Review Article

Challenges and opportunities to develop enediyne natural products as payloads for antibody-drug conjugates

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Abstract

Calicheamicin, the payload of the antibody-drug-conjugates (ADCs) gemtuzumab ozogamicin (Mylotarg®) and inotuzumab ozogamicin (Besponsa®), belongs to the class of enediyne natural products. Since the isolation and structural determination of the neocarzinostatin chromophore in 1985, the enediynes have attracted considerable attention for their value as DNA damaging agents in cancer chemotherapy. Due to their non-discriminatory cytotoxicity towards both cancer and healthy cells, the clinical utilization of enediyne natural products relies on conjugation to an appropriate delivery system, such as an antibody. Here, we review the current landscape of enediynes as payloads of first-generation and next-generation ADCs.

> STATEMENT OF SIGNIFICANCE: Enediyne natural products are potent cytotoxic DNA damaging agents with unique molecular and cellular mechanisms of action. This review highlights opportunities, challenges, and successful examples in advancing enediynes as payloads for antibody-drug conjugates.

INTRODUCTION

The cytotoxicity imparted by small molecule drugs is a key driver for the antibody-drug conjugate (ADC) concept. The rationale behind ADCs is to generate cytotoxin enrichment in target tissues, typically tumors, while simultaneously sparing nontarget tissues. Although the cytotoxins used for ADCs are frequently non-selective, prohibiting administration as stand-alone drugs, their chemical coupling to a monoclonal antibody (mAb) expands their therapeutic index for clinical utility.

Numerous complex properties are needed for a successful ADC payload. First, the cytotoxicity of the small molecule needs to be extraordinarily high. Factors such as ineffic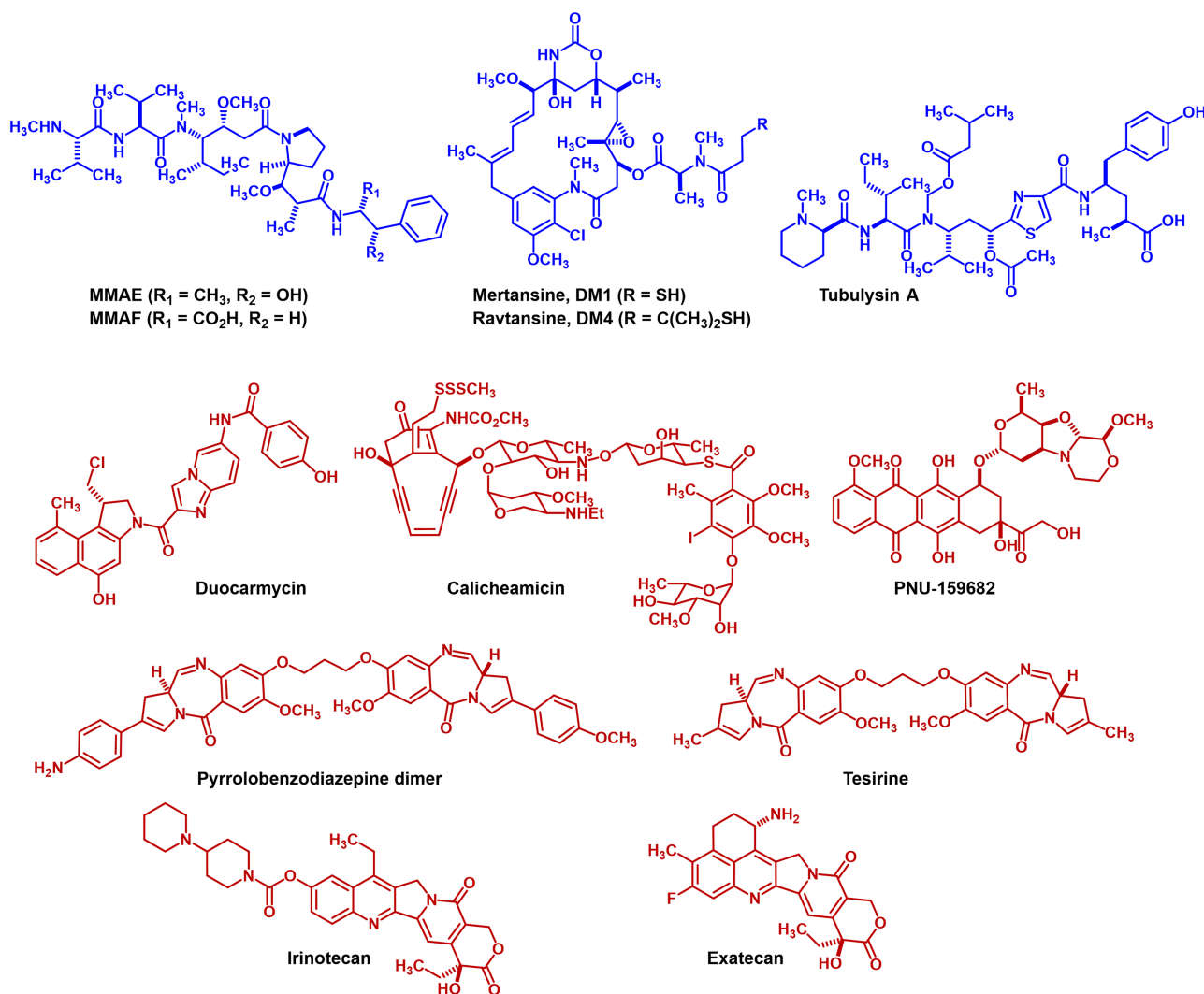ient tumor penetration of mAbs in IgG format, low antigen expression, inefficient internalization or cell surface recycling may result in very low concentrations of the small molecule at its destination. Therefore, suitable ADC payloads generally exhibit sub-nanomolar EC_{50} values against their target cells [1–4]. Second, the small molecule should be sufficiently stable in plasma to account for the long circulatory half-life of ADCs, and it should be sufficiently soluble in aqueous buffer to facilitate antibody conjugation and avoid ADC aggregation [5]. Finally, the small molecule must have a reactive handle to enable the covalent installation of a linker at a defined position, and the resulting active metabolite—depending on the site and type of linker used—should retain potency. Due to the difficulty in meeting these constraints, only a handful of cytotoxins—despite

Figure 1. Structures of small molecule payloads. Payloads are broadly classified into agents that disrupt tubulin polymerization (blue) or interact with DNA (brown), that have been conjugated to chimeric, humanized or fully human mAbs and translated clinically into ADCs.

the myriad known—have been found to be suitable ADC payloads. Representative payloads and their structures are shown in Fig. 1.

Clinically translated ADC payloads can generally be classified based on their modes of action. The first class are agents that interfere with tubulin polymerization, represented by the auristatin, maytansinoid and tubulysin families. The second class are DNA damaging agents represented by the duocarmycins, calicheamicin, camptothecins, anthracyclines and pyrrolobenzodiazepine dimers that generate DNA lesions through alkylation, crosslinking, strand scission or interference with the replication machinery. The major sources of ADC payloads are synthetic or semisynthetic variations of microbial natural products [6], with the exception of analogues of camptothecin, which is isolated from the tree *Camptotheca acuminata* [7]. Recent years have seen a tremendous boom in the ADC field, with five of the nine FDA-approved ADCs reaching the market since June 2019, including

entities against new targets that utilize new payloads and conjugation chemistries [8,9].

