Antibody Drug Conjugates for Cancer Therapy

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Abstract
Antibody drug conjugates (ADCs) constitute a family of cancer therapeutics designed to preferentially direct a cytotoxic drug to cells expressing a cell-surface antigen recognized by an antibody. The antibody and drug are linked through chemistries that enable release of the cytotoxic drug or drug adduct upon internalization and digestion of the ADC by the cell. Over 40 distinct ADCs, targeting an array of antigens and utilizing a variety of drugs and linkers, are undergoing clinical evaluation. This review primarily covers ADCs that have advanced to clinical investigation with a particular emphasis on how the individual targets, linker chemistries, and appended drugs influence their behavior.
I. Introduction

Most reviews on antibody drug conjugates (ADCs) begin by citing Paul Ehrlich’s prophetic “magic bullet” proposal from 1908 (Strehbhardt and Ulrich, 2008). In actuality, though, magic is an illusion, whereas the ragged, convoluted path that has led to the still incremental success of antibody drug conjugates today is painfully real. If one could administer a therapeutically effective dose of a highly pan-cytotoxic compound to a living organism without evoking toxicity, that would indeed be magic. So far, one cannot. It is fanciful to expect the pan-toxic compound to enter a cell of one type, in this case cancer, and kill it, but enter a cell of another type, such as liver, skin, or intestine, and remain benign. The idea that appending such a compound to a vehicle specifying its delivery would ameliorate said toxicity is highly intriguing, yet equally specious. The fact remains that only a very small portion of the ADC actually enters the intended target cell while the remainder goes elsewhere. That elsewhere includes normal tissues that catabolize the conjugate, releasing the active compound to wreak havoc on the catabolizing cell, as well as neighboring cells. The liberated drug can also diffuse into general circulation, resulting in systemic exposure to distant tissues.

That normal tissues lacking target are impacted by ADCs should not be too surprising. Antibodies exhibit extensive half-lives in vivo, but they do not last forever. Nor do they specifically home to their intended targets after systemic administration. The distribution and ultimate catabolic fate of antibodies has been shown to occur throughout the body in a variety of tissues (Henderson et al., 1982; Wright et al., 2000; Garg and Balthasar, 2007). The nonspecific pinocytotic uptake of ADCs into normal cells residing in these tissues may differ from target-dependent uptake, but in either case they are ultimately routed to the lysosome for degradation, whereupon the drug is unleashed. Although a monumental effort has been rightfully allotted to the identification of tumor-specific targets and the development of stable drug linkers, target-independent toxicity resulting from normal turnover of the antibody continues to vex the advancement of ADCs.

Although a forthright discussion regarding the current limitations of ADCs is warranted, significant advances have occurred, and with more on the horizon, there remains great cause for optimism. Two ADCs, Kadcyla (trastuzumab emtansine; Genentech, South San Francisco, CA) and Adcetris (brentuzimab vedotin; Seattle Genetics, Seattle, WA), have been Food and Drug Administration approved and enjoy widespread use in the oncology clinic (Jackson and Stover, 2015). These ADCs prove that the therapeutic index of an otherwise untenable cytotoxin can be elevated to a therapeutically beneficial level by affixing it to an antibody. Kadcyla and Adcetris are not without side effects, though, as thrombocytopenia and neuropathy, respectively, can limit their dosing, thus underscoring the need for further improvements. Because the therapeutic index of an ADC is a function of several components, there exist several entry points for potential improvements (Fig. 1). The choice of target, the antibody, the chemistry and site of drug attachment, and the nature of released drug all influence the risk benefit ratio of ADCs. Here I will attempt to address each of these components by outlining examples of their implementation and how they impact the behavior of ADCs.

