Antibody Therapeutics, 2020, Vol. 3, No. 4 ***237–245***
doi:10.1093/abt/tbaa024
Advance Access Publication on 25 November 2020

Review Article

Tackling solid tumour therapy with small-format drug conjugates

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Received: August 26, 2020; Revised: November 11, 2020; Accepted: November 13, 2020

ABSTRACT

The pharmacokinetic–pharmacodynamic relationship is extremely complex and tumour drug penetration is one key parameter influencing therapeutic efficacy. In the context of antibody–drug conjugates (ADCs), which has undergone many innovation cycles and witnessed many failures, this feature is being addressed by a number of alternative technologies. Immunoglobulin-based ADCs continue to dominate the industrial landscape, but smaller formats offer the promise of more-effective cytotoxic payload delivery to solid tumours, with a higher therapeutic window afforded by the more rapid clearance. To make these smaller formats viable as delivery vehicles, a number of strategies are being employed, which will be reviewed here. These include identifying the most-appropriate size to generate the larger therapeutic window, increasing the amount of functional, cytotoxic payload delivered through conjugation or half-life extending technologies or other ways of extending the dosing without inducing toxicity.

Statement of Significance: Antibody–drug conjugates are a clinically and commercially established modality of cancer therapy with five new agents approved over the last 2 years. Treating solid tumours remains a major challenge with many failures and small-format drug conjugates offer a solution to the tumour penetration issue.

KEYWORDS: antibody–drug conjugate; fragments; scaffolds; solid tumours

INTRODUCTION

Drug penetration into solid tumours as a factor influencing efficacy has been discussed at length over the years, but is it only now being actively addressed [1]. For biological therapies in particular, the relationship between drug dosing and tumour uptake is highly complex and very often, the micro-distribution across a whole tumour does not correlate with drug dose or plasma concentration and this underappreciated variability could explain poor responses due to suboptimal concentrations of therapeutic agents in the tumour micro-environment (TME) [1,2]. This is especially true with monoclonal antibodies (MAbs), which have to overcome numerous biological barriers [3,4] such as poor vascular supply, crossing the endothelium, overcoming tumour interstitial fluid pressure, diffusing through dense stroma and passing through tight epithelial barriers (Fig. 1). This typically results in <1% of the injected dose/gram of MAb/ADC reacting the target in solid tumours in humans [4–6].

These observations increasingly backed up by preclinical and clinical data are motivating researchers to look at smaller formats of targeted therapeutics, which (due to more rapid diffusion kinetics) are known to have superior tissue-penetrating (perfusion) properties compared with large proteins such as immunoglobulins [7]. Of course, lower molecular weight (MW) therapeutics brings with it a whole new set of issues on the positive side (e.g. reduced side effects due to decreased cross-reaction with

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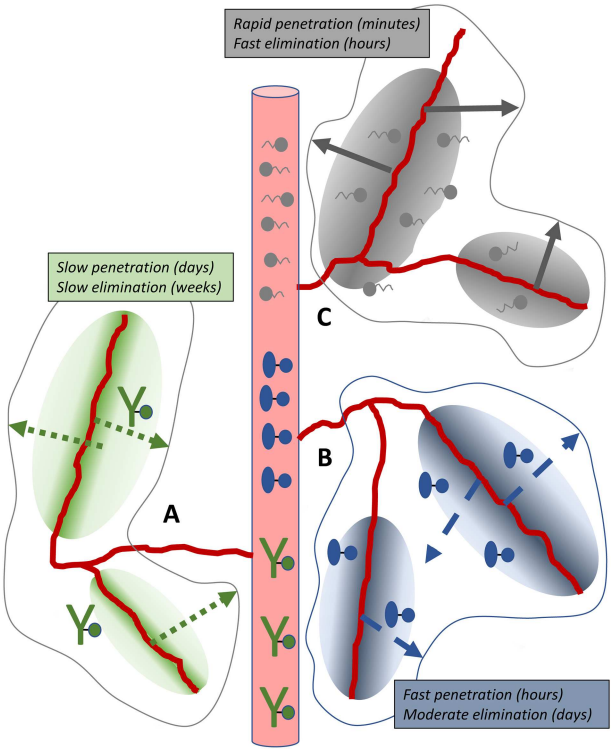

Figure 1. Drug conjugate delivery via the tumour vasculature and penetration can be illustrated with broadly three PK profiles. (A) Conventional ADCs with MWs of > 150 kDa accumulate and penetrate into tumours over days and eliminate from the body over weeks requiring less frequent dosing, but a higher risk of off-target/cumulative toxicity. (B) A wide range of smaller (5–100 kDa), protein-based binding scaffolds such as scFv and DARPins, which have uptake and penetration kinetics lasting hours, but are eliminated more rapidly (days), reducing non-specific exposure time, but may require strategies for higher drug delivery (e.g. higher DAR, HLE, more frequent or higher dosing). (C) Very small peptidic conjugates (<5 ka) that have very rapid and more complete uptake and penetration kinetics, but are eliminated in a matter of hours also requiring strategies to improve temporal exposure.

