antibody formation, and difficulties in their production. Nevertheless, over 25 immunoconjugates are currently under clinical development for use in hematologic malignancies, and the data emerging from clinical trials will provide a critical insight into the design of the next generation of immunoconjugate therapy.

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References

1. Wayne AS FitzGerald DJ, Kreitman RJ, Pastan I. Immunotoxins for leukemia. Blood. 2014;123:2470–7.
2. Robak T, Robak E. Current phase II antibody-dug conjugates for the treatment of lymphoid malignancies. Expert Opin Investig Drugs. 2014;3:911–24.
3. Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin treatment of cancer. Annu Rev Med. 2007;58, 221–37.
4. Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin therapy of cancer. Nature Rev Cancer. 2006;6:559–65.
5. Robak T, Wierzbowska A. Current and emerging therapies for acute myeloid leukemia. Clin Ther. 2009;31 Pt 2:2349–70.
6. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64:9–29.
7. Andrews RG, Takahashi M, Segal GM, Powell JS, Bernstein ID, Singer JW. The L4F3 Antigen is expressed by unipotent and multi-potent colony-forming cells but not by their precursors. Blood. 1986;68:1030–5.
8. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID. Expression of normal myeloid-associated antigens by acute leukemia cells. Blood. 1986;67:1048–53.
9. Andrews RG, Singer JW, Bernstein ID. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. J Exp Med. 1989;169:1721–31.
10. Larson RA, Boogaerts M, Estey E, Karanes C, Stadtmauer EA, Sievers EL, Mineur P, Bennett JM, Berger MS, Eten CB, Munteanu M, Loken MR, Van Dongen JJ, Bernstein ID, Appelbaum FR. Antibody-targeted chemotherapy of older patients with acute myeloid lukemia in first relapse using mylotarg (gemtuzumab ozogamicin). Leukemia. 2002;16:1627–36.
11. Sievers EL, Larson RA, Stadtmauer EA, Löwenberg B, Estey EH, Dombret H, Theobald M, Voliotis D, Bennett JM, Richie M, Leopold LH, Berger MS, Sherman ML, Loken MR, van Dongen JJ, Bernstein ID, Appelbaum FR. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. J Clin Oncol. 2001;19:3244–54.
12. Larson RA, Sievers EL, Stadtmauer EA, Löwenberg B, Estey EH, Dombret H, Theobald M, Voliotis D, Bennett JM, Richie M, Leopold LH, Berger MS, Sherman ML, Loken MR, van Dongen JJ, Bernstein ID, Appelbaum FR. Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. Cancer. 2005;104:1442–52.
13 Giles FJ, Kantarjian HM, Kornblau SM, Thomas DA, Garcia-Manero G, Waddelow TA, David CL, Phan AT, Colburn DE, Rashid A, Estey EH. Mylotarg (Gemtuzumab Ozogamicin) therapy is associated with hepatic venoocclusive disease in patients who have not received stem cell transplantation. Cancer. 2001;92:406–13.
14. Kell WJ, Burnett AK, Chopra R, Yin JA, Clark RE, Rohatiner A, Culligan D, Hunter A, Prentice AG, Milligan DW. A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. Blood. 2003;102:4277–83.
15. Burnett AK, Hills RK, Milligan D, Kjeldsen L, Kell J, Russell NH, Yin JA, Hunter A, Goldstone AH, Wheatley K. Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. J Clin Oncol. 2011;29:369–77.
16. Burnett AK, Hills RK, Hunter AE, Milligan D, Kell WJ, Wheatley K, Yin J, McMullin MF, Dignum H, Bowen D, Russell NH. The addition of gemtuzumab ozogamicin to low-dose ara-C improves remission rate but does not significantly prolong survival in older patients with acute myeloid leukaemia: results from the LRF AML14 and NCRI AML16 pick-a-winner comparison. Leukemia. 2013;27:75–81.

17. Burnett AK, Russell NH, Hills RK, Kell J, Freeman S, Kjeldsen L, Hunter AE, Yin J, Craddock CF, Dufva IH, Wheatley K, Milligan D. Addition of gemtuzumab ozogamicin to induction chemotherapy improves survival in older patients with acute myeloid leukemia. J Clin Oncol. 2012;30:3924–31.
18. Petersdorf SH, Kopecky KJ, Slovak M, Willman C, Nevill T, Brandwein J, Larson RA, Erba HP, Stiff PJ, Stuart RK, Walter RB, Tallman MS, Stenke L, Appelbaum FR. A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. Blood. 2013;121:4854–60.
19. Tang R, Cohen S, Perrot JY, Faussat AM, Zuany-Amorim C, Marjanovic Z, Morjani H, Fava F, Corre E, Legrand O, Marie JP. P-gp activity is a critical resistance factor against AVE9633 and DM4 cytotoxicity in leukaemia cell lines, but not a major mechanism of chemoresistance in cells from acute myeloid leukaemia patients. BMC Cancer. 2009;9:199. doi:10.1186/1471-2407-9-199.
20. Lapusan S, Vidriales MB, Thomas X, de Botton S, Vekhoff A, Tang R, Dumontet C, Morariu-Zamfir R, Lambert JM, Ozoux ML, Poncelet P, San Miguel JF, Legrand O, DeAngelo DJ, Giles FJ, Marie JP. Phase I studies of AVE9633, an anti-CD33 antibody-maytansinoid conjugate, in adult patients with relapsed/refractory acute myeloid leukemia. Invest New Drugs. 2012;30:1121–31.
21. Pagliaro LC, Liu B, Munker R, Andreeff M, Freireich EJ, Scheinberg DA, Rosenblum MG. Humanized M195 monoclonal antibody conjugated to recombinant gelonin: an anti-CD33 Immunotoxin with antileukemic activity. Clin Cancer Res. 1998;4:1971–6.
22. Scheinberg DA, Tanimoto M, McKenzie S, Strife A, Old LJ, Clarkson BD. Monoclonal antibody M195: a diagnostic marker for acute myelogenous leukemia. Leukemia. 1989;3:440–5.
23. Caron PC, Co MS, Bull MK, Avdalovic NM, Queen C, Scheinberg DA. Biological and immunological features of humanized M195 (Anti-CD33) monoclonal antibodies. Cancer Res. 1992;52:6761–7.
24. Rosenblum MG, Kohr WA, Beattie KL, Beattie WG, Marks W, Toman PD. Amino acid sequence analysis, gene construction, cloning, and expression of gelonin, a toxin derived from gelonium multiflorum. J Interferon Cytokine Res. 1995;15:547–55.
25. Borthakur G, Rosenblum MG, Talpaz M, Daver N, Ravandi F, Faderl S, Freireich EJ, Kadia T, Garcia-Manero G, Kantarjian H, Cortes JE. Phase 1 study of an anti-CD33 immunotoxin, humanized monoclonal antibody M195 conjugated to recombinant gelonin (HUM-195/rGEL), in patients with advanced myeloid malignancies. Haematologica. 2013;98:217–21.
26. Kung Sutherland MS, Walter RB, Jeffrey SC, Burke PJ, Yu C, Kostner H, Stone I, Ryan MC, Sussman D, Lyon RP, Zeng W, Harrington KH, Klussman K, Westendorf L, Meyer D, Bernstein ID, Senter PD, Benjamin DR, Drachman JG, McEarchern JA. SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML. Blood. 2013;122:1455–63.
27. Borthakur G. Precision ‘re’arming of CD33 antibodies. Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 1334.
28. Alexander DD. The non-hodgkin lymphomas: a review of the epidemiologic literature. Int J Cancer. 2007;120(Suppl 12):1–39.
29. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64:9–29.
30. Swerdlow SH, Campo E, Harris NL. World Health organization classification of tumors of haematopoietic and lymphoid tissues. Lyon: IARC; 2008.
31. Tedder TF, Tuscano J, Sato S, Kehrl JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. Annu Rev. Immunol.1997;5:481–504.
32. Tedder TF, Poe JC, Haas KM. CD22, a multifunctional receptor that regulates B lymphocyte survival and signal transduction. Adv Immunol. 2005;88:1–50.
33. Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. Immunol Rev. 2009;230:128–43.
34. Sullivan-Chang L, O’Donnell RT, Tuscano JM. Targeting CD22 in B-cell malignancies: current status and clinical outlook. BioDrugs. 2013;27:293–304.

35. Jain N, O'Brien S, Thomas D, Kantarjian H. Inotuzumab ozogamicin in the treatment of acute lymphoblastic leukemia. Front Biosci (Elite Ed). 2014;6:40–5.
36. Wong BY, Dang NH. Inotuzumab ozogamicin as novel therapy in lymphomas. Expert Opin Biol Ther. 2010;10:1251–8.
37. Advani A, Coiffier B, Czuczman MS, Dreyling M, Foran J, Gine E, Gisselbrecht C, Ketterer N, Nasta S, Rohatiner A, Schmidt-Wolf IG, Schuler M, Sierra J, Smith MR, Verhoef G, Winter JN, Boni J, Vandendries E, Shapiro M, Fayad L. Safety, pharmacokinetics, and preliminary clinical activity of inotuzumab ozogamicin, a novel immunoconjugate for the treatment of B-cell non-hodgkin's lymphoma: results of a phase I study. J Clin Oncol. 2010;28: 2085–93.
38. Kantarjian H, Thomas D, Jorgensen J, Jabbour E, Kebriaei P, Rytting M, York S, Ravandi F, Kwari M, Faderl S, Rios MB, Cortes J, Fayad L, Tarnai R, Wang SA, Champlin R, Advani A, O'Brien S. Inotuzumab ozogamicin, an anti-CD22-calecheamicin conjugate, for refractory and relapsed acute lymphocytic leukaemia: a phase 2 study. Lancet Oncol. 2012;13:403–11.
39. Kantarjian H, Thomas D, Jorgensen J, Kebriaei P, Jabbour E, Rytting M, York S, Ravandi F, Garris R, Kwari M, Faderl S, Cortes J, Champlin R, O'Brien S. Results of inotuzumab ozogamicin, a CD22 monoclonal antibody, in refractory and relapsed acute lymphocytic leukemia. Cancer. 2013;119:2728–36.
40. Jain N, O'Brien S, Thomas DA, Jabbour E, Faderl S, Ravandi F, Borthakur G, York S, Garris R, Cortes JE, Kantarjian HM. Inotuzumab ozogamicin in combination with low-intensity chemotherapy (Mini-hyper-CVD) as frontline therapy for older patients (≥60 Years) with acute lymphoblastic leukemia (ALL). Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 1432.
41. Rytting M, Triche L, Thomas D, O'Brien S, Kantarjian H. Initial experience with CMC-544 (Inotuzumab Ozogamicin) in pediatric patients with relapsed B-cell acute lymphoblastic leukemia. Pediatr Blood Cancer. 2014;61:369–72.
42. Ogura M, Hatake K, Ando K, Tobinai K, Tokushige K, Ono C, Ishibashi T, Vandendries E. Phase I study of anti-CD22 immunoconjugate inotuzumab ozogamicin plus rituximab in relapsed/refractory B-cell non-hodgkin lymphoma. Cancer Sci. 2012;103:933–8.
43. Ogura M, Tobinai K, Hatake K, Uchida T, Kasai M, Oyama T, Suzuki T, Kobayashi Y, Watanabe T, Azuma T, Mori M, Terui Y, Yokoyama M, Mishima Y, Takahashi S, Ono C, Ohata J. Phase I study of inotuzumab ozogamicin (CMC-544) in Japanese patients with follicular lymphoma pretreated with rituximab-based therapy. Cancer Sci. 2010;101:1840–5.
44. Fayad L, Offner F, Smith MR, Verhoef G, Johnson P, Kaufman JL, Rohatiner A, Advani A, Foran J, Hess G, Coiffier B, Czuczman M, Giné E, Durrant S, Kneissl M, Luu KT, Hua SY, Boni J, Vandendries E, Dang NH. Safety and clinical activity of a combination therapy comprising two antibody-based targeting agents for the treatment of non-hodgkin lymphoma: results of a phase I/II study evaluating the immunoconjugate inotuzumab ozogamicin with rituximab. J Clin Oncol. 2013;31:573–83.
45. Mansfield E, Amlot P, Pastan I, FitzGerald DJ. Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors. Blood. 1997;90:2020–6.
46. Kreitman RJ, Wilson WH, Bergeron K, Raggio M, Stetler-Stevenson M, FitzGerald DJ, Pastan I. Efficacy of the anti CD22 recombinant immunotoxin BL22 in chemotherapy resistant hairy cel leukemia. N Engl J Med. 2001;345:241–7.
47. Kreitman RJ, Pastan I. Antibody fusion proteins: anti-CD22 recombinant immunotoxin moxetumomab pasudotox. Clin Cancer Res. 2011;17:6398–405.
48. Park JH, Levine RL. Targeted immunotherapy for hairy cell leukemia. J Clin Oncol. 2012;30:1888–90.
49. Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler-Stevenson M, Fitzgerald DJ, Lechleider R, Pastan I. Phase I trial of anti-CD22 recombinant immunotoxin moxetumomab pasudotox (CAT-8015 or HA22) in patients with hairy cell leukemia. J Clin Oncol. 2012; 30:1822–8.
50. Li D, Poon KA, Yu SF, Dere R, Go M, Lau J, Zheng B, Elkins K, Danilenko D, Kozak KR, Chan P, Chuh J, Shi X, Nazzal D, Fuh F, McBride J, Ramakrishnan V, de Tute R, Rawstron

A, Jack AS, Deng R, Chu YW, Dornan D, Williams M, Ho W, Ebens A, Prabhu S, Polson AG. DCDT2980S, an anti-CD22-monomethyl auristatin E antibody-drug conjugate, is a potential treatment for non-hodgkin lymphoma. Mol Cancer Ther. 2013;12:1255–65.
51. Zolot RS, Basu S, Million RP. Antibody–drug conjugates. Nature Rev Drug Discovery. 2013;12:259–60.
52. Sullivan-Chang L, O'Donnell RT, Tuscano JM. Targeting CD22 in B-cell malignancies: current status and clinical outlook. BioDrugs. 2013;27:293–304.
53. Polson AG, Williams M, Gray AM, Fuji RN, Poon KA, McBride J, Raab H, Januario T, Go M, Lau J, Yu SF, Du C, Fuh F, Tan C, Wu Y, Liang WC, Prabhu S, Stephan JP, Hongo JA, Dere RC, Deng R, Cullen M, de Tute R, Bennett F, Rawstron A, Jack A, Ebens A. Anti-CD22-MCC-DM1: an antibody-drug conjugate with a stable linker for the treatment of non-hodgkin's lymphoma. Leukemia. 2010;24:1566–73.
54. Advani R, Lebovic D, Chen BM, Goy A, Chang, JE, Maeda LS, Ho W, Kahn R, Lu D, Su Z, Chu YW, Cheson BD. A phase I study of DCDT2980S, an antibody-drug conjugate (ADC) targeting CD22, in relapsed or refractory b-cell non-hodgkin's lymphoma (NHL). Blood (ASH Annual Meeting Abstracts). 2012;120:Abstract 59.
55. Advani R, Chen AI, Lebovic D, Brunvand MB, Chang A, Hochberg E, Yalamanchili S, Kahn R, Lu D, Chai A, Chu Y-W, Cheson BD. Final results of a phase i study of the anti-CD22 antibody-drug conjugate (ADC) DCDT2980S with or without Rituximab (RTX) in patients (Pts) with relapsed or refractory (R/R) B-cell non-hodgkin's lymphoma (NHL). Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 4399.
56. Robbins DH, Margulies I, Stetler-Stevenson M, Kreitman RJ. Hairy cell leukemia, a B-cell neoplasm that is particularly sensitive to the cytotoxic effect of anti-Tac(Fv)-PE38 (LMB-2). Clin Cancer Res. 2000;6:693–700.
57. Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, Waldmann TA, Pastan I. Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol. 2000;18:1622–36.
58. Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI. Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. Cancer Res. 2001;61:4483–9.
59. Hammer O. CD19 as an attractive target for antibody-based therapy. MAbs. 2012;4:571–7.
60. Blanc V, Bousseau1 A, Caron A, Carrez C, Lutz RJ, Lambert JM. SAR3419: an anti-CD19-maytansinoid immunoconjugate for the treatment of B-cell malignancies. Clin Cancer Res. 2011;17:6448–58.
61. Remillard S, Rebhun LI, Howie GA, Kupchan SM. Antimitotic sctivity of the potent tumor inhibitor maytansine. Science. 1975;189:1002–5.
62. Al-Katib AM, Aboukameel A, Mohammad R, Bissery MC, Zuany-Amorim C. Superior antitumor activity of SAR3419 to rituximab in xenograft models for non-hodgkin's lymphoma. Clin Cancer Res. 2009;15:4038–45.
63. Younes A, Kim S, Romaguera J, et al. Phase I multi-dose escalation study of the anti-CD19 maytansinoid immunoconjugate SAR3419 administered by intravenous infusion every 3 weeks to patients with relapsed/refractory B-cell lymphoma. J Clin Oncol. 2012;30:2776–82.
64. Ribrag V, Dupuis J, Tilly H, Morschhauser F, Laine F, Houot R, Haioun C, Copie C, Varga A, Lambert J, Hatteville L, Ziti-Ljajic S, Caron A, Payrard S, Coiffier B. A dose-escalation study of SAR3419, an anti-CD19 antibody maytansinoid conjugate, administered by intravenous infusion once weekly in patients with relapsed/refractory b-cell non-hodgkin lymphoma. Clin Cancer Res. 2014;20:213–20.
65. Coiffier B, Thieblemont C, de Guibert S, et al. Phase II study of anti-CD19 antibody drug conjugate (SAR3419) in combination with rituximab: clinical activity and safety in Patients with Relapsed/refractory Diffuse Large B-cell Lymphoma (NCT01470456). Blood (ASH Annual Meeting Abstracts). 2013;122:4395.
66. FitzGerald DJ, Wayne AS, Kreitman RJ, Pastan I. Treatment of hematologic malignancies with immunotoxins and antibody-drug conjugates. Cancer Res. 2011;71:6300–09.

67. Messmann RA, Vitetta ES, Headlee D, Senderowicz AM, Figg WD, Schindler J, Michiel DF, Creekmore S, Steinberg SM, Kohler D, Jaffe ES, Stetler-Stevenson M, Chen H, Ghetie V, Sausville EA. A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylatedricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22(+) B cell lymphoma. Clin Cancer Res. 2000;6:1302–13.
68. Herrera L, Bostrom B, Gore L, Sandler E, Lew G, Schlegel PG, Aquino V, Ghetie V, Vitetta ES, Schindler J. A phase 1 study of Combotox in Pediatric Patients with Refractory B-lineage Acute Lymphoblastic Leukemia. J Pediatr Hematol Oncol. 2009;31:936–41
69. Schindler J, Gajavelli S, Ravandi F, Shen Y, Parekh S, Braunchweig I, Barta S, Ghetie V, Vitetta E, Verma A. A phase i study of a combination of anti-CD19 and Anti-CD22 immunotoxins (Combotox) in adult patients with refractory B-lineage acute lymphoblastic leukaemia. Br J Haematol. 2011;154:471–6.
70. Barta SK, Zou Y, Schindler J, Shenoy N, Bhagat TD, Steidl U, Verma A. Synergy of sequential administration of a deglycosylated ricin a chain-containing combined anti-CD19 and anti-CD22 immunotoxin (combotox) and cytarabine in a murine model of advanced acute lymphoblastic leukemia. Leuk Lymphoma. 2012;53:1999–2003.
71. Vallera DA, Chen H, Sicheneder AR, Panoskaltsis-Mortari A, Taras EP. Genetic alteration of a bispecific ligand-directed toxin targeting human CD19 and CD22 receptors resulting in improved efficacy against systemic B cell alignancy. Leuk Res. 2009;33:1233–42.
72. Gerber HP, Kung-Sutherland M, Stone I, Morris-Tilden C, Miyamoto J, McCormick R, Alley SC, Okeley N, Hayes B, Hernandez-Ilizaliturri FJ, McDonagh CF, Carter PJ, Benjamin D, Grewal IS. Potent antitumor activity of the anti-CD19 auristatin antibody drug conjugate hBU12-vcMMAE against rituximab-sensitive and -resistant lymphomas. Blood 2009;113:4352–61.
73. Borate U, Fathi A T, Shah, BD, DeAngelo DJ, Silverman LB, Cooper TM, Albertson TM, O'Meara M M, Sandalic L, Stevison F, Chen R. A first-in-human Phase 1 study of the antibody-drug conjugate SGN-CD19A in relapsed or refractory b-lineage acute leukemia and highly aggressive lymphoma. Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 1437.
74. Boursalian TE, McEarchern JA, Law CL, Grewal IS. Targeting CD70 for human therapeutic use. Adv Exp Med Biol. 2009;647:108–19.
75. Shaffer DR, Savoldo B, Yi Z, Chow KK, Kakarla S, Spencer DM, Dotti G, Wu MF, Liu H, Kenney S, Gottschalk S. T cells redirected against CD70 for the Immunotherapy of CD70-positive malignancies. Blood. 2011;117:4304–14.
76. Israel BF, Gulley M, Elmore S, Ferrini S, Feng WH, Kenney SC. Anti-CD70 antibodies: a potential treatment for EBV+CD70-expressing Lymphomas. Mol Cancer Ther. 2005;4:2037–44.
77. Chu Y-W, Polson A. Antibody–drug conjugates for the treatment of B-cell non–hodgkin's lymphoma and leukemia. Future Oncol. 2013;9:355–68.
78. Thevanayagam L, Bell A, Chakraborty I, Sufi B, Gangwar S, Zang A, Rangan V, Rao C, Wang Z, Pan C, Chong C, Cardarelli P, Deshpande S, Srinivasan M. Novel detection of DNA-alkylated adducts of antibody-drug conjugates with potentially unique preclinical and biomarker applications. Bioanalysis. 2013;5:1073–81.
79. Derwin D, Passmore D, Sung J. Activation of antibody drug conjugate MDX-1203 by human carboxylesterase. Program and abstracts of the 101st meeting of the American Association for Cancer Research. Washington, DC, 17–21 April 2010 (Abstract 2575).
80. Oflazoglu E, Stone IJ, Gordon K, Wood CG, Repasky EA, Grewal IS, Law CL, Gerber HP. Potent anticarcinoma activity of the humanized anti-CD70 antibody h1F6 conjugated to the tubulin inhibitor auristatin via an uncleavable linker. Clin Cancer Res. 2008;14,6171–80.
81. Thompson JA, Forero-Torres A, Heath EI, Ansell SM, Pal SK, Infante JR, De Vos S, Hamlin PA, Zhao B, Klussman K, Whiting NC. The effect of SGN-75, a novel antibody–drug conjugate (ADC), in treatment of patients with renal cell carcinoma (RCC) or non-hodgkin lymphoma (NHL): a phase i study. J Clin Oncol, ASCO Annual Meeting Abstracts. Part 1. 2011;29 (Supplement):Abstract 3071.

82. Palanca-Wessels MC, Flinn IW, Sehn LH, SE, Flinn IW, Sehn LH, Patel M, Sangha R, Tilly H, Advani R, Casasnovas O, Press OW, Yalamanchili S, Kahn R, Lu D, Chai A, Chu Y-W, Morschhauser F. A phase I study of the anti-CD79b antibody-drug conjugate (ADC) DCDS4501A targeting CD79b in relapsed or refractory b-cell non-Hodgkin's lymphoma (NHL). Blood (ASH Annual Meeting Abstracts). 2012;120:Abstract 56.
83. Palanca-Wessels MC, Salles GA, Czuczman MS, Assouline SE, Flinn IW, Sehn LH, Patel M, Sangha R, Tilly H, Advani R, Casasnovas O, Press OW, Yalamanchili S, Kahn R, Lu D, Chai A, Chu Y-W, Morschhauser F. Final results of a phase i study of the anti-CD79b antibody-drug conjugate DCDS4501A in relapsed or refractory (R/R) B-cell non-hodgkin lymphoma (NHL). Blood (ASH Annual Meeting Abstracts). 2013;122:4400.
84. Siddiqi T, Thomas SH, Chen R. Role of brentuximab vedotin in the treatment of relapsed or refractory hodgkin lymphoma. Pharmgenomics Pers Med. 2014;7:79–85.
85. Bartlett NL, Sharman JP, Oki Y, Advani RH, Bello CM, Winter JN, Yang Y, Kennedy DA, Jacobsen ED. A phase 2 study of brentuximab vedotin in patients with relapsed or refractory CD30-positive non-hodgkin lymphomas: interim results in patients with DLBCL and other B-cell lymphomas. Blood (ASH Annual Meeting Abstracts). 2013;122:848.
86. Robak T, Robak P. Anti-CD37 antibodies for chronic lymphocytic leukemia. Expert Opin Biol Ther. 2014;14:651–61.
87. Barrena S, Almeida J, Yunta M, et al. Aberrant expression of tetraspanin molecules in B cell chronic lymphoproliferative disorders and its correlation with normal B-cell maturation. Leukemia. 2005;19:1376–83.
88. Deckert J, Park PU, Chicklas S, Yi Y, Li M, Lai KC, Mayo MF, Carrigan CN, Erickson HK, Pinkas J, Lutz RJ, Chittenden T, Lambert JM. A novel anti-CD37 antibody-drug conjugate with multiple anti-tumor mechanisms for the treatment of B-cell malignancies. Blood. 2013;122:3500–10
89. Beckwith KA, Frissora FW, Stefanovski MR, Towns WH, Cheney C, Mo X, Deckert J, Croce CM, Flynn JM, Andritsos LA, Jones JA, Maddocks KJ, Lozanski G, Byrd JC, Muthusamy N. The CD37-targeted antibody-drug conjugate IMGN529 is highly active against human CLL and in a novel CD37 transgenic murine leukemia model. Leukemia. 2014;28:1501–10.
90. Chan TS, Kwong YL, Tse E. Novel therapeutic agents for T-cell lymphomas. Discov Med. 2013;16:27–35.
91. Francisco JA, Cerveny CG, Meyer DL, Mixan BJ, Klussman K, Chace DF, Rejniak SX, Gordon KA, DeBlanc R, Toki BE, Law CL, Doronina SO, Siegall CB, Senter PD, Wahl AF. CAC10-vcMMAE, an Anti-CD30-monomethyl auristatin e conjugate with potent and selective antitumor activity. Blood 2003;102:1458–65.
92. Mak V, Hamm J, Chhanabhai M, Shenkier T, Klasa R, Sehn LH, Villa D, Gascoyne RD, Connors JM, Savage KJ. Survival of patients with peripheral t-cell lymphoma after first relapse or progression: spectrum of disease and rare long-term survivors. J Clin Oncol. 2013;31:1970–6.
93. Pro B, Advani R, Bartlett NL, Bartlett NL, Rosenblatt JD, Illidge T, Matous J, Ramchandren R, Fanale M, Connors JM, Yang Y, Sievers EL, Kennedy DA, Shustov A. Brentuximab Vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large cell lymphoma: results of a phase 2 study. J Clin Oncol. 2012;30:2190–6.
94. Pro B, Advani RH, Brice P, Bartlett NL, Rosenblatt JD, Illidge T, Matous J, Ramchandren R, Fanale MA, Connors JM, Yang Y, Huebner D, Kennedy DA, Shustov AR. Three-year survival results from an ongoing phase 2 study of brentuximab vedotin in patients with relapsed or refractory systemic anaplastic large cell lymphoma. Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 1809.
95. Bartlett NL, Chen R, Fanale MA, Brice P, Gopal A, Smith SE, Advani R, Matous JV, Ramchandren R, Rosenblatt JD, Huebner D, Levine P, Grove L, Forero-Torres A. Retreatment with brentuximab vedotin in patients with CD30-positive hematologic malignancies. J Hematol Oncol. 2014;7:24. doi:10.1186/1756-8722-7-24.
96. Duvic M, Tetzlaff M, Clos AL, Gangar P, Talpur R. Phase II trial of brentuximab vedotin for CD30+ cutaneous T-cell lymphomas and lymphoproliferative disorders. Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 367.

97. Manoukian G, Hagemeister F. Denileukin diftitox: a novel immunotoxin. Expert Opin Biol Ther. 2009;9: 1445–51.
98. Lansigan F, Stearns DM, Foss F. Role of denileukin diftitox in the treatment of persistent or recurrent cutaneous T-cell lymphoma. Cancer Manag Res. 2010;5:53–9.
99. Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, Jegasothy B, Wood G, Gordon M, Heald P, Oseroff A, Pinter-Brown L, Bowen G, Kuzel T, Fivenson D, Foss F, Glode M, Molina A, Knobler E, Stewart S, Cooper K, Stevens S, Craig F, Reuben J, Bacha P, Nichols J. Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol. 2001;19:376–88.
100. Prince HM, Duvic M, Martin A, Sterry W, Assaf C, Sun Y, Straus D, Acosta M, Negro-Vilar A. Phase III placebo-controlled trial of denileukin diftitox for patients with cutaneous T-cell lymphoma. J Clin Oncol. 2010;28:1870–7.
101. Duvic M, Geskin L, Prince HM. Duration of response in cutaneous T-cell lymphoma patients treated with denileukin diftitox: results from 3 phase III studies. Clin Lymphoma Myeloma Leuk. 2013;13:377–84.
102. Duvic M, Martin AG, Olsen EA, Fivenson DP, Prince HM. Efficacy and safety of denileukin diftitox retreatment in patients with relapsed cutaneous T-cell lymphoma. Leuk Lymphoma. 2013;54:514–19.
103. Foss FM, Sjak-Shie N, Goy A, Jacobsen E, Advani R, Smith MR, Komrokji R, Pendergrass K, Bolejack V. A multicenter phase II trial to determine the safety and efficacy of combination therapy with denileukin diftitox and cyclophosphamide, doxorubicin, vincristine and prednisone in untreated peripheral T-cell Lymphoma: the CONCEPT study. Leuk Lymphoma. 2013;54:1373–9137.
104. Frankel AE, Zuckero SL, Mankin AA, Grable M, Mitchell K, Lee YJ, Neville DM, Woo JH. Anti-CD3 recombinant diphtheria immunotoxin therapy of cutaneous T Cell lymphoma. Curr Drug Targets. 2009;10:104–9.
105. Woo JH, Bour SH, Dang T, Lee YJ, Park SK, Andreas E, Kang SH, Liu JS, Neville DM Jr, Frankel AE. Preclinical studies in rats and squirrel monkeys for safety evaluation of the bivalent anti-human T Cell immunotoxin, A-dmDT390–bisFv(UCHT1). Cancer Immunol Immunother. 2008;57:1225–39.
106. Falini B, Pileri S, Pizzolo G, Dürkop H, Flenghi L, Stirpe F, Martelli MF, Stein H. CD30 (Ki-1) molecule: a new cytokine receptor of the tumor necrosis factor receptor superfamily as a tool for diagnosis and immunotherapy. Blood. 1995;85:1–14.
107. Venkataraman G, Kamran Mirza M, Eichenauer DA, Diehl V. Current status of prognostication in classical hodgkin lymphoma. Br J Haematol. 2014;165:287–99.
108. Younes A, Gopal AK, Smith SE, Ansell SM, Rosenblatt JD, Savage KJ, Ramchandren R, Bartlett NL, Cheson BD, de Vos S, Forero-Torres A, Moskowitz CH, Connors JM, Engert A, Larsen EK, Kennedy DA, Sievers EL, Chen R. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory hodgkin's lymphoma. J Clin Oncol. 2012;30:2183–9.
109. Gopal AK, Chen R, Smith SE, Ansell SM, Rosenblatt JD, Savage KJ, Connors JM, EngertA, Larsen EK, Huebner D, Sievers EL, Younes A. Three-year follow-up data and characterization of long-term remissions from an ongoing phase 2 study of Brentuximab Vedotin in patients with relapsed or refractory hodgkin lymphoma. Blood (ASH Annual Meeting Abstracts). 2013;122:Abstract 4382.
110. Bartlett NL, Chen R, Fanale MA, Brice P, Gopal A, Smith SE, Advani R, Matous JV, Ramchandren R, Rosenblatt JD, Huebner D, Levine P, Grove L, Forero-Torres A. Retreatment with Brentuximab Vedotin in patients with CD30-positive hematologic malignancies. J Hematol Oncol. 2014;7:Abstract 24.
111. Pro B, Perini GF. Brentuximab vedotin in Hodgkin's lymphoma. Expert Opin Biol Ther. 2012;12:1415–21.
112. Lutz RJ, Whiteman KR. Antibody-maytansinoid conjugates for the treatment of myeloma. MAbs. 2009;1:548–51.
113. van de Donk NW, Lokhorst HM. New developments in the management and treatment of newly diagnosed and relapsed/refractory multiple myeloma patients. Expert Opin Pharmacother. 2013;14:1569–73.

114. Govindan SV, Cardillo TM, Sharkey RM, Tat F, Gold DV, Goldenberg DM. Milatuzumab-SN-38 conjugates for the treatment of CD74+ cancers. Mol Cancer Ther. 2013;12:968–78.
115. Witzig TE, Kimlinger T, Stenson M, Therneau T. Syndecan-1 expression on malignant cells from the blood and marrow of patients with plasma cell proliferative disorders and B-cell chronic lymphocytic leukemia. Leuk Lymphoma. 1998;31:167–75.
116. Ikeda H, Hideshima T, Fulciniti M, Lutz RJ, Yasui H, Okawa Y, Kiziltepe T, Vallet S, Pozzi S, Santo L, Perrone G, Tai YT, Cirstea D, Raje NS, Uherek C, Dälken B, Aigner S, Osterroth F, Munshi N, Richardson P, Anderson KC. The monoclonal antibody nBT062 conjugated to cytotoxic maytansinoids has selective cytotoxicity against CD138-positive multiple myeloma cells in vitro and in vivo. Clin Cancer Res. 2009;15:4028–37.
117. Lutz RJ, Whiteman KR. Antibody-maytansinoid conjugates for the treatment of myeloma. MAbs. 2009;1:548–51.
118. Tassone P, Goldmacher VS, Neri P, Gozzini A, Shammas MA, Whiteman KR, Hylander-Gans LL, Carrasco DR, Hideshima T, Shringarpure R, Shi J, Allam CK, Wijdenes J, Venuta S, Munshi NC, Anderson KC. Cytotoxic activity of the maytansinoid immunoconjugate B-B4-DM1 against CD138^{+} multiple myeloma cells. Blood. 2004;104:3688–96.
119. Heffner LT, Jagannath S, Zimmerman TM, Lee KP, Rosenblatt J, Lonial S, Lutz RJ, Czeloth N, Osterroth F, Markus Ruehle M, Beelitz MA, Wartenberg-Demand A, Haeder T, Anderson KC, Munshi NC. BT062, an antibody-drug conjugate directed against CD138, given weekly for 3 weeks in each 4 week cycle: sfety and further evidence of clinical activity. Blood (ASH Annual Meeting Abstracts). 2012;120:Abstract 4042.
120. Berdeja JG, Hernandez-Ilizaliturri F, Chanan-Khan A, Patel M, Kelly KR, Running KL, MurphyM, Guild R, Carrigan C, Ladd S, Wolf BB, O'Leary JJ, Ailawadhi S. Phase I study of Lorvotuzumab Mertansine (LM, IMGN901) in combination with Lenalidomide (Len) and Dexamethasone (Dex) in patients with CD56-positive relapsed or relapsed/refractory multiple myeloma (MM). Blood (ASH Annual Meeting Abstracts). 2012;120:Abstract 728.
121. Kelly KR, Chanan-Khan A, Somlo G, Leonard TH, David SS, Todd MZ, Jagannath S, Nikhil CM, Lonial S, Roy V, Ruehle M, Chavan S, Patel P, Rothenburger M, Wartenberg-Demand A, Haeder T, Kenneth C. Anderson Indatuximab Ravtansine (BT062) in combination with lenalidomide and low-dose dexamethasone in patients with relapsed and/or refractory multiple myeloma: clinical activity in len/dex-refractory patients. Blood (ASH Annual Meeting Abstracts). 2013;122:758.
122. Berdeja JG. Lorvotuzumab mertansine: antibody-drug-conjugate for CD56+ multiple myeloma. Front Biosci (Landmark Ed). 2014;19:163–70.
123. Lutz RJ, Whiteman KR. Antibody-maytansinoid conjugates for the treatment of myeloma. mAbs. 2009;1:548–51.
124. Ishitsuka K, Jimi S, Goldmacher VS, Ab O, Tamura K. Targeting CD56 by the Maytansinoid Immunoconjugate IMGN901 (huN901-DM1): a potential therapeutic modality implication against natural killer/T cell malignancy. Br J Haematol. 2008;141:129–31.
125. Chanan-Khan A, Wolf J, Garcia J, Gharibo M, Jagannath S, Manfredi D, Sher T, Martin C, Zildjian SH, O'Leary J, Vescio R. Efficacy analysis from a phase I study of Lorvotuzumab Mertansine (IMGN901) used as monotherapy in patients with heavily pre-treated CD56-positive multiple myeloma. Blood (ASH Annual Meeting Abstracts). 2010;116:819 (Abstract 1962).
126. Berdeja JG, Hernandez-Ilizaliturri F, Chanan-Khan A, Patel M, Kelly KR, Kelli L, Running KL, Murphy M, Guild R, Carrigan C, Ladd S, Wolf BB, O'Leary JJ, Ailawadhi SA. Phase I study of Lorvotuzumab Mertansine (LM, IMGN901) in combination with Lenalidomide (Len) and Dexamethasone (Dex) in patients with CD56-positive relapsed or relapsed/refractory multiple myeloma (MM). Blood (ASH Annual Meeting Abstracts). 2012;120:Abstract 728.

Chapter 6
Challenges for Therapeutic Application of *Pseudomonas* Exotoxin-Based Immunotoxins

Vladimir Dergachev and Itai Benhar

Abstract Immunotoxins are therapeutic molecules that belong to a class of biopharmaceuticals called "Armed antibodies". Immunotoxins are based on very potent toxins of bacterial or plant origin that lack target-cell specificity. To make them target-cell-specific, the non-specific cell binding domains of the original toxins are replaced with a target-cell-specific binding protein, in most cases a monoclonal antibody or a recombinant antibody fragment. The most clinically-advanced immunotoxins are currently being evaluated in phase II and III clinical studies. Like other targeted and non-targeted therapeutics, immunotoxins too suffer from several limitations that may hinder their therapeutic efficacy. Such limitations include, but are not limited to immunogenicity, modification of the extracellular target to which the targeting antibody binds, modification of the intracellular target upon which the toxin acts to cause cell growth inhibition, and insufficient potency as single agents and off-target toxicity, where non-target cells and organs are affected by the immunotoxin, severely impairing its therapeutic index. This chapter is devoted to a group of immunotoxins in which the toxic moiety is derived from exotoxin A (PE) of the bacterium *Pseudomonas aeruginosa*. The limitations to the efficacy of PE-based immunotoxins, as well as potential solutions for overcoming such limitations, will be presented. Chapter 2 of this book: "Resistance of tumor cells against antibody-targeted protein toxins" by Ulrich Brinkmann et al. is focused on factors that influence the sensitivity or potential resistances of cancer cells towards recombinant immunotoxins which carry truncated and/or mutated derivatives of Pseudomonas exotoxin as cytotoxic payloads.

Keywords Immunotoxin(s) · Pseudomonas exotoxin A · Immunotoxin · Monoclonal antibody · Immunogenicity · De-immunization

Abbreviations

ADC	Antibody-drug conjugate
APCs	Antigen presenting cells

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ATL	Adult T-cell leukemia
BBB	The blood brain barrier
CED	Convection-enhanced delivery
CLL	Chronic lymphocytic leukemia
CTCL	Cutaneous T-cell lymphoma
dsFv	Disulfide-stabilized Fv fragment of an antibody
DT	Diphtheria toxin; *E. coli*, *Escherichia coli* bacteria
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
HCL	Hairy cell leukemia
IL-13	Interleukin 13
IL-13R	Receptor for IL13
IL-4	Interleukin 4
IL-4R	Receptor for IL4
IT	Immunotoxin
Le^Y	$Lewis^Y$ carbohydrate antigen
mAb	Monoclonal antibody
NSAIDs	Non-steroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
PDAC	Pancreatic ductal adenocarcinoma
PE (or ETA)	Pseudomonas exotoxin A
PEG	Polyethylene glycol
RICs	Antibody-radionuclide conjugates (radioimmunoconjugates)
RIP	Ribosome-inactivating protein
scFv	Single-chain Fv fragment of an antibody
VLS	Vascular leak syndrome

6.1 Introduction

Many organisms living in nature are known to produce and secrete poisonous substances to their local environment. These toxins appear as secondary metabolites and virulence factors originating from animals/plants/bacteria and usually play notable roles in defense/prey strategies, necessary for maintaining the fitness and survival of the organism. Natural toxinsvary, with a wide range of chemical composition and molecular size, aiming at different cellular targets and operating in mechanisms of action. They have been perfected during evolution for efficient harming of potential enemies; some of them are lethal even in minute doses.

The majority of natural protein toxins can be classified into three groups by their mechanism of intoxication: (1) disruption of cell integrity, (2) disruption of electrical activity of the cell, and (3) disruption or interference with cellular processes mediated by enzymatic activity.

During the last three decades, monoclonal antibodies (mAbs) have become major promising “players” for anti-cancer therapy. Most of the approved mAbs, a little

over 30, inhibit growth of target cells by recruiting immune effector mechanisms or by interfering with a cell-survival signal transduction pathway, However, the majority of antibodies does not show sufficient cell-killing effects in their native "naked (un-armed)" form, and, thus, can only be used as "guided missiles", delivering a lethal cargo to the target cells to enhance killing activity [1]. These antibody-drug complexes (immunoconjugates) may bear a variety of potential killing agents and are usually classified into four groups: (1) antibody-radionuclide conjugates (RICs)—designed to deliver a sufficiently high dose of radiationlocally to eradicate the tumor while sparing the surroundingnormal tissue; (2) antibody—small-molecule drug conjugates (ADCs)—designed to selectively deliver potent anticancer drugs and, thus, improve their therapeutic index; (3) antibody-protein toxin conjugates (immunotoxins, ITs)—recombinant fusion proteins composed of antibodies and plant or bacterial-derived toxins; and (4) antibody–enzyme conjugates—designed to be administrated with prodrugs to provide them the required metabolism and activate the drug locally [2].The combination of antibody-provided specific targeting and a highly potent cytotoxic agent in a single molecule enables the crucial discrimination between healthy and cancer cells within the body. Another prominent advantage of immunoconjugates over the free drug is in their large molecular dimensions that provide them with in vivo stability, leading to a prolonged therapeutic effect [3]. In general, immunoconjugates suffer from several limitations such as immunogenicity, sub-optimal pharmacokinetics and biodistribution properties or decomposition before being delivered (premature cargo release)—all these are only several examples of challenging limitations. Thus, currently only three mAb-based immunoconjugates have been approved by the FDA: two murine radiolabeled anti-CD20 mAbs for treatment of B-cell lymphomas (ibritumomabtiuxetan and tositumomab) and humanized anti-CD33 antibody-drug conjugate for the treatment of leukemia (gemtuzumabzogamicin) [4].

In this chapter, we will focus on representatives from the immunotoxins group with a focus on the most clinically-advanced group, *Pseudomonas* exotoxin-based immunotoxins.

6.2 Immunotoxins

ITs are targeted toxins in which a specific target or, usually a monoclonal antibody or an antibody fragment, replaces the non-specific cell-binding domain of a bacterial or a plant toxin [5]. Many toxins were evaluated as candidates for development as ITs, still, the field is dominated by two bacterial ADP ribosylating toxins, Pseudomonas exotoxin and diphtheria toxins, and by one representative of the plant ribosomal-inactivating proteins (RIPs), ricin toxin. All of these toxins, once their catalytic domain reaches the cytosol, inhibit protein synthesis either by inhibiting translation elongation (ADP ribosylating toxins) or by inactivating eukaryotic ribosomes (RIPs), eventually leading to cell death [5].

The first generation of ITs, created in the mid to late 1980s, were composed of intactIgGs that werelinked to full-length toxins by disulfide bonds. These ITs

suffered from an enormous size, heterogeneous composition and lack of specificity, making them limited in their ability to penetrate into solid tumors. The second generation of ITs, investigated from the mid 1980s to the early 1990s, were composed of truncated versions of toxins that lack a cell binding domain that were chemically conjugated to a targeting moiety. The development of the third generation of ITs (recombinant ITs, RITs), started in the early 1990. This development was made possible due to break through in recombinant DNA and protein engineering technologies, and consisted of atoxin-encoding DNA sequence fused to the antigen-encoding DNA sequence (or antibody fragment, particularly variable region of the antibody engineered as a single chain Fv) on the same Expression vector and were mostly expressed in *E.coli* bacteria. These conjugates were much smaller in size and homogeneous [3]. Early recombinant RITs used mAb variable regions of the heavy and light chains in the single chain format (scFv) that were connected by a 15-amino acid peptide linker [6]. Later, to improve stability and binding activity, the peptide linker was replaced by a disulfide bond between the heavy and light chain Fv fragments (dsFv), and binding affinity was improved by in vitro affinity maturation [7].

The critical features of ITs are: (1) the cell-binding domain of the native toxin is replaced with an antibody fragment [8, 9]; (2) general size minimization is done by removing unnecessary parts of the toxin and by using small antibody fragments rather than full-size IgGs—this significantly improves the ability to penetrate into solid tumors and also protects Its from degradation by proteases [10, 11]; and (3) reducing or eliminating immunogenic epitopes that maybe recognized by the immune system and contribute to rapid neutralization and elimination of the therapeutic molecule [9, 12].

The most commonly used toxins may be divided into two groups by their origin: (1) bacterial exotoxins—diphtheria toxin from *Corynebacterium diptheriae* (DT) and Pseudomonas exotoxin A from *Pseudomonas aeruginosa* (PE); and (2) plant toxins—ricin (the prominent member of this group), saporin and pokeweed antiviral protein (PAP). All of the above have been tried in targeting a variety of tumor-associated surface markers [13, 14].

As this chapter is being written, searching PUBMED using “immunotoxin” as a query word returned 5364 hits. There are hundreds of publications related to PE-based ITs. Our chapter, focusing on limitations to clinical applicability of PE-based immunotoxins, cannot be comprehensive enough to allow the citation of most of them. We apologize to the authors of studies that were not cited in this chapter.

6.3 Brief Historical Overview of PE-Based ITs

Pseudomonasexotoxin A (abbreviated PE or ETA) is one of the virulent factors that are naturally secreted by the *Pseudomonas aeruginosa* bacterium and helps it invade animal tissues, including the tissues of humans. PE is a polypeptide composed of 613 amino acid residues and it belongs to the ADP-ribosylation toxins family.

The toxin itself contains three main structural and functional domains: (1) the N-terminal receptor ® binding domain Ia (aa 1-252) is required for target cell recognition; (2) domain II (aa 253–364) is responsible for retrograde translocation (T) of the toxin through cell membrane into the cytosol; and (3) the catalytic (C) domain III (aa 405–613), together with the last amino acids of domain Ib (aa 365–404),is the catalytic unit of the protein [8, 15]. After the toxin reaches the blood, carboxypeptidases remove the C-terminal lysine (#613) and expose the REDL sequence (that functions as a KDEL, ER retrieval sequence). Next (see Fig. 6.1), the toxin binds to CD91 (alpha2-macroglobulin receptor) on the cell surface via its cell-binding domain Ia, followed by the toxin's internalization via clathrin-coated pits into early endosomes. Acidification of the endosome leads to PE dissociation from its receptor, a conformational change and finally cleavage of the toxin by the cellular protease furin (within the furin-sensitive loop in domain II). This cleavage results in two products linked by an intradomain disulfide bond. This disulfide bond undergoes reduction and the C-terminal part, comprising part of domain II, domain I band domain III travels to the trans-Golgi network, binds to the KDEL receptor

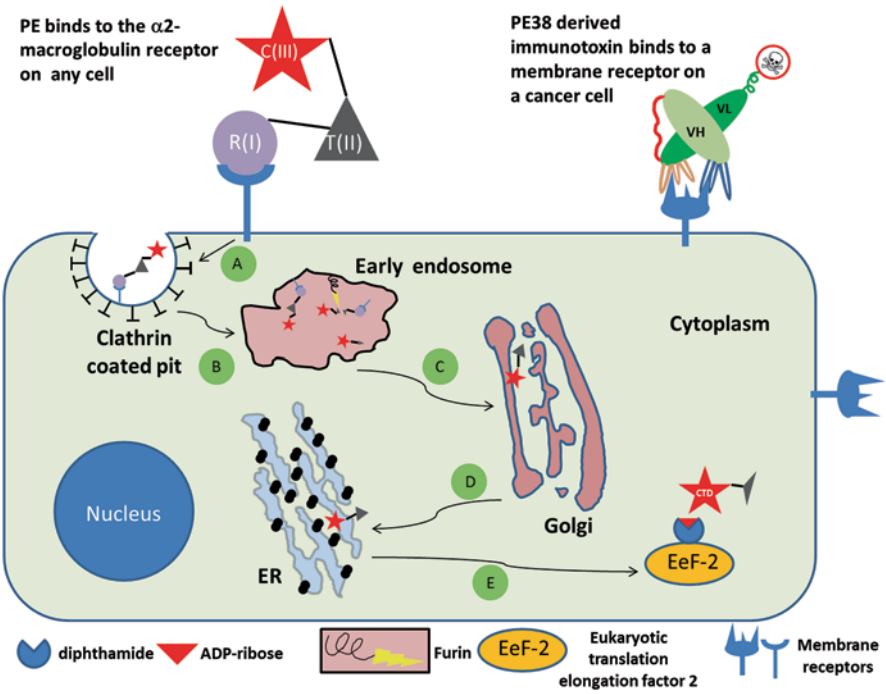

Fig. 6.1 How PE and PE-based ITs intoxicate cells. **a** PE or IT binds to their respective cell surface receptors. **b** They internalize into clathrin-coated pits that fuse with early endosomes, where they undergo furin-mediated cleavage and reduction of the interdomain disulfide bond. **c** The C-terminal part (CTD) retro-translocates via Golgi to the ER (**d**). **e** The CTD is further translocated from the ER to the cytosol where it binds to a diphthamide residue on the translation elongation factor 2 (EeF-2) and ADP ribosylates it. This step irreversibly-inactivates EeF-2, leading to arrest in protein synthesis and eventually to apoptotic cell death

(via its exposed REDL sequence) and finally routes to the endoplasmic reticulum (ER).In the ER, sequences in domain II mediate the translocation of the 37 kDa fragment to the cytoplasm via the Sec61p translocon. Once in the cytosol, the catalytic domain III blocks the eukaryotic translation elongation factor 2 (EF2) via an ADP-ribosylation mechanism, causing arrest of protein synthesis. This event initiates the apoptotic cascade by lowering Mcl-1 levels and unleashing Bak to promote apoptotic death [16–18].

