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Review

An Old Idea Tackling a New Problem: Targeted Toxins Specific for Cancer Stem Cells

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Abstract: Targeting and killing specific cells discriminately has been the goal of targeted therapy dating back to the era of Paul Ehrlich. The discovery of cancer stem cells has caused a paradigm shift within the cancer field and provided an opportunity to use targeted therapies such as targeted toxins to bind and kill these cells selectively. A number of targeted toxins have been developed against recently identified cancer stem cell markers. In this review we discuss the development and current status of these exciting novel drugs and their potential use to combat drug-refractory relapse.

Keywords: cancer stem cells; targeted toxins; immunotoxins; CD133; EpCAM; CD123; CD44

Abbreviations

dCD133KDEL: deimmunized pseudomonas exotoxin fused to anti-CD133 scFv with a KDEL terminus; KDEL: amino acid sequence Lys-Asp-Glu-Leu; mAb: monoclonal antibody; TT: Targeted toxin; PE: pseudomonas exotoxin; scFv: recombinant single chain VH and VL domain.

1. Introduction

The idea of specifically targeting and killing cells responsible for disease is not a new one. Paul Ehrlich discovered the first targeted therapy, Arsphenamine, in 1909. This work inspired scientists in a range of disciplines over the next 100 years to continue working and developing new therapeutics that specifically destroy target cells. Immunotoxins, hereafter referred to more generally as targeted toxins, descended from this early work and have been used in cancer therapy for decades [1,2].

Targeted toxins (TTs) are biological drugs consisting of a ligand linked to a protein toxin. Some of the most commonly used toxins are pseudomonas exotoxin (PE), diptheria toxin (DT), ricin, saporin, bouganin, and gelonin. These toxins act catalytically on target cells to induce apoptosis using a range of mechanisms from inhibiting protein translation via ADP-ribosylation of EF-2 (PE and DT) to inactivating ribosomes (ricin, saporin, bouganin, gelonin) [3]. While the mechanism of action may vary, the one constant is that the TT must reach the target cells and the ligand portion of the molecule must bind its specific receptor and then be internalized before it is able to induce apoptosis. These ligands are typically antibodies, antibody fragments, cytokines, or growth factors, all specific to their target receptor [3,4].

The past decade has witnessed a paradigm shift within the cancer field. There has been overwhelming evidence recently presented in a wide range of tumor types that there exists a typically small subpopulation of cells within the cancer that have been termed cancer stem cells (CSCs). These cells can enhance tumor initiation, self-propagation, and differentiation into all the phenotypically diverse cells found within the tumor population. Furthermore, CSCs have been shown to be more chemotherapy and radiation resistant than non-CSCs [5,6]. This explains the single most difficult problem in treating cancer patients, drug-refractory relapse. Thus, new therapeutic agents are necessary to target these cells in particular in order to prevent drug-resistant relapse. A logical approach to eliminate CSCs is to target their unique cell surface markers.

A number of markers have been identified that allow for the separation and ultimately targeting of CSCs. In 1997, Bonnet and Dick reported the first CSCs in acute myeloid leukemia. These cells were characterized as being CD34+/CD38− [7]. Since then a number of markers have been used to successfully identify CSCs in a broad range of tumor types. However, only cell surface markers, not intracellular markers, are useful when it comes to treatment using TTs. Thus, this review will focus only on TTs specific for these markers. Table 1 shows the CSC TTs discussed herein and their phase of development.

2. Cancer Stem Cell Targeted Toxins

2.1. CD123

CD123, also known as IL-3Rα, is the alpha subunit of interleukin-3 and is expressed on leukemic stem cells (LSCs) in Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Myelodysplastic Syndrome (MDS), and Systemic Mastocytosis [8]. An anti-IL3 Diphtheria toxin fusion protein (DT_{388}IL3) recently completed a phase I clinical trial [9]. Out of 45 patients, three had complete or partial responses with four more having minimal responses. Transaminasemia, vascular

leak syndrome, and fever were the main toxicities in this study. These toxicities point to the need to improve selectivity and potency in order to increase efficacy.

Table 1. Targeted Toxins directed against CSC markers currently under investigation.