Fc-receptors, reduced temporal exposure to normal tissues, higher tumour: plasma exposure ratio) and negative side (smaller window of bioavailability, reduced overall uptake) [8,9], so striking the balance to obtain a favourable window is key and probably none more so than in the field of antibody–drug conjugates (ADCs) [3,10].

With nine approved products and approaching 100 ADCs in clinical trials [10,11], this modality is again on an upward trend after numerous setbacks and innovation cycles. Effective treatment of solid tumours remains a significant challenge for the reasons outlined above with greater clinical successes seen in haematological cancers [11,12]. The ADC industry is firmly focused on the Immunoglobulin format with numerous approaches for refined conjugation and more a homogeneous product quality, but an evolving area is the use of smaller formats (i.e. antibody fragments or binding scaffolds smaller than 150 kDa), which promises to widen the therapeutic window by improving tumour kill efficacy whilst reducing normal organ toxicity. This review will focus on the emerging small-format 'biologics' from ~2 kDa peptide–drug conjugates to larger ~80 kDa immunoglobulin fragment derivatives, which will all have very different pharmacokinetic (PK) and pharmacodynamic properties (Fig. 1). For the smaller formats, the chemical linker–payload has a greater influence on these properties as it can make up 10–30% of the overall conjugate mass compared with a typical 2–3% for an IgG, therefore requires special consideration and bespoke design (Fig. 2; Table 1). This review will focus on non-radioactive and non-liposomal pharmaceutical conjugates.

RECOMBINANT ANTIBODY FRAGMENTS

Recombinant antibody fragments lend themselves to a wide range of engineering approaches [13,14] to facilitate linker–payload bioconjugation such as the introduction of conjugation friendly thiols. They are normally produced in prokaryotic systems removing the glycan-conjugation option utilized by some in the ADC field. Fragments such as single-chain Fvs (scFv) and single-domain antibodies tend to be more robust and stable having been subjected to stringent selection pressures during discovery compared with Fab-fragments [14]. The resulting antibody fragment drug conjugate (FDC) at a drug:antibody ratio (DAR) of ~2 has the feature of carrying more payload compared with a standard ADC of DAR4 on a mass basis but has a shorter half-life and thus lower systemic bioavailability compared with an ADC.

Fab-fragments have been superseded by formats such as scFvs but examples exist of conjugates demonstrating proof-of-principle. Early conjugates with moderately potent, chemotherapy-approved payload such as paclitaxel and doxorubicin have largely been ineffective, but a trastuzumab–mono-methyl auristatin E (MMAE) FDC DAR1 with 200–500 pM potency *in vitro* required alternate day dosing at 20 mg/kg to see any tumour regression [15]. This high dosing requirement was also seen more recently with an anti-CD-20 Fab appended with a sortase conjugation tag used for enzymic conjugation of an MMAE payload [16]. The FDC had to be dosed at 20 mg/kg every 3 days for four doses to obtain 4/6 cures, compared with complete cures for an equivalent ADC. Notably, the FDC had ~6× lower plasma exposure as measured by the PK area under curve. The FDC, however, was better tolerated. A similar but dual-linker-payload (DAR3, cleavable and non-cleavable auristatin) was also very potent (IC50 0.7–0.9 nM) but not evaluated *in vivo* [17]. Trastuzumab Fab-based conjugates based on the ultra-potent pyrrolobenzodiazepine (PBD) payload class (IC50 in the low pM range) were recently described where a novel dual maleimide disulphide rebridging technology previously applied to ADCs was applied to the native cysteines in a Fab [18]. The tesirine payload has been used in several clinical-stage ADCs but was also the cause of unacceptable toxicity in the discontinued Rova-T and others subsequently [19]. This was modified to be more hydrophilic with a symmetrical dual maleimide bridge. *In vitro* potencies were 6–7 pM for high human epidermal growth factor receptor-2 (HER2)-expressing cells and as potent as the trastuzumab-based ADC despite the reduced avidity and possibly reduced internalization kinetics (not determined). *In vivo* efficacy was not explored [18].

