Advances in anticancer antibody-drug conjugates and immunotoxins.

Title

Advances in Anticancer Antibody-Drug Conjugates and Immunotoxins

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Short running title: Advances in Antibody-Drug Conjugates and Immunotoxins

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Abstract

Antibody-delivered drugs and toxins are poised to become important classes of cancer therapeutics. These biopharmaceuticals have potential in this field, as they can selectively direct highly potent cytotoxic agents to cancer cells that present tumor-associated surface markers, thereby minimizing systemic toxicity. The activity of some conjugates is of particular interest receiving increasing attention, thanks to very promising clinical trial results in hematologic cancers. Over twenty antibody-drug conjugates and eight immunotoxins in clinical trials as well as some recently approved drugs, support the maturity of this approach. This review focuses on recent advances in the development of these two classes of biopharmaceuticals: conventional toxins and anticancer drugs, together with their mechanisms of action. The processes of conjugation and purification, as reported in the literature and in several patents, are discussed and the most relevant results in clinical trials are listed. Innovative technologies and preliminary results on novel drugs and toxins, as reported in the literature and in recently-published patents (up to February 2013) are lastly examined.

Keywords: Antibody drug conjugate, anticancer agents, auristatins immunotoxin, calicheamicins, cross-linkers, duocarmycins, maytansinoids

1. Introduction

Monoclonal antibodies (mAbs) are eminently suitable as drug carriers, thanks to their selectivity and flexibility The recent successful development of monoclonal antibodies that target key components of biological pathways has expanded the range of treatment options for patients with several cancers.

Antibody-based therapeutics are of growing significance in cancer therapy, as is shown by the fact that 33 such formulations (in the form of a mAb as such or conjugated with a drug) have now been approved for oncologic indications by the FDA and are marketed in the USA. Eight of these had global market revenues above US $1 billion, and their combined global revenues exceeded US $50 billion. These therapeutics are one of the fastest growing sectors in the pharmaceutical industry. The commercial pipeline of mAb-based therapeutics is still growing, and nearly 350 candidates [1], not only for oncological use, but also for several immunological indications and for Alzheimer's disease, are now in clinical development. Progress in the development of antibody-based therapeutics is dramatically accelerating.

Further, the traditional bivalent, monospecific, full-length IgG molecule only accounts for about half of the anticancer mAbs in the pipeline. The rest are compounds that can be conjugated to drugs, toxins or radiolabels; they may be multispecific or otherwise engineered for increased functionality [2]. Immunoconjugates have evolved over 40 years; with antibody-drug conjugates (ADCs) they recently reached significant clinical and regulatory milestones, with the marketing approval of brentuximab vedotin. Molecular engineering techniques have also had a significant impact on the efficacy/toxicity profiles of immunotoxins (ITs), for example denileukin diftitox, which is now registered for the treatment of cutaneous T-cell lymphomas.

This review will provide an overview of the more advanced ADCs and ITs in the industrial pipeline, or under development in academic research laboratories. The mechanisms underlying cell intoxication by toxins or by anticancer drugs will first be presented, followed by the evolution of the structure of conjugates obtained by molecular biology or chemical synthesis. On the basis of the targeting agent employed, the conjugates will be divided into two major groups: those targeting hematologic and those targeting solid cancers. Finally, the most advanced results obtained in preclinical or clinical trials will be described in some detail. Due to the impressive body of basic research in the field of ADCs and ITs, the review will concentrate chiefly on patented approaches.

Immunotoxins are protein-based therapeutics consisting of a targeting moiety linked or fused to a killing moiety. The target moiety can be an antibody or a ligand directed against a receptor or cell-surface antigen that is specific for the targeted disease, while the active moiety is a member of a class of highly toxic proteins or enzymes. Essentially, any molecule that induces cell death by directly interfering with the cell machinery, by modifying the cell membrane, or by inducing apoptotic proteins can be used. Because of the enzymatic potency of these proteins, a small number of toxin molecules successfully delivered to the cytoplasm (or to the ribosomal compartment) may be lethal to the cell [3].

Initially, ITs comprised mAb or growth factors chemically conjugated to cytotoxic plant or bacterial toxins [4], but these have largely been replaced by recombinant methods, thanks to their considerable design flexibility and product homogeneity.

Monoclonal antibody technology is now mature, fully humanized mAbs are in therapeutic use, and fragments of varying complexity are available. Thus the most strenuous and continuous efforts are directed to manipulating the killing moiety, where the success of delivering the cytotoxic domain of an IT to the cell cytoplasm depends on a series of steps, each having varying degrees of efficiency depending on the cell type, antigen density, binding affinity, internalization/recycling, and subcellular trafficking or endosomal escape. High anticancer activity is unfortunately usually contrasted by critical drawbacks, which are caused by varying degrees of nonspecific toxicity, mainly affecting hepatocytes, the kidneys and the vascular endothelium [5].

ADCs are designed to specifically transport small molecules after appropriate chemical modification. The payloads most frequently used for conjugation to the mAb include tubulin inhibitors, DNA double-strand break-inducing compounds, and DNA minor groove binder/alkylators, the majority of these were originally derived from natural products, are now prepared by synthesis. Conceptually, ADCs act as prodrugs minimizing the systemic toxicity of the free drug and augmenting the antitumor activity of the targeting vehicle. This review will discuss the various parameters affecting conjugation strategies and their potential consequences for obtaining potent and specific ADCs.

2. Immunotoxins

This section comprises a brief description of the toxin, followed by a detailed description of the applications of immunoconjugates/chimeric proteins that have reached advanced clinical trials. The mAb moiety and receptor used to target the payload will be described, classifying ITs against hematologic cancers and those targeting solid cancers. This will be followed by data related to the most recent IT-related approaches reported in the literature and protected by patents.

Basically, toxins contain a binding domain, a catalytic domain, and, in some cases, a domain responsible for translocation. Plants and fungi produce a number of molecules with defensive functions, to protect themselves against pathogens. These proteins include ribosome-inactivating proteins, which are capable of inactivating ribosomes by preventing attachment of elongation factors 1 and 2 to ribosomal RNA, conversely, bacterial toxins inactivate elongation factor-2 (EF-2) by ADP ribosylation. These toxins have been widely studied and are the subject of numerous reports. The most extensively-examined constructs comprise bacterial toxins, such as Pseudomonas exotoxin (PE) and diphtheria toxin (DT), as well as the plant-derived deglycosylated ricin A chain (dgA) and recombinant gelonin (rGel). Other more recent constructs, based on non-toxic Ribonuclease (RNases) of animal origin or proapoptotic endogenous proteins, of human origin, have been proposed to induce effective cell death [6]. The diagram in Fig. (**1**) illustrates the pathway involved in ITs comprising by PE, DT and RIPs from binding to cell intoxication [7].

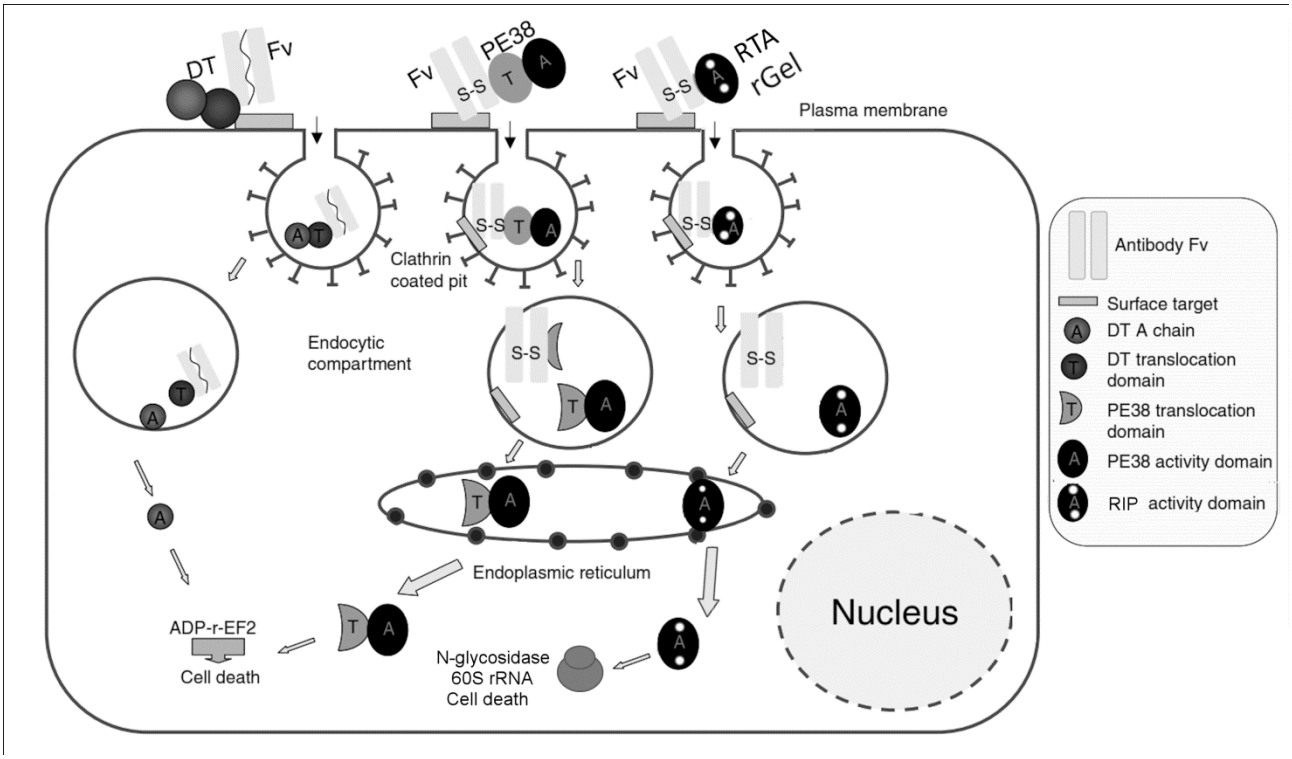

Figure 1. Immunotoxin endocytosis and trafficking within mammalian cells (modified from [7])

ITs experienced a first boom in the 1980s and 90s, but only with the development of fully-humanized mAbs did their clinical relevance increase. Their renewed importance is documented in a special issue of the FEBS Journal entitled ' Engineering toxins for 21st century therapies' [8] as well as in recent reviews [9, 10]. Table **1** summarizes the principal ITs that are currently in clinical development.

2.1 Pseudomonas exotoxin-based immunotoxins.

Pseudomonas exotoxin is a single-chain protein of 613-amino acids containing 3 functional domains (Fig. **2**). Domain Ia binds the low-density lipoprotein receptor-related protein 1. The function of domain Ib is still unknown, and domain II is responsible for translocation of the toxin to the cytosol. Domain III is the enzymatic portion that catalyzes the transfer of the ADP-ribose moiety from NAD to elongation factor 2 (EF-2), arresting protein synthesis and ultimately leading to cell death [11]. The first of two important amino acid motifs within the PE molecule is the fragment in domain II (aa 274-280, RHRQPRG) which is exposed on the exterior surface of the protein, where it is accessible for the cleavage by the eukaryotic protease furin. The second significant motif is the pentapeptide REDLK (aa 609-613) at the C-terminus of PE, which acts as an endoplasmic reticulum retention sequence. Both motifs are essential for the cytotoxicity of PE.

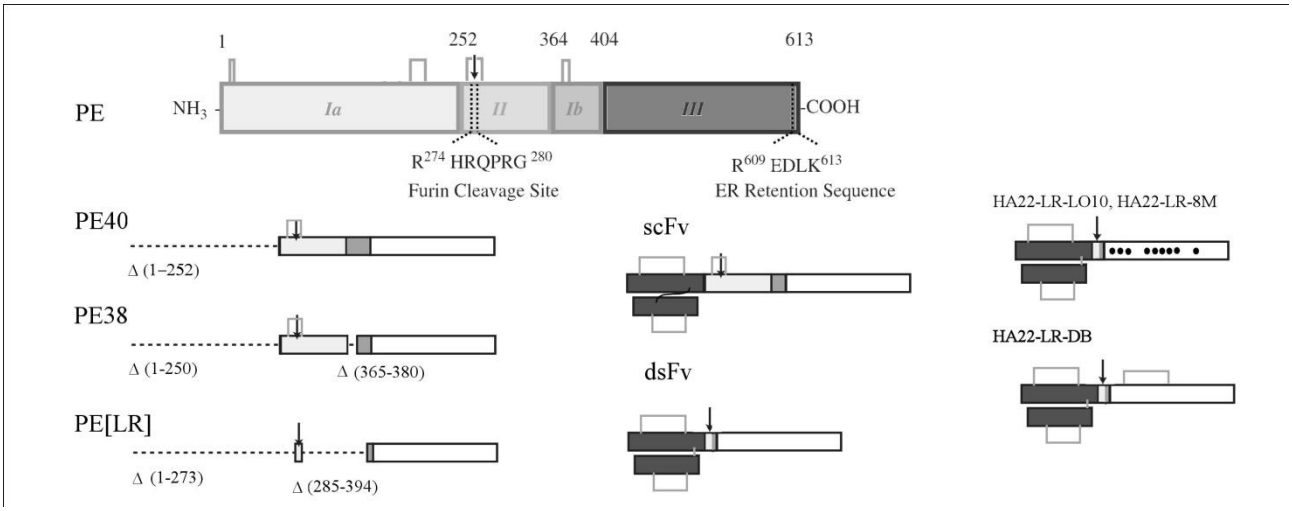

Figure 2. PE and PE-based immunotoxins. PE contains different domains with specified functions: binding (Ia), translocation (II), catalysis (III). From native PE to truncated fragments in which the binding domain is deleted (PE40), or domain Ib (PE38) is deleted, or also part of domain II (PE[LR]). The diagram shows the evolution of ITs structures maintaining the activity that reduces the immune response. The arrow indicates the furin cleavage site; the external lines represent disulfide bonds in PE and in mAb fragments.

The biggest contributor to IT-PE evolution has been the Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, USA. Based on 30 years of research, a large number of PE-based ITs directed against various surface tumor antigens have been constructed and tested in preclinical trials [11], and three chimeric PE-based ITs are now undergoing clinical trials.

The recombinant immunotoxin BL22 [RFB4(dsFv)-PE38 or CAT-3888], composed of Fv fragments of anti-CD22 mAb, undergone to several early-phase clinical trials for the treatment of B cell malignancies. CD22 is a lineage-restricted B-cell antigen expressed on the surface of many types of malignant B cells, including chronic B-lymphocytic cells leukemia (B-CLL), B lymphoma cells such as Burkitt's lymphomas, and hairy cell leukemias (HCL), as well as on normal mature B lymphocytes. CD22 is an attractive target because expression of CD22 is lineage-restricted and, in most cases, is not lost during neoplastic transformation. BL22 produced an excellent response when used to treat B cell malignancies. Advanced phase II clinical trials (ClinicalTrials.gov Identifier NCT00924040) in patients with HCL achieved complete regression rates of between 47% and 61% [12, 13].

Clinical trials of BL22 have been superseded by an improved form of mAb portion, initially known as HA22, CAT-8015 and now called Moxetumomab pasudotox (MedImmune LLC)[14]. This IT is better able to bind CD22, because three point mutations have been added to the complementarity-determining regions (CDR) of the heavy-chain domain, using phage display. In this case, improvement in activity against cells from patients with B-CLL and HCL was up to 50-fold [15, 16] (NCT00659425). Moxetumomab pasudotox exhibits some activity in different patient populations: refractory or relapsed ALL or NHL and HCL [17]. Recently, a phase I trial on relapsed/refractory HCL reported complete remission in 46% of 28 patients and relapse for 3 of 13 patients. At doses up to 50 μg/kg QOD x3, HA22 demonstrated a good safety profile. The durability of complete remission was assessed by clone-specific real-time

quantitative PCR [18]. HA22 and variants are also named in a recent patent, which claims a method for treating ALL in pediatric patients [19].

