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Review Article

Strategies to mitigate the on- and off-target toxicities of recombinant immunotoxins: an antibody engineering perspective

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ABSTRACT

Targeted cancer therapies using immunotoxins have achieved remarkable efficacy in hematological malignancies. However, the clinical development of immunotoxins is also faced with many challenges like anti-drug antibodies and dose-limiting toxicity issues. Such a poor efficacy or safety ratio is also the major hurdle in the research and development of antibody-drug conjugates. From an antibody engineering perspective, various strategies were summarized or proposed to tackle the notorious on-target off-tumor toxicity issues, including passive strategy (XTENylation of immunotoxins) and active strategies (modulating the affinity and valency of the targeting moiety of immunotoxins, conditionally activating immunotoxins in the tumor microenvironments and reconstituting split toxin to reduce systemic toxicity, etc.). By modulating the functional characteristics of the targeting moiety and the toxic moiety of immunotoxins, selective tumor targeting can be augmented while sparing the healthy cells in normal tissues expressing the same target of interest. If successful, the improved therapeutic index will likely help to address the dose-limiting toxicities commonly observed in the clinical trials of various immunotoxins.

Statement of Significance: Poor therapeutic index is the major hurdle in the development of targeted cancer therapies with immunotoxins and antibody-drug conjugates. In this review, from an antibody engineering perspective, various strategies to mitigate the on- and off-target toxicities of immunotoxins were reviewed. They may help to address the dose-limiting toxicities commonly observed in the clinical trials of various immunotoxins.

KEYWORDS: conditionally active biologics; split toxin; off-target toxicity; on-target toxicity; therapeutic index; antibody-drug conjugate; immunotoxin

INTRODUCTION

Cancer is a leading cause of human death worldwide, accounting for approximately one in six deaths. Conventional cancer treatments usually involve surgery, chemotherapy, radiotherapy and/or hormone therapy. Targeted cancer therapy is a type of cancer treatment that precisely target and kill cancer cells by damaging or interfering with specific molecular functions or signaling pathways critically involved in the process of human tumorigenesis. In the past 20 years, due to its favorable efficacy and safety profiles over conventional therapies, targeted cancer therapy became the forefront of cancer

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Table 1. List of immunotoxins that were previously evaluated in human clinical trials (as of 08/12/2021, clinicaltrials.org)

Drug/biological	Target antigens	Toxin	Representative clinical trials (NCT#)
LMB-100	Mesothelin	De-immunized PE24	02810418, 02798536, 03644550, 04034238, 04840615, 03436732
LMB-2	CD25	PE38	00321555, 00924170, 00080535, 00085150, 00295958, 00389506
Moxetumomab Pasudotox	CD22	PE38	03501615, 01829711, 03805932, 02338050, 00457860, 00462189
MOC31PE	CD326 (EpCAM)	PE	02219893
BL22	CD22	PE38	00021983, 00074048, 00126646, 00077493, 00924040, 00024115
LMB-9, LMB-7	Lewis Y	PE38	00019435, 00005858, 00010270, 00003020 (LMB-7)
Anti-CD19 and CD22	CD19 and CD22 (combo)	Deglycosylated ricin A chain	00450944, 01408160
Hum-195/rGel	CD33	Gelonin	00038051
IgG-RFB4-SMPT-dgA	CD22	Deglycosylated ricin A chain	00001271
A-dmDT390-bisFv(UCHT1)	CD3	Diphtheria toxin (DT390)	00611208
T-Guard	CD3 and CD7 (combo)	Ricin toxin A (RTA)	02027805, 04128319, 00640497
DT2219	CD19/CD22 (bispecific)	Diphtheria toxin (DT390)	02370160, 00889408
RFT5pdgA	CD25	Deglycosylated ricin A chain	00314093, 00667017
BM7PE	Mucin-1	PE	04550897
MR1–1	EGFRvIII	PE38KDEL	01009866
Denileukin diftitox	CD25	Diphtheria toxin	00117845
Transferrin-CRM107	Transferrin receptor	Diphtheria toxin	00052624
IL-4(38–37)-PE38KDEL	IL-4 receptor	PE38KDEL	00003842
SS1(dsFv)-PE38	Mesothelin	PE38	01445392, 00024687, 00024674, 00066651, 01362790, 01051934
Cintredekin besudotox	IL-13R	PE	00053040, 00006268, 00036972, 00064779
MT-3724	CD20	Shiga-like toxin-I A1	02556346, 02361346, 02715843

treatments [1]. The most developed class of targeted cytotoxic treatments includes antibody-drug conjugates (ADC) and immunotoxins [2]. ADC and/or immunotoxins took advantage of the affinity and binding specificity of biologics to more precisely deliver the cytotoxic payload to cancer cells. The significantly improved clinical benefits led to the approval of 12 ADC worldwide and three immunotoxins, including moxetumomab pasudotox (anti-CD22) for relapsed/refractory hairy cell leukemia [3, 4], denileukin difitox (targeting CD25) for cutaneous T cell lymphoma [5] and tagraxofusp (targeting CD123) for blastic plasmacytoid dendritic cell neoplasm [6]. Remarkable efficacy was shown for immunotoxins. For example, in a pivotal study of moxetumomab pasudotox in adults with relapsed/refractory hairy cell leukemia, the complete response rate was 41% and the objective response rate was 75%. About 85% of complete responders achieved minimal residual disease negativity on bone marrow biopsy immunohistochemistry [4]. All immunotoxins that were previously evaluated in the human clinical trials are listed in Table 1.

Bacteria-derived toxins such as *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT) or plant-derived toxins like ricin, gelonin and others are used in the design and construction of immunotoxins [7, 8]. Native bacterial A/B-type toxins like PE usually consist of a catalytic A subunit and a B subunit that mediates receptor binding and translocation. Proteolytic cleavage by the pro-protein convertase (furin) to separate A subunit from receptor-bound B subunit is required for the activation of bacterial toxins. Unlike bacterial toxins that are activated at the cell surface (anthrax toxin), the furin catalyzed activation of A/B-type toxins occurs in the endosomes (PE, DT and Shiga toxin). Following furin cleavage and reduction of a key disulfide bond, the active A subunits are translocated to the cytosol through different trafficking pathways [9]. The PE requires retrograde trafficking to the trans-Golgi network and then to the ER, where the A subunits are retro-translocated to the cytosol, though evidence suggesting direct cytosol translocation from endosomes does exist [10, 11]. The DT A subunits are shuffled directly to the cytosol through a channel formed by the DT B subunits in the endosomal membrane [12]. The reducing environment of the cytosol reduces the linking disulfide bond and frees the toxic DT A subunits [13]. In the cytosol, the catalytically active A subunits of PE and DT exert their functions to inhibit protein synthesis by ADP-ribosylating of elongation factor 2 [2, 14]. Unlike the efficient proteolytic cleavage of DT over a broad pH range, the native mature conformational PE can only be cleaved at an acidic pH and the cleavage is quite inefficient—only $\sim$5–10% of cell-associated PE is cleaved within cells [15]. Engineering residues surrounding the PE

