Chapter 1
Targeted Cancer Therapy: History and Development of Immunotoxins

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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*,

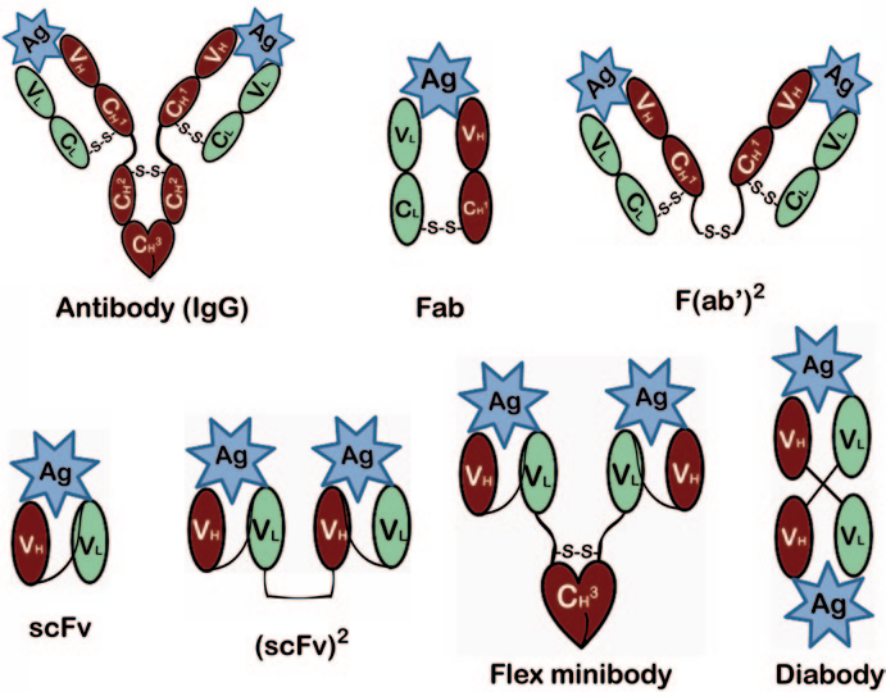

Fig. 1.1 Antibody-based targeting proteins: Different antibody-based molecules, produced by enzymatic digestion of intact antibodies, or using molecular engineering techniques, as recombinant proteins, are used to enhance the penetration of antibodies into solid tumors. IgG: Intact immunoglobulin; scFv 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

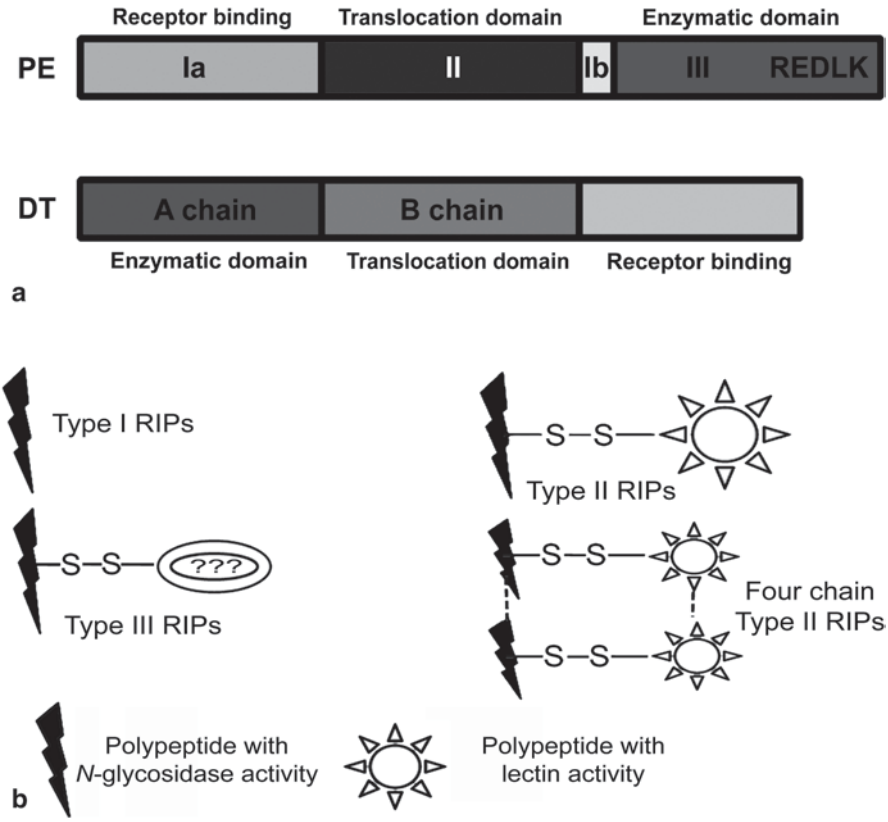

Fig. 1.2 Different types of toxins: **a** Bacterial toxins: The schematic representation depicting the different domains of the bacterial toxins, *Pseudomonas* enterotoxin and diphtheria toxin. **b** Different classes of RIPs: Diagrammatic representation of the different classes of ribosome inactivating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