Immunogenicity and non-specific toxicities continue to be problematic, and for this reason several patents protect the intensive research done to improve the PE moiety, with single mutants [20] or eight point mutations [21, 22], leading to a species named HA22-LR (LR = lysosome resistant) which showed reduced animal toxicity, due to the removal of a portion of domain II [23]. Furthermore, a unique IT (HA22-LR-8M) in which human B-cell epitopes of PE have been identified and silenced, yet that retains excellent cytotoxic and antitumor activity, is claimed [24]. To achieve this result, domain II of PE was deleted (except for the furin processing site) and eight point mutations were introduced into domain III, to remove large hydrophilic residues. Further, a disulfide bond, able to increase thermal stability and protease resistance, was introduced into domain III. HA22-LR-DB exhibited significantly lower immunogenicity in mice [25]. The most significant improvement led to the new recombinant IT HA22-LR-LO10, in which seven other point mutations produced the highest antitumoral efficacy of the series, plus very low reactivity with human antisera; this product can be administered in high doses without causing excessive toxicity [26].

In a complementary approach, the identification and elimination of T cell epitopes of PE (particularly four T-cell epitopes of PE38) were described in a patent [27]. Concerning the mAb moiety, studies have aimed to reduce immunogenicity and prolonging plasma life, by modifying the mAb portion. A reduction of the isoelectric point (from 10 to below 8) was achieved by introducing specific mutations in the Fv portion. In all three ITs studied, animal toxicity was reduced to below half by lowering the pI [28].

Another potential engineering target is the furin cleavage site of PE, which is very inefficiently cleaved by the enzyme. Patents have been filed describing the role of the peptide linker interposed between VH and PE domains (sequence KASGG) [29]. In addition to CD22, the receptor CD25 (also referred to as Tac, p55, and interleukin-2 receptor alpha subunit) has also been targeted for the treatment of various leukemias and lymphomas. CD25 is a lymphoid activation marker highly expressed on the surface of Hodgkin's lymphoma and in other hematologic malignancies cells, and only present on a minority of normal human cells. In the anti-CD25 IT LMB-2 [Anti-Tac(Fv)-PE38KDEL] the carboxyl terminus of the toxin is replaced from REDL to KDEL, in order to enhance trafficking to the endoplasmic reticulum [30]. LMB-2 is in trials for the treatment of patients with adult T-cell leukemia (NCT00924170) and HCL (NCT00321555) after pretreatment with fludarabine plus cyclophosphamide [31]. It is unfortunate that, although replacing the native sequence with KDEL might enhance the cytotoxicity of PE-based IT, the therapeutic benefit is offset by an accompanying increase in non-specific toxicity, as has been observed in *in vivo* preclinical tests [32].

Regarding non-specific toxicities, and in particular renal and liver toxicity, an interesting evaluation on the urine of patients treated with BL22 and LMB-2 demonstrated that BL22, in particular, is excreted into the urine in a potentially cytotoxic form, even after its plasma level declines; thus it may remain intact long enough to cause renal toxicity [33].

ITs have been developed comprising the PE-KDEL sequence fused with different scFv antibody fragments, directed against hematologic diseases targeted to the CD33 antigen [34]. CD33 is expressed during myeloid differentiation, and is present on the leukemic blast cells of 90% of patients with acute myeloid leukemia. It is significant that CD33 is also the target of Mylotarg (humanized IgG-chalicheamicin conjugate) [35]. The same approach targeting the CD19 antigen, a cell surface glycoprotein potentially attractive target for therapy of B-lymphoid malignancies, has also been described [36].

The CD7 antigen is T-cell specific and is expressed on most T-cell lymphomas and leukemias, but is absent on a portion of normal T-lymphocytes. A key property of CD7 for therapeutic applications which makes this antigen well suited for IT construction, is its rapid internalization after binding by an antibody or fragments. CD7 has been targeted with toxins including dgRTA, saporin and other RIPs, linked to mAb via chemical linkage [37-40]. CD7-PE40-KDEL has been produced and preliminary ex-vivo data, also protected by a patent, have been presented [41, 42].

Rhabdomyosarcoma (RMS) is the most common pediatric malignant soft tissue tumor; once metastasis or relapse has commenced it is highly resistant to all forms of treatment currently available. It has recently been determined that the acetylcholine receptor (AChR) γ-subunit, which defines the fetal AChR (fAChR) isoform, is almost exclusively expressed in RMS post partum, a single chain variable fragment (scFv) derived from a fully human anti-fAChR Fab-fragment was fused to PE, to generate an anti-fAChR IT named scFv35-ETA[43, 44]

A recent patent has reported the methods for optimizing a recombinant human PE38sBAFF immunotoxin (BAFF signifies B cell-activating factor receptor), directed to human B-lineage acute lymphoblastic leukemia [45].

2.2 Pseudomonas exotoxin-based ITs in treating solid tumors:

One of the more interesting ITs, claimed in recent NIH patents, is SS1P or antimesothelin (Fv)-PE38. Mesothelin is expressed on several different types of solid tumors including mesothelioma, ovarian, pancreatic, and lung cancers [46]. Further, SS1P is currently being evaluated for treatment of pleural mesothelioma. Unfortunately, immunogenicity appears to constitute the major obstacle to SS1P treatment, and high levels of neutralizing antibodies developed in 75%-88% of patients after a single treatment cycle. Mainly for this reason, SS1P has shown little response when tested alone, but when combined with chemotherapy it has produced many partial responses [47, 48]. Clinical trial NCT01362790, involving SS1P and pentostatin plus cyclophosphamide, in a treatment of mesothelioma, is currently recruiting participants. An interesting recent study [49], using SSP1 as IT model, describes the importance of antigen shedding and IT activity: free mesothelin competes with cell surface mesothelin for SS1P, acting as an unproductive sink for the IT. The study used experimental data with SS1P to develop a mathematical model that describes the relationship between tumor volume changes and doses of the IT administered.

To expand the usefulness of SS1P in therapy, and to further understand the mechanism underlying IT-induced apoptosis, the activity of SS1P or SS1P-KDEL on pancreatic cancer cells resistant to conventional treatments has been evaluated [50]. This study involved co-administering of the IT with TNF-related apoptosis-inducing ligand (TRAIL) agonist mAb (HGS-ETR2), which activate the apoptosis extrinsic pathway. *In vivo* evaluations only combining IT (0.3 mg/Kg, 3 x QOD doses) with HGS-ETR2 (1x 10 mg/Kg) demonstrated a very efficient anticancer effect; this may therefore be an effective approach to overcoming cell resistance.

Regarding solid tumors, Erb38 (e23(dsFv)-PE38), the first dsFv-PE based IT to enter clinical use is directed at HER2/neu, a receptor widely expressed on breast cancer cells [51]. In a phase I study, hepatotoxicity occurred in all patients; this effect was thought to depend on the presence of HER2/neu on hepatocytes. Furthermore, in a phase I trial with scFv(FRP5)-PE (scFv(FRP5)-ETA) involving 18 patients with Her2/neu-expressing cancers, dose-limiting liver toxicity and induction of antibodies against IT occurred in 13 patients [52].

After local administration into cutaneous lesions of melanoma and breast cancer, scFv(FRP5)-PE showed no systemic toxicity. The IT was administered by intratumoral injection once daily for 7-10 days (total daily doses from 60 to 900 μg), and total dose per treatment cycle ranged from 0.6 to 6.0 mg, with adverse reactions restricted to local symptoms. Complete regression of injected tumor nodules was achieved in four patients (40%) and partial reduction in tumor size in another two patients (20%) [52].

Viventia Biotech Inc. has been developing several ITs, and one of the most promising is referred to as VB4-845, a fusion protein directed against a human epithelial adhesion molecule (EpCAM or CD326), highly expressed in non-squamous carcinoma cells. VB4-845 (or oportuzumab monatox, Proxinium®) is a recombinant fusion protein that combines the specificity of an antiEpCAM scFv with the toxicity of PE 40. Patients with squamous cell carcinoma of the head and neck were treated with escalating doses (100 to 930 μg /week) of VB4-845, administered either from a single puncture site or from multiple sites, in one centimeter increments evenly distributed throughout the tumor. Repeated intratumoral delivery of VB4-845 was found not to be limited by immunogenicity and was well tolerated. Preliminary results also indicate a positive outcome of VB4-845 therapy in EpCAM-positive patients [53]. The same fusion protein was employed in a phase I study on bladder cancer refractory to or intolerant of bacillus Calmette-Guerin. VB4-845 was administered into the bladder through a catheter at doses in the range 0.1 mg to 30.16 mg once weekly. Adverse effects were generally mild and very manageable and although most patients developed antibody titers, the findings showed no evidence of detrimental effects on the clinical outcome associated with the immune response [54]. Recently, a phase II study demonstrated that instillation of 30 mg of VB4-845 (once a week for 6 consecutive weeks) had a favorable safety profile. Regarding efficacy, the complete response rate at 6 months was 27%, with 16% maintaining a disease-free status beyond 1 year [55].

In order to reduce the host response against PE-based IT, an approach has been attempted whereby the toxic moiety is comprised of fragments of cholera exotoxin (CET) and PE [56]. CET has a very similar three-dimensional structure to PE, and domains II and III are 36% and 50% similar. These have led to the development of HB21scFv-PE40 and HB21scFv-CET40 [50], where HB21 is an anti-Tfn receptor mAb.

Another recent patent claims the production of a fusion protein composed of an immunoglobulin Fc-binding protein, denoted ZZ, which is a mutated form of domain B of *Staphylococcus aureus* protein A, genetically fused to a truncated form of PE (precisely, PE38, PE38KDEL, PE40) [57-59]. Further, ZZ-PE was conjugated with chH23 (recognizing MUC1 expressing cells) or chFRP5 (anti Her2/neu) mAbs. *In vivo* results on A431 cells xenografted in nude mice were positive.

Again in connection with the target of mAb trastuzumab, Her2/neu (ErbB2), an invention based on an optimized DNA sequence encoding the scFv(FRP5), that is an anti-ErbB2 mAb has been reported; the goal was to improve production of a fusion protein IT with PE (domains II and III) [60, 61]. In addition to Her2/neu, a specific glycoprotein, recognized by mAb M4G3, is also well expressed by human breast cancer [62]; its application as carrier of PE40 has been claimed in a patent [63].

A promising approach involves another protein derived from Staphylococcus aureus (staphylococcal enterotoxin A-SEA) used as effector to target cytotoxic T cells to tumors. An ADC currently undergoing phase II/III studies is naptumomab estafenatox, (ABR-217620) (Active Biotech Research) which is a fusion protein, in which an anti-5T4 Fab (5T4 being a receptor found on a large number of solid tumors, including non-small-cell lung cancer, renal cell cancer,

and breast cancer) is fused to a mutated variant of low antigenicity and reduced systemic toxicity SEA [64]. The safety and effectiveness of the mAb when administered with interferon-alpha is being evaluated in patients with advanced renal cell carcinoma (NCT00420888).

Duke University claims two patents relating to the development of brain tumor therapy. The first is targeted against the epidermal growth factor receptor (EGFR) [65]. The activity of anti-EGFR-PE IT (MR1-1) is currently under evaluation in a phase I trial, in which it is delivered intracerebrally by convection-enhanced delivery (CED) in patients with supratentorial malignant brain tumors (NCT01009866). The second invention is directed against Podoplanin [66], a mucin-like sialoglycoprotein that is highly expressed in gliomas. Podoplanin is also reported to be a marker that enriches tumor-initiating cells, which are thought to resist conventional therapies and to be responsible for relapse: expression of podoplanin by cancer cells has been shown to promote tumor cell motility and tumor lymphangiogenesis in vitro [67].

The scientific community has directed considerable effort toward glioblastoma multiforme (GBM). GBM is the most prevalent primary brain tumor; it is responsible for a large percentage of brain-cancer-related deaths, owing to its grim mortality rate. Among target molecules overexpressed by this tumor and used to deliver cytotoxic agents, one of the most interesting is an IL-13 receptor fusion protein named cintredekin besudotox (IL13-PE38QQR or NK-408) (NeoPharm IL) [68] in which lysine residues at positions 590 and 606 are replaced by glutamines (Qs), and lysine at position 613 is replaced by arginine (R). Phase III evaluation was conducted on 296 patients with GBM (the trial PRECISE). This trial was the first and largest study using convection-enhanced diffusion (CED) with IT and the Gliadel wafer as active comparator. Although IL-13-PE38QQR was well tolerated, there was no survival advantage of IT administered via CED compared with the Gliadel wafer. Several parameters, including initial screening of patients for target antigen (IL-13Rα2) expression, optimal catheter positioning, and real-time drug delivery imaging, must thus be optimized to increase the success rate in future clinical trials [69]. A phase I trial (NCT00880061) on the treatment of pediatric brain cancers is currently recruiting participants.

Another interesting IT that targets EGFR is TP-38 (Teva Ltd.). This recombinant protein is a fusion of the toxin PE-38 with TGF-a. A phase I clinical trial was conducted on recurrent primary or metastatic malignant brain tumor patients, where dose escalation of TP-38 demonstrated a median survival of 28 weeks post TP-38 treatment, and a median survival of 20 weeks or 33 weeks for those with residual disease [70].

In order to improve potency by reducing side-effects, Vallera proposed a procedure that allows the preliminary administration of non-immunotoxic receptor-targeting reagents before active IT (PE or DT-based [71]. A fusion protein (GnRH-PTD-PE39KDEL) containing human LH-releasing hormone GnRH [72], PE fragment and a peptide vector-protein transport domain PTD has also been described.

2.3 Diphtheria toxin-based immunotoxins.

Diphtheria toxin (DT) is a single-chain protein 535 amino acids long. It comprises an enzymatic A domain (amino acids 1-193) and a binding B fragment divided into two functional domains: the translocation domain (T) (amino acids 202–

378) and the receptor binding domain (R) (amino acids 386–535) (Fig. **3**). In order to kill cells, DT is first proteolytically cleaved outside the cell into two domains, which remain covalently linked by a disulfide bond. After binding to its cell surface receptor, DT is internalized through the clathrin-coated pathway. The acidic pH in the endosome triggers a conformational change, and a reduction of the disulfide bond leads to insertion of the toxin in the membrane of the endosome, and forms a channel through which the enzymatic fragment translocates to the cytosol. Finally, the mechanism leading to cell intoxication is the enzymatic ADP-ribosylate of EF-2 in the cytosol. As with PE, cell death is facilitated by apoptosis. Concerning the extreme potency of this bacterial toxin, it has been shown that delivery of a single molecule of the catalytic domain into the cytosol is sufficient to kill a cell [73].

To improve its specificity, DT was point mutated by converting both Leu390 and Ser525 to phenylalanine, resulting in a derivative named CRM107 [74]. Further truncated forms of the DT A and B domains (DAB 486), containing the first 485 amino acids of DT, have also been produced [75], as has a shorter version named DT389, containing the first 388 amino acids of DT with deletion of cell-binding domains [76]. Unlike the procedure with PE, in order to enable the ADP-ribosylating domain to translocate to the cytosol without the ligand, the ligand is placed at the carboxyl terminus of DT (normally a furin-cleavable ligand).

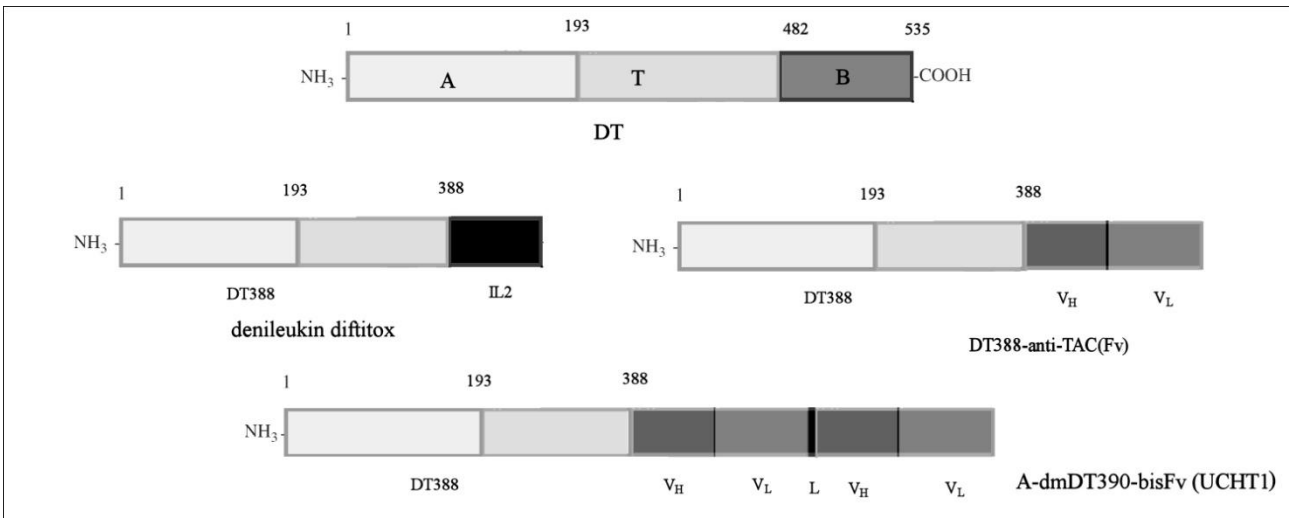

Figure 3. Schematic structure of DT and chimeric immunoconjugates. DT is composed of three different domains: enzymatic (A), binding (B) and a translocation or transmembrane (T) domain. The figure represents: a fusion protein between DT389 fragment and the targeting agent interleukin 2 (IL-2), (Denileukin diftitox), an example of DT fused with one sFv molecule (DT388-anti-TAC(Fv) and with two tandem sFv (A-dmDT390-bisFv(UCHT1). The linker is represented as L.