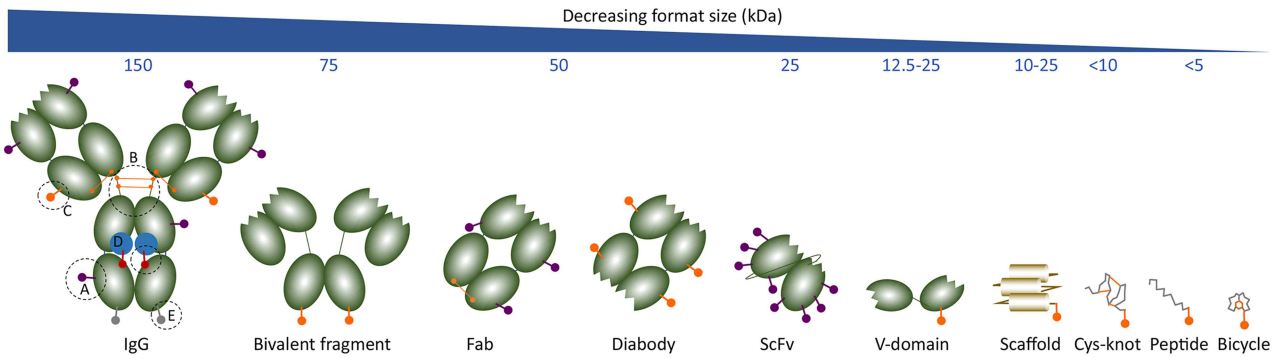

Figure 2. A size and format comparison of various drug conjugates. The archetypal IgG is shown with the common conjugation strategies (A) surface lysines, (B) hinge thiols, (C) site-specific thiols, (D) Fc-carbohydrate, (E) genetically engineered tag or non-natural amino acid. The same colour coding is used for the conjugation onto the alternative, smaller formats of decreasing size: bivalent antibody fragment (~75–80 kDa), Fab or diabody (~50 kDa), high-DAR ScFv (25 kDa), VH-domains (12.5–25 kDa), many types of scaffolds (10–25 kDa) and a variety of peptides.

Table 1. A list of drug-conjugate formats in order of increasing size with examples of the targets addressed and payloads used

Format size (kDa)	Format name	Example target	Cancer indication	Example payload	References
1.5–2	Bicycle (bicyclic peptides)	MMP14 EphA2 Nectin	Breast, lung multiple solid tumours	DM1 vcMMAE	[67–69, 71]
~3–5	Pentarin	Somatostatin receptor	Neuroendocrine Liver	DM1	[62–65]
~3.5–5	Cysteine knots	Integrin, MMP2	Pancreatic	Gemcitabine, MMAF, Cis-platin	[60, 61]
5–6.5	Affibody	HER2	Breast/gastric	Idarubicin, vcMMAE Photosensitizer	[43–45, 46]
~10–11	Centyrin Adnectin	EGFR Glypican	Multiple solid tumours Liver	vcMMAF Tubulysin	[48, 50]
~15–18	DARPIn	EpCAM	Multiple solid tumours	MMAF	[53]
~15	Abdurin	EphA2	Prostate	vcMMAE	[56]
~12.5–25	VH (like) domains	PSMA	Prostate	DGN549	[33]
~25–27	ScFv	HER2, EGFR CD41/61	Breast/gastric	Photosensitizers MMAF, vcMMAE, Auristain F vcMMAE	[21, 22, 24, 25, 28, 29]
~55–60	Diabody	CD30	Lymphoma	MMAF	[38]
~50	Fab	CD20, HER2	Lymphoma breast/gastric	vcMMAE PBD	[15, 18]
~80	SIP ScFv-Fc	Fibronectin, Tenascin-C FGFR-2	Multiple solid tumours	Cemadotin, DM1, vcMMAE	[35–37, 39]

ScFvs are artificially tethered, recombinant antibody structures but represent the preferred format for most antibody discovery programmes that utilize a display technology [13,14]. In specific applications where time-critical elimination was necessary (e.g. fast clearance ahead of a second step), they have proven useful. There are many reports on targeted photodynamic therapy where a conditionally cytotoxic photosensitizer payload is delivered to tumours but must be removed from the systemic circulation before laser illumination [20]. We and others have developed this technology and demonstrated tumour eradication *in vivo* with very few side effects [21,22], but the complex nature of such a two-step therapy has hampered commercial development. This has not put off some companies combining optically active payloads and conventional ADCs so that therapeutics can be simultaneously imaged and used for treatment, in a theranostic approach [23].