In the early 1980s, Ira Pastan's group at the NIH pioneered the development of PE-based immunotoxins. They were the first to propose the possibility of targeting PE to cancer cells by using antibodies. Like other first generation ITs, the first attempt to attach PE to antibody was done chemically, by treatment with iminothiolane, which reacts with lysine residues on proteins and generates free sulfhydryl groups that are used in the coupling reaction. PE was attached to anti-transferrin receptor and anti-Tac antibodies that binds to CD25 on T-cells and T-cell malignancies [19]. Because these antibody-toxin conjugates contained the non-specific cell-binding domain I, they remained capable of binding healthy cells, thus producing side effects in animals, and due to severe hepatotoxicity, clinical trials were little pursued. A first-generation PE-based immunotoxin that was tried in patients was OVB3-PE [20, 21].

Deletion of domain I was the first step in making the second generation, PE-based immunotoxins. Structure-function studies of PE that were carried out during the same time period have shown that a large part of domain Ib (a.a.365–380) can be deleted without effecting cytotoxicity, resulting in a smaller version of the modified toxin called PE38 (the name denotes the molecular weight of the protein, i.e. the truncated derivate is 38 kDa, while the domain I-deleted toxin is 40 kDa) [22]. To facilitate efficient site-specific chemical coupling, a small-lysine containing peptide was appended at the amino terminus (a molecule that was named LysPE38). A prototype second domain immunotoxin was a conjugate between the anti Lewis^Y (Le^Y, a carbohydrate antigen widely presented in colon, breast and many other epithelial cancers) carbohydrate antigen B3 mAb and LysPE38. This immunotoxin, designated LMB-1, has shown a much better therapeutic window in animals and was subsequently tested in phase I clinical studies. Such a clinical trial has shown a complete response in a patient with breast cancer and a striking partial response in a patient with colon cancer with extensive metastases to retroperitoneal lymph nodes [23]. This was a milestone study, since it demonstrated for the first time an antitumor response to an immunotoxin in epithelial tumors. In that study, the maximum tolerated dose (MTD) of LMB-1 was 75 μg/kg given intravenously three times every other day. The major toxicity was vascular leak syndrome—an example for off-target toxicity that will be further discussed below.

Enter the third generation immunotoxins; the immunotoxins that have been developed since the early 1990s were mostly designed and produced by using molecular biology techniques and thus were called recombinant immunotoxins (RITs). This enabled reducing the size from ~200 kDain chemical conjugated immunotoxins to 63 kDain RITs, and, in this manner, significantly increased penetration into solid tumors. The two RITs that were the first to be selected for clinical development

were B3 (Fv)-PE38 (LMB-7), targeting Le^Y, and anti-Tac (Fv)—PE38 (LMB-2), targeting CD25.

LMB-7 completed a long series of pre-clinical studies that will be discussed below. It underwent a single phase I clinical study (study NCT00003020 in WWW. clinicaltrials.gov)to testthe effectiveness in treating patients who have leptomeningeal metastases. During its preclinical development, this prototypic third generation immunotoxin demonstrated limitations that are typical of such molecules, such as immunogenicity, instability and off-target toxicity, which will be further discussed below. As for Le^Y as a therapeutic target, a PE-based IT developed by Bristol-Myers Squibb, BR96 sFv-PE40, was evaluated preclinically, but later abandoned [24].

Anti-Tac (Fv)—PE38 (LMB-2) is based on mAb Anti-Tac that binds the alpha subunit of the IL2 receptor (CD25, the Tac antigen) with high affinity. Many hematological malignancies express this receptor at a high level ranging from several hundred to a few thousand sites/cell. Daclizumab (trade name Zenapax) is a therapeutic humanized anti-Tac mAb antibody that is FDA approved to prevent rejection in organ transplantation and is undergoing clinical evaluation for treatment of multiple sclerosis [25–27]. ITs based on anti-Tac antibody fragments have been extensively studied by Ira Pastan's group and their collaborators since the early 1990s and have been evaluated in a number of clinical studies. Clinical trials were conducted in patients with hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), Hodgkin's disease and cutaneous T-cell lymphoma, showing partial and complete responses. Due to the problems of instability and aggregation at 37 °C, RITs were designed in away that light and heavy chains of the Fv are held together by an engineered disulfide bond. Anti-Tac sdFv-PE38 RITs were also evaluated following such stabilization (see below).

BL22 is an example of a disulfide-linked immunotoxin where the Fv of the anti-CD22 antibody (RFB4) was linked to PE38. Many B-cell malignancies express the CD22 antigen on their cell surface. The development of anti CD22, PE-based ITs began with RFB4 (Fv) PE38 [28], which underwent "disulfide stabilization", resulting in BL22. BL22 has shown remarkable activity in drug-resistant HCL in phase 1 clinical trials.

Another example for a disulfide-stabilized IT is SS1P [SS1 (dsFv) PE38], an anti-mesothelin RIT. Mesothelin is a 40-kDa cell surface membrane glycoprotein and its biological role in normal mesothelial cells is not clear. While showing limited expression in normal human tissues, it is known to be a solid tumor antigen that undergoes up-regulation in a number of epithelial cancers such as pancreatic, ovarian, lung and others [29]. This offers it as an attractive candidate for targeted therapy. SS1P was produced by the fusion of anti-mesothelin Fv (SS1) to PE38, a 38-kDa portion of *Pseudomonas* exotoxin, improved by affinity maturation and disulfide-stabilization [30–32]. The mechanism of action involves binding to the target, internalization by endocytosis and cell death by arrest of protein synthesis [9]. It is currently being evaluated in a phase I clinical trial in patients with mesothelin positive tumors. Currently, two anti-CD22-*Pseudomonas* exotoxin (PE) immunotoxins designed for treatment of B-cell malignancies are undergoing clinical phase II evaluation: BL22 [RFB4- (dsFv)-PE38] and its affinity matured version HA22

(moxetumomab pasudotox) that was developed due to low activity of the original BL22 in some other B-cell malignancies (i.e., CLL, ALL and non-Hodgkin's lymphoma). HA22 has a higher binding affinity for CD22 and greater in vitro potency. Phase I trials in patients with hairy cell leukemia ($n=32$) showed that moxetumomab pasudotox has a better complete response rate comparing to BL22 (31 vs. 25 %, respectively). Expanding its application to other hematological malignancies is also under evaluation [33].

While the study of PE-based RITs in the continental USA was carried out primarily by Ira Pastan's group and their collaborators, several groups in Europe have also studied similarly constructed RITs. Most of these studies were carried out in in vitro or in animal models and will not be discussed in detail here (a partial list of such studies can be found in references [34–40]. Winfried Wels and collaborators have been studying RITs that target ErbB2 and EGFR since the early 1990s. While most of their studies were pre-clinical [41–44], they did carry out a phase I clinical study with the RIT called scFv (FRP5)-ETA. ScFv (FRP5)-ETA is a recombinant single-chain antibody-toxin fusion protein with binding specificity for ErbB2/HER2. Previous studies from their group demonstrated potent antitumor activity of the molecule against ErbB2 overexpressing tumor cells in vitro and in animal models. The clinical study of scFv (FRP5)-ETA was reported in 2003, summarizing case reports from four different clinical centers. Eleven patients suffering from metastatic breast and colorectal cancers and from malignant melanoma were treated on a compassionate use basis by intratumoral injection of scFv (FRP5)-ETA into cutaneous lesions once daily for 7–10 days. Treatment caused the injected tumors to shrink in six of the tencases evaluated (60 %). Complete regression of injected tumor nodules was accomplished in four patients (40 %), and partial reduction in tumor size in another two patients (20 %). The authors suggested that their results demonstrated that local therapy with scFv(FRP5)-ETA can be effective against ErbB2 expressing tumors, justifying further clinical development of this reagent [45].

Despite success in treating hematologic malignancies, the therapeutic application and, in fact, the FDA approval of RITs has been hindered by a number of obstacles: immunogenicity of the murine antibody, immunogenicity of the protein toxin and of the targeting antibody, rapid clearance from the blood stream and systemic toxicity at very low doses (the maximum tolerated dose achievable with such immunotoxins is about 0.05 mg/kg). The combination of rapid clearance and low dose limits them from use in solid tumors. Biodistribution studies show that only negligible amounts of intravenously administrated RITs reach the tumor tissue (< 0.01 % of the injected dose per gram of tumor) and, thus, it is unlikely that therapeutic concentrations of such RITs can be delivered to solid tumors. In addition, recent trials have shown that despite toxin-mediated inhibition of protein synthesis, the action is not absolute, and in some cases mammalian cells that were treated by RITs appear to survive the treatment [46]. This phenomenon, referred to here as insufficient potency, points to the existence of some not fully understood/described toxin-resistance mechanisms. Current effort is directed at better understanding the complex regulation machinery at different stages of the pathway by which immunotoxins kill cells. This knowledge may be helpful to enhance the killing effect of these immunotoxins, or even to expand them to the treatment of different malignancies [47, 48].

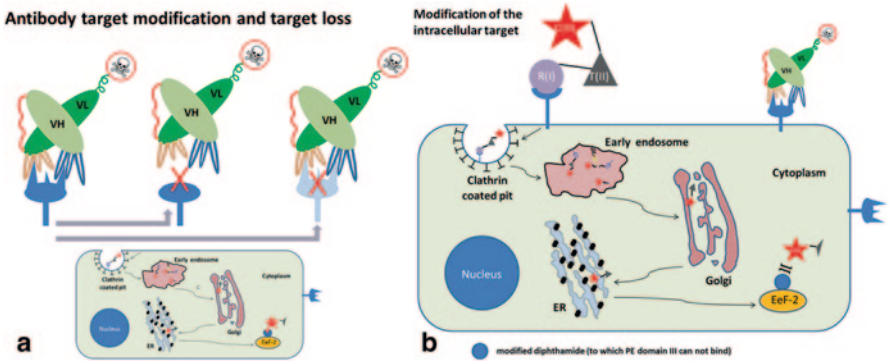

Fig. 6.2 How cancer cells may become resistant to RITs. **a** Cancer cells may down-regulate, mutate or lose altogether the cell-surface protein to which the RIT binds. This will result in the cells becoming resistant to the RIT. **b** Modification of the intracellular target leading to the cell becoming resistant to the toxin. In the case of PE, when EeF-2 is mutated in a way that it no longer binds the PE domain III, the cells become totally resistant to PE intoxication. Fortunately, such resistance has been observed only in the cell culture and not in the clinical setting

In summary, the major limitations of RITs are: immunogenicity, limited stability, insufficient potency, off-target toxicity and sub-optimal PK/PD profile. Additional obstacles that are also typical of other antibody-based therapeutic approaches are loss of target antigen on the cancer cells, receptor shedding and modification of the intracellular target molecule upon which the toxin acts (see Fig. 6.2). These limitations, and solutions that have been suggested or attempted to overcome them, are the subject of the following sections.

6.4 Immunogenicity of PE-based RITs and Solutions for Reducing it

Immunogenicity of protein therapeutics is presently considered a major obstacle to their successful clinical application. Immunogenicity is generally manifested by the appearance of anti-drug-antibodies (ADAs) and finding ways to "deimmunize" immunogenic biopharmaceutical (biologics) is a burgeoning field of study [49–52]. Therapeutic monoclonal antibodies, being the largest group of biologics, are no exception to that rule, and a lot of effort has and is still being invested in reducing their immunogenicity [53–56].The leading approach for reducing the immunogenicity of biologics involved the identification of B- or T-cell epitopes by computational or be experimental means (or combinations thereof) and eliminating amino acid residues that correspond to these epitopes while trying to maintain the activity of such "de-immunized" proteins [56–59].

The immunogenicity of PE-based RITs was recognized since the very early days of RIT development (see Fig. 6.3). This was in the early 1990s when the term "de-immunization" was net yet coined. Still, several studies were carried out to identify mouse, primate and human antibody epitopes of PE and PE-based RITs [60–62].

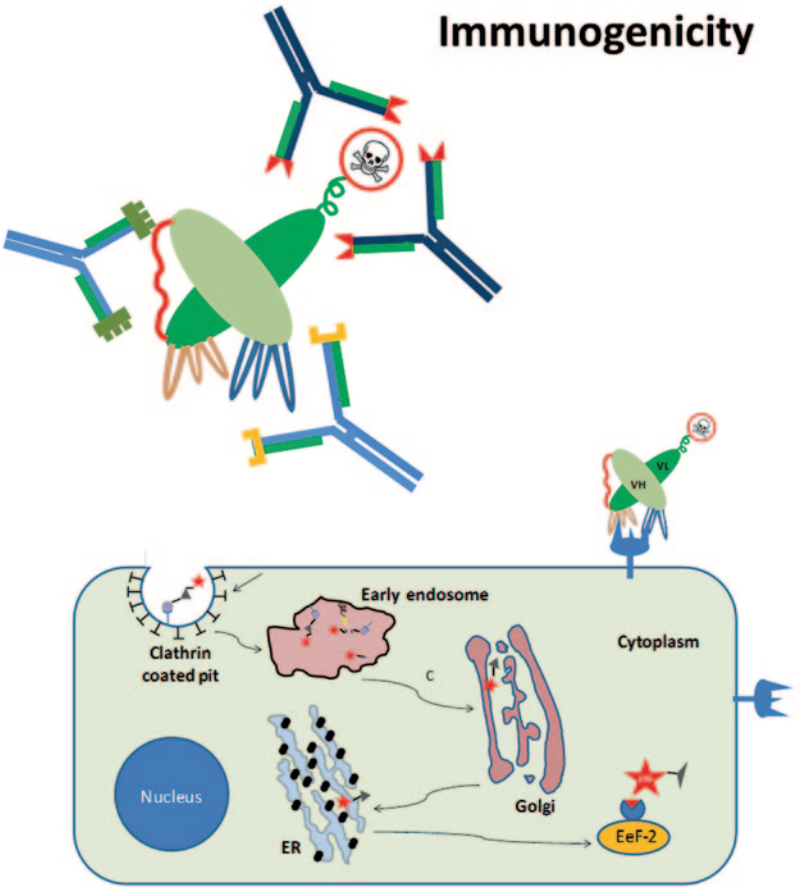

Fig. 6.3 Immunogenicity. When an IT is injected into an animal or human patient with an intact immune system, antibodies that recognize immunogenic epitopes on the targeting antibody and (primarily) on the toxin lead to IT neutralization upon repeated administration. This makes subsequent treatment not useful

Well before deimmunization of PE-based RITs was attempted, other approaches to reduce their immunogenicity were carried out. Initially, the attachment of polyethylene glycol (PEGylation) to PE and RITs was considered. PEGylation of biologicals as a means of modulating their PK/PD and/or their immunogenicity is known for over 30 years [63–65]. A number of studies were carried out to evaluate the effects of PEGylation on PE and RITs. Initially, detailed mapping of surface-exposed residues that can be mutated to cysteine (to facilitate chemical conjugated to PEG) on PE domains II and III were carried out [66, 67]. In these studies, a large number of residues were identified that could be mutated to cysteine with minimal loss of potency. Several PE cys domain III mutants that were conjugated to monomethoxy-polyethylene glycol (mPEG) via a disulfide or a thioether bond retained high cytotoxic activity. However, when a 20-kDa mPEG was used, there

was a decrease in cytotoxic activity with the disulfide-bonded molecules being more active. Positions 522 and 604 were good sites for PEGylation, but position 490 was not. The authors also found that PEGylation of PE 522C prolonged its in vivo circulation time in mice [67]. When PE domain II was studied, each of the five most exposed surface amino acids (H276, E282, N306, R313, and E327) were mutated to obtain PE-cys proteins that retained most of their cytotoxic activity. When the PE-cys proteins were conjugated with ovalbumin, using a cleavable disulfide linkage, cytotoxicity was retained, but it was lost with a non-cleavable thioether linkage. In contrast, cytotoxicity was maintained when PE-cys mutants were coupled to 5- or 20-kDa mPEG, using either a disulfide or a thioether linkage. Pharmacokinetic studies on one of the PEG-conjugated molecules (R313C) showed that the mean residence time ($t_{1/2}$) was prolonged to 72 min, compared to 20 min for un-PEGylatedPE-cys (R313C). The authors suggested that those studies showed that it is possible to derivatize PE at specific residues in domain II, maintain significant cytotoxic activity, and alter pharmacokinetics. Those studies also suggested that large mPEG molecules can be translocated to the cytosol while still attached to domain II of PE [66]. These two early studies of PE PEGylation did not evaluate the effect on immunogenicity.

Six years later, another study evaluated the effect of site-specific PEGylation, this time of a RIT. To make a PEGylated RIT with improved therapeutic properties, the authors prepared a mutant of anti-Tac (Fv)-PE38 (LMB-2). For site-specific PEGylation of LMB-2, one cysteine residue was introduced into the peptide connector (ASGCGPE) between the scFv and the toxin. This mutant LMB-2 (cys1-LMB-2), which retained full cytotoxic activity, was then site-specifically conjugated with 5 or 20 kDa of polyethylene glycol-maleimide. When it was compared with unmodified LMB-2, both PEGylated RITs showed similar cytotoxic activities in vitro, but superior stability at 37 °C in mouse serum, a 5- to 8-fold increase in plasma half-lives in mice, and a 3- to 4-fold increase in antitumor activity. This was accompanied by a substantial decrease in animal toxicity and immunogenicity [68].

The anti mesothelin RIT SS1P was also studied as a PEGylated RIT. The authors have modified this immunotoxin using several PEGylation strategies employing releasable linkages between the protein and the PEG polymers, and observed superior performance of these bioconjugates when compared to similar PEG derivatives bearing permanent linkages to the polymers. The releasable PEGylated RITs exhibited increased antitumor activity in A431-K5 xenografts in mice, with a diminished animal toxicity. Pharmacokinetic analysis of the releasable PEGylated derivatives in mice demonstrated an over 80-fold expansion of the area under the curve exposure of bioactive protein when compared to the un-modified (un-PEGylated) RIT [69].

To preserve potency, PEGylation of RITs by conjugating PEG to lysine residues should be site-specific and link the PEG to residues so that potency is not compromised. A mutant of PE with no lysine residues within PE38, designed for site-specific chemical conjugation was described in 1994 [70] (see also Sect. 5, below). However, in a RIT, the targeting antibody also contains lysine residues and accidental conjugation of PEG to some of them may compromise binding affinity.

Two studies looked at mutating lysine residues of scFvs that were used to target RITs. In the first study, the effect of chemical modification of lysine residues of the scFv of the anti Le^Y RIT B3 (Fv)-PE38 was carried out to study which of the scFv lysine residues could tolerate chemical modification while preserving biological activity. The authors found that derivatizing lysine residues of B3 (Fv)-PE38 causes a marked loss of specific target cell cytotoxicity and/or immunoreactivity. They also showed that two lysine residues in the antibody-combining region of B3 (Fv)-PE38 can be replaced with arginine residues, with only a small loss of cytotoxicity and no change in specificity. This mutant molecule is 3-fold more resistant to inactivation by derivatization with succinimidyl 4-(N-maleimidomethyl)cyclohexane 1-carboxylate (SMCC) or Bolton-Hunter reagent [71].

In the second study, to determine if a RIT could be produced with a diminished number of lysine residues so that chemical modification could be restricted to certain regions of the protein, the authors chose the RIT anti-Tac(dsFv)-PE38 that has 13 lysine residues in the Fv portion and 3 in the toxin. They prepared a series of mutants with 0–12 lysines in the Fv and 0 or 3 in the toxin. Almost all of these molecules retained full biological activity. Those data suggest that replacement of lysine residues can be achieve without loss of biological potency. These molecules could be a useful starting point to carry out site-specific PEGylation experiments of RITs [72].These studies demonstrated that chemical modification of RITs in general and their PEGylation in particular are very dependent on the antibody used for targeting, with some antibodies being more tolerant than others. As for PE itself, it can be chemically modified at a large number of positions with full preservation of potency. Presently, we are not aware of attempts to introduce PEGylated PE-based RITs into the clinic.

Regarding immunogenicity of PE-based RITs, it can originate from the targeting antibody, from the toxin or from artificial linkers used to connect together the parts of the molecule. PE itself has long been known to be a highly immunogenic protein and high titers of anti-PE binding, as well as neutralizing antibodies, were generated in rodents, primates and humans that were injected with RITs, following even a single injection [61, 73, 74]. Antibodies against the targeting Fvs also appeared in most cases, but their titers were much lower that the anti-toxin titers. In general, it is well documented that HAMA, human anti mouse antibodies that appear in humans injected with murine antibodies, are mostly directed at the antibody constant domains and less against the antibody variable domains.

There is a single study where a murine scFv used to target a RIT was humanized. In that study, the humanization of the scFv of the anti Le^Y RIT B3(Fv)-PE38 (LMB-7) by “framework exchange” was reported. The variable domains of the heavy (VH) and light (VL) chains were aligned with their best human homologs to identify framework residues that differ. Initially, 11 framework residues in VH and six in VL were changed by site-specific mutagenesis to human framework residues and introduced simultaneously into a preassembled single-chain Fv expression cassette. Six VH and five VL residues that differ were not changed because they were buried in the interdomain interface, or previously found to result in decreased affinity when mutated. As in many naïve initial attempts at antibody humanization [75], this

basic design resulted in some 20-fold loss of activity. To recover affinity, VL residues at the interdomain interfacial position 100 and at the buried position 104 were changed to the human sequence, which resulted in increasing the activity 8-fold. Changing the VH residue at position 82b from the human sequence back to that of the mouse restored the activity 2- to 3-fold to the full binding and cytotoxic activity of the mouse sequence. Humanized B3(Fv)-PE38 lost immunogenic epitopes recognized by sera from monkeys that had been immunized with B3(Fv)-PE38 [76].

Currently, no special effort is being invested in humanizing the scFvs or dsFvs that are used in clinical studies. The antibodies used to target LMB-2, SS1P, BL22 and HA22 are murine. As described below, most of the efforts for RIT deimmunization were focused on the toxin. We believe that now, when toxin deimmunization has met with apparent success, more attention will be directed at the targeting antibody, and future RITs, directed at new targets, will be based on humanized or fully human antibodies.

The largest strides toward reducing the immunogenicity with practical implications towards clinical development have been made during the past 10 years in a series of studies that were designed to identify and eliminate the immunogenic epitopes of PE itself. Because PE is of bacterial origin, it is highly immunogenic to animals and humans. As a result, all PE-based ITs are also highly immunogenic proteins. The recently conducted clinical trials include three RITs based on a 38-kDa fragment of PE, PE38 (Moxetumomab pasudotox, SS1P, and LMB-2) which is made up of domains II and III. Both domains II and III contain immunogenic B-cell epitopes [62]. Of note, over half of the patients with drug resistant HCL that were treated with BL22 achieved complete remission after 3–10 cycles of treatment [77]. However, patients having a normal immune system respond with production of anti-toxin antibodies after approximately 3 weeks, significantly limiting the number of cycles that could be applied for these patients. It should be noted that patients with leukemias and lymphomas (like in the case of drug resistant HCL) have a shattered immune system due to previous chemotherapy treatments, making their immune response to RITweaker than that of carcinoma patients. Destruction of immune cells by tumor cells infiltrating into the bone marrow also contributes to the weak immune system of such patients. However, to increase efficiency and expand immunotoxin therapy to other types of cancers, deimmunization is a necessary move to enable multiple cycles of treatment [58]. Clinical observations from patients treated with different derivatives of PE38 have shown that most of the antibodies were directed against PE38 and rarely to the Fv. It was understood that finding efficient ways to reduce the immunogenicity of PE38 have a crucial importance for clinical applications of PE38-based RITs in the future.

To briefly recount the order of events in the development of an anti-protein immune response: high affinity antibodies are produced and undergo affinity maturation in B cells. Initial antigen recognition happens between the antigen and a surface-displayed immunoglobulin, the B-cell receptor on pre-B cells. This is followed by internalization of the antigen, digestion to peptides and its further presentation on the surface by major histocompatibility complex class II. Next, specific helper T cells bind to those B cells that together promote class-switching, affinity

maturation and production of high affinity antibodies by mutual stimulation. This process is mediated by different intracellular signals and co-receptors, and occurs in secondary lymphoid organs, such as lymph nodes [78, 79]. It should be stressed that the location of B cell epitopes does not fully overlap with the locations of the T cell epitopes on an immunogenic protein [80].

Ira Pastan's group at the NIH has been working for about a decade exploring the field of PE38 deimmunization while applying several approaches. The leading approach that was evaluated first is based on identifying B-cell epitopes on the protein and their elimination by mutagenesis. One should bear in mind that to preserve killing abilities in mutated RITs, several features of the toxin should be preserved: (1) cleavage by furin, (2) translocation to the ER, (3) translocation from ER to cytosol, (4) binding to NAD, (5) binding to EF2 and (6) transfer of ADP ribose to EF2. Onda et al. performed a series of studies aimed to deimmunize the anti CD22 RIT HA22. These studies combined structural and functional analyses with identification and removal of B-cell epitopes. First, they isolated a panel of anti-PE38 mouse mAbs and categorized them into seven major epitope groups and 13 subgroups. Their working hypothesis was that changing large, surface-exposed, hydrophilic residues that are commonly involved in antibody binding, such as arginine, lysine, glutamine, and glutamate, to smaller residues such as alanine, glycine, or serine will reduce or eliminate the antibody reactivity with the mutated RIT. The derived RIT mutants were found to have a reduced immunogenicity in mice and retained antitumor activity [81]. Subsequently, in a study aimed at stabilizing the toxin by eliminating protease cleavage sites (carried out on SS1P, an anti-mesothelin RIT see Sect. 5, below), a large part of domain II was removed, resulting in the RITHA22-LR; HA22-lisosomal resistant). Since many B-cell epitopes are located in domain II, HA22-LR hada diminished immunogenicity compared to the parental molecule HA22-PE38. [10, 62, 81, 82]. In the more recent studies, the authors performed a detailed bioinformatic analysis and revealed a small number of discrete, putative B-cell epitopes that may be important for antibody recognition and are all located on the surface of domain III. Subsequent 8 point mutations enabled to abolish the identified epitopes. The obtained deimmunized HA22-LR-8M did not induce a primary or secondary response when repeatedly injected intravenously into mice, yet retained excellent cell killing of $CD22^+$ cells and antitumor activity in a mouse xenograft model. Since (until such RITs will enter clinical evaluation) it was not possible to evaluate the immunogenicity in humans, an antigenicity study that is closely related was done instead (binding of HA22-LR-8M to preexisting anti PE38 antibodies that were obtained from patients that were treated with RITsLMB-9 or SS1P during their clinical evaluation as a surrogate measure of the immune response [62]). HA22-LR-8M demonstrated consistently lower antigenicity than HA22-PE38 with patient serum samples in the competition assay as well as with mouse anti PE38 antibodies, hinting that mice and humans may share some B-cell epitopes [83].

To further reduce the immunogenicity of PE-based RITs, despite the fact that the previously obtained HA22-LR-8M with eliminated major mouse B-cell epitopes demonstrated no significant response with human serum, it was obvious that since not all human and mouse epitopes are identical additional human B-cell epitopes

need to be identified and eliminated. Liu et al. focused on the production of further improved variants of HA22-LR by identifying and silencing additional human B-cell epitopes. The authors constructed a phage-display library containing Fvs that react with the native PE38 toxin. RITs were then point mutated to locate B-cell epitopes reactive with these phage-displayed human Fvs. Using this approach, six human B-cell epitopes were identified in domain III. These epitopes were eliminated by mutations, and the best clone, HA22-LR-LO10. had a total of seven point mutations in domain III, had much better cytotoxic activity in vitro (when tested for killing $CD22^+$ lymphoma cell lines) compared to its predecessor HA22-LR, and same anti-tumor activity in an in vivo mouse xenograft model in which CA46 Burkitt's lymphoma cells were implanted subcutaneously. Antigenicity was measured by a competition assay and showed that binding of HA22-LR-LO10by sera of 22 out of 25 patients was reduced up to 10.000-fold.

SS1P, anti-mesothelin RIT, was a subject for additional attempts to deimmunize PE-based RITs. The clinical utility of SS1P is limited by inducing an immune response and by causing dose-limiting capillary leak syndrome (CLS) in patients. With SS1P treated patients that immunogenicity problem is more acute than HA22 treated patients as SS1P patients are not immunosuppressed, thus, they all mount an antibody response to the RIT already following the first administration. Weldon et al. tried to overcome these obstacles by redesigning the SS1P molecule. The authors implemented earlier observations that were made during the development of the much less immunogenic variant HA22-LR, where two major mouse B-cell epitope groups and antigen processing sites were removed from PE38, while in vitro activity on patients' CLL cells was dramatically increased [62, 82]. When those changes were introduced into SS1P, the LR-adapted SS1P, named SS1-LR/GGS/8M, carried the following modifications: (1) domain II was removed; (2) a Gly–Gly–Ser short peptide linker was added after the furin cleavage site; and (3) eight highly solvent exposed residues were replaced in the catalytic domain III of PE. SS1-LR/GGS/8M had significantly improved properties compared to its parental SS1P, had increased anti-tumor activity, and could be given in much higher doses to rats and mice without production of toxic side effects. Its immunogenicity was diminished greatly, suggested by lowered reactivity with human anti-sera against SS1P [84].

Taking into account the pivotal role of T cells for the immune response and the formation of neutralizing antibodies in particular, it was hypothesized that removal of T-cell epitopes may be also required to better meet the challenge of reducing the immunogenicity of PE-based RITs. Following this line of reasoning, Mazor et. al conducted a study that investigated $CD4^+$ T-cell epitopes in PE38 and used these data to produce RITs that do not stimulate T-cell responses in a majority of human donors. To identify peptides that result in T-cell activation, donor peripheral blood mononuclear cells (PBMCs) were incubated with RIT for initial stimulation, in order to allow processing of the RIT by antigen-presenting cells (APCs) and further presentation of its peptides to T cells. Then, these activated T cells were exposed to overlapping synthetic peptides corresponding to the PE38 sequence. T-cell response was measured by ELISpot assay for IL-2 secretion. The result was that samples from all 50 healthy donors (that had never been exposed to PE38)

responded to at least one peptide. This promiscuous HLA class II DRB1-restricted highly immunodominant epitope was found in 46% (23/50) of the donors with different HLA alleles and located in domain II of PE. Alanine-scanning mutagenesis revealed two amino acids that were found responsible for establishing of this T-cell immunodominant epitope, and their deletion/alanine exchange yielded elimination of this epitope. Subsequently, mutations in discovered positions were introduced into HA22. The obtained HA22- L297A and HA22-Y298A mutants were evaluated on four $CD22^+$ cell lines, showing a small decrease in cytotoxicity compared to the parental HA22 RIT. Novel mutant derivatives preserved the RIT stability and did not stimulate a T-cell response as was shown by in vitro expansion with whole protein (not immunogenic in 34% of donors and less immunogenic in an additional 42% of the donors). To rule out the possibility that by mutating PE new T-cell epitopes were generated, alanine mutagenesis was used to reduce the binding of peptides to HLA molecules. The authors discussed their plans to continue effort to find epitopes in domain III as well and combine them with already achieved deletions in domain II. This, they suggested, may yield a RIT with very low immunogenicity characteristics to apply in humans with a normal immune system [85].

Recently, the same approach was applied to prepare a further deimmunized HA22 RIT. HA22-PE38 (also known as Moxetumomab Pasudotox) is currently undergoing phase III clinical trials for the treatment of refractory hairy-cell leukemia (http://www.clinicaltrials.gov/ct2/show/NCT01829711?term=Moxetumomab+Pasudotox&rank=1). To perform a high-resolution mapping of the T-cell epitopes on PE38, Mazor et al. applied the same approach described in the previous paragraph [85]. It enabled the discovery of seven additional T-cell epitopes located on the domain III, additionally to the already known immunodominant epitope on the domain II. This knowledge was used to construct an RIT named LMB-T18 based on the HA22-LR scaffold (that lacks domain II) with six additional point mutations in domain III (R505A, R494A, L477H, R427A, L552E, and F443A) and incorporated a GGS peptide linker after the furin cleavage site. The authors found that the cell killing efficiency of the mutated RIT, evaluated in vitro in four $CD22^+$ cell lines, proved to bevery potent (IC_{50}s less than 10 pM). In vivo evaluations were carried out using SCID mice implanted with lymphoma cell xenografts showing complete remissions. LMB-T18 was also evaluated for killing cells freshly isolated from seven HCL and six CLL patients and found that it was extremely active. The T-cell response had a decrease of 90% compared with HA22-PE38 (tested with PBMCs from naive donors). The authors concluded that the next logical step wouldbe to produce RITs with combined B-cells and T-cells eliminated/mutated epitopes [86].

The RIT deimmunization studies described in this section involved bioinformatics tools as well as experimentation. In the B-cell epitope elimination campaign, the bioinformatics part was restricted mainly to using the 3D structure of PE to identify the putative surface-exposed residues that may be a part of the B-cell epitopes. There are no generally accepted algorithms for predicting B-cell epitopes. The situation is different with regard to predicting T-cell epitopes. One such approach was recently demonstrated in a report by King et al. who described a computational protein design method that can predict T-cell epitopes and maximize the content of human

peptide sequences without affecting protein stability. This method incorporates host genome information and MHC-binding prediction tools. Interestingly, when applied to predict T-cell epitopes of PE38, mutations that were predicted by the theoretical method partially matched the mutations observed in previous deimmunization experiments of Mazor and colleagues. To experimentally verify the computational predictions, the approach was applied on superfolderGFP and on PE38, resulting in successful prediction and elimination of known immunodominant T-cell epitopes. The work was focused only on eliminating the most immunoreactive epitopes for a given set of MHC alleles, therefore, to cover a breadth of HLA allotype diversity and testing of a larger number of patients should be tested [87].

To conclude this section, immunogenicity of PE-based immunotoxins, arguably the largest hurdle for their progress to clinical approval, has been addressed in a most impressive tour-de-force of epitope identification and elimination. One should also be at awe to the remarkable robustness of the PE protein that can tolerate so much "abuse"; deletion of large parts and many combined point mutations and still retain potency. We are confident that these, what we shall perhaps call "fifth generation PE-based RITs", will soon enter clinical studies. Such deimmunized RITs should be more effective in cancer treatment because more treatment cycles can be given.

6.5 Limited Stability of PE-Based RITs and How it Was Overcome by Antibody and Toxin Engineering

When the first third generation PE-based RITs were constructed they were based on single-chain antibody fragments (scFvs) fused to PE38. It soon became clear that scFv-based RITs suffer from limited stability, with a tendency to aggregate in solution fairly rapidly. It was quite clear that PE itself was not toblame, since recombinant forms of PE were already produced and had shown excellent stability. It was also known that scFvs are in general un-stable antibody fragments. Solutions for stabilizing Fv fragments were offered by the group of Andreas Plückthun already in 1990 [88]. In that publication, the authors compared how well the Fv of the mAb McPC603 can be stabilized by chemical crosslinking, by a peptide linker (as a scFv) or by the introduction of an artificial disulfide bond facilitated by mutating VH-VL interfacial residues to cysteines. In that study, disulfide stabilization proved to be very efficient in improving stability. However, the disulfide-stabilized McPC603-derived Fvs suffered from some loss in binding affinity.

About two years later, a "disulfide stabilization" campaign was initiated by Ira Pastan at NIH and collaborators. They undertook a systematic analysis of antibody structural analysis for designing the positions of the introduced cysteine mutations to fulfill in particular two major criteria: (1) that the selected positions would be universal interfacial positions, with a Cα-Cα distance suitable for the formation of the artificial disulfide bond, and (2) that the engineered disulfide bond would be distal from the CDR loops, so the binding affinity would not be compromised.

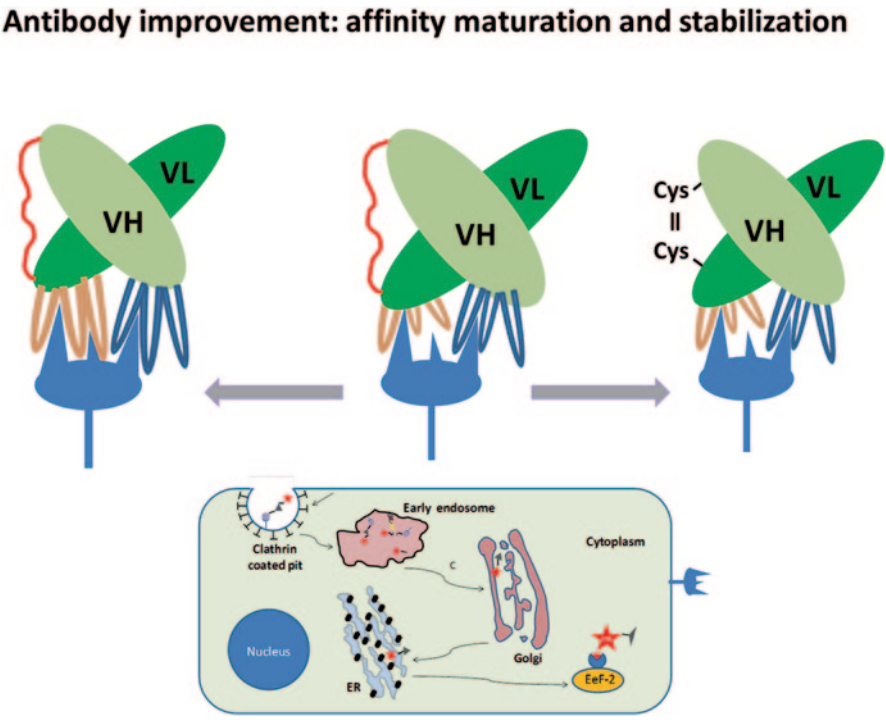

Fig. 6.4 Affinity maturation and disulfide stabilization. Affinity maturation of the targeting antibody (simulated on the *left* by increasing contact complementarity with the antigen) contributes to higher affinity, leading to increased potency. Stabilization of the targeting Fv (disulfide stabilization, simulated on the *right*) also contributes to improved RIT efficacy

The first article describing disulfide-stabilized Fvs (that became known as dsFvs, see Fig. 6.4) in the context of RITs was published in 1993 [89]. A series of articles followed, further evaluating several pairs of interfacial positions for disulfide stabilization, and testing the generality of the approach by constructing and evaluating RITs based on different antibodies [31, 90–96]. In those studies, it was found that all the tested antibodies were more stable as dsFvs in comparison to the corresponding scFvs, and that in almost all cases the affinity had not been compromised. As a result, disulfide stabilization became the "norm" for antibody fragments used as targeting moieties of RITs. In fact, all the PE-based RITs that were clinically evaluated since the mid-1990s, which include BL22, HA22 and SS1P (LMB-2 is the only one still based on a scFv) are all dsFv-based RITs [77, 97–99]. An additional RIT that has been evaluated (mostly preclinically, but soon to be tested in patients) as asdFv-PE38 fusion protein is MR1–1 [100], directed against a mutant form of the EGF receptor.

Fab fragments are universally known to be more stable than scFvs. A few Fab-based immunotoxins have been produced and evaluated pre-clinically [101, 102]. When third generation PE-based RITs were first constructed, their size was kept to a minimum to facilitate improved tumor penetration. Hence, scFvs and later dsFvs

were the targeting molecules of choice. More recently, smaller derivatives of PE were developed as part of the effort to deimmunize PE and to make it more resistant to proteolysis [10]. While sdFv-PE38 RITs are about 63 kDa in size (about the kidney infiltration size), the smaller RITs are cleared more rapidly from the circulation. Thus, their size is sub-optimal and will likely be re-increased by using Fabs to target them instead of dsFvs.

PE itself is a very robust protein with exceptional stability and solubility. This can be appreciated from one of the studies where the stability of scFvs and dsFvs was compared. In that study by Reiter et al., the stability of scFvs and dsFvs was compared by incubating them at temperatures from 25 ºC to 50 ºC or in the presence of increasing concentrations from 0–8 molar of the denaturing agent urea and measuring residual activity following the incubation. PE itself was included as a control. The results that were obtained clearly showed the increase stability of dsFvs compared to the corresponding scFvs. Still, even the more stable dsFvs were inactivated at some point while PE itself remained fully active even at the harshest tested conditions [94].

Nevertheless, PE underwent many modifications to make the derivatives suitable for particular challenges. Early studies compared the potency of PE derivatives in which the C-terminal REDLK ER retrieval sequence was changed to other sequences, including the "canonical ER retrieval sequence" KDEL, and their potencies in cell killing were compared. ER retrieval is a key step in the retrograde transport that PE (and PE-based RITs) undergoes on the route from receptor binding to cytosolic localization. This is because PE molecules that do not enter the pathway leading to the ER are delivered to the lysosome and destroyed. In such studies, it was found that PE and RITs that have the C-terminal sequence KDEL are more potent (by about 10 fold) than the REDLK, REDL of RDEL sequences [103, 104]. However, such toxins had a much higher liver toxicity in mice and, therefore, a potentially reduced therapeutic window. As a result, RITs that progressed to clinical development carry the wild-type C-terminal sequence.

An additional useful modification of PE was the removal of all the lysine residues in PE38 to make it (after the addition of an N-terminal peptide that contains a single lysine residue) most suitable for site-specific lysine-directed chemical conjugation. A few derivatives were made, like PE38QQR and PE38QQΔ, in which lysine at PE positions 590 and 606 were mutated to glutamine and the C-terminal lysine was either mutated to arginine (QQR) or deleted (QQΔ) [70].

As described above, during intracellular trafficking, PE and RITs can berouted to a "productive" route ending in the lysosome or a "destructive" route ending in lysosomal degradation. Several studies evaluated the possibility of producing PE derivatives that are less susceptible to lysosomal degradation. Notably, studies by Weldon et al. were focused on mapping residues within PE38 that, when mutated or deleted, reduce the extent of lysosomal degradation. The authors have investigated the proteolytic susceptibility of PE38 immunotoxins to lysosomal proteases and found that cleavage sites were clustered within a limited segment of PE38. Specifically, the lysosomal protease cleavage sites occurred between residues 260–261, 265–266, 297–298, 341–342, 342–343, 351- 352, 352–353, 353–354, 364–381, 390–391, and 391–392. All these clustersare located within domains II and Ib. Subsequently, RIT

deletion mutants were generated in this region using HA22, an anti-CD22 Fv-PE38 RIT currently undergoing clinical trials for B-cell malignancies. One of these mutants, HA22-LR (for "lysosome resistant), lacked all the identified cleavage sites (essentially most of domain II, leaving the "furin cleavage loop"), was resistant to lysosomal degradation, and retained excellent biological activity. HA22-LR killed CLL cells more potently and uniformly than did HA22, suggesting that lysosomal protease digestion may limit immunotoxin efficacy unless the susceptible domain is eliminated. Finally, a remarkable observation that was made during the study is that mice tolerated doses of HA22-LR at least 10-fold higher than lethal doses of HA22, and these higher doses exhibited markedly enhanced antitumor activity. The authors concluded that HA22-LR advances the therapeutic efficacy of HA22 by using an approach that may be applicable to other PE-based immunotoxins [10]. It is expected that PE-LR will be the toxin-of-choice for the RITs that will be developed in the future.

A bonus that resulted from the generation of HA22-LR is, as described above in the section discussing immunogenicity, that many B- and T-cell epitopes of PE are mapped to domain II. Thus, the LR version of PE-based RITs is inherently less immunogenic than PE38 [86].

In a follow-up study, Liu et al. tested the hypothesis that increased stability may result in reduced immunogenicity of RITs. The authors introduced a disulfide bond into domain III by identifying and mutating two structurally adjacent residues to cysteines at sites suggested by computer modeling. This RIT, HA22-LR-DB, displayed a remarkable increase in thermal stability and an enhanced resistance to trypsin degradation. In addition, HA22-LR-DB retained cytotoxic and anti-tumor activity, while exhibiting significantly lower immunogenicity in mice [105].

6.6 Insufficient Potency and Combining Therapies to Enhance Potency

In oncology, drug combinations are the mainstay of therapeutic intervention. One of the major potential strategies for the problem of insufficient potency is by combination of RITs with agents that enhance cell killing. In general, in cancer treatment, synergistic effects caused by combination therapies usually considered to be more effective comparing to single agents.

A case-in-point of overcoming insufficient potency can be demonstrated in the case of the anti-mesothelin RIT SS1P [SS1(dsFv)PE38]. SS1P is currently evaluated clinically in patients with mesothelin positive tumors. Despite successful phase I clinical trials, recent studies show that SS1P alone is limited in its efficiency [99, 106]. In fact, the major obstacle for treating patients with SS1P is the immunogenicity of PE, which was discussed above in Sect. 4. Still, these observations led scientists to examine SS1P in combination with other agents to improve its efficiency against different tumors. So far, such combination therapy studies were carried out mostly in vitro and in animal models.

In one study, SS1P was evaluated in combination with taxol (Paclitaxel, a drug whose action involves stabilization of cellular microtubules. As a result, it interferes with the normal breakdown of microtubules during cell division). Immuno-deficient mice were implanted with A431/K5 tumors expressing mesothelin antigen and were treated with SS1P alone, taxol alone, or the two agents together. The results showed that the combination treatment had a strong synergistic anti-tumor effect in the mice, but not in vitro [107]. An additionalstudy by the same group investigated the mechanism of synergy and comparedtaxol-sensitive and taxol-resistant KB tumors (both equally sensitive to SS1P alone). It turned out that KB tumors have high levels of shed mesothelin in their extracellular space (receptor shedding, see Fig. 6.5). Taxol treatment significantly lowered shed mesothelin levels in drug-sensitive but not in the drug-resistant KB tumors. The shed form of mesothelin antigen competes with the membrane form of the antigen for binding of SS1P, and as a result decreases its anti-tumor activity. Taxol-induced reduction in shed antigen levels can explain the synergy of immunotoxin and taxol in taxol-sensitive tumors and lack of synergy in taxol-resistant tumors [108].