DT-based ITs have been reviewed by Verma and coworkers [77]. One of the first patents describing IT application with anti-CD3 mAb [78] reports the IT anti T-cell and PE and DT, and the method to induce tolerance in primates. This patent has recently been extended.

Denileukin diftitox, also known as DAB389 IL-2 (Ontak® , Eisai Inc), is the first commercially-available genetically-constructed fusion protein. This IT comprises a fusion between DAB389 and the targeting agent interleukin 2 (IL-2), which can link to the IL-2 receptor and deliver the modified DT to many different hematologic malignancies [79] (Fig. **3**). Ontak was approved by the FDA in 2008 for the treatment of persistent or recurrent CD-25-positive cutaneous T-cell lymphoma (CTCL). In 2001, orphan designation (EU/3/01/075) was granted by the European Commission to denileukin diftitox for the treatment of CTCL.

The clinical activity of denileukin diftitox appears to be associated with the presence of CD-122, found only on high-affinity and intermediate-affinity IL-2 receptors. Various forms of IL-2R expression have also been reported on the surface of malignant hematopoetic cells, including Hodgkin's and non-Hodgkin's (NHL) lymphomas [80], adult T-cell leukaemia/ lymphoma (ATL) [81], and CLL; most of these diseases have high-affinity-receptor-expressing cells. Recent studies into Ontak have shown that it exerts a potent cytotoxic effect on natural T killer (NK) cells which play a critical role in antitumor immunity; this phenomenon might impair the product's antitumoral properties. However, the study authors opine that co-administration of IL-15 with Ontak, tested on primates, could alleviate this problem by selectively protecting potentially oncolytic NK cells, while allowing the depletion of immunosuppressive regulatory T cells in cancer patients [82]. More recently, Ontak demonstrated interesting activity as monotherapy [83] in the treatment of stage IV melanoma. The one-year survival rate was markedly improved in partial responders (NCT00299689). At the time of publication, 32 different clinical trials have been carried out; seven further trials, on melanoma, lymphoma, and ovarian cancer, are currently recruiting.

Other ITs targeting the uniquely-expressed low affinity IL2R (IL2Rα) have also been proposed [84]. ITs composed of a single protein of DT390 and IL2 without any extra amino acids at the junction unlike denileukin diftitox, are reported to possess in vitro activity. The specificity of IT to this low affinity receptor promises to ablate the toxicities of targeting high affinity receptor thus shows potential to be a good alternative to denileukin diftitox.

One of the most promising DT-based ITs for treating hematologic tumors is the bivalent anti-human-T-cell immunotoxin A-dmDT390-bisFv (UCHT1) (Fig. **3**), proposed for patients with T cell malignancies. It is a single-chain fusion protein comprising the catalytic domain and translocation domains of DT fused to two tandem sFv molecules reactive with human CD3ε via a (G4S)3 linker [85]. In this construct, two mutations were introduced for removing the potential N-glycosylation sites in the translocation domain of the DT B chain (dm indicating double mutation). A recent patent details the preparation of an anti CD3 diabody fused with mutants of DT [86]. A DT-human interleukin-3 conjugate has also been evaluated in ex vivo purging of bone marrow, useful for example in the case of autologous stem cell transplantation. Data from clinical evaluation, in patients affected by AML, indicated two partial remissions among 36 patients and one complete remission for 8 months [87].

Improvements in bispecific IT have been reported, comprising two scFv ligands, recognizing CD19 and CD22 plus catalytic DT390, genetically enhanced to give them increased *in vivo* anti-leukemia activity [88, 89].

Several studies report the use of DT fragments fused with targeting agents, using the recombinant human vascular endothelial growth factor-165 fragment (VEGF165) [90]. In toxin-based suicide gene therapy approaches, different selected targeting agents have been evaluated. Using the same DT390 fragment as toxin moiety, ITs were constructed with ligand interferon gamma-inducible protein 10 (IP-10) [91, 92], regulated on activation normal T cells expressed and secreted (RANTES) [93, 94], and on mouse macrophage inflammatory protein-1α gene [95, 96].

The transferrin (Tf) receptor is overexpressed on rapidly dividing cells and on various tumor cells, and is one of the preferred receptors for treating GBM. Considerable effort was made in the late 1990s to deliver DT fragments to GBM cells. In a phase II trial, Tf-CRM107 (in which CRM107 was linked by a thioester bond to human Tf) resulted in a 35% response rate at the maximum tolerated dose [97]. Further trial phases were not approved, because Tf-CRM107 was unlikely to improve overall patient survival compared with the current standard of care. More recently, a genetically engineered Tf has been proposed as carrier of DT; it offers improved performance, although only in vitro data are available at present [98, 99].

The bivalency of the targeting agent has also been exploited for delivering DT as anti-GBM agent, in the form of a conjugate comprising anti-IL-13 spliced to truncated diphtheria toxin (DT390), plus anti-EGF added to the same single-chain protein [100]. This same approach has more recently provided a bispecific diphtheria DT-based IT, named DTATEGF, which targets both EGFR and the urokinase type plasminogen activator (uPA) receptor (uPAR) [101]. When delivered by CED in a human metastatic non-small-cell lung cancer (NSCLC) brain-tumor mouse xenograft model, this IT showed high efficacy.

Other DT-based ITs have been produced by fusion with stem cell factor to target malignancies expressing c-kit; overexpression of c-kit has been reported in many types of cancers [102]. The activity of DT-stem cell factor was demonstrated over a range of malignancies in in vitro experiments. The same group proposed DT-HN-1 peptide fusion toxin targeting head and neck squamous cell carcinoma [103]

New modified toxins have recently been proposed, in the form of modified toxins exhibiting reduced immunogenicity and reduced binding to vascular endothelium or to vascular endothelial cells, thereby reducing the incidence of vascular leak syndrome (VLS). These innovations also produced polypeptide toxophores from a modified DT, the modification being in at least one amino acid residue (6-8, 28-30, or 289-291) of at least one T-cell epitope [104].

2.4 Ribosome inactivating proteins-based immunotoxins.

2.4.1 Ricin

Ribosome inactivating proteins (RIPs) are a group of glycosylated and non-glycosylated enzymes able to specifically and irreversibly inhibit protein synthesis; they were first detected in vascular plants, but also in fungi, algae and bacteria. RIPs exert their toxic effects through binding to the large 60S ribosomal subunit on which they act as an N-glycosidase by specifically cleaving the adenine base A4324 in the 28S ribosomal rRNA subunit.

Ricin (RT), one of the most potent RIPs employed in IT construction, is a glycosylated heterodimer comprising an enzymatically-active A chain and a lectin B chain that binds galactose or N-acetylgalactosamine residues, on the glycoproteins and glycolipids present on the surface of most eukaryotic cells. Several studies have examined the use of

full ricin carried by mAbs, but its major drawback, the relevant residual toxicity, has never been resolved. Coupling only the A chain of type I RIPs (which are equivalent to the enzymatically-active A chain of type II RIPs) to a carrier that is capable of binding cells renders the conjugate highly cytotoxic, but with less systemic toxicity than ITs that are whole-ricin based. To minimize nonspecific carbohydrate-receptor mediated uptake of the RT A chain by reticuloendothelial cells in the liver, ITs have also been constructed that consist of deglycosylated toxin (dgA) [105]. The lymphoid activation markers CD25, already evaluated for LMB-2 IT, and CD30 (a transmembrane glycoprotein receptor and member of the tumor necrosis factor (TNF) receptor superfamily 8 (TNFRSF8) [106] were the targets of IT-dgA-based fusion proteins. The ITs RFT5-dgA (IMTOX25) (anti-CD25) and ki-4.dgA (anti-CD30) were conjugated chemically using bifunctional reagents reacting on the lysine on the mAb surface. One clinical trial of IMTOX25 on refractory cutaneous T-cell non-Hodgkin lymphoma has now been completed [107], and another, on CD25 positive adult T Cell leukemia/lymphoma, is currently recruiting (NCT01378871).

As said above, CD22 and CD19 are two of the most interesting targets for ITs, because they are surface antigens that are highly expressed on malignant B cells. To target the B-cell lymphoma cells, two separate ITs, RFB4-dgA (anti-CD22 mAb)] and HD37-dgA (anti-CD19 mAb) have been constructed. Separate phase I trials using continuous infusion of RFB4-dgA or of HD37-dgA provide evidence of antitumor activity. An equimolar mixture of these two ITs (Combotox) is used to improve antitumor specificity in patients with minimal residual disease [108, 109]. Phase I trials of Combotox in pediatric patients with refractory B-lineage ALL have been reported [110]; treatment in combination with cytarabine in relapsed/refractory B-lineage ALL is now in trials [111].

As in the case of PE and DT, also for ricin-based ITs, normal tissue toxicity includes VLS, hemolytic uremic syndrome, and hepatotoxicity [112]. Different approaches have been attempted to reduce these side-effects, particularly VLS. Mutants of dgA have been constructed in which an 'LDV' motif, able to bind to the fibronectin receptor, was inserted [113]. However, the mutants seemed less potent than wild-type RTA. With the aim of reducing the blood half-life, and thus the VLS, the construction has recently been reported of an IT with reduced blood half-life: anti-CD22 IT, shown to have a shorter half-life, also possessed a reduced pulmonary vascular leak capacity in mice [114].

2.4.2 Recombinant constructs of RIP I toxins

Type I RIPs have been isolated from a variety of plants belonging to the Asteridae, Caryophyllidae, Liliidae, Magnoliidae, and Rosidae, the greatest number being isolated from the Rosidae, which comprises the families Cucurbitacea, Euphorbiaceae and Fabaceae [115]. Among these RIPs, gelonin (Gel) remains the most interesting and potent plant toxins, and is widely used in IT constructs [7]. Gel is a 29-kDa protein with a potency and mechanism of action similar to those of RTA, but with improved stability and reduced systemic toxicity.

Gel has been coupled to antibodies, scFv fragments, and growth factors, both by chemical linkage and as fusion protein (recombinant gelonin, rGel) [116]; the resulting ITs have been evaluated for selective tumoricidal activity in hematological and solid tumors. Several patents protect these inventions [116-118].

B lymphocyte stimulator (BLyS), also known as B cell activating factor, belongs to the TNF family (BAFF); it is a member of the TNF superfamily of cytokines. Recently, a recombinant fusion protein was generated between rGel and BLyS, for the specific delivery of cytotoxic payloads to malignant mature B cells expressing BAFF receptors [119].

Two other studies support the relevance of this approach: one demonstrates the specific and significant cytotoxicity against mantle cell lymphoma cells that overexpressed the BLys receptor [120], while the other improved the performance of this approach in *in vivo* evaluations [121]. Survival of mice in xenograft models of B cell precursor acute lymphocytic leukemia, mantle cell lymphoma, and diffuse large B cell lymphoma was significantly prolonged; although BLyS-rGel treatment efficiently eliminated disease within the spleen, prolonging survival, established disease within other organs remained refractory to BLyS-rGel in the model employed.

The CD33 antigen is markedly expressed on the surface of myeloid leukemia cells; HuM195, the humanized version of the mouse M195 anti CD33 mAb, was fused with rGel as early as the late 1990s [122]. Currently, this IT HuM195/rGel (Targa Therapeutics) is in phase I trials for the treatment of patients with refractory or relapsed leukemia [123] (NCT00038051).

Vascular endothelial growth factor (VEGF) plays a key role in the growth and metastasis of solid tumors. A fusion protein has been produced containing VEGF 121 linked by a flexible tether (sequence G4S) to rGel; it was expressed as a soluble protein in bacteria [124]. rGel may be the toxin of choice because it does not appear to cause the capillary leak syndrome. Recently, the fusion protein VEGF 121/rGel, comprising the smallest of the VEGF isoforms VEGF 121, demonstrated interesting anti-vascular effects [125]. VEGF121 /rGel is rapidly internalized into log-phase endothelial cells via VEGFR-2, and mediates a robust cytotoxic effect that is primarily necrotic. Several applications in prostate [126] and bladder cancers [127] have been reported.

Because of the high expression of Her2/neu on many breast cancer cells, Her2/neu based IT was the first to enter clinical practice as a candidate for IT therapy. As reported above, PE-based IT produced significant hepatotoxicity, a phenomenon that was also observed when Her2/neu mAb was used to target rGel; the antibody form with the highest affinity for the antigen was found, in preclinical tests, to increase hepatotoxicity due to immune complex formation with the shed antigen. The use of fusion constructs with an intermediate affinity appeared optimal, while toxicity was not impacted by the presence of soluble antigen [128].

An IT comprising rGel and mAbs directed at the extracellular domain of-Her2/neu was first produced through disulfide chemical linkage [129]. Further, anti Her2/neu scFv, designated C6.5, was fused to rGel using various linker configurations to examine how the use of different linker affects the *in vitro* and *in vivo* efficacy of fusion constructs. In particular, it was demonstrated that an inert linker (G4S) between the toxin and the mAb fragment was more effective than that containing a furin-cleavage site (derived from DT or PE sequence), in terms of both plasma stability and tumor-inhibitory activity against established human tumor xenografts [130]. The construct with the most susceptible cleavage site was also the least effective, in contrast to the need for a furin-cleavage site in other ITs, and also in PE. These findings show that, at a threshold internalization rate, the cytotoxicity of rGel-based ITs, with a G4S spacer, depends on the rate limiting endosomal escape of the toxin into the cytosol [3]. The same study also presented, for the first time, a rGel based IT comprising the tenth human fibronectin type III domain (Fn3) fragments, engineered for affinity toward CEA and EGFR. In a similar approach, some protein constructs containing the fibronectin domain have been filed [131, 132].

Very recently, significant inhibition of tumor growth in an *in vivo* non-small cell lung cancer model was demonstrated, using a dosage of 20 mg/Kg (four doses) [134]. Interesting, the effect of anti EGFR-rGel IT in inducing cell death appears to be mediated by autophagy. The novelty of this type of IT is protected by a patent [134].

In addition to VB4-845, reported above, Viventia Biotech Inc. has in its pipeline an IT comprising the RIP I bouganin [135, 136] a type I RIP isolated from the leaves of *Bougainvillea spectabilis* Willd. Like other type I RIPs, bouganin has the same mechanism of intoxication and picomolar potency [137]. However, when tested in mice, an LD in excess of 32 mg/kg was obtained, illustrating that bouganin is less toxic than other type I RIPs [137]. In particular, an IT named VB6-845 was developed with a variant T-cell epitope-depleted of bouganin (de-bouganin) fused to either the C-terminus or the N-terminus of the heavy and light chains of the anti EpCAM Fab, via a furin-sensitive linker. Intravenous administration of VB6-845 at 20 mg/kg to SCID mice bearing NIH:OVCAR-3 human ovarian tumor or MCF-7 breast cancer cell line led to complete regression of tumor growth by day 20, with most animals being tumor free at the end of the study (day 75). Phase I trials on solid tumors (NCT00481936) demonstrated that VB6-845 is well tolerated; a markedly attenuated antibody response against de-bouganin was also observed [138]

2.5 Ribonucleases-based IT

In an attempt to reduce the marked side effects of ITs, including immunogenicity and the vascular leak syndrome, and to mitigate the risk of hemolytic uremic syndrome, other sources of potent enzymes with anticancer activity have also been explored. Ribonucleases (RNAse) degrade RNA and, since the 1950s, bovine pancreatic RNase A has been thought to have therapeutic potential as an anticancer agent. Based on the IT principle, immuno-RNases have been produced in which the toxin moiety of IT is replaced by an active moiety that has less systemic toxicity [139]. The enzyme exerts its RNA degrading activity, which readily leads to cell death, only once it has been internalized by the target cell. Theoretically, choosing a human RNase and a human antibody fragment, an immuno-RNase could be made that would not only be non-toxic, but also non-immunogenic. The most widely studied RNAses, at least as far as *in vitro* studies are concerned, are Angiogenin and Ranpirnase (Onconase®).

