How can the potential of the duocarmycins be unlocked for cancer therapy?

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The duocarmycins belong to a class of agent that has fascinated scientists for over four decades. Their exquisite potency, unique mechanism of action, and efficacy in multidrug-resistant tumour models makes them attractive to medicinal chemists and drug hunters. However, despite great advances in fine-tuning biological activity through structure–activity relationship studies (SARS), no duocarmycin-based therapeutic has reached clinical approval. In this review, we provide an overview of the most promising strategies currently used and include both tumour-targeted prodrug approaches and antibody-directed technologies.

Introduction

Duocarmycins are a family of DNA minor groove-binding compounds with exquisite cytotoxicity originally identified in *Streptomyces* [1]. Cyclopropapyrroloindole-based duocarmycins, such as (+)-duocarmycin SA (DSA, **1**) and CC-1065 (**2**, Fig. 1a), comprise a DNA recognition motif (DNA-RM) and a pharmacophore responsible for alkylating DNA (Fig. 1a). In the AT-rich regions of the minor groove, the N3 position of adenine performs a nucleophilic attack on the least substituted carbon in the duocarmycin cyclopropane in a stereoelectronically dependant manner, forming DNA adducts [2]. Synthetic manipulation of the DNA alkylating subunit (variation in 'A', Figs 1c and 2) dramatically impacts the cellular potency in the pM–nM range, with most compounds forcing cells to undergo apoptosis [3] via direct S-phase inhibition and subsequent cell cycle arrest [4].

Seco-duocarmycins derived from common scaffolds, such as CI (**3**), DSA (**4**), CPI (**5**), and CBI (**6**), are precursor molecules (Fig. 1b) that contain a phenolic hydroxyl group that is responsible for initiating rearrangement of the pharmacophore to produce a cyclopropane-containing cytotoxin, via a process known as spirocyclisation (Fig. 1c). The natural duocarmycin products are produced in the enantiomerically *S* form at the chloromethane chiral centre. The chiral configuration of the duocarmycins has been demonstrated to influence both the sequence selectivity of DNA alkylation and the potency [5]. In regard to the latter, the *S* enantiomer is 100–1000-fold more potent than the *R* enantiomer in generating DNA damage, and synthetic approaches are typically aimed at producing the pure *S* form for this reason [6].

Despite their considerable potential as therapeutics, market approval for the clinical use of duocarmycins has not yet been granted. Clinical administration of adozelesin [7], bizelesin [8] and the two carbamate prodrugs carzelesin [9] and KW-2189 [10] (carbamate protection shown in Figure 11 and 12, respectively) has been associated with severe adverse effects and no therapeutic index, leading to all trials being discontinued. Despite this clinical failure, interest in these molecules has remained high and has led to a large body of work that has demonstrated fascinating insights in both biological and chemical explorations [6]. The inherent capacity of duocarmycins to evade traditional resistance mechanisms and retain exquisite potency in multidrug-resistant cells [11–13] still attracts considerable interest from both academia and industry to develop new approaches for this class of molecule, with a focus on expanding the therapeutic index for patient benefit. Accordingly, in this review, we outline and discuss the most promising drug discovery strategies over the past decade focussed on maximising the potential of these powerful compounds derived and inspired by nature.