II. The Drugs Conjoined With Antibodies

A. Doxorubicin

Some of the earlier iterations of ADCs tested in the clinic included the incorporation of antimetotics and antimetabolites approved for use in chemotherapy as unconjugated cytotoxins (Ford et al., 1983; Oldham et al., 1988; Elias et al., 1990; Petersen et al., 1991; Krauer et al., 1992; Takahashi et al., 1993). These early clinical ADCs suffered from a panoply of issues, not the least of which was a drug likely too impotent for targeted delivery. These studies also preceded the broad application of humanized or human therapeutic antibodies and reported frequent hypersensitivity reactions to the administered mouse monoclonal antibodies. Premature release of drug from the antibody also contributed to a loss of efficacy and, in some cases, toxicity. A subsequent iteration, again using a standard chemotherapeutic, doxorubicin, largely circumvented the hypersensitivity reactions with a human/mouse chimeric antibody. This immunoconjugate, referred to as BR96-doxorubicin, targeted the Lewis Y antigen and carried up to eight doxorubicin molecules per antibody conjugated through an acid labile linker (Hellstrom et al., 2001). Because of a lack of efficacy, BR96-doxorubicin failed to progress beyond a phase II metastatic breast cancer trial (Tolcher et al., 1999). A teachable element here was the significant difference in the nature of the toxicities—hematopoietic toxicity with free doxorubicin and gastrointestinal toxicity with the ADC. It was surmised that Lewis Y expressed in the gut—then a trigger to the toxici—was responsible for the contrasting toxicities. However, as discussed later in this review, toxicities not typically associated with a free drug can arise when it is conjugated to an antibody, irrespective of the antibody target.
the development of ADCs in the clinic are derived from C. Auristatins and Maytansinoids

leukemia (Shor et al., 2015). stage trials of relapsed/refractory acute lymphoblastic encouraging activity has been observed in earlier non-Hodgkin lymphoma failed to reach its endpoint, cies. Although a Phase III trial in relapsed/refractory CD22, has been investigated in hematologic malignan-
calicheamicin, inotuzamab ozogamicin, and targeting setting (Loke et al., 2015). A second ADC containing an evaluation of gemtuzumab ozogamicin in that AML patients with favorable cytogenetics prompting the human epidermal growth factor receptor II (HER2) showed remarkable activity in HER2-positive metastatic breast cancer in a Phase III clinical trial and was approved for use in this indication (Verma et al., 2012). The HER2 homolog HER1, commonly referred to as EGFR, has also been targeted by an ADC incorporating DM1 and is in early stage clinical testing.

B. Calicheamicin

The requirement for increased potency was met head on by conjugating the highly cytotoxic compound calicheamicin to a humanized antibody recognizing CD33 (Hinman et al., 1993). Calicheamicin is a bacterially derived antibiotic that binds somewhat specifically to oligopyrimidine-oligopurine sequences in the minor groove of DNA, leading to double strand scission. Calicheamicin exhibits cell killing potency that can exceed that of standard of care antimitotics by 1000-fold (Lee et al., 1987; Zein et al., 1988). A slightly less potent, but more stable, derivative of calicheamicin, N-acetyl-γ-calicheamicin dimethyl hydrazide, was used for incorporation into ADCs (Fig. 2). A calicheamicin ADC, gemtuzumab ozogamicin, targeted CD33 and was investigated for the treatment of relapsed and newly diagnosed acute myelocytic leukemia (AML) in a variety of single agent and combination clinical trials. Initial response rates appeared encouraging and led to market approval, but subsequent studies revealing excessive toxicity and a lack of improved overall survival when used in combination with standard of care therapy resulted in withdrawal from the market (Jurcic, 2012). Nevertheless, a retrospective meta-analysis uncovered an overall survival benefit in AML patients with favorable cytogenetics prompting an evaluation of gemtuzumab ozogamicin in that setting (Loke et al., 2015). A second ADC containing calicheamicin, inotuzumab ozogamicin, and targeting CD22, has been investigated in hematologic malignan-
cies. Although a Phase III trial in relapsed/refractory non-Hodgkin lymphoma failed to reach its endpoint, encouraging activity has been observed in earlier stage trials of relapsed/refractory acute lymphoblastic leukemia (Shor et al., 2015).