For the human RNAse angiogenin, the most significant results concern a CD30 ligand-based fusion toxin (Ang-CD30L) that has demonstrated significant toxicity against several CD30-positive cell lines [140]. Ranpirnase (Rap) is a basic amphibian RNase, with a molecular mass of 12 Kd. Although Rap is an amphibian protein, its repeated administration in humans has caused few problems. It has recently been shown that Onconase® selectively enhances activation-induced apoptosis of peripheral blood lymphocytes, which might go some way to explaining the apparent lack of significant adverse immunological reactions. Thus Rap has been extensively studied, both pre-clinically and clinically, as an antitumor agent [141] both alone and in combination therapy with standard chemo-radiotherapeutic agents [142]. Since Onconase® appears to be most effective against malignant pleural mesothelioma, efforts have been focused on that disease, and have progressed to phase IIIb confirmatory clinical trials in both the United States and Europe, although adverse effects including renal insufficiency, allergic reaction, arthalgia and peripheral edema have been reported [143].

Chemical linked, and fusion proteins of RNase, and antibody fragments or carrier proteins, such as Tf and cytokines, have all been proposed for use in producing innovative IT. Interesting activity has also been reported for LL2-Rap [144], which is obtained through chemical linkage with a murine anti-CD22 mAb. To improve selective cytotoxicity and pharmacokinetics, a diabody format 2L-Rap-hHLL1 composed of 2 Rap molecules, each fused to the N-terminus of the light chain of the anti-CD74 humanized mAb hH11, was also proposed [145, 146].

More recently, an antiTrop-2 Rap conjugate against Calu-3 (lung adenocarcinoma) demonstrated efficacy in an *in vivo* model [147]. Trop-2 is a type-I transmembrane protein which plays a role as tumor-associated calcium signal transducer, but its expression has been shown to be necessary for tumorigenesis and invasiveness of colon cancer cells. Interest in Trop-2 as a target for cancer therapy was enhanced by reports of its overexpression in breast, colorectal, ovarian, pancreatic and oral squamous carcinomas [148-151]

A recent patent describes a conjugate made using hRS7-rap(Q), a mutant Rap with its putative N-glycosylation site removed. The fusion protein consists of two copies of Rap linked to the N-terminal ends of humanized anti-Trop-2 mAb (hRS7). In alternative embodiments, the IT was described as being made using the dock-and-lock technology (DNL) and may comprise conjugates of mAb, fragments with Rap and other toxins [152].

The DNL technology is an interesting assembling method, based on the natural non-covalent interaction that occurs between the dimerization and docking domain (DDD) of cAMP-dependent protein kinase, and the anchoring domain (AD) of the A-kinase anchoring protein. The AD domain is derivatized with one functional moiety, while the DDD domain, which is naturally present in its dimeric form $(DDD)_2$, is derivatized to carry two copies of a second functional group. The non-covalent self assembly of AD and $(DDD)_2$ domains, or 'docking', is further stabilized by a covalent 'locking' by means of disulfide bonds between two sets of cysteines, strategically engineered onto the docking partners. The DNL approach provides a novel platform from which multivalent Rap constructs can be prepared [153]. In addition to the Rap construct, DNL technology has been used to make tri-Fab bispecific antibodies for pretargeting applications (and now in phase I clinical trials), as well as multivalent monospecific antibodies, immunocytokines, siRNA; all these inventions are covered by patents [154-158]. A recent review describes this powerful approach in detail [159].

2.6 Proapoptotic proteins used as targeted immunotoxins/chimeric proteins

To overcome several drawbacks due to the high toxicity and immunogenicity of the toxins, targeting approaches have been proposed for delivering proapoptotic proteins. Programmed cell death can then be activated through the extrinsic pathway, via death receptors on the cell surface, or through the intrinsic pathway, activated by cellular stress [160]. Although not as lethal as the above toxins, proapoptotic proteins (such as tBID, caspases, Granzyme A and B) can play a role as ITs, by the addition not only of a targeting moiety but also of a potentiating translocation unit.

Improved ITs containing human-derived pro-apoptotic effector proteins can be obtained by fusing anti-HER2 scFv and the active tBID [tBID is a proapoptotic BH3-domain-only member of the Bcl-2 family, which induces programmed cell death by stimulating the release of other proapoptotic factors]. As linker between moieties, the 10-residue furin cleavage sequence from DT's translocation domain was used [161]. The construct, named e23sFv-TD-tBID, showed interesting activity against human breast cancer xenografts, without any signs of aspecific toxicity after 4x10 mg administrations. The same group proposed the use of cell transfection by a gene that expresses and secretes a chimeric protein, comprising a scFv HER2, a PE translocation domain (from aa 253 to 412) and an active caspase (3 or 6). Caspases are vital elements in transferring apoptotic signals and executing apoptosis in mammalian cells [162]. Immunocasp-3 and 6 have been suggested for anticancer treatment, either as cell-based therapy or as a DNA vaccine against human gastric cancer [163] and lung metastasis from osteosarcoma [164].

The targeting of a potent human protein involved in the apoptosis process, Granzyme B (GzmB), has been widely reported. GzmB is used by cytotoxic lymphocytes as a molecular weapon for defense against virus-infected and malignantly transformed cells. ITs have been targeted to a variety of antigens, including gp240, VEGF-receptor, Lewis Y antigen, HER2, CD64 and CD22, expressed on different tumor cells [165]. Clayton Biotechnology Inc. has an interesting patent portfolio concerning the use of GzmB for tumor therapy [166, 167].

Table 1: Immunotoxins in Clinical Development.

IT	Targeting moiety	Target	Indication	Phase	Toxin	References
Denileukin diftitox (Ontak®)	IL-2	IL-2R	CTCL, T-CLL, B-CLL, NHL, melanoma	2008 approved	DT	[80-84]
BL22	Murine anti-CD22 dsFv fragment	CD22	HCL, B-CLL, NHL, ALL	Phase I,II	PE	[12-13]
Moxetumomab pasudodotox	Murine anti-CD22 dsFv fragment	CD22	HCL, B-CLL, NHL	Phase I,II	PE	[14-18]
LMB-2	Murine anti-CD25 scFv fragment	CD25	HCL, leukemia	Phase II	PE	[30,31]
SS1P	Murine anti-mesothelin dsFv fragment	Mesothelin	Solid cancers	Phase I	PE	[46-48]
Erb38, scFv(FRP5)-ETA	Murine anti-erbB2 dsFv fragment	erbB2/HER2	Solid cancers	Phase I	PE	[52]
Oportuzumab monatox	Humanized scFv fragment	EpCAM	Squamous cell head and neck and bladder cancer	Phase II,III	PE	[53-55]
Naptumomab estafenatox	Murine anti-5T4 Fab fragment	5T4	Renal cell carcinoma	Phase II,III	SEA	[64]
MR1-1	Murine anti-EGFRvIII scFv fragment	EGFRvIII	Brain cancer	Phase I	PE	[65]
Cintredekin besudotox	IL-13	IL-13R	Brain cancer	Phase III	PE	[68]
TP-38	TGFα	EGFR	Glioblastoma	Phase II	PE	[70]
UCHT1	Murine anti-CD3ε bis Fv	CD3ε	T-cell lymphoma/leukemia	Phase I,II	DT	[85]
DT388-IL3	Variant IL-3	IL-3R	AML, MDS	Phase I,II	DT	[87]
RFT5-dgA (IMTOX25)	Murine MAb	CD25	CTCL, NHL, melanoma	Phase I,II	dgA	[107]
Combotox®	Murine MAbs	CD19/CD22	ALL	Phase I	dgA	[108-111]
HuM195/rGel	Humanized anti-CD33 antibody	CD33	Leukemia	Phase I	r-gelonin	[123]

Abbreviations:

AML: acute myelogenous leukemia

B-CLL: B-cell chronic lymphocytic leukemia

CTCL: cutaneous T cell lymphoma

dgA: deglycosylated ricin A chain

DT: Truncated diphtheria toxin

HCL: hairy cell leukemias

MDS: myelodysplasia

PE: Truncated Pseudomonas exotoxin A

NHL: non-Hodgkin's lymphoma

SEA: Truncated staphylococcal enterotoxin A

T-CLL: T-cell chronic lymphocytic leukemia

2.7 Current & Future Developments of ITs.

Further progress and improved clinical responses depend on the identification of new antigenic targets on tumor cells, and on the strategy of administering a combination of ITs that target different tumor antigens. As described above, one of the major issues for ITs containing bacterial or plant proteins is the formation of antibodies, which prevents retreatment of patients with solid tumors in whom the immune system is intact. In many hematologic malignancies, the immune system has been damaged, and many cycles of IT therapy can be given without antibody formation.

Furthermore, many of the details of the PE/RIP intoxication process remain uncertain, and must be addressed before success may be claimed. Future advances in IT therapeutics will surely depend on a clear and full understanding of the mechanisms involved. Further progress will depend on the identification of new antigenic targets on tumor cells, and on the production of less immunogenic ITs, based on human or "humanized" cytotoxic proteins, in order to enable several treatment cycles to be administered.

3. Antibody-drug conjugates

Monoclonal antibodies have also been covalently linked to anticancer drugs, enabling high doses of the cytotoxic agent to be specifically delivered to cancer cells, largely sparing normal tissues [168]. As for ITs, the antibodies used are specific for antigens that are highly expressed on cancer cells; they are conjugated to drugs through different specialized chemical linkers. Potent cytotoxic agents may be needed to maximize the efficacy of drug conjugates. The drug must also be inactive and nontoxic in the conjugated form, to avoid systemic toxicity, and it should be released inside the cancer cell to exert its activity. A crucial point concerns the choice of linker-spacer that connects the drug molecules to the mAb. Several strategies have been developed to selectively release the therapeutic agent from a conjugate only after internalization, exploiting intracellular metabolism of tumor cells. The principal mechanisms involve the use of spacers that are cleavable by proteolysis of enzymes overexpressed in tumor tissues, or of acid-sensitive linkages cleavable under the acidic conditions present in tumors, endosomes, and lysosomes [169]. Furthermore, exploiting the tumor's hypoxic environment can lead to enhanced activity of reductive enzymes, and therefore even higher glutathione concentrations. Reduction reactions can then be used to efficiently release active drug from the non-toxic prodrug [170].

Self-immolative spacers have also been proposed, comprising drug, linker, and trigger. The tumor-specific cleavage reaction takes place between trigger and linker, to form a drug-linker derivative. This then degrades spontaneously by elimination or cyclization, to release the free drug [171], preferably inside the affected tissues. As a result, exposure of normal tissues is limited, at least in theory, leading to a more favorable toxicity profile. Furthermore, especially for ADCs that are not prepared as fusion constructs, the conjugation method has been shown to play a crucial role not only in pharmacokinetics, but also in activity, potency, and tolerability because it determines the drug loading stoichiometry and homogeneity [4].

Unlike ITs, several ADCs are currently in advanced clinical trials for a variety of tumors (both hematologic and solid type) and great effort is being made by pharmaceutical companies to reach the goal of high specificity, high potency and lower toxicity [172]. Table **2** summarizes the ADCs that are currently in clinical development.

Among the drugs used for this purpose are different classes of molecules, the main ones being the calicheamicins, duocarmycins, auristatins and maytansinoids; other potent molecules from different natural sources are also under investigation.

3.1 DNA interacting agents - Calicheamicins

The calicheamicins are a class of enediyne antibiotics derived from the bacterium Micromonospora echinospora. Isolated originally in the mid-1980s from the chalky soil, or "calichi pits", located in Kerrville (Texas), the calicheamicins target DNA and bind to the minor groove, causing strand scission [173]. The calicheamicins are active in the biochemical induction assay at concentrations below 1 pg/mL, extremely active against Gram-positive bacteria, and highly active against Gram-negative bacteria. Most interestingly, they have shown extraordinary potency against murine tumors, being approximately 4000 times more active than doxorubicin; optimal dose are in the 0.5-1.5 μg/kg range [174, 175].

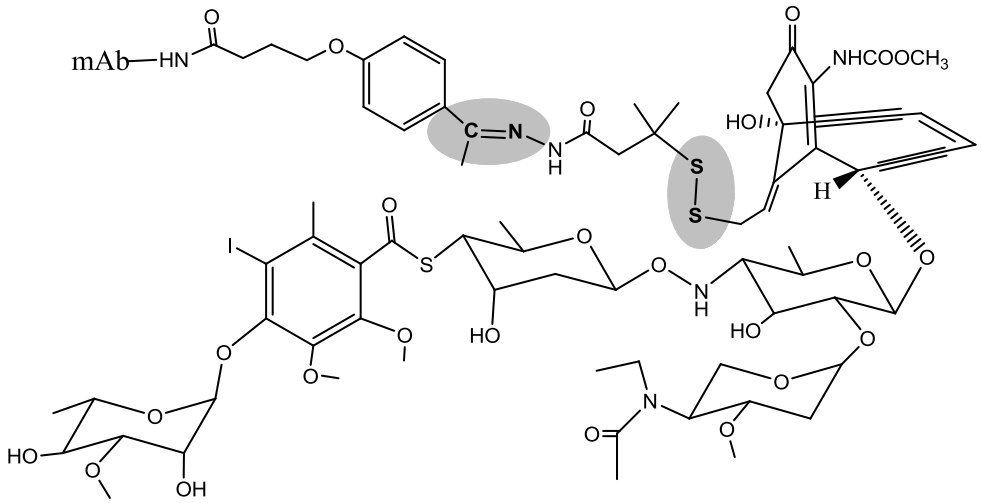

Figure 4. Structure of N acetyl, gamma calicheamicin conjugate Mylotarg. In grey are the two chemically labile linkers.

Calicheamicin gamma 1 contains two distinct structural regions, each playing a specific role in the compound's biological activity. The larger of the two consists of an extended sugar residue, comprising four monosaccharide units and one hexasubstituted benzene ring; these are joined together through a highly unusual series of glycosidic, thioester, and hydroxylamine linkages. The second structural region, the aglycon (known as calicheamicinone), contains a compact, highly functionalized bicyclic core, housing a strained enediyne unit within a bridging 10-member ring. The aryl tetrasaccharide serves to deliver the drug to its target, tightly binding to the minor groove of double helical DNA. The aglycon is a rigid, highly-functionalized bicyclic core, in which the enediyne moiety is locked within a rigid 10-member bridging ring, awaiting activation. Also forming part of the aglycon is an allylic trisulfide, which serves as a trigger: when a nucleophile (e.g. glutathione) attacks the central sulfur atom of the trisulfide group, it causes a significant change in structural geometry, which imposes a great deal of strain on the 10-member enediyne ring. This strain is completely relieved by the enediyne undergoing the cycloaromatization reaction, generating the highly-reactive 1,4-benzenoid diradical and leading, eventually, to DNA cleavage [176].

The first approved ADC was gemtuzumab ozogamicin (Mylotarg™, Pfizer Inc.), an anti-CD33 calicheamicin conjugate active against hematologic malignancies (Fig. **4**) [177]. Gemtuzumab ozogamicin consists of a humanized anti-CD33 antibody attached, via a bifunctional hydrazone linker, to the cytotoxic drug calicheamicin [178]. It was approved by the FDA in 2000 for the treatment of patients with CD33-positive acute myeloid leukemia, but was voluntarily withdrawn in 2010 when post-marketing studies indicated that the ADC did not improve survival and had greater toxicity than chemotherapy alone. However recent studies suggest that the use of fractionated lower doses of gemtuzumab ozogamicin may allow for the safer delivery of higher cumulative doses and lead to substantial improvement in outcomes for patients with acute myeloid leukemia [179, 180].