furin cleavage site revealed little correlation between furin cleavage efficiency and cytotoxicity [9, 11]. The reduction of furin-nicked PE suggested the involvement of additional subcellular membrane-associated proteins in downstream unfolding and further proteolytic activation of PE [16, 17]. Moxetumomab pasudotox utilized the 38 kDa fragment of PE, consisting of the processing and the catalytic subunits (domains II and III) including the native furin cleavage site [3]. In recombinant immunotoxins, the binding moiety and the toxic moiety are linked via a polypeptide in which the furin cleavage site is very elegantly designed and protected from extracellular proteolytic activation [14, 18]. Due to the strong immunogenic nature of bacteria- or plant-derived toxins, immunogenicity is the common challenge faced by early generations of immunotoxin development. In a clinical trial evaluating LMB-1, an immunotoxin composed of B3-targeting antibody chemically linked to PE38, all 38 patients developed antibodies against the toxin moiety [19]. Further deletion of the majority of domain II that is sensitive to lysosomal protease degradation resulted in PE24 [20]. Strategies to de-immunize the PE24 toxin moiety, extensively reviewed elsewhere [7, 21–24], led to T cell and/or B cell epitopes de-immunized variants of recombinant immunotoxins. Though T cell epitope de-immunized immunotoxins have not been tested in clinical settings, B cell epitope de-immunized immunotoxin LMB-100, an antibody-toxin conjugate with an anti-mesothelin Fab linked to PE24, did show reduced immunogenicity in a recent clinical trial [7, 25]. To overcome the notorious anti-drug antibody (ADA) challenge, the humanized immunotoxin concept was proposed [26] and the anti-tumor potential of human-derived cytolytic proteins like granulysin was evaluated [27]. Various natural or de novo-designed novel toxins were also explored for their potential for targeted cytotoxic delivery [28]. With the recent advances in accurate protein structure prediction and de novo protein design, it is now possible to design synthetic toxins to kill target cells of interest, or mini proteins to modulate cellular functions [29, 30].

Other than the aforementioned immunogenicity issue, the development of immunotoxins is faced with many challenges, including efficient trafficking and endosomal escape of immunotoxins, narrow therapeutic window and poor solid tumor penetration/retention [31]. Protein toxins are generally more potent than small-molecule cytotoxic payloads, but this really depends on the small molecule payloads. For example, pyrrolobenzodiazepine (PBD) dimers are highly potent DNA cross-linking agents used as the payload in cancer therapy across a broad range of prolactin receptor-positive breast cancers and other cancer types [32]. Only one molecule of diphtheria toxin fragment A was demonstrated to be sufficient to kill the cell [33]. In a side-by-side comparison of protein toxins (dianthin-30 or gelonin) with small molecule drug monomethyl auristatin E (MMAE), immunotoxins were found to be $\sim$250–300$\times$ more potent than MMAE-derived ADCs [34]. The drastic potency of immunotoxins also raised the concern of on-target toxicity. Ideally, combining the highly potent cytotoxic payload with highly tumor-specific antigens would lead to desired clinical benefits. However, tumor-specific antigens are very rare to find as target receptors more or less are also expressed in normal tissues. Highly potent immunotoxins, when killing target antigen-expressing tumor cells, also kill healthy cells in normal tissues expressing tumor-associated antigens. As demonstrated with a recombinant anti-mesothelin immunotoxin SS1P, binding to proximal tubular cells in the kidney led to quick clearance of SS1P from the blood, capillary leak syndrome and kidney damage [35]. In addition, the clinical failure of ROVA-T (a delta-like 3/DLL3 targeting ADC) in small-cell lung cancer suggests that targeting a highly tumor-specific antigen may not warrant a desired clinical outcome [36]. Lack of efficacy and dose-limiting toxicities are considered to be two major causes of clinical failure [37]. Undesired target-dependent and/or -independent uptake of immunotoxins by healthy cells also contributed significantly to the dose-limiting toxicities of immunotoxins [35]. In the cases of immunotoxins, the poor safety profile caused by on- and off-target toxicities became a hurdle in the development of immunotoxin therapeutics.

In this review, we focus on the strategies to increase the therapeutic index of immunotoxin treatments. By modulating the affinity, valency and/or both of the targeting moiety, by making the immunotoxin conditionally active or by other means like site-specific XTENylation or split toxin, immunotoxins can specifically and precisely target tumor cells with medium to high target antigen expression, while sparing healthy cells with medium to low antigen expression. Such strategies would likely create differentiated binding and killing, which might lead to an improved efficacy to toxicity ratio. Albeit focused on immunotoxins in this review, these strategies can also be applied to improve the therapeutic index of next-generation ADCs or immune-stimulating antibody conjugates (ISAC) [38], where binding to target cells of interest needs to be differentiated from that of non-target cells.

PEGYLATION OF IMMUNOTOXINS DID NOT PRODUCE THE DESIRED CLINICAL BENEFITS

Chemical modification with polyethylene glycol (PEGylation) is one strategy to improve the therapeutic efficacy of biotherapeutics including monoclonal antibodies, cytokines and immunotoxins. PEGylation is routinely used in the pharmaceutical industry to increase the serum half-life and stability of drugs, as evidenced by 14 PEGylated drugs approved by the FDA and many others in human clinical trials [39]. However, such a strategy did not lead to improved clinical efficacy in immunotoxin treatments, even though efficacies observed in preclinical models suggested it a viable option. Site-specific PEGylation of LMB-2 on the lysine residues (anti-CD25-PE38 immunotoxin) led to about a 20-fold increase in therapeutic efficacy, including a 3–4-fold higher anti-tumor activity and about 6-fold reduction in normal tissue toxicity in preclinical mouse models [40]. PEGylation, on one hand, reduced off-target toxicity caused by the nonspecific binding of LMB-2 to normal tissues such as liver cells [41]. PEGylation of the cytotoxic moiety shields the ionic interactions thus reducing the nonspecific cellular absorption and uptake by normal tissues. The increased molecule size