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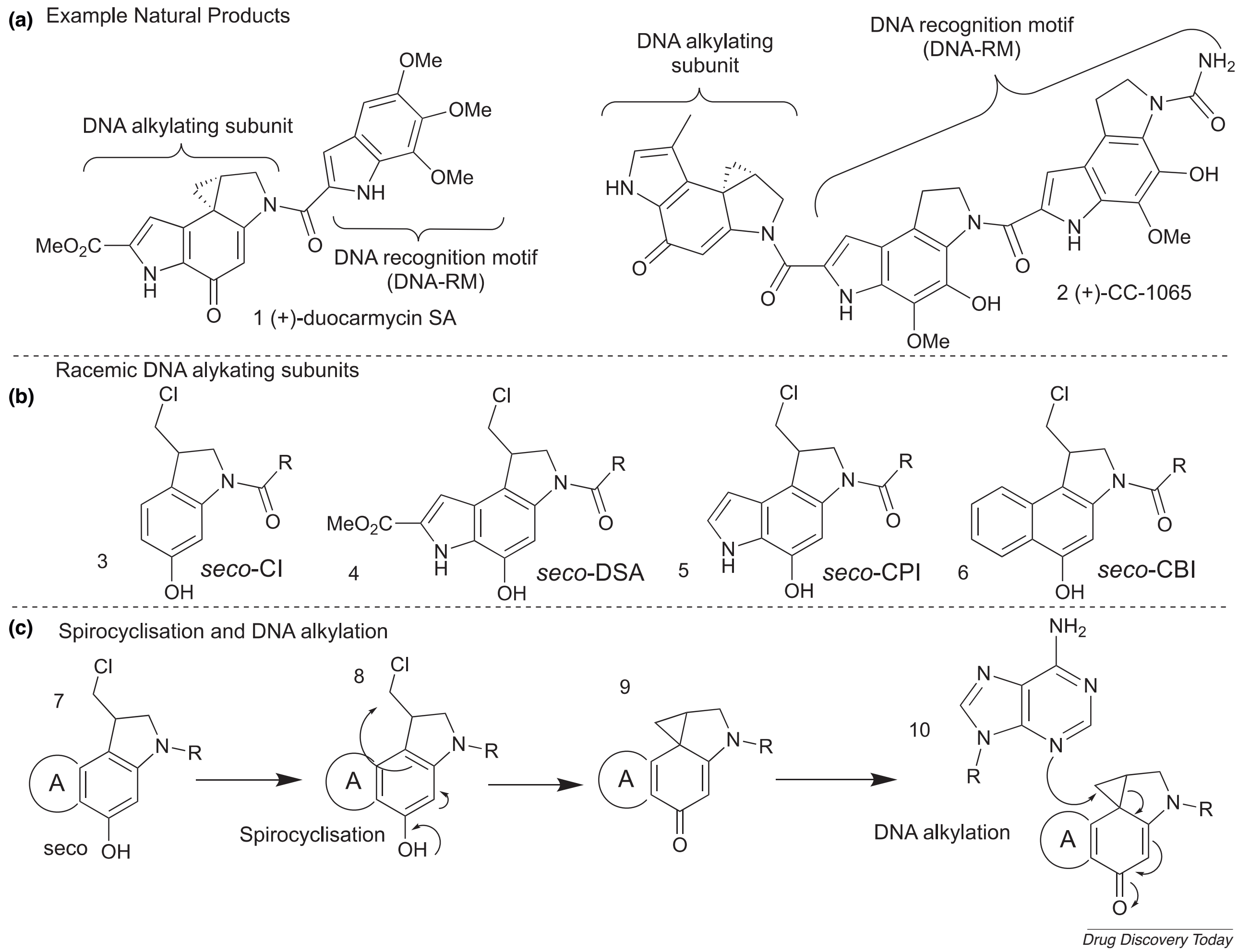

FIGURE 1

Duocarmycin SA, CC-1065, and common seco-duocarmycin pharmacophores.

Prodrug strategies and related chemical modifications

Hypoxia-activated duocarmycin prodrugs

Solid tumours typically contain regions of high cell density and poor vascularisation leading to low oxygenation and hypoxia. These regions are often therapy resistant and house some of the most aggressive cancerous cells [14]. Although hypoxic fractions remain an obstacle to effective treatment, they also offer opportunities for the development of hypoxia-activated prodrugs (HAPs). Generally, HAPs are designed to exploit the presence of one or two-electron oxidoreductases, which can catalyse the reduction of a trigger group crucial to prodrug activation and hypoxia selectivity.

Detailed mechanistic insight into duocarmycin-based HAPs using NO_2 as the trigger group in place of the *seco*-phenol positioned OH group, **13** (Fig. 2) has been described. The NO_2 group is bioactivated under hypoxic conditions by one-electron oxidoreductases to hydroxylamine or primary amine metabolites capable of undergoing spirocyclisation equivalent to the naturally configured *seco*-duocarmycins (Fig. 1c) [15]. Early proof-of-concept (PoC) studies using *seco*-amino CI-based duocarmycins provided evidence that these had significant biological potency and, hence, were suitable for prodrug derivatisation [6]. Through several interesting mechanistic studies, the hypoxic cytotoxicity ratios [HCR, IC_{50}(oxic)/IC_{50}(hypoxic)] were improved, ultimately leading to the development of sulfonamide-containing CBI analogues suitable as HAPs [16]. To increase aqueous solubility for *in vivo* investigations, phosphate preprodrugs, such as **20** and **21** (Fig. 3), were developed and demonstrated anticancer activity at nontoxic doses in murine xenograft models [17]. Significant potentiation was also observed between these compounds and radiotherapy or chemotherapy (gemcitabine and docetaxel), with full remission seen in some mice [17,18]. However, maximum tolerated doses could not be calculated because of sporadic acute toxicity [18]. Although there are several NO_2-containing drugs on the market [19], spontaneous bioreduction of the NO_2 group might lead to off-target effects [20] and, hence, further toxicity experiments would be of interest to progress such duocarmycins-based HAPs.