We later extended our work on scFv-targeted photodynamic therapy to conventional payloads with more commercial success, broadly calling them 'FDCs'. Using particular scFv VH–VL frameworks predisposed to chemical conjugation and high payload loading, DARs of 5–10 were obtainable via lysine conjugation whilst retaining the critical biophysical properties [24,25]. Although heterogeneous in nature, stochastic high DAR FDCs have fewer permutations than lysine-conjugated ADCs. As expected, the linker–payload structure had a major impact on biophysical properties such as aggregation, binding affinity and thermal stability leading us to tailor payloads specifically to match the scFv format. Superior tumour penetration compared with ADCs has been observed and nM–pM potencies observed *in vitro* on cell lines using auristatin and maytansine payloads [24–27]. A key finding when developing high DAR scFv-based FDCs was that although the MW was theoretically within the range for renal excretion, the chemical–physical properties of the linker–payload became a dominating feature that altered the PK to a predominantly hepatic clearance route and a slower-than-expected systemic elimination approaching albumin-binding half-life extension (HLE) methods [26,27]. This, in turn has made FDCs a viable option with dosing now approaching that of ADCs.

We have used lysine residues to achieve the high DAR, but site-specific conjugation, more aligned to the conventional ADC field can be achieved using C-terminal cysteine thiols or dedicated conjugation tags to obtain lower DARs [10]. One example is the SNAP technology that utilizes a small, engineered DNA–alkyltransferase enzyme as a recognition and conjugation domain to link benzylguanine-modified payloads. Low nM potencies against epidermal growth factor receptor (EGFR)-expressing cells lines were seen *in vitro* using the scFv derived from the clinically approved panitumumab MAb [28].

Specifically focussing on the TME, Yap *et al.* [29] developed a scFv- based FDC targeting an integrin glycoprotein (GPIIb/IIIa: CD41/CD61), which is found in an active–high-affinity conformation on activated platelets that are increasingly thought to be involved in mediating tumour growth and metastasis in the TME. Using a sortase-recognition tag, valine–citruline (vc)-MMAE with a Gly_3 linker was conjugated to a DAR1. *In vivo*, four doses of a 6 mg/kg scFv-GGG-vc-MMAE gave a moderate ~8-day tumour growth delay demonstrating proof-of-concept for this novel approach. Targeting the TME was further illustrated using a Cy5 dual-labelled conjugate [29]. An interesting twist on using scFvs was described by Wang *et al.* [30] aiming to capitalize on the increased macro-pinocytosis seen in ras-driven cancers such as pancreatic. An-anti-EGFR scFv recombinantly fused to domain III of human serum albumin (for HLE) and the apoprotein/carrier for the cytotoxic antibiotic lidamycin. The ~60 kDa conjugate effectively internalized and was highly potent across four pancreatic cancer cell lines (IC_{50} range 15–70 pM), although clear specificity was not shown. The concept of delivering an ADC via non-clatherin route was demonstrated and a well-tolerated, moderate tumour growth delay was shown at 0.4 mg/kg given twice [28]. Higher doses were not used. The modular design idea was exploited to build a nanobody–drug conjugate with a magnetic resonance imaging (MRI) contrast agent [30]. A biparatopic anti-EGFR nanobody was fused to a gadolinium-binding domain (imaging) and a C3 tag for payload conjugation. HLE was also incorporated through an anti-albumin nanobody. A maleimide-functionalized cis-platin chemotherapy drug was conjugated to the fusion protein's C-terminus and Gd^{3+} incorporated non-covalently via dialysis. Uptake and imaging were demonstrated but, not unexpected for a relatively moderately potent drug (IC_{50} ~ 1 mM); only moderate potency was seen *in vitro. In vivo,* the conjugate was as potent as free cisplatin (in terms of platinum content), but much better tolerated. This was due to the 4–5× higher accumulation in tumours, which was further supported by the T_1-weighted MRI contrast images [31].

Smaller antibody fragments such as Variable (V)-domains (Ablynx's nanobodies: VHH-domain antibodies derived from llamas, Crescendo Biologics' Humabodies: human VH-domains) require some sort of HLE technology to make them viable candidates. Their Humabody-Drug Conjugates (HDCs) platform is made up of 15kDa domains conjugated to a low-DAR, additionally half-life extended using albumin-binding domains. This retains the benefits of tumour penetration [32]. CB108, a biparatopic HDC against prostate-specific membrane antigen (PSMA) has been shown to be effective *in vivo*. A very nice study by Nessler *et al* [33] aimed to tease out some of the important features and benefits of smaller format drug conjugates. Low-affinity monovalent (VH1) and high-affinity, rapidly internalizing, biparatopic (VH1–VH2) HDCs were created with and without HLE domains against PSMA. These were conjugated to a DNA-alkylating payload DGN549, to a DAR1. In the absence of any drug delivery or mass transport limitations, rapid internalization led to the highest *in vitro* potency, but slower internalization aided tumour penetration and higher efficacy *in vivo*. HLE was needed for *in vivo* efficacy as these low-DAR conjugates would otherwise clear via renal filtration. Alexa-Fluor-680 labelling of the various formats (without the payload) confirmed the superior penetration of the VH1–HLE format, which was additionally backed up with tumour spheroid modelling data [33]. Elasmogen have a similar technology based on shark variable domains from new antigen receptors called soloMER™, which coupled with its HLE technology NDure™ [34] is being utilized to discover and develop soloMER™–drug conjugates.