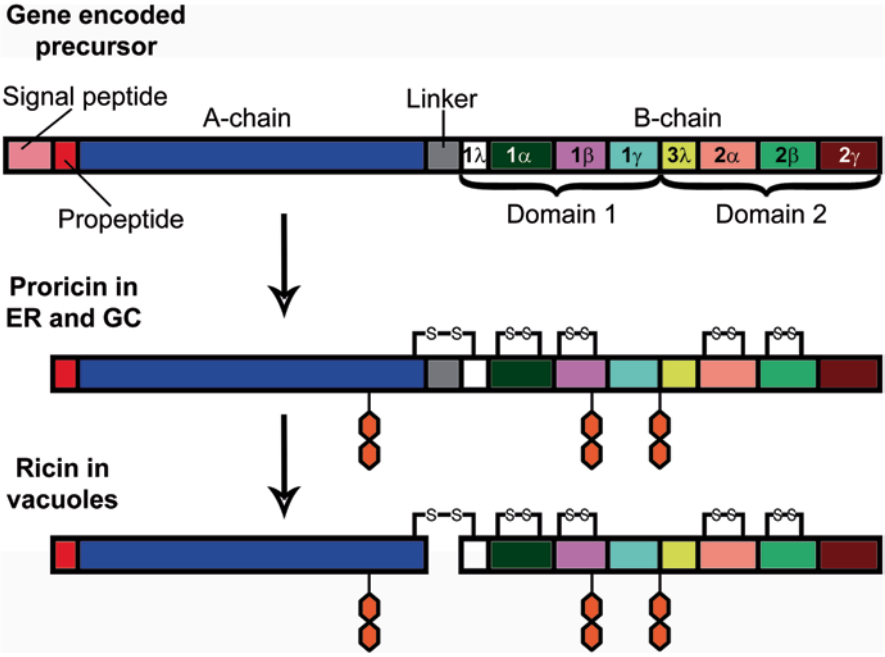

Fig. 1.3 Synthesis of Ricin: Ricin, a type II RIP, is synthesized and stored in the seeds of the plant *Ricinus communis*. The signal peptide directs co-translational translocation of the protein in the ER. This is cleaved in the ER to give a pre-pro form of ricin, which is targeted to the Golgi, where 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

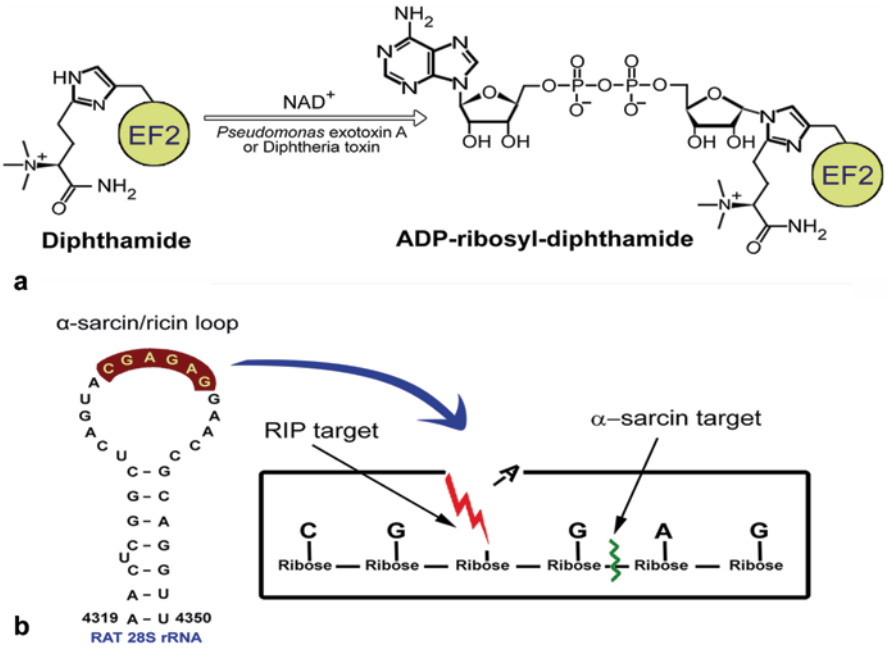

Fig. 1.4 Mechanism of action of proteins that inhibit translation **a** Inhibitors of translation: like PE and DT bring about their cytotoxic effect by ADP ribosylation of the modified diphthamide residue of eEF2. They use the cellular store of $NAD^+$for the substrate ADP that is required. This ADP-ribosyl-diphthamide 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