An additional study focused on studying the effect of modulating the concentration of shed antigen on RIT potency was carried out using LMB-2. While RITs have been shown with high effectiveness in malignancies where most of the tumor burden is suspended in the peripheral blood or spleen (like in case of HCL), they are very limited in their effectiveness against aggressive solid tumors [109].This study evaluated the potential to obtain a synergistic effect of LMB-2combined with the chemotherapeutic agent gemcitabine (a nucleoside analog) in case of ATL. LMB-2 was previously evaluated in patients with relapsed and refractory hematologic

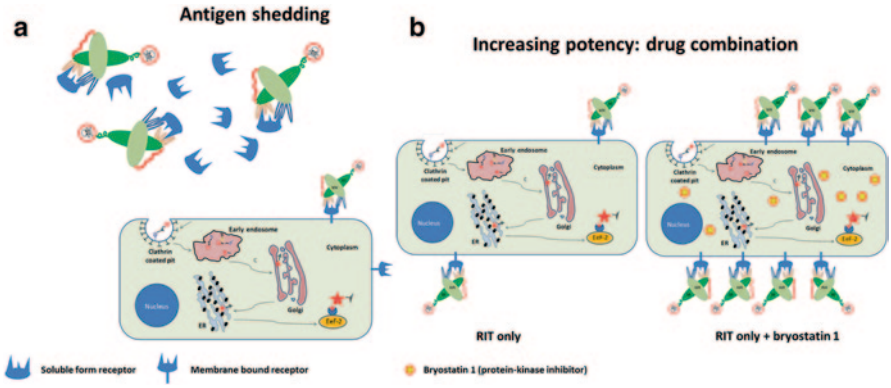

Fig. 6.5 Increasing potency by drug combination. **a** Many cell surface receptors, including RIT targets, shed the extracellular domain to the tumor interstitium and on to the circulation. The shed form of the RIT target competes with the membrane form for binding of the RIT, and as a result decreases its anti-tumor activity. In one example described in the text, when the anti-mesothelin RIT SS1P was combined with taxol, the taxol treatment significantly lowered shed mesothelin levels in drug-sensitive but not in the drug-resistant KB tumors. **b** A drug combination may increase the antigen density on the target cells, as is described in the case where BL22 RIT was combined with bryostatin 1, which led to an increase of CD22 expression on difficult-to-treat target cells, resulting in their becoming sensitive to RIT treatment

malignancies with most promising results obtained in Hodgkin's lymphoma, CTCL, HCL, CLL and ATL [98, 110]. However, its clinical benefits were limited because of immunogenicity and rapid disease progression, particularly in ATL. The authors proposed that in the case of solid tumors, the presence of high concentrations of soluble target antigen CD25 (sCD25) may potentially block the circulating LMB-2 and lower the effective concentration that can bind the tumor cells. To study if this is indeed the case, the levels of sCD25 in $CD25^+$ ATAC-4 tumor xenografts in nude mice were measured before and after administration of gemcitabine and then determined whether gemcitabine and LMB-2 would show in vivo or in vitro synergy. It was found that levels of interstitial sCD25 within the tumors were higher by100-fold than in the serum and that gemcitabine could reduce them by 10-fold. Additionally, a synergistic antitumor activity in vivo was shown by combination of gemcitabine and LMB-2, while in vitro their combined effect was only additive [109]. These two studies highlight the obstacle placed by the presence of soluble target proteins that compete with the tumor cells for RIT binding and the importance of treatments that reduce the concentration of soluble targets on potentiating RITs.

Another study of combination therapy involved mesothelin-expressing pancreatic cancers that are known to be resistant to most of the chemotherapeutic agents. The SS1P RIT inhibited protein synthesis in two of the pancreatic cancer cell lines, but did not significantly affect cell death. The resistance to RIT treatment was contributed to low levels of the pro-apoptotic protein Bak. The authors demonstrated that combining TRAIL or an anti-TRAIL receptor 2 agonist antibody, HGS-ETR2, with SS1P caused an effective synergistic effect leading to cell death and reduction of tumor size in xenograft-bearing nude mice [111].In yet another study, SS1P was applied in combination with several activated protein kinase (PKC) inhibitors. PKC enzymes contribute to cells survival, proliferation and angiogenesis and this is why the therapeutic application of PKC inhibitors are considered a potential strategy to improve cancer treatment [112]. Enzastaurin, but not two other tested PKCs in the study, has shown significant enhancement of SS1Pimmunotoxin action in combination treatment of cells that exhibited partial resistance to SS1P alone. Reductions of ATP levels, caspase activation and loss of attachment from culture dishes finally resulted in apoptotic cell death. This synergistic effect was concentration-dependent in the range of 4–10 μM enzastaurin and showed a 10-fold enhancement of immunotoxin action of KLM1 cells [113]. This combination treatment resulted in greater general reductions in protein synthesis and even in the complete loss of activation of caspases 3 and 7, and of several proteins, such as Mcl-1, Bcl2, AKT, considered pivotal in many immunotoxin-cell death models. The nature of this additional inhibitory action and the contribution of each component are not fully clear. However, the authors note that like many other kinase inhibitors, enzastaurin is known to be a multi-kinase inhibitor (not sufficiently specific) and it will be a problem to achieve required-for-treatment concentrations in patients with mesothelin-positive tumors. They suggested that looking for more specific kinase inhibitors that produce synergistic effects with RITs is a challenge for future studies.

In a recent study, the authors hypothesized that protein tyrosine kinases may have important roles in affecting their susceptibility of cancer cells to RITs. Tyrosine

kinases are known to be major players of protein phosphorylation within cells, and are hyperactivated during cancer processes. The anti mesothelin RIT SS1P and the anti CD22 RIT and HA22 (moxetumomab pasudotox) were chosen for the study. To examine their hypothesis, the authors used siRNAs to knock down the expression of 88 known tyrosine kinases in cancer cells while testing their response to SS1P or HA22. Only five of the siRNAs (that knocked down the expression of *INSR*, *HCK*, *SRC*, *PDGFRβ*and *BMX*) were found to enhance the activity of SS1P. Further investigation of the enhancement mechanism showed that *HCK* knockdown stimulated SS1P processing by furin-mediated cleavage, lowered levels of the anti-apoptotic protein Mcl-1 and raised the expression level of the pro-apoptotic protein Bax. Additionally, the authors demonstrated that *SRC* family inhibitors could mimic the effect of tyrosine kinase knockdown, resulting in a significant increase of SS1P and HA22 killing activity in A431/H9 and CA46 cells, respectively. One of these SRC inhibitors, SU6656 has been successfully evaluated in mouse xenograft tumor models, where it demonstrated a synergistic antitumor effect with both SS1P and with HA22 [114].Another recent study looked for additional agents that could induce an effective cell apoptosis of pancreatic ductal adenocarcinoma (PDAC) cell lines that are resistant to SS1P despite high mesothelin expression. In that study, SS1P was combined with the BH3-mimetic ABT-737 (which selectively targets and neutralizes three BCL2 family pro-survival proteins BCL-XL, BCL-2, and BCL-W, but now Mcl-1 [115]). The combination led to a significant increase in cell death, while the studied cells lines were resistant to each component alone. The effect had a variable extent in different cell lines (KLM-1, BxPc-3, Panc 3.014). The authors showed that RIT-mediated protein synthesis inhibition and the capability to down regulate Mcl-1 and Bcl2A1 were major factors that affected the efficacy of the combination treatment [116].

The following study is a case in point where drug combination was used to expand the utility of an RIT for treating additional malignancies than originally intended. BL22 is an RIT targeting CD22 molecules presented on the surface of certain B-cell malignancies such as lymphoma and leukemia [117, 118]. BL22 has shown significant therapeutic potency in patients with HCL [119], but failed in the treatment of less indolent leukemias and lymphomas [77], particularly CLL. This failure was apparently due to lower expression of the target CD22 on the surface of tumor cells of the unresponsive malignancies. In a recent study, it was found that pre-activation of primary CLL cells with the macrocyclic lactone bryostatin 1 (a potent modulator of protein kinase isolated from the marine bryozoan *Bugulaneritina*) overcame this issue. Primary CLL cells that were treated with bryostatin 1 followed by BL22 treatment showed significant induction of apoptosis. It turned out that bryostatin 1 works in two distinct pathways: (1) it strongly upregulates the surface expression of CD22 receptors on leukemic cells that cause a "hairy cell phenotype" in CLL cells, and (2) it depletes protein kinase C-β2. Additionally to CLL cells, the authors showed that BL22 and bryostatin 1 combined treatment exerts a strong apoptotic effect in large B-cell lymphomas and mantle cell lymphoma cells. The authors concluded that this drug synergism should be tested in vivo to evaluate if it can be a feasible therapeutic approach for CLL and B-cell malignancies [48].

6.7 Potentiation of RITs by Affinity Maturation of the Targeting Antibody

The antigen binding affinity of antibodies is one of the key factors contributing to-their therapeutic efficiency. In vitro affinity maturation of therapeutic antibodies is a commonly applied practice in their clinical development (see Fig. 6.4) [120–124].

Accordingly, affinity maturation of the targeting antibodies used to construct RITs may significantly improve their antitumor activity. There are several strategies for in vitro affinity maturation of antibodies. These include "non-targeted within the V-gene" approaches such as error-prone PCR, mutator *E. coli* strains, chain shuffling and DNA shuffling and "targeted within the V-gene approaches" such as CDR randomization, CDR walking, hotspot mutagenesis, PCR-based mutagenesis, parsimonious mutagenesis and saturation mutagenesis. Each approach has its own advantages and disadvantages. The affinity maturation approach introduces sequence diversity into the antibody genes, creating a repertoire of mutants derived from the original antibody. One of several display technologies is then applied to isolate the highest affinity clones for further study [125, 126].

Affinity maturation of an scFv as a part of a RIT was first reported in 1999. In that report, Chowdhury and Pastan reported the affinity maturation of the anti mesothelin scFv SS1. The process that was applied was called "mimicking somatic hypermutation *in vitro*", which may be classified as a "targeted within the V-gene" approach. In that study, DNA sequences were identified in the antibody variable domains that are naturally prone to hypermutations (as evident from the fact that they are frequently mutated during the natural in vivo affinity maturation process that antibodies undergo). The authors selected a few hot spots encoding non-conserved amino acids, and introduced random mutations to make libraries with a size of under 10^4 independent clones. Affinity selection of the hot spot libraries by phage display yielded several mutants with a 15- to 55-fold increase in affinity [32]. The best affinity matured clone later became the dsFv used to target the SS1P RIT [99].

Another study focused on the improvement of the anti-CD22 RIT BL22 for B-cell malignancies. In the previous section, it was already mentioned that BL22 was much less effective against CLL compared to HCL. Thus, to improve its affinity, hot spot mutagenesis combined with phage display using CD22-positive Daudi cells for affinity selection was carried out. The best affinity improved clone contained mutations in HCDR3, specifically amino acid residues Thr-His-Trp (THW) in place of Ser-Ser-Tyr (SSY) at positions 100, 100A, and 100B of the Fv and had an affinity improved from 85 nM to 6 nM. The THW mutant (that was named HA22) had a 5- to 10-fold increase in activity on various CD22-positive cell lines and was up to 50 times more cytotoxic to cells from patients with CLL and HCL [97, 127]. Later, in order to achieve a more productive intracellular trafficking and reduced immunogenicity (described above), most of the PE domain II was deleted, resulting in an RIT named HA22-LR. HA22LR was much more effective than HA22-PE38 in killing CLL cells, showing less general toxicity. In an attempt to further improve the affinity of HA22, Kawa et al. applied a different approach instead of the hotspot

mutagenesis that was used in HA22 evolution. In that case, a different antibody fragment format was used, consisting of an scFv further stabilized byan artificial disulfide bond (according to the dsFv approach). The resulting HA22 (scds) Fv-LR had the same cytotoxicity as that of HA22 (dsFv) LR. In order to identify functionally important residues for antigen binding, alanine scanning mutagenesis was implemented. Single asparagine to alanine exchange (N34A) in VL CDR1 resulted in a substantial increase in affinity and activity. Cell viability assays showed that the N34A mutanthad a 10-fold improvement in activity toward CD22-positive cell lines. The authors suggested that this may constitute a clinical benefit with a lower dose and in turn lead to a decrease in nonspecific toxicity in patients [128].

Recently, Kuan et al. isolated a high-affinity scFv against the glycoprotein NMB and showed how affinity maturation can improve its potency as a targeting antibody of an RIT. The glycoprotein NMB (GPNMB) is a transmembrane glycoprotein specifically associated and overexpressed in malignant gliomas, such as common primary adult brain tumor glioblastoma multiforme (GBM). The median survival after diagnosis of GBM is 14 months [129]. Glycoprotein NMB and other antigens presented in GBM are promising targets for immunotherapeutic cancer treatments. Initially, the anti-GPNMB scFv (G49) isolated from a human synthetic phage display library, showed high affinity to GPNMB-expressing cells and once converted to an RIT form was cytotoxic to GPNMB-positive glioma cells. The authors conducted in vitro affinity maturation (by a random mutagenesis approach) and introduced mutations into CDR3 of the light chain and CDR1 of the heavy chain. This introduction of sequence diversity combined with phage display made it possible to isolate a mutant scFv (902V) with an 11-fold increase in affinity. Clone 902V was further improved by sequence randomization throughout the whole scFv by error-prone PCR, and one mutant, F6V, was selected by yeast-surface display. Finally, this affinity-matured scFv was fused with PE38. The obtained F6V-PE38 targets HGGs, medulloblastomas, and melanomas; it has shown significant activity in in vitro cell-killing assays and in vivo models of GPNMB-expressing xenografts in nude mice [130].

The most recent study involved other common cell surface targets for glioblastoma; gangliosides 3′-isoLM1 and 3′,6′-isoLD1. Gangliosides are a group of sialic acid-containing glycosphingolipids with extending extracellular section. They are overxpressed in over 60% of glioblastomas, making them attractive therapeutic targets for brain malignancies [131]. Piao et al. developed a novel RIT, DmAb14m-(scFv)-PE38KDEL (DmAb14m-IT) that specifically binds these gangliosides. First, the authors isolated ganglioside-targeting mAbs as murine hybridomas. Next, they cloned DmAb14 that exhibited high reactivity towards both 3′-isoLM1 and 3′,6′-isoLD1. Since the original hybridoma was of the IgM isotype, which is not ideal for fusion with ITs, cloning of its VH and VL domainsas a scFv was carried out. Subsequently, the DmAb14-scFvs were subjected to in vitro affinity maturation by CDR hotspot random mutagenesis combined with phage display. The best resulting clone was fused with PE38KDEL (PE38 that carries KDEL sequence at C-terminus to improve ER retrieval, leading to RIT potentiation). In vitro evaluation of the resultantRIT showed significantly improved abilities compared to the parental

molecule: increased cytotoxicity, better cell internalization and better affinity to the ganglioside targets. It showed potent killing activity on glioma cell lines and tumor xenograft-derived cells expressing 3′-isoLM1 and 3′,6′-isoLD1 [132].

6.8 Reducing Off-Target Toxicity and Overcoming Physical Barriers

In general, RIT dose-limiting toxicity is regarded in most cases as a part of "off-target" toxicity, resulting from interactions of the RIT with cells and tissues other than the target malignant cells. Regarding PE-based RITs, in the pre-clinical studies the dose-limiting toxicity in mice of PE and RITs was mostly liver toxicity [10, 133, 134]. Interestingly, it has been reported that the non-specific mouse liver toxicity of PE-based RITs can be significantly reduced by engineering the targeting Fvs to lower the isoelectric point of the Fv. The authors hypothesized that this may be due to modified pharmacokinetics of the differently-charged RITs, however, this has not been established experimentally. An additional modification of the PE component of RITs that reduced mouse toxicity was the deletion of most of the domain II in the context of HA22-LR (10-fold lower than HA22-PE38) [10]. The authors suggested that the observations that were made during the studies of lowering the toxicity by lowering the pI of the targeting Fvs probably did not account for the difference between HA22 and HA22-LR. This is because HA22-PE38 and HA22-LR have an identical Fv and the pI of HA22-LR is slightly increased relative to the pI of HA22 ($pI_{HA22\text{-}PE38} = 5.26$ and $pI_{HA22\text{-}LR} = 5.63$). In addition, the 2- to 3-fold difference in toxicity observed for this pI lowering strategy is also much smaller than the more than 10-fold difference between HA22 and HA22-LR [10]. It is still unknown whether the lower non-specific toxicity of lower pI RITs or using the LR toxin instead of PE38 is relevant to toxicity in humans [134, 135].

As for RITs that were tested clinically, dose limiting toxicity in humans varied between different RITs and so did off-target toxicities. In some cases the cause of the off-target toxicity was identified but in other cases it is still unknown. When the "second generation" IT LMB-1 (the anti Le^Y IgG B3 chemically conjugated to PE38) was tested in a phase I clinical study, the dose-limiting toxicity was mainly vascular leak syndrome (VLS, manifested by hypoalbuminemia, fluid retention, hypotension and, in one case, pulmonary edema) [23].Kuan et al. investigated the effects of several PE-based ITs on different human endothelial cell lines to elucidate the mechanism of VLS induced by ITs containing PE and found that anti Le^YITs, including LMB-1 and also the RIT LMB-7 (B3(Fv)-PE38 were toxic to several of the endothelial cell lines that were tested. This effect was unique to the B3-targeted ITs as other RITs that target different antigens were not toxic to endothelial cells. The authors further found that the cytotoxicity of B3-containing ITs is due to specific B3 binding to endothelial cells comes from the fact that the cytotoxicity can be blocked by excess free mAb B3 as competitor [136].

LMB-7 was also tested in a phase I clinical trial, in which, as in the case of LMB-1, VLS was also observed (although it was not dose limiting) [137]. In fact, in that clinical trial, two significant toxicities were observed. The first toxicity was a targeted toxicity: severe gastritis caused by the killing of normal cells that expressed Le^Y antigen in the stomach. This toxicity could be prevented by blocking acid secretion with the proton pump inhibitor Omeprazole accompanied by antacids. At higher dose levels renal toxicity developed and was dose limiting. This toxicity was probably because of the small amounts of Le^Y present on some tubular cells in the kidney [9].

The anti Le^Y RIT SGN-10 (BR96 sFv-PE40) was developed by Seattle Genetics and tested in a phase I clinical trial in 46 patients with Le^Y-positive metastatic carcinoma that was published in 2002. In that study, the dose limiting toxicities were gastrointestinal (including diarrhea, nausea, and vomiting) and VLS [138]. During the pre-clinical evaluation of SGN-10, it was tested in a rat VLS model. It was reported that intravenous administration of BR96 sFv-PE40 resulted in symptoms that closely resemble VLS seen in human immunotoxin trials. Prophylactic administration of the corticosteroid dexamethasone resulted in the prevention of VLS and survival of rats injected with what would otherwise be lethal doses of the RIT without changing the therapeutic efficacy [139, 140]. It should be noted that dose-limiting toxicities and adverse effects that were observed in most of the clinical trials used to evaluate RITs could be managed by simple medical interventions with i.e. blood-pressure lowering drugs or NSAIDs.

The anti mesothelin RIT SS1P was evaluated in two phase I study in which toxicity was also evaluated. In one study, SS1P was given by continuous infusion over 10 days. Continuous infusion was tested as an approach to increase tumor uptake. In the other trial, SS1P was given by a 30 min infusion every 2 days for 3–6 doses. The significant dose-limiting toxicity in both trials was pleuritis ascribed to the targeted killing of normal mesothelial cells in the pleura. VLS characterized by weight gain and a fall in serum albumin also occurred, but was not dose limiting [9, 141].

A group of ITs was developed in which PE38 or PE38KDEL is not targeted by an antibody but, rather, by a ligand (or part of a ligand) binding to a cell-surface receptor that is overexpressed in cancer. As a matter of fact, the only FDA approved IT (which is not PE-based) is denileukin diftitox (Ontak). Ontak, an IL-2-DT fusion protein, was approved in 1999 the for patients with persistent or relapsed CD25-positive cutaneous T-cell lymphoma (CTCL) [142].

As for ligand-PE fusion proteins, IL-4, IL-13 and EGFR were the tested ligands. Although overexpressed in malignancies, the receptors for these cytokines are present on many normal cells so that systemic therapy results in unacceptable toxic side effects. For example, in a phase I trial of IL-4(38–37)–PE38KDEL in patients with advanced solid tumors that expressed IL-4R, the dose-limiting toxicity was liver damage and no objective responses were observed. These agents are better suited for local therapy, and three of these agents that target the EGF, IL-4 and IL-13 receptors have been evaluated for the therapy of glioblastoma. During treatment, the proteins were slowly infused into or next to the brain tumor by continuous infusion over many hours. Phase I and II trials of IL-4(38–37)–PE38KDEL showed a few

complete and partial responses, but the associated toxicity was unacceptable (the dose-limiting toxicity was liver damage) and the development of this IT was abandoned [143]. By contrast, both TGFalpha–PE38 and IL-13–PE38QQR were much better tolerated and have shown complete responses in some patients during phase I and phase II trials. The development of these ITs is ongoing [144–146].

The toxic side effects of immunotoxins in animals and humans are of two types. One type arises from the targeted killing of normal cells that have the same antigen as the tumor cells. Unfortunately, the best solution to overcome this toxicity is to find a different target antigen that is not expressed on normal cells (and, of course, a different antibody). The second type of toxicity arises from undefined nonspecific binding to normal cells which is probably driven by the toxin itself or from physicochemical properties of the RIT. The studies that were described in this section highlight the importance of carefully assessing the target specificity of antibodies that are used to target RITs. Because RITs are so potent, differences in target expression level that may be sufficient for "naked" therapeutic antibodies (the anti EGFR mAb cetuximab and the anti ErbB2 mAb trastuzumab are such antibodies) may not provide a sufficient therapeutic window for RITs.

Finally, another critical issue for the success of treatment with immunotoxins is overcoming physical barriers within the body to gain access to the tumor target. Reducing the size of ITs was described in the introduction as a general solution for improving tumor penetration. The blood brain barrier (BBB) is the major barrier for systemically delivering chemotherapeutics from the circulation to brain tumors, for example the brain tumor glioblastoma multiforme (GBM) [147]. Traditional procedures like surgical resection and radiation therapy have little effectiveness for targeting brain tumor tissues due to their highly invasive nature. Catheter placement has many disadvantages in the context of drug delivery, such as leakage of the infusate into the interventricular and subarachnoid spaces and results in poor drug delivery and distribution [148]. As an alternative novel approach, convection-enhanced delivery (CED) was proposed to circumvent the BBB through direct intracerebral injection (by using a hydrostatic pressure gradient) of large drug molecules throughout the interstitial spaces of infiltrated brain tumors. The proposed advantages of CED drug delivery platform are: (1) minimizing the exit of the drug from the brain while enhancing the drug delivery and reducing systemic toxicity effects; (2) therapeutic agent distribution may be controlled by the applied pressure and, thus, it enables to deliver constant concentrations of the RITs for a predictable distance before a drop-off [149]; and (3) CED enables limited neurotoxicity yet provides effective drug therapy to the tumor upon accurate catheter placement [150]. As part of the need for more accurate methods of validation and drug distribution, Mehta et al. demonstrated the ability of monitoring CED of RITs in humans by in vivo imaging. The imaging for CED was performed by combined the infusion of the MR1-1 RIT [MR1-1(dsFv)-PE38KDEL] and the MRI contrast agents: iodine-labeled albumin (124I-HSA) and gadolinium conjugated diethylenetriaminepentaacetic acid in patients with supratentorial recurrent malignant gliomas. This method enabled high-resolution monitoring of large molecule distribution with the tissue [151]. The authors concluded that CED-infusion approaches offer a promising platform for therapy in patients with GBM.

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References

1. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol. 2010;10:317–27.
2. Teicher BA, Chari RV. Antibody conjugate therapeutics: challenges and potential. Clin Cancer Res. 2011;17:6389–97.
3. Baskar S, Muthusamy N. Antibody-based therapeutics for the treatment of human B cell malignancies. Curr Allergy Asthma Rep. 2013;13:33–43.
4. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol. 2005;23:1137–46.
5. Frankel AE, Kreitman RJ, Sausville EA. Targeted toxins. Clin Cancer Res. 2000;6:326–34.
6. Liang Shan YL, Wang P. Recombinant immunotoxin therapy of solid tumors: challenges and strategies. J Basic Clin Med. 2013;2:1–6.
7. Nagata S, Onda M, Numata Y, Santora K, Beers R, Kreitman RJ, Pastan I. Novel anti-CD30 recombinant immunotoxins containing disulfide-stabilized Fv fragments. Clin Cancer Res. 2002;8:2345–55.
8. Siegall CB, Chaudhary VK, FitzGerald DJ, Pastan I. Functional analysis of domains II, Ib, and III of Pseudomonas exotoxin. J Biol Chem. 1989;264:14256–61.
9. Pastan I, Hassan R, Fitzgerald DJ, Kreitman RJ. Immunotoxin therapy of cancer. Nat Rev Cancer. 2006;6:559–65.
10. Weldon JE, Xiang L, Chertov O, Margulies I, Kreitman RJ, FitzGerald DJ, Pastan I. A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity. Blood. 2009;113:3792–800.
11. Pastan I. Immunotoxins containing Pseudomonas exotoxin A: a short history. Cancer Immunol Immunother. 2003;52:338–41.
12. Liu W, Onda M, Lee B, Kreitman RJ, Hassan R, Xiang L, Pastan I. Recombinant immunotoxin engineered for low immunogenicity and antigenicity by identifying and silencing human B-cell epitopes. Proc Natl Acad Sci USA. 2012;109:11782–7.
13. Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin treatment of cancer. Annu Rev Med. 2007;58:221–37.
14. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J. 2006;8:E532–51.
15. Liu PV. Extracellular toxins of Pseudomonas aeruginosa. J Infect Dis. 1974;130(Suppl):S94–9.
16. Hwang J, Fitzgerald DJ, Adhya S, Pastan I. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. Cell. 1987;48:129–36.
17. Du X, Youle RJ, FitzGerald DJ, Pastan I. Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Mol Cell Biol. 2010;30:3444–52.
18. Shapira A, Benhar I. Toxin-based therapeutic approaches. Toxins. 2010;2:2519–83.
19. FitzGerald DJ, Waldmann TA, Willingham MC, Pastan I. Pseudomonas exotoxin-anti-TAC. Cell-specific immunotoxin active against cells expressing the human T cell growth factor receptor. J Clin Invest. 1984;74:966–71.
20. FitzGerald D, Idziorek T, Batra JK, Willingham M, Pastan I. Antitumor activity of a thioether-linked immunotoxin: OVB3-PE. Bioconjug Chem. 1990;1:264–8.
21. Pai LH, Bookman MA, Ozols RF, Young RC, Smith JW, Longo DL, Gould B, Frankel A, Mcclay EF, Howell S, Reed E, Willingham MC, Fitzgerald DJ, Pastan I. Clinical-evaluation of intraperitoneal Pseudomonas exotoxin immunoconjugate OVB3-PE in patients with ovarian-cancer. J Clin Oncol. 1991;9:2095–103.
22. Siegall CB, Ogata M, Pastan I, FitzGerald DJ. Analysis of sequences in domain II of Pseudomonas exotoxin A which mediate translocation. Biochemistry. 1991;30:7154–9.

23. Pai LH, Wittes R, Setser A, Willingham MC, Pastan I. Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to Pseudomonas exotoxin. Nat Med. 1996;2:350–3.
24. Siegall CB. Targeted therapy of carcinomas using BR96 sFv-PE40, a single-chain immunotoxin that binds to the Le(y) antigen. Semin Cancer Biol. 1995;6:289–95.
25. Martin R. Humanized anti-CD25 antibody treatment with daclizumab in multiple sclerosis. Neurodegener Dis. 2008;5:23–6.
26. Martin R. Anti-CD25 (daclizumab) monoclonal antibody therapy in relapsing-remitting multiple sclerosis. Clin Immunol. 2012;142:9–14.
27. Pfender N, Martin R. Daclizumab (anti-CD25) in multiple sclerosis. Exp Neurol. 2014;262:44–51 XXX (Epub ahead of print).
28. Mansfield E, Chiron MF, Amlot P, Pastan I, FitzGerald DJ. Recombinant RFB4 single-chain immunotoxin that is cytotoxic towards CD22-positive cells. Biochem Soc Trans. 1997;25:709–14.
29. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. Clin Cancer Res. 2004;10:3937–42.
30. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc Natl Acad Sci USA. 1998;95:669–74.
31. Reiter Y, Brinkmann U, Kreitman RJ, Jung SH, Lee B, Pastan I. Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions. Biochemistry. 1994;33:5451–9.
32. Chowdhury PS, Pastan I. Improving antibody affinity by mimicking somatic hypermutation in vitro. Nat Biotechnol. 1999;17:568–72.
33. Kreitman RJ, Pastan I. Antibody fusion proteins: anti-CD22 recombinant immunotoxin moxetumomab pasudotox. Clin Cancer Res. 2011;17:6398–405.
34. Brehm H, Niesen J, Mladenov R, Stein C, Pardo A, Fey G, Helfrich W, Fischer R, Gattenlohner S, Barth S. A CSPG4-specific immunotoxin kills rhabdomyosarcoma cells and binds to primary tumor tissues. Cancer Lett. 2014;352(2):228–35.
35. Hristodorov D, Nordlohne J, Mladenov R, Huhn M, Fischer R, Thepen T, Barth S. Human microtubule-associated protein tau mediates targeted killing of CD30(+) lymphoma cells in vitro and inhibits tumour growth in vivo. Br J Haematol. 2014;164:251–7.
36. Pardo A, Stocker M, Kampmeier F, Melmer G, Fischer R, Thepen T, Barth S. In vivo imaging of immunotoxin treatment using Katushka-transfected A-431 cells in a murine xenograft tumour model. Cancer Immunol Immunother. 2012;61:1617–26.
37. Ribbert T, Thepen T, Tur MK, Fischer R, Huhn M, Barth S. Recombinant, ETA'-based CD64 immunotoxins: improved efficacy by increased valency, both in vitro and in vivo in a chronic cutaneous inflammation model in human CD64 transgenic mice. Br J Dermatol. 2010;163:279–86.
38. Klimka A, Barth S, Drillich S, Wels W, van Snick J, Renauld JC, Tesch H, Bohlen H, Diehl V, Engert A. A deletion mutant of Pseudomonas exotoxin-A fused to recombinant human interleukin-9 (rhIL-9-ETA') shows specific cytotoxicity against IL-9-receptor-expressing cell lines. Cytokines Mol Ther. 1996;2:139–46.
39. Barth S, Huhn M, Wels W, Diehl V, Engert A. Construction and in vitro evaluation of RFT5(scFv)-ETA', a new recombinant single-chain immunotoxin with specific cytotoxicity toward CD25 + Hodgkin-derived cell lines. Int J Mol Med. 1998;1:249–56.
40. Klimka A, Barth S, Matthey B, Roovers RC, Lemke H, Hansen H, Arends JW, Diehl V, Hoogenboom HR, Engert A. An anti-CD30 single-chain Fv selected by phage display and fused to Pseudomonas exotoxin A (Ki-4(scFv)-ETA') is a potent immunotoxin against a Hodgkin-derived cell line. Br J Cancer. 1999;80:1214–22.
41. Schmidt M, Wels W. Targeted inhibition of tumour cell growth by a bispecific single-chain toxin containing an antibody domain and TGF alpha. Br J Cancer. 1996;74:853–62.
42. Schmidt M, Hynes NE, Groner B, Wels W. A bivalent single-chain antibody-toxin specific for ErbB-2 and the EGF receptor. Int J Cancer. 1996;65:538–46.

43. Jeschke M, Wels W, Dengler W, Imber R, Stocklin E, Groner B. Targeted inhibition of tumor-cell growth by recombinant heregulin-toxin fusion proteins. Int J Cancer. 1995;60:730–9.
44. Wels W, Harwerth IM, Mueller M, Groner B, Hynes NE. Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB-2 receptor. Cancer Res. 1992;52:6310–7.
45. Azemar M, Djahansouzi S, Jager E, Solbach C, Schmidt M, Maurer AB, Mross K, Unger C, von Minckwitz G, Dall P, Groner B, Wels WS. Regression of cutaneous tumor lesions in patientsintratumorally injected with a recombinant single-chain antibody-toxin targeted to ErbB2/HER2. Breast Cancer Res Treat. 2003;82:155–64.
46. Mattoo AR, FitzGerald DJ. Combination treatments with ABT-263 and an immunotoxin produce synergistic killing of ABT-263-resistant small cell lung cancer cell lines. Int J Cancer. 2013;132:978–87.
47. Traini R, Ben-Josef G, Pastrana DV, Moskatel E, Sharma AK, Antignani A, Fitzgerald DJ. ABT-737 overcomes resistance to immunotoxin-mediated apoptosis and enhances the delivery of pseudomonas exotoxin-based proteins to the cell cytosol. Mol Cancer Ther. 2010;9:2007–15.
48. Biberacher V, Decker T, Oelsner M, Wagner M, Bogner C, Schmidt B, Kreitman RJ, Peschel C, Pastan I, Meyer ZumBuschenfelde C, Ringshausen I. The cytotoxicity of anti-CD22 immunotoxin is enhanced by bryostatin 1 in B-cell lymphomas through CD22 upregulation and PKC-betaII depletion. Haematologica. 2012;97:771–9.
49. Nechansky A, Kircheis R. Immunogenicity of therapeutics: a matter of efficacy and safety. Expert Opin Drug Discov. 2010;5:1067–79.
50. De Groot AS, Scott DW. Immunogenicity of protein therapeutics. Trends Immunol. 2007;28:482–90.
51. Kelley M, Ahene AB, Gorovits B, Kamerud J, King LE, McIntosh T, Yang J. Theoretical considerations and practical approaches to address the effect of anti-drug antibody (ADA) on quantification of biotherapeutics in circulation. AAPS J. 2013;15:646–58.
52. Chirmule N, Jawa V, Meibohm B. Immunogenicity to therapeutic proteins: impact on PK/PD and efficacy. AAPS J. 2012;14:296–302.
53. Chamberlain P. Assessing immunogenicity of biosimilar therapeutic monoclonal antibodies: regulatory and bioanalytical considerations. Bioanalysis. 2013;5:561–74.
54. Hwang WY, Foote J. Immunogenicity of engineered antibodies. Methods. 2005;36:3–10.
55. Niebecker R, Kloft C. Safety of therapeutic monoclonal antibodies. Curr Drug Saf. 2010;5:275–86.
56. Holgate RG, Baker MP. Circumventing immunogenicity in the development of therapeutic antibodies. IDrugs. 2009;12:233–7.
57. De Groot AS, Moise L. Prediction of immunogenicity for therapeutic proteins: state of the art. Curr Opin Drug Discov Devel. 2007;10:332–40.
58. Nagata S, Pastan I. Removal of B cell epitopes as a practical approach for reducing the immunogenicity of foreign protein-based therapeutics. Adv Drug Deliv Rev. 2009;61: 977–85.
59. Parker AS, Choi Y, Griswold KE, Bailey-Kellogg C. Structure-guided deimmunization of therapeutic proteins. J Comput Biol. 2013;20:152–65.
60. Roscoe DM, Pai LH, Pastan I. Identification of epitopes on a mutant form of Pseudomonas exotoxin using serum from humans treated with Pseudomonas exotoxin containing immunotoxins. Eur J Immunol. 1997;27:1459–68.
61. Roscoe DM, Jung SH, Benhar I, Pai L, Lee BK, Pastan I. Primate antibody response to immunotoxin: serological and computer-aided analysis of epitopes on a truncated form of Pseudomonas exotoxin. Infect Immun. 1994;62:5055–65.
62. Onda M, Nagata S, FitzGerald DJ, Beers R, Fisher RJ, Vincent JJ, Lee B, Nakamura M, Hwang J, Kreitman RJ, Hassan R, Pastan I. Characterization of the B cell epitopes associated with a truncated form of Pseudomonas exotoxin (PE38) used to make immunotoxins for the treatment of cancer patients. J Immunol. 2006;177:8822–34.

63. Francis GE, Fisher D, Delgado C, Malik F, Gardiner A, Neale D. PEGylation of cytokines and other therapeutic proteins and peptides: the importance of biological optimisation of coupling techniques. Int J Hematol. 1998;68:1–18.
64. Goodson RJ, Katre NV. Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. Biotechnology (N Y). 1990;8:343–6.
65. Delgado C, Francis GE, Fisher D. The uses and properties of PEG-linked proteins. Crit Rev Ther Drug Carrier Syst. 1992;9:249–304.
66. Kuan CT, Wang QC, Pastan I. Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. J Biol Chem. 1994;269:7610–6.
67. Benhar I, Wang QC, FitzGerald D, Pastan I. Pseudomonas exotoxin A mutants. Replacement of surface-exposed residues in domain III with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. J Biol Chem. 1994;269:13398–404.
68. Tsutsumi Y, Onda M, Nagata S, Lee B, Kreitman RJ, Pastan I. Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. Proc Natl Acad Sci USA. 2000;97:8548–53.
69. Filpula D, Yang K, Basu A, Hassan R, Xiang L, Zhang Z, Wang M, Wang QC, Ho M, Beers R, Zhao H, Peng P, Zhou J, Li X, Petti G, Janjua A, Liu J, Wu D, Yu D, Longley C, FitzGerald D, Kreitman RJ, Pastan I. Releasable PEGylation of mesothelin targeted immunotoxin SS1P achieves single dosage complete regression of a human carcinoma in mice. Bioconjug Chem. 2007;18:773–84.
70. Debinski W, Pastan I. An immunotoxin with increased activity and homogeneity produced by reducing the number of lysine residues in recombinant Pseudomonas exotoxin. Bioconjug Chem. 1994;5:40–6.
71. Benhar I, Brinkmann U, Webber KO, Pastan I. Mutations of two lysine residues in the CDR loops of a recombinant immunotoxin that reduce its sensitivity to chemical derivatization. Bioconjug Chem. 1994;5:321–6.
72. Onda M, Vincent JJ, Lee B, Pastan I. Mutants of immunotoxin anti-Tac(dsFv)-PE38 with variable number of lysine residues as candidates for site-specific chemical modification. 1. Properties of mutant molecules. Bioconjug Chem. 2003;14:480–7.
73. Roscoe DM, Pai LH, Pastan I. Identification of epitopes on a mutant form of Pseudomonas exotoxin using serum from humans treated with Pseudomonas exotoxin containing immunotoxins. Eur J Immunol. 1997;27:1459–68.
74. Wang QC, Pai LH, Debinski W, FitzGerald DJ, Pastan I. Polyethylene glycol-modified chimeric toxin composed of transforming growth factor alpha and Pseudomonas exotoxin. Cancer Res. 1993;53:4588–94.
75. Riechmann L, Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. Nature. 1988;332:323–7.
76. Benhar I, Padlan EA, Jung SH, Lee B, Pastan I. Rapid humanization of the Fv of monoclonal antibody B3 by using framework exchange of the recombinant immunotoxin B3(Fv)-PE38. Proc Natl Acad Sci USA. 1994;91:12051–5.
77. Kreitman RJ, Squires DR, Stetler-Stevenson M, Noel P, FitzGerald DJ, Wilson WH, Pastan I. Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. J Clin Oncol. 2005;23:6719–29.
78. Abbas AK. Antigen presentation by B lymphocytes: mechanisms and functional significance. Semin Immunol. 1989;1:5–12.
79. Parker DC.T cell-dependent B cell activation. Annu Rev Immunol. 1993;11:331–60.
80. Brons NH, Blaich A, Wiesmuller KH, Schneider F, Jung G, Muller CP. Hierarchic T-cell help to non-linked B-cell epitopes. Scand J Immunol. 1996;44:478–84.
81. Onda M, Beers R, Xiang L, Nagata S, Wang QC, Pastan I. An immunotoxin with greatly reduced immunogenicity by identification and removal of B cell epitopes. Proc Natl Acad Sci USA. 2008;105:11311–6.

82. Hansen JK, Weldon JE, Xiang L, Beers R, Onda M, Pastan I. A recombinant immunotoxin targeting CD22 with low immunogenicity, low nonspecific toxicity, and high antitumor activity in mice. J Immunother. 2010;33:297–304.
83. Onda M, Beers R, Xiang L, Lee B, Weldon JE, Kreitman RJ, Pastan I. Recombinant immunotoxin against B-cell malignancies with no immunogenicity in mice by removal of B-cell epitopes. Proc Natl Acad Sci USA. 2011;108:5742–7.
84. Weldon JE, Xiang L, Zhang J, Beers R, Walker DA, Onda M, Hassan R, Pastan I. A recombinant immunotoxin against the tumor-associated antigen mesothelin reengineered for high activity, low off-target toxicity, and reduced antigenicity. Mol Cancer Ther. 2013;12:48–57.
85. Mazor R, Vassall AN, Eberle JA, Beers R, Weldon JE, Venzon DJ, Tsang KY, Benhar I, Pastan I. Identification and elimination of an immunodominant T-cell epitope in recombinant immunotoxins based on Pseudomonas exotoxin A. Proc Natl Acad Sci USA. 2012;109:E3597–603.
86. Mazor R, Eberle JA, Hu X, Vassall AN, Onda M, Beers R, Lee EC, Kreitman RJ, Lee B, Baker D, King C, Hassan R, Benhar I, Pastan I. Recombinant immunotoxin for cancer treatment with low immunogenicity by identification and silencing of human T-cell epitopes. Proc Natl Acad Sci U S A. 2014;111:8571–6.
87. King C, Garza EN, Mazor R, Linehan JL, Pastan I, Pepper M, Baker D. Removing T-cell epitopes with computational protein design. Proc Natl Acad Sci U S A. 2014;111:8577–82.
88. Glockshuber R, Malia M, Pfitzinger I, Plückthun A. A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry. 1990;29:1362–7.
89. Brinkmann U, Reiter Y, Jung SH, Lee B, Pastan I. A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Proc Natl Acad Sci USA. 1993;90:7538–42.
90. Benhar I, Reiter Y, Pai LH, Pastan I. Administration of disulfide-stabilized Fv-immunotoxins B1(Dsfv)-Pe38 and B3(Dsfv)-Pe38 by continuous-infusion increases their efficacy in curing large tumor xenografts in nude-mice. Int J Cancer. 1995;62:351–5.
91. Reiter Y, Brinkmann U, Jung SH, Lee B, Kasprzyk PG, King CR, Pastan I. Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide stabilization of the Fv fragment. J Biol Chem. 1994;269:18327–31.
92. Reiter Y, Brinkmann U, Jung SH, Pastan I, Lee B. Disulfide stabilization of antibody Fv: computer predictions and experimental evaluation. Protein Eng. 1995;8:1323–31.
93. Reiter Y, Brinkmann U, Lee B, Pastan I. Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. Nat Biotechnol. 1996;14:1239–45.
94. Reiter Y, Brinkmann U, Webber KO, Jung SH, Lee B, Pastan I. Engineering interchain disulfide bonds into conserved framework regions of Fv fragments: improved biochemical characteristics of recombinant immunotoxins containing disulfide-stabilized Fv. Protein Eng. 1994;7:697–704.
95. Reiter Y, Pai LH, Brinkmann U, Wang QC, Pastan I. Antitumor-activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Cancer Res. 1994;54:2714–8.
96. Webber KO, Reiter Y, Brinkmann U, Kreitman R, Pastan I. Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-Tac antibody: comparison with its single-chain analog. Mol Immunol. 1995;32:249–58.
97. Salvatore G, Beers R, Margulies I, Kreitman RJ, Pastan I. Improved cytotoxic activity toward cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. Clin Cancer Res. 2002;8:995–1002.
98. Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, Waldmann TA, Pastan I. Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol. 2000;18:1622–36.
99. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, Pastan I. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. Clin Cancer Res. 2007;13:5144–9.

100. Jiang H, Xie Y, Burnette A, Roach J, Giardina SL, Hecht TT, Creekmore SP, Mitra G, Zhu J. Purification of clinical-grade disulfide stabilized antibody fragment variable—Pseudomonas exotoxin conjugate (dsFv-PE38) expressed in Escherichia coli. Appl Microbiol Biotechnol. 2013;97:621–32.
101. Bera TK, Pastan I. Comparison of recombinant immunotoxins against LeY antigen expressing tumor cells: influence of affinity, size, and stability. Bioconjug Chem. 1998;9:736–43.
102. Park JH, Kwon HW, Chung HK, Kim IH, Ahn K, Choi EJ, Pastan I, Choe M. A divalent recombinant immunotoxin formed by a disulfide bond between the extension peptide chains. Mol Cells. 2001;12:398–402.
103. Kreitman RJ, Pastan I. Importance of the glutamate residue of KDEL in increasing the cytotoxicity of Pseudomonas exotoxin derivatives and for increased binding to the KDEL receptor. Biochem J. 1995;307:29–37.
104. Seetharam S, Chaudhary VK, FitzGerald D, Pastan I. Increased cytotoxic activity of Pseudomonas exotoxin and two chimeric toxins ending in KDEL. J Biol Chem. 1991;266:17376–81.
105. Liu W, Onda M, Kim C, Xiang L, Weldon JE, Lee B, Pastan I. A recombinant immunotoxin engineered for increased stability by adding a disulfide bond has decreased immunogenicity. Protein Eng Des Sel. 2012;25:1–6.
106. Kreitman RJ, Hassan R, Fitzgerald DJ, Pastan I. Phase I trial of continuous infusion anti-mesothelin recombinant immunotoxin SS1P. Clin Cancer Res. 2009;15:5274–9.
107. Zhang Y, Xiang L, Hassan R, Paik CH, Carrasquillo JA, Jang BS, Le N, Ho M, Pastan I. Synergistic antitumor activity of taxol and immunotoxin SS1P in tumor-bearing mice. Clin Cancer Res. 2006;12:4695–701.
108. Zhang Y, Xiang L, Hassan R, Pastan I. Immunotoxin and Taxol synergy results from a decrease in shed mesothelin levels in the extracellular space of tumors. Proc Natl Acad Sci USA. 2007;104:17099–104.
109. Singh R, Zhang Y, Pastan I, Kreitman RJ. Synergistic antitumor activity of anti-CD25 recombinant immunotoxin LMB-2 with chemotherapy. Clin Cancer Res. 2012;18:152–60.
110. Kreitman RJ, Wilson WH, Robbins D, Margulies I, Stetler-Stevenson M, Waldmann TA, Pastan I. Responses in refractory hairy cell leukemia to a recombinant immunotoxin. Blood. 1999;94:3340–8.
111. Du X, Xiang L, Mackall C, Pastan I. Killing of resistant cancer cells with low Bak by a combination of an antimesothelin immunotoxin and a TRAIL Receptor 2 agonist antibody. Clin Cancer Res. 2011;17:5926–5934.
112. Mochly-Rosen D, Das K, Grimes KV. Protein kinase C, an elusive therapeutic target? Nat Rev Drug Discov. 2012;11:937–57.
113. Mattoo AR, Pastan I, Fitzgerald D. Combination treatments with the PKC inhibitor, enzastaurin, enhance the cytotoxicity of the anti-mesothelin immunotoxin, SS1P. PLoS ONE. 2013;8:e75576.
114. Liu XF, Xiang L, FitzGerald DJ, Pastan I. Antitumor effects of immunotoxins are enhanced by lowering HCK or treatment with SRC kinase inhibitors. Mol Cancer Ther. 2014;13:82–9.
115. Billard C. BH3 mimetics: status of the field and new developments. Mol Cancer Ther. 2013;12:1691–700.
116. Hollevoet K, Antignani A, Fitzgerald DJ, Pastan I. Combining the antimesothelin immunotoxin SS1P with the BH3-mimetic ABT-737 induces cell death in SS1P-resistant pancreatic cancer cells. J Immunother. 2014;37:8–15.
117. Kreitman RJ, Margulies I, Stetler-Stevenson M, Wang QC, FitzGerald DJ, Pastan I. Cytotoxic activity of disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) toward fresh malignant cells from patients with B-cell leukemias. Clin Cancer Res. 2000;6:1476–87.
118. Choudhary S, Mathew M, Verma RS. Therapeutic potential of anticancer immunotoxins. Drug Discov Today. 2011;16:495–503.