The Boger lab described *N*-acyl *O*-amino phenol HAPs that are sensitive to cleavage at the weak O-N bond, but which were reported to be preferentially activated under hypoxic conditions. Although early-stage compounds suffered from poor *in vivo* stability [21,22], cyclic counterparts (**14**, Fig. 2) were shown to have

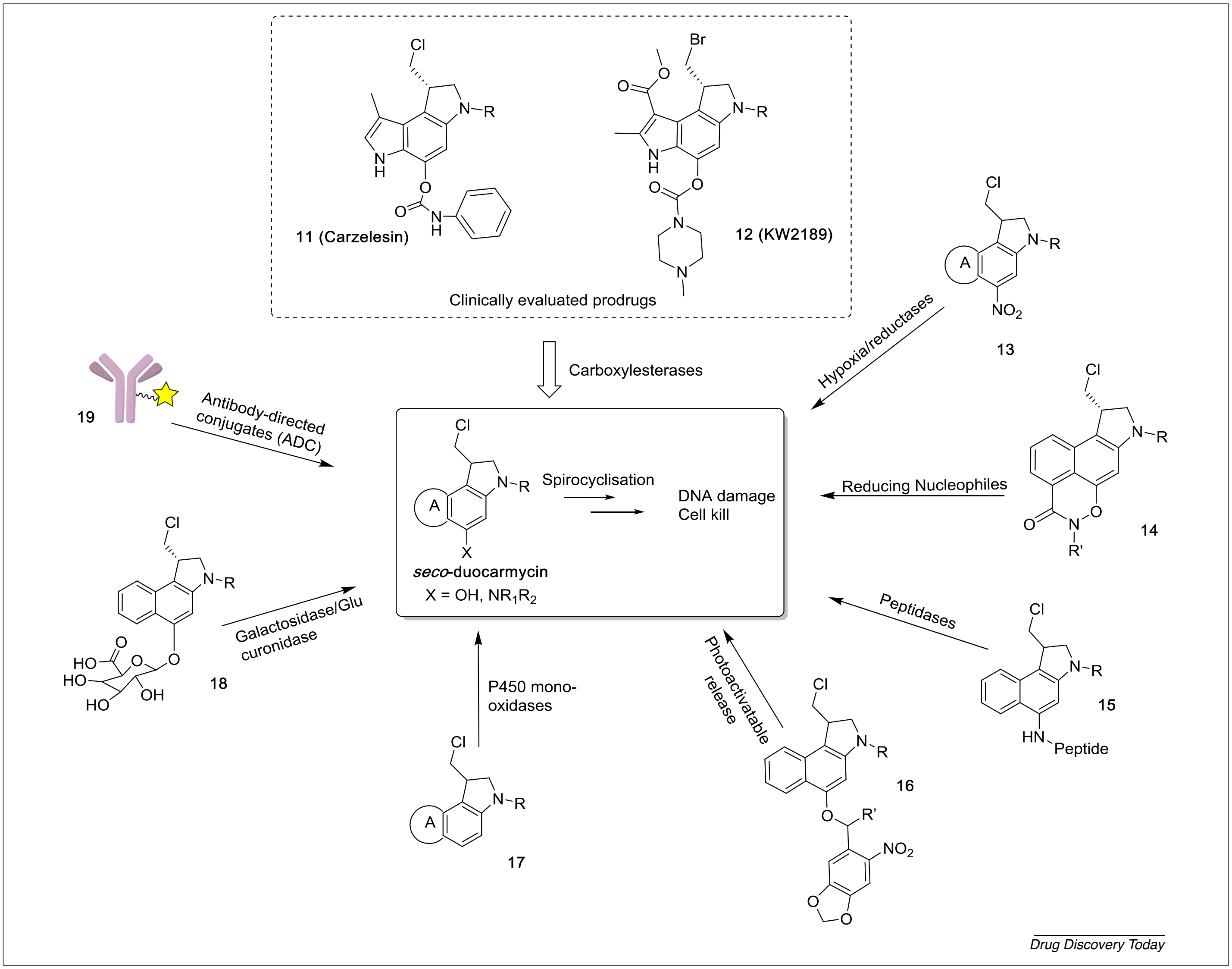

FIGURE 2

Strategies to selectively bioactivate duocarmycins in tumour tissue.

greater stability and still undergo rapid cleavage using chemical reductants. Prodrug **22** (Fig. 3) was shown to be hundreds of times less active than the activated cytotoxin, which in turn had comparable toxicity to the *sec*o-CBI equivalent without substituent in position 6 [23].