C. Auristatins and Maytansinoids

Currently, the most widely implemented cytotoxins in the development of ADCs in the clinic are derived from the natural product dolastatin 10, originally isolated from a sea hare and later found to be synthesized by cyanobacteria ingested by it (Pettit et al., 1987; Luesch et al., 2002). Comprised of a 4-mer peptide of unconventional amino acids capped with a C-terminal dolaphenine (Fig. 2), dolastatin 10 disrupts microtubules and kills cells with a potency 20–50 times greater than that of vinblastine (Bai et al., 1990). Several clinical trials investigating the antineoplastic activity of dolastatin 10, and a synthetic derivative TZT-1027, were performed but objective responses were not achieved (Singh et al., 2008a). Further derivatives of dolastatin 10, containing substitutions at the C-terminal dolaphenine with nor-ephedrine or phenylalanine, yielded auristatins E and F, respectively (Maderna et al., 2014). Omission of a methyl group from the N terminus afforded the respective monomethyl analogs monomethylauristatin E/F (MMAE and MMAP, respectively), containing a secondary amine amenable to chemical derivatization with linkers for conjugation to antibodies (Doronina et al., 2003). These two compounds, predominantly MMAE, are the most common cytotoxic agents employed in ADCs currently under clinical investigation (Table 1). One of the MMAE conjugates, Adcetris (brentuximab vedotin), received approval by the Food and Drug Administration in 2011 for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma (Younes, 2014).

Like dolastatin 10, maytansines was originally isolated as a natural product that displayed highly potent cytotoxic activity resulting from its ability to disrupt microtubule polymerization (Kupchan et al., 1972; Lopus et al., 2010) (Fig. 2). However, a paucity of objective responses in the cancer clinic hampered further develop-
men of maytansine as a chemotherapeutic (Ravry et al., 1985; Cassady et al., 2004). Interest in its anti-
neoplastic activity was reinvigorated by the incorpora-
tion of maytansinoid thiol derivatives into antibody drug conjugates (reviewed by Lambert, 2013). Two maytansine derivatives, termed DM1 and DM4, differing primarily by the degree of methylation on the carbon atom adjacent to the disulfide bond (Kellogg et al., 2011), are undergoing clinical investigation as ADC warheads attached to various antibodies (Table 1). One of the DM1 ADCs, ado-trastuzumab emtansine (Kadcyla), target-
ging the human epidermal growth factor receptor II (HER2) showed remarkable activity in HER2-positive metastatic breast cancer in a Phase III clinical trial and was approved for use in this indication (Verma et al., 2012). The HER2 homolog HER1, commonly referred to as EGFR, has also been targeted by an ADC incorporating DM1 and is in early stage clinical testing.

D. Pyrrolobenzodiazepine

Although maytansines and auristatins, both of which disrupt microtubule dynamics, dominate the ADC clinical landscape, additional early stage clinical studies have been initiated with ADCs containing highly
potent DNA damaging agents. In particular, a synthetic dimerized derivative of pyrrolobenzodiazepine (PBD), an anthramycin class of antibiotic conjugated to an antibody targeting CD33 (Kung Sutherland et al., 2013), has recently advanced into the clinic for testing against acute myeloid leukemia (Stein et al., 2014) (Fig. 2, Table 1). As a free drug, PBD dimers are among the most potent cytotoxic compounds ever identified, exhibiting potency in cell killing assays reaching GI50 values in the low to even subpicomolar range (Hartley, 2011).