also limits the transport of PEGylated immunotoxin from blood to normal tissues like the lung, kidney and liver [42]. On the other hand, PEGylation increases serum half-life and stability of immunotoxin while reducing immunogenicity. The same strategy was used to reduce the antigen-independent toxicity of a non-binding ADC with a hydrophobic MMAE payload [43]. Similarly, shielding of the hydrophobic payload with optimal PEGylation reduced systemic toxicity by slower clearance and dramatically decreased nonspecific cellular uptake in normal tissues (Fig. 1A) [43]. Even though PEGylation improves the therapeutic efficacy of a drug, this technology is also facing many challenges [44]. The steric hindrance caused by long PEG chains may either interfere with the targeting moiety binding to its receptor or limit the furin cleavage efficiency of pseudomonas exotoxin. More importantly, PEGylation sometimes leads to inefficient endosomal escape, which may adversely limit the therapeutic efficacy of immunotoxin. Therefore, many parameters, including the site of PEGylation, the degree of PEGylation, the radius of hydration, and the size and format of targeting moiety impact the therapeutic efficacy of PEGylated immunotoxins. Zheng et al. developed a maleimide-based site-specific PEGylation method to precisely control the degree of PEGylation for improved therapeutic efficacy. A cysteine residue was introduced and PEGylated in the linker region between the Fv and the toxin domains [39]. Unlike the lysine-specific PEGylation of LMB-2 [40], the heterogeneity of PEGylated immunotoxin was properly addressed by cysteine-specific PEGylation on spatially and functionally distinct domains on various LMB immunotoxins specifically targeting the human Mesothelin. Two PEGylated LMB variants (LMB-244-PEG and LMB-163-PEG) showed substantial tumor regression in murine models [39]. Beyond lysine and cysteine tagging, residues like tyrosine, serine, threonine and histidine can also be used for site-specific PEGylation, which represents unexplored opportunities in the immunotoxin field [45]. Some other strategies like releasable PEGylation or albumin-binding were also attempted and had demonstrated preclinical efficacy [46–48]. Notably, the weight loss of treated mice, indicative of drug treatment-related toxicity, was still commonly observed with PEGylated immunotoxins [39, 46]. In the case of recombinant immunotoxins, LMB-244-PEG and LMB-163-PEG treatment, an approximately 10% weight loss was still observed when the immunotoxins were administrated using a dose and schedule that previously did not cause weight loss over 3 weeks [39].

Despite many attempts with elegant drug designs and preclinical experiments, site-specific PEGylation of immunotoxins did not translate into expected clinical success. Inadequate PEGylation of immunotoxin does not help to reduce immunogenicity, while over PEGylation obviously kills the toxin activity. Other than finding the "sweet spot" of PEGylation, the drug development process is further complicated by site-specific mutagenesis of immunotoxins, production of engineered immunotoxins, maleimide PEGylation and subsequent re-purification steps. Site-specific introduction of a single cysteine, on one hand, may help the site-specific PEGylation to reduce immunogenicity. On the other hand, it also creates aggregation and product heterogeneity issues in the downstream development process. Besides, PEGylation technology itself is also facing substantial challenges. The presence of anti-PEG antibodies in roughly 25% of the healthy population and the increased IgM titers following repeated dosing of PEGylated drugs might limit the potential of PEGylated immunotoxins in cancer therapy [49, 50]. PEGylation could be very difficult to achieve selective delivery of immunotoxins due to many challenges in the technology itself and the engineering of immunotoxins. Indeed, very limited clinical success was achieved with PEGylated immunotoxins in the past. To overcome the limitations of PEG, alternative biodegradable bulking agents like polysaccharides and unstructured polypeptide polymers were developed and tested in human clinical trials [51]. Of particular interest is the Pro-XTEN (Protease-releasable XTEN mask) technology developed by Amunix (now a Sanofi company). The Pro-XTEN technology combines half-life extension using a class of tunable unstructured polypeptide polymers to act as spatial shields with off-tumor toxicity mitigation by exploiting the tumor-specific protease activity in the tumor microenvironment to conditionally activate therapeutic candidates. Such a switchable biologics concept will be further discussed in the conditionally activatable immunotoxin section.

MODULATING THE AFFINITY OF BINDING MOIETY FOR SELECTIVE TUMOR TARGETING

Immunotoxins must target tumor tissues so that cytotoxic agents can be delivered into the cytosol of the tumor cells to achieve clinical benefits. Aside from many factors, the anti-tumor efficacy of biotherapeutics is a function of antibody binding affinity and target antigen density [52, 53]. It is well accepted in the field that high-affinity antibodies penetrate solid tumors poorly due to the "binding site effect," while antibodies with moderate or low binding affinity could effectively penetrate tumors and achieve uniform diffusion [54]. High-affinity antibodies with slow dissociation can bind to target antigens in a monovalent form (Fab arm/receptor interaction), thus narrow binding curve differentiation is achieved when binding to tumor cells with high antigen density from normal cells with low to medium antigen density. In contrast, antibodies with low to medium affinity tend to fall off normal cells with low antigen density but are retained on tumor cells with high antigen density via the avidity effect (Fig. 1B) [55]. The same principle was applied to Her2-based ADC to achieve a better safety profile by screening for antibody candidates with "just right" selective binding, internalization and cytotoxicity. Herceptin-based immunotoxins targeting Her2 were also designed and optimized for cytotoxic activity in Her2-positive SKBR-3 cells, but not in Her2-low expressing MCF-7 cells [56]. The right affinity helps tumor-specific targeting and cytosolic delivery. Tight binding to the target receptor usually leads to the lysosomal degradation of internalized immunotoxins [31]. Optimal affinity to shed antigen in the circulation is also critically important for the efficacy of targeted therapeutics like immunotoxins [57]. To determine the impact of affinity on anti-tumor efficacy, Cao et al. evaluated various anti-Her2/neu scFv