Enediyne natural products are among the most cytotoxic natural products ever discovered and are a promising source of next-generation ADC payloads. Enediynes are highly cytotoxic DNA damaging agents produced through microbial secondary metabolism. They are classified into two subcategories based on the presence of a nine- or 10-membered unsaturated carbocycle that drives their molecular and cellular mechanisms of action (Fig. 2). Their indiscriminate cytotoxicity requires conjugation to appropriate delivery systems to ensure cytotoxin enrichment at the desired site. When linked to an appropriate delivery system, enediynes have an excellent clinical track record, with two of the thirteen known enediynes constituting components of three FDA-approved drugs. This includes two ADCs, gemtuzumab ozogamicin (Mylotarg®) and inotuzumab ozogamicin (Besponsa®), utilizing the enediyne calicheamicin (CAL), and a polymer-conjugate

(zinostatin stimalamer, SMANCS®) utilizing the enediyne neocarzinostatin (NCS).

The potential of enediynes as ADC payloads has prompted numerous investigations into their chemistry, biology and biosynthesis [10]. Recent decades have seen an expansion in the number of known enediynes and revealed insights into their molecular and cellular mechanisms of action. Advancements in the biosynthesis of enediyne natural products coupled with technological developments in microbial genome sequencing, assembly and mining have predicted a vast landscape of yet to be discovered enediynes. This review surveys the major opportunities offered by enediynes as next-generation ADC payloads. We discuss how an understanding of their biosynthesis facilitates expanding the arsenal of enediyne natural products. Finally, we present some of the hurdles to their development and an overview of successful strategies used to clinically advance enediynes as ADC payloads.

STRUCTURE AND REACTIVITY

The enediyne natural products share a common structural motif containing two acetylenic groups conjugated to a double bond or incipient double bond within a 9- or 10-membered carbocycle. The 9-membered enediynes include neocarzinostatin (NCS) [11], kedarcidin (KED) [12], maduropeptin (MDP) [13], C-1027 [14] and N1999A2 [15]. These molecules, except for N1999A2, generally are found in complex with a cognate apoprotein that binds the enediyne chromophore to generate a stable chromoprotein. The 10-membered enediynes can be divided into two subfamilies: the CAL-like enediynes including the calicheamicins (CALs) [16], the esperamicins (ESPs) [17], namenamicin (NAM) [18] and shishijimicins (SHIs) [19], and the anthraquinone-fused enediynes including dynemicin (DYN) [20,21], uncialamycin (UCM) [22], the tiancimycins (TNMs) [23] and the yangpumicins (YPMs) [24,25]. Representative members of the enediynes are shown in Fig. 2A.

The conjugated carbocycle drives the shared mode of action for the enediynes. Electronic rearrangement of the enediyne carbocycle generates a benzenoid diradical that drives enediyne-induced cytotoxicity. Enediyne natural products with a 9-membered carbocycle can proceed through either the Myers-Saito or Bergman rearrangement pathways depending on their architecture, whereas all known 10-membered enediynes proceed through the Bergman rearrangement.

While the 10-membered enediynes are generally stable as discrete small molecules, their cycloaromatization is "triggered" by chemical transformations of key motifs—the allylic trisulfide in the case of the CAL family [26] and the C16-C25 epoxide within the anthraquinone-fused enediynes [27,28]. Triggering occurs by reactions with cellular thiols or reducing agents, while the anthraquinone-fused enediynes possess an additional cyclization pathway under acidic conditions [29,30]. For the 9-membered enediynes, cycloaromatization can be induced spontaneously upon dissociation from their cognate apoproteins or by reaction with cellular thiols depending on the specific enediyne core structures [31,32].

The structural complexity and intrinsic reactivity of the enediyne natural products presents a bottleneck to their development as ADC payloads. Indeed, a large number of enediyne natural products have been isolated as their cycloaromatized congeners [33–37]. This hurdle has been overcome for representatives of selected enediynes using a combination of synthetic, semisynthetic and metabolic engineering efforts discussed in the following.

MOLECULAR AND CELLULAR MECHANISMS OF ACTION

The biological activity of enediyne natural products is driven by their interactivity with their target, DNA. Cycloaromatization of the enediyne core yields a nascent benzenoid diradical, which, in the proximity of DNA, induces hydrogen atom abstraction from the deoxyribose backbone, and the resulting deoxyribose centered radical can react with molecular oxygen to generate single strand breaks (SSBs), double-strand breaks (DSBs) or interstrand crosslinks (ICLs). These DNA lesions are responsible for the cytotoxicity of enediyne natural products. The precise modes of interactivity with DNA differ depending on the selected enediyne (Fig. 2B), and the specific cellular responses are determined by the types of DNA damage induced as well as the propensity of treated cells to follow specific biological pathways.

The modes of DNA binding have been investigated using classical biochemical and structural techniques for selected representative enediynes. Seminal studies using CAL, NCS and their aglycon analogues have revealed the role that carbohydrates play to facilitate DNA binding or confer sequence specificity [38,39]. Complexes of the NCS chromophore and CAL with DNA have also been well studied, revealing multiple modes of DNA interactivity mediated by the presentation of sugars into the minor groove [40–43]. Other enediynes, including C-1027, SHI and DYN utilize additional intercalative binding modes through peripheral benzoxazolinate [44], β-carboline [45] or anthraquinone moieties [29], respectively. Electrophoretic mapping of cleavage sites on B-form DNA has suggested that the sequence preference for enediynes towards specific loci can vary significantly both between and within each subfamily.

Enediynes vary broadly in the types of DNA lesions they induce *in vitro*, particularly with respect to their sequence specificity and propensity towards generating SSBs or DSBs, the latter of which are especially cytotoxic. Studies using the radiomimetic drug bleomycin suggest that between 500 and 50,000 DSB per cell can induce cytotoxicity in cultured fibroblast cells whereas SSBs are approximately 300-fold less toxic [46,47]. CAL notably has a relatively strong sequence preference for its cleavage site (TCCT•AGGA), inducing nearly exclusively DSBs [48], whereas NCS and C-1027 induce DSB:SSB ratios of 1:5 and 1:2, respectively [49,50]. Despite this diversity of enediynes and their sequence preference, a common characteristic is the rapid induction of DNA breaks under cell-free and cellular conditions.