Although eight HAPs have progressed to clinical evaluation, none have yet been approved. Any future successes of duocarmycin-based HAPs will likely be linked to the design of: (i) better drug candidates with an increased bystander effect; and (ii) better clinical trials that involve patients harbouring severely hypoxic tumours as validated via a reliable biomarker approach [24].

Cytochrome P450 oxidation

Cytochrome P450 (CYP) enzymes are a superfamily of mixed function oxidases where especially. CYP1–3 subfamily members are unique in their ability to oxidise drugs and xenobiotics. Given that several isoforms, including CYP1A1, 1B1, 2S1, and 2W1, are overexpressed in many human tumour types compared with normal tissue [25], there is an opportunity to design prodrugs that are dependent on a crucial functional group that can be unmasked or restored by CYP oxidation. In this context, the *seco*-duocarmycins lend themselves as candidate molecules because of the importance of the phenolic OH group in the central chloromethylindoline trigger unit; the removal of the OH group in CBI and CPI scaffolds leads to more than a 1000-fold loss in potency [26]. In PoC studies, it was demonstrated that both CYP1A1 [27] and CYP2W1 [28] are capable of restoring the phenolic OH group vital for biological activity (pharmacophore **17**; Fig. 2). Lead bioprecursors **23** and **24** have been investigated *in vivo* and were shown to significantly delay tumour growth in xenograft models engineered to express CYP1A1 and CYP2W1, respectively [28,29]. Importantly, bodyweight changes were kept to within 10%, indicating that such re-engineered duocarmycin bioprecursors can be safely administered while retaining anticancer activity.

Phenol-masking prodrugs

This collection of approaches shares the common feature of masking the phenol (or primary amine) group with a cleavable moiety