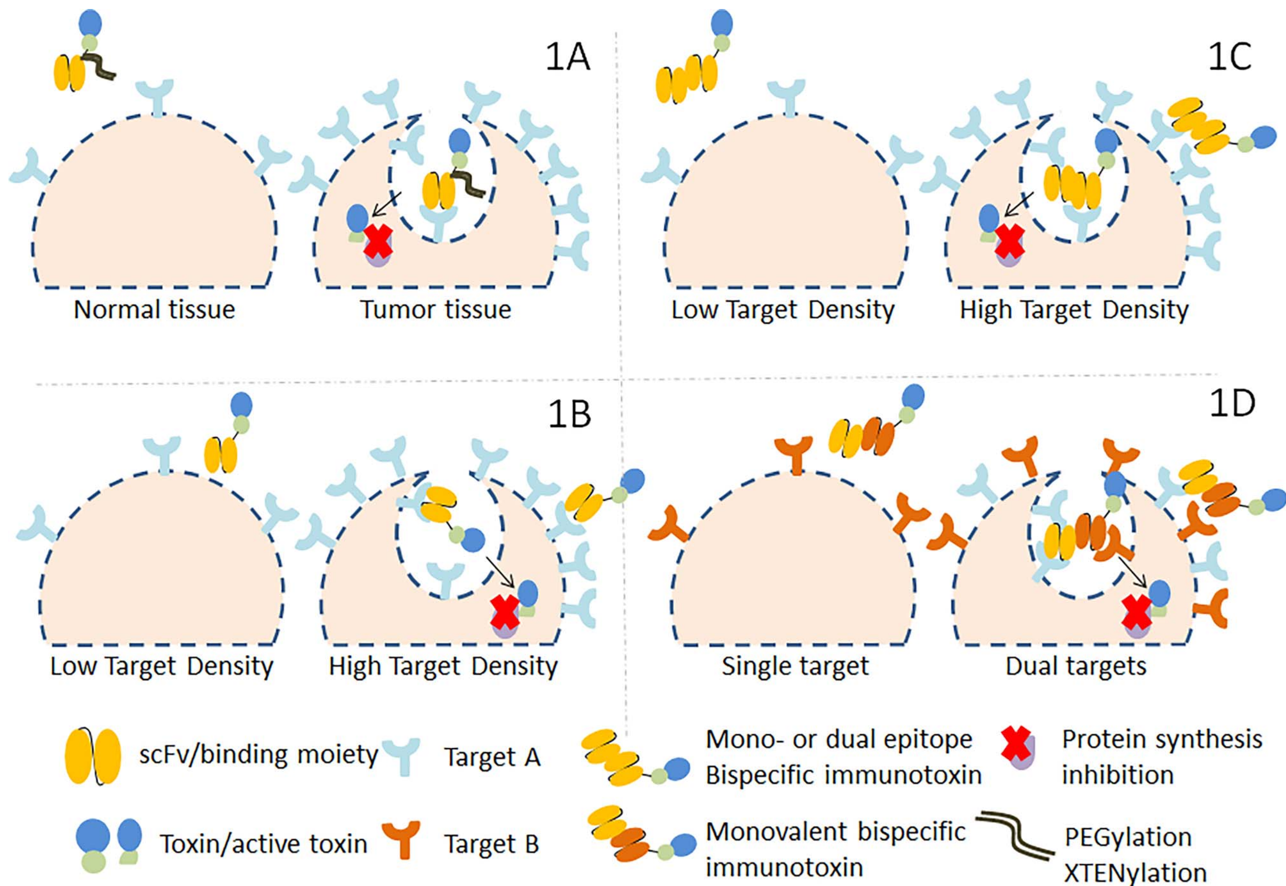

Figure 1. Reducing on- and off-target toxicities by PEGylation of immunotoxins (A) or by modulating the binding affinity (B) and/or valency (C, D) of the targeting moiety. (A) PEGylation or XTENylation is a passive mechanism for selective tumor targeting by reducing normal tissue absorption of PEGylated immunotoxins. (B) By taking advantage of target antigen density on tumor cells, immunotoxins with the optimal binding affinity get retained on tumor cells, while they fall off normal cells with less target antigen expression. (C) By modulating the valency of the binding moiety, bispecific immunotoxins targeting the same antigen (same or dual epitope) for tumor selectivity. (D) By modulating the valency of the binding moiety, monovalent bispecific immunotoxins target the co-expression of two different antigens on tumor cells, while sparing healthy cells only expressing one target antigen. *Pseudomonas* exotoxin A PE24 inhibition of protein synthesis by ADP-ribosylating elongation factor 2 was illustrated here.

with a wide affinity range fused to recombinant gelonin. High-affinity B1D3-rGel immunotoxin induced significant liver toxicity and weight loss, while intermediate MH3-B1/rGel immunotoxin showed effective tumor growth inhibition without hepatotoxicity [57]. This suggested off-target hepatotoxicity induced by immune complexes formed when high-affinity immunotoxin binds to the shed antigen in the circulation. In addition, the presence of shed antigen in the circulation or extracellular environment serves as a target sink, which leads to poor delivery of recombinant immunotoxins to the tumor and reduced anti-tumor effect. Indeed, reducing Mesothelin (MSLN) shedding by 80% using an MSLN mutant cell line showed a 2–3-fold increase in MSLN-targeted immunotoxin uptake [58]. The affinity of immunotoxins likely needs to be optimized in an optimal range to achieve selective tumor targeting for better anti-tumor efficacy and to avoid on- and off-target toxicities. Cell-based immunotoxin screening system was established to provide a rapid and direct approach for screening functional antibodies with internalization capacities [59]. Such a screening system, when applied to cell lines with different target protein densities, helps to identify functional antibody hits with selective tumor targeting and cytotoxicity.

By modulating antibody affinity, valency and target antigen density, increased therapeutic index could be achieved by selective tumor targeting while limiting normal tissue toxicity [55, 60–62]. The biological nature of the target antigens such as receptor density on the cell surface or the internalization rate upon antibody–ligand binding is equally important for optimal tumor targeting [52, 60]. For optimal anti-tumor efficacy and tumor targeting by immunotoxins, an ideal target must meet the following criteria: (1) target antigen expression is highly tumor-specific with no or very low expression in normal tissues [63], (2) high expression level on tumor cells [64] and (3) high internalization rate or recycling rate that is largely unaffected by the binding affinity of immunotoxins [32]. These will ensure sufficient immunotoxins enter tumor cells and help to avoid normal tissue toxicity [65].

MODULATING THE VALENCY OF THE BINDING MOIETY FOR IMPROVED THERAPEUTIC INDEX

For improved solid tumor penetration and better anti-tumor efficacy, the targeting moiety of immunotoxins usually takes the format of single-chain variable fragments (scFv) or novel scaffolds [66]. scFv, while maintaining binding specificity to the target antigen, is faced with limitations like lower tumor retention due to its monovalency. To overcome such low tumor retention challenges, immunotoxins with bi- or trivalent targeting moieties against the same epitope or non-competing epitopes were designed and evaluated [67–69]. For target antigens like CD64 whose internalization is facilitated by receptor cross-linking, modulating the valency of the binding moiety to facilitate receptor cross-linking is a viable approach to increase the cytotoxicity of immunotoxins (Fig. 1C) [70]. The bivalent anti-CD64 immunotoxin H22(scFv)2-ETA′ showed 10-fold increased efficacy compared with the monovalent H22(scFv)-ETA′ [70]. A trivalent immunotoxin targeting carcinoembryonic antigen (CEA) (IMTXTRICEAαS) also showed superior anti-tumor activity in mice bearing human colorectal cancer xenografts compared with the conventional monovalent counterpart [67]. Increased valency of the binding moiety to the singular target antigen often leads to higher binding affinity due to the target avidity effect, which inevitably raises the aforementioned need to modulate the binding affinity to balance anti-tumor activity and on-target normal tissue toxicity.