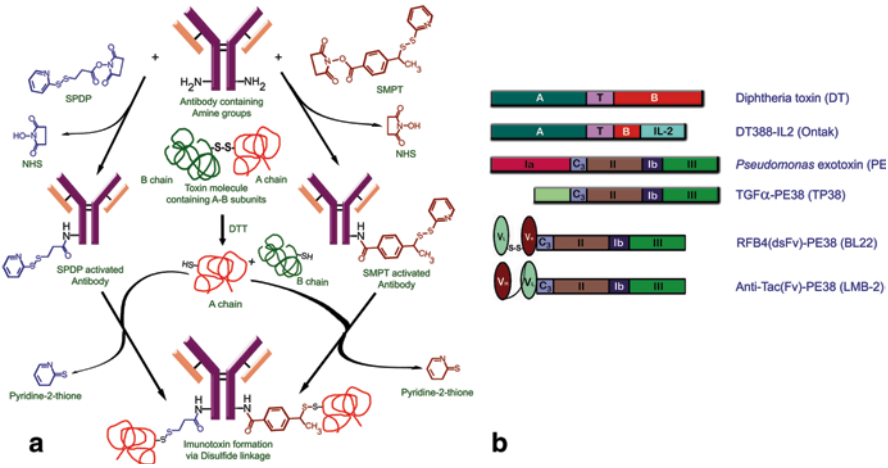

Fig. 1.5 **a** Construction of an immunotoxin by chemical conjugation: Heterobifunctional cross-linkers like SPDP and SMPT are used in the chemical conjugation for an immunotoxin preparation. They have an NHS-ester group at one end and a disulfide group at the other. The antibody used for IT preparation 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

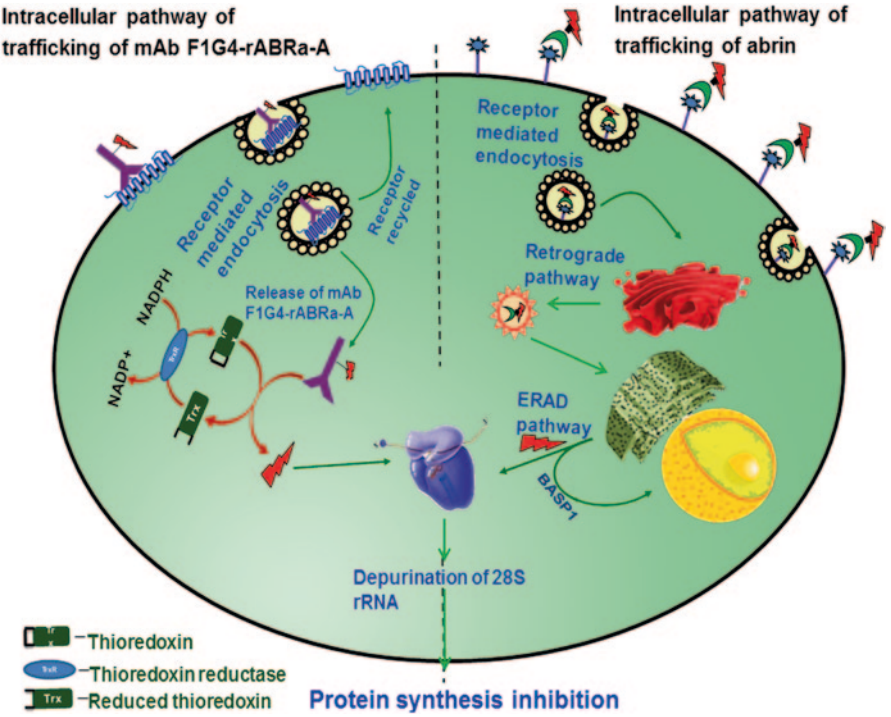

Fig. 1.6 Proposed model for the internalization and cytotoxicity of ITs: The IT binds to the receptor via the antibody. Once bound, it is internalized via receptor-mediated endocytosis through clathrin coated pits. The protein is then released from the vesicles into the cytosol. In the cytosol, the S–S 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