119. Kreitman RJ, Wilson WH, Bergeron K, Raggio M, Stetler-Stevenson M, FitzGerald DJ, Pastan I. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. N Engl J Med. 2001;345:241–7.
120. Adams GP, Schier R. Generating improved single-chain Fv molecules for tumor targeting. J Immunol Methods. 1999;231:249–60.
121. Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res. 1998;58:485–490.
122. Irving RA, Coia G, Roberts A, Nuttall SD, Hudson PJ. Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. J Immunol Methods. 2001;248:31–45.
123. Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. Adv Drug Deliv Rev. 2006;58:640–56.
124. Maynard J, Georgiou G. Antibody engineering. Annu Rev Biomed Eng. 2000;2:339–76.
125. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K. High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules. 2011;16:3675–700.
126. Levin AM, Weiss GA. Optimizing the affinity and specificity of proteins with molecular display. Mol Biosyst. 2006;2:49–57.
127. Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler-Stevenson M, Fitzgerald DJ, Lechleider R, Pastan I. Phase I trial of anti-CD22 recombinant immunotoxin moxetumomab pasudotox (CAT-8015 or HA22) in patients with hairy cell leukemia. J Clin Oncol. 2012;30:1822–8.
128. Kawa S, Onda M, Ho M, Kreitman RJ, Bera TK, Pastan I. The improvement of an anti-CD22 immunotoxin: conversion to single-chain and disulfide stabilized form and affinity maturation by alanine scan. MAbs. 2011;3:479–86.
129. Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, Yan H, Adamson DC. Glioblastoma multiforme: a review of therapeutic targets. Exp Opin Ther Targets. 2009;13:701–18.
130. Kuan CT, Wakiya K, Keir ST, Li J, Herndon JE 2nd, Pastan I, Bigner DD. Affinity-matured anti-glycoprotein NMB recombinant immunotoxins targeting malignant gliomas and melanomas. Int J Cancer. 2011;129:111–21.
131. Hedberg KM, Mahesparan R, Read TA, Tysnes BB, Thorsen F, Visted T, Bjerkvig R, Fredman P. The glioma-associated gangliosides 3′-isoLM1, GD3 and GM2 show selective area expression in human glioblastoma xenografts in nude rat brains. Neuropathol Appl Neurobiol. 2001;27:451–64.
132. Piao H, Kuan CT, Chandramohan V, Keir ST, Pegram CN, Bao X, Mansson JE, Pastan IH, Bigner DD. Affinity-matured recombinant immunotoxin targeting gangliosides 3′-isoLM1 and 3′,6′-isoLD1 on malignant gliomas. MAbs. 2013;5:748–62.
133. Schumann J, Angermuller S, Bang R, Lohoff M, Tiegs G. Acute hepatotoxicity of Pseudomonas aeruginosa exotoxin A in mice depends on T cells and TNF. J Immunol. 1998;161:5745–54.
134. Onda M, Kreitman RJ, Vasmatzis G, Lee B, Pastan I. Reduction of the nonspecific animal toxicity of anti-Tac(Fv)-PE38 by mutations in the framework regions of the Fv which lower the isoelectric point. J Immunol. 1999;163:6072–7.
135. Onda M, Nagata S, Tsutsumi Y, Vincent JJ, Wang Q, Kreitman RJ, Lee B, Pastan I. Lowering the isoelectric point of the Fv portion of recombinant immunotoxins leads to decreased nonspecific animal toxicity without affecting antitumor activity. Cancer Res. 2001;61:5070–7.
136. Kuan CT, Pai LH, Pastan I. Immunotoxins containing Pseudomonas exotoxin that target Le(Y) damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome. Clin Cancer Res. 1995;1:1589–94.
137. Pai LH, Wittes R, Sester A, Pearson D, Willingham MC, Pastan I. Phase I trial of recombinant immunotoxin LMB-7 (B3 (Fv) PE38) for adult solid tumors. Proc Am Assoc Cancer Res. 1997;38:85.

138. Posey JA, Khazaeli MB, Bookman MA, Nowrouzi A, Grizzle WE, Thornton J, Carey DE, Lorenz JM, Sing AP, Siegall CB, LoBuglio AF, Saleh MN. A phase I trial of the single-chain immunotoxin SGN-10 (BR96 sFv-PE40) in patients with advanced solid tumors. Clin Cancer Res. 2002;8:3092–9.
139. Siegall CB, Liggitt D, Chace D, Mixan B, Sugai J, Davidson T, Steinitz M. Characterization of vascular leak syndrome induced by the toxin component of Pseudomonas exotoxin-based immunotoxins and its potential inhibition with nonsteroidal anti-inflammatory drugs. Clin Cancer Res. 1997;3:339–45.
140. Siegall CB, Liggitt D, Chace D, Tepper MA, Fell HP. Prevention of immunotoxin-mediated vascular leak syndrome in rats with retention of antitumor activity. Proc Natl Acad Sci USA. 1994;91:9514–8.
141. Hassan R, Bullock S, Kreitman RJ, Kindler HL, Pastan I. Targeted therapy of mesothelin expressing mesotheliomas (MM), ovarian cancer (OC) and pancreatic cancer (PC): Results of phase I study of SS1(dsFv)PE38 (SS1P). J Clin Oncol. 2004;22:203s.
142. Manoukian G, Hagemeister F. Denileukin diftitox: a novel immunotoxin. Exp Opin Biol Ther. 2009;9:1445–51.
143. Rand RW, Kreitman RJ, Patronas N, Varricchio F, Pastan I, Puri RK. Intratumoral administration of recombinant circularly permuted interleukin-4-Pseudomonas exotoxin in patients with high-grade glioma. Clin Cancer Res. 2000;6:2157–65.
144. Sampson JH, Akabani G, Archer GE, Bigner DD, Berger MS, Friedman AH, Friedman HS, Herndon JE, 2nd, Kunwar S, Marcus S, McLendon RE, Paolino A, Penne K, Provenzale J, Quinn J, Reardon DA, Rich J, Stenzel T, Tourt-Uhlig S, Wikstrand C, Wong T, Williams R, Yuan F, Zalutsky MR, Pastan I. Progress report of a Phase I study of the intracerebral microinfusion of a recombinant chimeric protein composed of transforming growth factor (TGF)-alpha and a mutated form of the Pseudomonas exotoxin termed PE-38 (TP-38) for the treatment of malignant brain tumors. J Neurooncol. 2003;65:27–35.
145. Sampson JH, Reardon DA, Friedman AH, Friedman HS, Coleman RE, McLendon RE, Pastan I, Bigner DD. Sustained radiographic and clinical response in patient with bifrontal recurrent glioblastoma multiforme with intracerebral infusion of the recombinant targeted toxin TP-38: case study. Neuro Oncol. 2005;7:90–6.
146. Parney IF, Kunwar S, McDermott M, Berger M, Prados M, Cha S, Croteau D, Puri RK, Chang SM. Neuroradiographic changes following convection-enhanced delivery of the recombinant cytotoxin interleukin 13-PE38QQR for recurrent malignant glioma. J Neurosurg. 2005;102:267–75.
147. Muldoon LL, Soussain C, Jahnke K, Johanson C, Siegal T, Smith QR, Hall WA, Hynynen K, Senter PD, Peereboom DM, Neuwelt EA. Chemotherapy delivery issues in central nervous system malignancy: a reality check. J Clin Oncol. 2007;25:2295–305.
148. Sampson JH, Raghavan R, Provenzale JM, Croteau D, Reardon DA, Coleman RE, Rodriguez Ponce I, Pastan I, Puri RK, Pedain C. Induction of hyperintense signal on T2-weighted MR images correlates with infusion distribution from intracerebral convection-enhanced delivery of a tumor-targeted cytotoxin. Am J Roentgenol. 2007;188:703–9.
149. Ding D, Kanaly CW, Bigner DD, Cummings TJ, Herndon JE 2nd, Pastan I, Raghavan R, Sampson JH. Convection-enhanced delivery of free gadolinium with the recombinant immunotoxin MR1–1. J Neurooncol. 2010;98:1–7.
150. Sampson JH, Archer G, Pedain C, Wembacher-Schroder E, Westphal M, Kunwar S, Vogelbaum MA, Coan A, Herndon JE, Raghavan R, Brady ML, Reardon DA, Friedman AH, Friedman HS, Rodriguez-Ponce MI, Chang SM, Mittermeyer S, Croteau D, Puri RK. Poor drug distribution as a possible explanation for the results of the PRECISE trial. J Neurosurg. 2010;113:301–9.
151. Mehta AI, Choi BD, Raghavan R, Brady M, Friedman AH, Bigner DD, Pastan I, Sampson JH. Imaging of convection enhanced delivery of toxins in humans. Toxins. 2011;3:201–6.

Chapter 7
Drug Resistance to Calicheamicin Conjugated Monoclonal Antibody Therapy

Miwa Adachi and Akihiro Takeshita

Abstract Many new agents have been introduced in the treatment of acute myeloid leukemia (AML), and around 80 % of AML achieve complete remission (CR). However, a considerable number of patients relapse, which is mainly associated with drug resistance. Gemtuzumab ozogamicin (GO) is a conjugate of a cytotoxic agent, a calicheamicin derivative, linked to a recombinant humanized monoclonal antibody (mAb) directed against the CD33 antigen, which is expressed on leukemia cells from more than 90 % of patients with AML. GO was approved with promising results from phase I and II studies. However, the initial phase III study failed to confirm the merit of GO compared to conventional chemotherapies. One of the reasons is explained by the drug resistance acquired in leukemia cells before and during the treatments. Several resistance mechanisms against GO have been proposed. Among them, the most important resistant mechanism is the multidrug resistant (MDR) P-glycoprotein (P-gp). Some MDR modifiers removed the resistance of GO i*n vitro*. However, one of the MDR modifiers, cyclosporine A (CyA), did not improve the response rate or survival, despite considerable number of adverse effects. Several investigators have reported promising results with the use of GO in acute promyelocytic leukemia (APL), which commonly expresses a larger amount of CD33 and a lower amount of P-gp than that of AML. Recent results show the efficacy of GO in a favorable risk of AML, such as core binding factor leukemia and APL. Another calicheamicin immunoconjugate, inotuzumab ozogamicin (IO), also introduced in B cell malignancies, provides us with promising results. However, IO also reportedly has similar resistant mechanisms to GO.

Keywords Calichamicin · Immunoconjugate · Gemtuzumab ozogamicin · Inotuzumab ozogamicin · Acutemyeloid leukemia · B cell malignancies · P-glycoprotein

Abbreviations

AML	Acute myeloid leukemia
mAb	Monoclonal antibody

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CR	Complete remission
GO	Gemtuzumab ozogamicin
MDR	Multidrug resistant
P-gp	P-glycoprotein
CyA	Cyclosporine A
APL	Acute promyelocytic leukemia
IO	Inotuzumab ozogamicin
OS	Overall survival
DFS	Disease-free survival
MRP1	MDR-associated protein 1
CRp	Complete remission with thrombocytopenia
RFS	Relapse-free survival
OR	Overall response
SOS	Sinusoid obstructed syndrome
HSCT	Hematopoietic stem cell transplantation
Ara-C	Cytosine arabinoside
DNR	Daunorubicin
RAEB	Refractory anemia with excess blasts
EFS	Event-free survival
pBzRs	Peripheral benzodiazepine receptors
HiDAC	High dose AraC
ETP	Etoposide
WHO	World Health Organization
ATRA	All-*trans* retinoic acid
Am80	Tamibarotene
ATO	Arsenic trioxide
MR	Molecular remission
ITAM	Immunoreceptor tyrosine activation motifs
ITIM	Immunoreceptor tyrosine inhibitory motifs
BCR	B-cell antigen receptor
CLL	Chronic lymphocytic leukaemia
NHL	Non-Hodgkin lymphoma
CBF	Core binding factor

7.1 Introduction

Acute myeloid leukemia (AML), one of the most representative hematological malignancies [1], constitutes approximately 25–30% of adult leukemias in the Western countries. The age-adjusted incidence rate of AML is approximately 3–4 per 100,000 people, and the incidence increases with aging. AML is characterized by the clonal proliferation of hematopoietic precursor cells and impairment of normal hematopoiesis. Many agents have been introduced in the treatment of AML, and around 80% of AML cases achieve complete remission (CR) [2, 3]. However, a

considerable number of patients relapse, and as a result, the 5-year-overall survival (OS) and disease-free survival (DFS) remain at around 40 and 20%, respectively. The reasons have been explored mainly by the genomic methods, which showed that AML was genetically more heterogeneous than expected. Moreover, the specificity of molecular diagnosis does not necessarily result in a specific molecular targeted therapy. Several promising agents have failed to win through randomized trials in AML [4, 5]. Monoclonal antibody therapy against CD33 was also introduced and developed despite such a background.

Gemtuzumab ozogamicin (GO), whose development code was CMA676, is a conjugate of a calicheamicin derivative and a recombinant humanized antibody (IgG_4) directed against the CD33 antigen [6]. Calicheamicin is a highly potent antitumor antibiotic [7–10], which binds to DNA, breaks double-stranded DNA, and induces cell death. It is classified under the same category as toxin-conjugated antibody against surface antigen of tumor cells. Here, we try to understand the action and resistant mechanism of calicheamicin immune-conjugates by GO. In addition, we introduce several means to overcome the drug resistance.

7.2 CD33

The CD33 antigen, a 67-kDa trans-membrane glycoprotein, belongs to the immunoglobulin (Ig) superfamily subgroup of sialic acid-binding Ig-like lectins (siglecs). [6, 11, 12]. It consists of two Ig-like extracellular domains and two cytoplasmic domains, [13] which have tyrosine residues similar to the immune-receptor tyrosine-based inhibitory motifs. Several protein tyrosine phosphatase inhibitors or the bridge formation by immunoglobulins result in phosphorylation of the tyrosine. While the molecular reaction stream after the phosphorylation of the tyrosine and the precise function of CD33 have not been well elucidated, it has been thought to be associated with cell adhesion and interaction. It could suppress cell proliferation and function, and induce apoptosis *in vitro* [14], but these functions have not been clarified *in vivo*.

CD33 is normally expressed on myelocyte and myelomonocytic precursor cells, as well as mature myeloid lineage cells, macrophages, monocytes, and dendritic cells [15–17]. The amount of CD33 reaches highest in promyelocytes and myelocytes, and decreases with maturation of the myeloid lineage. CD33 is also expressed on erythroblasts, megakaryoblasts, and Kupffer cells at some level, [11, 12] but not on normal hematopoietic stem cells and lymphocytes [18, 19].

Eighty to 90% of AML are reportedly considered as CD33-positive [17, 20–22]. The amount of CD33 on AML cells is estimated at 10,000–20,000 copies/cell, which is 3–5 times more than normal bone marrow cells [23]. CD33 is sometimes determined on acute lymphoblastic leukemia (ALL), but the amount is relatively smaller (5–26%) than AML [22, 24] and differs among the ALL subtypes. These facts suggest that CD33 is a useful target for the development of therapeutic agents for AML and limited ALL.

Fluorescence conjugated with anti-CD33 antibody, hP67.8, which was detected on the cell surface just after incubation, moved to intracellular location after 3–5 h and disappeared after 24 h [25]. The data supports that CD33 is rapidly internalized after anti-CD33 antibody binding, and then moved to the lysosome where the immunoconjugates undergodegradation and quenching of the fluorochrome. The internalization process indicated that antibody-cytotoxic agent complexes can effectively be taken up by CD33 positive leukemia cells. Consequently, radio- and toxin-conjugated anti-CD33 antibodies have been developed, such as conjugates of radioisotopes, calicheamicin, gelonin, and ricin [26–29]. Of these, GO has drawn attention with the encouraging results.

Many surface antigens are reportedly co-expressed on CD33-positive AML cells [24]. However, only CD34 reportedly relates to the efficacy of GO. In the previous study, GO was less effective on CD34-positive leukemia cells, even when they expressed a sufficient amount of CD33; this effect was independent of the amount of CD34 [30]. Sievers et al [31] reported in their clinical study that the expression of CD34 was associated with a shorter survival after treatment with GO. These might be explained by that CD34-positive cells have more defensive mechanisms including P-glycoprotein (P-gp) than CD34-negative cells.

7.2.1 Gentuzumab Ozogamicin (GO)

GO is a humanized IgG4 anti-CD33 monoclonal antibody (hP67.6) conjugated to NAc-gamma calicheamicin DMH, a hydrazide derivative of calicheamicin (Fig. 7.1) [32]. Approximately half of antibodies are conjugated by calicheamicin, with an average load of 4–6 molecules of calicheamicin per antibody. Calicheamicin, a hydrophobic enediyne antibiotic agent, was first isolated from the actinomycete *Micromonospora echiospora ssp. Calichensis* [7, 8]. The hydrazone function in the AcBut linker, which links the antibody and calicheamicin, releases calicheamicin divertive from its conjugated state under acidic conditions.

After GO binds to CD33 on the cells, CD33-antibody complexes are rapidly internalized and transferred into lysosomes [25]. The calicheamicin derivative is released via hydrolysis in the acid environment of the lysosome. Then it moves to the nucleus, and binds to the minor groove of DNA in a sequence-specific manner. It cleaves single and double-stranded DNAs by the removal of specific hydrogen atoms from the deoxyribose rings of DNAs [9]. DNA damage leads to apoptotic or non-apoptotic cell death due to mitochondrial damage [33–35]. Naito et al [36] observed cell morphology after the incubation of GO by video-microscopy, which revealed some cells exhibited apoptotic changes, while the remaining cells showed non-apoptotic features. The cytotoxic mechanism of GO is the same as that of free calicheamicin, except for the internalization via CD33. Cells incubated with calicheamicin undergo either temporary or permanent cell cycle arrest depending on the concentration [31, 36]. Transient G_2/M arrest was observed prior to the increase of the hypodiploid portion in cell lines incubated with GO. Several molecular pathways, such as Chk1 and Chk2 phosphorylation and caspase 3, reportedly played roles in this process [37].

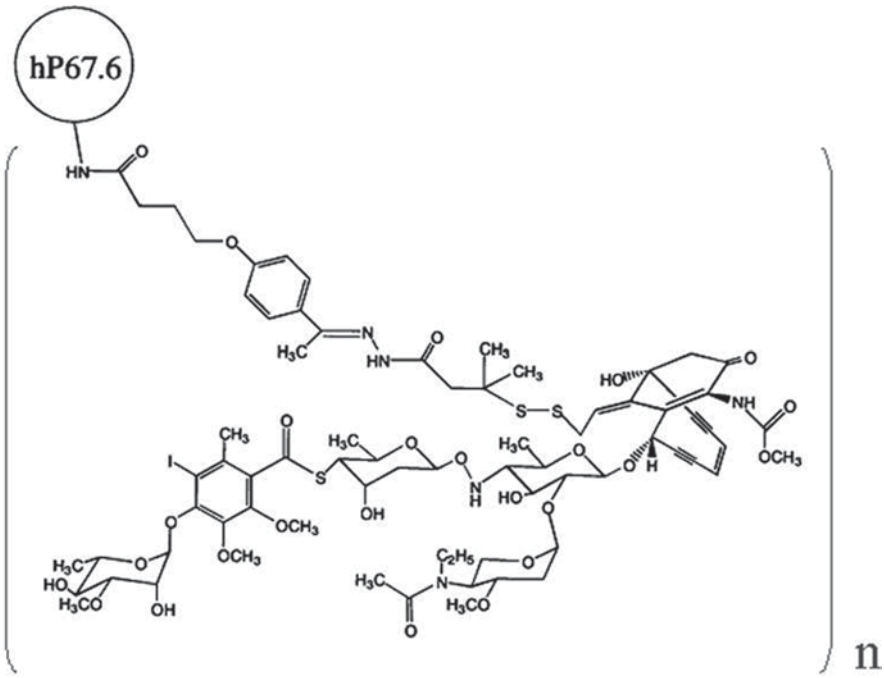

Fig. 7.1 GO is a humanized IgG4 anti-CD33 monoclonal antibody (hP67.6) conjugated to NAc-gamma calicheamicin DMH, a hydrazide derivative of calicheamicin

Cells expressing higher levels of CD33 were reportedly more susceptible to GO [38]. On the other hand, several patients with CD33-negative leukemia have also responded to GO [39]. Several studies have tried to explain the efficacy of GO on CD33-negative leukemia. One proposed explanation is that GO is partially moved into cell by CD33-independent endocytosis [39]. Another is that CD33-negative leukemia cells may have a sub-threshold low amount of CD33, which reacts substantially with GO [40].

7.2.2 GO Monotherapy, Phase I Study

In a phase I study conducted in the U.S., 40 patients with relapsed or refractory (relapsed/refractory) AML were treated by GO (0.25–9 mg/m^2) [41]. Leukemia cells were eliminated from the blood and bone marrow of 8 (20%) of the 40 patients. Neutrophil counts recovered in five of these eight patients, but platelet count recovered in only three. Patients who achieved complete remission (CR) without recovering the platelet count more than 100×10^9/L were entered to the concept of CR with thrombocytopenia (CRp), which has been subsequently used in the evaluation of GO.

7.2.3 Phase II Study

Phase II trials with GO were started at a dose of 9 mg/m^2 (2-week intervals for two doses) [42]. A total of 142 patients with AML in first relapse were enrolled in the study. Of these, 30 % achieved overall response (OR), including CR and CRp. The median relapse-free survival (RFS) was 5.3 months [43]. Grade 3 or 4 bilirubinemia was observed in 23 %, and hepatic transaminitis in 17 %. Hepatic sinusoid obstructed syndrome (SOS) was observed in seven patients (3 %), and three of these were fatal. Five patients, who received hematopoietic stem cell transplantation (HSCT) before the treatment of GO, did not have apparent SOS. However, 3 of 27 patients, who received HSCT after the treatment of GO, died of SOS. Based on these results, the Food and Drug Administration of U.S. approved GO for relapsed CD33-positive AML in patients 60 years of age or older [45].

7.2.4 Drug Resistance via P-glycoprotein

MDR is a phenomenon in which malignant cells acquire cross-resistance to a variety of unrelated cytotoxic drugs. P-gp, one of the most potent MDR mechanisms, is a membrane glycoprotein that actively pumps cytotoxic agents out from cells, and decreases intracellular drug accumulation [44, 45]. Various agents have been introduced to overcome P-gp-associated drug resistance. They include calcium blocker, quinidine, cyclosporine, cepharantin, carotenoids and soforth. Naito et al [36] analyzed the cytotoxic effect of GO on NOMO-1 and NB4 cell lines as well as their multidrug resistant sublines, NOMO-1/MDR and NB4/MDR. They analyzed it by a video-microscopic system, DNA fragmentation, dye exclusion and ^{3}H-thymidine uptake after analysis of CD33, CD34 and P-gp expressions. A concentration-dependent cytotoxic effect of GO was observed in cell lines that expressed CD33. Sensitive cells were temporally arrested at the G2/M phase of the cell cycle before undergoing morphological changes. GO was not effective on the multidrug-resistant sublines compared with the parental cell lines. MDR modifiers, MS209 and PSC833, restored the cytotoxic effect of GO in P-gp-expressing sublines. They concluded that calicheamicin derivatives, which are internalized with GO via CD33 and detached from GO in lysosomes, could be pumped out by P-gp from the cells (Fig. 7.2) [36]. Matsui et al [31] continuously analyzed the *in vitro* effects of GO on leukemia cells from 27 AML patients in relation to the amount of P-gp, MDR-associated protein 1 (MRP1), CD33 and CD34. The effect of GO, estimated by the amount of hypodiploid portion on the cell cycle, was inversely related to the amount of P-gp estimated by the MRK16 monoclonal antibody, and to the P-gp function assessed by intracellular rhodamine-123 accumulation in the presence of MDR modifiers. They showed that MDR modifiers reversed GO resistance in P-gp-expressing CD33$^+$leukemia cells. GO was less effective on CD33$^+$CD34$^+$ than CD33$^+$CD34$^-$ cells. Interestingly, similar results were obtained in studies using inotuzumab ozogamicin (IO), a calicheamicin-conjugated anti-CD22 antibody, for lymphoid malignancies [46, 47]. It will, herein, subsequently be described in detail. Another study showed the cells that

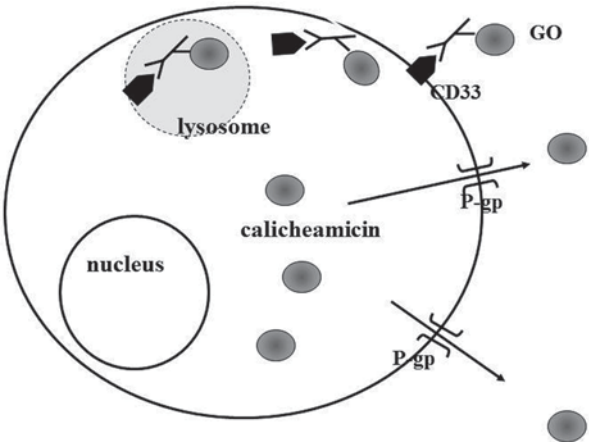

Fig. 7.2 After GO binds to CD33 on the cells, CD33-antibody complexes are internalized and transferred into lysosomes, in which calicheamicin is detached. Intracellularly released calicheamicin derivatives are pumped out via P-gp in multidrug-resistant cells. MDR modifiers recover the effect of GO

were persistently exposed to low-dose GO acquired resistance to GO and expressed P-gp [48]. GO-sensitiveHL-60 cells, which were persistently exposed to low concentrations of GO, changed to GO-resistant HL-60(HL-60/GOR) cells. P-gp was significantly expressed in HL-60/GOR cells, but not in parental HL-60 cells.

These *in vitro* results were confirmed imperviously mentioned phase I studies of GO [40, 41]. Good responders were more frequently observed in leukemia patients characterized by low dye efflux *in vitro*. Any kind of screening tests for P-gp before the treatment of GO might be helpful to have a better clinical outcome. Naito et al [36] suggested that the combination use of GO and MDR modifiers may be an ideal therapeutic approach for P-gp-expressing leukemia, assuming that the hematologic and non-hematologic toxicities are not worsened. This idea has been tried clinically in relapsed/refractory AML.

7.2.5 GO Treatment with MDR Modifier, CyA

Cyclosporin A (CyA), which has been easily-available and widely used as an immunosuppressant, has a considerable effect as an MDR modifier on the other hand. It has, in fact, been administered as an adjunct to GO-containing chemotherapy in the treatment of AML (Table 7.1) [49–51]. Apostolidou et al [49] treated with GO (6 mg/m^2 on day 6), cytosine arabinoside (Ara-C)(1 g/m^2 on days 1–5), liposome-encapsulated daunorubicin (DNR) (75 mg/m^2 on days 6–8) and CyA (on day 6) (MDAC regimen)for 11 patients with relapsed/refractory AML. One (9%) patient achieved a transient CR, and one achieved CRp. Grade 3/4 toxicities included sepsis in 7 patients(63%); hyperbilirubinemia in 6 (54%), and mucositis in 3 (27%).

Tsimberidou et al [50] evaluated the efficacy and toxicity of a combination regimen of GO (6 mg/m^2 on day 1),fludarabine (15 mg/m^2 on days 2–6), AraC (0.5 g/m^2 on days 2–6) and CyA (6 mg/kg on days 1 and 2) (MFAC regimen) in 59 patients with previously untreated AML, refractory anemia with excess blasts (RAEB), or

RAEB in transformation (RAEBT): 39 patients (66%) were AML and 20 patients (34%) were RAEB/RAEBT. CR was achieved in 27 patients (46%) and CRp was achieved in patient (2%). The 1-year OS was 38% and the event-free survival (EFS) in patients with CR/CRp was 27%. Grade 3/4 toxicity included hyperbilirubinemia in 31% and transaminitis in 7% of the patients. Four patients (7%) developed SOS. They conducted a Phase II study of the MFAC regimen in 32 patients with resistant/relapsed AML [51]. Nine (28%) patients achieved CR, and 2 (6%) CRp. The 1-year OS was 19%. Fourteen patients (44%) developed grade 3/4 hyperbilirubinemia, 6 (18%) grade 3/4 hepatic transaminitis, and 3 (9%) SOS.

CyA did not improve the response rate nor survival, although SOS was observed in a considerable number of patients. The unsuccessful attempt of the treatment may be explained by the possibility that CyA ablates the function of P-gp, which is widely distributed across critical organ systems, resulting in increased adverse effects, and that the clinical outcome from the P-gp negative cases assumed influence on the non-significance of the results [52]. Several transporters other than P-gp have also been suggested. MRP1, another well-known transporter protein, is sometimes expressed in AML [53]. However, the clinical importance of MRP1 was relatively limited among the mechanisms of resistance to GO [54]. Other transporters reportedly have further limited effects.

7.2.6 Drug Resistance Other Than P-glycoprotein

The roles of βcl-2 and βcl-x, anti-apoptotic proteins, in the resistance to GO have been reported [55, 56]. GO induced proapoptotic activation of Bak and Bax and stress-activated protein kinase in sensitive AML cells, but not in resistant ones, KG1a AML cells. The effect of GO was enhanced by βcl-2 antisense oligonucleotide, oblimersen sodium, but reduced by over-expression of βcl-2 and βcl-x. Bax, Bak and stress-activated protein kinase may play a role in resistance to GO [57]. The resistance mechanism is not specific for GO, but considerable. Oblimersen (7 mg/kg, days 1–7 and 15–21) was administered with GO (9 mg/m^2 on days 4 and 18) in 48 elderly patients with relapsed AML (Table 7.1) [55]. Twelve patients (25%) achieved OR. The median OS for all patients enrolled was 2.3 months. Grade 3/4 toxicities were sepsis (12%) urinary tract infection (8%), pneumonia (6%) and respiratory events (31%).

The peripheral benzodiazepine receptors (pBzRs) locate in the multiprotein mitochondrial pore complex which regulates mitochondrial membrane potential. Bcl-2 and related anti-apoptotic proteins block apoptosis by keeping the pores closed, but pBzR ligands promote the opening of pores and induce apoptosis. The pBzRs ligand, PK11195, increased the sensitivity of AML cells to standard chemotherapeutics both by inhibiting P-gp and by promoting mitochondrial apoptosis [56]. It increased the sensitivity to GO in AML cells *in vitro*.

Rosen et al [58] reported that the activation of survival signaling pathways, such as PI3K/AKT, MEK/ERK and JAK/STAT, is reportedly associated with GO

Table 7.1 Treatment with GO in combination with multidrug resistant modifiers for the relapsed/refractory AML

Regimen	Combination therapy	GO (/m^2)	No. of cases	Median age	CR (CRp) %	Refractory case (%)	VOD (%)	Authors
MDAC	DNA, AraC, CyA	6 mg × 1	11	37 (16–67)	9 (9)	37	0	Apostlidou et al [49]
MFAC	F, AraC, CyA	4.5 mg × 1	32	53 (18–78)	28 (6)	34	9	Tsimberidou et al [50]
Anti-βcl2	Oblimersen	9 mg × 1	48	67 (>60)	10 (15)	0	0	Moore et al [55]

AraC cytrabine, *CyA* cyclosporine A, *DNR* daunorubicin, *F* fludarabine

resistance *in vitro* in AML cells. An AKT inhibitor, MK-2206, restored the resistance of GO and calicheamicin in resistant AML cells.

The transport of GO into the bone marrow may be important for intensifying the effect of GO [27, 38]. An excess of circulating CD33-positive cells decreased the effect of GO, and resulted in worse outcomes [26, 59]. GO may be spent in the circulation before it reaches the bone marrow [31, 32, 36]. This suggests that GO might be made more effective by the reduction of CD33 in peripheral blood by proceeding chemotherapy [46]. Therefore, GO is often managed several days after the start of induction chemotherapy. However, we understand that a high blast cell count is equally an adverse prognostic factor in leukemias treated with other antileukemic agents.

Several agents may also enhance the effect of GO. G-CSF increased the effect of GO, and induced AML cells to enter G_2/M and a hypodiploid phase [60, 61]. Valproic acid, a histone deacetylase inhibitor, strengthened the effect of GO [62]. However, the synergistic effect of GO with these agents has not been confirmed in clinical studies. Clinically, multiple mechanisms may simultaneously arise in the development of resistance to GO.

7.2.7 Phase III Study with GO for AML and Disappearance from the Market

The Southwest Oncology Group (SWOG) studyS0106 reported the benefit and toxicity of adding GO to standard therapy in 627 patients with *de novo* AML [63]. Patients were randomized to receive induction therapy with DNR (45 mg/m^2 on days 1–3) and AraC (100 mg/m^2 on days 1–7) and GO (6 mg/m^2 on day 4) (AD+GO) or standard induction therapy with DNR (60 mg/m^2 on days 1–3) and AraC (100 mg/m^2 on days 1–7) (AD). After patients achieved CR, they received consolidation therapy with 3 courses of high dose AraC (HiDAC). Patients in remission were re-randomized to the treatment of GO (5 mg/m^2 every 28 days, 3 doses) or observation.

The OR rate was 74 % in both induction arms. The RFS was not significantly different between two arms. Adverse effects were significantly increased in the AD+GO arm. The results of SWOG-S0106 triggered Pfizer Corp. to voluntarily withdraw GO from the market in 2010.

7.2.8 Subsequent Phase III Study for AML

In a subsequent study, 238 patients with *de novo* AML and an intermediate karyotype were treated with standard chemotherapy with or without GO [64]. GO (6 mg/m^2) was added to standard 3 + 7 induction, and to a consolidation of mitoxantrone (MIT) and AraC. The CR rate and early death rate were not different between both groups. Grade 3/4 hepatic toxicities were increased in the GO arm. The EFS and the OS were not changed in both treatment arms. In patients who did not receive HSCT, EFS was significantly higher in the GO arm (54 *vs* 27 %) while OS was not improved.

In the MRC-AML15 trial, 1113 patients with *de novo* AML, excluding APL, were randomly assigned to receive either of the following 3 induction treatments: DNR and AraC; DNR, etoposide (ETP) and AraC; or fludarabine, IDA, AraC and G-CSF; with or without GO (3 mg/m^2) [65]. After achieving remission, 948 patients were randomly assigned to GO (3 mg/m^2) in combination with amsacrine, AraC and ETP or HiDAC (1.5 g or 3 g/m^2). The CR rate or the OS were not significantly different between both groups. Survival benefit of GO was observed in patients with favorable cytogenetics, but not in patients with high-risk cytogenetics. GO did not increase toxicity.

In other results from the UK and Denmark, 1115 patients with AML or high-risk MDS were randomly assigned to receive induction chemotherapy with either DNR (50 mg/m^2 on days 1, 3, 5) and AraC (100 mg/m^2 twice a day on days 1–10) or DNR and clofarabine (20 mg/m^2 on days 1–5), with or without GO (3 mg/m^2) [66]. The OR rates were not different between both groups. GO did not increase toxicity and mortality. Three-year cumulative incidence of relapse was significantly lower, and 3-year OS was significantly better in the patients treated with GO.

Two hundred and seventy-eight elderly patients with *de novo* AML received DNR (60 mg/m^2 on days 1–3) and AraC (200 mg/m^2 for 7 days) without (control group) or with GO (3 mg/m^2 on days 1, 4, and 7) [67]. The OR rate was not different between the two groups. The 2-year-EFS, OS, and RFS were significantly improved by the addition of GO. GO did not increase the risk of death from toxicity.

These recent results demonstrated some advantage for patients treated with GO. In addition, induction mortality was not increased in these studies. Efficacy was observed, typically in patients with favorable-risk, and sometimes in intermediate-risk. The reason for this has not been elucidated. However, multiple resistant mechanisms observed in high-risk could explain it.

7.2.9 The Efficacy of GO for Acute Promyelocytic Leukemia (APL)

APL, which is classified asAML-M3 in the FAB classification system and as APL with t(15;17)(q22;q12) and *PML-RARA* transcript within myeloid malignancies according to the World Health Organization (WHO) classification system [68]. This disease is characterized by differentiation arrest in myeloid precursor cells and their uncontrolled proliferation. All-*trans* retinoic acid (ATRA) has dramatically decreased these complications, and around 90 % of newly-diagnosed patients achieved CR and more than 60 % survived long-term with subsequent post-remission chemotherapy [69–72]. While ATRA combined with chemotherapy has been the standard treatment for patients with APL, approximately 20 % undergo relapse [73–75]. Several salvage therapies, including tamibarotene (Am80), arsenic trioxide (ATO), and stem cell transplantation, have been introduced for the treatment of APL [76, 77]. GO was also administered to APL, and the successful outcome of this therapy has been reported for patients with newly diagnosed or relapsed APL [78–80].

Several reasons have been proposed to explain the efficacy of GO for APL [81, 82]. First, a large amount of CD33 is commonly expressed on the surface of APL cells. Second, the level of P-gp on the surface of APL cells is significantly lower than that of AML. Third, APL cells are highly sensitive for free calicheamicin. Lo-Coco et al [79] reported that 14 of 16 patients with molecularly relapsed APL achieved molecular remission (MR) after GO monotherapy (6 mg/m^{2}at 2-week intervals for three doses). Of 14 responders, seven (50 %) remained in sustained MR for a median of 15 months. GO was administered again in two patients with relapse, and both obtained a new MR.

Another study reported that two patients in a third morphologic relapse with a considerable number of APL cells were treated by GO monotherapy (9 mg/m^2 on days 1 and 15) and achieved CR [80]. One of the patients was treated with consolidation chemotherapy, but the other was not. Both patients had a considerably long CR. GO may represent another treatment option if stem cell transplantation is not being considered in APL.

Aribi et al [81] reported the efficacy of a combination therapy consisting of ATO, ATRA and GO in eight patients with APL in first relapse. Patients were treated with ATO until CR, and then received the consolidation therapy including ATO, ATRA and GO (9 mg/m^2) once a month for 10 months. The second CR was longer than the first CR in 75 %. Moreover, all patients achieved MR. Grade 3/4 non-hematological toxicities were not observed. These reports show that GO is effective for APL patients with molecularly relapsed and advanced relapsed forms of the disease. These data also support the use of GO treatment for APL, which usually have low levels of P-gp and high levels of CD33.

7.3 CD22

CD22, a 140 kD a transmembrane sialo-adhesion glycoprotein, is widely distributed in mature B cells.[83–85] CD22 is a member of the Ig super-family and has seven extracellular Ig-like domains, which mediate cell adhesion tosialic-acid-bearing ligands. The cytoplasmic regions of CD22 have the immune receptor tyrosine activation motifs (ITAM) and tyrosine inhibitory motifs (ITIM). CD22 ITAMs are phosphorylated after BCR activation, and enhance the recruitment of protein tyrosine phosphatases to CD22. The CD22-associated phosphatases then dephosphorylate BCR components resulting in the attenuation of BCR signaling. The function of CD22 is reportedly to modulate the B-cell antigen receptor (BCR) signaling and to regulate cell-cell interactions. The activation of CD22 by ligand binding and cross-linking send negative signals and result in cytotoxicity for B-cell lymphoma [86–89].

7.3.1 Inotuzuma Bozogamicin

Calicheamicin conjugated antibody-targeted chemotherapy strategy has been also applied to B cell malignancies. Because the expression of CD22 is restricted to the B cell lineage and CD22 has a characteristic of internalising molecules, anti-CD22 antibody can be used for targeted delivery of calicheamicin. IO is the calicheamicin conjugated to a humanized IgG4 anti-CD22 mAb, G544, with the linker containing an acid-labile hydrazone. Therefore, the action mechanisms of IO are similar to GO, except that these conjugates recognize distinct molecular targets. Clinical efficacies have been reported in several B cell malignancies [90–92].

7.3.2 Drug Resistance of IO

The reports about the resistant mechanism of IO have not be more frequently found than those of GO. However, the similar resistant mechanisms observed in the studies of GO can be found in IO. The effect of IO was analyzed in relation to CD22 and P-gp in B-cell chronic lymphocytic leukaemia (CLL) and non-Hodgkin lymphoma (NHL) *in vitro* [47]. The cell lines used were the CD22-positive parental Daudi and Raji and their P-gp positive sublines, Daudi/MDR and Raji/MDR. The effect of IO was analyzed by morphology, annexin-V staining, and cell cycle distribution. A dose-dependent, selective cytotoxic effect of IO was observed in cell lines that expressed CD22. CMC-544 was not effective on Daudi/MDR and Raji/MDR cells compared with their parental cells. The MDR modifiers, PSC833 and MS209, restored the cytotoxic effect of CMC-544 in P-gp-expressing sublines. In clinical samples, the cytotoxic effect of CMC-544 was inversely related to the amount of P-gp, and to intracellular rhodamine-123 accumulation. The effect positively correlated with the amount of CD22.

7.4 Conclusion

Antibody-targeted chemotherapy using immunoconjugates of calicheamicin is theoretically an effective therapeutic method in the treatment of cancers. They have improved the specificity and therapeutic effects. They have been used as a single agent or in combination with conventional chemotherapies or other molecular target therapies, and several successes have been reported. However, the immunoconjugates of calicheamicin also acquire drug resistance and, hence, it should be used with understanding of their characteristic features.

GO has introduced a new perspective into the treatment of AML. However, the second evaluation of this treatment did not yield positive results mainly due to MDR. Recent studies have shown the efficacy of GO in AML, with a favorable risk in APL as well. Subsequent evaluations should focus on the efficacy of GO in the core binding factor (CBF) leukemia and its mechanism of action, which may lead to the re-approval of GO. IO is a very potent agent against B cell malignancies. IO action and resistant mechanisms will be similar to GO. Combination therapies with other agents will be promising.

Conflict of Interest No potential conflicts of interest were disclosed.

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References

1. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood. 2005;106:1154–1163.
2. Estey EH. Acute myeloid leukemia: 2013 update on risk-stratification and management. Am J Hematol. 2013;88:318–27.
3. Stein EM, Tallman MS. Remission induction in acute myeloid leukemia. Int J Hematol. 2012;96:164–70.
4. Kuhnl A, Grimwade D. Molecular markers in acute myeloid leukaemia. Int J Hematol. 2012;96:153–63.
5. Naoe T, Kiyoi H. Gene mutations of acute myeloid leukemia in the genome era. Int J Hematol. 2013;97:165–74.
6. Freeman SD, Kelm S, Barber EK, Crocker PR. Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood. 1995;85:2005–12.
7. Lee MD, Dunne TS, Siegel MM, Chang CC, Morton GO. Borders DB. Calicheamicins, a novel family of antitumor antibiotics. 1: Chemistry and partial structure of calicheamicinγ_1. J Am Chem Soc. 1987;109:3464–6.
8. Lee MD, Dunne TS, Chang CC. Calicheamicins, a novel family of antitumor antibiotics. 2: Chemistry and structure of calicheamicinγ1I. J Am Chem Soc. 1987; 109:3466–8.
9. Zein, N, Poncin M, Nilakantan, R, Ellestad, GA. Calicheamicin gamma 1I and DNA: molecular recognition process responsible for site-specificity. Science. 1989;244:697–9.

10. Hangeland JJ, De Voss JJ, Heath JA, Townsend CA, Ding WD, Ashcroft JS, Ellestad GA. Specific abstraction of the 5'(S)- and 4'-deoxyribosyl hydrogen atoms from DNA by calicheamicinγ_1^I. J Am Chem Soc. 1992;114:9200–2.
11. Tchilian EZ, Beverley PC, Young BD, Watt SM. Molecular cloning of two isoforms of the murine homolog of the myeloid CD33 antigen. Blood.1994; 83: 3188–98.
12. Gao, Z, McAlister VC, Williams GM. Repopulation of liver endothelium by bone-marrow-derived cells. Lancet. 2001;357:932–3.
13. Crocker PR. Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling. Curr Opin Struct Biol. 2002;12:609–15.
14. Taylor VC, Buckley CD, Douglas M, Cody AJ, Simmons DL, Freeman SD. The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. J Biol Chem. 1999;274: 11505–12.
15. Andrews RG, Torok-Storb B, Bernstein ID. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood. 1983;62:124–32.
16. Andrews RG, Takahashi M, Segal GM, Powell JS, Bernstein ID, Singer JW. The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. Blood. 1986;68:1030–5.
17. Griffin, J. D, Linch, D, Sabbath, K, Larcom, P, and Schlossman, S. F. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res. 1984;8:521–34.
18. Robertson MJ, Soiffer RJ, Freedman AS, Rabinowe SL, Anderson KC, Ervin TJ, Murray C, Dear K, Griffin JD, Nadler LM, Human bone marrow depleted of CD33-positive cells mediates delayed but durable reconstitution of hematopoiesis: clinical trial of MY9 monoclonal antibody-purged autografts for the treatment of acute myeloid leukemia. Blood. 1992;79:2229–36.
19. Wagner JE, Collins D, Fuller S, Schain LR, Berson AE, Almici C, Hall MA, Chen KE, Okarma TB, Lebkowski JS. Isolation of small, primitive human hematopoietic stem cells: distribution of cell surface cytokine receptors and growth in SCID-Hu mice. Blood. 1995;86:512–23.
20. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID. Expression of normal myeloid-associated antigens by acute leukemia cells. Blood. 1986;67:1048–53.
21. Terstappen, LW, Safford, M, Konemann, S, Loken, MR, Zurlutter, K, Buchner, T, Hiddemann, W, and Wormann, B. Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis. Leukemia. 1992;6:70–80.
22. Putti MC, Rondelli R, Cocito MG, Arico M, Sainati L, Conter V, Guglielmi C, Cantu-Rajnoldi A, Consolini R, Pession A, Zanesco L, Masera G, Biondi A, Basso G. Expression of myeloid markers lacks prognostic impact in children treated for acute lymphoblastic leukemia: Italian experience in AIEOP-ALL 88–91 studies. Blood. 1998;92:795–801.
23. Jilani I, Estey E, Huh Y, Joe Y, Manshouri T, Yared M, Giles F, Kantarjian H, Cortes J, Thomas D, Keating M, Freireich E, Albitar M. Differences in CD33 intensity between various myeloid neoplasms. Am J Clin Pathol. 2002;118: 560–6.
24. Iwamoto S, Deguchi T, Ohta H, Kiyokawa N, Tsurusawa M, Yamada T, Takase K, Fujimoto J, Hanada R, Hori H, Horibe K, Komada Y. Flow cytometric analysis of de novo acute lymphoblastic leukemia in childhood: report from the Japanese Pediatric Leukemia/Lymphoma Study Group. Int J Hematol. 2011;94: 185–92.
25. McGrath MS, Rosenblum MG, Philips MR, Scheinberg DA. Immunotoxin resistance in multidrug resistant cells. Cancer Res. 2003;63:72–9.
26. Scheinberg DA, Lovett D, Divgi CR, Graham MC, Berman E, Pentlow K, Feirt N, Finn RD, Clarkson BD, Gee TS, et al. A phase I trial of monoclonal antibody M195 in acute myelogenous leukemia: specific bone marrow targeting and internalization of radionuclide. J Clin Oncol. 1991;9:478–90.
27. Caron PC, Co MS, Bull MK, Avdalovic NM, Queen C, Scheinberg DA. Biological and immunological features of humanized M195 (anti-CD33) monoclonal antibodies. Cancer Res. 1992;52:6761–7.

28. Borthakur G, Rosenblum MG, Talpaz M, Daver N, Ravandi F, Faderl S, Freireich EJ, Kadia T, Garcia-Manero G, Kantarjian H, Cortes JE. Phase 1 study of an anti-CD33 immunotoxin, humanized monoclonal antibody M195 conjugated to recombinant gelonin (HUM-195/rGEL), in patients with advanced myeloid malignancies. Haematologica. 2013;98:217–21.
29. La Russa VF, Griffin JD, Kessler SW, Cutting MA, Knight RD, Blattler WA, Lambert JM, Wright DG. Effects of anti-CD33 blocked ricin immunotoxin on the capacity of $CD34^+$ human marrow cells to establish in vitro hematopoiesis in long-term marrow cultures. Exp Hematol. 1992;20:442–8.
30. Matsui H, Takeshita A, Naito K, Shinjo K, Shigeno K, Maekawa M, Yamakawa Y, Tanimoto M, Kobayashi M, Ohnishi K, Ohno R. Reduced effect of gemtuzumab ozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers. Leukemia. 2002;16:813–9.
31. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H, Karanes C, Theobald M, Bennett JM, Sherman ML, Berger MS, Eten CB, Loken MR, van Dongen JJ, Bernstein ID, Appelbaum FR. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. J Clin Oncol. 2001;19:3244–54.
32. Hamann PR, Hinman LM, Hollander I, Beyer CF, Lindh D, Holcomb R, Hallett W, Tsou HR, Upeslacis J, Shochat D, Mountain A, Flowers DA, Bernstein I. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconjug Chem. 2002;13:47–58.
33. Zhao B, Konno S, Wu JM, Oronsky AL. Modulation of nicotinamide adenine dinucleotide and poly(adenosine diphosphoribose) metabolism by calicheamicin gamma 1 in human HL-60 cells. Cancer Lett. 1990;50:141–7.
34. Nicolaou KC, Pitsinos EN, Theodorakis EA, Saimoto H, Wrasidlo W. Synthetic calicheamicin mimics with novel initiation mechanisms: DNA cleavage, cytotoxicity, and apoptosis. Chem Biol. 1994;1:57–66.
35. Lode HN, Reisfeld RA, Handgretinger R, Nicolaou KC, Gaedicke G, Wrasidlo W. Targeted therapy with a novel enediyene antibiotic calicheamicin theta(I)1 effectively suppresses growth and dissemination of liver metastases in a syngeneic model of murine neuroblastoma. Cancer Res. 1998;58:2925–8.
36. Naito K, Takeshita A, Shigeno K, Nakamura S, Fujisawa S, Shinjo K, Yoshida H, Ohnishi K, Mori M, Terakawa S, Ohno R. Calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (gemtuzumab zogamicin, CMA-676) shows cytocidal effect on CD33-positive leukemia cell lines, but is inactive on P-glycoprotein-expressing sublines. Leukemia. 2000;14:1436–43.
37. Amico D, Barbui AM, Erba E, Rambaldi A, Introna M, Golay J. Differential response of human acute myeloid leukemia cells to gemtuzumab ozogamicin in vitro: role of Chk1 and Chk2 phosphorylation and caspase 3. Blood. 2003;101: 4589–97.
38. Walter RB, Raden BW, Kamikura DM, Cooper JA, Bernstein ID. Influence of CD33 expression levels and ITIM-dependent internalization on gemtuzumab ozogamicin-induced cytotoxicity. Blood. 2005;105:1295–302.
39. Jedema I, Barge RM, van der Velden VH, Nijmeijer BA, van Dongen JJ, Willemze R, Falkenburg JH. Internalization and cell cycle-dependent killing of leukemic cells by Gemtuzumab Ozogamicin: rationale for efficacy in CD33-negative malignancies with endocytic capacity. Leukemia. 2004;18:316–25.
40. Linenberger ML. CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. Leukemia. 2005;19:176–82.
41. Sievers EL, Appelbaum FR, Spielberger RT, Forman SJ, Flowers D, Smith FO, Shannon-Dorcy K, Berger MS, Bernstein ID. Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a phase I study of an anti-CD33 calicheamicin immunoconjugate. Blood. 1999;93:3678–84.