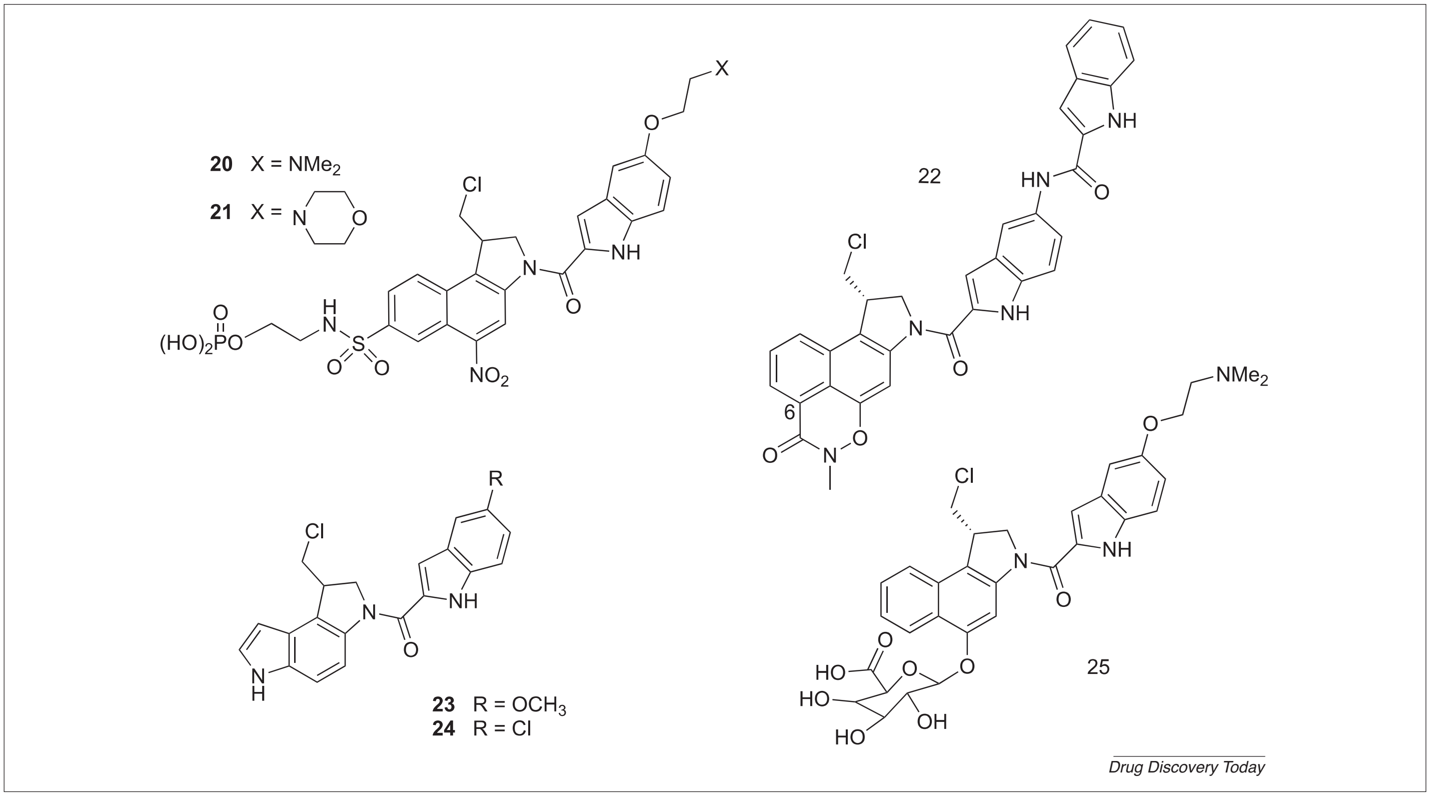

FIGURE 3

Lead duocarmycin prodrug molecules.

to prevent activation by spirocyclisation. Previous approaches explored masking the active phenol with carbamates (**11** and **12)** [9,10], vinyl ethers [30], Fe (II) reactive endoperoxides [31], human serum albumin reactive 6-maleimidocaproic [32], and gastrin receptor-targeting pentagastrin carbamate [33]. Some encouraging approaches are discussed herein.

Two recent studies investigated using *seco*-CBIs as photoactivable chemotherapeutics (PACTs) by masking the phenol with an *O*-linked 2-nitropiperonyl UV-labile group (**16**, Fig. 2) [34,35]. In both cases, the studies demonstrated efficient *in vitro* activation after exposure to UV light at 365 nm. A drawback of this therapeutic approach is that light has to be directed to a known tumour mass, which currently limits its use in metastatic cancers. However, PACTs could be useful in unresectable tumours or where removal of tumour mass is incomplete [36]. In addition, a 2017 study combined near-infrared photo-activation of a duocarmycin derivative with antibody-directed targeting in a theranostic system [37]. This approach is further discussed in the antibody–drug conjugate (ADC) section.

Extensive work has been undertaken to develop glycosidic-modified duocarmycins suitable for use in antibody-directed enzyme prodrug therapy (ADEPT) technology. In this system, the phenol in the DNA alkylating subunit is chemically modified via an *O*-linked glycosidic group, leading to inactivation through an inability to spirocyclise (pharmacophore **18**, Fig. 2) (reviewed in [38]). Potency in A549 cells was shown to be more than 1000-fold higher in the presence of the target glycosidic enzyme [39].

A study by Tietze, Roffler and colleagues also used a gene-directed enzyme prodrug therapy (GDEPT) strategy [40]. To demonstrate the potential of this approach, intratumoural injections of adenovirus expressing β-glucuronidase fused to an optimised transmembrane domain were administered to human CL1-5 xenografts in mice alongside compound **25**. Median survival was extended from 35 days with no treatment to over 150 days for mice treated with the prodrug and vector, with seven out of nine mice reaching full remission. This experiment represented a PoC in terms of enzyme delivery using a non-antibody approach and indicated the potential in using a delivery option such as a viral expression system [40].

Both irradiation and chemotherapeutic treatment have been reported to result in senescent cell formation [41], which is effective at inhibiting tumour growth, but might also function as a means of escaping apoptosis and allowing a possible reversal out of senescence [42]. Interestingly, galactose-protected bifunctional CBI-dimers were recently reported as potential senolytics on the basis that β-galactosidase is more prevalent in senescent cells [43]. Although a senolytic function was demonstrated *in vitro* and *in vivo*, no toxicity data were reported in this study. However, given the extreme toxicity of duocarmycins and the ubiquitous expression of lysosomal β-galactosidase in tissues [44], systemic toxicity might be anticipated and, hence, would need to be carefully managed in the clinic.