Fig. 2. Structures of the drugs and their derivatives used in ADCs. Linkers and drug release mechanisms (red lines) are illustrated in the context of the ADC.
PBD dimers bind in a semisequence selective manner to the minor groove of duplex DNA. Their cytotoxicities are attributed to the formation of adducts to guanine residues on opposing strands of DNA, resulting in interstrand crosslinks (Gregson et al., 2001; Hartley et al., 2004). One of the PBD dimers, SG2000 (SJG-136), entered clinical investigation for the treatment of epithelial ovarian, primary peritoneal, or fallopian tube cancer, and although some of these studies were terminated, its assessment in advanced chronic lymphocytic leukemia and acute myeloid leukemia currently remain open (ClinicalTrials.gov Identifier: NCT02034227).

E. SN-38

As discussed above, most of the drug components so far employed in the ADCs that have advanced to the
The camptothecin analogs promote DNA strand breaks, their mechanism of action differs from calicheamicin and the PBDs, which interact directly with the DNA duplex. Camptothecin analogs, by contrast, cannot bind to DNA alone, but interact with the topoisomerase-DNA complex (Redinbo et al., 1998; Liu et al., 2000) resulting in stalled DNA replication forks.

**F. Duocarmycins**

An additional class of DNA damaging agents, CC-1065 and the related duocarmycins, has also been applied in ADC technology (Shor et al., 2015) (Fig. 2). These agents interact with the minor groove of duplex DNA and alkylate adenine residues, consequently promoting strand breaks (Boger and Johnson, 1995). A first in human Phase I clinical study of SYD985 was recently opened in which an anti-HER2 antibody conjugated to a duocarmycin is being investigated in HER2-positive breast cancer (ClinicalTrials.gov # NCT02277717). It is
anticipated that this ADC might impact HER2-positive tumors expressing lower levels of HER2 than that associated with the diagnostically positive 3+ tumors targeted by Kadcyla (van der Lee et al., 2015). An additional ADC, MDX-1203, contained an analog of CC-1065 coupled to an antibody targeting CD70 (Thevanayagam et al., 2013). A Phase I clinical investigation of its potential utility in renal cell carcinoma and non-Hodgkin’s lymphoma was recently completed (ClinicalTrials.gov # NCT00944905). In this dose escalation study, a relatively high dose of 15 mg/kg was reached, whereupon a dose-limiting toxicity of hypersensitivity in 2 of 16 patients was encountered, but an Maximum Tolerated Dose was not defined (Owonikoko et al., 2014). Additional adverse events included fatigue (85%) and nausea (54%), as well as other common constitutional symptoms, whereas delayed toxicities, described as facial edema and/or pleural or pericardial effusions, were observed in 6/16 (38%) subjects treated at the 15 mg/kg dose. Best response of stable disease was reported for 18 of 26 patients, although it did not correlate with dose level.

G. Other Chemotypes

Preclinical studies describing additional microtubule disrupting agents, such as novel taxoids and tubulysins, that have been conjugated to antibodies also appear interesting (Ojima, 2008; Cohen et al., 2014). Particularly noteworthy is the preclinical assessment of the cytotoxin amanitin as an ADC payload (Fig. 2). Amanitin represents a significant deviation from the microtubule disrupting and DNA damaging agents most commonly used in ADCs. Derived from poisonous mushrooms, amanitin is an octomer of cyclized amino acids that binds to mammalian RNA polymerase II with high affinity, thereby disrupting DNA transcription and causing cell death (Coche t-Meilhac and Chambon, 1974). Free amanitin is only poorly diffusible across cell plasma membranes and requires specific organic anion transporters, with restricted tissue expression, to facilitate its transport into cells (Letschert et al., 2006). Thus conjugating amanitin to an antibody results in highly specific toxicity to cells bearing the antibody target antigen. A comparison of free amanitin toxicity to that of an amanitin ADC targeting Epithelial Cell Adhesion Molecule (EpCAM), resulted in IC₅₀ values that were up to 10,000-fold lower on EpCAM-positive cell lines relative to free amanitin (Moldenhauer et al., 2012). In vivo antitumor activity was also apparent at doses considerably lower than that which induced toxicity in mice.