To avoid limitations in targeting a singular receptor in complex and multifactorial diseases like cancer and inflammatory diseases, dual targeting strategies using bispecific antibodies were contemplated [71]. The initial proof-of-concept in the immunotoxin field was actually achieved using monoclonal antibodies with dual binding specificity. Antibodies D2C7 and 14E1 bind to the same epitope on both wild-type EGFR and the truncated EGFRvIII. D2C7- and 14E1-based immunotoxins, D2C7(scdsFv)-PE38KDEL and scFv(14E1)-ETA, showed the effective killing of glioblastoma cells overexpressing both forms of EGFR [72, 73]. To further increase tumor selectivity and therapeutic index, monovalent bispecific antibody targeting of EGFR and Her2 double-positive tumor cells over single-positive normal tissue was evaluated using a dual-flank tumor xenograft model system [55]. This demonstrated the feasibility of efficient tumor selectivity by targeting two tumor-associated antigens co-expressed on the same tumor cell using affinity-modulated monovalent bispecific antibodies in cancer therapy (Fig. 1D). However, one must keep in mind that challenges still remain with regards to shedding antigens in the circulation or the tumor microenvironment. Dual targeting of urokinase-type plasminogen activator receptor (uPAR) and EGFR receptor using a de-immunized bispecific diphtheria toxin showed improved anti-tumor efficacy than targeting uPAR alone using a rhabdomyosarcoma cell line RH30-derived xenograft murine model [74]. Compared with singular targeting of uPAR, the increase of therapeutic index by dual targeting is marginal, which suggests dual targeting alone is not sufficient enough for tumor selectivity [74]. OXS-1550, a CD19XCD22 bispecific diphtheria immunotoxin designed to overcome cancer resistance mechanisms induced by loss or down-regulation of either CD19 or CD22, was evaluated in phase I clinical trial (NCT00889408) for relapsed or refractory CD19+, CD22+ B-lineage leukemia or lymphoma [75]. The trial was later discontinued due to dose-limiting toxicity issues. A de-immunized version of DT2219 is currently explored to address such dose-limiting toxicity encountered in phase I clinical trials [76]. Such a marginal therapeutic index and dose-limiting toxicity further emphasize the importance of fine-tuning the affinity of each Fab arm to improve its overall toxicity profile. Such a “just right” affinity usually involves the extensive screening of a large panel of bispecific antibody variants to achieve desired clinical efficacy and safety profile. For example, both JNJ-61186372 (EGFR/c-Met bispecific antibody) and zenocutuzumab were obtained by functionally screening hundreds of variants [77, 78]. Cell-based immunotoxin screening system could facilitate such a process [59]. However, one must also keep in mind that in vitro cell-based screening sometimes shows poor correlation with primary tumor cells residing in a heterogeneous and complex disease setting. Such lack of correlation could be partially due to the fact that cell lines differ from primary tumors transcriptionally or biologically, thus not all cell lines serve as appropriate models of primary tumors. The clinical relevance of in vitro cell line models needs to be scrutinized on-target receptor density, especially when screening for antibodies with “just right” binding affinity or screening for avidity-based binders. Patient-derived xenograft (PDX) models are considered better preclinical cancer models than cell culture models with regards to molecular characteristics, disease mechanisms and clinical relevance [79]. In the establishment of PDX models, serial transplantation of human patient-derived tumors led to inconsistency in molecular characteristics, genomic instability over passages and altered tumor microenvironment, which raises the concern of cancer cell fidelity in PDX models [80]. If applicable, primary tumor samples are recommended in the early discovery stage. In vitro cell lines with various target receptor densities should also be established and used as alternatives in the screening process for more informed decision-making.

Beyond affinity and valency modulation, it will be more impactful to develop new technologies for cancer-selective antibody discovery. Cancer-selective antibodies can be generated using an antibody engineering approach like conditionally active biologics (reviewed later) or using a discovery approach by directly selecting antibodies with tumor-specific binding properties. Conventional antibody discovery utilizes animal immunization or rationally designed antibody libraries to generate a panel of binders with pre-defined requirements. The panel of binders is then vigorously screened using in vitro cell-based assays for tumor binding preference over normal tissues. Such a workflow only showed limited success with tumor-specific targets. For tumor-associated antigens, results of in vitro cell-based screening could not effectively translate into the cancer-selective binding in vivo. In addition, antibodies are usually discovered at a physiological pH, whereas

tumor-targeting antibodies are expected to function at an acidic pH in the tumor microenvironment. These warrant an antibody discovery need to generate cancer-selective antibodies in the tumor microenvironment, preferably in vivo. Recent advances in the rational design of synthetic antibody-mimetic or alternative scaffold libraries made it possible for biologics to penetrate solid tumors deeper [81, 82]. When such phage libraries are injected into mice carrying PDXs or tumor organoids, cancer-selective antibodies or antibody mimetics can be enriched and isolated directly from the native tumor microenvironment. Such an in vivo panning concept, pioneered by Ruoslahti and Schnitzer [83, 84], should generate cancer-selective antibodies that function in the tumor microenvironment and recognize tumor-intrinsic features that do not exist in normal tissues.