In addition, selected enediyne natural products can induce altered DNA damage patterns under altered
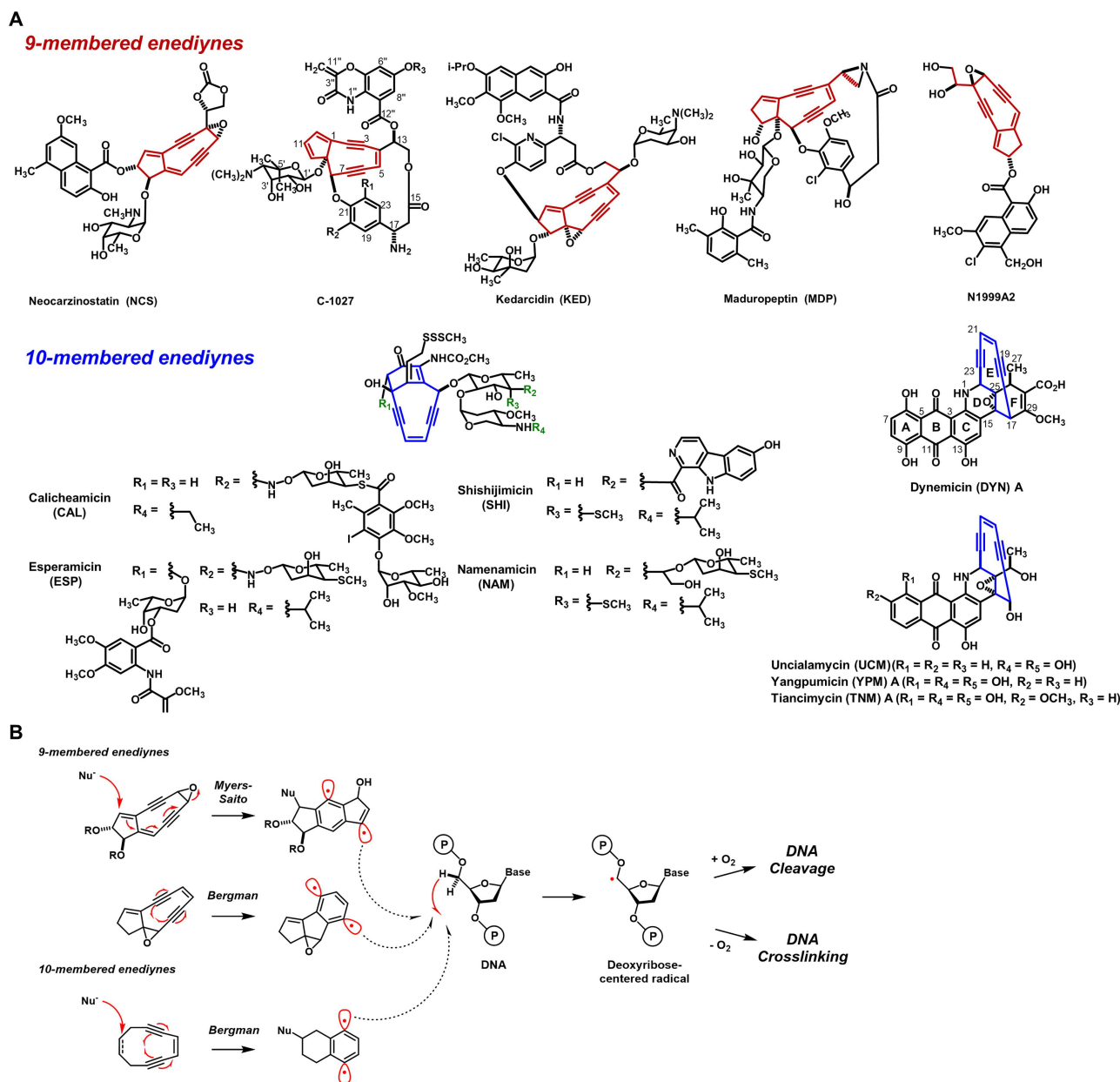
Figure 2. Structures and mechanism of action of enediyne natural products. (A) Structures of the 9- and 10-membered enediynes. The characteristic 9- and 10-membered enediyne cores are highlighted in red and blue, respectively. (B) Mechanism of action of 9- and 10-membered enediynes.

conditions. This has been most extensively demonstrated for C-1027, which exhibits threefold enhanced cytotoxicity towards hypoxic cells. In contrast, NCS is approximately fourfold less cytotoxic under hypoxic conditions [51]. This feature has been attributed to the ability of C-1027 to generate oxygen-independent ICLs in addition to conventional DSBs. While both DSBs and ICLs operate under normoxic conditions, ICL generation is markedly enhanced under hypoxic conditions that suppress DSBs, providing a strategy to treat recalcitrant tumors in oxygen-deprived microenvironments.

Structural variations engineered into the peripheral moieties of C-1027 alter the propensity of the resulting analogues to generate either DSBs or ICLs. Genetic manipulation of the C-1027 biosynthetic machinery enabled the production of 7″-desmethyl, 20-deschloro and 22-deshydroxy-C-1027 analogues (Fig. 2A) that displayed markedly distinct DNA interactivity compared to the parent compound. Removal of either the 20-chloro or 22-hydroxy group on the β-tyrosine moiety abolishes ICL activity, while conversion of the 7″-methoxy group to a hydroxyl group abolishes DSB activity, providing a rational approach to design new antitumor agents with fine-tuned DNA damage properties [52]. These findings may prove useful to engineer antitumor agents active against DSB-suppressing hypoxic tumor microenvironments.

Different types of DNA lesions contribute uniquely to enediyne induced cytotoxicity. The primary transducers of cell cycle checkpoints of DNA damage are members of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs), including ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR), whose general roles are to activate responses to DSBs and ICLs, respectively [53]. Under normoxic conditions where DSBs predominate following drug treatment, enediynes robustly activate ATM-mediated DNA repair pathways, and cells deficient in ATM remain hypersensitive to enediyne treatment. As selected enediynes, notably members of the C-1027 family, can utilize DNA crosslinking in the absence of oxygen, ATR pathways are also upregulated when cells are treated under hypoxic conditions and ICLs dominate [52,54].

The molecular and cellular mechanisms of the enediynes present appealing features for an ADC payload. First, because enediynes act at loci extended across a genome, tumor cells are unable to evade susceptibility by mutations to the target, thereby limiting the emergence of resistance mechanisms.

Second, compared to microtubule disruptors that induce aberrant disruptions to cell cycle phasing allowing quiescent or dormant cells to evade susceptibility, enediyne treatment results in faster and more complete killing of cells [23]. This rapid cytotoxicity may be more effective in promoting immunogenic apoptosis (also called immunogenic cell death), a form of cell death characterized by secretion of damage-associated molecular patterns (DAMPs) and an activation of an immune response [55]. While the immunogenicity rendered by enediyne-induced cytotoxicity is poorly studied, DNA damage agents are known to induce immunogenic apoptosis [56–58], and treatment of B16-F1 melanoma cells with C-1027 has been reported to induce translocation of calreticulin—a canonical DAMP—to the cell surface to facilitate phagocytosis [59].

Third, the diversity of DNA lesions that can be induced—DSBs or ICLs—depending on the tumor microenvironment allows for robust cytotoxicity in broad tumor types as ICL generation under hypoxic conditions compensates for the lack of robust DSB generation [52]. Induction of ICLs under anaerobic conditions extends opportunities for ADCs targeting solid tumors with notoriously hypoxic cores. Tuning this property for a given enediyne scaffold by subtle perturbations to its structure further enables the engineering of designer enediynes with defined DNA damage properties.

Finally, the ability to control cyclization by possessing structural motifs as a built-in "trigger" presents opportunities to tune the reactivity of both 9- and 10-membered enediynes to promote diradical generation only once the payload has reached its destination. As discussed subsequently, this feature has been exploited to engineer CAL analogues that are triggered hydrolytically rather than reductively [60].

ENEDIYNE DISCOVERY IN THE GENOMIC ERA

The discovery of new enediyne natural products has fundamentally changed in recent decades with a revelation that there are many enediynes in Nature that remain to be discovered. This understanding changes the approach to programs for novel enediyne discovery and is driven by two primary fundamental reasons related to conceptual and technological advancements of enediyne biosynthesis.