In closing this section, it should be noted that a primary distinction between microtubule disrupting drugs, such as the auristatins and maytansines, and some of the aforementioned DNA damaging agents, is the increased propensity of the latter drugs to kill nonproliferating cells (Shor et al., 2015). This may be viewed as an advantage in the context of treating indolent cancers or exterminating so-called cancer stem cells that may undergo slow rates of cell division. However, the destruction of normal stem cells, as well as any nonregenerating cell types, particularly endothelial cells, could yield unacceptable toxicities.

III. Antibody-Drug Linkers

A. Stability and Drug Release

The term "stability" is frequently bandied about when discussing ADCs, where it typically refers to retention of drug by the antibody either ex vivo in buffers, plasma, or blood or in vivo after administration. However, the antibody itself can be destabilized by drug conjugation, resulting in faster clearance of total antibody postdosing (Fig. 3A). Finally, the term "stability" can also apply to the ultimate liberation of drug upon cellular uptake and catabolism of the ADC. For the sections here on drug linkers and their sites of attachment, discussions on stability will primarily refer to extracellular release of drug from antibody.

![Fig. 3](image-url)
The rapid release of drug after administration of the ADC seems counterintuitive with respect to fulfilling the "magic bullet" prophecy. Accordingly, the evolution of antibody-drug linker chemistry was largely guided by the desire to maintain stability during systemic circulation followed by release upon internalization of the ADC into cancer cells. In many respects, the ADC linker tenets echo the general ambitions of prodrug therapy, aptly described by Singh et al. (2008b), “Thus, the major objective of prodrug design is to temporarily alter the physicochemical properties of drugs to accomplish modification of drug pharmacokinetics, prolongation of action, reduce toxicities and side effects, increased selectivity, and resolve formulation challenges.” Upon internalization, the ADC is routed to the lysosome, where in contrast to plasma, the highly hydrolytic environment is acidic and replete with proteases. Accordingly, acid labile and protease susceptible linkers were implemented (Hamann et al., 2002; Doronina et al., 2003). The high levels of reducing equivalents in the cell cytoplasm, relative to that in plasma, have also been exploited by using linkers with reducible disulfide bonds (Chari et al., 1992). These three modalities of drug release—proteolysis, reduction, and pH-catalyzed hydrolysis—account for all of the ADCs in clinical testing in which cleavable linker technology is employed (Table 1).

B. Noncleavable Linkers

Quite surprisingly, it is not always necessary to release the drug from the amino acid it is appended to in the antibody. Kadcyla, approved for use in human cancer, is prepared using the crosslinking agent succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), leaving the nonreducible thioether MCC linker positioned between antibody lysine residues and DM1 (Fig. 2). With this linker, the resulting drug remains attached to a lysine residue upon internalization and digestion of the antibody in the lysosome yet retains its ability to kill cells (Erickson et al., 2010). In the case of Kadcyla, preclinical efficacy was comparable to that observed with the anti-HER2 ADC containing maytansinoids coupled through reducible linkers (Lewis Phillips et al., 2008). A noncleavable linker, maleimidocaproyl (MC), is also used with the auristatin ADCs in which MMAF is the indicated cytotoxin (Table 1). Here, the resulting drug retains the linker plus a cysteine residue derived from the antibody (Doronina et al., 2006). It should be noted that although the MCC and MC linkers described here do not contain a cleavable bond, a retro-Michael reaction involving the maleimide moiety is possible and can result in deconjugation of drug from antibody in vivo or in other matrices (Alley et al., 2008; Pillow et al., 2014).

A common feature of the noncleavable linkers used for DM1 and MMAF is that the released drug derivatives are hydrophilic and thus not very potent when added to intact cells (Doronina et al., 2006; Erickson et al., 2010). This suggests that the free drug released from cells expressing antibody target may be less impactful to neighboring cells not expressing the target. This "bystander effect," or lack thereof, could have implications for treating tumors heterogeneously expressing the target. This was borne out in preclinical studies using xenograft tumors that homogenously or heterogeneously expressed the target of maytansinoid ADCs containing the cleavable or noncleavable linkers (Kovtun et al., 2006). Tumors with homogenous expression could be eradicated with either ADC, whereas only the ADC with the cleavable (reducible) linker was effective against heterogeneous tumors. This implies that Kadcyla, which contains the noncleavable linker, does not require bystander killing, perhaps owing to the exceptionally high level of amplified HER2 expression on the selected breast cancers for which it is indicated.