CONDITIONALLY ACTIVATABLE IMMUNOTOXINS FOR IMPROVED OVERALL SAFETY PROFILE

Conditionally activatable biologics represent a class of novel biologics targeting solid tumors with a favorite therapeutic window. This concept takes advantage of the unique biological conditions in the tumor microenvironment such as acidic pH or tumor tissue-specific protease activity to activate biologics in tumor tissues to achieve tumor-selective targeting while keeping them inactive in normal tissues (Fig. 2A) [85, 86]. A variety of stimuli can be applied to activate biologics at the tumor site, including light, temperature, high reducing potential, pH, oxygen level, tumor-specific protease activity and ion concentration [87–89]. Some activatable antibodies have advanced into the early stages of human clinical trials like "probodies" or "recycling antibodies," including the probody of Yervoy BMS-986249 and CX-2029 targeting previously undruggable CD71 [90–92]. Probody therapeutics, a class of new protein therapeutics, are specifically designed to restrict the drug activity in the tumor microenvironment to enhance the therapeutic index. The targeting domain of the probody is usually masked by a masking peptide via a protease-cleavable substrate linker. De-masking by tumor-associated protease cleavage releases the masking peptide and enables the target binding [91, 93]. Probodies take advantage of the deregulated tumor-associated protease activity to conditionally activate the targeting domain, whereas conditionally active biologics (CAB) rely on the acidic tumor microenvironment for tumor-selective targeting. Tumor-selective conditionally active biologic anti-CTLA4 antibodies that are only active in the acidic tumor microenvironment were developed and demonstrated equivalent tumor inhibition as Ipilimumab in a human CTLA-4 knock-in mouse model [85, 94]. Such CAB molecules bind to targets on tumor cells in an acidic tumor microenvironment. However, the binding to targets in normal tissues under physiological conditions was reversibly inhibited by a novel mechanism called protein-associated chemical switches [85], which leads to reduced normal tissue toxicity and a widened therapeutic index. Nevertheless, from an antibody engineering point of view, both the probody and the CAB concepts require extensive customized antibody engineering to make the biologics conditionally functional in the specific tumor microenvironment of interest. Such extensive engineering efforts will likely incur incomplete in vivo masking, poly-specificity, immunogenicity, poor pharmacokinetics and/or other developability issues that the industry would, by all means, like to avoid. For example, in a phase I first-in-human study of CX-2029, a probody drug conjugate targeting CD71, anemia was frequently reported [90]. Early clinical data suggested undesired dose-dependent on-target toxicity in the bone marrow, although MMAE might complicate the observed toxicity [90]. To overcome such customized engineering needs and to avoid the undesired consequences of binding to the functional region of therapeutic candidates, the Pro-XTEN technology by Amunix provides a class of universal and tunable unstructured polypeptide masks with favorable physiochemical properties. In the Pro-XTEN concept, the low immunogenic XTEN masks reduce systemic exposure by serving as spatial shields that can be removed by the intrinsically high protease activity in the tumor [51]. Beyond such a genetic engineering approach, biocompatible polymers were also used to enlarge the therapeutic window by limiting the systemic exposure of therapeutic candidates [95]. In the concept of switchable immune modulators (Sw-IM), biologics were reversibly blocked by biocompatible polymers via a tumor microenvironment responsive covalent chemical linker. The redox-responsive and/or pH-responsive stimuli in the tumor microenvironment degrade the chemical linker to achieve selective activation [95]. Such a concept was validated with several immune-modulating antibodies against immune checkpoints like 4-1BB, PD-1 and CTLA-4 [95].

Taking advantage of the glioma-associated metalloproteinase activity, blood–brain barrier (BBB)-penetrating nanohybrid protein toxin was constructed for the cross-BBB delivery of trichosanthin to the glioma cells [96]. This was achieved by the construction of a fusion protein toxin consisting of lactoferrin (targeting moiety), MMP-2 substrate peptide, cell-penetrating peptide and trichosanthin toxin. Lactoferrin, via binding to low-density lipoprotein receptor-related protein-1, facilitated cross-BBB delivery of nanohybrid toxin. On the tumor surface, MMP-2 cleavage of the substrate peptide led to the release of CPP-trichosanthin. The cell-penetrating peptide then delivered the trichosanthin to the cytosol of glioma to kill the tumor cells [96]. A favorable in vivo efficacy and toxicity profile was observed using an orthotopic GL261-bearing immunocompetent C57BL/6 mouse model but the clinical application of such a multistage booster delivery strategy was limited by the immunogenicity of the nanohybrid protein toxin. Pore-forming immunotoxin with caged cytotoxicity was also explored. Activation of toxins by proteolysis on the tumor cell surface triggers pore formation and cell killing [97]. Whether or not this caged cytotoxicity strategy can be translated into a better therapeutic index is still unknown due to the lack of in vivo efficacy data.

Among the abovementioned conditionally active technologies, the Pro-XTEN technology could be a very impactful approach for the systemic delivery of immunotoxins to achieve both an enlarged therapeutic window