42. Larson RA, Sievers EL, Stadtmauer EA, Lowenberg B, Estey EH, Dombret H, Theobald M, Voliotis D, Bennett JM, Richie M, Leopold LH, Berger MS, Sherman ML, Loken MR, van Dongen JJ, Bernstein ID, Appelbaum FR. Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. Cancer. 2005;104:1442–52.
43. Bross PF, Beitz J, Chen G, Chen XH, Duffy E, Kieffer L, Roy S, Sridhara R, Rahman A, Williams G, Pazdur R. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin Cancer Res. 2001;7: 1490–6.
44. Kartner N, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature. 1985;316:820–3.
45. Gottesman MM, Pastan I, Ambudkar SV. P-glycoprotein and multidrug resistance. Curr Opin Genet Dev. 1996;6:610–7.
46. Takeshita A, Yamakage N, Shinjo K, Ono T, Hirano I, Nakamura S, Shigeno K, Tobita T, Maekawa M, Kiyoi H, Naoe T, Ohnishi K, Sugimoto Y, Ohno R. CMC-544 (inotuzumab ozogamicin), an anti-CD22 immuno-conjugate of calicheamicin, alters the levels of target molecules of malignant B-cells. Leukemia. 2009;23:1329–36.
47. Takeshita A, Shinjo K, Yamakage N, Ono T, Hirano I, Matsui H, Shigeno K, Nakamura S, Tobita T, Maekawa M, Ohnishi K, Sugimoto Y, Kiyoi H, Naoe T, Ohno R. CMC-544 (inotuzumab ozogamicin) shows less effect on multidrug resistant cells: analyses in cell lines and cells from patients with B-cell chronic lymphocytic leukaemia and lymphoma. Br J Haematol. 2009;146:34–43.
48. Matsumoto T, Jimi S, Hara S, Takamatsu Y, Suzumiya J, Tamura K. Importance of inducible multidrug resistance 1 expression in HL-60 cells resistant to gemtuzumab ozogamicin. Leuk Lymphoma. 2012;53:1399–405.
49. Apostolidou E, Cortes J, Tsimberidou A, Estey E, Kantarjian H, Giles FJ. Pilot study of gemtuzumab ozogamicin, liposomal daunorubicin, cytarabine and cyclosporine regimen in patients with refractory acute myelogenous leukemia. Leuk Res. 2003;27:887–91.
50. Tsimberidou A, Estey E, Cortes J, Thomas D, Faderl S, Verstovsek S, Garcia-Manero G, Keating M, Albitar M, O'Brien S, Kantarjian H, Giles F. Gemtuzumab, fludarabine, cytarabine, and cyclosporine in patients with newly diagnosed acute myelogenous leukemia or high-risk myelodysplastic syndromes. Cancer. 2003;97:1481–7.
51. Tsimberidou A, Cortes J, Thomas D, Garcia-Manero G, Verstovsek S, Faderl S, Albitar M, Kantarjian H, Estey E, Giles FJ. Gemtuzumab ozogamicin, fludarabine, cytarabine and cyclosporine combination regimen in patients with $CD33^{+}$primary resistant or relapsed acute myeloid leukemia. Leuk Res. 2003;27: 893–7.
52. Takeshita A. Efficacy and resistance of gemtuzumab ozogamicin for acute myeloid leukemia. Int J Hematol. 2013;97:703–16.
53. Legrand O, Zittoun R, Marie JP. Role of MRP1 in multidrug resistance in acute myeloid leukemia. Leukemia. 1999;13:578–84.
54. Cianfriglia M, Mallano A, Ascione A, Dupuis ML. Multidrug transporter proteins and cellular factors involved in free and mAb linked calicheamicin-gamma1 (gentuzumab ozogamicin, GO) resistance and in the selection of GO resistant variants of the HL60 AML cell line. Int J Oncol. 2010;36:1513–20.
55. Moore, J, Seiter, K, Kolitz, J, Stock, W, Giles, F, Kalaycio, M, Zenk, D, and Marcucci, GA Phase II study of Bcl-2 antisense (oblimersen sodium) combined with gemtuzumab ozogamicin in older patients with acute myeloid leukemia in first relapse. Leuk Res. 2006;30:777–83.
56. Walter RB, Raden BW, Cronk MR, Bernstein ID, Appelbaum FR, Banker DE. The peripheral benzodiazepine receptor ligand PK11195 overcomes different resistance mechanisms to sensitize AML cells to gemtuzumab ozogamicin. Blood. 2004;103:4276–84.
57. Haag P, Viktorsson K, Lindberg ML, Kanter L, Lewensohn R, Stenke L. Deficient activation of Bak and Bax confers resistance to gemtuzumab ozogamicin-induced apoptotic cell death in AML. Exp Hematol. 2009;37:755–66.
58. Rosen DB, Harrington KH, Cordeiro JA, Leung LY, Putta S, Lacayo N, Laszlo GS, Gudgeon CJ, Hogge DE, Hawtin RE, Cesano A, Walter RB. AKT signaling as a novel factor associ-

ated with in vitro resistance of human AML to gemtuzumab ozogamicin. PLoS One. 2013;8: e53518.
59. van der Velden VH, Boeckx N, Jedema I, te Marvelde JG, Hoogeveen PG, Boogaerts M, van Dongen JJ. High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. Leukemia. 2004;18:983–8.
60. Kell WJ, Burnett AK, Chopra R, Yin JA, Clark RE, Rohatiner A, Culligan D, Hunter A, Prentice AG, Milligan DW. A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. Blood. 2003;102:4277–83.
61. Fianchi L, Pagano L, Leoni F, Storti S, Voso MT, Valentini CG, Rutella S, Scardocci A, Caira M, Gianfaldoni G, Leone G. Gemtuzumab ozogamicin, cytosine arabinoside, G-CSF combination (G-AraMy) in the treatment of elderly patients with poor-prognosis acute myeloid leukemia. Ann Oncol. 2008;19:128–34.
62. ten Cate B, Samplonius DF, Bijma T, de Leij LF, Helfrich W, Bremer E. The histone deacetylase inhibitor valproic acid potently augments gemtuzumab ozogamicin-induced apoptosis in acute myeloid leukemic cells. Leukemia. 2007; 21:248–52.
63. Petersdorf SH, Kopecky KJ, Slovak M, Willman C, Nevill T, Brandwein J, Larson RA, Erba HP, Stiff PJ, Stuart RK, Walter RB, Tallman MS, Stenke L, Appelbaum FR. A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. Blood. 2013;121:4854–60.
64. Delaunay J, Recher C, Pigneux A. Addition of gemtuzumab ozogamycin to chemotherapy improves event-free survival but not overall survival of AML patients with intermediate cytogenetics not eligible for allogeneic transplantation. Results of the GOELAMS AML 2006 IR study (Abstract#79). Blood. 2011;118:37–8.
65. Burnett AK, Hills RK, Milligan D, Kjeldsen L, Kell J, Russell NH, Yin JA, Hunter A, Goldstone AH, Wheatley K. Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. J Clin Oncol. 2011;29:369–77.
66. Burnett AK, Russell NH, Hills RK, Kell J, Freeman S, Kjeldsen L, Hunter AE, Yin J, Craddock CF, Dufva IH, Wheatley K, Milligan D. Addition of gemtuzumab ozogamicin to induction chemotherapy improves survival in older patients with acute myeloid leukemia. J Clin Oncol. 2012;30:3924–31.
67. Castaigne S, Pautas C, Terre C, Raffoux E, Bordessoule D, Bastie JN, Legrand O, Thomas X, Turlure P, Reman O, de Revel T, Gastaud L, de Gunzburg N, Contentin N, Henry E, Marolleau JP, Aljijakli A, Rousselot P, Fenaux P, Preudhomme C, Chevret S, Dombret H. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. Lancet. 2012;379:1508–16.
68. Swerdlow SH, Campo E, Harris NL. Acute myeloid leukaemia with recurrent genetic abnormalities: WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008. pp. 110–23
69. Warrell RP, Jr, de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med. 1993;329:177–89.
70. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008;111:2505–15.
71. Fenaux P, Le Deley MC, Castaigne S, Archimbaud E, Chomienne C, Link H, Guerci A, Duarte M, Daniel MT, Bowen D et al. Effect of all transretinoic acid in newly diagnosed acute promyelocytic leukemia. Results of a multicenter randomized trial. European APL 91 Group. Blood. 1993;82:3241–9.
72. Ohno R, Yoshida H, Fukutani H, Naoe T, Ohshima T, Kyo T, Endoh N, Fujimoto T, Kobayashi T, Hiraoka A et al. Multi-institutional study of all-trans-retinoic acid as a differentiation therapy of refractory acute promyelocytic leukemia. Leukaemia Study Group of the Ministry of Health and Welfare. Leukemia. 1993;7:1722–7.

73. Asou N, Adachi K, Tamura U, Kanamaru A, Kageyama S, Hiraoka A, Omoto E, Akiyama H, Tsubaki K, Saito K, Kuriyama K, Oh H, Kitano K, Miyawaki S, Takeyama U, Yamada O, Nishikawa K, Takahashi M, Matsuda S, Ohtake H, Ohno R. Analysis of prognostic factors in newly diagnosed patients with acute promyelocytic leukemia: the APL92 study of the Japan Adult Leukemia Study Group (JALSG). Cancer Chemother Pharmacol. 2001;48(Suppl. 1):S65–71.
74. Ohno R, Asou N, Ohnishi K. Treatment of acute promyelocytic leukemia: strategy toward further increase of cure rate. Leukemia. 2003;17:1454–63.
75. Douer D. New advances in the treatment of acute promyelocytic leukemia. Int J Hematol. 2002;76(Suppl. 2):179–87.
76. Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A, Karasuno T, Takeuchi M, Miyawaki S, Ueda R, Naoe T, Ohno R. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. Blood. 1997;90:967–73.
77. Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA, Pandolfi PP, Warrell RP Jr. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med. 1998;339:1341–8.
78. Estey EH, Giles FJ, Beran M, O'Brien S, Pierce SA, Faderl SH, Cortes JE, Kantarjian HM. Experience with gemtuzumab ozogamycin ("mylotarg") and all-trans retinoic acid in untreated acute promyelocytic leukemia. Blood. 2002;99:4222–4.
79. Lo-Coco F, Cimino G, Breccia M, Noguera NI, Diverio D, Finolezzi E, Pogliani EM, Di Bona E, Micalizzi C, Kropp M, Venditti A, Tafuri A, Mandelli F. Gemtuzumab ozogamicin (Mylotarg) as a single agent for molecularly relapsed acute promyelocytic leukemia. Blood. 2004;104:1995–9.
80. Takeshita A, Shinjo K, Naito K, Matsui H, Sahara N, Shigeno K, Suzumura T, Horii T, Shirai N, Maekawa M, Yada Y, Teshima H, Takeuchi J, Ohnishi K, Ohno R. Two with all-trans retinoic acid-resistant acute promyelocytic leukemia treated successfully with gemtuzumab ozogamicin as a single agent. Int J Hematol. 2005;82:445–8.
81. Takeshita A, Shinjo K, Naito K, Matsui H, Sahara N, Shigeno K, Horii T, Shirai N, Maekawa M, Ohnishi K, Naoe T, Ohno R. Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells. Leukemia. 2005;19:1306–11.
82. Aribi A, Kantarjian HM, Estey EH, Koller CA, Thomas DA, Kornblau SM, Faderl SH, Laddie NM, Garcia-Manero G, Cortes JE. Combination therapy with arsenic trioxide, all-trans retinoic acid, and gemtuzumab ozogamicin in recurrent acute promyelocytic leukemia. Cancer. 2007;109:1355–9.
83. Collins BE, Blixt O, Han S, Duong B, Li H, Nathan JK, Bovin N, Paulson JC. High-affinity ligand probes of CD22 overcome the threshold set by cis ligands to allow for binding, endocytosis, and killing of B cells. J Immunol. 2006;177:2994–3003.
84. Engel P, Wagner N, Miller AS, Tedder TF. Identification of the ligand-binding domains of CD22, a member of the immunoglobulin superfamily that uniquely binds a sialic acid-dependent ligand. J Exp Med. 1995; 181:1581–6.
85. Haas KM, Sen S, Sanford IG, Miller AS, Poe JC, Tedder TF. CD22 ligand binding regulates normal and malignant B lymphocyte survival in vivo. J Immunol. 2006;177:3063–73.
86. Tedder TF, Poe JC, Haas KM. CD22: a multifunctional receptor that regulates B lymphocyte survival and signal transduction. Adv Immunol. 2005; 88:1–50.
87. Tedder TF, Tuscano J, Sato S, Kehrl JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. Annu Rev Immunol. 1997;15:481–504.
88. Tuscano J, Engel P, Tedder TF, Kehrl JH. Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22 L interactions impairs T-cell proliferation. Blood. 1996;87:4723–30.
89. Tuscano JM, Riva A, Toscano SN, Tedder TF, Kehrl JH. CD22 cross-linking generates B-cell antigen receptor-independent signals that activate the JNK/SAPK signaling cascade. Blood. 1999;94:1382–92.

90. Kantarjian H, Thomas D, Jorgensen J, Jabbour E, Kebriaei P, Rytting M, York S, Ravandi F, Kwari M, Faderl S, Rios MB, Cortes J, Fayad L, Tarnai R, Wang SA, Champlin R, Advani A, O'Brien S. Inotuzumab ozogamicin, anti-CD22-calecheamicin conjugate, for refractory and relapsed acute lymphocytic leukaemia: a phase 2 study. Lancet Oncol. 2012;13:403–11.
91. Advani A, Coiffier B, Czuczman MS, Dreyling M, Foran J, Gine E, Gisselbrecht C, Ketterer N, Nasta S, Rohatiner A, Schmidt-Wolf IG, Schuler M, Sierra J, Smith MR, Verhoef G, Winter JN, Boni J, Vandendries E, Shapiro M, Fayad L. Safety, pharmacokinetics, and preliminary clinical activity of inotuzumab ozogamicin, a novel immunoconjugate for the treatment of B-cell non-Hodgkin's lymphoma: results of a phase I study. J Clin Oncol. 2010;28:2085–93.
92. Ogura M, Tobinai K, Hatake K, Uchida T, Kasai M, Oyama T, Suzuki T, Kobayashi Y, Watanabe T, Azuma T, Mori M, Terui Y, Yokoyama M, Mishima Y, Takahashi S, Ono C, Ohata J. Phase I study of inotuzumab ozogamicin (CMC-544) in Japanese patients with follicular lymphoma pretreated with rituximab-based therapy. Cancer Sci. 2010;101:1840–5.

Chapter 8
Engineered Versions of Granzyme B and Angiogenin Overcome Intrinsic Resistance to Apoptosis Mediated by Human Cytolytic Fusion Proteins

Christian Cremer, Grit Hehmann-Titt, Sonja Schiffer, Georg Melmer, Paolo Carloni, Stefan Barth and Thomas Nachreiner

Abstract The use of therapies based on antibody fusion proteins for the selective elimination of tumor cells has increased markedly over the last two decades because the severe side effects associated with conventional chemotherapy and radiotherapy are reduced or even eliminated. However, the initial development of *immunotoxins* suffered from a number of drawbacks such as nonspecific cytotoxicity and the induction of immune responses because the components were non-human in origin. The most recent iteration of this approach is a new class of targeted *human cytolytic fusion proteins* (hCFPs) comprising a tumor-specific targeting component such as a human antibody fragment fused to a human effector domain with pro-apoptotic activity. Certain tumors resist the activity of hCFPs by upregulating the intracellular expression of native inhibitors, which rapidly bind and inactivate the human effector domains. Higher doses of the hCFPs are, therefore, required to improve therapeutic efficacy. To circumvent these inhibitory processes, novel isoforms of the enzymes *granzyme B* and *angiogenin* have been designed to increase their intrinsic activity and reduce their interactions with native inhibitors resulting in more potent hCFPs that can be applied at lower doses. This chapter summarizes the basic scien-

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tific knowledge that can facilitate the rational development of human enzymes with novel and beneficial characteristics, including the ability to avoid neutralization by native inhibitors.

Keywords Targeted therapy · Human cytolytic fusion protein · Apoptosis · Effector domain · Angiogenin · Granzyme B · Tumor-specific binding domain · Natural inhibitor · Serpin B9 · PI-9 · RNH1

Abbreviations

Å	Angström
ADC	Antibody drug conjugate
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukemia
AMML	Acute myelomonocytic leukemia
APAF 1	Apoptoticproteaseactivatingfactor 1
AV	Annexin V
BID	BH3 interacting domain death agonist
CASM	Computer-aided simulation modeling
CMML	Chronic myelomonocytic leukemia
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
Cyt c	Cytochrome C
DAPK2	Death-associated proteinkinase 2
dATP	Deoxyadenosine triphosphat
DC	Dendritic cell
DFF45	DNA fragmentation factor-45
DFG	Deutsche Forschungsgemeinschaft
DNA	Deoxyribonucleicacid
DNA PK	DNA-dependentproteinkinase
DNMT2	DNA methyltransferase 2
DPPI	Dipeptidyl peptidase 1
EBV	Epstein-Barr virus
EC50	Half maximal effective concentration
EFRE	European Fund for Regional Development
ER	Endoplasmicreticulum
ETA	Pseudomonas aeruginosaexotoxin A
ETA’	Truncated version of the Pseudomonas aeruginosa exotoxin A
FDA	Food and Drug Administration
GrB	Granzyme B
HAMA	Human anti-mouse antibody
hCFP	Human cytolytic fusion protein
HEK	Human embryonic kidney
hLHR	Human luteinizing hormone receptor
ICAD	Inhibitor of caspase-activated DNase

IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entrysite
LeY	Lewis Y antigen
LPS	Lipopolysaccharide
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
mRNP	mRNA-based ribonucleoproteins
NKcells	Natural killer cells
NLS	Nuclear localization signal
NRW	North-Rhine Westphalia
NuMA	Nuclear mitotic apparatus protein
PARP	Poly (ADP-ribose) polymerase
PEG	Polyethylene glycol
PI	Propidium iodide
PI 9	Proteinase inhibitor-9
raPIT5a	Rat pituitary gland
RCL	Reactive center loop
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNH1	Ribonuclease/angiogen ininhibitor 1
RPMI	Roswell Park Memorial Instiute
rRNA	Ribosomal RNA
scFv	Single chain fragment variable
SDS	Sodium dodecyl sulfate
tiRNA	tRNA-derived stress-induced RNA
TNF	Tumor necrosis factor
tRNA	Transfer RNA
XIAP	X-linked inhibitor of apoptosis protein
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

8.1 Introduction: From Classical Immunotoxins to Human Cytolytic Fusion Proteins

The treatment of cancer is still dominated by the classical approaches of surgery, chemotherapy and radiotherapy. These techniques are notorious for their off-target activity, leading to serious and often life-threatening side effects as well as the development of drug resistance and high relapse rates due to the survival of residual tumor cells. Targeted cancer therapy can reduce the side effects associated with conventional treatments, thus, providing versatile tools for the continuing fight against cancer.

The first targeted cancer therapies were monoclonal antibodies, which implemented Paul Ehrlich's concept of using 'magic bullets' to selectively fight disease-causing organisms. At least 12 therapeutic antibodies have already entered the market [1] and many more are currently undergoing clinical development [2]. However, full-size antibodies often lack therapeutic efficacy due to their poor tumor penetration and the need for high doses to compete with serum immunoglobulins [3, 4].

Further development involved the coupling of antibodies to toxic molecules to increase their potency, resulting in two distinct concepts: antibody drug conjugates (ADCs) and immunotoxins [5]. Whereas ADCs are antibodies chemically conjugated to a toxin, drug or radionuclide, the toxic component of immunotoxins is a naturally occurring protein originating from bacteria or plants. The most prominent examples are *Pseudomonas aeruginosa* exotoxin A (ETA) [6] and the castor bean ricin A chain [7]. Thus far, four generations of immunotoxins have been described. The first generation was a subset of the ADCs, essentially full-size antibodies chemically conjugated to whole-protein toxins. The technological and pharmacological disadvantages of these molecules led to their replacement by second and third generations of immunotoxins which were genetic fusions comprising native ligands or single-chain variable fragments (scFv) as the targeting component and truncated bacterial or plant-derived toxins with the natural cell-binding domain deleted (Fig. 8.1). These fusion proteins are easier to manufacture, they have fewer side effects, and they are more efficacious due to enhanced tumor penetration and stability. The therapeutic potential of ADCs and immunotoxins has been demonstrated in several clinical trials [3, 8, 9] and a handful have already entered the market: Brentuximab vedotin, an ADC comprising the anti-CD30 antibody brentuximab coupled to the antimitotic agent monomethyl auristatin E for the treatment of Hodgkin's lymphoma [10], trastuzumab emtansine, an ADC comprising the Her-2 specific antibody trastuzumab (Herceptin) linked to the maytensine derivative DM1 for the treatment of Her2-positive metastatic breast cancer [11], and finally Denileukin diftitox (Ontak®), a fusion protein comprising the diphtheria toxin and interleukin-2 for the treatment of non-Hodgkin's lymphoma [12].

One major drawback of the classic immunotoxins is their immunogenicity, which is caused by both their component domains. The use of murine antibodies or fragments thereof induces a human anti-mouse antibody (HAMA) response in some patients, which reduces the efficacy of molecules containing murine antibody components and causes allergic reactions with life-threatening complications in extreme cases [13, 14]. The early generation of immunotoxins is, therefore unsuitable for long-term treatment regimens involving multiple doses, which is often necessary for cancer therapy. Complete 'humanization' is therefore a prerequisite for future clinical development [15, 16]. Numerous strategies have been developed to reduce the immunogenicity of immunotoxins, including the co-administration of immunosuppressive drugs [17, 18], derivatization of the antibody with polyethylene glycol (PEG) to mask the protein from immune surveillance [19], and the removal or modification of major B-cell and T-cell epitopes [20, 21]. More recent antibody-engineering techniques have allowed the development of humanized or fully human antibodies by grafting the complementarity-determining regions onto a

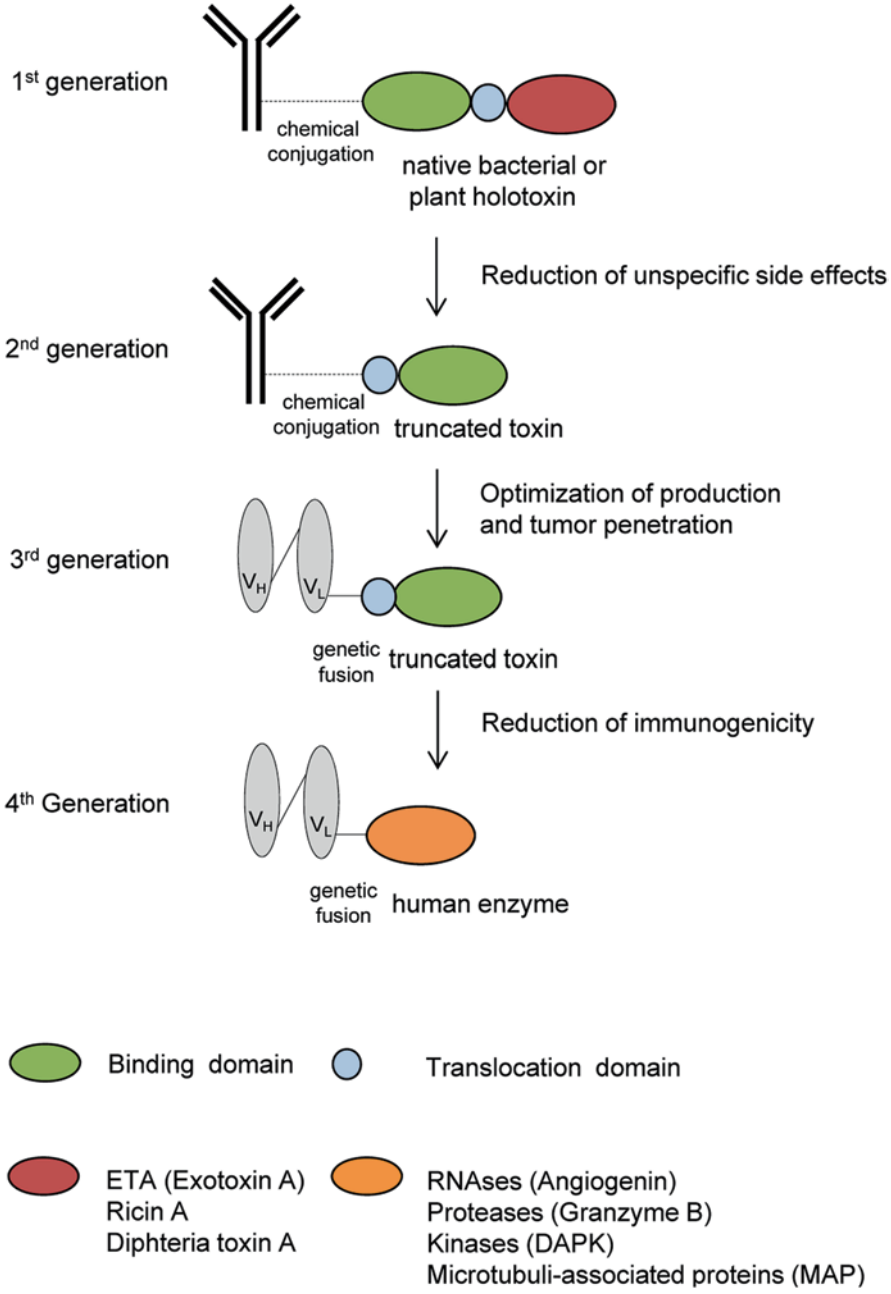

Fig. 8.1 Generations of immunotoxins. First generation immunotoxins were created by the chemical conjugation of antibodies and intact native toxins. Removal of the non-specific binding domain while retaining the native translocation domain of the toxin moiety led to second generation immunotoxins with markedly increased specificity. Recombinant DNA techniques allowed the production of third generation immunotoxins in which the native cell binding domain of toxins

human framework, using transgenic mice expressing human immunoglobulin genes or screening human-antibody phage display libraries [22, 23].

Even if the antibody component is upgraded, the bacterial and plant-derived toxins can still elicit an immune response. Complete humanization is, therefore, achieved by replacing such toxins with pro-apoptotic human proteins that induce cell death. This step has yielded the fourth and most recent generation of immunotoxins, which are known as *human cytolytic fusion proteins* (hCFPs). We have developed a series of hCFPs using effector domains based on the human serine protease granzyme B [24], human ribonucleases (RNases) such as angiogenin [25], human kinases such as death-associated protein kinase 2 (DAPK2) [26], and the microtubule-associated proteins (tau) [27]. Further examples of human effector domains include death receptor ligands and pro-apoptotic members of the BCL-2 family [16, 28]. Several hCFPs have already been tested to confirm their selective activity and efficiency *in vitro* and *in vivo* [16, 29].

8.2 Human Granzyme B

8.2.1 The Role of Granzyme B in Immune Surveillance

Several key features of the immune system help to prevent the development of cancer. Cytotoxic lymphocytes, which include natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), are the main defense against cells infected with intracellular pathogens or transformed into a tumorigenic phenotype [30]. Cytotoxic lymphocytes use two major pathways to induce apoptosis in infected or transformed cells. The first is the death receptor pathway mediated by the stimulation of death receptors on the tumor cell surface, i.e. CD95 (Fas receptor), the tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2, and the death receptors DR4 and DR5 [31, 32]. The second is the granule exocytosis pathway, mediated by the pore-forming protein perforin and a family of pro-apoptotic serine proteases (granzymes) [33]. Five human genes have been identified thus far encoding structurally related granzymes (*B, A, H, K and M*) with unique expression profiles and substrates specificities [34–37].

Granzyme B, a 32-kDa serine protease, is one of the key effector molecules in granule-mediated apoptosis during both the specific and non-specific host rejection of tumors and virus-infected cells by cytotoxic lymphocytes [38, 39]. The protease is expressed as an inactive enzyme precursor carrying an N-terminal signal peptide sequence. After cleavage of the signal peptide in the endoplasmic reticulum (ER),

is genetically replaced by shorter antibody fragments (e.g. scFvs) or natural ligands, thus, improving manufacturing and tumor cell penetration. Within the fourth generation immunotoxins (human cytolytic fusion proteins), the toxin moiety, an endogenous cell death inducing protein of human origin, is genetically fused to humanized/human antibody fragments or natural ligands in order to reduce immunogenicity

granzyme B is glycosylated with two mannose-6-phosphate groups. The glycosylated protein is then directed into the secretory granules of NK cells and CTLs where it is activated by dipeptidyl peptidase I (DPPI)/Cathepsin C [40, 41]. Within the granules, granzyme B and perforin are stored as a multimeric complex using the proteoglycan serglycin as a scaffold [42]. Following target cell recognition using an elaborate repertoire of receptors, and then effector cell activation, the cytotoxic granules containing granzyme B/perforin complexes (as well as other granzymes) are released by calcium-dependent exocytosis into the submicroscopic intercellular cleft (immunological synapse) formed with the target cell [43, 44]. The physiological substrates of granzyme B are located predominantly in the cytosol but also in the nucleus [45]. The enzyme must, therefore, be translocated from the immunological synapse across the target cell plasma membrane to the specific site of action, and this step is not fully understood despite numerous investigations. At least two models of granzyme B uptake have been proposed, both based on the membrane-penetrating properties of perforin [46].

Once released, granzyme B activates several pro-apoptotic pathways by cleaving and activating multiple protein substrates, including procaspases and structural proteins of the cytoskeleton, as well as proteins involved in protein folding and DNA repair (Fig. 8.2) [47]. It displays an unusual specificity for aspartate or glutamate at the P1 site within a specific P4–P1 tetrapeptide motif (Ile-Glu-Pro-Asp/Glu) in its substrates [36]. Granzyme B cleaves the initiator procaspase-8, promoting its homodimerization and subsequent activation of the mitochondrial pathway. It also directly activates procaspase-3 and procaspase-7, thus triggering apoptosis at multiple points along the caspase-dependent pathway. Likewise, granzyme B directly activates the mitochondrial pathway by cleaving the BH3-interacting domain death agonist (BID) [48]. Activation of the mitochondrial apoptotic machinery by granzyme B, either indirectly by caspase-8 or by the direct generation of truncated BID (tBID), is driven by the translocation of tBID into the mitochondria and the local activation of the pro-apoptotic BCL-2 family members BAX and BAK. Once cleaved, these proteins promote mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c (cyt c) from the mitochondrial intermembrane space, accompanied by further pro-apoptotic proteins such as Smac/DIABLO and OMI/HTRA2, which promote caspase activation by blocking the inhibitor of apoptosis proteins (IAP) [49].

In the presence of dATP, the cytosolic release of cytochrome c results in the binding of the apoptotic protease activating factor 1 (APAF-1) and the formation of the heptameric apoptosome complex [50]. Apoptosome formation promotes the autocatalytic activation of procaspase-9 and subsequent processing of procaspase-3 and procaspase-7 [51], which coordinate the execution phase of programmed cell death by cleaving multiple intracellular death substrates (Fig. 8.2). This leads to the typical morphological changes associated with apoptosis including DNA fragmentation, chromatin condensation, cell shrinkage, plasma membrane blebbing and the formation of apoptotic bodies. Granzyme B is also capable of activating the downstream death substrates of caspases, reflecting its caspase-like preference for an aspartate in the P1 position [52–56]. This mitochondria/caspase-independent pathway

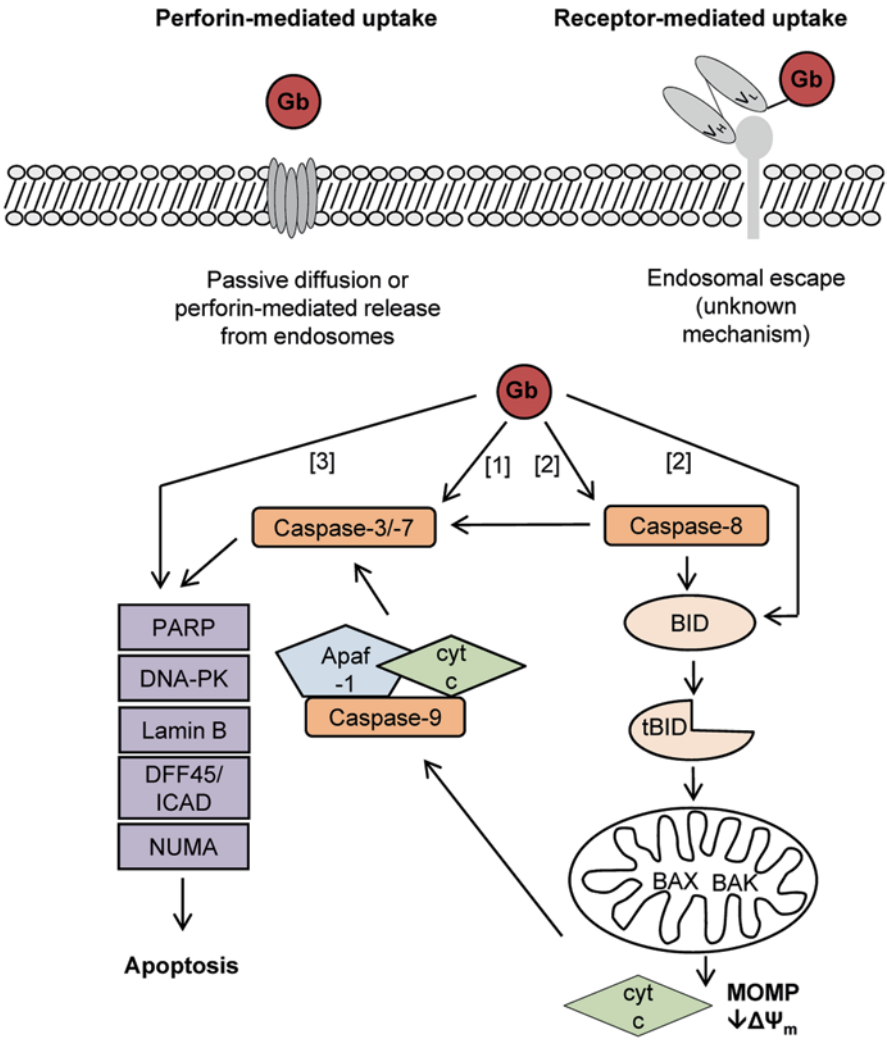

Fig. 8.2 Multiple pathways of granzyme B-mediated apoptosis induction. Two proposed mechanisms of perforin-facilitated delivery of native granzyme B to the target cell cytosol are described: Passive diffusion in response to perforin-induced Ca^{2+}-dependent membrane pore formation or cytosolic release by perforin-mediated endosomal membrane disruption. Fusion of granzyme B to a targeting moiety directs specific binding to a cell surface receptor which is constitutively endocytosed. Once released into the cytosol, granzyme B utilizes three major mechanisms to induce apoptosis in target cells: [1] direct cleavage of effector caspases (*caspase pathway*) [2] activation of the mitochondrial pathway, either directly by the cleavage or Bid, or indirectly by caspase-8 activation or [3] direct cleavage of cellular death substrates like the nuclear mitotic apparatus protein (NuMA), PARP (poly(ADP-ribose) polymerase), DNA-PK (*DNA-dependent protein kinase*), Lamin B and DFF45 (*DNA fragmentation factor-45*)/ICAD (*inhibitor of caspase-activated DNase*) (*mitochondria/caspase-independent pathway*)

bypasses the strict regulatory control of the caspase cascade allowing the reliable induction of apoptosis in target cells.

Granzyme B may also have an extracellular, perforin-independent non-toxic role that contributes to innate and adaptive immunity by processing cytokines and disrupting endothelial cell–cell contacts allowing the extravasation of leukocytes into affected tissues [57]. The degradation of extracellular matrix proteins by granzyme B is likely to play a significant role in the pathophysiology of many chronic inflammatory diseases, including autoimmunity and transplant rejection [39].

8.2.2 Therapeutic Potential and Limitations of Granzyme B for the Treatment of Cancer

The inherent advantages of granzyme B as an immunotherapeutic toxin include its role as a pleiotropic effector molecule for NK cells and CTLs, its broad portfolio of apoptosis-inducing mechanisms, its human origin (reducing its immunogenicity) and the ease with which hCFPs based on this enzyme can be synthesized.

The functional expression of granzyme B and fusion proteins containing it has been achieved in several heterologous systems, including *Escherichia coli*, *Pichia pastoris*, insect cells and mammalian cell lines such as HEK293T, HeLa, Jurkat and COS [24, 58, 64]. The enzymatic activity of granzyme B depends on correct processing and the generation of a free N-terminus. Several strategies addressing this issue have been developed, such as inserting an enterokinase cleavage site upstream of the mature polypeptide sequence allowing *in vitro*-processing [24, 61, 65, 66], and the direct fusion of a *Saccharomyces cerevisiae* mating factor α leader sequence allowing *in vivo*-processing by Kex2 protease in the Golgi apparatus [58, 67]. *In vivo*-processing was also achieved by expressing the native granzyme B precursor protein with the propeptide deleted in insect and COS cells, the insertion of a furin site [63, 64] and the co-expression of rat DPPI [68].

The potential of granzyme B in targeted cancer therapy has been demonstrated *in vitro* by coupling it to antibody fragments or natural ligands targeting CD64 [24], gp240 [65, 69], ErbB2/Her2 (human epidermal growth factor receptor 2) [58, 60, 70, 71], human luteinizing hormone receptor (hLHR) [61], the Lewis Y antigen (LeY) [59], and CD30 [72].

Despite encouraging data demonstrating the potential of human granzyme B for targeted cancer therapy, the involvement of granzyme B as the main effector molecule in natural cellular defenses against transformed tumor cells is both a blessing and a curse. It is a blessing because granzyme B achieves the efficient and reliable induction of apoptosis via multiple pathways, thus potentially evading the anti-apoptosis mechanisms of tumors. However, it is also a curse because native granzyme B is strictly regulated, which means that the over-expression of anti-apoptotic molecules occurs not only in cells directly or indirectly involved in immune surveillance but also in transformed cells as an escape strategy.

We recently summarized examples of tumor cells, which have been found to express PI-9 to evade granule-mediated killing and might, therefore, be difficult to treat with granzyme B-based immunotherapeutics [73]. Indeed, several studies have confirmed the direct correlation between PI-9 expression and the loss of granzyme B pro-apoptotic activity and cytotoxic lymphocyte activity *in vitro* and *in vivo*. Immune system evasion promoted by PI-9 could, therefore, have a significant impact on cancer therapy based on granzyme B. The overexpression of recombinant PI-9 in a primarily PI-9-negative prostate cancer cell line conferred protection against the induction of granule-mediated apoptosis by NK92 cells [74]. Furthermore, boosting PI-9 expression in the breast cancer cell line MCF-7, either using estrogens or by stable transfection with a PI-9 transgene, conferred resistance to NK cell cytotoxicity [75, 76]. The knockdown of PI-9 expression by RNA interference (RNAi) abolished the estrogen-mediated inhibition of NK cell-mediated apoptosis [75]. Similar results were obtained in earlier studies with the hepatoma cell line HepG2ER7 [77]. Granule-mediated apoptosis was also progressively blocked in HeLa cells stably transfected with a functional PI-9 transgene but not in those stably transfected with an inactive mutant. PI-9 expression has been confirmed in a variety of human tumor cell lines and the murine homologue Spi-6 is likewise expressed in murine tumor cells, revealing a direct correlation between Spi-6/PI-9 expression and resistance to CTL-mediated killing [78]. PI-9-positive cancer cells are generally resistant against granzyme B-mediated cytotoxicity, including the myeloblastic K562 cells, the Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines LCL-1 and LCL-2, the Burkitt's lymphoma cell line Daudi, AMLR cells and the U937 subclone U937PI-9^{+} [79]. Nevertheless, the overall impact of PI-9-mediated resistance against granzyme B remains controversial. For example, CTL and NK cell cytotoxicity was observed in various lymphoma cell lines regardless of PI-9 expression [80] and hCFPs based on granzyme B were toxic towards the PI-9-positive breast cancer cell lines SK-BR3 and MCF-7, which upregulate PI-9 expression in the presence of estrogen [59, 75, 78]. Despite this controversy, the strictly regulated nature of granzyme B activity has been shown to interfere with the cytotoxicity of granzyme B-based hCFPs in a series of studies, encouraging the development of hCFPs that can circumvent the anti-apoptotic activity of PI-9.

8.2.3 Regulation of Granzyme B Activity by PI-9

The exact manner in which tumor cells and virus-infected cells become resistant to granule-mediated killing by cytotoxic lymphocytes remains unclear. Immune surveillance based on granule-mediated cytotoxicity is tightly controlled at the post-translational level by serine proteinase inhibitors (serpins), among which PI-9 is the only known endogenous human antagonist of granzyme B [81]. PI-9 is a 42-kDa intracellular protein from the ovalbumin family of intracellular serine protease inhibitors (clade B serpin superfamily, member 9), a group of structurally related proteins that regulate protease activity in the vertebrate adaptive and innate immune systems

by irreversible complex formation with the protease, i.e. they act as a suicide substrate [82–84]. PI-9 accumulates not only in the cytosol of lymphocytes to protect them from granule leakage, but also in bystander cells such as B cells, monocytes, mast cells, and antigen-presenting cells, which are likely to be affected by granzyme B during an immune response [85–88]. PI-9 expression is likewise upregulated in cells at immune privileged sites, including the eye lens capsula, ovaries, placenta, testis and embryonic stem cells [88, 89]. During inflammation it is expressed by pro-inflammatory cells and cells infected by diverse viruses, including cytomegalovirus and EBV [87, 90, 91]. PI-9 expression can be upregulated by cytokines and inflammatory modulators such as lipopolysaccharides (LPS), interferons (IFNα and IFNγ), TNFα, and interleukins IL-1β and IL-2 [90, 92, 95], as well as estradiol-17β and other estrogens in the human liver, human HepG2 hepatoblastoma cells and MCF-7 breast cancer cells [75, 96–98]. Furthermore, PI-9 expression can be upregulated in renal tubular cells following allograft rejection, suggesting a protective role against granzyme B-mediated cytotoxicity that results in improved graft survival [99].

Cancer cells can evolve multiple intrinsic and extrinsic mechanisms to evade the granule exocytosis and death receptor apoptosis pathways [100]. This not only contributes to the transformation of tumor cells but also represents one of the major challenges in both targeted cancer therapy/immunotherapy and standard chemotherapy [101]. There is strong evidence that the upregulation of PI-9 gene expression in tumor cells confers resistance against granule-mediated cytotoxicity and allows tumors to escape immune surveillance [76, 78, 102]. Highly variable PI-9 expression has been reported in different human cancers, including carcinomas of the lung, prostate gland, breast, cervix, colon, nasopharynx and esophagus [74, 75, 78, 103, 106] as well as melanomas [107], leukemias [108] and lymphomas [80, 102, 109]. High levels of PI-9 are also associated with a poor prognosis in some cancers [105, 107, 110, 111].

A number of mouse tumors has been shown to express Spi-6, the murine homolog of PI-9 [112, 113]. The upregulation of Spi-6 in dendritic cells (DCs) and tumors inhibits granule-mediated cytotoxicity conferred by granzyme B [78, 113]. Murine and human granzyme B show substantial differences in terms of structure, substrate preferences and cytotoxicity (human granzyme B is 30 times more potent) [84, 114], therefore, granzyme B inhibition by Spi-6 may play only a minor role in the development of murine tumors. However, serpina3n is an inhibitor of human granzyme B secreted by murine Sertoli cells [115]. Like PI-9, serpina3n acts as a suicide inhibitor by forming irreversible SDS-stable inhibitor-protease complexes. An intracellular serpin derived from the rat pituitary gland (raPIT5a) is also structurally related to PI-9 and can also form SDS-stable complexes with human granzyme B suggesting a regulatory function [116].

Although a direct correlation between PI-9 expression and the failure of many immunotherapeutic approaches has not been confirmed, the upregulation of PI-9 in tumor cells must be taken into account when developing effective immunotherapeutic strategies involving granzyme B. These not only include targeted therapies using

granzyme B as a pro-apoptotic effector molecule but also active immunotherapy aiming to induce tumor-specific immune responses [107].

Tumor cells likewise regulate apoptosis by the overexpression of different classes of anti-apoptotic proteins, primarily blocking death receptor and ER stress-induced apoptosis, but indirectly influencing granzyme B-mediated cytotoxicity by interfering with apoptotic signaling pathways. These include the anti-apoptotic BCL-2 family members, BCL-2, BCL-x_L, MCL-1, BCL-W, BFL-1 and BCL-B, which block the induction of apoptosis at the mitochondrial level and might interfere with the promotion of MOMP by granzyme B [117]. The dysregulation of anti-apoptotic BCL-2 family proteins is one of the key features of cancer and often underlies the poor response to current treatments [118–122]. BCL-x_L and MCL-1 are often upregulated in tumors and may confer resistance by blocking multiple apoptotic pathways [123, 124].

The direct activation of caspases by granzyme B is negatively regulated by inhibitor of apoptosis proteins (IAPs), a family of structurally-related caspase inhibitors that blocks the execution phase of apoptosis. IAPs are upregulated in many cancers [125–128] and this confers resistance to several apoptosis-based therapies [129–132]. Blocking the caspase and mitochondrial pathways by overexpressing BCL-2 and the X-linked inhibitor of apoptosis protein (XIAP) confers resistance to granule-mediated cell death induced by human NK cells [133]. IAPs and anti-apoptotic BCL-2 family members are, therefore, attractive targets for cancer therapy [134, 135].

8.2.4 Therapeutic Options to Restore the Sensitivity of PI-9-Positive Tumors Against Granzyme B

8.2.4.1 Downregulation of PI-9 Expression and Activity

Therapeutic concepts that increase the specific cytotoxicity of granzyme B against PI-9-positive cancer cells include the downregulation of PI-9 gene expression by RNAi or antisense RNA. In this context, PI-9 siRNA can restore the sensitivity of allogeneic mesenchymal stem cells [136] and breast cancer cells [75] but as a clinical practice RNAi is challenging because the delivery of siRNAs is generally inefficient and off-target effects are common place [137].

Granzyme M is a regulatory protease co-secreted with granzyme B in cytotoxic granules, and this enzyme has been shown to cleave PI-9 and, therefore, inhibits its activity at the protein level [138]. The co-application of granzyme M genetically fused to tumor cell-specific ligands and granzyme B hCFPs should allow both enzymes to be directed to the same target cells, resulting in the inactivation of endogenous PI-9 and the promotion of granzyme B-mediated cytotoxicity. We recently generated a fusion construct comprising granzyme M and the CD64-specific antibody H22(scFv) [139, 140], resulting in specific *in vitro* cytotoxicity towards the AML cell line HL60 [141, 142].

8.2.4.2 Design of Granzyme B Variants that are Insensitive Towards PI-9

The strategies described above are indirect approaches that require the co-administration of a separate therapeutic modality to inhibit PI-9. A more elegant and ambitious solution is the identification of critical contact residues between the protease and its substrate and the generation of a modified granzyme B variant that retains its pro-apoptotic activity but is no longer sensitive to PI-9. In an analogous approach, a human tissue-type plasminogen activator was mutated to achieve resistance against a complex mixture of endogenous serpins [143].

Based on *in silico* analysis carried out by the German Research School for Simulation Sciences in Jülich, we recently identified the molecular contacts between granzyme B and PI-9 by molecular modeling [144]. The inhibition of granzyme B by PI-9 involves the generation of a reversible Michaelis complex followed by a covalent stoichiometric 1:1 interaction, resulting in the irreversible inactivation of both the enzyme and the inhibitor [81, 145, 146]. The crystal structure of PI-9 is not yet available, but classical inhibitory serpins are unique among active site protease inhibitors because they share a characteristic, metastable tertiary structure comprising nine α-helices (A-I), three β-helices (A-C) and a variable exposed RCL [147, 148]. The latter is the primary interaction interface with granzyme B because it acts as a pseudosubstrate with the typical P4–P1 recognition motif featuring a glutamate residue at position P1 [81, 149, 150].