Target	Name	Toxin	Cancer	Phase of Development	Reference
IL3	$DT_{388}IL3$	Diphtheria Toxin	AML	Phase I	[9]
CD123	26292(Fv)-PE38-KDEL	Pseudomonas Exotoxin A	AML	Preclinical	[10]
CD44	Bivatusumab Mertansine	Maytansine Derivitive	HNSCC	Phase I	[16,17]
EpCAM	chiHEA125-Ama	α-Amanitin	Pancreas	Preclinical	[24]
EpCAM	Ec4-ETA	Pseudomonas Exotoxin A	Colon	Preclinical	[26]
EpCAM	Opportuzumab Monatox	Pseudomonas Exotoxin A	Bladder HNSCC	Phase II Phase I	[27,28] [29]
EpCAM	VB6-845	deBouganin	Breast	Preclinical	[30]
EpCAM/Her2	DTEpCAM23	Diphtheria Toxin	Colon	Preclinical	[23]
CD133	$CdtA^{C149A, C178A}BC$-CD133MAb	Cytolethal Distending Toxin	HNSCC	Preclinical	[35]
CD133	dCD133KDEL	Deimmunized Pseudomonas Exotoxin A	HNSCC Breast	Preclinical Preclinical	[36] [38]

Notes: Several laboratories are now investigating a range of different approaches and toxins targeting cancer stem cell associated markers. In several of the preclinical studies, more than one cancer type was investigated.

A TT called 26292(Fv)-PE38-KDEL was developed to target LSCs that shows a promising increase in selectivity and potency compared to $DT_{388}IL3$. This TT combines a single chain variable fragment (scFv) with a truncated form of pseudomonas exotoxin, which was mutated to increase activity and has shown good activity against several leukemia lines [10]. The activity appears to be dependent on a threshold level of CD123 expression on the leukemia cells, which can vary greatly from patient-to-patient. Additional studies are needed to further assess efficacy and toxicity of this TT.

2.2. CD44

CD44 has become a useful marker for identifying CSCs in AML, colon, head and neck squamous cell carcinoma (HNSCC), and other cancers as well [11,12]. CD44 is a member of a cell adhesion molecule (CAM) family of proteins involved with regulating growth, differentiation, survival, and migration. Bivatuzumab is a humanized monoclonal antibody specific for the anti-CD44v6 isoform of CD44. This isoform in particular correlates with poor prognosis in a number of cancers, including gastric, breast, and colorectal cancer [13,14].

Researchers have recently tried targeting CD44 with Bivatuzumab conjugates with some promising results [15–17]. One of these conjugates is a targeted toxin named Bivatusumab mertansine (or BIWI 1), and it has recently been tested clinically to treat head and neck squamous cell carcinoma (HNSCC). BIWI 1 consists of a monoclonal antibody conjugated to a potent maytansine derivitive. In the phase 1 study of patients with advanced multi-drug-refractory HNSCC, three patients out of 31 showed

significant improvement in the study with visible tumor regression [16,17]. However, the study was terminated prior to completion following the death of a patient in a parallel study due to toxic epidermal necrolysis. While this study's objective was to determine toxicity and safety, the fact that some efficacy was observed suggests potential of targeting CD44. However, more work is needed to reduce the limiting toxicities.

2.3. EpCAM

Epithelial cell adhesion molecule (EpCAM, also known as CD326 and ESA) is a well-known overexpressed marker on many carcinomas. Initially thought to simply be a cell-cell adhesion molecule, more recently it has been discovered that EpCAM has diverse roles within cancer cells that range from cell signaling and migration to proliferation and differentiation [18]. Even more interesting are the findings that EpCAM is expressed at an even higher level on CSCs and correlates with increased tumorigenesis *versus* non-EpCAM positive cells in breast, pancreatic, hepatocellular, HNSCC, and other carcinomas as well [19–21]. This finding may not be completely surprising based on the recent evidence showing EpCAM is a direct target in the Wnt/β-catenin signaling pathway, which is a critical pathway in both normal adult stem cells and CSCs [6,22]. Furthermore, EpCAM is an attractive receptor for TTs because it efficiently internalizes when bound by an antibody or an scFv enabling toxin bearing molecules easy access to the interior of the target cells where they can act to induce apoptosis [23]. Recently, a number of anti-EpCAM TTs have been developed and show great promise in both pre-clinical testing and in recent clinical trials.

One of these EpCAM TTs was created by conjugating α-amanitin (a mushroom toxin) to the anti-EpCAM chimerized monoclonal antibody chiHEA125. This targeted toxin called chiHEA125-Ama was then tested against pancreatic carcinoma *in vitro* and in a mouse xenograft model with significant efficacy. Six of ten and nine of ten in the highest concentration groups (50 and 100 μg/kg) showed no visible tumors at day 16 after only two injections of chiHEA125-Ama. It was also found to be potently active against other EpCAM positive carcinoma lines *in vitro* as well. At the doses tested, no observable toxicity was detected in an analysis of blood liver enzymes levels [24]. Pancreatic cancer is the fourth leading cause of death in the United States and with extremely limited therapeutic options available chiHEA125-Ama warrants further development as a potential anticancer agent for pancreatic (and other EpCAM expressing) cancer [25].