Inotuzumab ozogamicin (CMC-544, Pfizer Inc.) is an antibody-drug conjugate that targets CD22, a cell surface antigen expressed on approximately 90% of B-cell malignancies [181, 182]. A fully humanized monoclonal antibody (G5/44, IgG4) is linked to calicheamicin with an acid labile linker (acetylphenoxy-butanoic) [177]. Inotuzumab ozogamicin is currently being investigated in several clinical trials for B-cell malignancies. In particular, a recently-completed phase III study compares the combination of inotuzumab ozogamicin and rituximab with a drug regimen selected by the investigators for adult patients with follicular NHL (NCT00562965). Other phase III studies are ongoing, and explore the use of inotuzumab ozogamicin in combination with rituximab for relapsed/refractory aggressive NHL patients who are not candidates for intensive high-dose chemotherapy (NCT01232556) and in patients with relapsed/refractory acute lymphoblastic leukemia (NCT01564784).

Another member of the enediyne family, characterized by two acetylenic groups conjugated to a double bond within a 9- or 10-member ring, is C-1027 or Lidamycin (Fig. **5**). C-1027 is produced by *Streptomyces globisporus* in a complex consisting of a 1:1 non-covalently associated mixture of an apoprotein and a 9-member enediyne chromophore, resembling neocarzinostatin. Although ADCs based on neocarzinostatin have been reported, it is worth remembering the importance of this compound which is marketed as Zinostatin stimalamer (ZSS). ZSS is an anticancer agent derived from neocarzinostatin by conjugation with two molecules of poly(styrene-co-maleic acid), and now in use to treat hepatocellular carcinoma [183] (ZSS is registered in Japan).

The chromophore can undergo a rearrangement to form a transient benzenoid diradical species, in the same way as does calicheamycin, and initiate a cascade leading to DNA breaks [176]. An interesting property of C-1027 is that it preferentially targets hypoxic cells [184]. C-1027 has now entered phase II clinical trials in China [185].

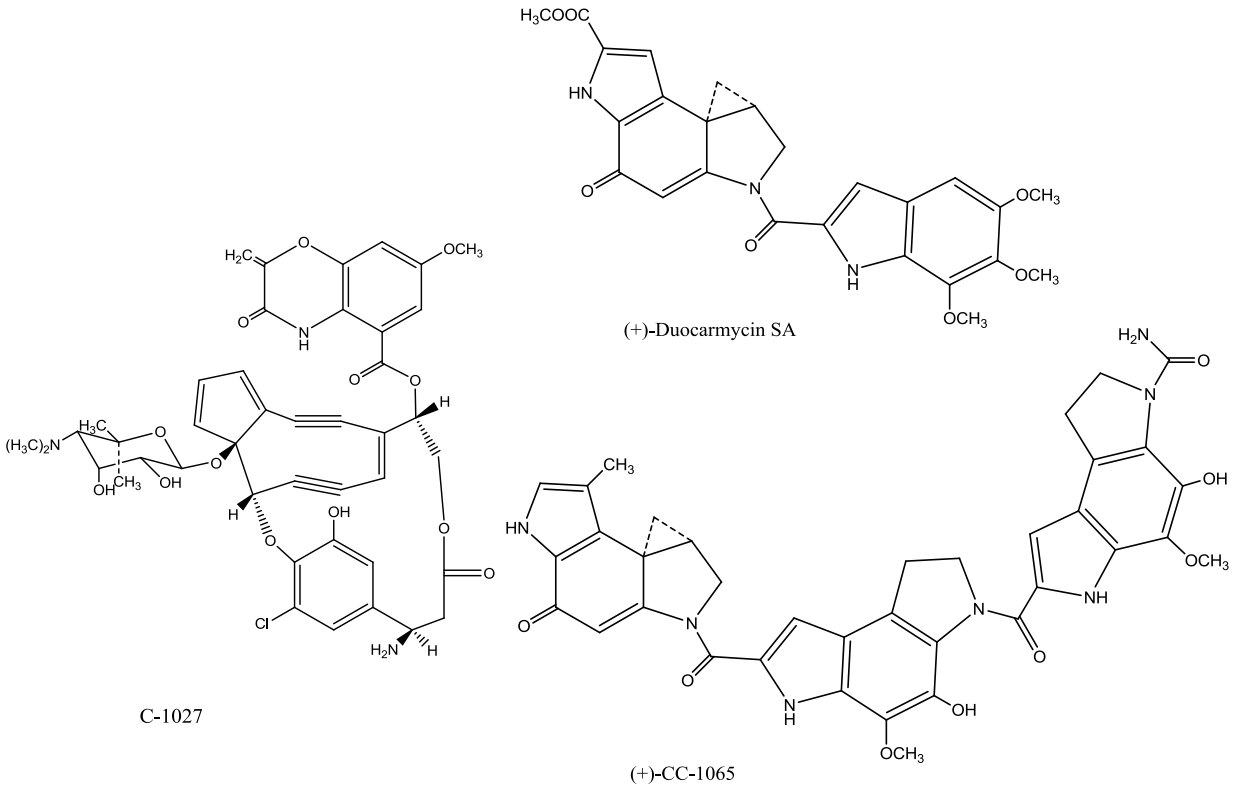

Figure 5. Chemical structures of C-1027 chromophore (lidamycin) and the natural compounds duocarmycin SA and CC-1065.

Different targeting approaches for C-1027 are being developing in China. Conjugates with human Factor VII light chain (hlFVII) [186], cell penetrating $(Arg)_9$ peptide [187], anti-type IV collagenase antibody Fab′ fragment [188, 189], anti-CD20 scFv fragment [190] as targeting domain, both by fusion proteins and chemical conjugates, have been developed using different linking systems. *In vivo* data has shown interesting activity in several tumor models.

3.2 DNA interacting agents - Duocarmycins.

The duocarmycins SA and CC-1065 (Fig. **5**) are two parent members of a family of highly potent naturally-occurring anti-tumor antibiotics found in Streptomyces species. The broad spectrum of antitumor activity and high potency of

these candidate drugs is, unfortunately, contrasted by their toxicity in experimental animals [191, 192]. Numerous investigations have sought to synthesize alkylating pharmacophores and analogues with improved biological properties, namely possessing similar cytotoxicity but reduced hepatoxicity [193-195]. The exceptionally potent cytotoxic activity of these drugs derives from their ability to bind and alkylate DNA in adenine thymine-rich regions of the minor groove, forming a covalent linkage to the N3 of adenine [196]. The duocarmycins comprise cyclopropylindole (CPI) tricyclic, which is the alkylating moiety, attached via an amide bond to a pyrroloindole unit that exerts DNA-binding activity. The strained cyclopropyl group allows the covalent linkage to adenine to form, while rotation of the second indole unit, once in the minor groove, can help to activate the molecule. Significantly, these compounds are relatively unreactive until they reach their biological target, where they are selectively activated for DNA alkylation.

The relationships between structure, reactivity, and biological potency of the CPI subunit and methyl substituted cyclopropylbenzoindole (CBI) derivatives have been intensively investigated [195]. The CBI subunit was initially developed to replace the alkylating CPI subunit, obtaining a chemically more stable (~4-fold), biologically more potent (~4-fold), and considerably more synthetically accessible unit [197].

Due to their picomolar activity, duocarmycin derivatives have been selected as payload, linked to protein carriers such as human serum albumin and mAbs [198, 199] in order to achieve selectivity against tumor cells. Recent patents have described in detail the application of CBI units- linked to mAb , the role and choice of spacer and the releasing mechanism (Syntarga (now Synthon), Medarex Co.(Bristol-Myers Squibb) and ImmunoGen Inc.). Figure **6** shows the structure of duocarmycin derivatives developed and patented over recent years. The compounds are locked in their racemic seco chloride amino-CBI forms, and linkage with mAb can be achieved through the phenolic hydroxyl group of CBI, or after the DNA-linking moiety.

Research has lead to compounds locked in their racemic seco chloride amino-CBI forms, by protecting the phenolic hydroxyl group as a carbamate. This carbamate forms part of a cathepsin-labile linker, ending in a caproyl maleimide connecting group (Seattle Genetics Inc.) [199]. However, most of these prodrugs, in particular the phenyl, piperazino, and piperidino carbamates, possess low solubility. Hydrophilic polyethylene glycol (PEG) spacers can be incorporated in the linker to increase solubility of the derivatives and reduce aggregation to mAb dimers and higher species during conjugation.

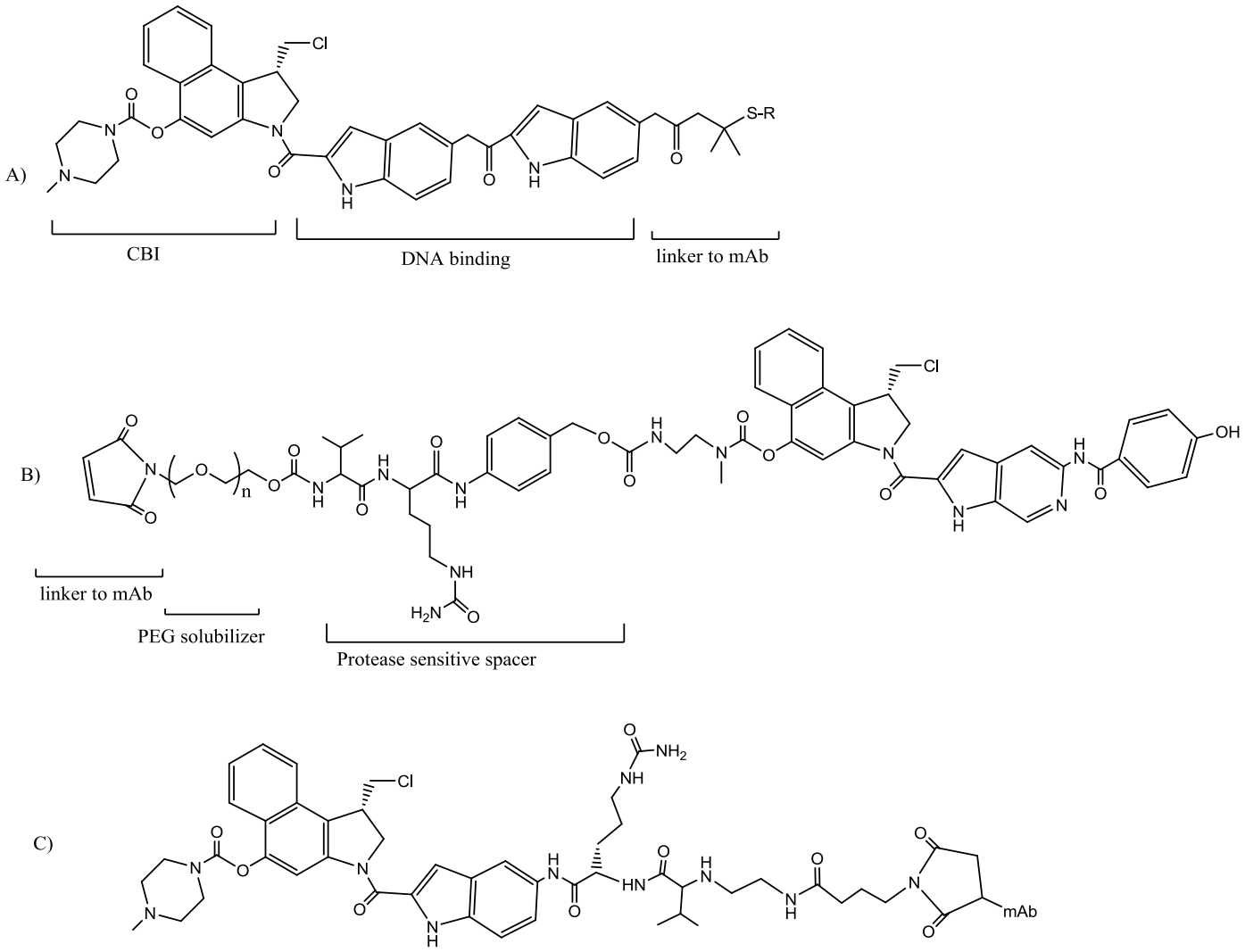

Figure 6. Stuctures of some CBIs, their specific moieties and the linkers/spacers described in some patents. In structure A the methylpiperazinyl group is used to increase the water solubility, making the compound chemically stable and slow reacting. Structure B shows a protease sensitive Val-iCitr-Phe lnkage and a self immolative linkage. In structure C the cleavable dipeptide (maleimidebutaryl valine-citrulline) is linked to mAb anti-CD70 (see text).

Preliminary studies have examined the activity of Trastuzumab conjugates against N87 tumor s.c. xenografted in mice; the animals were treated with a single dose of 12 mg/Kg (19 days after implantation). The ADC containing a relatively short linker increased the survival time compared to the corresponding conjugate with longer spacers [200]. The CBI unit can alternatively be attached to the antibody via the auxiliary indolecarboxamide moiety, again using cathepsin-labile linkers: in this case, a water-solubilizing N-methylpiperazino carbamate is used to protect the phenolic group of the CBI moiety (Fig. **6**, structure C) [201]. A similar strategy has been pursued with other CBI duocarmycins [202, 203]. In the late 1990s, DC1 compounds were conjugated with mAb [204], but were not developed further due to instability and poor solubility of the DC1 component in aqueous solutions Immunogen) [205] .

In order to improve solubility, prodrugs of DC1 have recently been produced in which the seco-CBI hydroxyl group is protected by a phosphate group, and DNA binding comprises a bis-indolyl moiety, followed by attachment of a thiol-containing linker. *In vivo* results of conjugates obtained with mAbs that react with tumor-associated antigen CD19 and CanAg have shown that the more labile disulfide bond produces efficient release of the phosphate prodrug and higher antitumor activity [206].

Based on promising *in vivo* experiences, the compound MDX-1203 began clinical trials on renal cell carcinoma and relapsed/refractory B-cell NHL (Bristol-Myers Squibb) (NCT00944905) although the study has now been suspended. In MDX-1203, the duocarmicin analogue was linked through a cleavable dipeptide (maleimidebutaryl valine-citrulline) to the anti-CD70 mAb (Figure 6, structure C). CD70, a member of the tumor necrosis factor superfamily, is expressed on a variety of solid tumors, including renal cell carcinoma, pancreatic, ovarian and lung cancer, as well as multiple myeloma and several types of non-Hodgkin's lymphoma [207]. The relevance of drug release through proteolytic mechanism has recently been discussed [208].

3.3 DNA interacting agents - Pyrrolo[1,4]benzodiazepines

Among other anticancer agents used to obtain ADCs is tomaymycin, an antitumor antibiotic produced by Streptomyces tomaymyceticus and belonging to the pyrrolo[1,4]benzodiazepine (PBD) group of antibiotics [209] (Fig. **7**, structure A). Tomaymycin and other members of its class, such as anthramycin, neothramycin and DC-81, bind covalently through the N2 of guanine, and lie within the minor groove of DNA. Tomaymycin antitumor activity is, however, limited by its non-specific toxicity towards normal cells and by its low potency. Thus, to increase its therapeutic activity and diminish the non-specific toxic effects, several derivatives of tomaymycin and other PBDs have been synthesized; their development has recently been reviewed [210]. Among them, PBD dimers are particularly interesting, since they are capable of forming sequence-selective, non-distorting and potently cytotoxic DNA interstrand cross-links in the minor groove of DNA. Based on these new findings, conjugates between new derivatives of tomaymycin as dimers and an antibody, via non-cleavable linkers such as an amide bond, were recently patented (Sanofi-Aventis) (Figure 7 structure A) [211]; in early 2012 Spirogen Ltd. announced they would collaborate with Genentech on the discovery and development of ADCs, using Spirogen's PBD drugs and associated linker technology. A single Spirogen PBD was recently reported as a potent anti-CD70 ADC in models of renal cell carcinoma (Seattle Genetics Inc.) (Fig. **7**, structure B) [212]. More recently, another PBD-dimer based ADC, SGN-CD33A (Seattle Genetics Inc.) exhibits antitumor activity against a broad panel of primary AML. Furthermore SGN-CD33A appeared to overcome multidrug resistance in *in vitro* experiments [213].