Antibody–drug conjugates

ADCs have gained prominence over the past decade, with nine approved drugs and >100 in clinical trials. ADCs combine the excellent targeting properties of antibodies with the cell-killing power of conjugated payloads [45]. Currently, none of the clini-

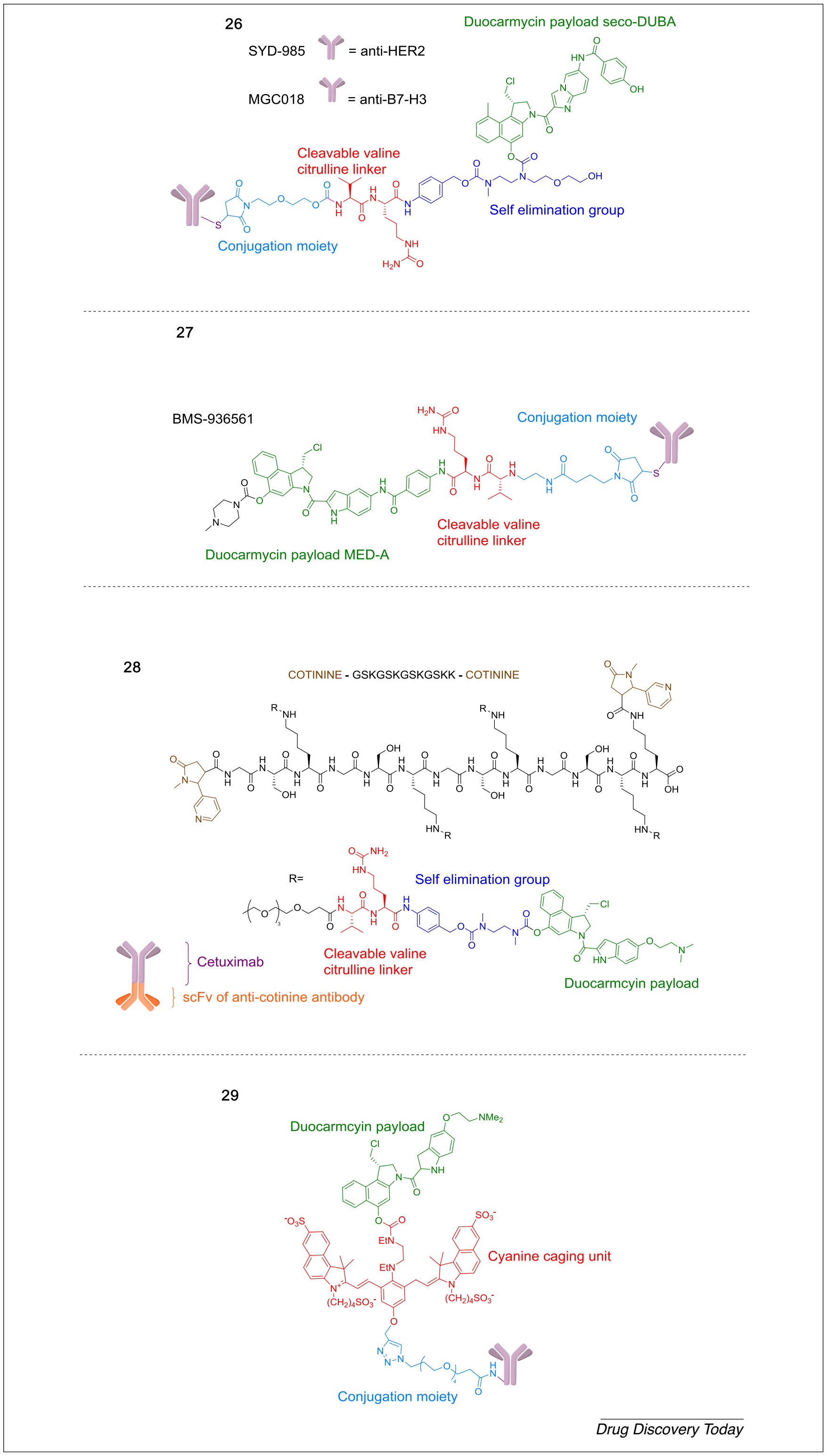

FIGURE 4

Duocarmycin payloads in antibody–drug conjugates (ADCs).

cally approved ADCs have a duocarmycin as payload. Investigations to this end are discussed further herein.