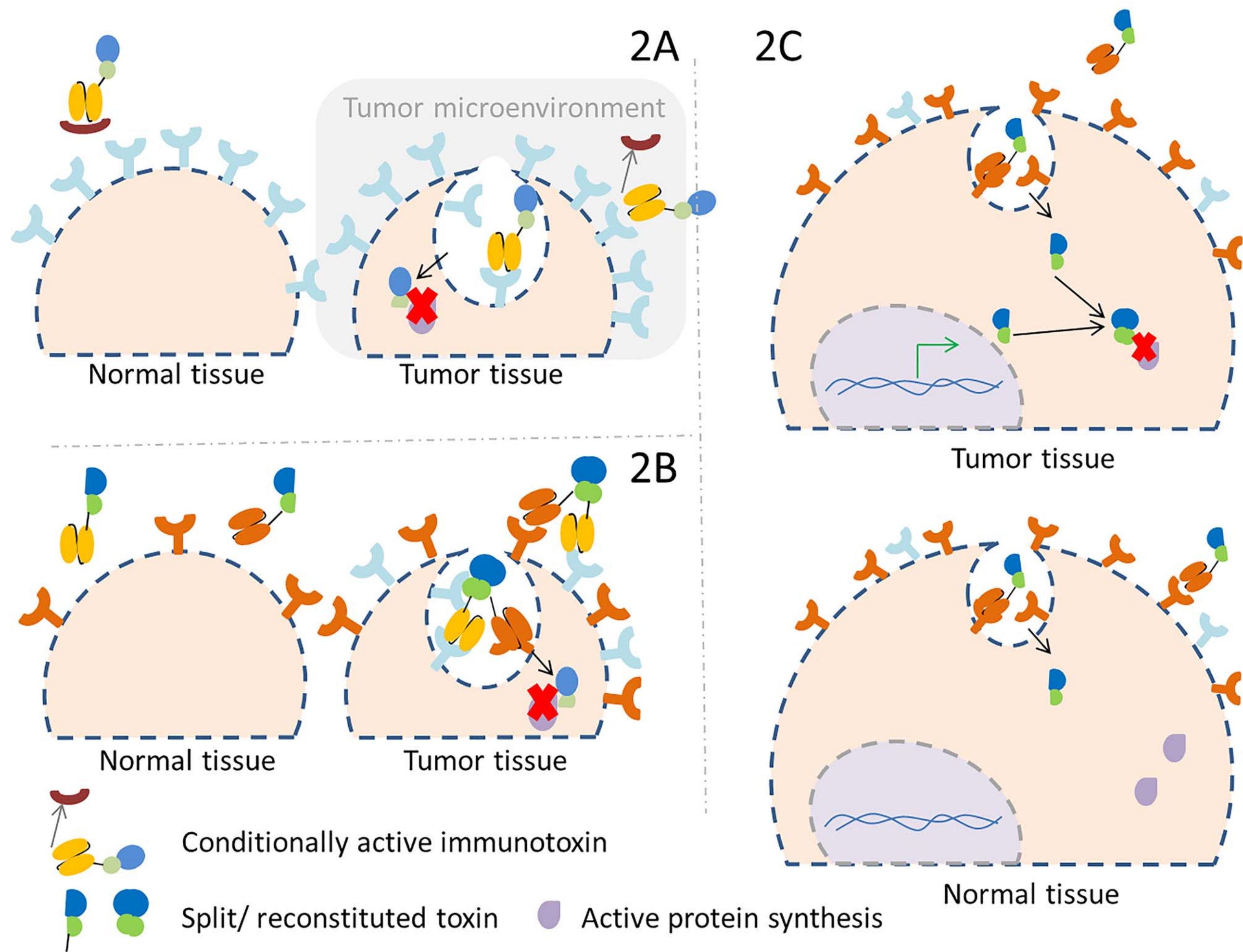

Figure 2. Tumor selective targeting by conditionally active immunotoxins (A) or by the split toxin technology of various mechanisms (B, C). (A) Conditionally active immunotoxins rely on the tumor microenvironment to activate the tumor targeting, while remaining inactive in normal tissues. The tumor microenvironment herein includes but not limited to tumor-specific protease activity. (B) Cell surface reconstitution of split toxin targets the co-expression of two different tumor-associated antigens, while sparing normal cells only expressing one of them. (C) Cytosolic reconstitution of split toxin utilizes different routes of tumor targeted delivery to improve the tumor selectivity. *Pseudomonas* exotoxin A PE24 inhibition of protein synthesis by ADP-ribosylating elongation factor 2 was illustrated here.

and reduced immunogenicity. The Pro-XTEN technology has the following advantages: First, spatial shielding by unstructured polypeptide polymers reduces the need for customized protein engineering and avoids the undesired consequences of engineering the binding site. XTENylation can be achieved by direct fusion or site-specific conjugation to immunotoxins. Second, XTENylation not only prolongs the half-life of immunotoxins but also reduces the immunogenicity of XTENylated immunotoxins. Immunogenicity is currently a significant challenge in immunotoxin research and development. Last, the XTEN polymers are highly soluble, stable and tunable, direct fusion to immunotoxins might help the large-scale production of immunotoxins. Scaled-up production of immunotoxins is another imminent challenge the field is facing. Moreover, the tunability of XTENylation enables the modulation of immunotoxins to improve the tumor-to-normal-tissue ratio for optimal biodistribution and bioavailability. At the same time, the unknown clinical risks of delivering unstructured polypeptide polymers to the tumor microenvironment need to be identified and managed properly if necessary.

The conditional activation that heavily relies on the components of the tumor microenvironment is also faced with many challenges. Both Probody and Pro-XTEN technologies need intrinsically high protease activity in the tumor microenvironment to turn the inactive toxin from the pro-drug form to the active form. The interpatient heterogeneity of tumor protease expression levels makes it difficult to balance clinical efficacy and safety [91, 98]. Low tumor protease activity generates inadequate active toxins to be clinically efficacious, whereas high-dose to compensate for low tumor protease activity will inevitably result in off-target toxicity. Thus, a clinically relevant biomarker and patient stratification strategy are needed for the desired clinical benefits of conditionally active immunotoxins. The intratumor and intermetastatic heterogeneity of the tumor microenvironment pose another

challenge [99]. Large lesions with intrinsically high protease activity are favored killing by conditionally active immunotoxins. Micro-metastatic lesions without an established tumor microenvironment likely will be spared killing, which inevitably leads to cancer relapse. To further augment the clinical efficacy of conditionally active immunotoxins, combination therapies targeting multiple pathways simultaneously should be explored to prevent the selection of resistant populations [99].

RECONSTITUTED SPLIT TOXIN FOR WIDENED THERAPEUTIC INDEX

Beyond tumor microenvironment-assisted conditionally active strategy, other concepts of conditional activation like protein fragmentation or split toxin were also explored [100, 101]. PE38, the 38KDa protein of *Psuedomonas* exotoxin A, was split into two inactive fragments at residues 407–408. The resulting fragments were fused to Npu DnaE intein. Upon binding to the same tumor cells expression of human Her2/neu, PE38 toxin was reconstituted via the intein mediated trans-splicing reaction (Fig. 2B). The reconstituted PE38 toxin showed comparable but slightly lower cytotoxicity than the original immunotoxin [100]. Noncovalent transcomplementation of *Pseudomonas aeruginosa* exotoxin A via a hetero-specific coiled-coil interaction also reconstituted toxin with reduced cytotoxic activity [102]. Splitting toxins into dysfunctional fragments reduced the on- and off-target toxicities induced by the target-specific and nonspecific absorption of immunotoxins. When combined with a dual-targeting strategy, split immunotoxin can further improve the safety profile by binding to dual targets co-expressed on the same tumor cell. The advantage of such approaches need to be further tested and likely will face the immunogenicity challenge. To improve the reconstitution efficiency and to properly address the immunogenicity challenges, Purde et al. adopted the prodrug concept [103, 104] and improved the tumor-targeting precision by intein-mediated cytoplasmic reconstitution of diphtheria toxin (split-DTA_C/PA) via independent, selective pathways (Fig. 2C) [101]. One dysfunctional part of the toxin (PA/LF_N-I_C-DTA_C, protective antigen C-terminal DTA split fragment) was delivered to the cytosol via a receptor-mediated pathway, while the cells stably expressing the matching dysfunctional part (tdTomato-DTA_N-I_N-p66α). In tumor xenografts harboring tdTomato-DTA_N-I_N-p66α, cytoplasmic reconstitution of split toxin strongly delayed tumor growth without side toxicity [101]. This concept needs to be further validated in human clinical trials to demonstrate that such cytoplasmic reconstitution of split toxin can translate into favorable efficacy and safety profiles. The short 3-hour half-life of PA/LF_N-I_C-DTA_C, the dysfunctional partial toxin delivered via receptor-mediated endocytosis, may render immunogenicity problems after repeated dosing. Nevertheless, the combinatorial therapy using both protein and viral therapeutics to achieve widened therapeutic index might provide unprecedented benefits in human clinical trials.

Overall, the improved therapeutic index can be achieved using the above strategies, including XTENylation, modulating the binding affinity of a targeting moiety, modulating valency of the targeting moiety, conditional activation of immunotoxins and reconstitution of split toxins. Other strategies for improved efficacy include local application of immunotoxins like H22(scFv)2-ETA′ or MOC31PE to avoid vascular leak syndrome that is commonly observed with systemic application of immunotoxins [70, 105]. In combination with CTLA-4 blockade, Leshem Y. et al demonstrated that local delivery of recombinant immunotoxin SS1P or LMB-100 initiated immunotoxin-mediated cell death. The immunogenic cell death induced anti-tumor T cell responses, which leads to the elimination of malignant cells at distant sites in a cell line-derived xenograft murine model [106]. This presents new opportunities for recombinant immunotoxins to treat patients with multiple lesions or metastatic distant sites. Dual targeting cocktail therapy of anti-CD3 immunotoxin and anti-CD7 immunotoxin in steroid-refractory acute graft-versus-host disease showed an overall response rate of 60% (12 of 20), with 10 patients (50%) achieving a complete response [107]. Combination therapy with immune checkpoint inhibitors or small molecule drugs like endosomal escape enhancers also helped to improve the efficacy of immunotoxins [108–110]. Engineered protein toxin variant with reduced vascular toxicity also showed a superior activity window in preclinical studies. For example, the Denileukin diftitox V6A variant s-DAB-IL-2(V6A) led to a 3.7-fold less lethality in mice with no weight loss observed [110]. These are largely irrelevant to the main topic here and will not be reviewed further.