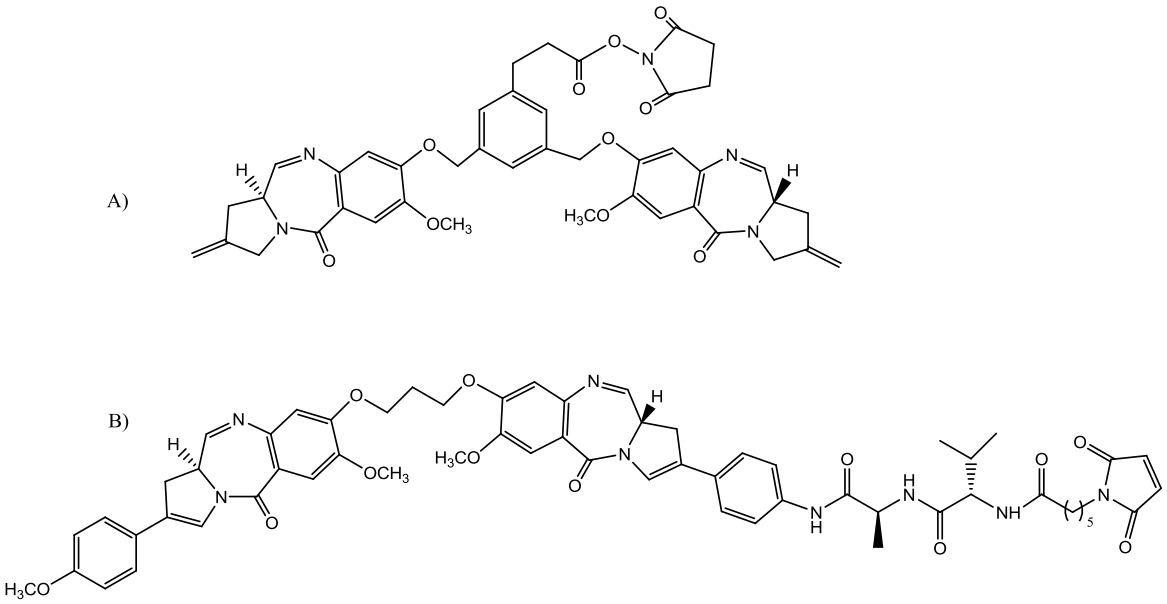

Figure 7. A pyrrolobenziodiazepine payload joined with a spacer and reactive linkers. A) Tomaymycin dimer, claimed in a Sanofi-Aventis patent [209]; B) compounds SG3211, described in the Seattle Genetics patent [213].

3.4 Tubulin interacting agents -Auristatins

Dolastatins are natural cytotoxic pseudopeptides extracted from the marine shell-less mollusk Dolabela auricularia [214]. The dolastatin family has demonstrated antineoplastic, bactericidal, and fungicidal properties [215, 216]. Within the family, dolastatin-10 and dolastatin-15 act as potent disruptors of tubulin polymerization, inhibiting the binding of vinca alkaloids to tubulin in a noncompetitive manner, and also stabilizing the binding of colchicines to tubulin. Although very potent, dolastatin-10 did not give noteworthy results in phase II trials [217, 218], and for this reason, together with its complex chemical synthesis, low yields, and poor water solubility, attempts have been made to develop analogues, such as auristatin E (AE) and monomethylauristatin E (MMAE) [219, 220]. These derivatives are 200-times more potent than vinblastine, and are good candidates for ADC active moieties (Fig. **8**).

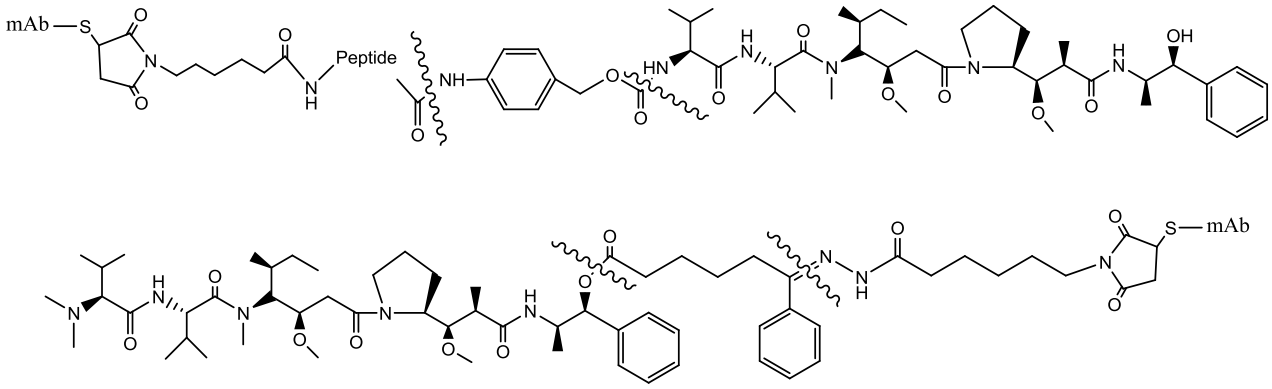

Figure 8. Structure of auristatin E and monomethylauristatin E conjugates. The arrows indicate the site of hydrolysis (enzymatic or pH-dependent).

Since the development of gemtuzumab ozogamicin, several second-generation ADCs based on auristatins have been proposed, and new linkage technologies have been introduced. Among these, brentuximab vedotin (SGN-35, ADCETRIS™, Seattle Genetics Inc. and Millenium Pharmaceuticals) [221] which was approved by the FDA in 2011, is a CD30-directed antibody-drug conjugate consisting of an IgG1 monoclonal chimeric antibody cAC10, which is specific for human CD30 and MMAE; it inhibits tubulin polymerization, also causing intratumoral vascular damage [222]. The protease-cleavable linker comprising a maleimidocaproyl-valine-citrulline-p-aminobenzoyloxycarbonyl (MC-vc-PAB) chain covalently attaches MMAE to newly-inserted cysteines of cAC10, by means of heterobifunctional reagents [223]. On average, each mAb molecule is conjugated to four molecules of MMAE. The drug-antibody linkage is stable in the bloodstream, but, once inside the cell, MMAE is released in the lysosomal compartment via proteolytic cleavage. Brentuximab vedotin is indicated for the treatment of Hodgkin's lymphoma (HL), after failure of autologous stem cell transplant (ASCT), or of HL in patients who are not ASCT candidates after failure of at least two multiagent chemotherapy regimens, or in systemic anaplastic large cell lymphoma (sALCL) after failure of at least one multiagent chemotherapy regimen. Brentuximab vedotin demonstrated an unprecedentedly high rate of response in early studies, in relapsed and refractory HL and sALCL, also showing tolerability and manageable toxicity [224]. A very recent review (2013) detailed the impressive efficacy and favorable toxicity profile of this ADC [225].

The anticancer drug MMAE can also be conjugated to a humanized anti-CD79b antibody via the THIOMAB technology [226] for the treatment of NHL (Genentech, Inc.) [227, 228]. CD79 is a signaling component of the B-cell receptor, with a narrow expression pattern that minimizes the effect on normal tissues [229]. The THIOMAB technology enabled the antibody to be engineered, with cysteine substitution at positions on light or heavy chains which provide reactive thiol groups for linkage with MC-vc-PAB-MMAE. This linker-drug moiety has also been conjugated via engineered cysteines to another antibody, anti-MUC16, expressed on ovarian cancer cells and tested *in vivo* (Genentech Inc.) [226, 230].

Glembatumumab vedotin (also known as CDX-011 and CR011-vc-MMAE, Celldex Therapeutics) is an antibody-drug conjugate in which the fully human monoclonal antibody glembatumumab (CR011) is linked to MMAE; it targets cancer cells expressing the transmembrane glycoprotein NMB (GPNMB) [231]. GPNMB is a protein overexpressed by many tumor types, including melanoma, breast cancer and glioma. GPNMB has been shown to be associated with cancer cells' ability to invade and metastasize; it has been correlated with reduced time to progression and survival in breast cancer [231]. In July 2010, a phase IIb clinical study started on glembatumumab vedotin in 120 patients with advanced GPNMB-expressing breast cancer, including patients with triple-negative breast cancer (NCT01156753). Glembatumumab vedotin has been investigated in phase I/II clinical trials for the treatment of advanced melanoma and breast cancer (NCT00412828, NCT00704158). Preliminary results show that glembatumumab vedotin has clinical activity (promoting tumor shrinkage) in both cancer types. Patients whose tumors express GPNMB respond better to glembatumumab and have longer progression-free survival than those whose tumors do not express GPNMB.

MMAE has also been linked via the valine-citrulline maleimidocaproyl linker to a fully human IgG2k monoclonal antibody, which binds to the transmembrane antigen AGS-5 with high affinity; the result is ASG-5ME (Seattle Genetics Inc and Agensys Inc.). AGS-5 is highly expressed in various epithelial tumors. Phase I studies of ASG-5ME in pancreatic or gastric and prostate cancer are ongoing (NCT01166490) [232].

ASG-22ME (Seattle Genetics Inc. and Agensys Inc) is an ADC targeting nectin-4, which is expressed on many cancers including bladder, breast, lung and pancreatic cancer [233]. The antibody is attached to MMAE via an enzyme-cleavable linker. ASG-22ME is in phase I trials for solid tumors (NCT01409135).

A monoclonal antibody directed against guanylyl cyclase C (GCC or GUCY2C) has also been conjugated to MMAE obtaining MLN0264 (Millenium Pharmaceuticals). GCC is a transmembrane receptor normally found on intestinal cells and dopamine neurons in the brain, but it is also overexpressed on the surface of gastrointestinal cancers. An open-label, dose escalation, phase I, first-in-human study of MLN0264, in adult patients with advanced gastrointestinal malignancies expressing guanylyl cyclase C, started in 2012 and is now recruiting (NCT01577758) [234].

In PSMA ADC (Progenics Pharmaceuticals Inc.), MMAE is linked to a fully human monoclonal antibody that binds to prostate-specific membrane antigen, PSMA, a protein that is abundantly expressed on the surface of prostate cancer cells, as well as on cells in the newly-formed blood vessels of major solid tumors [235, 236]. The conjugate is under investigation in a dose-escalation phase I study, administered i.v. in subjects with progressive, castration-resistant, metastatic prostate cancer that has progressed after prior taxane therapy (NCT01414283), and also in an extended study in subjects with prostate cancer (NCT01414296). Robust antitumor activity is reported across a range of doses, and durable responses were seen in heavily pre-treated patients. In September 2012, Progenics thus began enrolment in a phase II study of PSMA ADC in prostate cancer patients (NCT01695044).

There are promising new applications in the field of PSMA ADC-targeting of the neovasculature of non-prostatic cancers. An immunohistochemical analysis of numerous solid tumor samples showed that PSMA is expressed in large amounts in the tumor neovascolature, indicating the potential utility of these conjugates against a broad range of solid tumors (Progenics Pharmaceuticals Inc.). The prostate cancer antigen Six-Transmembrane Epithelial Antigen of the Prostate-1 (STEAP-1) has recently been reported [237]. Genentech Inc have developed ADCs with MMAE and described the preliminary data in a recent patent [238].

A recent patent [239] describes another antibody-MMAE conjugate that binds to 191P4D12 protein, an antigen overexpressed in colon, pancreas, ovarian, breast, bladder and lung cancers. For this conjugate, too, the selected linker comprises vc(Val-Cit) protease cleavable components.

Similarly to MMAE, monomethyl auristatin F (MMAF) was used to obtain ADCs against different targets in solid tumors, as AGS-16M8F and AGS-16C3F (Agensys Inc.). In these conjugates, MMAF is linked via a non-cleavable maleimidocaproyl linker to an antibody directed against ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3), a cancer target upregulated in the majority of renal cancers. Phase I trials to assess the safety, pharmacokinetics and effectiveness of AGS-16M8F (NCT01114230) and AGS-16C3F (NCT01672775) monotherapy, in subjects with renal cell carcinoma of clear cell or papillary histology, are now recruiting [240].

SGN-75 (Seattle Genetics Inc) is a humanized anti-CD70 monoclonal antibody attached to MMAF. A phase I study of SGN-75 in patients with CD70-positive relapsed or refractory non-Hodgkin's lymphoma or metastatic renal cell carcinoma was recently completed (NCT01015911). The phase Ib clinical trial for renal cell carcinoma in combination with Everolimus began in August 2012 (NCT01677390) [241].

RG7593 (DCDT2980S, Genentech Inc.) is a humanized IgG1 anti-CD22 monoclonal antibody conjugated to MMAE. A Phase II clinical trial evaluating either anti-CD22 ADC or anti-CD79b ADC in combination with Rituxan® (Rituximab), for relapsed or refractory follicular non-Hodgkin's lymphoma, and relapsed or refractory diffuse large B-cell lymphoma, and a Phase I clinical trial evaluating anti-CD22 ADC in combination with Rituxan for hematologic malignancies, are ongoing. This molecule is being developed utilizing Seattle Genetics' ADC technology.

BAY79-4620 is a humanized IgG1 directed to carbonic anhydrase IX conjugated to MMAE, which is currently in phase I on patients with advanced solid tumors (NCT01028755, NCT01065623) [242].

3.5 Tubulin interacting agents -Maytansinoids

Another class of potent drugs that has been used to prepare ADCs are the maytansinoids. Maytansine is an ansamycin antibiotic originally isolated from the Ethiopian shrub *Maytenus ovatus*, subsequently renamed *Maytenus serrata* [243, 244]. Maytansine binds to tubulin at the vinca-domain binding site, as do the auristatins. However, due to its potency and the resultant high toxicity, it was not used clinically until more active synthetic derivatives of maytansine were developed for conjugation to antibodies specific for tumor antigens, by employing the ester side chain of the molecule; this approach led to a number of conjugates (ImmunoGen Inc., Genentech (Roche), Amgen, Bayer, Sanofi, Biotest) [245, 246].

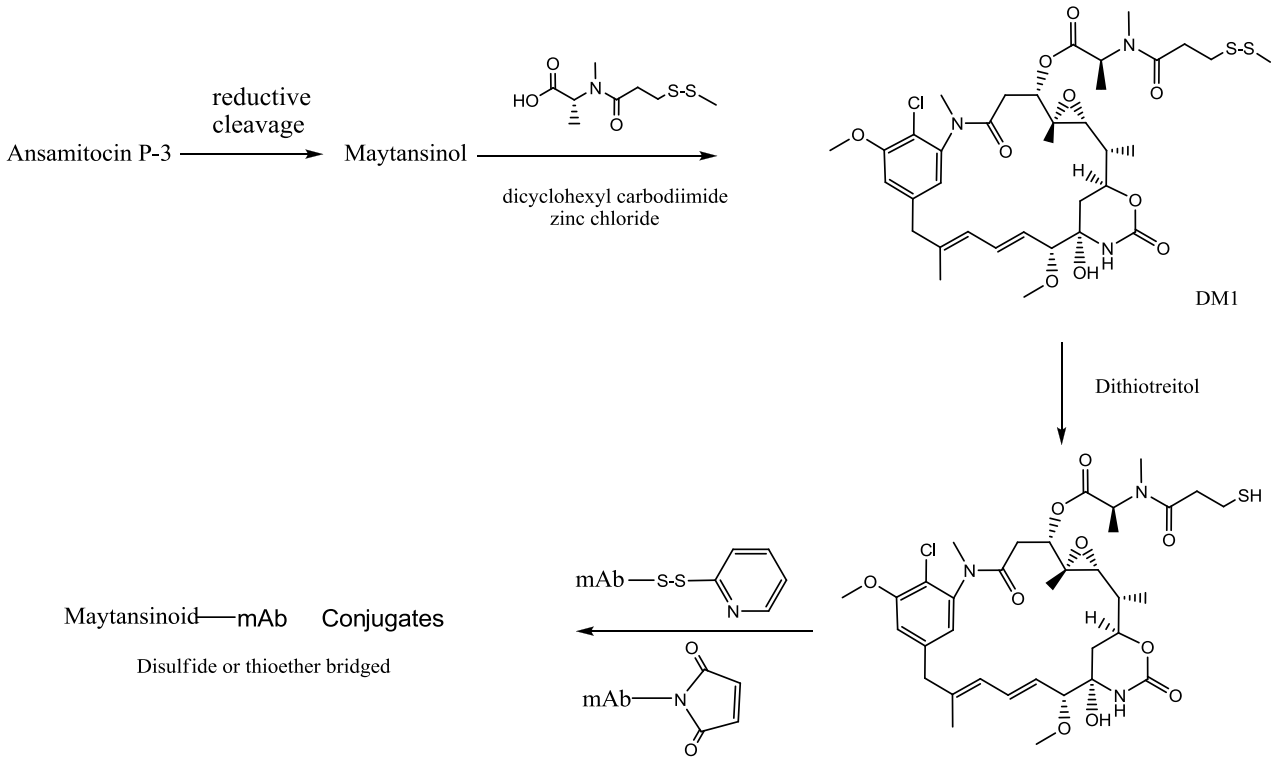

Figure 9. Synthesis of maytansinoid and the approach to linking it to a mAb via an easily reducible or stable linkage.