Duocarmycin-based ADCs under clinical evaluation

Human epidermal growth factor receptor 2 (HER2) is the target of two clinically approved ADCs (trastuzumab emtansine and trastuzumab deruxtecan) and a duocarmycin-based ADC (trastuzumab duocarmazine, also known as SYD985), which is currently in Phase III clinical trials for breast cancers [46–48]. SYD985 uses a duocarmycin-derived payload (duocarmycin-hydroxybenzamide-azaindole, DUBA), which is shown in ADC **26** (Fig. 4). The DUBA is *O*-linked to a self-elimination group designed to undergo spontaneous breakdown after cleavage at an adjacent valine citrulline (VC) site by cathepsin B. This elimination releases a *seco*-DUBA, which undergoes spirocyclisation to an active form. The drug linker is conjugated to the antibody through a thiol-reactive maleimide to cysteines exposed through interchain disulfide bond reductions. The drug: antibody ratio (DAR) can be partially controlled by adjusting the amount of reducing agent [49]. The significance of the DAR is highlighted by SYD983, a forerunner of SYD985, which has a range of DARs from 0 to 8. Hydrophobic interaction chromatography (HIC) was used to separate the mixture and the resultant SYD985, with an average DAR of 2.7, was twofold more potent in killing HER2-expressing SK-BR-3 breast cancer cells compared with SYD983 [50,51].

In a recent study, it was demonstrated that SYD985 was effective against patient-derived xenografts (PDXs) with resistance to trastuzumab emtansine (T-DM1) [13]. The encouraging results are likely a consequence of the presence of the cleavage point in the linker of SYD985 for lysosomal release, low levels of HER2 being sufficient for cytotoxicity and drug efflux pumps having minimal impact [13,52]. SYD985 was found to be 3-50 times more effective in cell lines expressing low levels of HER2 compared with T-DM1, whereas the results were similar when HER2 was expressed at high levels [53].

MGC018 is an ADC that uses a DUBA drug linker identical to that in SYD985 (compound **26**; Fig. 4) conjugated to a monoclonal antibody (mAb) that targets B7-H3, an immune check point antigen that is highly expressed in most solid tumours [54]. MGC018 recently entered Phase I/II clinical trials for patients with solid tumours as a monotherapy or combination with MGA012, a PD-1-targeting immune check point inhibitor. Preliminary safety reporting from the trial suggested that adverse effects are mostly tolerable and demonstrated early signs of efficacy in some patients [55].

BMS-936561 (MDX-1203), a CD70-targeting ADC incorporating a duocarmycin as a warhead, has also reached clinical trials [56]. BMS-936561 (**27**, Fig. 4) is conjugated through a linker with a maleimide and VC cleavage site attached to the DNA-RM of the duocarmycin. The phenol is protected by a carbamate group, which can be removed by endogenous carboxylesterases [57], in the same way as KW-2189. However, despite completion of a Phase I clinical trial targeting renal cell cancer and non-Hodgkin's lymphoma with well-tolerated doses identified, the programme has been discontinued [58].

Preclinical duocarmycin-based ADCs in development

The duocarmycin scaffold has been modulated in several ways with the purpose of exploring them for use as payload ADCs. For detailed understanding of chemical and biological explorations, we refer to recent reviews on redesigned duocarmycins, including the application of duocarmycin-based dimers [59–61]. Several clinical and preclinical duocarmycin ADC candidates are antibody target variants of SYD985 [54,62]; however, here we cover some other approaches that have not yet yielded clinical progression.

A recent study reported on an anti-EGFR bispecific antibody complexed with a tetra-duocarmycin cotinine compound, **28** (Fig. 4). Similarly, to SYD985, the duocarmycin payload was conjugated via an equivalent *O*-linked VC self-elimination linker, but had a homogenous DAR of 4 [63]. The conjugate was produced by bridging two cotinine modules with the peptide linker GSKGSKGSKGSKK and then tethering the drug linker to each of the four first lysines (Fig. 4). Subsequently, this part of the conjugate was complexed with the cotinine-specific single-chain variable fragment (scFv) of the bispecific antibody. This ADC elicited significant tumour growth inhibition in human adenocarcinoma xenograft models in mice, with no detectable weight loss during the 5-week treatment period [63].