The first factor driving the change in enediyne discovery is related to the recent advance in fundamental understanding of enediyne biosynthesis. The biosynthesis of enediyne natural products is discussed extensively elsewhere [10]. Herein, we summarize the major highlights that have significantly impacted enediyne discovery. The first is that enediyne biosynthetic gene clusters (BGCs) share a common five-gene cassette critical for enediyne core biosynthesis, and screening for this conserved locus enables identification of novel enediyne producers. This strategy has been applied *in silico* to identify enediyne producers from public genome databases and through a real-time PCR-based approach to identify enediyne producers from yet-to-be sequenced microorganisms [23,25]. Second, BGCs encoding for enediynes enable prediction of whether the encoded metabolite is a 9-membered or 10-membered enediyne, as well as its peripheral moieties [61]. This streamlines novel enediyne discovery by enabling prioritization based on desired structural features as payload candidates. Finally, advancements in understanding the enediyne biosynthetic machinery enables genetic manipulations *in vivo*, or biochemical reconstitutions of pathways and steps *in vitro*, to generate analogues and install linkers, as demonstrated with the TNM family of enediynes [62,63].

The second major change to the landscape of enediyne discovery is driven primarily by technological advancements in microbial genome sequencing, assembly, and mining that have revealed a vastly underappreciated biosynthetic potential for the enediynes among microbes. Genome mining of microbial genomes available at public databases as of 2016 uncovered 192 microbes, mainly within the phylum *Actinobacteria*, which harbored 271 distinct enediyne BGCs but whose production was yet to be realized [61,64]. The underappreciated enediyne BGCs in the public domain, coupled with the fundamental knowledge from enediyne biosynthesis enabling structural predictions, provide a new genomics-based approach to enediyne discovery that contrasts starkly with the traditional grind-and-find approach that relies on bioassay-guided fractionation of microbial extracts [64]. A challenge remains today to discover and produce the desired novel enediynes in scale and speed in sufficient quantities for their development as ADC payloads.

ACCESS TO ENEDIYNE NATURAL PRODUCTS

The complex molecular architectures of the enediyne natural products present a barrier to accessing and advancing these molecules as ADC payloads. Often, the only available means of production requires lengthy total syntheses or microbial fermentation of native producers. Moreover, the isolated natural products typically require additional semisynthetic strategies for conjugation as the parent natural products may lack appropriate handles for linker installation.

Substantial efforts have streamlined the syntheses of enediyne natural products to enable the advancement of their conjugates. A major advancement has been the synthesis of enediyne analogues with reactive handles appropriately placed to facilitate downstream semisynthetic modifications. For example, the installation of an ethylenediamine group to C8 of UCM analogues provided a foundation for ADC construction to the A-ring of UCMs [65,66] (Fig. 2A). In addition, accessing the final natural products through total synthesis can have the added advantage of protecting the reactivity of certain groups to enable regioselective linker installation at a position that is normally unfavored in the final payload molecule. This advantage was utilized in the synthesis of UCM analogues to install linkers to the C-ring during the synthetic route [67]. While synthetic studies have proven significant to advance enediynes to preclinical investigation, this approach has yet to demonstrate scalability for subsequent clinical translation and supply.

Biosynthetic pathway engineering has laid the groundwork to produce enediynes from microbial fermentation using a combination of classical and contemporary methods. Traditional medium and strain optimization techniques have generated high-producing strains and robust conditions for production through scalable fermentation [68–70]. Contemporary techniques to enhance production have also taken advantage of the recent advance in our understanding of enediyne biosynthesis. Manipulating the regulation governing C-1027 biosynthesis has afforded recombinant strains with up to seven-fold higher titers [71,72]. Moreover, transcriptional profiling of antibiotic-resistant mutant strains that overproduce TNM has enabled the identification of global regulators, the subsequent manipulation of which paved the way to engineer strains with simultaneous higher production and improved growth characteristics [73]. Access to enediyne natural products by microbial fermentation of genetically amenable producer also enables their structural diversification through genetic pathway engineering. This has been utilized extensively to structurally diversify all subfamilies of enediyne natural products [62,74].

Engineered production of designer enediynes in their native hosts has also uncovered strategies to enable antibody-drug conjugation. The difficulties in functionalizing enediyne natural products can be overcome by exploiting biosynthetic enzymes as biocatalysts to install reactive handles for linker conjugation. This has been demonstrated using an *S*-adenosylmethionine (SAM)-dependent methyltransferase in the TNM biosynthetic pathway for installation of orthogonal functional groups using *S*-alkylated analogues of SAM [63]. These functionalized TNMs were shown to be potent against DNA and human cancer cells and conjugated as a proof-of-concept to antibody-reactive linkers. As microbial genome sequencing continues to reveal novel enediyne BGCs to expand the toolbox of biocatalysts, this strategy will continue to be exploited for functionalizing enediynes.

Finally, the genomics revolution, which has profoundly influenced enediyne discovery, has also facilitated access to desired enediyne natural products by enabling bioinformatic identification of alternative producers with superior titers, desired growth characteristics, or a combination thereof. The strength of this approach is best exemplified for C-1027. Genetic screening of a large (>3500 members) strain collection for enediyne BGCs led to the identification of four additional *Streptomyces* species harboring the C-1027 BGC, one of which produced C-1027 chromoprotein at an impressively high titer of ∼900 mg/L [75]. This approach highlights the ability to leverage expansive microbial genome sequencing for addressing the bottleneck of the practical supply of enediynes.
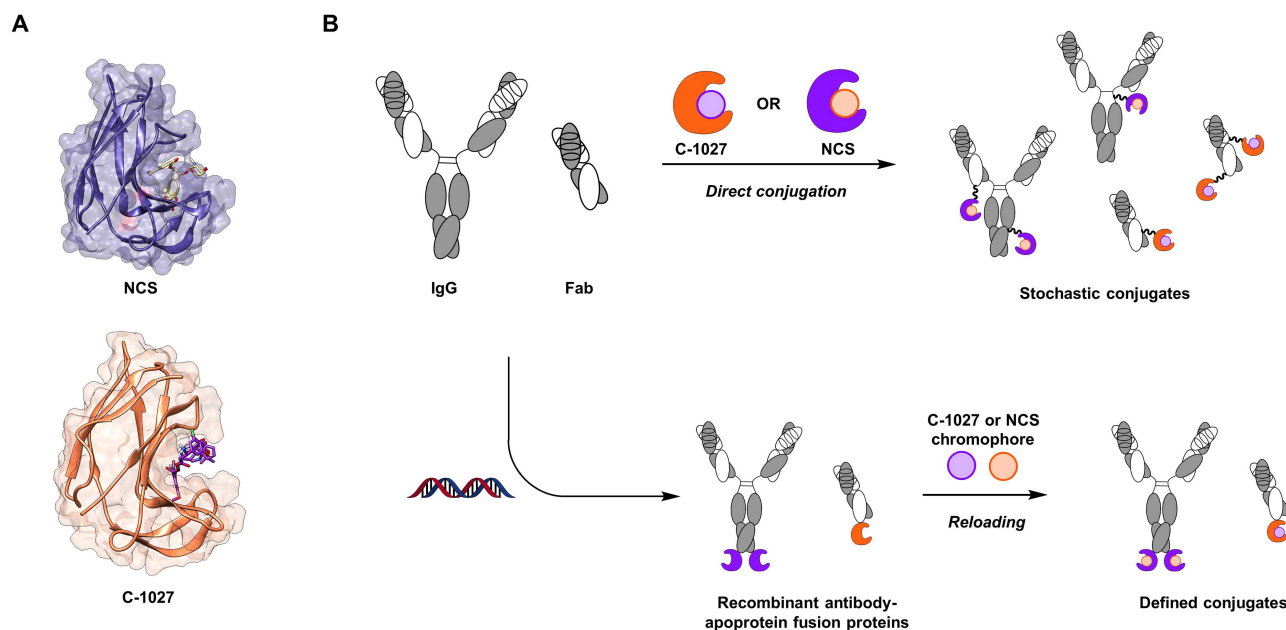
ANTIBODY-DRUG CONJUGATION STRATEGIES FOR THE 9-MEMBERED ENEDIYNES