An interesting TT was recently developed that combines the same truncated form of pseudomonas exotoxin with an anti-EpCAM DARPin and is named Ec4-ETA [26]. DARPins, or designed ankyrin repeat proteins, are a new class of binding molecules that can be engineered to be highly specific and have very high affinity for a given receptor. They are very stable, easily manipulated, and produce high yields when expressed in *Escherichia coli*. Ec4-ETA is the first known example of a DARPin targeted toxin. Ec4-ETA was tested *in vitro* against a number of EpCAM positive carcinomas and showed IC_{50} values in the sub-picomolar range. It also efficiently localized to colon carcinoma xenografts when given systemically and significantly inhibited tumor growth. Furthermore, Ec4-ETA was well tolerated and showed no detectable liver toxicity in the mice. This study shows that DARPins could be used in place of antibodies or antibody fragments in TTs as successful, high affinity targeting ligands.

Oportuzumab Monatox, also known as VB4-485, is an anti-EpCAM TT produced by Viventia Biotechnologies that has recently completed three clinical trials [27–29]. Oportuzumab Monatox (OM) was produced by fusing a truncated form of Pseudomonas exotoxin A (ETA) to an scFv of the humanized anti-EpCAM antibody MOC31. The first two clinical trials were in patients with grade 2 or 3 stage drug-refractory transitional cell carcinoma of the bladder. In the phase one study, 64 patients were enrolled in dose escalating cohorts where the highest dose given was 30.16 mg. Patients received intravesically administered OM once a week for six consecutive weeks, and were followed for an additional 4–6 weeks and then reassessed. During the study no dose-limiting toxicity was identified and so a maximum tolerated dose (MTD) was not determined. They did find that 77% of the patients did develop human anti-toxin antibodies by the end of the study, with 16% developing human anti-human antibodies. However, upon reassessment at the 12-week time point 24 of the patients (39%) achieved a complete response defined as a nonpositive urinary cytology with either a normal cystoscopy or an abnormal cystoscopy with a negative biopsy [27]. This is impressive especially since the doses in this study were not optimized for efficacy. In the phase two study 46 patients received either 6 or 12 weekly 30 mg treatments. At the end of the study 20 patients (44%) achieved a complete response and 7 patients (16%) were still disease free 18–25 months following the end of the study. Furthermore, no patients had to discontinue treatment due to an adverse affect [28]. OM may prove to be a valuable agent for bladder carcinoma in monotherapy or possibly in combination with other therapies.

In another phase one clinical trial in twenty patients with advanced recurrent HNSCC, OM was administered intratumorally weekly for four weeks. The MTD was determined to be 930 μg with elevated liver enzymes being the dose limiting toxicity. All patients at the end of the study had detectable anti-toxin antibodies and neutralizing antibodies developed in seven of the patients. However, there was a positive response in 87.5% of EpCAM-positive patients. Four patients showed tumor growth stabilization, while 10 others had a notable regression. Another four patients exhibited complete responses to the treatment [29].

Because neutralizing antibodies are a problem for OM, Viventia has developed a new variation of this drug with reduced immunogenicity. This new variant, VB6-845 uses the same anti-EpCAM scFv, but the pseudomonas exotoxin fragment is swapped out for a deimmunized version of Bouganin, a plant toxin that acts through deadenylation of rRNA thus blocking protein translation. Preclinical testing has shown good efficacy and safety and it will be interesting to see how this drug performs in upcoming clinical trials [30]. Overall, OM has exhibited very promising results and may be an effective therapeutic in the treatment of HNSCC as well as transitional cell carcinoma of the bladder.

Our laboratory has developed a bispecific TT that targets both EpCAM and erbB2, the gene product of Her2 that is overexpressed on 30–40% of ovarian and breast cancers [23]. This bispecific, called DTEpCAM23, showed potent picomolar activity *in vitro* against a range of carcinomas including breast, colon, ovarian, lung, and prostate cancer. DTEpCAM was more effective than either monospecific TT alone or in combination. Furthermore, in two tumor models of colon cancer, DTEpCAM23 significantly inhibited tumor growth. Bispecific TTs may prove to be very useful moving forward because they can target both CSC and the more differentiated tumor cell populations as well.

2.4. CD133

Another major cancer stem cell marker that has been targeted is CD133, also known as Prominin-1. CD133 is a pentaspan membrane glycoprotein and has been shown to be a marker of the CSC populations in many carcinomas including breast, colon, prostate, liver, pancreatic, lung and HNSCC [11,31]. It is known to be associated with the Wnt signaling pathway because down-regulation of CD133 results in corresponding degradation of β-catenin and decreased proliferation *in vitro* and *in vivo* [32–34]. However, the specific function of this cell surface receptor is still unknown.

The first known TT that selectively inhibits the CD133+ cell population is called $CdtA^{C149A, C178A}$BC-CD133MAb. This TT uses the anti-CD133 antibody AC133 conjugated to cytolethal distending toxin (Cdt). Cdt acts as a nuclease and damages host DNA leading to growth arrest and subsequent cell death. In this study, the proliferation of CD133+ HNSCC cells was arrested thus providing a necessary proof of concept showing that CD133 is internalized (a necessary step in TT function) and can be specifically targeted to inhibit cell growth [35].