Bivalent antibody-derived fragments have met with greater preclinical success as seen with small immunoproteins (SIP-Philochem) [35–37] and diabodies (Seattle Genetics) [38]. Neri's SIP technology uses the CHε4 domain to dimerize scFvs yielding a fragment that is ~50% the size of an IgG, with a faster elimination time due to the absence of neonatal FcR binding. Using primarily non-internalizing, tumour neovasculature targets such as fibronectin and tenascin-C, excellent uptake and tumour/blood contrast ratios were obtained and the availability of two C-termini presented two cysteine thiol conjugation positions [35]. The aim is to destroy tumour vasculature to starve the tumour of nutrients and this removes the tumour penetration hurdle, but the

payload is released extracellularly and diffuses into the nearby cells with a resulting bystander killing effect. If thiol-bearing payloads are used, practically no linker is required ('traceless') as long as the disulphide is hindered or buried/protected within the protein architecture to reduce the risk of inadvertent release [35]. The release mechanism is via extracellular thiols (e.g. glutathione), which is amplified upon more cells dying. Using a DM1 payload on an anti-fibronectin–EDA SIP, well-tolerated cured were seen in murine F9 teratocarcinoma animal models dosed at 7 mg/kg three times [36]. An anti-tenascin C SIP coupled to a more commonly used vc-MMAE linker–payload (DAR2) also demonstrated tumour growth inhibition at 7 mg/kg four times but was not as effecting as the IgG version that was more stable. The IgG-based ADC was again more stable in a side-by-side comparison of IgG vs. SIP using the F8 antibody and DM1 payload conjugated at a DAR2 as a C-terminal disulphide [35]. As expected, the SIP–drug conjugate accumulated into the tumour and cleared more rapidly and the 24-h uptake levels were more than four times higher for the IgG ADC. Although the payload on the ADC was ∼10× more stable, the SIP conjugate was more effective on a molar basis with authors attributing this to the faster payload release leading to higher tumour payload exposure over a shorter period of time compared with the slow-release of an ADC. Toxicity, which may be higher for a less stable drug-conjugate, was not evaluated [37]. A similarly configured scFv–Fc format ADC was made from an anti-fibroblast growth factor receptor-2 (FGFR2) antibody discovered by phage display. Using the vc-MMAE payload ∼nM potency was seen [39].

The most comprehensive analysis of a potent antibody FDC was described by Seattle Genetics [38] using an anti-CD30 diabody with four cysteine thiols conjugated to MMAE and mono-methyl auristatin F (MMAF) payloads with maleimide linkers. The diabody ADC (MMAF DAR ∼4) was compared with an equivalent IgG ADC. In this example, the two formats had comparable DARs and valency. The diabody–drug conjugate had a faster blood clearance reflected by its smaller size, but the 30× lower exposure level only led to a 3× drop in *in vivo* efficacy (7.2 vs. 2 mg/kg needed for comparable tumour growth inhibition). Interestingly, the renal clearance expected for such fragment sizes was not evident, suggesting that the payload had a major influence diverting the conjugate to the liver for metabolism [38].

The above research and development makes observations based on therapeutic efficacy without direct evidence that tumour penetration is having a significantly positive impact. This has been difficult to quantify for drug conjugates. Direct correlations have been made between antibody size and tumour perfusion [7] but a more recent analysis in a SKOV3–HER2 model examining uptake and tumour penetration homogeneity of monovalent and bivalent nanobodies (MW ∼ 15–30 kDa) size and affinity was carried out using intravital fluorescence microscopic imaging [40]. This nicely showed that the smaller format gave rapid and more homogeneous tumour uptake, during the 1- to 3-h time frame, compared with the trastuzumab IgG that was restricted to around the vasculature, and also show that a too high affinity for the nanobodies hindered penetration (binding site barrier). The IgG, as shown by many, gave higher overall uptake by 24 h.