The 9-membered enediynes, existing as chromoproteins (apoprotein–chromophore complex), present unique obstacles and opportunities for their development. The most notable of these is the need to use conjugation chemistries compatible with the chromoprotein. This has led to the development of ADCs utilizing two distinct conjugation strategies (Fig. 3). The first has been a "direct" conjugation approach in which antibodies are conjugated to the chromoproteins using heterobifunctional linkers. The second "reloading" strategy has used recombinantly produced chimeric fusions of antibodies (or antibody fragments) with enediyne apoproteins in bacterial or mammalian expression hosts, which are then reloaded in a subsequent step by incubation with the enediyne chromophores, resulting in a non-covalent complex with the antibody-apoprotein fusion protein. To date, ADCs have been reported utilizing two 9-membered enediynes: NCS and C-1027.

Neocarzinostatin-based ADCs

NCS is a 9-membered enediyne, first reported in 1965 from *Streptomyces carzinostaticus* as a proteinaceous antitumor antibiotic [76] and decades later structurally characterized as a chromoprotein with a 9-membered enediyne chromophore [77]. As the first member of the enediyne family to be structurally elucidated, NCS has served as an early archetype for the chemistry and biology of the enediynes. The biological activity of NCS has been utilized clinically for the treatment of liver cancer with the approval of the NCS-polymer (poly-[styrene-co-maleic acid (SMA)]) conjugate zinostatin stimalamer (SMANCS®) [78]. NCS has also served as the payload for several immunoconjugates under previous clinical investigation as discussed below.

The conjugation chemistry used for NCS generally used heterobifunctional crosslinkers in a direct conjugation approach. All reported immunoconjugates were prepared using the NCS chromoprotein and used the reactivity of native amino or thiol groups present on the 11.2-kDa (113 amino acids) NCS apoprotein, NcsA, to append reactive groups for stochastic conjugation to surface lysine or reduced hinge cysteine residues present on antibodies. In practice, conjugating the drug to an antibody-reactive linker is highly parallel to the methods used for small molecule conjugation, with a key distinction being the enhanced aqueous solubility of enediyne chromoproteins such as NCS compared to typical small molecule payloads.

Figure 3. Antibody drug conjugation strategies for the 9-membered enediynes. (A) X-ray crystallographic structures of the NCS (PDB: 1NCO) and C-1027 (PDB: 1HZL) chromoproteins. (B) Conjugation strategies employed for the 9-membered enediynes. Direct conjugation of the C-1027 chromoproteins to whole IgG or antibody fragments such as Fab generates stochastic conjugates in a single step. A second strategy of chimeric reloading generates recombinant fusions of IgG or antibody fragments with the enediyne apoproteins that can be purified and loaded with the cognate chromophores.

The most clinically advanced NCS-immunoconjugates utilized the murine anti-adenocarcinoma antibody A7 targeting an unknown 45-kDa glycoprotein on the cell surface of human colon cancer cells [79–82]. Conjugates of A7 in IgG1 format were prepared using succinimidyl 3-(2-pyridyldithio) propionate (SPDP) heterobifunctional crosslinking chemistry to couple reduced cysteines on the apoprotein to lysine residues on the mAb for an average drug-to-antibody ratio (DAR) of 2–3 [83]. These conjugates were generally well tolerated and efficacious in patients with advanced colorectal cancer, but most mounted strong and long lasting human anti-mouse antibody (HAMA) responses that hampered the utility of this immunoconjugate [84–86]. Notably, anti-NCS antibodies were not detected in any examined patients. A chimeric mouse/human IgG1 version of A7-NCS as well as a chimeric Fab format have also been prepared and advanced to the clinic. However, no anticancer responses were observed, and several issues were identified in a subset of patients, including a strong HAMA response and rapid clearance of the Fab-NCS conjugate [87]. The immunogenicity of these constructs supports the wisdom of re-evaluating NCS-based conjugates using newer generation humanized or fully human mAbs. However, the potential immunogenicity of the bacterial chromoproteins warrants caution for developing 9-membered enediynes such as NCS as ADC payloads. Important insights into their therapeutic utility may come from the clinical development of immunotoxins such as moxetumomab pasudotox (Lumoxiti®), which utilizes a 38-kDa fragment of *Pseudomonas aeruginosa* exotoxin A [88]. Subsequent generations of immunotoxins have been engineered for decreased immunogenicity [89], and this strategy may be prudent to advance 9-membered enediyne chromoproteins as ADC payloads.

C-1027-based ADCs

C-1027 is a 9-membered enediyne discovered in 1989 from fermentation broths of *Streptomyces globisporus*. The remarkable potency of C-1027, with EC_{50} values five orders of magnitude lower than that of chemotherapeutics such as mitomycin and doxorubicin [90,91], prompted considerable efforts to clinically translate C-1027 for oncology.

C-1027 has been utilized for direct conjugation using SPDP crosslinkers through reduced cysteine residues on its apoprotein to lysine residues on mAbs in IgG and Fab formats analogous to the preparation of antibody-NCS conjugates. This approach yielded conjugates with potent inhibitory activity against liver cancer *in vitro* and in xenograft mouse models [92–94]. Other examples have utilized *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) crosslinkers to conjugate through lysine residues on the chromoprotein to chemically installed thiols on mAbs targeting type IV collagenase in IgG1 and Fab format [95–98], generating similarly potent immunoconjugates in preclinical models. These approaches highlight the functional versatility and utility of amino acid residues on the apoproteins of 9-membered enediynes to be used for chemical conjugation.

However, most C-1027-based ADCs have been prepared using the two-step reloading strategy (Fig. 3B). This approach involves recombinantly produced chimeric fusions of mAbs with the C-1027 apoprotein, CagA. Once purified, the proteins are then reloaded in a subsequent step by incubation with the C-1027 chromophore to regenerate the chromoprotein complex. Due to the labile nature of the chromophore once unbound from its cognate apoprotein, the dissociation has been performed using organic solvents at cold temperatures (-70 °C) after which the extracted

chromophore is incubated with the recombinant chimeric fusion protein to reconstitute the ADC [92].

The reloading approach prepares C-1027-based ADCs in diverse formats against a variety of disease-relevant antigens. All of these fuse the C-1027 apoprotein to single-domain antibodies or antibody fragments and produce the chimeric fusion proteins in bacterial expression systems [99–103]. Reloaded ADCs using mAbs in IgG format have yet to be reported. Notably, as the chromoprotein is fused at a distinct site on the mAb, i.e. at an N- or C-terminus, this approach provides a more uniform ADC relative to stochastic direct conjugation approaches.

ANTIBODY-DRUG CONJUGATION STRATEGIES FOR THE 10-MEMBERED ENEDIYNES

The 10-membered enediyne natural products can be broadly classified into the CAL family and anthraquinone-fused enediynes. These compounds, in contrast with their 9-membered counterparts, are generally stable as discrete small molecules in the absence of any associated protein. This feature enables broader semisynthetic modifications to diversify the 10-membered enediynes and install antibody-reactive linkers. To date, ADCs have been reported utilizing three 10-membered enediynes: CAL, SHI and UCM.