C. The Impact of Linker on Toxicity

The impact of released drug substance on cells lacking ADC target also has obvious safety implications. Despite having comparable efficacy, ADCs with maytansinoids linked through reducible linkers produced greater weight loss in rats than an ADC conjugated via the noncleavable thioether bond (Lewis Phillips et al., 2008). Moreover, in clinical testing, some of the adverse events associated with maytansinoid ADCs appear to be a function of the linker used for drug attachment. For example, thrombocytopenia is a common dose-limiting toxicity associated with Kadcyla, containing a nonreducible bond to DM1 (Krop and Winer, 2014). By contrast, hematologic toxicities were unremarkable with lorvotuzumab mertansine (hu901DM1), containing the highly reducible N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP) linkage to DM1 and targeting CD56. Instead, peripheral neuropathy, unremarkable with Kadcyla, was a common adverse event with lorvotuzumab mertansine (Berdeja, 2014). SAR3419, containing DM4 conjugated through the hindered reducible SPDB linker, also did not evoke serious hematologic toxicity, but instead ocular toxicity was frequently noted (Younes et al., 2012a).

Although these maytansinoid ADCs target different antigens, the target is not likely responsible for the distinct, linker-related, aforementioned toxicities. Other ADCs, sharing the same drug and linker but targeting divergent antigens, exhibit overlapping adverse events. Neuropathy was again a frequent side effect with MLNM2704, containing SPP-DM1 and targeting Prostate Specific Membrane Antigen (PSMA) (Galsky et al., 2008), yet the expression patterns of PSMA and CD56 differ significantly. The neuropathy occurring in response to a cell-permeable tubulin binding agent is not surprising because this is commonly associated with standard of care chemotherapeutic taxanes and vinca alkaloids. Accordingly, one might anticipate peripheral neuropathy with MMAE ADCs.
containing cleavable peptide linkers that release cell-permeable free MMAE. This is indeed a common toxicity with the VC-MMAE conjugates, but not frequently noted with the noncleavable MC-MMAF auristatin ADCs, which release a free drug substance with reduced potency on intact cells. Together, this implies that the peripheral neuropathy associated with maytansinoid and auristatin-based ADCs containing cleavable linkers may be a bystander effect resulting from the release of cell-permeable drug products originating from the catabolized ADC.

The ocular toxicity associated SAR3419 is again inconsistent with the expression pattern of the target CD19, which is largely restricted to blood cells but is common to the SPDB-DM4 linker-drug configuration. Similar ocular toxicities, variably described as epitheliopathy, keratitis, dry eye, and blurred vision, have been observed with IMGN-853 and BT062, targeting the folate receptor and CD138, respectively (Jagannath et al., 2011; Kurkjian et al., 2013). Although cleavable through reduction, the SPDB linker is highly stabilized relative to SPP, thereby prolonging retention of drug by the antibody during circulation. Thus, the resulting enhancement to overall exposure of normal tissues, such as eye, to intact ADC could contribute to toxicities not observed with more labile linkers. Grades 1 and 2 eye disorders were also noted for Kadcyla, containing the highly stable SMCC linkage to DM1 (Burris et al., 2011a).