Our prediction of the structure of the Michaelis complex was based on the X-ray structures of the related complex of rat trypsin and *Manduca sexta* serpin B1 [145], and a 2 Å resolution crystal structure of human granzyme B bound to a five-residue peptide [151]. The unsolved structure of human PI-9 was predicted by homology modeling, building on the rat trypsin/serpin B1 complex, and the backbone of human granzyme B was adapted to rat trypsin followed by alanine-scanning calculations [152] and molecular dynamics simulations. This allowed us to identify R28 and R201 as the most important amino acids at the interface of the enzyme and its substrate, but because these were not part of the active site they were not expected to affect its catalytic activity. Substitution of the arginine residue with alanine (neutral charge), glutamate (opposite charge) and lysine (identical charge) resulted in seven variants that were potentially resistant to PI-9, namely R28A, R28K, R28E, R201A, R201K, R201E and the double mutant R28A-R201A.

Based on these *in silico* findings, we used Baker's computer-aided simulation modeling (CASM) procedure [152] followed by molecular dynamics simulations of PI-9 complexes with wild-type and mutated granzyme B in aqueous solution. Three variants (GrBR28K, GrBR201A and GrBR201K) appeared most likely to cause complex destabilization without affecting catalytic activity. These *in silico* calculations were confirmed by comparative *in vitro* assays measuring the proteolytic cleavage of the synthetic substrate AC-IEDT-*p*NA, which mimics the cleavage site of the granzyme B substrate procaspase-3 [153], by wild-type and mutant granzyme B in the presence and absence of recombinant PI-9 [144]. Mutations were introduced into granzyme B by site-directed mutagenesis and the resulting variants were transiently expressed in HEK293T cells before affinity-purification from the culture

supernatant. In the presence of PI-9, the GrBR28K and GrBR201A mutants retained 76 and 46% of their original activity, respectively, whereas the GrBR201K mutant retained 94% of its activity and was, therefore, the most promising derivative. This combination of *in silico* and *in vitro* techniques allowed us to identify granzyme B mutants that were potentially much more suitable for the treatment of resistant and relapsing PI-9-positive tumors. However, the PI-9 insensitivity of a granzyme B variant (K27A) described at an earlier stage was not confirmed in our *in silico* and *in vitro* studies [149].

8.2.4.3 Therapeutic Efficacy of hCFPs Based on GrBR201K

The encouraging *in vitro* data inspired us to generate hCFPs containing each of the three most promising granzyme B mutants (GrBR28K, GrBR201A and GrBR201K) paired with scFvs targeting CD30 for the treatment of Hodgkin's lymphoma or CD64 for the treatment of $CD64^+$ malignancies. CD64, the high-affinity receptor for human IgG (FcγRI), is constitutively expressed on macrophages, monocytes and their progenitors [154]. CD64 is upregulated on cancer cells of the monocyte/macrophage lineage such as AML [155, 156], and on macrophages during inflammation [157, 158]. CD64 expression on myeloid cells is induced by cytokines such as IFNγ [159]. CD64 is an excellent candidate target for immunotherapy because it is overexpressed on AML subtypes M4 and M5 (according to the French-American-British classification) [156, 160] but is not expressed on pluripotent stem cells or $CD34^+$ hematopoietic progenitor cells [161].

AML is a heterogeneous group of malignancies characterized by the hyperproliferation of hematopoietic stem cells of the myeloid lineage [162]. The accumulation of malignant progenitor cells in the bone marrow suppresses normal hematopoiesis and disturbs the balance of blood cell production. Standard AML chemotherapy is associated with high relapse rates due to repopulation by residual cells and/or the development of multi-drug resistance [163, 164]. Patients with relapsed AML can be treated with radiotherapy or allogeneic hematopoietic stem cell transplantation, but these options are associated with the risk of complications in elderly patients and those suffering from comorbidity [165]. In 2000, targeted therapy with gemtuzumab ozogamycin (Mylotarg), an ADC comprising a CD33-specific antibody chemically linked to the antitumor antibiotic calicheamicin, was approved by the FDA for the treatment of relapsed AML, but it was withdrawn from the market in 2010 because the safety risks outweighed the clinical benefits [166].

Wild-type granzyme B fused to the CD64-specific H22(scFv) (GrB-H22(scFv)) was shown to induce apoptosis *in vitro* against PI-9-negative U937 cells (an AML-related cell line stimulated by IFN-γ) and *ex vivo* against AML primary cells [24]. Next we carried out a *proof-of-concept* study to determine whether hCFPs based on mutant granzyme B could achieve the selective elimination of PI-9-positive tumor cells. The PI-9-resistant human granzyme B protein GrBR201K was fused to the CD64-specific H22(scFv) to generate the hCFP GrbBR201K-H22(scFv). Subsequent comparative *ex vivo* studies using $CD64^+$ primary cells from AMML and

CMML patients treated with wild-type and mutant versions of granzyme B revealed that GrBR201K-H22(scFv) was much more cytotoxic than its wild-type counterpart in the presence of PI-9 as demonstrated by both the XTT-assay and the Annexin V/propidium iodide (AV/PI) staining [167]. Western blot analysis of primary cell lysates showed that PI-9 was upregulated in cells from three of four CMML patients and one of three tested AMML patients after incubation for 14 h in RPMI medium containing 10 % fetal calf serum. There was no significant difference in the cytotoxicity of the wild-type and mutant granzyme B constructs against PI-9-negative cells from AMML and CMML patients as well as the PI-9-negative human promyeloic leukemia cell line HL-60. The GrBR201K mutant can, therefore, restore the sensitivity of PI-9-positive target cells towards granzyme B and may significantly improve the clinical outcome.

We have also generated hCFPs comprising wild-type granzyme B or GrBR201K fused to the antibody Ki4(scFv), which targets CD30 overexpressed on classical Hodgkin's lymphoma cells [72]. AV/PI staining of PI-9-positive L428 and PI-9-negative L540cy Hodgkin's lymphoma cells showed that PI-9 expression in the L428 cells nearly completely abolished the cytotoxic activity of the wild-type granzyme B construct, which showed potent cytotoxicity towards L540cy cells, whereas the mutant granzyme B construct was cytotoxic against both cell lines. A comparative study of the wild-type and mutant versions was then carried out using a mouse subcutaneous tumor model based on L428 cells transfected with the red fluorescent protein katushka2 [168]. The evaluation of tumor growth by optical imaging using the CRi Maestro system revealed that GrBR201K-Ki4(scFv) but not the wild-type construct was able to reduce tumor size with a statistical significance of $P < 0.005$ compared to control mice treated with PBS. These results demonstrated for the first time that the resistance of cancer cells against granzyme B conferred by PI-9 can be overcome *in vitro, ex vivo* and *in vivo* by using the PI-9 insensitive variant GrBR201K which was developed by *in silico* molecular modeling.

8.3 Human Angiogenin

Human angiogenin (Ang) is a 14-kDa stress-activated ribonuclease also known as RNase 5, which acts as a potent stimulator of neovascularization [169, 170] and shares 33 % sequence identity with pancreatic RNase A [171]. The enzyme is unique in humans and is encoded by a gene on chromosome 14q11 in close proximity to the *α/λ T-cell receptor* gene [169, 172]. Angiogenin is expressed by mast cells and fibroblasts with an N-terminal signal peptide that facilitates its secretion into the blood stream [171, 173, 174] where it circulates at a concentration of 250–360 ng/ml [175]. It specifically targets smooth muscle cells, endothelial cells and motor neurons, stimulating proliferation, cell migration and tubular development in response to environmental conditions [176, 177]. The expression of angiogenin is upregulated in several types of cancer and it promotes the establishment, growth and metastasis of tumors [178, 179].

Angiogenin plays an important role in the central nervous system (CNS) and is strongly expressed in developing neurons to promote neurite outgrowth and neuronal pathfinding [180]. Some loss-of-function angiogenin mutants are strongly associated with the development of the motor neuron disease amyotrophic lateral sclerosis (ALS) in certain pools of patients [176]. The administration of functional angiogenin has a neuroprotective effect in transgenic ALS mice and primary ALS-related motoneuron cell cultures [181]. Mutations in the *angiogenin* gene have also been linked to Parkinson's disease although the activity of the enzyme in this context has yet to be characterized [182].

Smooth muscle cells and endothelial cells are targeted via specific binding sites on the cell surface displaying actin [183], heparin, plasminogen, elastase or angiostatin [175, 184]. After binding to the cell surface, angiogenin is internalized by receptor-mediated endocytosis [185]. The detailed mechanism of endosomal escape is unknown but it is thought that angiogenin translocates into the cytosol from early endosomes [186]. Under physiological conditions, it undergoes rapid nuclear translocation immediately after endosomal escape triggered by a nuclear localization signal (NLS) spanning amino acids 55–59 [187, 188]. In the nucleus, angiogenin enhances the transcription of ribosomal RNA (rRNA) genes by epigenetic activation at the promoter, thereby facilitating the protein synthesis necessary for blood vessel growth, primary tumor development and metastasis [189, 190]. The cellular activity of angiogenin strongly depends on cell density and environmental stress. Under normal conditions, the ribonuclease is inhibited in the cytosol by the ubiquitous ribonuclease inhibitor RNH1 after internalization, but it remains free in the nucleus and can interact with the DNA as described above [191]. However, angiogenin in the cytoplasm is released from RNH1 under oxidative stress conditions [191]. This reflects oxidation of several RNH1 thiol groups followed by its rapid degradation [192, 193]. Oxidative stress also causes most of the nuclear angiogenin to return to the cytosol and enables inhibition of the remaining nuclear enzyme to avoid further rRNA production [191]. Angiogenin inhibits cell-free protein synthesis by cleaving 5S, 18S and 28S rRNAs as well as the transfer RNA (tRNA) at the 3′-CCA position and within the anticodon loop [194, 195]. The latter can be prevented by the DNA methyltransferase DNMT2 because tRNA methylation protects it from degradation by angiogenin [196]. Substrate cleavage is species-dependent because human angiogenin does not prevent the proliferation of murine cells [197]. The degradation processes described above result in the synergistic inactivation of ribosomes and tRNAs to block translation, simultaneously inducing the production of tRNA-derived stress-induced RNAs (tiRNAs). The 5′-tiRNA segment can displace eIF4A/G from capped and uncapped mRNAs as well as eIF4F from the m7G-cap [193]. These events work in concert and ultimately result in translational repression [198–202]. The tiRNAs can also trigger the phospho-eIF2α-independent assembly of stress granules, containing complexes of concentrated and untranslated mRNA-based ribonucleoproteins (mRNPs) [193, 198, 203]. Single-stranded tiRNA fragments are also incorporated into the RNA-induced silencing complex [204], which results in the specific cleavage of complementary mRNA strands before translation can be initiated [205]. The shutdown of protein synthesis by angiogenin is believed

to be a mechanism of active cellular stress response that blocks energy-intensive processes. Interestingly, cytochrome c released from mitochondria can interact with tiRNA, thereby preventing the assembly with APAF-1 to form the apoptosome. This phenomenon probably represents a mechanism to avoid the premature induction of apoptosis [206].

Several mechanisms have been proposed to explain how angiogenin induces apoptosis, including sensitization based on the suppression of translation of mRNAs encoding anti-apoptotic proteins [207]. However, anti-apoptotic proteins are often synthesized from internal ribosome entry sites (IRES) that are not inhibited by tiRNAs [191, 208]. The stress granules that assemble following the production of tiRNAs are thought to modulate the balance between pro-apoptotic and anti-apoptotic signaling pathways [209].

8.3.1 Targeted Cell Depletion Using Human Angiogenin

The ability of angiogenin to shut down protein synthesis makes it a promising effector component for hCFPs (Fig. 8.3). The targeted depletion of specific cell populations using angiogenin-based hCFPs has already been demonstrated *in vitro* and *ex vivo* [25, 197, 210, 214].

Nevertheless, further modifications are required to increase the cytotoxicity of such constructs because conventional immunotoxins have EC_{50} values several orders of magnitude lower than hCFPs, in the picomolar range [215–218]. This may be explained, in part, by the fact that human enzymes lack a natural translocation domain, which restricts their capacity for endosomal escape [219]. Another drawback is the presence of intrinsic inhibitors in the target cells, as described above for granzyme B and PI-9 [220, 221]. In the case of angiogenin, the natural inhibitor is the above mentioned cytosolic protein RNH1 (also called RI, RNH and PRI) which binds the ribonuclease and, indeed, all monomeric members of the pancreatic RNase family with an extraordinarily high affinity [222]. RNH1 is found mainly in the cytosol but small amounts are also present in the mitochondria and nucleus [223]. The interaction between RNH1 and angiogenin is one of the strongest protein interactions discovered thus far, with a K_i value of $10^{-13} - 10^{-16}$ correlating with a complex half-life of several days [220, 221, 224]. The inhibition of angiogenin completely abolishes its angiogenic and RNase activities, the latter being necessary for apoptosis [225]. RNH1 is ubiquitous in human cells and it accounts for more than 0.01 % of the total intracellular protein content [226–228]. The cytotoxicity of hCFPs based on angiogenin and other RNases is, thus, dependent on the delivery of adequate amounts of the fusion protein into the cytosol to overcome RNH1-mediated inhibition [222]. When small amounts are delivered, cytotoxicity correlates with the ability of the ribonuclease to evade RNH1, as discovered during the characterization of ranpirnase (ONCONASE®) from the northern leopard frog *Rana pipiens*. [229]. This enzyme has a low catalytic activity but remarkable cytotoxicity in the picomolar range when fused to an anti-CD22 antibody [230]. This efficacy is

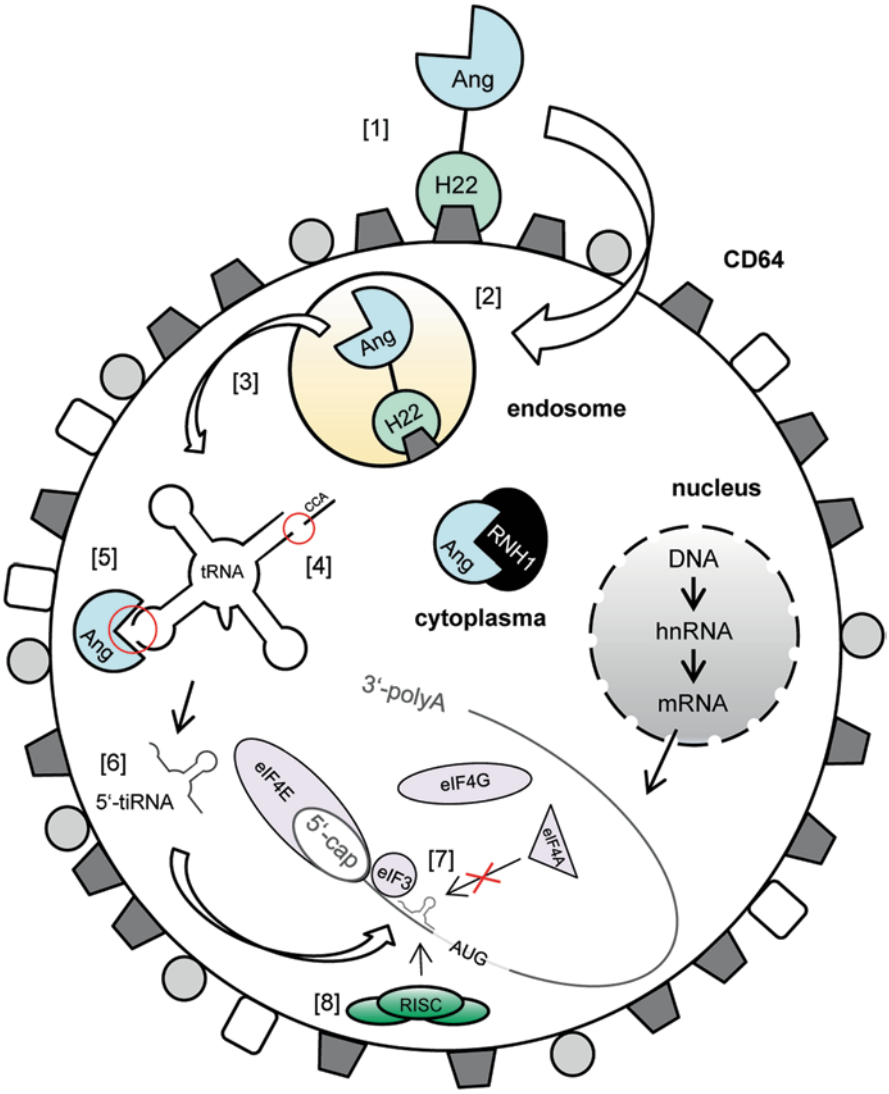

Fig. 8.3 Cytotoxic activity of the H22(scFv)-AnghCFP. The cytolytic fusion protein H22(scFv)-Ang specifically targets overexpressed CD64 on the target cell surface via the H22(scFv) [1]. After receptor-mediated internalization [2], the early endosome gets acidified finally leading to the release of the hCFP from the receptor and the escape from the endosome [3]. Once in the cytosol, angiogenin cleaves tRNA molecules at the 3′-CCA termini [4] and the anticodon-loop [5] to generate tiRNAs [6]. The generated fragments induce a translational arrest because they are able to block the binding sites for the translation initiation factors eIF4A and eIF4G [6]. In addition, tRNA fragments can be incorporated into the RNA-induced silencing complex (RISC) leading to efficient mRNA degradation and down-regulation of protein biosynthesis [7]. All events synergistically promote translational arrest and apoptosis induction

largely dependent on its insensitivity towards RNH1. In contrast, an hCFP containing human pancreatic RNase usually achieves EC_{50} values in the nanomolar range [231]. In a phase II clinical trial, 43 % of patients with unresectable malignant mesothelioma experienced stabilization of their formerly progressive disease following a single dose of ONCONASE®. This drug is currently undergoing a phase III clinical trial for the treatment of malignant mesothelioma [232–235]. This example clearly demonstrates the therapeutic potential of targeted RNases and shows that their efficacy mainly depends on their ability to induce apoptosis even in the presence of RNH1. Wild-type human RNases must be administered in large doses to overcome intrinsic inhibition, presenting a higher risk of side effects. As discussed above for granzyme B, it has therefore been necessary to develop angiogenin mutants to circumvent RNH1 inhibition and achieve satisfactory therapeutic potency at low doses.

8.3.2 Generation of Angiogenin Mutants with Improved Cytotoxicity

The successful induction of apoptosis by angiogenin requires cytosolic localization, potent RNase activity and low sensitivity towards RNH1. All of these properties can be improved using the strategies discussed in the following three sections.

8.3.2.1 Enhancing Cytosolic Translocation and Retention

Most human enzymes lack a natural translocation domain and the first obstacle affecting the cytotoxicity of hCFPs is, therefore, inefficient endosomal release following internalization. One solution is to incorporate artificial adapter sequences between the binding moiety and the human effector domain [214]. The cytotoxicity of hCFPs can be enhanced using this strategy but the proteins were found to be less stable in serum due to the proteolytic cleavage sites of the adapter, making such proteins unsuitable for therapeutic applications. Alternatively, the initial immunotoxin concept featured toxins with their own natural translocation domains which can be incorporated into hCFPs. For example, the *P. aeruginosa* ETA′ translocation domain has been combined with human granzyme B to improve its endosomal release [62, 236]. However, an incorporation of bacterial or plant-derived peptides into hCFPs removes the advantage of using human effector proteins because the molecule once again becomes immunogenic.

Another way to address this issue is to consider the intracellular routing of hCFPs containing human RNases. Typically, hCFPs bind to cell-surface receptors and are taken up by receptor-mediated endocytosis into early endosomes, which are acidified before the protein is released. This route is dependent on the signal peptide present on all human RNases, and it is important to ensure that hCFPs are not directed to the lysosomal compartments. For cytotoxic variants of RNase A, it was shown that > 95 % of the internalized enzyme was degraded in the endosomal-

lysosomal pathway before the protein reached the cytosol [221]. The amino acid sequence KFERQ was identified as a signal peptide for lysosomal delivery [237]. Angiogenin does not contain a lysosomal signal peptide but does contain a NLS that directs it efficiently to the nucleus. In pulmonary artery endothelial cells under *in vitro* conditions, it accumulates in the nucleus after just 15 min [185]. Even if nuclear localization is not desirable, nuclear import requires the enzyme to first pass through the cytosol, where tRNA degradation can take place to induce apoptosis. However, the removal of the angiogenin NLS to promote cytosolic retention was unfavorable because the mutant was less cytotoxic than its wild-type counterpart although it retained the normal enzymatic activity [185]. This indicates that nuclear routing using an NLS and the avoidance of lysosomal compartments is essential for the cytotoxicity of angiogenin.

8.3.2.2 Increasing the Enzymatic Activity of Angiogenin

The shutdown of protein synthesis depends on the RNase activity of angiogenin. Consequently, another way to increase the cytotoxicity of hCFPs containing this enzyme is to increase its catalytic activity, thus inactivating tRNAs more efficiently and producing more tiRNA. Angiogenin has a relatively low catalytic activity compared to other RNases, but this activity is nevertheless required to stimulate angiogenesis [238]. The reason for the low ribonucleolytic activity was explained when the crystal structure of the enzyme was solved [239]. In contrast to pancreatic RNase A, the active center of angiogenin is obstructed by a glutamine residue at position 117 (Q117) [240] and the C-terminal segment of the protein must, therefore, undergo a conformational change to allow substrate binding and cleavage [240, 241]. Substitution mutations Q117G and Q117A disrupt the hydrogen bond between Q117 and T44, which stabilizes the native enzyme conformation [241]. Therefore, the removal of Q117 was believed to favor the accessibility and cleavage of tRNA, producing more tiRNA and increasing the cytotoxicity of the enzyme. The angiogenin Q117G variant was therefore fused to H22(scFv) doubling the cytotoxicity of the hCFP compared to the equivalent construct containing wild-type angiogenin [242].

8.3.2.3 Reducing the Susceptibility of Angiogenin to Inhibition

As discussed above, angiogenin in the cytosol is exposed to RNH1, which sequesters the ribonuclease into an inactive complex and efficiently prevents the induction of apoptosis [225]. Current developments are focusing on the generation of angiogenin mutants that retain their ability to cleave tRNA even in the presence of the inhibitor. Similar observations have been made while modifying the human pancreatic RNase: the substitution of four amino acids reduced its affinity for RNH1 by more than 10^3-fold without affecting its catalytic activity, and the cytotoxicity of the mutant was only eight-fold lower than that of ONCONASE® [243]. Furthermore, inhibition-resistant dimeric mutants have been developed for human pancreatic

RNase [244] and BS-RNase [245]. However, the modification of human enzymes for therapeutic applications is restricted by the possibility that each modification may change the native protein conformation or even form novel epitopes on the surface that can be targeted by neutralizing antibodies. Therefore, the optimization strategy should focus on the generation of a limited number of mutations that improve cytotoxicity without causing overt structural changes.

Some angiogenin mutants with a reduced affinity towards RNH1 have already been identified by *in vitro* protein interaction experiments and *ex vivo* within the scope of neovascularization studies [178, 246, 247]. We adapted this knowledge to our requirements and assumed that hyper-angiogenic variants with a lower inhibitor affinity would be more suitable as hCFP effector domains, given that neovascularization requires RNase activity even in the presence of the inhibitor. This would also allow the formation of tiRNA and would therefore shut down protein synthesis. The Ang G85R/G86R variant was shown to have a 10^6-fold lower affinity for RNH1 than the wild-type enzyme [178]. Further analysis showed that this variant was 10–25 times more cytotoxic than the wild-type enzyme towards the human promyelocytic leukemia cell line HL-60 when fused to H22(scFv) [242]. EC_{50} values in the picomolar range were observed against human pro-inflammatory macrophages, which represents a five-fold higher efficacy than ETA′-based immunotoxins [197, 242]. This example clearly demonstrates the potential of modified human angiogenin variants that are resistant to the inhibitor RNH1.

8.3.2.4 Angiogenin Variants with Several Modified Properties

Angiogenin mutants that combine two or all three of the improvements described above would be promising effector domains of hCFPs, but the development of such combined variants is challenging because the mutations may be mutually incompatible. Mutations that reduce the affinity of angiogenin for RNH1 would be unsuitable if they were too close to the active site because this might interfere with substrate binding or cleavage. Even if the separate mutations were located in distinct domains, their combined effect might alter the overall conformation of the enzyme resulting in a reduction or loss of catalytic activity. Finally, even if the different mutations were mutually compatible, they might not act synergistically and may, therefore, represent only a marginal improvement over the single-mutant variants.

To generate a variant with multiple improved properties, we combined the Q117G and G85R/G86R versions of the enzyme described above. Based on our previous studies, we expected a synergistic improvement by combining the lower affinity for RNH1 with the higher RNase activity. Instead, the resulting enzyme showed extraordinary RNase activity but lower cytotoxicity than the individual Ang G85R/G86R mutant because the affinity for RNH1 was restored to a near wild-type level [242]. The advantage of more efficient substrate cleavage was therefore offset by the greater susceptibility to inhibition. These data highlight the need for *in silico* modeling to circumvent the need for trial-and-error mutagenesis. This approach is indispensable in the field of protein design and will help to optimize the functionality of therapeutically relevant enzymes.

8.4 Conclusion

Human cytolytic fusion proteins (hCFPs) have been developed to overcome the drawbacks of former generations of immunotoxins, whose efficacy is extraordinary but whose clinical applications are limited by potential immunogenicity and side effects. Human pro-apoptotic enzymes offer a promising alternative to bacterial and plant-derived toxins but human cells are equipped with several inhibitor proteins that reduce the efficacy of such effectors. The development of mutants that escape these cellular protection mechanisms while maintaining their catalytic properties could, therefore, improve the therapeutic efficacy of hCFPs. The novel isoforms of granzyme B and angiogenin discussed in this chapter were generated by site-directed mutagenesis to increase their pro-apoptotic efficacy, highlighting the potential of mutant human proteins as improved effector domains. The resulting hCFPs can compete with immunotoxins in terms of efficacy and can even outperform them in certain target cells. Furthermore, hCFPs are better tolerated by the immune system and, therefore, offer the prospect of long-term therapeutic efficacy, particularly where conventional immunotoxins have failed. For example, a deletion in the *WDR85* gene, encoding a WD-repeat protein that plays a role in the first step of diphthamide biosynthesis, has recently been shown to confer resistance against ETA′-based immunotoxins in some children affected by acute lymphoblastic leukemia [248]. Similar resistance mechanisms might develop against each toxin regardless of its origin. However, these patients may still be sensitive towards human pro-apoptotic enzymes, particularly if they are optimized as an effector domain. *In silico* modeling and simulations will facilitate the design of novel mutants in the future. Based on these advantages, hCFPs containing improved enzyme mutants promise to become a powerful and innovative class of novel anti-cancer immunoconjugates in the future.

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References

1. Scott AM, Allison JP, Wolchok JD. Monoclonal antibodies in cancer therapy. Cancer Immun. 2012;12:14.
2. Reichert JM. Antibody-based therapeutics to watch in 2011. MAbs. 2011;3(1):76–99.
3. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov. 2006;5(2):147–59.
4. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer. 2001;1(2):118–29.

5. Dosio F, Brusa P, Cattel L. Immunotoxins and anticancer drug conjugate assemblies: the role of the linkage between components. Toxins (Basel). 2011;3(7):848–83.
6. Weldon JE, Pastan I. A guide to taming a toxin-recombinant immunotoxins constructed from Pseudomonas exotoxin A for the treatment of cancer. FEBS J. 2011;278(23):4683–700.
7. Schnell R, Borchmann P, Staak JO, Schindler J, Ghetie V, Vitetta ES, Engert A. Clinical evaluation of ricin A-chain immunotoxins in patients with Hodgkin's lymphoma. Ann Oncol. 2003;14(5):729–36.
8. Lambert JM. Drug-conjugated monoclonal antibodies for the treatment of cancer. Curr Opin Pharmacol. 2005;5(5):543–9.
9. Becker NB I. Antibody-based immunotoxins for the treatment of cancer. Antibodies. 2012;1:39–69.
10. Younes A, Yasothan U, Kirkpatrick P. Brentuximab vedotin. Nat Rev Drug Discov. 2012;11(1):19–20.
11. Sawaki M. Trastuzumab emtansine in the treatment of HER2-positive metastatic breast cancer in Japanese patients. Breast Cancer (Dove Med Press). 2014;6:37–41.
12. Dang NH, Pro B, Hagemeister FB, Samaniego F, Jones D, Samuels BI, Rodriguez MA, Goy A, Romaguera JE, McLaughlin P, Tong AT, Turturro F, Walker PL, Fayad L. Phase II trial of denileukin diftitox for relapsed/refractory T-cell non-Hodgkin lymphoma. Br J Haematol. 2007;136(3):439–47.
13. Mischak RP, Foxall C, Rosendorf LL, Knebel K, Scannon PJ, Spitler LE. Human antibody responses to components of the monoclonal antimelanoma antibody ricin A chain immunotoxin XomaZyme-MEL. Mol Biother. 1990;2(2):104–9.
14. Scadden DT, Schenkein DP, Bernstein Z, Luskey B, Doweiko J, Tulpule A, Levine AM. Immunotoxin combined with chemotherapy for patients with AIDS-related non-Hodgkin's lymphoma. Cancer. 1998;83(12):2580–7.
15. Madhumathi J, Verma RS. Therapeutic targets and recent advances in protein immunotoxins. Curr Opin Microbiol. 2012;15(3):300–9.
16. Mathew M, Verma RS. Humanized immunotoxins: a new generation of immunotoxins for targeted cancer therapy. Cancer Sci. 2009;100(8):1359–65.
17. Oratz R, Speyer JL, Wernz JC, Hochster H, Meyers M, Mischak R, Spitler LE. Antimelanoma monoclonal antibody-ricin A chain immunoconjugate (XMMME-001-RTA) plus cyclophosphamide in the treatment of metastatic malignant melanoma: results of a phase II trial. J Biol Response Mod. 1990;9(4):345–54.
18. Siegall CB, Haggerty HG, Warner GL, Chace D, Mixan B, Linsley PS, Davidson T. Prevention of immunotoxin-induced immunogenicity by coadministration with CTLA4Ig enhances antitumor efficacy. J Immunol. 1997;159(10):5168–73.
19. Tsutsumi Y, Onda M, Nagata S, Lee B, Kreitman RJ, Pastan I. Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. Proc Natl Acad Sci U S A. 2000;97(15):8548–53.
20. Hansen JK, Weldon JE, Xiang L, Beers R, Onda M, Pastan I. A recombinant immunotoxin targeting CD22 with low immunogenicity, low nonspecific toxicity, and high antitumor activity in mice. J Immunother. 2010;33(3):297–304.
21. Liu W, Onda M, Lee B, Kreitman RJ, Hassan R, Xiang L, Pastan I. Recombinant immunotoxin engineered for low immunogenicity and antigenicity by identifying and silencing human B-cell epitopes. Proc Natl Acad Sci U S A. 2012;109(29):11782–7.
22. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature. 1986;321(6069):522–5.
23. Lonberg N. Fully human antibodies from transgenic mouse and phage display platforms. Curr Opin Immunol. 2008;20(4):450–9.
24. Stahnke B, Thepen T, Stocker M, Rosinke R, Jost E, Fischer R, Tur MK, Barth S. Granzyme B-H22(scFv), a human immunotoxin targeting CD64 in acute myeloid leukemia of monocytic subtypes. Mol Cancer Ther. 2008;7(9):2924–32.

25. Huhn M, Sasse S, Tur MK, Matthey B, Schinkothe T, Rybak SM, Barth S, Engert A. Human angiogenin fused to human CD30 ligand (Ang-CD30L) exhibits specific cytotoxicity against CD30-positive lymphoma. Cancer Res. 2001;61(24):8737–42.
26. Tur MK, Neef I, Jager G, Teubner A, Stocker M, Melmer G, Barth S. Immunokinases, a novel class of immunotherapeutics for targeted cancer therapy. Curr Pharm Des. 2009;15(23):2693–9.
27. Hristodorov D, Mladenov R, Pardo A, Pham AT, Huhn M, Fischer R, Thepen T, Barth S. Microtubule-associated protein tau facilitates the targeted killing of proliferating cancer cells in vitro and in a xenograft mouse tumour model in vivo. Br J Cancer. 2013;109(6):1570–8.
28. Rosenblum MG, Barth S. Development of novel, highly cytotoxic fusion constructs containing granzyme B: unique mechanisms and functions. Curr Pharm Des. 2009;15(23):2676–92.
29. Lorberboum-Galski H. Human toxin-based recombinant immunotoxins/chimeric proteins as a drug delivery system for targeted treatment of human diseases. Expert Opin Drug Deliv. 2011;8(5):605–21.
30. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance. Immunol Rev. 1995;146:95–115.
31. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ. 2003;10(1):26–35.
32. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol. 2007;39(7–8):1462–75.
33. Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol. 2001;2(12):reviews3014.1–3014.7
34. Hudig D, Gregg NJ, Kam CM, Powers JC. Lymphocyte granule-mediated cytolysis requires serine protease activity. Biochem Biophys Res Commun. 1987;149(3):882–8.
35. Edwards KM, Kam CM, Powers JC, Trapani JA. The human cytotoxic T cell granule serine protease granzyme H has chymotrypsin-like (chymase) activity and is taken up into cytoplasmic vesicles reminiscent of granzyme B-containing endosomes. J Biol Chem. 1999;274(43):30468–73.
36. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem. 1997;272(29):17907–11.
37. Kam CM, Hudig D, Powers JC. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. Biochim Biophys Acta. 2000;1477(1–2):307–23.
38. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002;20:323–70.
39. Boivin WA, Cooper DM, Hiebert PR, Granville DJ. Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. Lab Invest. 2009;89(11):1195–220.
40. Bots M, Medema JP. Granzymes at a glance. J Cell Sci. 2006;119(Pt 24):5011–4.
41. Pham CT, Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc Natl Acad Sci U S A. 1999;96(15):8627–32.
42. Raja SM, Wang B, Dantuluri M, Desai UR, Demeler B, Spiegel K, Metkar SS, Froelich CJ. Cytotoxic cell granule-mediated apoptosis. Characterization of the macromolecular complex of granzyme B with serglycin. J Biol Chem. 2002;277(51):49523–30.
43. Jenkins MR, Griffiths GM. The synapse and cytolytic machinery of cytotoxic T cells. Curr Opin Immunol. 2010;22(3):308–13.
44. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225–74.
45. Pinkoski MJ, Winkler U, Hudig D, Bleackley RC. Binding of granzyme B in the nucleus of target cells. Recognition of an 80-kilodalton protein. J Biol Chem. 1996;271(17):10225–9.
46. Kurschus FC, Jenne DE. Delivery and therapeutic potential of human granzyme B. Immunol Rev. 2010;235(1):159–71.
47. Rousalova I, Krepela E. Granzyme B-induced apoptosis in cancer cells and its regulation (review). Int J Oncol. 2010;37(6):1361–78.

48. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol. 2008;26:389–420.
49. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007;87(1):99–163.
50. Kim HE, Du F, Fang M, Wang X. Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. Proc Natl Acad Sci U S A. 2005;102(49):17545–50.
51. Guerrero AD, Chen M, Wang J. Delineation of the caspase-9 signaling cascade. Apoptosis. 2008;13(1):177–86.
52. Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen L. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity. 1998;8(4):451–60.
53. Goping IS, Sawchuk T, Underhill DA, Bleackley RC. Identification of {alpha}-tubulin as a granzyme B substrate during CTL-mediated apoptosis. J Cell Sci. 2006;119(Pt 5):858–65.
54. Sebbagh M, Hamelin J, Bertoglio J, Solary E, Breard J. Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. J Exp Med. 2005;201(3):465–71.
55. Sharif-Askari E, Alam A, Rheaume E, Beresford PJ, Scotto C, Sharma K, Lee D, De Wolf WE, Nuttall ME, Lieberman J, Sekaly RP. Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. EMBO J. 2001;20(12):3101–13.
56. Zhang D, Beresford PJ, Greenberg AH, Lieberman J. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. Proc Natl Acad Sci U S A. 2001;98(10):5746–51.
57. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev. 2010;235(1):105–16.
58. Dalken B, Giesubel U, Knauer SK, Wels WS. Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition. Cell Death Differ. 2006;13(4):576–85.
59. Kurschus FC, Kleinschmidt M, Fellows E, Dornmair K, Rudolph R, Lilie H, Jenne DE. Killing of target cells by redirected granzyme B in the absence of perforin. FEBS Lett. 2004;562(1–3):87–92.
60. Wang T, Zhao J, Ren JL, Zhang L, Wen WH, Zhang R, Qin WW, Jia LT, Yao LB, Zhang YQ, Chen SY, Yang AG. Recombinant immunoproapoptotic proteins with furin site can translocate and kill HER2-positive cancer cells. Cancer Res. 2007;67(24):11830–9.
61. Kanatani I, Lin X, Yuan X, Manorek G, Shang X, Cheung LH, Rosenblum MG, Howell SB. Targeting granzyme B to tumor cells using a yoked human chorionic gonadotropin. Cancer Chemother Pharmacol. 2011;68(4):979–90.
62. Zhao J, Zhang LH, Jia LT, Zhang L, Xu YM, Wang Z, Yu CJ, Peng WD, Wen WH, Wang CJ, Chen SY, Yang AG. Secreted antibody/granzyme B fusion protein stimulates selective killing of HER2-overexpressing tumor cells. J Biol Chem. 2004;279(20):21343–8.
63. Dalken B, Jabulowsky RA, Oberoi P, Benhar I, Wels WS. Maltose-binding protein enhances secretion of recombinant human granzyme B accompanied by in vivo processing of a precursor MBP fusion protein. PLoS One. 5(12):e14404.
64. Caputo A, Garner RS, Winkler U, Hudig D, Bleackley RC. Activation of recombinant murine cytotoxic cell proteinase-1 requires deletion of an amino-terminal dipeptide. J Biol Chem. 1993;268(24):17672–5.
65. Liu Y, Zhang W, Niu T, Cheung LH, Munshi A, Meyn RE Jr, Rosenblum MG. Targeted apoptosis activation with GrB/scFvMEL modulates melanoma growth, metastatic spread, chemosensitivity, and radiosensitivity. Neoplasia. 2006;8(2):125–35.
66. Liu Y, Cheung LH, Thorpe P, Rosenblum MG. Mechanistic studies of a novel human fusion toxin composed of vascular endothelial growth factor (VEGF)121 and the serine protease granzyme B: directed apoptotic events in vascular endothelial cells. Mol Cancer Ther. 2003;2(10):949–59.

67. Giesubel U, Dalken B, Mahmud H, Wels WS. Cell binding, internalization and cytotoxic activity of human granzyme B expressed in the yeast Pichia pastoris. Biochem J. 2006;394(Pt 3):563–73.
68. Smyth MJ, McGuire MJ, Thia KY. Expression of recombinant human granzyme B. A processing and activation role for dipeptidyl peptidase I. J Immunol. 1995;154(12):6299–305.
69. Liu Y, Cheung LH, Hittelman WN, Rosenblum MG. Targeted delivery of human pro-apoptotic enzymes to tumor cells: in vitro studies describing a novel class of recombinant highly cytotoxic agents. Mol Cancer Ther. 2003;2(12):1341–50.
70. Zhang L, Zhao J, Wang T, Yu CJ, Jia LT, Duan YY, Yao LB, Chen SY, Yang AG. HER2-targeting recombinant protein with truncated pseudomonas exotoxin A translocation domain efficiently kills breast cancer cells. Cancer Biol Ther. 2008;7(8):1226–31.
71. Cao Y, Marks JW, Liu Z, Cheung LH, Hittelman WN, Rosenblum MG. Design optimization and characterization of Her2/neu-targeted immunotoxins: comparative in vitro and in vivo efficacy studies. Oncogene. 2014;33(4):429–39.
72. Schiffer S, Hansen HP, Hehmann-Titt G, Huhn M, Fischer R, Barth S, Thepen T. Efficacy of an adapted granzyme B-based anti-CD30 cytolytic fusion protein against PI-9-positive classical Hodgkin lymphoma cells in a murine model. Blood Cancer J. 2013;3:e106.
73. Hehmann-Titt G, Schiffer S, Berges N, Melmer G, Barth S. Improving the therapeutic potential of human Granzyme B for targeted cancer therapy. Antibodies. 2013;2:19–49.
74. Ray M, Hostetter DR, Loeb CR, Simko J, Craik CS. Inhibition of Granzyme B by PI-9 protects prostate cancer cells from apoptosis. Prostate. 2012;72(8):846–55.
75. Jiang X, Ellison SJ, Alarid ET, Shapiro DJ. Interplay between the levels of estrogen and estrogen receptor controls the level of the granzyme inhibitor, proteinase inhibitor 9 and susceptibility to immune surveillance by natural killer cells. Oncogene. 2007;26(28):4106–14.
76. Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, Trapani JA, Bird PI. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. Mol Cell Biol. 1998;18(11):6387–98.
77. Jiang X, Orr BA, Kranz DM, Shapiro DJ. Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells. Endocrinology. 2006;147(3):1419–26.
78. Medema JP, de Jong J, Peltenburg LT, Verdegaal EM, Gorter A, Bres SA, Franken KL, Hahne M, Albar JP, Melief CJ, Offringa R. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A. 2001;98(20):11515–20.
79. Fritsch K, Finke J, Grullich C. Suppression of granzyme B activity and caspase-3 activation in leukaemia cells constitutively expressing the protease inhibitor 9. Ann Hematol. 2013;92(12):1603–9.
80. Godal R, Keilholz U, Uharek L, Letsch A, Asemissen AM, Busse A, Na IK, Thiel E, Scheibenbogen C. Lymphomas are sensitive to perforin-dependent cytotoxic pathways despite expression of PI-9 and overexpression of bcl-2. Blood. 2006;107(8):3205–11.
81. Sun J, Bird CH, Sutton V, McDonald L, Coughlin PB, De Jong TA, Trapani JA, Bird PI. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J Biol Chem. 1996;271(44):27802–9.
82. Law RH, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, Whisstock JC. An overview of the serpin superfamily. Genome Biol. 2006;7(5):216.
83. Bots M, Medema JP. Serpins in T cell immunity. J Leukoc Biol. 2008;84(5):1238–47.
84. Bots M, Van Bostelen L, Rademaker MT, Offringa R, Medema JP. Serpins prevent granzyme-induced death in a species-specific manner. Immunol Cell Biol. 2006;84(1):79–86.
85. Buzza MS, Hirst CE, Bird CH, Hosking P, McKendrick J, Bird PI. The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. Cell Immunol. 2001;210(1):21–9.

86. Bladergroen BA, Strik MC, Wolbink AM, Wouters D, Broekhuizen R, Kummer JA, Hack CE. The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells. Eur J Immunol. 2005;35(4):1175–83.
87. Classen CF, Bird PI, Debatin KM. Modulation of the granzyme B inhibitor proteinase inhibitor 9 (PI-9) by activation of lymphocytes and monocytes in vitro and by Epstein-Barr virus and bacterial infection. Clin Exp Immunol. 2006;143(3):534–42.
88. Bladergroen BA, Strik MC, Bovenschen N, van Berkum O, Scheffer GL, Meijer CJ, Hack CE, Kummer JA. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. J Immunol. 2001;166(5):3218–25.
89. Buzza MS, Hosking P, Bird PI. The granzyme B inhibitor, PI-9, is differentially expressed during placental development and up-regulated in hydatidiform moles. Placenta. 2006;27(1):62–9.
90. Kannan-Thulasiraman P, Shapiro DJ. Modulators of inflammation use nuclear factor-kappa B and activator protein-1 sites to induce the caspase-1 and granzyme B inhibitor, proteinase inhibitor 9. J Biol Chem. 2002;277(43):41230–9.
91. Rowshani AT, Strik MC, Molenaar R, Yong SL, Wolbink AM, Bemelman FJ, Hack CE, Ten Berge IJ. The granzyme B inhibitor SERPINB9 (protease inhibitor 9) circulates in blood and increases on primary cytomegalovirus infection after renal transplantation. J Infect Dis. 2005;192(11):1908–11.
92. Horie O, Saigo K, Murayama T, Ryo R. Differential expression of proteinase inhibitor-9 and granzyme B mRNAs in activated immunocompetent cells. Tohoku J Exp Med.2005;205(2):103–13.
93. Barrie MB, Stout HW, Abougergi MS, Miller BC, Thiele DL. Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6. J Immunol. 2004;172(10):6453–9.
94. Hirst CE, Buzza MS, Bird CH, Warren HS, Cameron PU, Zhang M, Ashton-Rickardt PG, Bird PI. The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. J Immunol. 2003;170(2):805–15.
95. Majima T, Ichikura T, Chochi K, Kawabata T, Tsujimoto H, Sugasawa H, Kuranaga N, Takayama E, Kinoshita M, Hiraide H, Seki S, Mochizuki H. Exploitation of interleukin-18 by gastric cancers for their growth and evasion of host immunity. Int J Cancer. 2006;118(2):388–95.
96. Jiang X, Orr BA, Kranz DM, Shapiro DJ. Estrogen induction of the granzyme B inhibitor, proteinase inhibitor 9, protects cells against apoptosis mediated by cytotoxic T lymphocytes and natural killer cells. Endocrinology. 2006;147(3):1419–26.
97. Jiang X, Patterson NM, Ling Y, Xie J, Helferich WG, Shapiro DJ. Low concentrations of the soy phytoestrogen genistein induce proteinase inhibitor 9 and block killing of breast cancer cells by immune cells. Endocrinology. 2008;149(11):5366–73.
98. Kanamori H, Krieg S, Mao C, Di Pippo VA, Wang S, Zajchowski DA, Shapiro DJ. Proteinase inhibitor 9, an inhibitor of granzyme B-mediated apoptosis, is a primary estrogen-inducible gene in human liver cells. J Biol Chem. 2000;275(8):5867–73.
99. Rowshani AT, Florquin S, Bemelman F, Kummer JA, Hack CE, Ten Berge IJ. Hyperexpression of the granzyme B inhibitor PI-9 in human renal allografts: a potential mechanism for stable renal function in patients with subclinical rejection. Kidney Int. 2004;66(4):1417–22.
100. Bhatia A, Kumar Y. Cellular and molecular mechanisms in cancer immune escape: a comprehensive review. Expert Rev Clin Immunol. 2014;10(1):41–62.
101. Indran IR, Tufo G, Pervaiz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim Biophys Acta. 2011;1807(6):735–45.
102. Bladergroen BA, Meijer CJ, ten Berge RL, Hack CE, Muris JJ, Dukers DF, Chott A, Kazama Y, Oudejans JJ, van Berkum O, Kummer JA. Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system? Blood. 2002;99(1):232–7.

103. Soriano C, Mukaro V, Hodge G, Ahern J, Holmes M, Jersmann H, Moffat D, Meredith D, Jurisevic C, Reynolds PN, Hodge S. Increased proteinase inhibitor-9 (PI-9) and reduced granzyme B in lung cancer: mechanism for immune evasion? Lung Cancer. 2012;77(1):38–45.
104. Rousalova I, Krepela E, Prochazka J, Cermak J, Benkova K. Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues. Int J Oncol. 2010;36(1):275–83.
105. Oudejans JJ, Harijadi H, Kummer JA, Tan IB, Bloemena E, Middeldorp JM, Bladergroen B, Dukers DF, Vos W, Meijer CJ. High numbers of granzyme B/CD8-positive tumour-infiltrating lymphocytes in nasopharyngeal carcinoma biopsies predict rapid fatal outcome in patients treated with curative intent. J Pathol. 2002;198(4):468–75.
106. Tanaka K, Harashima N, Niiya F, Miyagi Y, Hida N, Ochi M, Imai N, Harada M, Itoh K, Shichijo S. Serine proteinase inhibitor 9 can be recognized by cytotoxic T lymphocytes of epithelial cancer patients. Jpn J Cancer Res. 2002;93(2):198–208.
107. van Houdt IS, Oudejans JJ, van den Eertwegh AJ, Baars A, Vos W, Bladergroen BA, Rimoldi D, Muris JJ, Hooijberg E, Gundy CM, Meijer CJ, Kummer JA. Expression of the apoptosis inhibitor protease inhibitor 9 predicts clinical outcome in vaccinated patients with stage III and IV melanoma. Clin Cancer Res. 2005;11(17):6400–7.
108. Classen CF, Ushmorov A, Bird P, Debatin KM. The granzyme B inhibitor PI-9 is differentially expressed in all main subtypes of pediatric acute lymphoblastic leukemias. Haematologica. 2004;89(11):1314–21.
109. Tiacci E, Doring C, Brune V, van Noesel CJ, Klapper W, Mechtersheimer G, Falini B, Kuppers R, Hansmann ML. Analyzing primary Hodgkin and Reed-Sternberg cells to capture the molecular and cellular pathogenesis of classical Hodgkin lymphoma. Blood. 2012;120(23):4609–20.
110. Bossard C, Belhadj K, Reyes F, Martin-Garcia N, Berger F, Kummer JA, Briere J, Baglin AC, Cheze S, Bosq J, Ribrag V, Gisselbrecht C, Mounier N, Gaulard P. Expression of the granzyme B inhibitor PI9 predicts outcome in nasal NK/T-cell lymphoma: results of a Western series of 48 patients treated with first-line polychemotherapy within the Groupe d'Etude des Lymphomes de l'Adulte (GELA) trials. Blood. 2007;109(5):2183–9.
111. ten Berge RL, Meijer CJ, Dukers DF, Kummer JA, Bladergroen BA, Vos W, Hack CE, Ossenkoppele GJ, Oudejans JJ. Expression levels of apoptosis-related proteins predict clinical outcome in anaplastic large cell lymphoma. Blood. 2002;99(12):4540–6.
112. Kaiserman D, Knaggs S, Scarff KL, Gillard A, Mirza G, Cadman M, McKeone R, Denny P, Cooley J, Benarafa C, Remold-O'Donnell E, Ragoussis J, Bird PI. Comparison of human chromosome 6p25 with mouse chromosome 13 reveals a greatly expanded ov-serpin gene repertoire in the mouse. Genomics. 2002;79(3):349–62.
113. Medema JP, Schuurhuis DH, Rea D, van Tongeren J, de Jong J, Bres SA, Laban S, Toes RE, Toebes M, Schumacher TN, Bladergroen BA, Ossendorp F, Kummer JA, Melief CJ, Offringa R. Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells. J Exp Med. 2001;194(5):657–67.
114. Kaiserman D, Bird CH, Sun J, Matthews A, Ung K, Whisstock JC, Thompson PE, Trapani JA, Bird PI. The major human and mouse granzymes are structurally and functionally divergent. J Cell Biol. 2006;175(4):619–30.
115. Sipione S, Simmen KC, Lord SJ, Motyka B, Ewen C, Shostak I, Rayat GR, Dufour JM, Korbutt GS, Rajotte RV, Bleackley RC. Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. J Immunol. 2006;177(8):5051–8.
116. Hill RM, Morresey KS, Coates LC, Mezey E, Fell B, Bratt T, Trapani JA, Birch NP. A new intracellular serine protease inhibitor expressed in the rat pituitary gland complexes with granzyme B. FEBS Lett. 1998;440(3):361–4.
117. Sutton VR, Wowk ME, Cancilla M, Trapani JA. Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. Immunity. 2003;18(3):319–29.