As referenced in the photoactivatable subsection, a near-infrared photo-controllable theranostic duocarmycin antibody-conjugate **29** (Fig. 4) has been reported [37]. The CBI-based duocarmycin is *O*-linked to a cyanine caging group by *N,N'*-diethylethylendiamine at the C4' position. The caging group is then conjugated to panitumumab (anti-EGFR antibody) through nonspecific reactions between *N*-hydroxysuccinimide and lysines. The payload is released through exposure to light at 780 nm. Additionally, the conjugate can be visualised by external illumination, which was used to determine maximum tumour accumulation and, hence, the optimal time for activation. The conjugate demonstrated *in vitro* pM cytotoxicity after activation using external light exposure. Re-accumulation was also monitored, facilitating an informed second dose of activating light [37]. A benefit of this tool is that haemoglobin and water have low absorbance in the 600–850 nm range, reducing noise and enabling moderately deeper tissue imaging [64].

Two studies reported on the shielding of the active phenol with a phosphate group to increase compound solubility [65,66]. Zhao *et al.* published a study where a CBI-based payload was conjugated to a CD19 antibody [65], whereas Su *et al.* published a study in which two phosphate-protected CBIs joined by a linker were conjugated to a CD22 antibody [66]. Both studies reported that the addition of the phosphate group greatly enhanced solubility and, therefore, the efficiency of the conjugation reaction between the payload and the antibody [65,66]. Both lead ADCs demonstrated tumour growth inhibition in mice with xenografts expressing the relevant antibody target, reporting 38 days of tumour growth inhibition after 75 μg/kg $5 \times$ qd doses [65] and 21 days of tumour growth inhibition after a single dose of 1 mg/kg [66], respectively.

Small-molecule drug conjugate and peptide–drug conjugate

Small-molecule drug conjugates (SMDC) and peptide–drug conjugates (PDC) are attractive because they are smaller than ADCs and, therefore, are likely to confer superior tumour penetration. Both SMDCs and PDCs are designed to exploit the specificities of protein-targeting small molecules or peptides and the cytotoxicity of nonspecific chemotoxins by combining them with a cleavable linker [67].

Initial work published in 2014 demonstrated that duocarmycins could be viable payloads for SMDCs by combining inhibitors for carbonic anhydrase IX (CA9), which is over expressed in hypoxic cancer cells, with a *seco*-duocarmycin *O*-linked to a self-eliminating group [68]. However, the conjugate only demonstrated modest tumour growth inhibition after 7 days of treatment in mice with SKRC52 renal cell carcinoma grown subcutaneously.

More recently, a PoC study that combined a glutamate carboxylpeptidase II (GCPII, also known as prostate-specific membrane antigen)-targeting molecule (DUPA) and a DSA DNA alkylation subunit derived from duocarmycin with a VC cleavable linker (DUPA-DSA) was reported [69]. GCPII is a cell surface protein that undergoes endocytosis when bound to a ligand and is reportedly 1000-fold more prevalent in cancerous cells, in particular in prostate cancers [70]. DUPA-DSA has been demonstrated to be significantly potent in GCPII-expressing cells *in vitro* [69].

The tumour targeting of duocarmycins utilising a PDC approach has been explored in several studies [33,71–73]. Most recently, a peptide recognising the glycan Thomsen-Friedenreich Antigen (TFα), frequently displayed on the surface of cancer cells, was conjugated to a *seco*-DSA with a VC cleavage linker [73]. This PDC was shown to exert high nanomolar toxicity in TFα-positive cell lines, compared with no measurable toxicity in TFα-negative cell lines.

Future perspectives

Nature has undoubtedly produced intricate compound architectures that have fascinated and inspired drug hunters for medicinal use. Despite the promise of the powerful cell-killing duocarmycins, they have yet to be approved for clinical use. As reviewed here, several promising tumour-selective prodrug and ADC approaches are underway, with the latter strategy especially gaining momentum. For both prodrug and ADC strategies, low enzyme or antigen expression as well as tumour penetration are possible obstacles to effective treatment. Further refinement of duocarmycin-based therapies could include combining structural features successfully implemented in prodrug and ADC approaches to develop smarter, more efficacious, and better-tolerated therapies. Another area for exploration could be through judicious choice of combination treatment based on tumour biopsies to address target availability. Intriguingly, despite >40 years of research, their mode of action is not well understood. A better mechanistic understanding of how duocarmycin–-DNA adduct formation leads to apoptosis could reveal potential synthetic lethality partners for duocarmycins. Regardless of the approach used, market approval of a duocarmycin-based medicine would offer clinicians an opportunity to use a new type of agent with capacity to treat aggressive and resistant forms of cancers.

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