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

U. Brinkmann (✉) · S. Stahl
Roche Pharma Research & Early Development pRED, Large Molecule Research, Roche Innovation Center Penzberg, Nonnenwald 2, 82377 Penzberg, Germany
e-mail: ulrich.brinkmann@roche.com

F. Mueller · I. Pastan
Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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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

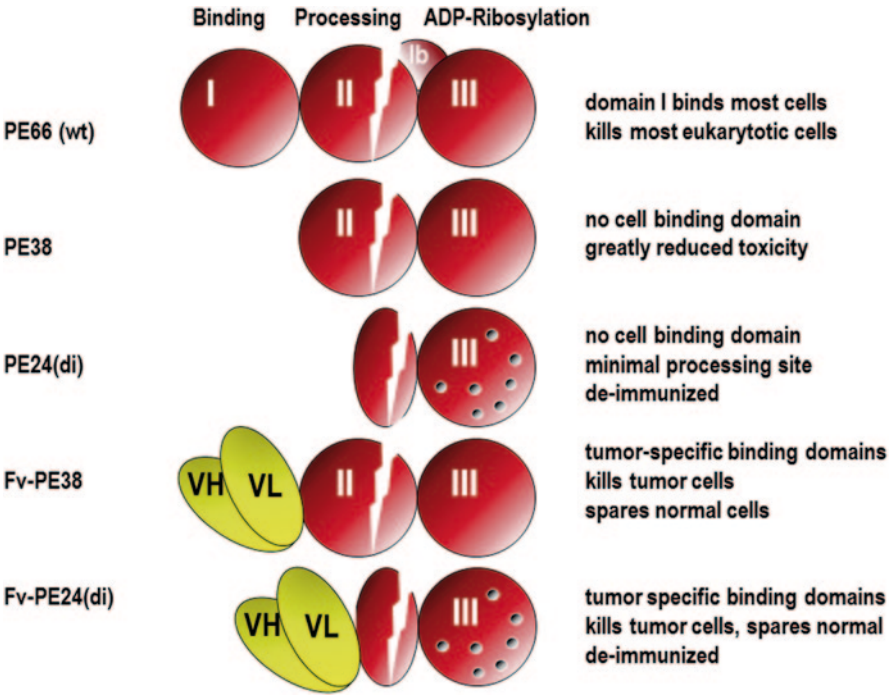

Fig. 3.1 Pseudomonas exotoxin A-and PE-derived immunotoxins. Removal of the non-specific cell binding domain I from Pseudomonas toxin (PE66, MW = 66 kDa) results in truncated derivatives PE38 (38 kDa) or PE24 (24 kDa) which can be de-immunized by removing B- and T cell epitopes [16, 17]. These toxin 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

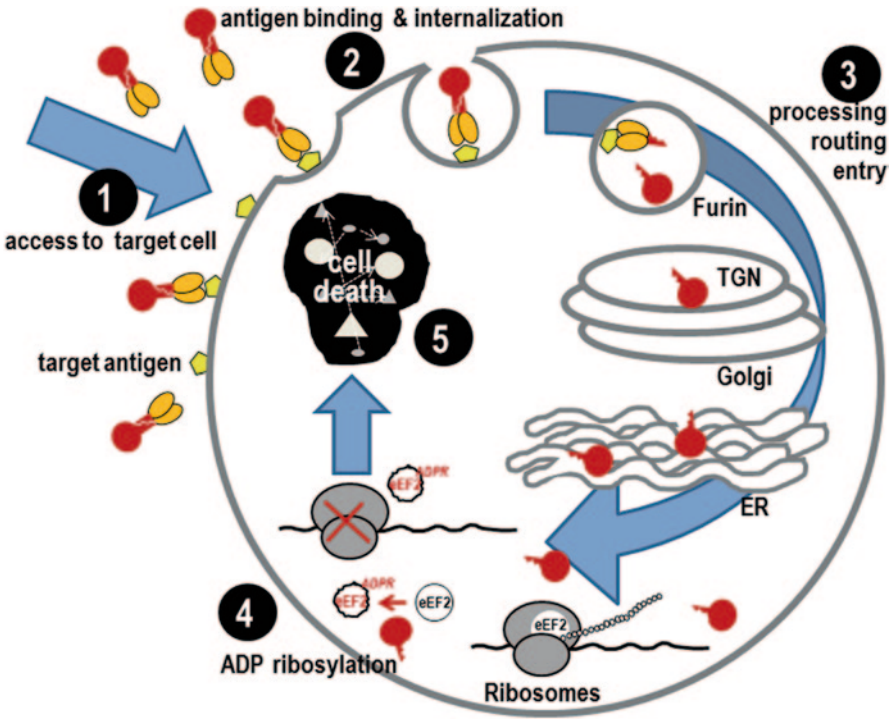

Fig. 3.2 **'Toxin pathway'**. After binding of the immunotoxin to the target antigen and internalization, the toxin fragment is released from the cell-targeting antibody and routed to the ER. Then, the toxin enters the cytoplasm where it ADP-ribosylates eEF2 which results in the inhibition of protein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

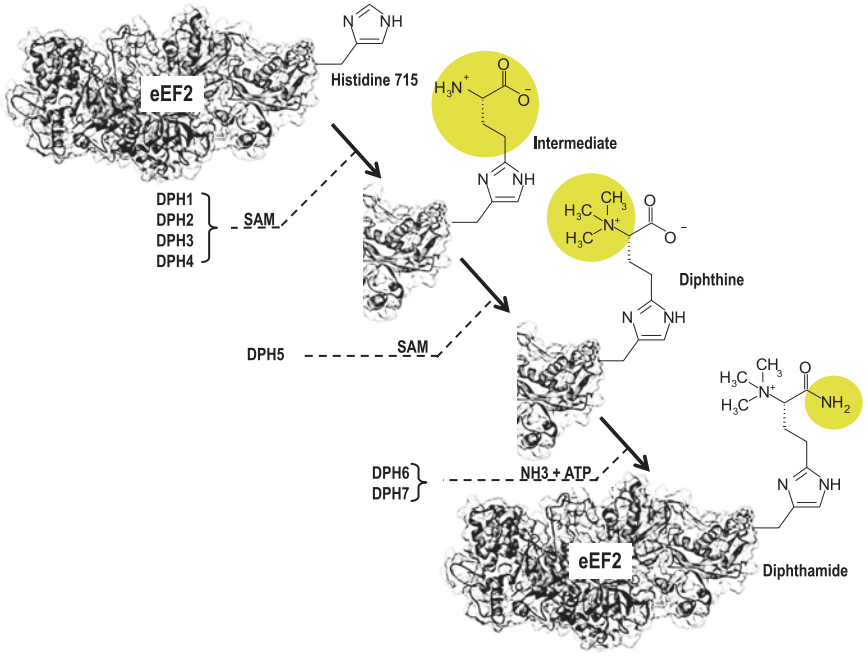

Fig. 3.3 Diphthamide synthesis. The diphthamide pathway was adapted from the model by Liu et al. and Lin et al. [39, 40] for yeast. *SAM* S-adenosyl-methionine. The role of DPH5 and/or alternative pathways and products in cells that lack one or more enzymes may need further clarification. [41]

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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