118. Kitada S, Pedersen IM, Schimmer AD, Reed JC. Dysregulation of apoptosis genes in hematopoietic malignancies. Oncogene. 2002;21(21):3459–74.
119. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Guyotat D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 1993;81(11):3091–6.
120. Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood. 1993;82(6):1820–8.
121. Tothova E, Fricova M, Stecova N, Kafkova A, Elbertova A. High expression of Bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Neoplasma. 2002;49(3):141–4.
122. Minn AJ, Rudin CM, Boise LH, Thompson CB. Expression of bcl-xL can confer a multidrug resistance phenotype. Blood. 1995;86(5):1903–10.
123. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 1993;74(4):597–608.
124. Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood. 1997;89(2):630–43.
125. Vucic D, Stennicke HR, Pisabarro MT, Salvesen GS, Dixit VM. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. Curr Biol. 2000;10(21):1359–66.
126. Imoto I, Tsuda H, Hirasawa A, Miura M, Sakamoto M, Hirohashi S, Inazawa J. Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. Cancer Res. 2002;62(17):4860–6.
127. Imoto I, Yang ZQ, Pimkhaokham A, Tsuda H, Shimada Y, Imamura M, Ohki M, Inazawa J. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res. 2001;61(18):6629–34.
128. Yang L, Cao Z, Yan H, Wood WC. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res. 2003;63(20):6815–24.
129. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. 2002;3(6):401–10.
130. Nachmias B, Ashhab Y, Ben-Yehuda D. The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. Semin Cancer Biol. 2004;14(4):231–43.
131. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. 2004;64(20):7183–90.
132. Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis. 2007;12(9):1543–68.
133. Sedelies KA, Ciccone A, Clarke CJ, Oliaro J, Sutton VR, Scott FL, Silke J, Susanto O, Green DR, Johnstone RW, Bird PI, Trapani JA, Waterhouse NJ. Blocking granule-mediated death by primary human NK cells requires both protection of mitochondria and inhibition of caspase activity. Cell Death Differ.2008;15(4):708–17.
134. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. Oncogene. 2008;27(48):6252–75.
135. Weyhenmeyer B, Murphy AC, Prehn JH, Murphy BM. Targeting the anti-apoptotic Bcl-2 family members for the treatment of cancer. Exp Oncol. 2012;34(3):192–9.
136. El Haddad N, Moore R, Heathcote D, Mounayar M, Azzi J, Mfarrej B, Batal I, Ting C, Atkinson M, Sayegh MH, Ashton-Rickardt PG, Abdi R. The novel role of SERPINB9 in cytotoxic protection of human mesenchymal stem cells. J Immunol. 2011;187(5):2252–60.
137. Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. Nat Rev Cancer. 2011;11(1):59–67.
138. Mahrus S, Kisiel W, Craik CS. Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B. J Biol Chem. 2004;279(52):54275–82.

139. Graziano RF, Tempest PR, White P, Keler T, Deo Y, Ghebremariam H, Coleman K, Pfefferkorn LC, Fanger MW, Guyre PM. Construction and characterization of a humanized anti-gamma-Ig receptor type I (Fc gamma RI) monoclonal antibody. J Immunol. 1995;155(10):4996–5002.
140. de Kruif J, Tijmensen M, Goldsein J, Logtenberg T. Recombinant lipid-tagged antibody fragments as functional cell-surface receptors. Nat Med. 2000.6(2):223–7.
141. Ball ED, Guyre PM, Shen L, Glynn JM, Maliszewski CR, Baker PE, Fanger MW. Gamma interferon induces monocytoid differentiation in the HL-60 cell line. J Clin Invest. 1984;73(4):1072–7.
142. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, Gallo R. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood. 1979;54(3):713–33.
143. Madison EL, Goldsmith EJ, Gerard RD, Gething MJ, Sambrook JF. Serpin-resistant mutants of human tissue-type plasminogen activator. Nature. 1989;339(6227):721–4.
144. Losasso V, Schiffer S, Barth S, Carloni P. Design of human granzyme B variants resistant to serpin B9. Proteins. 2012;80(11):2514–22.
145. Ye S, Cech AL, Belmares R, Bergstrom RC, Tong Y, Corey DR, Kanost MR, Goldsmith EJ. The structure of a Michaelis serpin-protease complex. Nat Struct Biol. 2001;8(11):979–83.
146. Hedstrom L. Serine protease mechanism and specificity. Chem Rev. 2002;102(12):4501–24.
147. Stein PE, Leslie AG, Finch JT, Turnell WG, McLaughlin PJ, Carrell RW. Crystal structure of ovalbumin as a model for the reactive centre of serpins. Nature. 1990;347(6288):99–102.
148. Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. Nature. 2000;407(6806):923–6.
149. Sun J, Whisstock JC, Harriott P, Walker B, Novak A, Thompson PE, Smith AI, Bird PI. Importance of the P4' residue in human granzyme B inhibitors and substrates revealed by scanning mutagenesis of the proteinase inhibitor 9 reactive center loop. J Biol Chem. 2001;276(18):15177–84.
150. Poe M, Blake JT, Boulton DA, Gammon M, Sigal NH, Wu JK, Zweerink HJ. Human cytotoxic lymphocyte granzyme B. Its purification from granules and the characterization of substrate and inhibitor specificity. J Biol Chem. 1991;266(1):98–103.
151. Rotonda J, Garcia-Calvo M, Bull HG, Geissler WM, McKeever BM, Willoughby CA, Thornberry NA, Becker JW. The three-dimensional structure of human granzyme B compared to caspase-3, key mediators of cell death with cleavage specificity for aspartic acid in P1. Chem Biol. 2001;8(4):357–68.
152. Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein-protein complexes. Proc Natl Acad Sci U S A. 2002;99(22):14116–21.
153. Metkar SS, Wang B, Ebbs ML, Kim JH, Lee YJ, Raja SM, Froelich CJ. Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. J Cell Biol. 2003;160(6):875–85.
154. Daeron M. Fc receptor biology. Annu Rev Immunol. 1997;15:203–34.
155. Krasinskas AM, Wasik MA, Kamoun M, Schretzenmair R, Moore J, Salhany KE. The usefulness of CD64, other monocyte-associated antigens, and CD45 gating in the subclassification of acute myeloid leukemias with monocytic differentiation. Am J Clin Pathol. 1998;110(6):797–805.
156. Ball ED, McDermott J, Griffin JD, Davey FR, Davis R, Bloomfield CD. Expression of the three myeloid cell-associated immunoglobulin G Fc receptors defined by murine monoclonal antibodies on normal bone marrow and acute leukemia cells. Blood.1989;73(7):1951–6.
157. Frasnelli M, So A. Toll-like receptor 2 and toll-like receptor 4 expression on CD64+ monocytes in rheumatoid arthritis: comment on the article by Iwahashi et al. Arthritis Rheum. 2005;52(7):2227–8.
158. van Roon JA, Bijlsma JW, van de Winkel JG, Lafeber FP. Depletion of synovial macrophages in rheumatoid arthritis by an anti-FcgammaRI-calicheamicin immunoconjugate. Ann Rheum Dis. 2005;64(6):865–70.

159. van de Winkel JG, Anderson CL. Biology of human immunoglobulin G Fc receptors. J Leukoc Biol. 1991;49(5):511–24.
160. Dunphy CH, Tang W. The value of CD64 expression in distinguishing acute myeloid leukemia with monocytic differentiation from other subtypes of acute myeloid leukemia: a flow cytometric analysis of 64 cases. Arch Pathol Lab Med. 2007;131(5):748–54.
161. Menendez P, del Canizo MC, Orfao A. Immunophenotypic characteristics of PB-mobilised CD34+ hematopoietic progenitor cells. J Biol Regul Homeost Agents. 2001;15(1):53–61.
162. Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. Hematol Am Soc Hematol Educ Program. 2004;1:98–117.
163. Robak T, Wierzbowska A. Current and emerging therapies for acute myeloid leukemia. Clin Ther. 2009;31(Pt 2):2349–70.
164. Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukaemia. Crit Rev Oncol Hematol. 2004;50(3):197–222.
165. Fritsch S, Buske C, Wormann B, Wedding U, Hiddemann W, Spiekermann K. Therapy of acute myeloid leukemia (AML) for medically non-fit patients. Med Klin (Munich). 2007;102(4):324–9.
166. Walter RB, Appelbaum FR, Estey EH, Bernstein ID. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. Blood. 2012;119(26):6198–208.
167. Schiffer S, Rosinke R, Jost E, Hehmann-Titt G, Huhn M, Melmer G, Barth S, Thepen T. Targeted ex vivo reduction of CD64-positive monocytes in chronic myelomonocytic leukemia and acute myelomonocytic leukemia using human granzyme B-based cytolytic fusion proteins. Int J Cancer. 2014;135(6):1497–508.
168. Shcherbo D, Merzlyak EM, Chepurnykh TV, Fradkov AF, Ermakova GV, Solovieva EA, Lukyanov KA, Bogdanova EA, Zaraisky AG, Lukyanov S, Chudakov DM. Bright far-red fluorescent protein for whole-body imaging. Nat Methods. 2007;4(9):741–6.
169. Weremowicz S, Fox EA, Morton CC, Vallee BL. Localization of the human angiogenin gene to chromosome band 14q11, proximal to the T cell receptor alpha/delta locus. Am J Hum Genet. 1990;47(6):973–81.
170. Fett JW, Strydom DJ, Lobb RR, Alderman EM, Bethune JL, Riordan JF, Vallee BL. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry. 1985;24(20):5480–6.
171. Strydom DJ, Fett JW, Lobb RR, Alderman EM, Bethune JL, Riordan JF, Vallee BL. Amino acid sequence of human tumor derived angiogenin. Biochemistry. 1985;24(20):5486–94.
172. Zhang J, Zhang YP. Pseudogenization of the tumor-growth promoter angiogenin in a leaf-eating monkey. Gene. 2003;308:95–101.
173. Kulka M, Fukuishi N, Metcalfe DD. Human mast cells synthesize and release angiogenin, a member of the ribonuclease A (RNase A) superfamily. J Leukoc Biol. 2009;86(5):1217–26.
174. Liote F, Champy R, Moenner M, Boval-Boizard B, Badet J. Elevated angiogenin levels in synovial fluid from patients with inflammatory arthritis and secretion of angiogenin by cultured synovial fibroblasts. Clin Exp Immunol. 2003;132(1):163–8.
175. Hu GF, Riordan JF, Vallee BL. A putative angiogenin receptor in angiogenin-responsive human endothelial cells. Proc Natl Acad Sci U S A. 1997;94(6):2204–9.
176. Gao X, Xu Z. Mechanisms of action of angiogenin. Acta Biochim Biophys Sin (Shanghai). 2008;40(7):619–24.
177. Kishikawa H, Wu D, Hu GF. Targeting angiogenin in therapy of amyotropic lateral sclerosis. Expert Opin Ther Targets. 2008;12(10):1229–42.
178. Dickson KA, Kang DK, Kwon YS, Kim JC, Leland PA, Kim BM, Chang SI, Raines RT. Ribonuclease inhibitor regulates neovascularization by human angiogenin. Biochemistry. 2009;48(18):3804–6.
179. Sadagopan S, Veettil MV, Chakraborty S, Sharma-Walia N, Paudel N, Bottero V, Chandran B. Angiogenin functionally interacts with p53 and regulates p53-mediated apoptosis and cell survival. Oncogene. 2012;31(46):4835–47.
180. Subramanian V, Feng Y. A new role for angiogenin in neurite growth and pathfinding: implications for amyotrophic lateral sclerosis. Hum Mol Genet. 2007;16(12):1445–53.

181. Skorupa A, King MA, Aparicio IM, Dussmann H, Coughlan K, Breen B, Kieran D, Concannon CG, Marin P, Prehn JH. Motoneurons secrete angiogenin to induce RNA cleavage in astroglia. J Neurosci. 2012;32(15):5024–38.
182. Steidinger TU, Standaert DG, Yacoubian TA. A neuroprotective role for angiogenin in models of Parkinson's disease. J Neurochem. 2011;116(3):334–41.
183. Hu GF, Strydom DJ, Fett JW, Riordan JF, Vallee BL. Actin is a binding-protein for angiogenin. Proc Natl Acad Sci U S A. 1993;90(4):1217–1221.
184. Strydom DJ. The angiogenins. Cell Mol Life Sci. 1998;54(8):811–24.
185. Moroianu J, Riordan JF. Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity. Proc Natl Acad Sci U S A. 1994;91(5):1677–81.
186. Wiedlocha A. Following angiogenin during angiogenesis: a journey from the cell surface to the nucleolus. Arch Immunol Ther Exp (Warsz). 1999;47(5):299–305.
187. Hu G, Xu C, Riordan JF. Human angiogenin is rapidly translocated to the nucleus of human umbilical vein endothelial cells and binds to DNA. J Cell Biochem. 2000;76(3):452–62.
188. Moroianu J, Riordan JF. Identification of the nucleolar targeting signal of human angiogenin. Biochem Biophys Res Commun. 1994;203(3):1765–72.
189. Olson KA, Fett JW, French TC, Key ME, Vallee BL. Angiogenin antagonists prevent tumor growth in vivo. Proc Natl Acad Sci U S A. 1995;92(2):442–6.
190. Sheng J, Yu W, Gao X, Xu Z, Hu GF. Angiogenin stimulates ribosomal RNA transcription by epigenetic activation of the ribosomal DNA promoter. J Cell Physiol. 2014;229(4):521–9.
191. Pizzo E, Sarcinelli C, Sheng J, Fusco S, Formiggini F, Netti P, Yu W, D'Alessio G, Hu GF. Ribonuclease/angiogenin inhibitor 1 regulates stress-induced subcellular localization of angiogenin to control growth and survival. J Cell Sci. 2013;126(Pt 18):4308–19.
192. Blazquez M, Fominaya JM, Hofsteenge J. Oxidation of sulfhydryl groups of ribonuclease inhibitor in epithelial cells is sufficient for its intracellular degradation. J Biol Chem. 1996;271(31):18638–42.
193. Emara MM, Ivanov P, Hickman T, Dawra N, Tisdale S, Kedersha N, Hu GF, Anderson P. Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly. J Biol Chem. 2010;285(14):10959–68.
194. St Clair DK, Rybak SM, Riordan JF, Vallee BL. Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes. Proc Natl Acad Sci U S A. 1987;84(23):8330–4.
195. Czech A, Wende S, Morl M, Pan T, Ignatova Z. Reversible and rapid transfer-RNA deactivation as a mechanism of translational repression in stress. PLoS Genet. 2013;9(8):e1003767.
196. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, Lyko F. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev. 2010;24(15):1590–5.
197. Schiffer S, Hristodorov D, Mladenov R, Aslanian E, Huhn M, Fischer R, Barth S, Thepen T. Species-dependent functionality of the human cytolytic fusion proteins granzyme B-H22(scFv) and H22(scFv)-angiogenin in macrophages. Antibodies. 2013;2(1):9–18.
198. Ivanov P, Emara MM, Villen J, Gygi SP, Anderson P. Angiogenin-induced tRNA fragments inhibit translation initiation. Mol Cell. 2011;43(4):613–23.
199. Rybak SM, Vallee BL. Base cleavage specificity of angiogenin with Saccharomyces cerevisiae and Escherichia coli 5S RNAs. Biochemistry. 1988;27(7):2288–94.
200. Saxena SK, Rybak SM, Davey RT Jr, Youle RJ, Ackerman EJ. Angiogenin is a cytotoxic, tRNA-specific ribonuclease in the RNase A superfamily. J Biol Chem. 1992;267(30):21982–6.
201. Shapiro R, Riordan JF, Vallee BL. Characteristic ribonucleolytic activity of human angiogenin. Biochemistry. 1986;25(12):3527–32.
202. Yamasaki S, Ivanov P, Hu GF, Anderson P. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J Cell Biol. 2009;185(1):35–42.
203. Anderson P, Kedersha N. Stress granules. Curr Biol. 2009;19(10):R397–8.
204. Nawrot B, Sochacka E, Duchler M. tRNA structural and functional changes induced by oxidative stress. Cell Mol Life Sci. 2011;68(24):4023–32.

205. Pratt AJ, MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem. 2009;284(27):17897–901.
206. Saikia M, Jobava R, Parisien M, Putnam A, Krokowski D, Gao XH, Guan BJ, Yuan Y, Jankowsky E, Feng Z, Hu GF, Pusztai-Carey M, Gorla M, Sepuri NB, Pan T, Hatzoglou M. Angiogenin-cleaved tRNA halves interact with cytochrome c, protecting cells from apoptosis during osmotic stress. Mol Cell Biol. 2014;34(13):2450–63.
207. Saelens X, Kalai M, Vandenabeele P. Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation. J Biol Chem. 2001;276(45):41620–8.
208. Liu B, Qian SB. Translational reprogramming in cellular stress response. Wiley Interdiscip Rev RNA. 2014;5(3):301–15.
209. Thomas MG, Loschi M, Desbats MA, Boccaccio GL. RNA granules: the good, the bad and the ugly. Cell Signal. 2011;23(2):324–34.
210. Newton DL, Xue Y, Olson KA, Fett JW, Rybak SM. Angiogenin single-chain immunofusions: influence of peptide linkers and spacers between fusion protein domains. Biochemistry. 1996;35(2):545–53.
211. Yoon JM, Han SH, Kown OB, Kim SH, Park MH, Kim BK. Cloning and cytotoxicity of fusion proteins of EGF and angiogenin. Life Sci. 1999;64(16):1435–1445.
212. Stocker M, Tur MK, Sasse S, Krussmann A, Barth S, Engert A. Secretion of functional anti-CD30-angiogenin immunotoxins into the supernatant of transfected 293 T-cells. Protein Expr Purif. 2003;28(2):211–9.
213. Reiners KS, Hansen HP, Krussmann A, Schon G, Csernok E, Gross WL, Engert A, Von Strandmann EP. Selective killing of B-cell hybridomas targeting proteinase 3, Wegener's autoantigen. Immunology. 2004;112(2):228–36.
214. Hetzel C, Bachran C, Fischer R, Fuchs H, Barth S, Stocker M. Small cleavable adapters enhance the specific cytotoxicity of a humanized immunotoxin directed against CD64-positive cells. J Immunother. 2008;31(4):370–6.
215. Tur MK, Huhn M, Thepen T, Stocker M, Krohn R, Vogel S, Jost E, Osieka R, van de Winkel JG, Fischer R, Finnern R, Barth S. Recombinant CD64-specific single chain immunotoxin exhibits specific cytotoxicity against acute myeloid leukemia cells. Cancer Res. 2003;63(23):8414–9.
216. Barth S, Huhn M, Matthey B, Tawadros S, Schnell R, Schinkothe T, Diehl V, Engert A. Ki-4(scFv)-ETA', a new recombinant anti-CD30 immunotoxin with highly specific cytotoxic activity against disseminated Hodgkin tumors in SCID mice. Blood. 2000;95(12):3909–14.
217. Bruell D, Bruns CJ, Yezhelyev M, Huhn M, Muller J, Ischenko I, Fischer R, Finnern R, Jauch KW, Barth S. Recombinant anti-EGFR immunotoxin 425(scFv)-ETA' demonstrates anti-tumor activity against disseminated human pancreatic cancer in nude mice. Int J Mol Med. 2005;15(2):305–13.
218. Singh R, Samant U, Hyland S, Chaudhari PR, Wels WS, Bandyopadhyay D. Target-specific cytotoxic activity of recombinant immunotoxin scFv(MUC1)-ETA on breast carcinoma cells and primary breast tumors. Mol Cancer Ther. 2007;6(2):562–9.
219. Berges N, Hehmann-Titt G, Hristodorov D, Melmer G, Thepen T, Barth S. Human cytolytic fusion proteins: modified versions of human granzyme B and angiogenin have the potential to replace bacterial toxins in targeted therapies against CD64+ diseases. Antibodies. 2014;3:92–115.
220. Haigis MC, Kurten EL, Raines RT. Ribonuclease inhibitor as an intracellular sentry. Nucleic Acids Res. 2003;31(3):1024–32.
221. Rutkoski TJ, Raines RT. Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity. Curr Pharm Biotechnol. 2008;9(3):185–9.
222. De Lorenzo C, Di Malta C, Cali G, Troise F, Nitsch L, D'Alessio G. Intracellular route and mechanism of action of ERB-hRNase, a human anti-ErbB2 anticancer immunoagent. FEBS Lett. 2007;581(2):296–300.
223. Furia A, Moscato M, Cali G, Pizzo E, Confalone E, Amoroso MR, Esposito F, Nitsch L, D'Alessio G. The ribonuclease/angiogenin inhibitor is also present in mitochondria and nuclei. FEBS Lett. 2011;585(4):613–7.

224. Lee FS, Vallee BL. Binding of placental ribonuclease inhibitor to the active site of angiogenin. Biochemistry. 1989;28(8):3556–61.
225. Shapiro R, Vallee BL. Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin. Proc Natl Acad Sci U S A. 1987;84(8):2238–41.
226. Erickson HA, Jund MD, Pennell CA. Cytotoxicity of human RNase-based immunotoxins requires cytosolic access and resistance to ribonuclease inhibition. Protein Eng Des Sel. 2006;19(1):37–45.
227. Lee FS, Vallee BL. Structure and action of mammalian ribonuclease (angiogenin) inhibitor. Prog Nucleic Acid Res Mol Biol. 1993;44:1–30.
228. Leland PA, Raines RT. Cancer chemotherapy-ribonucleases to the rescue. Chem Biol. 2001;8(5):405–13.
229. Bretscher LE, Abel RL, Raines RT. A ribonuclease A variant with low catalytic activity but high cytotoxicity. J Biol Chem. 2000;275(14):9893–6.
230. Newton DL, Hansen HJ, Mikulski SM, Goldenberg DM, Rybak SM. Potent and specific antitumor effects of an anti-CD22-targeted cytotoxic ribonuclease: potential for the treatment of non-Hodgkin lymphoma. Blood. 2001;97(2):528–35.
231. Zewe M, Rybak SM, Dübel S, Coy JF, Welschof M, Newton DL, Little M. Cloning and cytotoxicity of a human pancreatic RNase immunofusion. Immunotechnology. 1997;3(2):127–136.
232. Mikulski S, Grossman A, Carter P, Shogen K, Costanzi J. Phase-I human clinical-trial of onconase(r) (p-30 protein) administered intravenously on a weekly schedule in cancer-patients with solid tumors. Int J Oncol. 1993;3(1):57–64.
233. Mikulski SM, Costanzi JJ, Vogelzang NJ, McCachren S, Taub RN, Chun H, Mittelman A, Panella T, Puccio C, Fine R, Shogen K. Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresectable malignant mesothelioma. J Clin Oncol. 2002;20(1):274–81.
234. Pavlakis N, Vogelzang NJ. Ranpirnase—an antitumour ribonuclease: its potential role in malignant mesothelioma. Expert Opin Biol Ther. 2006;6(4):391–9.
235. Reck M, Krzakowski M, Jassem J, Eschbach C, Kozielski J, Costanzi JJ, Gatzemeier U, Shogen K, von Pawel J. Randomized, multicenter phase III study of ranpirnase plus doxorubicin (DOX) versus DOX in patients with unresectable malignant mesothelioma (MM). ASCO Meet Abstr. 2009;27(15 S):7507.
236. Zhao J, Wang Z, Yu CJ, Cao YX, Zhang L, Wang CJ, Yang AG. Growth inhibitory effects of recombinant granzyme B containing different N-terminal translocating peptides. Sheng Wu Gong Cheng Xue Bao. 2004;20(4):501–6.
237. Haigis MC, Kurten EL, Abel RL, Raines RT. KFERQ sequence in ribonuclease A-mediated cytotoxicity. J Biol Chem. 2002;277(13):11576–81.
238. Smith BD, Raines RT. Genetic selection for peptide inhibitors of angiogenin. Protein Eng Des Sel. 2008;21(5):289–94.
239. Acharya KR, Shapiro R, Allen SC, Riordan JF, Vallee BL. Crystal structure of human angiogenin reveals the structural basis for its functional divergence from ribonuclease. Proc Natl Acad Sci U S A. 1994;91(8):2915–9.
240. Leonidas DD, Shapiro R, Subbarao GV, Russo A, Acharya KR. Crystallographic studies on the role of the C-terminal segment of human angiogenin in defining enzymatic potency. Biochemistry. 2002;41(8):2552–62.
241. Russo N, Shapiro R, Acharya KR, Riordan JF, Vallee BL. Role of glutamine-117 in the ribonucleolytic activity of human angiogenin. Proc Natl Acad Sci U S A. 1994;91(8):2920–4.
242. Cremer C, Vierbuchen T, Hein L, Fischer R, Barth S, Nachreiner T. Angiogenin mutants as novel effector molecules for the generation of fusion proteins with increased cytotoxic potential. J Immunother. 2014. doi:10.1097/CJI.0000000000000053.
243. Leland PA, Staniszewski KE, Kim BM, Raines RT. Endowing human pancreatic ribonuclease with toxicity for cancer cells. J Biol Chem. 2001;276(46):43095–102.
244. Piccoli R, Di Gaetano S, De Lorenzo C, Grauso M, Monaco C, Spalletti-Cernia D, Laccetti P, Cinatl J, Matousek J, D'Alessio G. A dimeric mutant of human pancreatic ribo-

nuclease with selective cytotoxicity toward malignant cells. Proc Natl Acad Sci U S A. 1999;96(14):7768–73.
245. Murthy BS, De Lorenzo C, Piccoli R, D'Alessio G, Sirdeshmukh R. Effects of protein RNase inhibitor and substrate on the quaternary structures of bovine seminal RNase. Biochemistry. 1996;35(13):3880–5.
246. Chen CZ, Shapiro R. Site-specific mutagenesis reveals differences in the structural bases for tight binding of RNase inhibitor to angiogenin and RNase A. Proc Natl Acad Sci U S A. 1997;94(5):1761–6.
247. Chen CZ, Shapiro R. Superadditive and subadditive effects of "hot spot" mutations within the interfaces of placental ribonuclease inhibitor with angiogenin and ribonuclease A. Biochemistry. 1999;38(29):9273–85.
248. Wei H, Bera TK, Wayne AS, Xiang L, Colantonio S, Chertov O, Pastan I. A modified form of diphthamide causes immunotoxin resistance in a lymphoma cell line with a deletion of the WDR85 gene. J Biol Chem. 2013;288(17):12305–12.

Chapter 9
Therapeutic Impact of Immune Responses in Cancer

Michael Bette

Abstract Therapeutic targeted oncoimmunology has a long history reaching back to the nineteenth century and represents the basis of modern tumor immunology. Cell biological and molecular genetic techniques have uncovered crucial cellular and molecular mechanisms underlying effective cancer immunotherapies used in the clinic. To illustrate the scientific way that led to actual insights into the molecular and cellular approaches realized in recent cancer therapies, this chapter introduces into the history of oncoimmunology. Experimental findings of adoptive cell transfer-based cancer therapy are summarized under functional, immunological aspects. An actual overview of the antitumor prosperity of all genetically engineered tumor cells expressing recombinant cytokines which were characterized by animal experiments is given. The application of antigen-presenting cells which are triple transgenic for immune stimulatory cytokines, tumor specific antigens, and the correlated major histocompatibility complex class I necessary for tumor antigen presentation is explained exemplarily. A recent experimental animal model characterizing critical parameters for preconditioning the host prior to ACT of transgenic T cells and essential therapeutic conditions is described.

Keywords Adoptive cell transfer · Animal model · Cytokine · Effector cells · Immune evasion · Immune surveillance · Transgene · Tumor-specific antigen

Abbreviations

ACT	Adoptive cell transfer
DCs	Dendritic cells
GM-CSF	Granulocyte macrophage colony stimulating factor
HSCT	Hematological stem cell transplantation
IFN-γ	Interferon-gamma
IL	Interleukin
LAK cells	Lymphokine activated killer cells

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MCA	Methylcholanthrene
NK cells	Natural killer cells
MHC	Major histocompatibility complex
M-MuLV	Moloney murine leukemia virus
MMTV	Mouse mammary tumor virus
RAG-2	Recombination-activating gene-2
SCID	Severe combined immunodeficiency
TILs	Tumor-infiltrating leukocytes
TAAs	Tumor-associated antigens
T_{cyt}	Cytotoxic T cells
T_H cells	T Helper cells
T_{H2} cells	T_H type 2
T_{SCM} cells	T memory cells
T_{CM} cells	T central memory cells
T_{EM} cells	T effector memory cells
TSAs	Tumor-specific antigens

9.1 Introduction: Milestones in Cancer Research

Cancer immunology is a field of immunology that comprises all aspects of interactions between the immune system and cancer cells that are functionally involved in the generation, course and control of tumors. The cornerstone for cancer immunology was laid in 1891, when Coley described a successful therapeutic approach for treatment of cancer by generating an inflammatory immune response [1]. Encouraged by observations from Martha Tracy and S. P. Beebe of the Huntington Cancer Research Fund, who demonstrated that large multiple sarcomas in dogs rapidly disappeared under local or systemic injections of bacterial toxins, Coley applied a mixture of bacterial toxins derived from the Streptococcus erysipelas and the *Bacillus prodigiosus* in cancer patients. The thus generated erysipelas infection resulted in tumor clearance in ~30 % of patients with lymphoma or sarcoma. Based on the success of this cancer therapy, Coley concluded that infections "…may have an important bearing upon the whole cancer problem, since, if by the administration of certain bacterial toxins we can cause the degeneration, death, and absorption of living tumor cells of one variety of cancer—sarcoma—it is not unreasonable to suppose that by the use of some other forms of bacterial toxins we may succeed in destroying or inhibiting the growth of the other and more common variety—carcinoma." [2] Based on his clinical observations he formulated the theory that post-surgical infections can help patients to recover better from their cancer by provoking an inflammatory response. But while Coley assumed that in most cases the etiology of cancer is associated with an acute injury [3, 4], Paul Ehrlich proposed the hypothesis that nascent transformed cells arise continuously in our bodies and he further suggested that the immune system continuously scans for transformed cells and can suppress and finally eradicate such cells [5]. As a possible mechanism to combat transplanted neoplastic cells, Paul Ehrlich assumed an antibody-mediated athrepsy

of nutritive substances essentially required for the development of the neoplastic cells but was unable to identify any tumor-specific nutrition factor, due to technical limitations at that time.

The first experimental proof demonstrating the generation of a tumor-specific immune response was provided by Richmond Prehn and Joan Main in 1957 who showed that tumors induced by chemical carcinogens in mice could stimulate tumoricidal responses leading to rejection of this kind of experimentally induced tumor [6]. Surprisingly, spontaneously arising tumors were not rejected when tested in the same experimental manner. From this and subsequent studies it was a deducted option that naturally originating tumors were not immunogenic due to a given immune tolerance against the body's own antigens rendering the immune system powerless in the control of spontaneously developing neoplasms.

However, this assumption was challenged by Burnet and Thomas. Burnet implied that neo-antigens specific for degenerated cells can arise in tumors and might provoke an effective immune response that would eliminate developing cancers [7–9]. Thomas incorporated the age of an organism and the thus increasing probability for cancer development due to somatic mutations. The existence of somatic mutations rendering normal cells to become tumorigenic was shown by Sanford and colleagues in 1954 [10]. They verified that a normal fibroblast cell develops spontaneous mutations by *in vitro* culturing over several passages and acquires the propensity to generate sarcomata when injected into mice of the homologous strain.

Based on the experimental verification of spontaneous mutations as a natural source of cancer, Thomas suggested that complex long-lived organisms must possess mechanisms to protect against neoplastic disease similar to those mediating homograft rejection [11]. Describing the existence of mouse tumor-specific antigens and their functional role for immune system based tumor defense [12], represented a further crucial brick in the composition of the cancer immunosurveillance hypothesis, first conceptualized by Burnet in 1957. Citation: "*The failure in cancer is due not to any weakness of the organism but to a change in the character of the cells rendering them in one way or another insusceptible to the normal control.*" Interestingly, this prediction includes also phenomena in oncoimmunology not known in former times but today termed as "immune evasion".

In 1984, Snyder and Bishop observed that a single mutation in the oncogenic tyrosine kinase *v-src* of the *Rous-Sarcom*-Virus resulted in the loss of tumorigenicity of the virus in immunocompetent mice [13]. They explained this result with the presumption that due to the mutation the virus evasion of the host immune response was no longer possible and concluded that evasion of the host immune response is a necessary step in tumorigenesis by *v-src*. In 1985, Mullen and Schreiber observed, in an UV-induced fibrosarcoma mouse model, that tumor cells can actively suppress the specific tumoricidal immune response without suppressing the immune system in general [14] and termed this effect a "tumor-induced evasion from the immune system" [15]. At the same time, Rooney and colleagues demonstrated that Epstein-Barr virus-positive Burkitt's lymphoma can evade from the cytotoxic T cell response [16]. Based on these observations the hypothesis of an immune evasion strategy of neoplastic cells was introduced to the field of tumor immunology.

So far, the immune system was regarded as a tumor defense system only. But in the late 1990s, it was reported that tissue sites with inflammation were more frequently associated with tumor formation, leading to the assumption that leukocytes might also contribute to tumor growth. In 1986, Dvorak and colleagues indicated tumor sites as "wounds that never heal" [17] and in the last 20 years numerous immune cell derived factors with pro-tumorigenic properties have been identified. But to cope with the history of tumor science, it has to be pointed out, that this "novel" insight in tumor development was previously recognized by Virchow in 1863, who postulated during one of his lecture at the University of Berlin [18], that a chronic irritation and previous injuries are preconditions for tumorigenesis. In addition, multiple resident stromal cell types which are part of the tumor microenvironment can collectively contribute to tumor progression. The interplay of neoplastic cells, stromal cells and invading leukocytes leads to a miscellaneous activity pattern of the various cell types and a complex cocktail of soluble mediators, which altogether sculpture the developing tumor. By unraveling the tumor sculpting effects of the immune system on developing tumors, the term cancer immunosurveillance used as a host-protecting mechanism was no longer appropriate in its original form. To describe more accurately the dual host-protecting and tumor-sculpting interplay between immune and tumor cells, Dunn, Bruce, Ikeda, Old and, Schreiber proposed the use of the broader term "cancer immunoediting" [19]. They envisaged the model of cancer immunoediting, which includes three phases of cancer development termed as "Elimination, Equilibrium and Escape" [20, 21]. The outcome of the cancer immunoediting process is uncertain and depends on whether the immune system is able to completely eliminate all neoplastic cells during the "Elimination phase," or tumor sculpting processes successfully interfere with leukocyte functions by induction of immunologic anergy, tolerance or indifference against the neoplastic cells.

The "Elimination phase" starts at the point when cells of the innate immune system recognize the presence of a growing tumor, due to the tumor dependent local remodeling and damage of stromal tissue. As a consequence, inflammatory signals are generated leading to the attraction and activation of cells of the innate immune system to the local tumor site. These tumor-infiltrating leukocytes (TILs) produce various cytokines and chemokines, which further promote infiltration and activation of more TILs, in a self-enhancing circuit. Some mediators of this complex cytokine/chemokine storm carry inhibitory effects on the formation of new blood vessels. Other cytokines/chemokines function as activators of cytotoxic cells or promote tumor death via apoptosis. The resulting tumor cell debris become ingested by dendritic cells (DCs) which afterwards migrate to the draining lymph nodes, where they function as antigen-presenting cells (APCs). In the case of the presence of tumor-specific antigens (TSAs) these dendritic cells are able to recruit cells of the adaptive immune system. In the final phase of elimination, tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site and the TSA-specific killer T cells then destroy the antigen-bearing tumor cells. The anti-tumorigenic immune response runs out and terminates the "Elimination phase".

However, in rare cases some neoplastic cells survive the “Elimination phase” and enter the “Equilibrium phase” which is clinically characterized by a dormancy of the tumor cells. This immune-mediated tumor dormancy is sustained by a fragile balance between the presence of tumoricidal and tumor promoting cytokines and might persist over several years. During this time, tumor editing occurs and it gradually becomes likely, that tumor cell variants develop, which are able to avoid immune surveillance due to their loss of antigenicity. Additionally, tumor cell variants might actually become more and more capable to use leukocytes or stromal cells of the tumor microenvironment to support tumor development. At this stage the tumor cells pass into the “Escape phase”, in which the balance of the tumor microenvironment is skewed towards tumor progression by generating a complex immune suppressive milieu and enhancing the vascularization of the growing solid tumor. In fact, the “Elimination phase” and the “Escape phase” share many similarities, except that the novel tumor cell variants have acquired an enhanced malignancy during their functional dormancy at the “Equilibrium phase”. Interestingly, the period of dormancy of tumor cells can last over many years, as observed for human melanoma. In 2003, two cases of malignant melanoma have been reported in kidney allograft recipients, that received the organs from a donor which had been cured from melanoma over a period of 16 years and was classified as melanoma free [22]. In both cases the donor kidney originated from the same donor.

9.2 Immunotherapies at the Beginning of the Twenty-First Century

The most important message of tumor medicine, starting with early observations by Coley, Ehrlich and, Virchow in the nineteenth century up to actual insights into the molecular and the cellular mechanisms underlying the hallmarks of cancer is: “Cancer can be overcome by the body’s own immune system at all phases of tumor editing.” Enormous effort has been made in the past 30 years to identify suitable cytokines, TSAs and tumor-associated antigens (TAAs) to develop biochemical or molecular biological tools for targeting tumor cells for inhibiting tumor growth supporting factors and/or for triggering antitumorigenic immune responses. To date, three main groups of immunotherapies are accepted for cancer treatment and used in the clinic: (i) antibody therapies (ii) cytokine/chemokine therapies, and (iii) cell-based therapies.

The following sections will discuss current cancer immunotherapeutic approaches and their underlying cellular mechanisms. An overview about the therapeutic potential and challenges of up to current antibody-based strategies used in human cancer therapies is given in another topic of this book.

Cytokine-based therapies use specific cytokines or cytokine cocktails to manipulate and direct immune responses to generate tumoricidal effector cells able to eradicate existing tumors. The rationale for this approach is based on the observation that during the phase of immunoediting tumor cells can exploit cytokines to

reduce immune effector functions, increase resistance against apoptosis, or even support tumor growth and dissemination. The therapeutic application of cytokines might counteract the tumor controlled editing process mediated by a sophisticated cytokine milieu and reverse the tumor microenvironment from tumor permissive to tumoricidal. The first clinical results of cytokine-based cancer therapies, which used partially purified cytokines, started in the late 1970s by the application of leukocytes derived interferons [23, 24]. In the following years, further trials demonstrated the therapeutic benefits of leukocyte derived interferons on non-Hodgkin's lymphomas, myeloma, malignant melanoma, and other malignancies even if only with moderate success. Additionally, cytokines from the group of interleukins known to exhibit immune stimulatory properties were found to be effective in cancer treatment. The major advantage of using cytokines in cancer treatment is the unlimited availability of recombinant cytokines. The huge disadvantage of using systemically applied cytokines or cytokine cocktails are the associated severe side effects [25–31]. A novel approach to deliver cytokines to cancer patients is the usage of genetically modified cells which express and release one or more different cytokines. Transfer of such cells into the tumor microenvironment seems to be more effective for tumor treatment with simultaneously reduced adverse effects observed in association with systemic application of high dosages of purified cytokines. This approach combines classical approaches of cytokine/chemokine therapies, but also represents a cell-based cancer therapy.

9.3 Cell-Based Cancer Immunotherapies

The method of cell-based cancer therapies consists of adoptive cell transfer (ACT) of isolated viable autogenic, allogenic or syngenic cells into a cancer patient. Application of ACT for tumor eradication might be effective during all phases of cancer immune-editing but typically, treatment starts after a tumor disease has been diagnosed. This mostly implies, that neoplastic cells have successfully undergone dysplasia probably followed by anaplasia and have reached the phase of "Escape". At this stage genetically/ functionally modified cells become transferred with the purpose to sensitize, reactivate and, support remaining tumoricidal immune cells from the equilibrium phase. Figure 9.1 summarizes current ACT strategies in cancer medicine based on the transferred cell type. The application of hematological stem cell transplantation (HSCT) can be included to the method of ACT but, in fact, it consists of a therapeutic option for post tumor treatment of hematopoietic and lymphoid malignancies after elimination of the tumor cells by various lymphoablating schedules (Fig. 9.1). The main goal of the non-tumor targeting strategy of HSC transfer is to re-establish a functional immune system rather than to eradicate existing tumor cells. All other ACT strategies are tumor targeted and are directed to transfer the immunologic capability to generate an effective tumoricidal response against

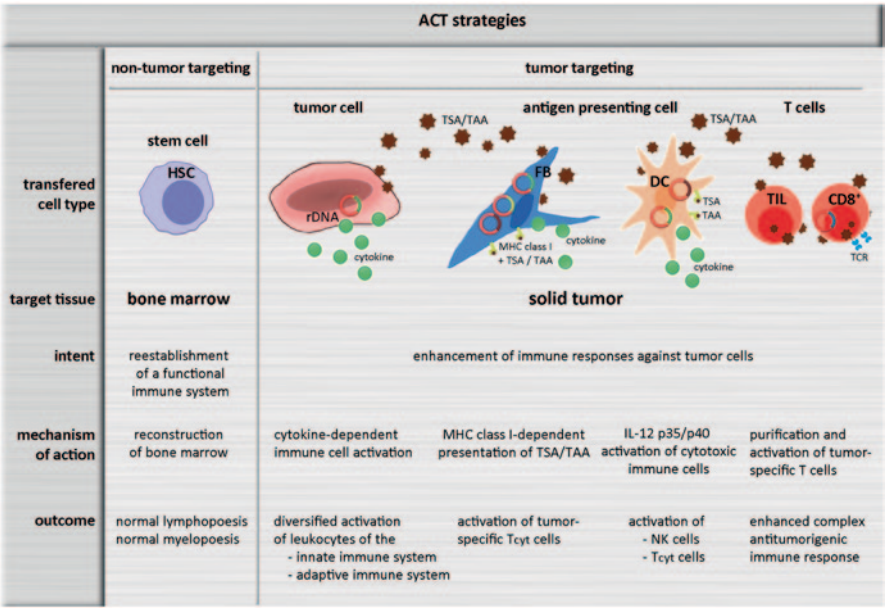

Fig. 9.1 Illustration of Adoptive Cell Transfer strategies. ACT strategies are arranged on the basis of the different immune cell sub-populations used for non-tumor targeting approaches such as hematopoietic stem cells (*HSC*) and targeted tumor therapies such as tumor cells fibroblasts (*FB*), dendritic cells (*DC*) or T cells either purified directly from the tumor stroma (tumor-infiltrating leukocyte, *TIL*) or from blood ($CD8^+$). To summarize the various strategies for which the specified cell types are used only the most representative manipulations are depicted. To show, when one of the specified cell types has been used genetically modified, is illustrated as follows: a transgenic vector is depicted by a *circle*; the transfected genes in the vector are color coded with *green*: representing a gene encoding for a cytokine; *yellow*: for molecules relevant in antigen presentation such as the MHC class I molecule; *blue*: for a T cell receptor (*TCR*); and *brown*: for tumor cell specific/associated antigens (*TSA/TAA*). When cells were loaded with antigens prior to adoptive transfer, *brown stars* are depicted. The target tissue, the intent, the most representative and proven mechanism of action observed in different tumor entities and the underlying mode of action are listed below each cell type

present neoplastic cells back into the recipient. In most cases, the stimulation of tumoricidal responses became realized by genetical manipulation of the transfected cells with various expression vectors for recombinant cytokines, TSAs or TAAs, or major histocompatibility complex (MHC) class I antigens. Eligible cells for delivering the different recombinant tools into the tumor stroma are resident cells of the tumor stroma such as fibroblasts, professional APCs of the immune systems such as DCs, and even tumor cells by themselves (Fig. 9.1). Recently, the autologous cell transfer of *ex vivo* isolated and *in vitro* TILs has been established as an additional ACT-based tumor therapy (Fig. 9.1) and actually the usage of recombinant TILs is under investigation. Altogether, each cell type used for ACT fulfills a specific point of action within the tumor-immune-microenvironment network.

9.4 Tumor Cells Transgenic for Cytokines Used in ACT

One of the first ACT tumor strategies used genetically modified tumor cells to deliver immune stimulatory cytokines into the tumor microenvironment in a way of a Trojan horse. Based on the assumption that an immune suppressive milieu within the tumor microenvironment is responsible for tumor persistence, the rationale of this approach is to break open the immune suppressive milieu and to elicit or increase a cell-mediated antitumoral response. Based on the experience of some tumor therapeutic effects of purified cytokines systemically applied at high doses, the first recombinant genes delivered into the tumor via transgenic cells are cytokines. For this, the coding sequence of the cytokine of interest became cloned, ligated into an expression vector, which is then transferred into cells of a defined cell line or primary cells isolated from the host. The transgenic cells are then applied to the host either systemically or directly into the solid tumor. Once these cells have reached the tumor, they start to express and release the recombinant cytokine which leads to an enhanced immune stimulatory milieu within the tumor microenvironment and, hopefully, to an induction of an effective anti-tumorigenic immune response. The functional characterization of a still increasing number of cytokines, chemokines and other immune response modulating, soluble mediators, has helped to identify appropriate candidates for recombinant cell-based ACT strategies. The advantage of using tumor cells as supplier of recombinant cytokinesis that the necessary cytokine dose is much lower in comparison to systemic application and, thus, adverse side effects might be reduced. Additionally, a long lasting, local expression of immune mediators is assured, which will not cease before all tumor cells, *de novo* arisen as well as transgenic tumor cells become eradicated in equal measure. Hence, various tumor cell lines were genetically modified to express recombinant cytokines known to exert tumoricidal effects directly upon tumor cells or to induce or enhance host-mediated mechanisms (for overview see Table 9.1).