FUTURE PERSPECTIVES

Targeted cancer therapy with immunotoxins showed remarkable efficacy in hematological malignancies as evidenced by the FDA approval of moxetumomab pasudotox [4]. However, poor safety profile due to on- and off-target toxicities is still a major hurdle in the development of immunotoxin cancer therapies. In many ways, such on- and off-target toxicity could likely be mitigated by modulating the affinity, valency and/or both to precisely target the immunotoxins to tumor cells of interest, by making the immunotoxins to be conditionally active in the tumor microenvironment, by modulating the cytotoxic killing potency of the toxin moiety and by site-specific XTENylation to reduce binding to normal tissues. Such measures, if clinically successful, might improve the therapeutic index of immunotoxins and therefore bring clinical benefits to the patients to fulfill their unmet medical needs. One also has to keep in mind that the toxicity of targeted therapy goes beyond early research and development. Beyond what's been described above, emerging masking/demasking and conformational activation technologies were developed to make antibodies responsive to various stimuli to achieve targeted tumor delivery. Antibody function can be allosterically regulated by circularly permutated calmodulins [111] or by chemical rescue using small molecules [112]. Antibody binding can be inactivated by various masking strategies, including epitope-mimetics, anti-idiotypic masks, allosteric disruption of the binding conformation and masking by steric hindrance [113, 114]. If transferrable to immunotoxins after fine-tuning binding

specificity, affinity and immunogenicity, these strategies could further help to widen the therapeutic index.

For ADCs with cleavable linkers, the cytotoxic membrane-permeable payload, upon release from the cytosol, can trigger the killing of nearby proliferating tumor cells in a target-independent manner [115]. Such a bystander-killing effect is not expected with immunotoxins as the protein toxin payload is only expected to exert its functions in the cytosol of the tumor cells it enters. The lack of bystander killing effect, on one side, curbs the off-target toxicity of immunotoxins [116]. On the other side, it also limits the anti-tumor efficacy of immunotoxins as a sufficient amount of protein toxins need to enter the cancer cells. Efficient delivery of immunotoxins to each cancer cell in a complex, heterogeneous, hypoxic, acidic and immune-suppressive tumor microenvironment remains challenging. Additional immune-modulating mechanisms of action like combo therapy with immune checkpoint inhibitors could further boost the efficacy of immunotoxins [108, 109].

Inefficient endosomal escape remains a rate-limiting step as a single potency curve was observed regardless of variations in antigen expression level, intracellular trafficking kinetics, exposure time and extracellular immunotoxin concentrations. With the help of a recently established flow cytometric method to quantify the endosomal escape [117], new strategies and designs to facilitate the cytosolic delivery efficiency of protein toxins should be explored [31]. One has to keep in mind that such in vitro cell-based functional screening system may not fully recapitulate primary tumor cells with regard to the receptor expression level, internalization rate, lysosomal degradation, redundant signaling pathways and tumor microenvironment [118]. Therefore, a disease indication-relevant animal model serves as a better screening tool for efficacy and safety profiles to avoid the loss of translation between preclinical studies and human clinical trials. One of the pain points in the industry is the loss of translation between disease animal models and human clinical trials as animal testing did not sufficiently identify human safety and toxicity [119]. This is exactly the case with immunotoxin therapy for solid tumors. Immunotoxin therapy for hairy cell leukemia has achieved a remarkable clinical success but it is currently wrestling with solid tumors and other hematologic malignancies like non-Hodgkin lymphoma or B cell chronic lymphocytic leukemia due to toxicities, immunogenicity issues and multiple mechanisms of resistance including the resistance to cell death via the elevated expression of pro-survival signaling pathways [120]. Such a loss of translation may also need to be considered in immunotoxins research and development and potential alternatives like artificial intelligence or machine learning-based approaches should be explored [121].

Efficient production of immunotoxins is yet another hurdle that limits the clinical potential of immunotoxins. Early generations of immunotoxins rely on chemical conjugation to link targeting domains to protein toxins, which inevitably leads to heterogeneity and low stability issues in final products. PEGylation, when applicable, makes it even worse [39, 40]. A newer generation of immunotoxins takes advantage of appropriate expression hosts for recombinant expression of immunotoxins in a single-step procedure. So far, various expression hosts like bacteria, yeast, plant cells and animal cells like Chinese hamster ovary (CHO) cells were investigated. Please refer to the review article by Zuppone et al. [122]. A universal expression host for efficient recombinant immunotoxin production is still in the look. Generally speaking, CHO cell-based expression platforms are preferred industrial scaled-up production platforms to ensure the high quality of final products. Potential endotoxin contamination and heterogeneous N-glycosylation patterns in the final products limited the therapeutic potential of bacteria- and yeast-derived recombinant immunotoxins, respectively. Other technical challenges remain. New strategies beyond "cytosolic immunization" are needed to avoid auto-intoxication of expression host cells [123]. Further engineering of the CHO cell lines to eliminate the retro-translocation of newly synthesized immunotoxins to the cytosol from ER. Alternatively, knockout host factors involved in the ER protein quality control check might boost yield by reducing proteolysis. Advanced protein design might help to ensure the quality, yield and developability of immunotoxins.

Tremendous efforts have been put into the early discovery/engineering of immunotoxins. For clinical and translational success, pharmacokinetics and biodistribution studies of immunotoxins might help to further define the clinical dosing strategy, to understand how the drug is metabolized in the body, and to profile the target antigen expression beyond tumors for overall safety profiles [124]. To fill the gap between preclinical studies and human clinical trials, protein toxin payload-sensitive biomarkers need to be identified to stratify patients, to further improve the therapeutic index of immunotoxins, and augment the probability of clinical success [125].

DATA AVAILABILITY

Not applicable.

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CONFLICT OF INTEREST STATEMENT

M.L., S.M., Y.Y., Y.S. and L.C. are current employees of Biocytogen and declare no conflict of interest.

ETHICS AND CONSENT STATEMENT

Not applicable.

ANIMAL RESEARCH STATEMENT

Not applicable.

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