Adverse ocular events are not typically associated with cleavable VC-MMAE ADCs but are noted with auristatin ADCs employing the stable MC-MMAF linker drug configuration (Forero-Torres et al., 2014; Tannir et al., 2014). In the case of the auristatin ADCs, it is more difficult to ascribe the ocular toxicity simply to increased stability of the intact ADC. Despite the lack of a cleavable bond, the MC-MMAF conjugates liberate their drugs in vivo at a rate comparable to their VC-MMAE counterparts (Alley et al., 2008). This is because pharmacological deconjugation occurs through maleimide/thioether fragmentation, resulting in loss of the entire linker-drug structure, a mechanism common to both the cleavable VC- and noncleavable MC-linker auristatin conjugates. Nevertheless, increased overall exposure of the MC-MMAF ADCs may still account for ocular toxicity, because they are typically more tolerable and thus dosed at higher levels than VC-MMAE ADCs. Finally, the enhanced exposure argument may also be consistent with side effects associated with Abraxane (nab-paclitaxel; Celgene, Summit, NJ), a protein-bound form of paclitaxel, relative to free paclitaxel. Interestingly, although ocular toxicities are not characteristic of free paclitaxel, ocular and visual disturbances occurred in 13% (n = 366) of patients treated with Abraxane (Package Insert, 12/2014). Overall, this suggests that the ocular events associated with stable ADC linkers are, in part, an outcome of increased exposures unattainable with less stable linkers that may be limited by other toxicities occurring at lower exposures.

D. Limitations of Linker Stability

The means to achieve near complete stability of the antibody drug bond exist, but whether this is even beneficial remains unclear. As a proof of concept, Alley et al. (2008) incorporated a bromoacetamidocaproyl linker to obtain auristatin-based ADCs that underwent no measurable systemic loss of drug for 2 weeks. However, despite a 25% increase in exposure, relative to the reference ADC containing the maleimidocaproyl linker, no appreciable gain in efficacy was observed. This implies an upper limit to the advantages of increased linker stability. Conversely, a measure of instability may be advantageous in some cases. A requirement for an unstable linker was recently described for IMMU-130, an ADC targeting CEACAM-5 and containing a pH-sensitive carbonate bond to SN-38 (Govindan et al., 2015) (Fig. 2). Nearly half of the eight drugs appended to the antibody were liberated within 20 hours in human serum. Nevertheless, preclinical antitumor activity was demonstrated, albeit with a relatively generous dosing schedule of 25 mg/kg of protein, twice weekly. The requirement for an unstable linker was made evident by testing a similar conjugate containing a systemically stable protease cleavable linker, which proved to be ineffective. This strategy, which results in considerable systemic release of free drug, may be particularly amenable to ADCs containing drugs, like SN-38, that have reduced potencies relative to those used in most ADCs.

IV. Sites of Conjugation

A. Stochastic Conjugation

The vast majority of the ADCs currently under clinical investigation are comprised of linker-drug moieties that are conjugated to either lysine or cysteine residues resident to the native composition of the antibody. The maytansinoids are derivatized with a succinimide ester and reacted with antibody in a specified molar ratio, randomly derivatizing up to 20 different lysines in the heavy and light chain subunits (Wang et al., 2005). The resulting ADC may have an average drug-to-antibody ratio (DAR) of approximately four, whereas any individual molecule may have a DAR ranging from zero to eight (Dere et al., 2013). The auristatins are derivatized with maleimide, which enables a reaction with free thiols, made available by reduction of the cystine disulfide bonds that normally link together the antibody subunits (Doronina et al., 2003). An IgG1 contains four such disulfides, two between heavy-heavy chain and one each for heavy-light chain connections, yielding eight possible free cysteine residues for drug conjugation. Although maximizing the DAR is intuitively tempting, if overloaded...
the hydrophobic nature of maytansinoids and auristatins can drive antibody aggregation and negatively impact pharmacokinetics (Hamblett et al., 2004; Chari, 2008). Despite derivatization of all eight interchain cysteine residues with MMAE, these purified ADCs appear identical in size to underderivatized native IgG, bind target, and kill cells in vitro with potency commensurate with their high drug load (Hamblett et al., 2004; Adem et al., 2014). However, ionic or thermal stress revealed a disproportionate tendency for the high DAR species to aggregate and fragment (Adem et al., 2014). Importantly, potency in vivo was severely compromised by rapid clearance of the DAR8 ADC, whereas DAR4 appeared more optimal (Hamblett et al., 2004). The implied relationship between DAR, clearance, and efficacy is illustrated in Fig. 3B.