One of the first cytokines used for proving the novel concept, that delivery of a recombinant expressed cytokine via genetically transfected tumor cells can be an effective therapeutic approach in tumor treatment, was the T Helper (T_H) cells-derived interleukin (IL)-4. This T_H type 2 (T_{H2}) synthesized-derived lymphokine was previously characterized as a multifunctional cytokine which exhibits a broad range of activities on B- and T cells and on hematopoietic cell lineages *in vitro* [32]. For construction of IL-4 expressing tumor cell lines, a genomic DNA fragment that contained the entire murine IL-4 coding region and about 3.5 kb of its 3′ flanking sequences was cloned from an embryonic BALB/c library, placed under the control of the promoter/enhancer from the LTR of either the Moloney murine leukemia virus (M-MuLV) or the mouse mammary tumor virus (MMTV) [33]. Transfection of these plasmids into mammary adenocarcinoma cells (K485) resulted in the generation of several transgenic mammary adenocarcinoma cell lines, which differ in their amount of released IL-4. The IL-4-transgenic mammary adenocarcinoma cell lineK485/D2B-1, which was found to express the highest levels of IL-4 when compared to other cell lines, was used for tumor transplantation into *nu/nu* mice [33]. The results showed that IL-4 expression substantially reduced the tumor growth,

Table 9.1 Overview of antitumor effects of ACT approaches using tumor cell lines transgenic for cytokines in various animal models

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for			Outcome[a]			References
IL-1α	Bladder carcinoma	MBT2	Irradiated	Development	–	Therapy	Delayed	–	Delayed	[41]
IL-1β	Bladder carcinoma	MBT2	Irradiated	Development	–	Therapy	Delayed	–	Delayed	[41]
IL-2	Fibrosarcoma	CMS-5	Living	Development	Vaccination	–	Rejected	Protected	–	[42–44]
	Melanoma	B16	Living	Development	Vaccination	–	Rejected	Protected not effective	–	[45, 46]
	Melanoma	B16	Irradiated	–	Vaccination	–	–	Protected	–	[45]
	Melanoma	Cloudman S91	Irradiated	–	Vaccination	Therapy	–	Protected	Eliminated	[47, 48]
	Myeloma	X63-Ag8.653	Living	Development	–	Therapy	Rejected	–	Eliminated	[49]
	Mammary tumor	4T07	Irradiated	Development	Vaccination	–	Rejected	Protected	–	[50]
	Mammary adenocarcinoma	TS/a	Living	Development	Vaccination	–	Rejected	Protected	–	[51]
	Lewis lung carcinoma	D122	Irradiated	Development	Vaccination	Therapy	Delayed	Protected	Eliminated	[52]
	Lewis lung carcinoma	LLC	Living	Development	Vaccination	–	Rejected	Delayed	–	[53]
	Colon carcinoma	CT-26	Living	Development	Prevention	–	Rejected	Rejected	–	[46]
	Bladder carcinoma	MBT2	Irradiated	Development	Vaccination	Therapy	Rejected	Protected	Eliminated	[41, 54]
	Renal cancer	SK-RC-29	Living	Development	–	–	Rejected	–	–	[55]
	Prostate cancer	R3327-Mat-LyLucell	Irradiated	–	Vaccination	Therapy	–	Protected	Eliminated	[56]
IL-4	Melanoma	B16	Living	Development	–	–	Delayed	–	–	[45]
	Melanoma	B16	Irradiated	–	Vaccination	–	–	Protected	–	[45]
	Mammary adeno-carcinoma	K485	Living	Development	Prevention	–	Rejected	Rejected	–	[33]
	Plasmacytoma	J558L	Living	Development	Prevention	–	Rejected	Rejected	–	[33, 35]
	Lewis lung carcinoma	LLC	Living	Development	Vaccination	–	Rejected	Delayed	–	[53]
	Renal cell carcinoma	Renca	Living	Development	Vaccination	Therapy	Rejected	Protected	Eliminated	[40]

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for			Outcome[a]			References
IL-5	Melanoma	B16	Irradiated	–	Vaccination	–	–	Not effective	–	[45]
IL-6	Melanoma	B16	Living	Development	–	–	Delayed	–	–	[45]
	Melanoma	B16	Irradiated	–	Vaccination	–	–	Protected	–	[45]
	Lewis lung carcinoma	D122	Irradiated	Development	Vaccination	Therapy	Delayed	Protected	Eliminated	[57]
	Lewis lung carcinoma	LLC	Living	Development	–	–	Shortened	–	–	[58]
IL-7	Ependymo-blastoma	203-glioma	Living	Development	–	Therapy	Rejected	–	Eliminated	[59]
	Plasmacytoma	J558L	Living	Development	–	–	Rejected	–	–	[60]
	Leukemia	WEHI-3	Irradiated	–	Vaccination	–	–	Protected	–	[61]
1L-12 p35/p40	Fibrosarcoma	MCA 205	Living	Development	–	Therapy	Rejected	–	Eliminated	[62]
	Colon carcinoma	CT-26	Living	Development	Vaccination	–	Rejected	Protected	–	[63]
IL-23	Colon carcinoma	CT-26	Living	Development	Vaccination	–	Rejected	Protected	–	[63]
IFN-γ	Fibrosarcoma	CMS-5	living	Development	Vaccination	–	–	Protected	–	[44, 64]
	Melanoma	B16	Living	Ddevelop-ment	Vaccination	–	Delayed	Not effective	–	[45]
	Melanoma	B16	Irradiated	–	Vaccination	–	–	Protected	–	[45]
	Bladder carcinoma	MBT2	Irradiated	Development	Vaccination	Therapy	Delayed	Protected	Eliminated	[41, 54]
	Neuroblastoma	C1300	Living	Development	Vaccination	–	Rejected	Protected	–	[65]
	Renal cancer	SK-RC-29	Living	Development	–	–	Not effective	–	–	[55]
	Prostatecancer	R3327-Mat-LyLucell	Irradiated	–	–	Therapy	–	–	Eliminated	[56]

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for			Outcome[a]			References
TNFα	Melanoma	B16	Living	Development	–	–	Delayed	–	–	[45]
	Melanoma	B16	Irradiated	–	Vaccination	–	–	Protected	–	[45]
	Skin tumor	1591-RE	Living	Development	–	–	Delayed	–	–	[66]
	Bladder carcinoma	MBT2	Living	Development	Prevention vaccination	Therapy	Rejected	Rejected protected	No effect	[54, 67]
	Plasma cytoma	J558L	Living	Development	–	–	Rejected	–	–	[68]
	Fibrosarcoma	MCA 205	Living	Development	–	–	Rejected	–	–	[69]
G-CSF	Colon adenocarcinoma	C-26	Living	Development	Prevention	–	Rejected	Delayed	–	[70]
GM-CSF	Melanoma	B16	Living	Development	–	–	Toxic for the animal	–	–	[45]
	Melanoma	B16	Irradiated	–	Vaccination	Therapy	–	Protected	Eliminated	[45]
	Bladder carcinoma	MBT2	Irradiated	–	–	Therapy	Delayed	–	Eliminated	[41]
	Renal cell carcinoma	Renca	Irradiated	–	Prevention	–	–	Rejected	–	[45]
	Colon carcinoma	CT-26	Irradiated	–	Prevention	–	–	Rejected	–	[45]
	Leukemia	WEHI-3	Irradiated	–	Vaccination	–	–	Protected	–	[61]
	Prostatecancer	R3327-Mat-LyLucell	Irradiated	–	Vaccination	Therapy	–	No effect	Eliminated	[56]
	Fibrosarcoma	CMS-5	Living	Development	–	–	Delayed	–	–	[44]

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for			Outcome[a]			References
IL-2+IFN-γ	Melanoma	B16	Living	–	Vaccination	–	–	Protected	–	[45]
	Fibrosarcoma	CMS-5	Living	Development	Vaccination	–	Rejected	Protected	–	[44]
GM-CSF+IL-2	Melanoma	B16	Living	–	Vaccination	–	–	Protected	–	[45]
JE/MCP-1	Chinese hamster ovary	CHO	Living	Development	Prevention	–	Rejected	Rejected	–	[71]

Definition of terms used:
"irradiated": mitotic activity of cells was inactivated by X-irradiation; "living": cells were vital;
"development": experimental approach, in which exclusively transgenic cytokine-expressing tumor cells were injected into the animal; "vaccination": transgenic tumor cells were injected prior to wild-type tumor cells; "prevention": transgenic tumor cells were injected in parallel with wild-type tumor cells; "therapy": transgenic tumor cells were injected after a wild-type tumor had been formed in an animal;
"rejected" (term is used in context of prevention): transgenic tumor cells become completely eradicated; "delay": expansion rate of tumor cells was reduced compared to wild type tumor cells; "protected" (term is used in context of vaccination): transgenic tumor cells become completely eradicated; "eliminated" (term is used in context of vaccination): existing tumor cells are killed by the transgenic tumor cells

[a] Note, that the tabulated rating in most cases does not reflect 100 % efficacy but the successful therapeutic outcome

when compared to non-transfected K485 cells, which in fact implies a tumor suppressive effect rather than a tumor therapeutic effect. However, a more pronounced tumor therapeutic effect was found, when murine plasmacytoma cells (J558L) [34] were transfected with an expression vector containing the *IL-4* gene under the control of a promoter/enhancer derived from the mouse immunoglobulin heavy chain genes (transgenic plasmacytoma cell lines: J558L-I3L6 [33]; J558L-XEPIL4 [35]). While subcutaneous injection of non-transfected plasmacytoma cells in *nu/nu* mice as well as in BALB/c mice led to the formation of large tumors, the injection of the IL-4 recombinant plasmacytoma cell lines J558L-I3L6 or J558L-XEPIL4 never led to any tumor formation [33, 35].

Furthermore, a paracrine antitumorigenic effect of IL-4 was found, as a mixture of non-transfected plasmacytoma cells with IL-4 expressing plasmacytoma cells prevented *nu/nu* mice and also BALB/c mice from tumor formation. This paracrine-mediated tumor suppressive property of IL-4 was not limited to syngenic plasmacytoma cells but also transferable to a variety of other murine tumor cell types. Mixed inoculation of IL-4 transgenic plasmacytoma cells with SMF cells, a mammary adenocarcinoma line [36] or with A.6R.1 cells, an Abelson virus-transformed fibroblast cell line [37], prevented *nu/nu* mice from tumor formation, and mixture with B16-FO, a C57BL/6-derived melanoma [38] or the sarcoma 180 [39] markedly inhibited growth of the melanoma tumors or the sarcoma tumor, respectively [33]. These experiments suggest a general tumor suppressive effect of IL-4 on diverse tumor cells of epithelial and mesenchymal origin, which is mediated in a paracrine fashion.

In vivo experiments using an IL-4 transgenic cell line derived from a spontaneously arising renal cell carcinoma of BALB/c mice (Renca-IL-4C) exhibited similar local tumor suppressive effects on the parental Renca cells in BALB/c mice and *nu/nu* mice [40] as observed with the mammary adenocarcinoma and the plasmacytoma cell lines [33]. In all of these tumor models histological analyses revealed an influx of primarily macrophages and granulocytes and only few T cells at the site of mixed (non-transfected- and IL-4 expressing) tumor cell injection. This might reflect a mainly innate immune system mediated tumoricidal response. Interestingly, when non-transfected- and IL-4 expressing Renca-cells were inoculated at distant sites, a mainly $CD8^+$ T cell-dependent systemic immune response was generated and was responsible for eradication of the renal tumor [40]. This systemic tumoricial T cell response was blocked when $CD8^+$ T cells were eliminated prior to injection of the parental Renca cells. Additionally, when the cured mice were injected with parental Renca cells, about 50% rejected the challenge [40], indicating some level of immune memory had been generated. In summary, the animal experiments of lL-4 expressing tumor cells show that the location at which IL-4 becomes synthesized determines the kind of tumoricidal immune response. If IL-4 is locally expressed within the tumor microenvironment, macrophages and granulocytes of the adaptive immune system mainly contribute to the tumor eradication whereas systemically synthesized IL-4 outside the tumor microenvironment mainly leads to an activation of $CD8^+$ cytotoxic T (T_{cyt}) cells, which belong to the adaptive arm of the immune system. A further important finding of these data is that different immune cell types

including macrophages, granulocytes and/or T_{cyt} cells can mediate the cytokine-induced tumoricidal response. Notably, activation of cytotoxic cells of the adaptive immune system seems to generate a significant and, most important, a long lasting antitumor response.

One cytokine having the propensity to activate cytotoxic cells is the T cell-derived lymphokine IL-2. This lymphokine has been found to stimulate the proliferation of T_{cyt} cells [72], T_H cells [73] and natural killer (NK) cells [74] and is able to transform resting lymphocytes into lymphokine activated killer (LAK) cells [75, 76]. All these cell types are known to participate in the antitumor response which is the reason whyIL-2 is the most frequently used cytokine in tumor cell-based ACT studies. Tumor cells transgenic for IL-2 were successfully applied in tumor models of fibrosarcoma, melanomas, myeloma, mammary carcinomas, carcinomas of the lung, colon and bladder, as well as renal and prostate cancers. The therapeutic benefit was mostly associated with high levels of cytotoxic cells. Immunohistochemical analyses by using antibodies directed against various leukocyte differentiation antigens (e.g. anti-CD4 detecting T_H cells, anti-CD8 for T_{cyt} cells, CD11b/CD18 for NK cells) revealed an enhanced presence of T cells [41], NK cells [41, 42], monocytes/macrophages and granulocytes [47] at the site of IL-2 transgenic tumor cell injection. The local presence of these cells suggests their effector role in antitumor immunity, but the distinct functional proof of the tumoricidal potency of distinct leukocyte subpopulations was provided by either depletion of defined immune cell populations *in vivo* or by the usage of animal strains, which genetically lack functional T cells (e.g. SCID mice, RAG-2 mice).For depletion experiments, antibodies able to kill a defined cell subpopulation were injected into the animal before application of IL-2 transgenic tumor cells. Depletion of $CD4^+$—and $CD8^+$ T cells in the IL-2 CMS-5 fibrosarcoma mouse model did not bypass the IL-2 mediated rejection of the transgenic tumor cells suggesting that the protective immune response is not mediated by T cells [42].The rejection of IL-2 transgenic CMS-5 fibrosarcoma cells in T cell deficient BALB/c *nu/nu* mice further supports the T cell independent antitumor immunity [42]. Additionally, the growth reduction of IL2 transgenic MBT2 bladder tumor cells in Swiss *nu/nu* mice also supports a T cell independent antitumorigenic effect of IL-2, at least when expressed at higher concentrations [52]. Nevertheless, IL-2-activated T cells exhibit a pivotal role in the establishment and long-lasting protection against tumor reimplantation. In various tumor models it was found, that once animals were cured from the tumor, an immunological protection in the sense of a vaccination developed in most cases, which protected animals of the reimplantation of the tumor [45, 48, 50, 54]. This protection was only possible, when the animals possessed functional T cells and the protection was associated with the generation of lytic cells such as LAK cells [41–43, 46, 47, 49, 52, 56].

The most promising observation of the IL-2-based ACT using transgenic tumor cells is that this approach was also successful in the therapeutic treatment of wild-type tumors [41, 47, 48, 52, 54, 56] which reflects the clinical situation in human cancer treatment. Other cytokines were cloned and transfected in tumor cells, from which IL-4, IL-6, IL-7 and granulocyte macrophage colony stimulating

factor (GM-CSF) showed some and interferon (IFN)-γ highly effective therapeutic properties (Table 9.1). In addition, the combined transfer of two recombinant cytokines into the same tumor cell may enhance the antitumor immunity [44, 54].

In summary, the findings raised by the various animal tumor models of ACT of cytokine-transfected tumor cell lines revealed that, depending on the cytokine or cytokine-mix expressed, an effective tumoricidal response can be induced by activation of nonspecific cytotoxic effector cells and/or tumor-specific T cells. Furthermore, for the acquisition of a long-lasting antitumor immunity, the activation of $CD8^+$ T cells is essential. The central role of tumor-specific lymphocytes of the adaptive immune system in the tumoricidal immune response further supports the existence of a specialized APC system which enables/enhances the presentation of TAAs or TSAs after the ACT of living or irradiated transgenic tumor cells. This led to the concept to transfer MHC class I genes or genetic information of TAAs and TSAs into transferable cells. For the gene transfer of such factors, cells of the tumor stroma such as fibroblasts or DCs are most applicable, because both cell types are known to function as efficient APCs and long lasting presentation of translated TAAs via the MHC class I complex seems most likely. Furthermore, the danger that living auto- or allogenic transferred tumor cells could spread and metastasize within the patient can be avoided.

9.5 Antigen Presenting Cells Transgenic for Tumor Antigens Used in Adoptive Cell Transfer

Based on genetic alterations neoplastic cells express specific antigens which are either present only on tumor cells—in case of TSAs—or on both tumor cells and also some normal cells—in case of TAAs—within the tumor microenvironment. The presentation of these antigens together with MHC class I molecules to effector T cells has been found to be a critical step in the generation of an efficient T_{cyt} cell-dependent response against the tumor. Insufficient MHC class I-dependent presentation of TSAs/TAAs by either tumor cells or host professional APCs can be a basic cause for a failure of the immune response in the tumor bearing host, even when TSAs or TAAs are expressed [77]. However, within the scope of immune evasion most neoplastic cells down regulate their expression of MHC class I molecules and, thereby, reduce the level of MHC class I-mediated activation of T_{cyt} cells [78–80]. For example, human papilloma viruses (HPVs) express the oncoprotein E5 which is implicated in MHC-class I downregulation [81, 82] and was shown to affect MHC class II maturation in IFN-γ-treated keratinocytes [83]. The therapeutic stimulation of MHC class I expression within the tumor entity via application of recombinant cytokines known to enhance the MHC expression such as IFN-γ [84] is one possibility to overcome this status. In fact, the use of IFN-γ transgenic tumor cells in ACT strategies, as listed above, is a proven possibility to elicit an efficient antitumor response mediated by TILs.

Recent strategies to induce or augment the host antitumor immune response included the transfer of genes encoding MHC class I, costimulatory molecules or cytokines, and TAAs into tumor cells and APCs. A functional proof of the correlation between MHC class I expression, generation of cytotoxic cells within the tumor microenvironment and the clearance of existing metastases by adoptive immunotherapy was given by Restifo and coworker [85]. They showed that transfection of methylcholanthrene (MCA)-induced sarcoma cells with recombinant IFN-γ led to enhanced MHC class I expression on the surface of the tumor cell line which converted it from poor presenter of antigen to high antigen presenter cells. Tumors derived from high presenter cells made it possible to isolate and clone $CD8^+$ TILs, which, when transferred *in vivo,* revealed a tumoricidal response against present metastases from the wild-type MCA-induced sarcoma [85]. These data prove that the presence of a sufficient level of MHC class I and, thus, it is appropriate to assume that an enhanced presentation of TSAs/TAAs is required to achieve therapeutic effects.

Many other animal experimental models used the strategy of enhancing the presentation of TSAs/TAAs by ACT strategies. Because tumor cells are genetically instable [86] and thus cannot be stably transfected with viral vectors, the usage of cells which are known to be efficient in antigen presentation came into the focus of cancer immunotherapies. However, this requires the identification of therapeutic relevant TSAs/TAAs for a given type of tumor, which is indeed, one of the major challenges in tumor medicine. Various methods, such as differential gene analysis, exome sequencing and proteomics are actually used methods to identify genes, peptides or proteins specifically expressed in neoplastic cells or solid tumors and, therefore, possibly they are usable as prognostic or therapeutic factors. Promising experimental results have been observed when fibroblasts and DC were used to transfer TSAs/TAAs into the tumor. To deliver or express TSAs/TAAs into APCs to this day cells were pulsed with unfractionated tumor-derived peptides [87], tumor cell lysates [88], apoptotic cell bodies [89, 90] and mRNA [91, 92] or cDNA libraries [93, 94] derived from tumor cells. Fibroblasts are readily available to be cultured, transfected and selected, and were found to produce physiologically relevant levels of cytokines after the introduction of cytokine genes [95–97]. Furthermore, these cell types can provide a useful manipulation of key aspects of antigen presentation, such as epitope choice, antigen density, and selection of immune- stimulating molecules, to promote the induction of potent cytotoxic T lymphocyte responses.

One sophisticated strategy of using fibroblasts as APCs of TSAs/TSAs is to generate double or triple transgenic fibroblast cell lines which possess transgenic vectors expressing MHC class I molecules, a stimulatory cytokine and either one or more TSAs or TSAs previously identified to be specific for the corresponding tumor entity. Using the murine model of highly malignant SB5b breast carcinoma, Cohen and coworker genetically engineered a fibroblast cell line (LM cells) of C3H/He ($H\text{-}2^k$) mouse origin which expressed an allogenic ($H\text{-}2^d$) MHC class I determinant, the immune stimulatory cytokine IL-2, and a cDNA library derived form a small pool of SB5b breast cancer cells [93, 94]. To enrich the pool of tumor DNA-transgenic cells with those cells, which synthesize tumor relevant antigens in association with

MHC class I, the whole transfected LM cell pool was subdivided in several sub-pools, which were tested on their potency to induce a cytotoxic response against SB5b cells *in vitro*. Only pools of high responders were used for a second round of this immunoselection and the most effective sub-pool of these triple transfected cells was used for ACT against the breast cancer cells. As shown, by further *in vitro* and *in vivo* testing, the tumoricidal response of triple transgenic high responder fibroblasts was mediated by the activation of $CD8^+$ T_{cyt} cells [94]. The immunization of breast cancer bearing mice with these cells had a therapeutic effect and led to eradication of the SB5b tumors in some animals and prolonged survival in others [98]. Furthermore, an immunological long-term immunity against the tumor cells had developed in the cured mice, protecting the animals from re-transplantation of tumor cells [93]. In the related model of intracerebral metastatic breast cancer such triple transgenic cells were also effective in eradication of the intracerebral SB5b metastases [99] suggesting that the tumoricidal immune protection was independent from the organic location of the tumor cells.

9.6 T Cells Used in Adoptive Cell Transfer

The most recent transgenic cell-based ACT strategy is the usage of genetically modified TILs. TILs have been found to serve as a good prognostic marker for many human tumor entities [100–107]. Isolation of TILs from tumor tissues with subsequent *in vitro* stimulation, expansion of tumor specific T cells and transfer back into the patient is hence a promising therapeutic approach. Until now, TIL populations that become therapeutically effective after *in vitro* stimulation were primarily isolated from melanomas thereby limiting the therapeutic usage of TILs to this tumor type. Nevertheless, beside TILs, *in vitro* modified T cells genetically redirected to recognize TSAs/TAAs on the surfaces of tumor cells are powerful therapeutic tools that can be used against virtually all types of tumors [108–113]. For the generation of T cells, expressing TCRs with a high affinity and specificity for TAAs, various techniques are currently available that are reviewed in detail by Restifo and colleagues [114]. A novel approach, termed “Chimeric Antigen Receptor” (CAR) therapy, genetically engrafts the gene sequence encoding the variable region of a target cell-specific antibody onto the TCR intracellular domain that is capable of activating T cells. The resulting transgenic T cells then become activated *in vivo* when the CAR binds to the tumor target antigen, which can take place independent from MHC class I or II. It is also possible to isolate TCR RNA from humanized mice, which bear T cells transgenic for human MHC class I. The immunization of such mice with human tumor antigens results in the generation of T cells specific for human MHC class I-restricted tumor antigens. As a third method, T cells isolated from a patient, found to be a high responder against a defined tumor type, are used as a genetic source to transfect autologous T cells of low responder patients. Numerous clinical trials using T cell based ACTs are on their way and the plethora of individual treatment schemes makes it nearly impossible to identify

critical parameters such as preconditioning treatment and therapeutic parameters like cell dose and differentiation phenotypes of the T cell. Similarly, the impact of other factors such as vaccination against the tumor or cytokine delivery in parallel to the T cell transfer remains unclear. For systematical characterization of such critical therapeutic conditions, animal studies are indispensable.

One animal model that has been extensively used to define some of the above mentioned critical key determinants for successful ACT immunotherapy is the Pmel-1 $CD8^+$ T cell receptor transgenic mouse model [115]. One animal model that has been extensively used to define some of the above mentioned critical key determinants for successful ACT immunotherapy is the Pmel-1 $CD8^+$ T cell receptor transgenic mouse model. This animal model uses the mouse melanoma B16 cell line to induce solid melanomas, which are in concordance with human melanomas, since they share the melanocyte/melanoma (self/tumor) -antigen gp100 [116]. The shared self/tumor-antigen gp100 also known as Pmel can be used in both, human (Pmel-17) and mice (Pmel-1) as a target for T cell-based tumor treatment. To generate murine Pmel1-TCR-transgenic T cells for evaluation in the B16 C57BL/6 mouse model, splenocytes were isolated from Pmel1 transgenic mice and incubated *in vitro* in the presence of gp100 (human $gp100_{25–33}$) and recombinant IL-2 [117]. This priming led to the expansion of mainly Pmel1-TCR-transgenic T cells. For testing T cells which have reached a defined differentiation status, $CD8^+$ T cells or $CD8^+$ T memory (T_{SCM}) cells, $CD8^+$ T central memory (T_{CM}) cells, or $CD8^+$ T effector memory (T_{EM}) cells were isolated [118]. The rationale for testing different developmental stages of $CD8^+$ T memory populations for ACT treatment is based on clinical and preclinical observations showing that the success of ACT-based approaches depends on the differentiation state of the transferred T cell population. Less differentiated T_{SCM} and T_{CM} were found to be more effective in tumor patients than more differentiated T_{EM} cells [119, 120].

Studies using the Pmel-1 mouse model observed a superior tumoricidal effect of T_{SCM} cells on the elimination of existing primary tumors [117]. However, Pmel1-TCR-transgenic $CD8^+$ T cells were able to eliminate the tumor and enhance the survival rate of the animals (up to 100%), large numbers (1×10^7) of these cells were necessary to reach this therapeutic effect. When using low numbers of Pmel1-TCR-transgenic $CD8^+$ T cells tumor eradication was incomplete. In contrast, the number of Pmel1-TCR-transgenic T_{SCM} cells necessary to reveal comparable tumor protection was only 1×10^4. The antitumor efficacy of the more differentiated T memory cells decreased in correlation to a more differentiated status in the order $T_{SCM} > T_{CM} > T_{EM}$. These results show that next to the absolute number of adoptively transferred cells, the T cell differentiation status significantly contributes to the efficacy of the tumor therapy. The aspect of T cell differentiation has to be taken into account particularly within the context of the cell expansion time during the priming and expansion of T cells *in vitro* prior to ACT. Longer culturing time leads to a higher number of transferable T cells but the cellular differentiation status increases too, resulting in a less effective tumor-directed T cells [121]. A second critical parameter is the amount of *in vivo* applied antigen used for re-stimulation of the transferred cells. The Pmel-1 mouse model revealed a strong correlation between

the amount of vaccine and the therapeutic outcome [117]. This observation underlines the importance of identifying effective TSAs/TAAs to enhance clinical outcomes. Thirdly, recombinant cytokines were used to support the success of ACT. For example, in the Pmel-1 mouse escalating dosages of cytokines known to activate, expand, or promote the survival of T cells such as IL-2, IL-7, IL-15, and IL-21 were used in parallel to ACT of Pmel1-TCR-transgenic $CD8^+$ T cells, demonstrating only moderate effects on the therapeutic outcome [117]. These results are challenging the requirement of cytokines in ACT treatment.

Targeting antibody therapies, unspecific cytokine/chemokine therapies as well as cell-based therapies are the three central pillars of modern oncoimmunology. The variety of possible treatment schemes makes it often difficult to recognize the central mode of action. On the other hand, the large number of paths that can be used to artificially influence immune responses gives us hope to develop highly effective immune-based anti-tumor strategies in the future. ACT therapies will help us to better understand basic immunological processes, including the role of various immune cell types in the antitumor inflammatory response.

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References

1. Coley WB. II. Contribution to the knowledge of sarcoma. Ann Surg. 1891;14:199–220.
2. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med. 1910;3:1–48.
3. Coley WB. II. Injury as a causative factor in cancer (continued). Ann Surg. 1911;53:615–50.
4. Coley WB. I. Injury as a causative factor in cancer. Ann Surg. 1911;53:449–88.
5. Ehrlich P. Über den jetzigen Stand der Karzinomforschung. Ned Tijdschr Geneeskd. 1909;5:273–90.
6. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. J Natl Cancer Inst. 1957;18:769–78.
7. Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res. 1970;13:1–27.
8. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957;1:779–86.
9. Burnet M. Immunological factors in the process of carcinogenesis. Br Med Bull. 1964;20:154–8.
10. Sanford KK, Likely GD, Earle WR. The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells in vitro. J Natl Cancer Inst. 1954;15:215–37.
11. Thomas L. Discussion. In: Lawrence HS, editor. Cellular and humoral aspects of the hypersensitive state. New York: Harper Lawrence, H. S.; 1959. p. 529–30.
12. Old LJ, Boyse EA. Immunology of experimental tumors. Annu Rev Med. 1964;15:167–86.
13. Snyder MA, Bishop JM. A mutation at the major phosphotyrosine in pp60v-src alters oncogenic potential. Virology. 1984;136:375–86.
14. Mullen CA, Urban JL, Van Waes C, Rowley DA, Schreiber H. Multiple cancers. Tumor burden permits the outgrowth of other cancers. J Exp Med. 1985;162:1665–82.
15. Mullen CA, Schreiber H. Tumor growth and evasion of immune destruction: UV-induced tumors as a model. Surv Immunol Res. 1985;4:264–70.

16. Rooney CM, Rowe M, Wallace LE, Rickinson AB. Epstein-Barr virus-positive Burkitt's lymphoma cells not recognized by virus-specific T-cell surveillance. Nature. 1985;317: 629–31.
17. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315:1650–9.
18. Virchow R, Virchow R. Aetiologie der neoplastischen Geschwulste/Pathogenie der neoplastischen Geschwulste. Die Krankhaften Geschwulste. Berlin: August Hirschwald; 1863.
19. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8.
20. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity. 2004;21:137–48.
21. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22:329–60.
22. MacKie RM, Reid R, Junor B. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. N Engl J Med. 2003;348:567–8.
23. Merigan TC, Sikora K, Breeden JH, Levy R, Rosenberg SA. Preliminary observations on the effect of human leukocyte interferon in non-Hodgkin's lymphoma. N Engl J Med. 1978;299:1449–53.
24. Priestman TJ. Interferon: an anti-cancer agent? Cancer Treat Rev. 1979;6:223–37.
25. Foon KA, Sherwin SA, Abrams PG, Longo DL, Fer MF, Stevenson HC, Ochs JJ, Bottino GC, Schoenberger CS, Zeffren J, et al. Treatment of advanced non-Hodgkin's lymphoma with recombinant leukocyte A interferon. N Engl J Med. 1984;311:1148–52.
26. Gutterman JU, Blumenschein GR, Alexanian R, Yap HY, Buzdar AU, Cabanillas F, Hortobagyi GN, Hersh EM, Rasmussen SL, Harmon M, Kramer M, Pestka S. Leukocyte interferon-induced tumor regression in human metastatic breast cancer, multiple myeloma, and malignant lymphoma. Ann Intern Med. 1980;93:399–406.
27. Knost JA, Sherwin SA, Abrams PG, Ochs JJ, Foon KA, Williams R, Tuttle R, Oldham RK. The treatment of cancer patients with human lymphoblastoid interferon. A comparison of two routes of administration. Cancer Immunol. 1983;15:144–8.
28. Quesada JR, Hawkins M, Horning S, Alexanian R, Borden E, Merigan T, Adams F, Gutterman JU. Collaborative phase I-II study of recombinant DNA-produced leukocyte interferon (clone A) in metastatic breast cancer, malignant lymphoma, and multiple myeloma. Am J Med. 1984;77:427–32.
29. Quesada JR, Reuben J, Manning JT, Hersh EM, Gutterman JU. Alpha interferon for induction of remission in hairy-cell leukemia. N Engl J Med. 1984;310:15–8.
30. Priestman TJ. Initial evaluation of human lymphoblastoid interferon in patients with advanced malignant disease. Lancet. 1980;2:113–8.
31. Retsas S, Priestman TJ, Newton KA, Westbury G. Evaluation of human lymphoblastoid interferon in advanced malignant melanoma. Cancer. 1983;51:273–6.
32. Paul WE. Interleukin 4/B cell stimulatory factor 1: one lymphokine, many functions. FASEB J. 1987;1:456–61.
33. Tepper RI, Pattengale PK, Leder P. Murine interleukin-4 displays potent anti-tumor activity in vivo. Cell. 1989;57:503–12.
34. Oi VT, Morrison SL, Herzenberg LA, Berg P. Immunoglobulin gene expression in transformed lymphoid cells. Proc Natl Acad Sci U S A. 1983;80:825–9.
35. Li WQ, Diamantstein T, Blankenstein T. Lack of tumorigenicity of interleukin 4 autocrine growing cells seems related to the anti-tumor function of interleukin 4. Mol Immunol. 1990;27:1331–7.
36. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell. 1988;54: 105–15.
37. Patek PQ, Collins JL, Cohn M. Transformed cell lines susceptible or resistant to in vivo surveillance against tumorigenesis. Nature. 1978;276:510–1.

38. Fidler IJ, Nicolson GL. Organ selectivity for implantation survival and growth of B16 melanoma variant tumor lines. J Natl Cancer Inst. 1976;57:1199–202.
39. Dunham LJ, Stewart HL. A survey of transplantable and transmissible animal tumors. J Natl Cancer Inst. 1953;13:1299–377.
40. Golumbek PT, Lazenby AJ, Levitsky HI, Jaffee LM, Karasuyama H, Baker M, Pardoll DM. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. Science. 1991;254:713–6.
41. Saito S, Bannerji R, Gansbacher B, Rosenthal FM, Romanenko P, Heston WD, Fair WR, Gilboa E. Immunotherapy of bladder cancer with cytokine gene-modified tumor vaccines. Cancer Res. 1994;54:3516–20.
42. Bannerji R, Arroyo CD, Cordon-Cardo C, Gilboa E. The role of IL-2 secreted from genetically modified tumor cells in the establishment of antitumor immunity. J Immunol. 1994;152:2324–32.
43. Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. J Exp Med. 1990;172:1217–24.
44. Rosenthal FM, Cronin K, Bannerji R, Golde DW, Gansbacher B. Augmentation of antitumor immunity by tumor cells transduced with a retroviral vector carrying the interleukin-2 and interferon-gamma cDNAs. Blood. 1994;83:1289–98.
45. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci U S A. 1993;90:3539–43.
46. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell. 1990;60:397–403.
47. Zatloukal K, Schneeberger A, Berger M, Schmidt W, Koszik F, Kutil R, Cotten M, Wagner E, Buschle M, Maass G, et al. Elicitation of a systemic and protective anti-melanoma immune response by an IL-2-based vaccine. Assessment of critical cellular and molecular parameters. J Immunol. 1995;154:3406–19.
48. Maass G, Schweighoffer T, Berger M, Schmidt W, Herbst E, Zatloukal K, Buschle M, Birnstiel ML. Tumor vaccines: effects and fate of IL-2 transfected murine melanoma cells in vivo. Int J Immunopharmacol. 1995;17:65–73.
49. Bubenik J, Simova J, Jandlova T. Immunotherapy of cancer using local administration of lymphoid cells transformed by IL-2 cDNA and constitutively producing IL-2. Immunol Lett. 1990;23:287–92.
50. Tsai SC, Gansbacher B, Tait L, Miller FR, Heppner GH. Induction of antitumor immunity by interleukin-2 gene-transduced mouse mammary tumor cells versus transduced mammary stromal fibroblasts. J Natl Cancer Inst. 1993;85:546–53.
51. Cavallo F, Giovarelli M, Gulino A, Vacca A, Stoppacciaro A, Modesti A, Forni G. Role of neutrophils and $CD4^+$ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. J Immunol. 1992;149:3627–35.
52. Porgador A, Gansbacher B, Bannerji R, Tzehoval E, Gilboa E, Feldman M, Eisenbach L. Anti-metastatic vaccination of tumor-bearing mice with IL-2-gene-inserted tumor cells. Int J Cancer. 1993;53:471–7.
53. Ohe Y, Podack ER, Olsen KJ, Miyahara Y, Ohira T, Miura K, Nishio K, Saijo N. Combination effect of vaccination with IL2 and IL4 cDNA transfected cells on the induction of a therapeutic immune response against Lewis lung carcinoma cells. Int J Cancer. 1993;53:432–7.
54. Connor J, Bannerji R, Saito S, Heston W, Fair W, Gilboa E. Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells. J Exp Med. 1993;177:1127–34.
55. Gastl G, Finstad CL, Guarini A, Bosl G, Gilboa E, Bander NH, Gansbacher B. Retroviral vector-mediated lymphokine gene transfer into human renal cancer cells. Cancer Res. 1992;52:6229–36.

56. Vieweg J, Rosenthal FM, Bannerji R, Heston WD, Fair WR, Gansbacher B, Gilboa E. Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. Cancer Res. 1994;54:1760–5.
57. Porgador A, Tzehoval E, Katz A, Vadai E, Revel M, Feldman M, Eisenbach L. Interleukin 6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metastatic cells. Cancer Res. 1992;52:3679–86.
58. Ohe Y, Podack ER, Olsen KJ, Miyahara Y, Miura K, Saito H, Koishihara Y, Ohsugi Y, Ohira T, Nishio K, et al. Interleukin-6 cDNA transfected Lewis lung carcinoma cells show unaltered net tumour growth rate but cause weight loss and shortened survival in syngeneic mice. Br J Cancer. 1993;67:939–44.
59. Aoki T, Tashiro K, Miyatake S, Kinashi T, Nakano T, Oda Y, Kikuchi H, Honjo T. Expression of murine interleukin 7 in a murine glioma cell line results in reduced tumorigenicity in vivo. Proc Natl Acad Sci U S A. 1992;89:3850–4.
60. Hock H, Dorsch M, Diamantstein T, Blankenstein T. Interleukin 7 induces $CD4^+$ T cell-dependent tumor rejection. J Exp Med. 1991;174:1291–8.
61. de Vos S Kohn DB Cho SK McBride WH Said JW Koeffler HP. Immunotherapy against murine leukemia. Leukemia. 1998;12:401–5.
62. Tahara H, Zitvogel L, Storkus WJ, Zeh HJ 3rd, McKinney TG, Schreiber RD, Gubler U, Robbins PD, Lotze MT. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. J Immunol. 1995;154:6466–74.
63. Lo CH, Lee SC, Wu PY, Pan WY, Su J, Cheng CW, Roffler SR, Chiang BL, Lee CN, Wu CW, Tao MH. Antitumor and antimetastatic activity of IL-23. J Immunol. 2003;171:600–7.
64. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated gamma-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. Cancer Res. 1990;50:7820–5.
65. Watanabe Y, Kuribayashi K, Miyatake S, Nishihara K, Nakayama E, Taniyama T, Sakata T. Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. Proc Natl Acad Sci U S A. 1989;86:9456–60.
66. Teng MN, Park BH, Koeppen HK, Tracey KJ, Fendly BM, Schreiber H. Long-term inhibition of tumor growth by tumor necrosis factor in the absence of cachexia or T-cell immunity. Proc Natl Acad Sci U S A. 1991;88:3535–9.
67. Asher AL, Mule JJ, Kasid A, Restifo NP, Salo JC, Reichert CM, Jaffe G, Fendly B, Kriegler M, Rosenberg SA. Murine tumor cells transduced with the gene for tumor necrosis factor-alpha. Evidence for paracrine immune effects of tumor necrosis factor against tumors. J Immunol. 1991;146:3227–34.
68. Blankenstein T, Qin ZH, Uberla K, Muller W, Rosen H, Volk HD, Diamantstein T. Tumor suppression after tumor cell-targeted tumor necrosis factor alpha gene transfer. J Exp Med. 1991;173:1047–52.
69. Karp SE, Hwu P, Farber A, Restifo NP, Kriegler M, Mule JJ, Rosenberg SA. In vivo activity of tumor necrosis factor (TNF) mutants. Secretory but not membrane-bound TNF mediates the regression of retrovirally transduced murine tumor. J Immunol. 1992;149:2076–81.
70. Colombo MP, Ferrari G, Stoppacciaro A, Parenza M, Rodolfo M, Mavilio F, Parmiani G. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma in vivo. J Exp Med. 1991;173:889–97.
71. Rollins BJ. JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. Cancer Cell. 1991;3:517–24.
72. Erard F, Corthesy P, Nabholz M, Lowenthal JW, Zaech P, Plaetinck G, MacDonald HR. Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. J Immunol. 1985;134:1644–52.
73. Mosmann TR, Coffman RL. Two types of mouse helper T-cell clone Implications for immune regulation. Immunol Today. 1987;8:223–7.
74. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187–376.

75. Smith KA. Interleukin-2: inception, impact, and implications. Science. 1988;240:1169–76.
76. Rosenberg SA, Lotze MT. Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. Annu Rev Immunol. 1986;4:681–709.
77. Tirapu I, Huarte E, Guiducci C, Arina A, Zaratiegui M, Murillo O, Gonzalez A, Berasain C, Berraondo P, Fortes P, Prieto J, Colombo MP, Chen L, Melero I. Low surface expression of B7–1 (CD80) is an immunoescape mechanism of colon carcinoma. Cancer Res. 2006;66:2442–50.
78. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121:1–14.
79. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA, Bennink JR. Identification of human cancers deficient in antigen processing. J Exp Med. 1993;177: 265–72.
80. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331:1565–70.
81. Campo MS, Graham SV, Cortese MS, Ashrafi GH, Araibi EH, Dornan ES, Miners K, Nunes C, Man S. HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells. Virology. 2010;407:137–42.
82. Marchetti B, Ashrafi GH, Dornan ES, Araibi EH, Ellis SA, Campo MS. The E5 protein of BPV-4 interacts with the heavy chain of MHC class I and irreversibly retains the MHC complex in the Golgi apparatus. Oncogene. 2006;25:2254–63.
83. Zhang B, Li P, Wang E, Brahmi Z, Dunn KW, Blum JS, Roman A. The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. Virology. 2003;310:100–8.
84. Fruh K, Yang Y. Antigen presentation by MHC class I and its regulation by interferon gamma. Curr Opin Immunol. 1999;11:76–81.
85. Restifo NP, Spiess PJ, Karp SE, Mule JJ, Rosenberg SA. A nonimmunogenic sarcoma transduced with the cDNA for interferon gamma elicits $CD8^+$ T cells against the wild-type tumor: correlation with antigen presentation capability. J Exp Med. 1992;175:1423–31.
86. Zhu W, Qin W, Sauter ER. Large-scale mitochondrial DNA deletion mutations and nuclear genome instability in human breast cancer. Cancer Detect Prev. 2004;28:119–26.
87. Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, Yong WH, Incardona F, Thompson RC, Riedinger MS, Zhang W, Prins RM, Black KL. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res. 2001;61:842–7.
88. Kass R, Agha J, Bellone S, Palmieri M, Cane S, Bignotti E, Henry-Tillman R, Hutchins L, Cannon MJ, Klimberg S, Santin AD. In vitro induction of tumor-specific HLA class I-restricted $CD8^+$ cytotoxic T lymphocytes from patients with locally advanced breast cancer by tumor antigen-pulsed autologous dendritic cells. J Surg Res. 2003;112:189–97.
89. Labarriere N, Bretaudeau L, Gervois N, Bodinier M, Bougras G, Diez E, Lang F, Gregoire M, Jotereau F. Apoptotic body-loaded dendritic cells efficiently cross-prime cytotoxic T lymphocytes specific for NA17-A antigen but not for Melan-A/MART-1 antigen. Int J Cancer. 2002;101:280–6.
90. Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective $CD4^+$ and $CD8^+$ T cell immunity against B16 melanoma. J Immunol. 2003;171:5940–7.
91. Muller MR, Grunebach F, Nencioni A, Brossart P. Transfection of dendritic cells with RNA induces CD4- and CD8-mediated T cell immunity against breast carcinomas and reveals the immunodominance of presented T cell epitopes. J Immunol. 2003;170:5892–6.
92. Muller MR, Tsakou G, Grunebach F, Schmidt SM, Brossart P. Induction of chronic lymphocytic leukemia (CLL)-specific CD4- and CD8-mediated T-cell responses using RNA-transfected dendritic cells. Blood. 2004;103:1763–9.
93. de Zoeten E Carr-Brendel V Markovic D Taylor-Papadimitriou J Cohen EP. Treatment of breast cancer with fibroblasts transfected with DNA from breast cancer cells. J Immunol. 1999;162:6934–41.

94. Sung Kim T Cohen EP. Immunity to breast cancer in mice immunized with fibroblasts transfected with a cDNA expression library derived from small numbers of breast cancer cells. Cancer Gene Ther. 2005;12:890–9.
95. Kim TS, Jung MY, Cho D, Cohen EP. Prolongation of the survival of breast cancer-bearing mice immunized with GM-CSF-secreting syngeneic/allogeneic fibroblasts transfected with a cDNA expression library from breast cancer cells. Vaccine. 2006;24:6564–73.
96. Peron JM, Bureau C, Gourdy P, Lulka H, Souque A, Calippe B, Selves J, Al Saati T, Bernad J, Cordelier P, Couderc B, Pradayrol L, Pipy B, Buscail L, Vinel JP. Treatment of experimental murine pancreatic peritoneal carcinomatosis with fibroblasts genetically modified to express IL12: a role for peritoneal innate immunity. Gut. 2007;56:107–14.
97. Tahara H, Zeh HJ 3rd, Storkus WJ, Pappo I, Watkins SC, Gubler U, Wolf SF, Robbins PD, Lotze MT. Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. Cancer Res. 1994;54:182–9.
98. Kim TS, Chopra A, IS OS, Cohen EP. Enhanced immunity to breast cancer in mice immunized with fibroblasts transfected with a complementary DNA expression library from breast cancer cells: enrichment of the vaccine for immunotherapeutic cells. J Immunother. 2006;29:261–73.
99. Lichtor T, Glick RP, Lin H, I OS, Cohen EP. Intratumoral injection of IL-secreting syngeneic/allogeneic fibroblasts transfected with DNA from breast cancer cells prolongs the survival of mice with intracerebral breast cancer. Cancer Gene Ther. 2005;12:708–14.
100. Golumbek PT, Azhari R, Jaffee EM, Levitsky HI, Lazenby A, Leong K, Pardoll DM. Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design. Cancer Res. 1993;53:5841–4.
101. Dunn GP, Dunn IF, Curry WT. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human glioma. Cancer Immun. 2007;7:12.
102. Oble DA, Loewe R, Yu P, Mihm MC, Jr. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma. Cancer Immun. 2009;9:3.
103. Ohtani H. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer. Cancer Immun. 2007;7:4.
104. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, Richardson AL, Brock J, Criscitiello C, Bailey H, Ignatiadis M, Floris G, Sparano J, Kos Z, Nielsen T, Rimm DL, Allison KH, Reis-Filho JS, Loibl S, Sotiriou C, Viale G, Badve S, Adams S, Willard-Gallo K, Loi S. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2014.
105. Schatton T, Scolyer RA, Thompson JF, Mihm MC Jr. Tumor-infiltrating lymphocytes and their significance in melanoma prognosis. Methods Mol Biol. 2014;1102:287–324.
106. Shirabe K, Motomura T, Muto J, Toshima T, Matono R, Mano Y, Takeishi K, Ijichi H, Harada N, Uchiyama H, Yoshizumi T, Taketomi A, Maehara Y. Tumor-infiltrating lymphocytes and hepatocellular carcinoma: pathology and clinical management. Int J Clin Oncol. 2010;15:552–8.
107. Uppaluri R, Dunn GP, Lewis JS Jr. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in head and neck cancers. Cancer Immun. 2008;8:16.
108. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011;365:725–33.
109. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C, Rosenberg SA. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol. 2011;29:917–24.
110. Chinnasamy N, Wargo JA, Yu Z, Rao M, Frankel TL, Riley JP, Hong JJ, Parkhurst MR, Feldman SA, Schrump DS, Restifo NP, Robbins PF, Rosenberg SA, Morgan RA. A TCR

targeting the HLA-A*0201-restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. J Immunol. 2011;186: 685–96.

111. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM, Rosenberg SA. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther. 2011;19:620–6.
112. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, Rossig C, Russell HV, Diouf O, Liu E, Liu H, Wu MF, Gee AP, Mei Z, Rooney CM, Heslop HE, Brenner MK. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. Blood. 2011;118:6050–6.
113. Kochenderfer JN, Yu Z, Frasheri D, Restifo NP, Rosenberg SA. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. Blood. 2010;116:3875–86.
114. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12:269–81.
115. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA, Restifo NP. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive $CD8^+$ T cells. J Exp Med. 2003;198:569–80.
116. Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013;26:300–15.
117. Klebanoff CA, Gattinoni L, Palmer DC, Muranski P, Ji Y, Hinrichs CS, Borman ZA, Kerkar SP, Scott CD, Finkelstein SE, Rosenberg SA, Restifo NP. Determinants of successful $CD8^+$ T-cell adoptive immunotherapy for large established tumors in mice. Clin Cancer Res. 2011;17:5343–52.
118. Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, Wrzesinski C, Boni A, Cassard L, Garvin LM, Paulos CM, Muranski P, Restifo NP. Wnt signaling arrests effector T cell differentiation and generates $CD8^+$ memory stem cells. Nat Med. 2009;15:808–13.
119. Gattinoni L, Powell DJ Jr., Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol. 2006;6:383–93.
120. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE, Dudley ME. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res. 2011;17:4550–7.
121. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred $CD8^+$ T cells. J Clin Invest. 2005;115:1616–26.

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