Calicheamicin-based ADCs

CAL γ_1 is a 10-membered enediyne isolated in 1987 from *Micromonospora echinospora* and served as an archetype for early studies of enediyne chemistry and biology [16]. CAL was found to be highly potent (1 pg/mL) in antibacterial assays against Gram-positive and Gram-negative bacteria and with picomolar cytotoxicity against diverse cancer cell lines. Although CAL and synthetic analogues show potent cytotoxicity and DNA cleavage activity [60], no members of this family have been evaluated in the clinic as free drugs, and instead they have been translated as ADC payloads.

Access to CAL for ADCs has largely relied on microbial fermentation of *M. echinospora*. A major enabling methodology involved utilizing an *N*-acetyl derivative of CAL that displaces the allylic trisulfide with a sterically hindered disulfide. This transformation enhances the serum stability of the resulting compounds and provides a handle through which linkers can be installed for antibody conjugation. Conjugations of CAL derivatives to mAbs through thiol displacement have utilized four distinct strategies (Fig. 4A). The first and simplest of these installed an activated disulfide for direct conjugation to cysteine residues on antibodies through a disulfide linkage [104]. The second of these installed a hydrazine group through a sterically hindered disulfide to couple to aldehyde groups on antibodies generated through mild oxidation of the carbohydrates, resulting in a pH-labile hydrazone linkage ("carbohydrate conjugate"). The third strategy installed an activated (*N*-hydroxysuccinimide) ester to CAL for stochastic conjugation to lysine residues on antibodies, leaving the disulfide as the site of drug release ("amide-based conjugate"). The fourth method also installs an activated ester for conjugation to lysine residues but adds an additional pH-labile hydrazone as the site of drug release ("hybrid conjugate").

An additional set of notable preclinical conjugates of CAL has used a synthetic analogue, CAL θ, which differs from γ_1 only by a thioester replacement for the methyl trisulfide, generating a hydrolytically triggered compound [105] (Fig. 4A). In contrast with the thiol displacement strategy, CAL θ could be functionalized through alkylation of the *N*-ethyl amino sugar to install thiol-reactive handles for conjugation to mAbs modified with 2-iminothiolane [106]. This strategy has been subsequently also applied to CAL γ_1 to install reactive linkers to the *N*-ethyl amino sugar [107]. For these analogues, an optional displacement of the allylic trisulfide to functionalized or isopropyl disulfides is also reported to improve the stability and aqueous solubility of the payloads. Ultimately, the extensive structure–activity relationship (SAR) examined for CAL payloads and their resulting ADCs provided many lessons that have been applied to other members of this family, including the shishijimicins.

Many CAL-based ADCs have advanced to clinical investigation. The first amide-based construct, designated CMB-401, advanced as far as phase II clinical trials for refractory or relapsed (R/R) ovarian cancer patients after standard chemotherapy and consisted of a conjugate to the humanized mAb CTM01 targeting MUC1 [108,109]. Of the 19 evaluable patients, none had objective responses. The authors speculated that the inefficient mechanism of drug release, relying solely on the sterically hindered disulfide, may be a reason for the lack of efficacy. Subsequent clinical trials all utilized hybrid conjugates.

The second CAL-based ADC, gemtuzumab ozogamicin, entered clinical trials for AML concomitant with CMB-401 and consisted of a hybrid conjugate to a humanized mAb targeting CD33. Gemtuzumab ozogamicin became the first FDA-approved ADC based on phase II data demonstrating a complete remission in approximately 30% of AML patients [110]. However, a post-approval study found a higher rate of toxicity for gemtuzumab ozogamicin in combination with chemotherapy compared to chemotherapy alone, leading to voluntary withdrawal of the ADC from the market in 2010. Gemtuzumab ozogamicin remained in clinical use on a compassionate use basis during which it was observed that a fractionated treatment regimen demonstrated significant clinical benefit leading to resubmission for FDA approval in 2017. Two weeks prior to the re-approval of gemtuzumab ozogamicin, another CAL-based ADC, inotuzumab ozogamicin, was FDA approved for R/R acute lymphoblastic leukemia (ALL) [111]. Inotuzumab ozogamicin consists of a humanized mAb targeting CD22 with an identical drug-linker construction as gemtuzumab ozogamicin. Both antibody carriers use an IgG4 format.

Shishijimicin-based ADCs

The SHIs are a family of 10-membered enediynes discovered in 2003 from the ascidian (sea squirt) *Didemnum proliferum* and were found to possess extraordinarily potent (pM) cytotoxicity [19]. As no genetic information is currently available for the biosynthetic origin of SHI, the
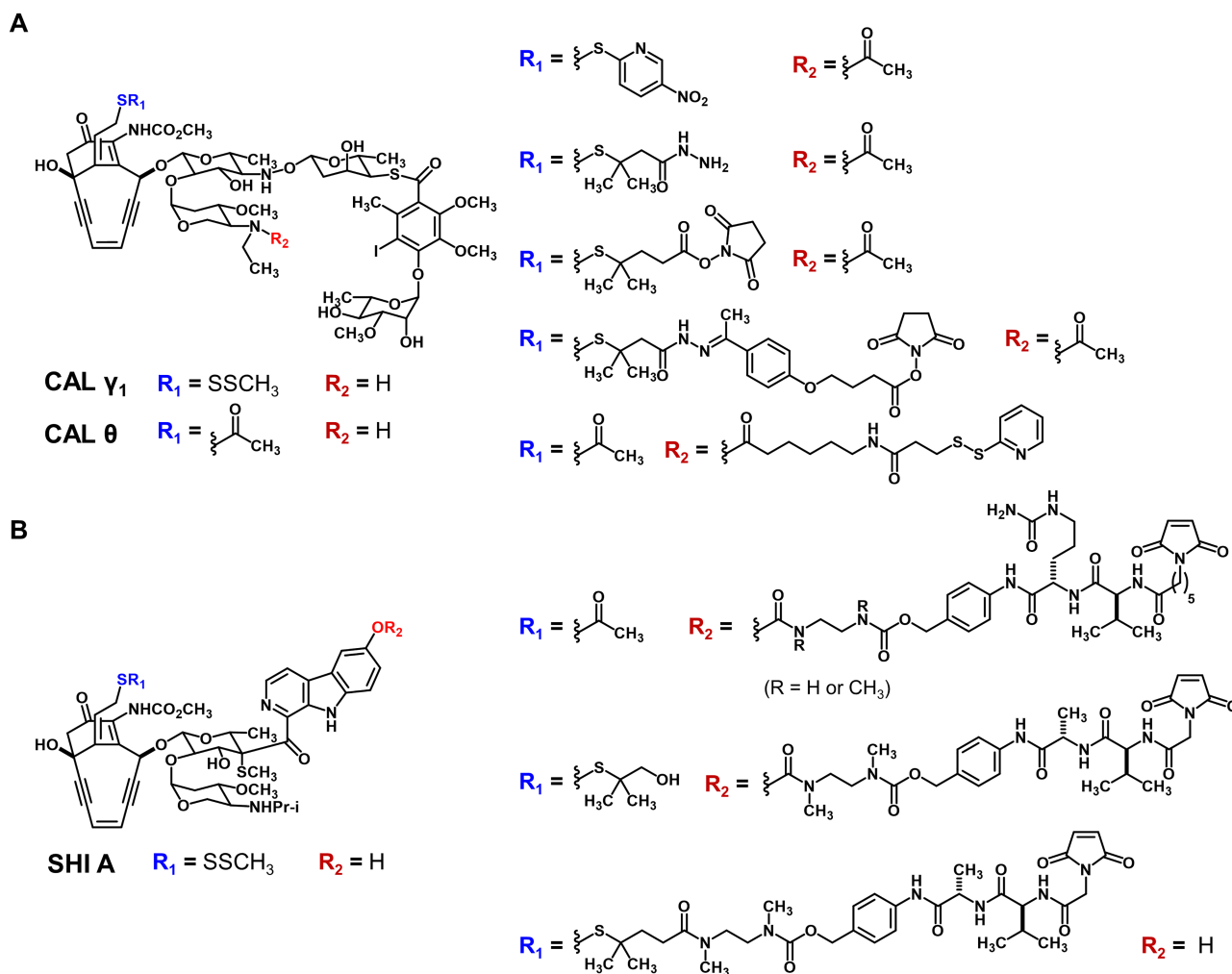
Figure 4. Conjugation strategies used for the CAL family of enediyne natural products. (A) Modification of CAL γ_1 and CAL Θ to install antibody-reactive linkers to generate diverse conjugates. (B) Installation of linkers to SHI to generate four linker-drug constructs amenable to antibody conjugation.