Concerning linkers, maytansinoids are usually conjugated to antibodies through disulfide-containing chains (Fig. **9**), which are stable at physiological pH but can be cleaved inside the cell. Among maytansinoids, DM1 (N20-deacetyl-N20-(3-mercapto-1-oxopropyl)-maytansine or emtansine) is one of the drugs most widely used to obtain antibody-drug conjugates. In particular, DM1 was linked via the non-cleavable heterobifunctional N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) linker to trastuzumab, a humanized IgG1 monoclonal antibody, to give trastuzumab emtansine T-DM1 (Genentech, Roche) [247].

Trastuzumab is a humanized monoclonal antibody directed against domain IV of the extracellular domains of HER2 receptors [248]. As a drug itself, trastuzumab targets and inhibits HER2 signaling, and it has significantly improved the prognosis for patients with HER2-positive breast cancer. It increases disease-free and overall survival in patients with metastatic breast cancer and, in the adjuvant setting, in patients with early breast cancer. However, not all patients will respond to trastuzumab, and even those who do almost inevitably experience tumor progression [247]. T-DM1 combines the therapeutic effect of the mAb with specific intracellular delivery of the potent cytotoxic drug DM1. T-DM1 is inactive until it is in the conjugated form; then, when T-DM1 binds to HER2, a proportion of the receptors are thought to be internalized by the process of receptor endocytosis; this is followed by the intracellular release of an active form of DM1, which kills the tumor cell [247]. T-DM1 is currently in clinical development for HER2-positive breast cancer, and several ongoing phase II and III trials are investigating the use of T-DM1 in early lines of therapy and in combination with other anticancer agents. In particular, for the EMILIA study, a phase III trial is comparing T-DM1 with capecitabine plus lapatinib in HER2-positive locally advanced or metastatic breast cancer patients, previously treated with trastuzumab and a taxane; preliminary results show that T-DM1 has greater efficacy and safety

than capecitabine/lapatinib [249, 250]. Very recently (February 2013), the US Food and Drug Administration has approved T-DM1 (Kadcyla ™) for patients with HER2-positive, late-stage breast cancer.

Besides trastuzumab, other monoclonal antibodies have recently been conjugated to the maytansinoid DM1 including some patented compounds. Among them, lorvotuzumab mertansine (IMGN901, ImmunoGen Inc.) is a CD56-targeting antibody-drug conjugate for the treatment of small-cell lung cancer and other cancers that express the neural cell adhesion molecule CD56 (Merkel cell carcinoma, many ovarian cancers, carcinoid and other tumors of neuroendocrine origin) [251]. IMGN901 consists of the anti-CD56 monoclonal antibody huN901 conjugated via a disulfide bond to DM1 [252]. It is now in clinical trials for small-cell lung cancer in combination with carboplatin/etoposide (NCT01237678), for other relapsed or refractory solid tumors (NCT00346385) and against multiple myeloma, in combination with lenalidomide plus dexamethasone (NCT00991562). ImmunoGen Inc. received FDA orphan drug designation for IMGN901 in treatment of Merkel cell carcinoma.

IMGN529 (ImmunoGen Inc.) is a conjugate that contains K7153A, an anti-CD37 antibody, linked to DM1. The B-cell surface antigen CD37 is an attractive target for antibody-drug conjugate-mediated therapies, as it is widely expressed on malignant B cells in non-Hodgkin's lymphoma and in chronic lymphocytic leukemia. In normal human tissues, high CD37 expression is mainly restricted to B cells, with substantially lower expression in T cells, NK cells, monocytes and granulocytes [253]. Similarly to trastuzumab in T-DM1, K7153A was conjugated to the drug via the SMCC linker, giving IMGN529 as a potential tool to treat B-cell malignancies such as non-Hodgkin's lymphoma and chronic lymphocytic leukemia [253, 254]. IMGN529 advanced into phase I clinical testing in patients with relapsed or refractory non-Hodgkin's lymphoma in April 2012 (NCT01534715).

AMG595 (Amgen Inc.) is an antibody-drug conjugate that binds to epidermal growth factor receptor variant III (EGFRvIII), which is a constitutively-active mutant of EGFR, in which exons 2-7 have been deleted. EGFRvIII is present in 30% to 50% of glioblastoma cases, but is not significantly expressed in normal tissues [255]. AMG595 is composed of a fully human anti-EGFRvIII specific antibody conjugated to the maytansinoid DM1 via a non-cleavable peptide linker, patented by Amgen [256, 257]. A phase I study on AMG595 administered in subjects with recurrent glioblastoma multiforme and/or anaplastic astrocytomas started in November 2011, but is not yet open for participant recruitment (NCT01475006).

Similarly to DM1, another maytansine derivative, DM4 (N20-deacetyl-N20-(4-mercapto-4-methyl-1-oxopentyl)-maytansine or mertansine), has been used to form several ADCs. IMGN853 is another drug-antibody conjugate developed by ImmunoGen Inc. [258]. This compound comprises a humanized monoclonal antibody, M9346A, specific for the folate receptor 1 (FOLR1, also known as folate receptor alpha) conjugated via a bifunctional disulfide-containing linker to DM4. FOLR1 is a protein often overexpressed in solid tumors, such as epithelial ovarian cancer and NSCLC tissues [259]. The high levels of folate receptor alpha in lung adenocarcinomas may be associated to these tumors' better response to antifolate chemotherapy, and folate receptor alpha is a potential novel target for this tumor type [259]. IMGN853 advanced into clinical testing for FOLR1-positive tumors in July 2012 (NCT01609556).

IMGN388 (ImmunoGen Inc.) is an immunoconjugate consisting of an anti-integrin monoclonal antibody covalently attached to DM4 [260, 261]. The conjugate binds to tumor cell surface integrins, a class of transmembrane cell surface receptors that link the extracellular matrix to intracellular signaling pathways controlling cell proliferation and differentiation [262]; upon internalization, the DM4 moiety is released from the conjugate, binding to tubulin and

disrupting microtubule assembly/disassembly dynamics, which may inhibit cell growth. A phase I dose-escalation study in patients with solid tumors is ongoing (NCT00721669).

DM4 has also been conjugated via the SPDB linker (succinimidyl-3-(2-pyridyldithio)butyrate) to an anti-CD19 humanized antibody, huB4, (huB4-DM4). This compound (SAR3419) was developed by Immunogen Inc. and now is licensed to Sanofi as part of a broad collaboration between the two companies [263]. SAR3419 showed activity against CD19-positive non-Hodgkin's lymphoma (NHL); it is now in phase II testing to evaluate activity against diffuse large B-cell lymphoma (DLBCL, the most common type of NHL) and B-cell acute lymphoblastic leukemia (B-ALL) (NCT01470456, NCT01472887 and NCT01440179). In a recent patent, Morariu described the phase I clinical evidences of SAR3419 [264].

Another DM4-conjugate, BIIB015 (Biogen Idec), consists of the humanized IgG1 monoclonal antibody huB3F6 against the Cripto protein conjugated with the drug via the SPDB linker [265, 266]. Cripto is a GPI-linked protein required for signal transduction of nodal, a transforming growth factor (TGF)-beta ligand. Cripto has been described as an oncogene and fits the classic pattern of an embryonic gene that is re-expressed in a transformed tumor cell. Cripto expression is markedly prevalent in a number of solid tumors, including more than 75% of breast, lung, and colorectal tumors. The phase I study of BIIB015 in relapsed/refractory solid tumors has been completed (NCT00674947).

BAY 94-9343 is a DM4-conjugate being developed by Bayer HealthCare under an agreement with ImmunoGen Inc. In this antibody-drug conjugate, DM4 has been linked to a fully human IgG1 antibody that targets mesothelin, a tumor differentiation antigen that is highly expressed in several malignant human diseases, including malignant mesothelioma and pancreatic, ovarian and lung adenocarcinomas [267, 268]. The limited expression of mesothelin in normal human tissues makes it an attractive candidate for mesothelin-expressing tumor therapy. BAY 94-9343 demonstrated potent, targeted anticancer activity against mesothelin-expressing tumors in preclinical testing, and thus in September 2011 it advanced into phase I dose escalation trials, to evaluate its safety, tolerability, pharmacokinetics, and pharmacodynamics in patients with advanced solid tumors (NCT01439152). In July 2012, Bayer HealthCare received orphan drug designation from the FDA for BAY 94-9343 in the US for the treatment of mesothelioma.

IMGN242 (ImmunoGen Inc.) comprises DM4 conjugated to the humanized antibody huC242, which binds to a cell surface superantigen, CanAg, expressed on colorectal, pancreatic and gastric cancer cells [269]. A phase I study on DM4-conjugated huC242 in patients with solid tumors has been completed (NCT00352131). In mid-2007, the compound began phase II testing for gastric cancer in a trial designed to include 23 patients. Only a small portion of these patients have been recruited and, as a result, trial enrollment was stopped (NCT00620607).

ImmunoGen’s research into novel maytansinoid conjugates continues and includes formulations in which the thioether (SMCC) linkage between DM1 and the mAb is partially oxidized to sulfoxide [270]. *In vitro* testing has shown a 3 fold increase of activity of the anti-EpCAM-SMCC-sulfoxide-DM1 compared to the parent conjugate. This research on charged (sulfonic) crosslinkers has resulted in a interesting recent patent in which the ocular toxicity of IMGN242 was reduced using the linker sulfo-SPDB [271].

3.6 Other anticancer drugs conjugated to mAbs

Besides calicheamicins, auristatins and maytansinoids, other anticancer drugs may be conjugated to specific monoclonal antibodies. In a recent patent, camptothecin was conjugated to anti-CD22 antibodies for the treatment of B-cell diseases (Immunomedics) [181, 272].

Doxorubicin was used to obtain the antibody-drug conjugate hLL1-DOX (Immunomedics) [273] in which doxorubicin was linked to the humanized antibody hLL1, also known as milatuzumab or IMMU-115, which targets CD74, an integral membrane protein that functions as a major histocompatibility complex (MHC) class II chaperone (Figure 10, structure A). CD74 has been shown to act as an accessory-signaling molecule, and has been implicated in malignant B-cell proliferation and survival. These biological functions, combined with expression of CD74 on malignant B cells and limited expression on normal tissues, point to CD74 as a potential therapeutic target [274]. hLL1-DOX is now in a phase I/II study in patients with recurrent multiple myeloma (NCT01101594).

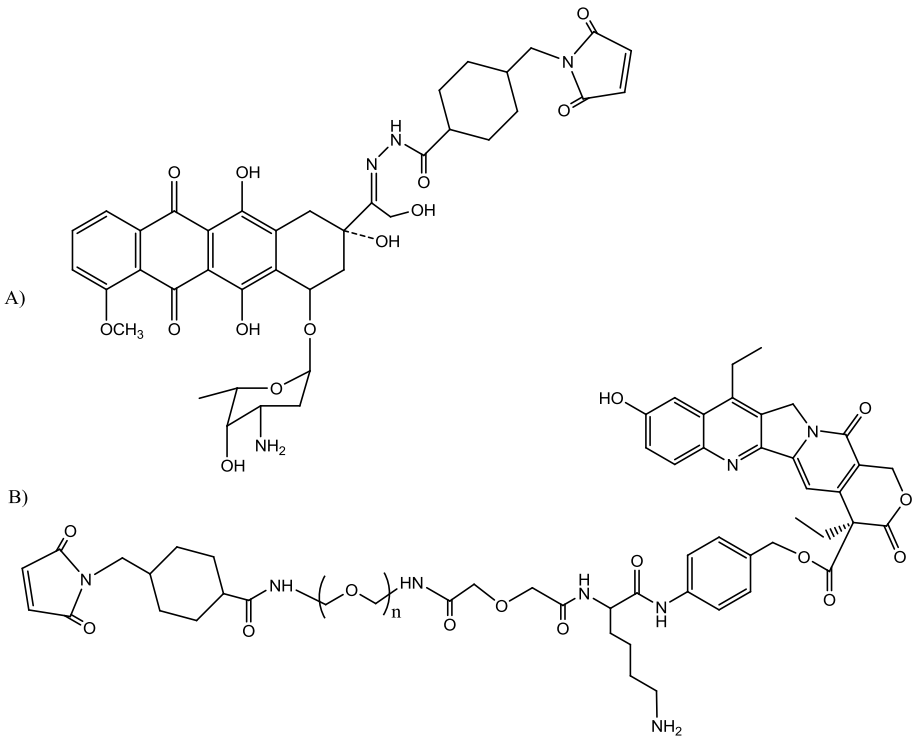

Figure 10. ADC can be obtained by linking doxorubicin properly activated with a maleimido group (as for hLL1-DOX) (A) or by conjugation of thiolated mAb with a derivative of SN-38 (B) a potent inhibitor of topoisomerase I, through an ester releasable spacer/linker.

Another antibody-drug conjugate (IMMU-132, Immunomedics Inc.) contains SN-38, the active metabolite of the topoisomerase I inhibitor irinotecan. SN-38 is linked to the antibody RS7 (Fig. **10**, structure B), which is specific for the Trop2 antigen (also known as EGP-1, epithelial glycoprotein-1), a cell-surface glycoprotein that is expressed on a variety of cancers, having conversely little or no expression in normal tissues [275]. Trop2 overexpression is associated

with decreased patient survival, as well as with increased tumor aggressiveness and metastasis. Its overexpression in metastatic tissue makes it a very attractive potential therapeutic target for late-stage disease [276]. IMMU-132 entered a phase I dose escalation study in patients with advanced epithelial cancers in October 2012 (NCT01631552).

Similarly to IMMU-132, IMMU-130 (Immunomedics Inc.) is formed of SN-38 linked to hMN-14 (also known as labetuzumab), a humanized monoclonal antibody that targets the carcinoembryonic antigen (CEA) [277]. A phase I study of IMMU-130 in patients with relapsed/refractory colorectal cancer is ongoing (NCT01270698); IMMU-130 also entered phase I trials in September 2012, for patients with metastatic colorectal cancer who have previously been treated with at least one irinotecan-containing regimen (NCT01605318). Advances in cross linkage chemistry between SN-38 and mAb are reported in a recent patent [278].

Table 2: ADCs in Clinical Development.