NON-ANTIBODY SCAFFOLDS

The 'non-antibody' binding format field continues to thrive because they promise to solve the problems presented by conventional antibodies such as expensive manufacturing, formulation/concentration, glycosylation, thermostability and tissue penetration. These scaffolds tend to range from ∼2 to 20 kDa (smaller than most antibody fragments), can be expressed at exceptionally high yield in *Escherichia coli*, selected by *in vitro* display, demonstrate higher stability and can be multimerized and built up according to the desired properties [41]. A few scaffold companies have published or disclosed intentions to develop SDCs, but other formats such as Anticalins, Avimers, Fynomers, Kunitz domains and Affilins have not gone down this route.

Affibody–Drug conjugates

Affibodies, based on the 6 kDa Staphylococcus protein-A, Z-domain can be engineered and displayed by phage to generate high-affinity binders. These are being developed as therapeutics by Swedish enterprise Affibody AB and are in Phase 2 clinical trials with a psoriasis therapeutic and a breast cancer positron emission tomography imaging agent [42]. No commercial affibody–drug conjugates have been disclosed, but conjugates have been described targeting HER2 (ZHER2891) with a vcMMAE payload (DAR1) with low nM potencies on high HER2-expressing cells lines [43]. Higher affinity, longer half-life, Fc-fusions had increased potency *in vitro* (130 pM) on SKBr3 cells [44]. More recently, the same affibody formats were coupled to the non-releasable DM1 payload (DAR1) resulting in higher *in vitro* potencies (270–470 pM, comparable to the trastuzumab ADC) and significant *in vivo* efficacy. Conjugates radiolabelled with ^{99}Tc showed marginally higher tumour uptake at 4 h at the expense of higher blood and normal organ uptake. Doses of 8.5 mg/kg, weekly five times were needed to see well-tolerated tumour growth delay of ∼20 days but no cures were seen in this first *in vivo* proof-of-principle of this scaffold format [45]. The same affibody was conjugated with photosensitizer payload pyropheophorbide-a (DAR1) with 12–23 nm IC_{50} potency on HER2-expressing cells. Well-tolerated cures were seen with a single injection of 20nMol of conjugate (∼0.2 mg dose/8 mg/kg) upon laser illumination with the rapid clearance being optimal to allow photo-activation without skin toxicity [46].

Fibronectin type III–drug conjugates

These popular scaffolds have been reviewed extensively [47] with a number investigated as drug conjugates. Immunoglobulin-like centyrins are ∼100-residue (11 kDa), thermal/chemical stable domains being developed by Janssen/J&J. Extensive surface cysteine scanning mutagenesis identified suitable conjugation positions and an anti-EGFR DAR1 MMAF conjugate demonstrated ∼0.2 nM IC_{50} *in vitro* potency [48]. No *in vivo* data have been

presented but a bioanalytic workflow was developed for centyrin–drug conjugate analysis in tissues in a collaboration between Janssen and Immunogen [49]. Clinical-stage adnectins are also based on the fibronectin domains and are being developed by BMS. Using a tubulysin analogue payload with a cleavable cathepsin B linker against the hepatocellular carcinoma antigen glypican-3, a DAR1 (via a maleimide moiety to a C-terminal cysteine thiol) adnectin–drug conjugate was made and evaluated [50]. One candidate conjugate had high thermostability ($T_m \sim 80°C$), 32 nM K_d binding affinity and 0.3 nM IC_{50} cell-kill potency on Hep3B cells *in vitro*. The payload conjugation had no deleterious effect on the conformation of the adnectin structure as supported by detailed hydrogen–deuterium exchange mass spectrometry [51]. No HLE strategy was employed as the authors favoured the rapid renal clearance (half-life approx. ½ h). Quantitative biodistribution showed very specific tumour uptake with renal exposure in the first few hours but low liver and other normal organ exposure. By 7 days, it was undetectable in all tissues other than the tumour. Most impressive was the well-tolerated, complete tumour cures at 0.12 mmol/kg (~1.4 mg/kg), despite the moderate affinity and rapid clearance given three times weekly. The authors acknowledge that this observation bucks the trend seen with small-format binders and suggest that the rapid internalization of the glypican-3 target may account for these promising results. It remains to be seen if such frequent dosing remains a viable option.