producing organism, putatively a host-associated microbe, has not been identified. The DNA interactivity and bioactivities of the SHI A and congeners have been extensively studied [45,112]. Notably, SHI binds at the minor groove of DNA, similar to CAL, but also possesses an intercalative mode of binding imbued through the β-carboline moiety, a nitrogen containing heterocycle.

Access to SHI for biological evaluation has been achieved through total synthesis [112]. These studies have provided insight into the SAR and modes of DNA cleavage for SHI A and structurally simplified analogues. Notably, the synthetic route to access this family utilized a precursor with excellent utility for installing linkers for antibody conjugation through the β-carboline moiety and allylic trisulfide.

The installation of linkers to SHI through the β-carboline moiety and allylic trisulfide enabled the construction of site-specific ADCs targeting undisclosed antigens [113]. Three drug-linker constructs were achieved through the β-carboline moiety using either a primary or secondary carbamate, with two replacing the allylic trisulfide with a thioacetyl group, and the third utilizing a sterically

hindered disulfide (Fig. 4B). A fourth drug linker was conjugated through a sterically hindered disulfide rather than the β-carboline motif. All utilize protease cleavable linkers and a maleimide for site-specific conjugation to engineered cysteines of thiomabs [114] for an approximate DAR of 2.

Biological evaluation of SHI-based ADCs revealed the critical importance of the site of linker attachment and the linker used. Constructs utilizing primary carbamates showed no specific activity *in vitro* against any cell lines examined, a finding attributed to the instability of the carbamate. While this conclusion was supported by restoring the *in vitro* cytotoxicity of the ADC by replacing the primary carbamate groups with secondary carbamates, all constructs prepared using the hydrolytically labile thioacetyl analogues were shown to be unstable in plasma over 7 days. Conjugation through a sterically hindered disulfide—a strategy employed successfully for CAL-based ADCs—ultimately succeeded to generate stable SHI-based ADCs with potent *in vitro* activity [113]. *In vivo* evaluation of SHI-based ADCs has yet to be reported.
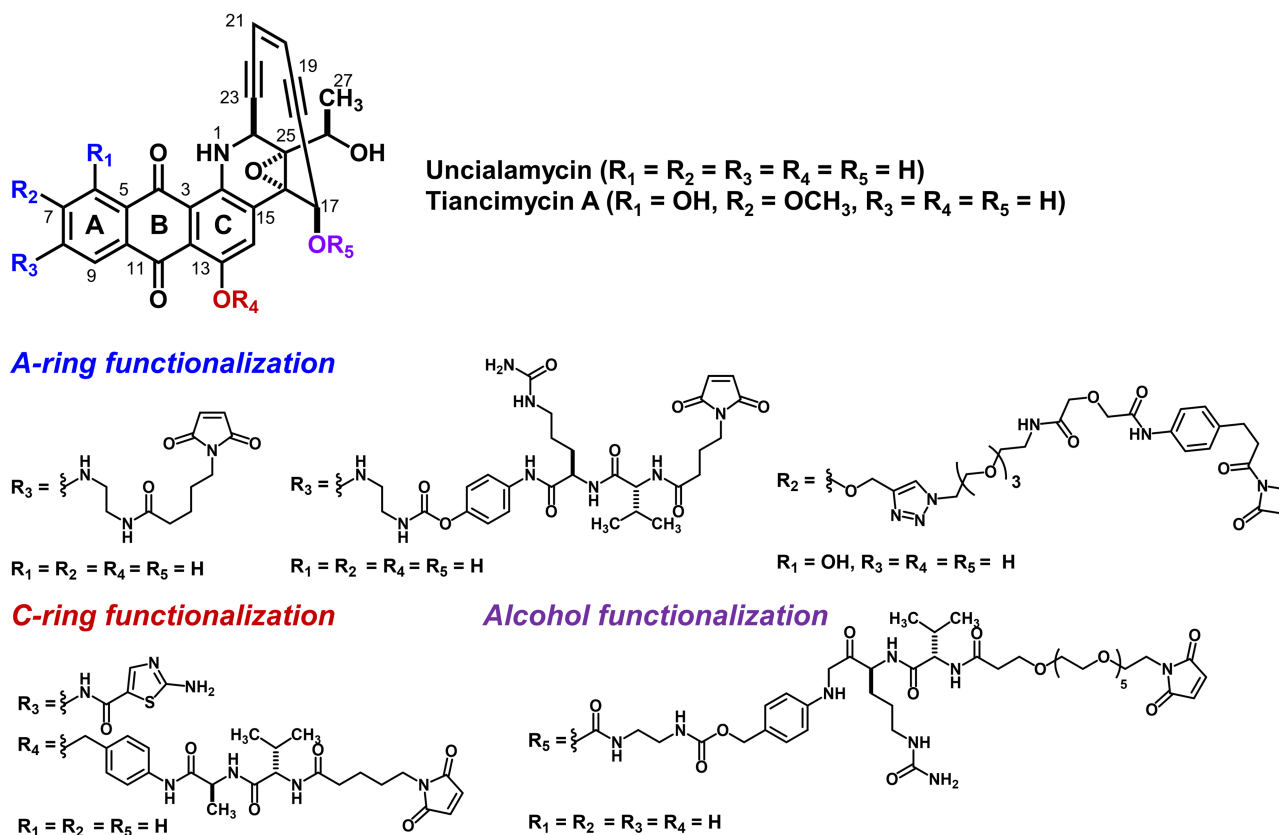
Figure 5. Heterobifunctional linker installation to the anthraquinone-fused enediynes. Linkers have been installed to multiple sites on the A-ring of both UCM and TNMs, and to the C-ring, and C17 alcohol present on UCM.

Anthraquinone-fused enediyne-based ADCs

The anthraquinone-fused enediynes, exemplified by the DYNs, UCM, TNMs and YPMs, represent the second subclass of 10-membered enediynes that feature an anthraquinone moiety fused to the enediyne carbocycle that enables DNA interactivity. Like the CAL family of enediynes the anthraquinone-fused enediynes contain a "trigger" in the form of an epoxide attached to the 10-membered enediyne core that—when opened under reductive or acidic conditions—induces the Bergman cyclization for members of this family.