Our group has recently developed a deimmunized anti-CD133 TT called dCD133KDEL. It combines an scFv from a novel monoclonal antibody (clone 7) with a deimmunized truncated form of pseudomonas exotoxin A [36]. This new monoclonal antibody is unique in that it binds all isoforms of CD133 thus avoiding the controversy surrounding AC133 resulting from its binding to only some undifferentiated epitopes and restricting its use [37]. By mutating immunogenic epitopes on the pseudomonas exotoxin, we significantly reduced the risk of dCD133KDEL having the same problem many other TTs have had in the clinic: that is the development of neutralizing antibodies against the toxin portion of the protein. In our first paper on dCD133KDEL, we showed its ability to inhibit the proliferation of two HNSCC cell lines *in vitro*, suppress tumor initiation, and cause significant tumor regression *in vivo* while only killing the small subpopulation of CD133+ cells. A therapeutic window was demonstrated between dCD133KDEL's killing of CD133+ cancer stem cells in our time course viability assay and killing of normal CD133+ hematopoietic stem cells in a colony formation assay [36]. In a second paper we showed dCD133KDEL is effective against breast carcinoma *in vitro* and in a systemic mouse model as well [38]. We have also tested this TT against the ovarian cancer line OVCAR5 *in vitro* and *in vivo* with very good preliminary results [39]. It also has an excellent safety profile that makes it possible to give multiple courses of 20 μg injection per nude mouse, where one course is 3 weekly injections given Monday, Wednesday, and Friday. The MTD in athymic nude mice was determined to be 2.0 mg/kg (5 times the dose used *in vivo*) and the limiting toxicity at this dose is liver toxicity indicated by elevated Alanine Transaminase levels (unpublished data). Table 2 summarizes the various tumors and model systems we have investigated to date using dCD133KDEL. This new CSC specific TT shows significant potential as a possible therapeutic for carcinomas where CD133 has been shown to be a marker for CSCs.

Table 2. A summary of the cancer and model types used to date by our group in evaluating the efficacy of dCD133KDEL.

Cell Line	Cancer Type	Model Type	Response Obtained	Reference
UMSCC-11B	HNSCC	Flank	Regression	[36]
MDA-MB-231	Breast	Systemic	Partial Regression	[38]
OVCAR-5	Ovarian	Intraperitoneal	Regression	[39]

Notes: Our group has published independent reports using 3 different xenograft models to assess the efficacy of dCD133KDEL in immunodeficient mice.

3. Conclusions

CSCs have been shown to be more resistant to chemotherapy and irradiation than more differentiated cancer cells that make up the bulk of the tumor. CSC then may be at the root of our most serious problem in cancer, drug refractory tumor relapse. Thus, it would be perilous to ignore them as therapeutic targets. TTs may prove to be uniquely qualified to fill this role because of their extremely high potency and exquisite selectivity.

A major concern of drugs that target CSC surface markers is that they would also kill normal adult progenitor cells that also express these same markers. In our work on dCD133KDEL, we partially addressed this concern by testing umbilical cord blood cells that were 76% CD133+. At a concentration 100 times greater than used to inhibit the proliferation of HNSCC cells *in vitro*, the normal human hematopoietic stem cells were not inhibited [36]. There are several hypotheses that could explain this therapeutic window. First, the CD133+ cells could have been killed and then replaced by the CD133- cell population. Two different groups have demonstrated this type of plasticity in progenitor cells recently [40,41]. Second, normal hematopoietic progenitors may be more quiescent and have a slower endocytic uptake than CSC and thus not be as effected by the toxin. Finally, the CSC may express CD133 at a higher level then the normal progenitor cells, which is true in colorectal, pancreatic, gastric, and hepatocellular carcinomas [42]. Each of these separately or in combination could explain the ability for dCD133KDEL to specifically kill CSC and not normal hematopoietic progenitors.

In the broader picture, each target will have a different therapeutic window, but our work shows significant promise that CSCs can be selectively eliminated. However, several strategies exist to limit the toxicity of systemically administered drugs. We described a method called ToxBloc where an intraperitoneal pre-dose of the ligand without toxin was given prior to injection with the targeted toxin. This allowed us to give doses 15-fold higher than the maximal tolerated dose [43]. Other possible methods include photochemical internalization and ultrasound triggered drug delivery via microbubbles [44,45].

Since many tumors are phenotypically diverse, the use of multiple drugs in combination may be necessary to successfully increase the percentage of tumor regressions in patients. It is already established that TTs work synergistically with chemotherapy [46,47]. So, new TTs that selectively attack CSCs could be an important weapon to combat drug refractory relapse.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2073-4468/2/1/82/s1.

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