DARPin–Drug conjugates

The Designed Ankyrin Repeat (DARPin) class of scaffold proteins are well-established with five clinical-stage products, one (abicipar) recently completing a Phase 3 trial in ophthalmology [52]. Drug conjugates are much further away. Using bi-orthogonal chemistry, an anti-EpCAM DARPin with a (i) C-terminal cysteine residue and a (ii) non-natural amino acid azidohomo-alanine was used to attach a half-life extending albumin-binding domain and MMAF payload (DAR1). This generated a DARPin–MMAF conjugate with an IC_{50} 400 pM, which had extended plasma half-life (17.4 h *in vivo*) [53]. No *in vivo* or commercial developments have been disclosed, but there may be issues with this format given a recent FDC rejection setback [54].

Abdurin–Drug conjugates

Abdurins can be diversified to form libraries of binders as they are based on engineered IgG CH2 domains (~15 kDa); similar to the larger Fcabs being developed by F-Star, these retain the ability to bind to the neonatal Fc receptor and thus have an inherent extended serum half-life [55]. Recently, Abdurin–drug conjugates were described using Abzena's CyPEG and HiPEG conjugation technologies and vcMMAE payload (DAR1). Moderate *in vivo* efficacy (tumour regression at 5 mg/kg, six doses) was seen in PC3 xenograft studies. A DAR2 conjugate led to some cures but significant loss of target and FcRn-binding affinity was observed in various combinations most likely due to the small size taking an impact upon chemical modification [56].

PEPTIDE–DRUG CONJUGATES

Small peptides have even faster penetration and more rapid elimination properties compared with the above examples. Their totally synthetic nature promises many benefits as drug conjugates. This topic is covered extensively by He *et al* [57]. There are many reports, for example with low-potency payloads such as doxorubicin. These conjugates have micromolar potencies and are not usually more potent than the free drug, but generally more specific [58]. More recent innovations with potent payloads demonstrate more promising approaches including some at the clinical stage of development.

Cystine knot–drug conjugates

Cystine knots (30–50 amino acids, also known as knottins) are at the larger end of the peptide scale, but like peptides, are amenable to scalable solid-phase synthesis and incorporation of useful stabilizing and functionalizing non-natural amino acids. They have enhanced chemical, protease and thermal stability properties compared with conventional antibody domains, due to their highly compact structure and stabilizing disulphide bridges [59]. Conjugation to cytotoxic payloads was achieved via solid-phase synthetic incorporation of a non-natural amino acid followed by azide–alkyne conjugation of a gemcitabine payload. The Knottin–drug conjugate was able to overcome drug resistance in PANC-1 pancreatic cancer cells, increasing the potency of gemcitabine 25-fold [60]. A more 'ADC-like 'molecule was generated using cell-free protein synthesis with click chemistry (DAR2) and an appended Fc-domain. The MMAF payload DAR2 was used resulting in potencies similar to the gemcitabine conjugates but tumour growth delay was seen *in vivo* at 10 mg/kg given twice/week for 3 weeks [61].

Pentarins–drug conjugates

Pentarins and bicyclic peptides represent the shorter end of the peptide scale (2–5 kDa) and are worth mentioning due to the advanced clinical stage of their drug conjugates.

The pentarin (penetrate, target) portfolio developed by Tarveda, consists of small peptides that can be made into pentarin–drug conjugates (PDC). Their lead compound, PEN-221, is a somatostatin receptor-2 (SSTR2, expressed on neuroendocrine tumours) targeted DM1 maytansine. The payload is conjugated to the disulphide-cyclized Tyr3-octreotate, which had high affinity (~51 pM) and rapid internalization. *In vivo*, 1–2 mg/kg PEN221 were enough to cure HCC33 (liver) and H524MD (lung) cancer tumour models given four times on a weekly schedule with maximal payload uptake achieved within 2 h [62]. Results presented at the American Society for Clinical Oncology in 2018 showed that PEN221 was well-tolerated at doses up to 18 mg every 3 weeks with evidence of efficacy in the Phase 1 arm [63]. This product is now in Phase 2 clinical trials

for SSTR2-expressing neuroendocrine and lung tumours. A follow-up compound, PEN866, is a PDC carrying the SN38 payload targeting the heat-shock protein chaperone HSP90. This is currently in a Phase 1/2a clinical trial for advanced solid cancers sensitive to topoisomerase I inhibitors and recent updates at the European Society for Medical Oncology (ESMO) and American Association for Cancer Research conferences suggested good tolerability, signs of clinical efficacy [64] and promising clinical uptake and good PK profile [65].