Access to the anthraquinone-fused enediynes has been achieved through both total synthesis and microbial fermentation. Both strategies have generated analogues with diverse substitutions to enable SAR studies [62,65,115].

Clinical translation of ADCs utilizing anthraquinone-fused enediynes has yet to be achieved, but preclinical studies have reported UCM analogues generated through total synthesis conjugated to mAbs targeting CD70 and mesothelin. The first generation of these constructs installed thiol-reactive non-cleavable and cleavable linkers through an ethylenediamine group installed at the C8 position (Fig. 5), revealing potent antigen-specific activity *in vitro* only when the cleavable linker was used [66]. Another set of constructs installed cleavable linkers through the C13 hydroxyl group and demonstrated potent antigen-specific activity *in vitro* and in xenograft mouse models [67].

Several additional publications and patents report drug-linker or ADC constructs using the anthraquinone-fused enediynes but have yet to fully report their results. Researchers at Pfizer [116] and Bristol-Myers Squibb [117] have independently reported site-specific UCM conjugates or drug-linker constructs prepared through the secondary alcohols at C26 and C17. While the biological evaluation of ADCs generated from these constructs has yet to be disclosed, the drug-linker constructs displayed strikingly different stabilities in mouse and human serum—putatively due to unique serum esterases in mouse serum—that were difficult to rationalize [117–119].

RESISTANCE TO ENEDIYNE-BASED ADCS

The general mechanisms of resistance to ADCs include antigen downregulation, upregulation of drug efflux transporters, defects in cellular trafficking and lysosomal degradation, and alterations to the payload target [120,121]. Clinical resistance to CAL-based ADCs has largely been found to be conferred by upregulation of multidrug transporters such as P-glycoprotein (MDR1/ABCB1), a mechanism also observed in patients treated with ADCs utilizing other payloads such as microtubule disruptors [122,123].

In the case of gemtuzumab ozogamicin, chronic exposure *in vitro* to the AML cell line HL-60, generated resistant clones overexpressing MDR1 [124]. Combined treatment with inhibitors of MDR1 increased the cytotoxicity of the ADC on both resistant cell lines and patient samples, supporting the notion that efflux occurring prior to drug translocation to the nucleus was the primary resistance mechanism [125,126]. *In vitro* studies support an additional mode of resistance mediated by the ability of cells to repair DNA damage and activate downstream anti-apoptotic factors [127,128], supported by synergistic enhancement in cytotoxicity when treatment is combined with an mTORC1 inhibitor [129]. While upregulation of DNA damage responses has conferred resistance *in vitro*, these findings have not been supported by comparable mechanisms in patients. Moreover, resistance to inotuzumab ozogamicin so far has been primarily reported to occur by upregulation of drug transporters, with the cytotoxicity of the ADC inversely correlated to MDR1 expression levels [130]. These results support drug efflux as the dominant mechanism of resistance to clinically used CAL-based ADCs.

The use of enediyne payloads—which can act broadly on many genomic loci—provides fewer opportunities for the development of resistance relative to other target-based ADC payloads. In the case of the auristatins and other agents targeting microtubule dynamics, resistance has been demonstrated by mutations to tubulin monomers. For example, resistance to an analogue of hemiasterlin was found to be conferred by single nucleotide changes to either β- or α-tubulin that conferred enhanced microtubule stability [131]. This modification of the target also conferred cross-resistance to other tubulin targeting agents that bind at the *Vinca* alkaloid site, including hemiasterlin A, vinblastine and dolastatin 10. These resistant cell lines did not overexpress MDR1, suggesting that target modification is sufficient to confer resistance *in vitro*. Similarly, resistance against α-amanitin and camptothecin analogues has been demonstrated through mutations to their targets, a subunit of RNA polymerase II and topoisomerase I, respectively [132–134]. In addition to changes at the amino acid level, targets can be post-translationally modified to confer resistance, as observed with doxorubicin [135]. These results demonstrate liabilities when using ADC payloads acting upon protein targets and provide opportunities to overcome resistance using DNA damaging agents including enediynes as alternative ADC payloads.

Little is known about clinical resistance to the anthraquinone-fused enediynes and their corresponding ADCs. An unusual finding of resistance to this family of compounds—conferred by small proteins that bind and sequester the free drug—has been identified in the human microbiome of healthy patients with no clinical exposure to these molecules [136]. This identical mode of resistance is utilized by the anthraquinone-fused enediyne producing organisms themselves, which contain genes encoding for homologous binding proteins within their BGCs. Moreover, the clinical implications of this finding are currently unclear but should be considered when developing this family as ADC payloads.

CONCLUSION

Enediyne natural products represent a rich source of novel payloads for ADC development with distinct advantages. The biosynthetic potential of novel enediyne natural products is largely untapped, and many remain to be discovered. The discovery of novel enediynes is likely to contribute to fundamentally new insights into their chemistry and biology for ADC payload development and provides diverse opportunities at the interface of cancer biology and natural products chemistry. Discovery and production of novel enediynes in scale and speed remain challenging. An understanding of successful strategies to functionalize and conjugate these scaffolds is critical to the future development of this field. The advances made and groundwork laid to access representative members of the 9- and 10-membered enediynes and translate them as ADC payloads are likely to be applicable to the advancement of yet-to-be discovered enediynes as next-generation ADC payloads.

Finally, ADCs are increasingly being evaluated in combination with small molecule chemotherapeutics and immuno-oncology drugs and enediyne-based ADCs should be evaluated in this context [137]. The premise here is that immunostimulatory effects of enediyne-induced cytotoxicity can synergize with immune checkpoint inhibitors, T-cell agonists, and other immunostimulatory agents. As site-specific antibody–drug conjugation chemistries continue their march to and through clinical development, dual-drug ADCs are also likely to play a prominent role in the ADC landscape of the future, and the unique mode of action of enediynes is likely to be applicable [138]. Preclinical studies suggest synergism when combining microtubule inhibitors, still the most clinically prevalent payload class, with drugs utilizing distinct modes of action [139,140]. The ability to diversify their structures—both synthetically and through metabolic pathway engineering—is a crucial factor that will facilitate the combination of enediynes with existing payloads.

ABBREVIATIONS

ADC, antibody–drug conjugate; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; mAb, monoclonal antibody; BGC, biosynthetic gene cluster; CAL, calicheamicin; DAMP, damage associated molecular patterns; DAR, drug-to-antibody ratio; DNA, deoxyribonucleic acid; DSB, double-strand break; DYN, dynemicin; EC_{50}, half-maximal effective concentration; ESP, esperamicin; HAMA, human anti-mouse antibody; ICL, interstrand crosslink; KED, kedarcidin; MDP, maduropeptin; mTORC1, mammalian target of rapamycin complex 1; NAM, namenamicin; NCS, neocarzinostatin; PCR, polymerase chain reaction; PIKK, phosphatidylinositol 3-kinase-like protein kinase; RNA, ribonucleic acid; SAM, *S*-adenosylmethionine; SAR, structure–activity relationship; SHI, shishijimicin; SMB, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; SSB, single-strand break; TNM, tiancimycin; UCM, uncialamycin; YPM, yangpumicin

CONFLICT OF INTEREST STATEMENT

C.R. holds the position of Editorial Board Member for Antibody Therapeutics and is blinded from reviewing or making decisions for the manuscript.

FUNDING

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