ADC	Antibody	Target	Indication	Phase	Linker	Drug	References
Gemtuzumab ozogamicin (Mylotarg®)	Humanized IgG4	CD33	CD33-positive acute myeloid leukemia	2000 approved 2010 withdrawn	Acid-labile hydrazone linker	Calicheamicin	[177, 179, 180]
Inotuzumab ozogamicin	Humanized IgG4	CD22	CD22-positive B-cell malignancies	Phase III	Acid-labile acetylphenoxy-butanoic linker	Calicheamicin	[181, 182]
MDX-1203	Human IgG1	CD70	Renal cell carcinoma and relapsed/refractory B-cell NHL	Phase I. (suspended May 24 2012)	Cleavable linker (maleimidebutaryl valine-citrulline)	Duocarmycins	[207, 208]
Brentuximab vedotin (Adcetris®)	Chimeric IgG1	CD30	Relapsed CD30-positive Hodgkin's lymphoma and systemic anaplastic large-cell lymphoma	2011 approved, Phase III and I,II combinations	Cleavable linker (maleimido caproyl-valine-citrulline PABC)	MMAE	[223-225]
Anti-CD79b ADC	Humanized	CD79b	Non-Hodgkin's lymphoma and diffuse large B-cell lymphoma, hematologic malignancies	Phase I,II	THIOMAB, cleavable linker (maleimido caproyl-valine-citrulline PABC)	MMAE	[227, 228]
Glembatumumab vedotin (CDX-011)	Human IgG2	GPNMB (glycoprotein nonmetastatic melanoma protein B)	Metastatic breast cancer and melanoma	Phase II	Cleavable peptide linker	MMAE	[231]
ASG-5ME	Human IgG2	AGS-5	Prostate, pancreatic and	Phase I	Cleavable linker maleimido caproyl-valine-	MMAE	[232]

			gastric cancers		citrulline		
ASG-22ME	Human IgG	Nectin-4	Solid tumors	Phase I	Cleavable linker maleimidocaproyl-valine-citrulline	MMAE	n.a.
MLN0264	Human IgG	Guanylyl cyclase C (GCC or GUCY2C)	Advanced gastrointestinal malignancies	Phase I	Cleavable peptide linker	MMAE	[234]
PSMA ADC	Human IgG1	Prostate-specific membrane antigen (PSMA)	Prostate cancer	Phase I,II	Cleavable peptide linker	MMAE	[236, 237]
RG7593	Humanized IgG1	CD22	Non-Hodgkin's lymphoma and diffuse large B-cell lymphoma, hematologic malignancies	Phase I,II	Stable linker	MMAE	n.a.
AGS-16M8F	Human IgG2	ENPP3	Renal cell carcinoma	Phase I	Non-cleavable maleimidocaproyl linker	MMAF	[240]
SGN-75	Humanized IgG1	CD70	Non-Hodgkin's lymphoma and metastatic renal cell carcinoma	Phase I	Non-cleavable maleimidocaproyl linker	MMAF	[241]
BAY79-4620	Humanized IgG1	carbonic anhydrase IX	Solid tumors	Phase I	Cleavable linker maleimidocaproyl-valine-citrulline	MMAE	[242].
Trastuzumab emtansine (T-DM1)	Humanized IgG1	HER2	HER2-positive breast cancer	2013 approved	Non-cleavable thioether SMCC linker	DM1	[248, 249, 250]
Lorvotuzumab mertansine (IMGN901)	Humanized IgG1	CD56	Multiple myeloma, Merkel cell carcinoma, ovarian cancer, SCLC	Phase I/II	Cleavable hindered disulfide linker	DM1	[252]
IMGN529	Humanized IgG1	CD37	Relapsed or refractory non-Hodgkin's	Phase I	Non-cleavable SMCC	DM1	[253, 254]

			lymphoma		linker		
AMG595	Human	EGFRvIII	Advance malignant glioma, anaplastic astrocytomas, glioblastoma multiforme	Phase I	Non-cleavable peptide linker	DM1	[256, 257]
IMGN853	Humanized	FOLR1	Ovarian cancer, NSCLC	Phase I	Cleavable disulfide linker	DM4	[258]
IMGN388	Human IgG1	Integrin αv	Solid tumors	Phase I	Cleavable disulfide linker	DM4	[260, 261]
SAR3419	Humanized IgG1	CD19	Non-Hodgkin's lymphoma, B-ALL	Phase II	Cleavable hindered disulfide linker	DM4	[264, 265]
BAY 94-9343	Human IgG1	Mesothelin	Malignant mesothelioma, pancreatic, ovarian, lung adenocarcinoma	Phase I	Cleavable hindered disulfide linker	DM4	[268, 269]
hLL1-DOX	Humanized IgG	CD74	Recurrent multiple myeloma	Phase I/II	Cleavable hydrazone linker	Doxorubicin	[274]
IMMU-132	Humanized IgG	Trop2	Advanced epithelial cancer	Phase I	Cleavable peptide linker	SN-38	[276]
Labetuzumab-SN-38 (IMMU-130)	Humanized IgG1	Carcinoembryonic antigen (CEA)	Relapsed/refractory and metastatic colorectal cancer	Phase I	Cleavable peptide linker	SN-38	[277, 278]

n.a. = Not available

4. Current & Future Developments of ADC

As reported above, antibody-drug conjugates have become one of the most active areas in oncology. Pharmaceutical companies' research groups are continuing their explorations, essentially in the hope of obtaining more potent conjugates, through specific linkages that can release the drug only after internalization, avoiding reduction of the mAb's specificity.

In the search for more potent impacting agents, a number of different sources have been explored. From vascular plants to marine sponges and bacteria, thousands of natural products with potential anticancer applications have been identified [279-281]. Some of them are described, or claimed, in several of the patents regarding ADC, mentioned above. Almost all the promising candidates were initially tested but failed clinical trials because of their very high toxicity; these include discodermolide (binding the tubulin's taxane domain), which is currently proposed as a prodrug [282, 283]. Other compounds acting on tubulin's vinca domain-binders or 'depolymerizers', other than dolastatin/auristatin and maytansinoids, are the cryptophycins [284]. These are among the most potent antimitotic agents known, and bind very strongly and irreversibly to tubulin, making them relatively exempt from efflux by the multi-drug resistance mechanism. A patent applied for by Sanofi reports conjugation of the mAb hu2H11 through a sterically hindered disulfide, by mean of a PEG spacer or through a Glu-Val-Cit spacer to position 3 phenyl cryptophycin. *In vitro* data demonstrated picomolar cytotoxic activity of several conjugates [285]. Several families of compounds extracted from natural sources act on the same target: ustiloxin, rhizoxin, and the spongistatins. Compounds inhibiting tubulin polymerization through colchicine-domain binders are: the combretastatins, podophyllotoxin, and colchicine, already investigated as prodrugs in clinical use. Further, also the anticancer activity of tubuline-binder compounds having low toxicity, such as noscapine, may be improved by conjugation with folic acid [286]. This approach has been used for other interesting molecules, the tubulysins (Fig. **11**, structure A), which are available from natural sources (myxobacteria Archangium gephyra and Angiococcus disciformis). Tubulysins have structural similarity to dolastatin-10, and present very high potency as tubulin modifiers (pM range). Several approaches have been tried to probe novel potent derivatives (pretubulysins [287], tubugins [288]), as well as nanotechnological approaches (pegylated dendrimers [289], cyclodextrin-based nanoparticles [290], folate-targeted compounds [291]); a recent review extensively reports on this field [292]

Another important class of anticancer agents, some of which are claimed in ADC-related patents, is that of the DNA minor groove binding and alkylating agents (chalicheamycin, duocarmycins, PDB and others). This class is becoming increasingly large as compounds are discovered from natural sources, such as trabectedin (registered as Yondelis), or others having a distamycin-like frame, e.g. brostallicin, tallimustine, or bearing alkylating head groups, although the potency of the compounds reported above remains for the moment unsurpassed [293]. Another area, containing both known and novel compounds, concerns compounds able to bind and alkylate on the DNA major groove, such as neocarzinostatin; these have recently been reviewed [294]

A class of potent drugs recently investigated as ADC components comprises the RNA polymerase II inhibitors, and one of the major successes appears to have been achieved by alpha-amanitin. α-Amanitin, a bicyclic octapeptide, is a major component of the amatoxins, which are produced by basidiomycetes mushrooms, particularly of the genus Amanita, but also by some species of the genera Galerina and Lepiota [295] (Fig. **11**, structure B). Her-DSC-30.0134 is one of the ADCs in the pipeline of Heidelberg Pharma/ Wilex, where the drug is attached to the antibody trastuzumab via a stable linker, with a drug: antibody ratio of 4:1. This conjugate exhibits picomolar activity against SKOV33 and SK-Br-3 cell

lines and shows a reduction in the liver toxicity associated with hepatocyte uptake of the free drug [296, 297]. α-Amanitin has been conjugated to a chimerized monoclonal antibody, chiHEA125, directed against the human epithelial cell adhesion molecule (EpCAM) to generate the conjugate chiHEA125-Ama [298]. Both *in vitro* and *in vivo* tests suggested that anti-EpCAM antibody conjugates with α-amanitin may be highly effective therapeutic agents for pancreatic carcinomas and various EpCAM-expressing malignancies.

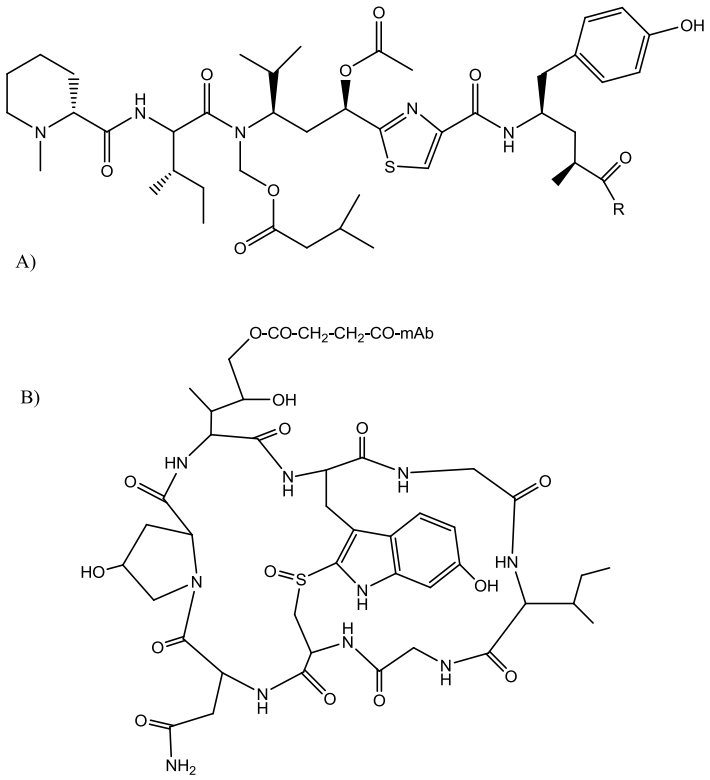

Figure 11. Structure of two highly active compounds. (A) Tubulysin, where R stands for different linkers able to crosslink mAb. (B) Structure of alpha-amanitin linked, after reaction with glutaraldehyde, to the mAb.

Considering the targeting moiety portion of ADCs, one of the most crucial points is to select a target antigen that is expressed and properly internalized in tumor cells, and less expressed if at all in normal tissues. This allows significant side-effects to be minimized. The abundance of target antigens on the cell surface, and their distribution in tumors, may be important determinants of ADC efficacy [299]. Among such compounds, a recent patent describes ADC with antiangiogenic property directed against endoglin [300] or against the 5T4 antigen expressed in tumor-initiating cells [301]. The effectiveness of an ADC ultimately reflects a combination of target antigen properties, including exposure (secreted or shed antigen), antigen density, internalization process, and sensitivity to the cytotoxic payload [302]. A modern and interesting approach to reduce the binding to normal tissues is proposed by CytomX Therapeutics with the

term 'Probodies' and is covered by several patents [303-305]. The antibodies bear a masking moiety positioned so as to cover the binding sites. The masking moiety is linked to a cleavable moiety that can be removed by disulfide reduction or by enzyme action. In particular it can be cleaved by matrix metalloproteinase 9, which are abundant around tumors, in high-inflammation environment and is known to enhance the invasion and metastasis of tumor cells [306].

After selecting the cytotoxic payload and the mAb, another important issue is related to the conjugation sites [307]. The majority of ADC described are obtained by attaching drug moieties to the targeting antibody, either at lysines or thiols. This approach leads to multiple ADC species that differ in potency and pharmacokinetics and must be precisely detected [309]. A significant improvement in homogeneity was achieved by immobilizing the mAbs on Sepharose G gel [310]. Site-specific introduction of cysteine residues (the THIOMAB approach [230, 310] or the "Trout/Novartis" approach [311, 312]) into the antibody structure has been used to make highly-uniform ADCs that not only have pronounced activities, but may also provide enhanced stability. Using the THIOMAB approach, an in-depth understanding of the role played by the structural and chemical environment surrounding the conjugation site has been achieved [313]. Using an *in vivo* model with cysteine-maleimide linker chemistry, it has been shown that the stability and therapeutic activity of an antibody conjugate is positively affected by succinimide ring hydrolysis, and negatively affected by maleimide exchange with thiol-reactive constituents in the plasma. Recombinant DNA technology enables further increase in ADC quality by introducing modified amino acids into antibody structures, for the purpose of specific drug attachment. Very recently (2013) was reported a pharmacokinetic study able to underlying the causes of target-mediated clearance of a THIOMAB MMAE conjugate directed to prostate cancer target TENB2 [314]. Another approach described by Trout and coworkers aims at rationally designing cysteine variants so as to increase yield of conjugates by reducing their aggregation. The cross-linking propensity of IgG1 cysteine variants was balanced in silico to the spatial-aggregation propensity parameter. The results indicated the utility of spatial-aggregation propensity technology for selecting antibody-cysteine variants with desired properties [312, 315]. Genetic modifications can lead to the introduction of unpaired selenocysteine, whose reduction is compatible with retention of native disulphide bonds [316]. Alongside thiol-maleimido linkage, the technology may also allow for incorporation of unnatural amino acids as sites of drug attachment. For example, the core technology of Ambrx is based in site-specific conjugation (by insertion of non natural amino acid such as p-acetyl-phenylalanine) with anticancer agents [317, 318]. Auristatin F containing a terminal alkoxy-amine group was used to generate ADC where the moieties are bound by an oxime linkage.

An enzymatic approach, using transglutaminase for protein- conjugation, was presented in a recent patent [319]. The transglutaminases (EC 2.3.2.13) belong to a family of enzymes that catalyze the formation of a covalent bond between a free amine group (e.g., protein- or peptide-bound lysine) and the gamma-carboxamide group of protein- or peptide-bound glutamine. Selecting an engineered Fc portion, with an acyl-donor glutamine-containing tag through derivatization with drugs, toxins, fluorescent agents etc. containing and amine group, can lead to a site-specific conjugation. The patent described the conjugation process of a mAb to aminocaproyl-monomethyl auristatins and reported preliminary *in vivo* activities.

Spacer/linker chemistry involving aminoacid components, and that involving Peg components were described in very recent patents. Widdison (Immunogen Inc.) applied the approach described above for duocarmicyns and auristatins, and proposed for the first time the use of dipeptide spacer (D, L) di-alanine or valine-citrulline-PAB in DM conjugates. Preliminary *in vivo* evaluations have shown improved activity [320]. A similar approach was used to conjugate

doxorubicin through branched peptidyl linkers [321]. Further, alanine-Peg spacers linked to maytansinol were also proposed in another patent [322] in which the novel ADC showed improved activity.

The synthetic process improved significantly in recent years. Two recent patents from Immunogen described the increased homogeneity achieved by using a lower reaction temperature and also the reduction in the steps required that can be achieved by using 'in situ' administration of cross-linkers [323, 324].

Many novel approaches are now mature and are starting to enrich the pipelines of several small companies. Centrose, for example, is validating its new ADC concept of an extracellular drug conjugate with a dual targeting approach, combining the cancer cell specific receptor and the drug receptor; this may lead to higher antitumor activity and greater safety, without the need to internalize the drug. The drug now being tested is a sugar-modified scillarenin with increased toxicity against cancer cells [325].

In conclusion, although this review does not claim to examine the entire field, it presents an overview, showing the significant developments in the IT and ADC fields, and the wealth of patents protecting the strong pipelines both large pharmaceutical companies and specialized start-ups. The use of ADCs as therapeutics is a new field and on that is driven by innovation at all levels of development. The interest in this field, reflecting ADCs' clinical efficacy in oncology, is such that an annual World Summit has now been instituted. It is currently in its 4th year; in 2012 it was held in Frankfurt [326]. ADCs have already led to significant improvements in clinical activity over unconjugated mAbs, and the considerable research efforts offer promise that the next few years will deliver a number of exciting new approved ADCs to treat both hematologic and solid cancers. At the same time criticisms on the advantages and limits (mostly related to the side effects) of therapeutic mAb, ADCs, ITs are debating [5] as well as critical comparisons with other targeted approaches such as peptide-drug conjugates [327] or targeted nanomedicines [328-330]. All these analysis should contribute to improve the development of targeted strategies and then eventually reaching more effective therapies for cancer patients.

CONFLICTS OF INTEREST

The authors declare no potential conflict of interest.

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