Bicyclic peptide (Bicycle)–drug conjugates

The phage-displayable bicyclic peptide ('bicycles') technology discovered and developed by Heinis *et al*. [66] is being commercialized by Bicycle Therapeutics Ltd, including a major programme on Bicycle–drug (toxin) conjugates (BTCs). MT1-matrix metalloprotease (MMP) is overexpressed in multiple cancers including triple negative breast, non-small cell lung and soft tissue sarcoma. An anti-MT1-MMP BTC (BT1718) carrying a DM1 payload via a hindered disulphide linker has ~2 nM affinity, rodent cynomolgus species cross-reactivity and plasma stability of >20 h. It demonstrated efficacy in tumour models at 3 and 5 mg/kg BDC given twice weekly for 2–4 weeks. Complete cures were seen at 10 mg/kg with good tolerability as measured by body weight [67]. This product is currently in a Phase 1/2 clinical trial. An update from the ESMO identified a recommended Phase 2 dosing of 7.2 mg/m^2, once weekly with demonstratable tumour uptake and signs of efficacy [68]. A follow-up clinical candidate, BT5528 addresses the ephrin A2 receptor (EphA2) receptor (target for MEDI-547, a discontinued ADC that showed severe toxicity). Using a different payload, vc-MMAE, rapid tumour uptake was seen with persistent accumulation and rapid renal clearance in xenograft models. Payload conjugation had no adverse effect on the bicyclic peptide affinity (5.7 vs. 1.9 nM) and a rapid renal clearance was observed (half-life ~0.4–0.6 h in rodents and non-human primates). A weekly dose of 0.5 mg/kg (equivalent to 10–15 mg/kg of a similar ADC DAR2) gave rise to tumour regressions with tumours as large as 1000 mm^3 being treatable at doses of 3 mg/kg demonstration the penetration advantage over an ADC. As expected, non-cleavable variants were ineffective. A nice correlation was seen between EphA2 receptor level and tumour cure efficacy and none of the previously observed toxicities were seen when compared with a MEDI-547 equivalent ADC in rat or non-human primate toxicology studies [69]. BT5528 is in a Phase 1/II trial for solid tumours as a monotherapy and combination with checkpoint inhibitor nivolumab [70]. Other preclinical targets under commercial development include nectin-4 (BT8009) [71].

DISCUSSION

Antibody–drug conjugates are complex therapeutics to develop and those challenges remain into manufacturing. Non-IgG formats, as discussed here offer the possibilities of reduced manufacturing costs due to the easier chemistry manufacture control processes afforded by bacterial production, higher yields, lack of glycosylation and generally simplified analytics due to the smaller size. It remains to be seen if these features translate into economic or patient benefits.

Precision medicine is often a buzzword used loosely to describe tailoring a drug therapy to a patient's genetic profile but is increasingly being used in terms of other patient characteristics. Personalized dosing schemes to improve tumour penetration could be one key element [1] and having available formats to maximize tumour penetration will add to the clinical armoury. The increasing preclinical use of payload imaging technologies such as matrix-assisted laser desorption ionization mass spectrometry imaging [72] could inform this at the preclinical animal model level. Other strategies to aid penetration, such as addition of modulators to enhance penetration (e.g. RGD (Arginine-Glutamate-Aspartate) peptides, ligands to endothelial/epithelial cells that increase vascular permeability such as NRP-1, Lys/Arg-rich peptides [73] or TEM8 targeting for targeting stroma in solid tumours [74] or LRRC15, cancer-associate fibroblasts marker [75]) will require knowledge of an additional receptor making tailoring even more complex.

Collateral exposure through non-targeted deposition within normal tissues is recognized as a key driver to ADC–payload toxicity [76,77] with the well-characterized example of dose-limiting toxicity of trastuzumab–emtansine caused by Fc-mediated binding to platelets (thrombocytopenia) [78]. Most of these small-format drug conjugates promise to overcome this due to abolished Fc-receptor binding and reduced chronic exposure, but a clear correlation between improved tolerability and conjugate size would be difficult to demonstrate given the wide variation in formats.

Tumour spheroid technology is becoming more accessible and used in the discovery workflow and evaluating penetration can help to prioritize candidates. It is acknowledged that *in vitro* cell kill potency (IC_{50}) is a poor indicator of tumour cure efficacy as we and others find that it's not necessarily that the most potent conjugates make the best *in vivo* candidate [33]. Shah *et al* [79] modelled the correlation between *in vitro* IC_{50} and *in vivo* ID_{50} and shown that 27× more ADC was needed in the plasma compared with cell culture medium to achieve tumour growth 'stasis'. This shows that, in these models, there remain transfer barriers to solid tumour therapy and that smaller formats could make the real difference needed to address some of these difficult-to-treat solid tumours.

CONFLICT OF INTEREST STATEMENT

MD is an employee and shareholder in Antikor Biopharma Ltd and QX is an employee and shareholder in Essex Biotechnology Ltd.

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