B. Uniform Site-specific Conjugation

To circumvent some of the issues associated with nonuniform drug loading, site-directed conjugation was developed. The prototypical version involved substitution of a specified amino acid in the IgG1 heavy or light chain with a cysteine, resulting in two exclusive sites of conjugation per antibody tetramer (Junutula et al., 2008). The resulting THIOMAB-MMAE conjugate surprisingly exhibited efficacy comparable to a stochastically drugged ADC containing nearly twice as much MMAE after dosing of equimolar amounts of antibody protein. Improvements in safety were also noted, culminating in an enhanced apparent therapeutic index. A comparison of THIOMABs containing MMAE appended to distinct sites, revealed a pronounced impact of conjugation site on linker-drug stability, which in turn, dramatically impacted efficacy (Shen et al., 2012). Thus proper conjugation site selection offers an alternative approach to chemical modification as a means to achieve enhanced stability. Cysteines may also be engineered into the carboxy- or amino-termini of IgG polypeptides where they can be reacted with drugs derivatized with a sulfhydryl or aldehyde group (Bernardes et al., 2013).

Several alternatives to the THIOMAB technology, facilitating conjugation of drugs at specific sites, have recently emerged (reviewed by Panowksi et al., 2014). One of them involves the use of the amino suppressor tRNA/aminocyl-tRNA synthethase pair that enables coded translation of an unnatural amino acid into a specified position in the antibody. Incorporation of p-acetylphenylalanine provides a keto group as a reactive site for formation of a stable oxime with an alkoxyamine derivatized drug (Axup et al., 2012). Tested as an anti-HER2-p-acetylphenylalanine-auristatin conjugate, the ADC inhibited xenograft tumor growth. The clearance and exposure parameters of the ADC were very similar to the corresponding naked antibody. An additional embodiment in which an orthogonal reactive group is introduced involves the incorporation of a selenocysteinic amino acid at the C terminus of the IgG polypeptide chain (Hofer et al., 2009). This was accomplished by engineering a 3’ TGA codon along with a proximal selenocysteine insertion sequence element derived from the thioredoxin reductase 1 cDNA. The TGA then codes for incorporation of the naturally occurring amino acid selenocysteine at the C terminus of the modified IgG chain. The seleno group offers a unique site on the antibody for electrophilic attack, resulting in covalent conjugation of appropriately derivatized compounds.

Additional methods for site-specific conjugation include aldehyde tagging, wherein a short consensus sequence, CXPXR, is engineered into eight different regions of the IgG heavy and light chain polypeptides (Drake et al., 2014). The sequence is recognized by formyl glycine generating enzyme, resulting in the conversion of the consensus cysteine to a formyl glycine containing a reactive aldehyde group. Hydrazino-isopictet-spengler chemistry was then used to generate a stable bond to the reactive aldehyde group. An example conjugate containing maytansine appended to anti-HER2 was found to be stable and efficacious in vivo. Three independent insertion sites were evaluated, and similar to the THIOMAB, the site of conjugation impacted the stability and corresponding efficacy of the ADCs. In another approach using a transposable consensus sequence, Strop et al. (2013) exploited microbial transglutaminase (mTG), which catalyzes cross-linking of glutamine side chains to primary amines. Hence, a primary amine acyl acceptor can be covalently coupled to the glutamine in the presence of mTG. The glutamine tag, LLQG, was inserted into a variety of positions in the IgG chains, and a number of highly reactive sites were identified. One of the reactants tested was acyl-lysine-monomethyl dolastatin 10 (MMAD), resulting in a potent ADC with an approximate DAR of two. Preclinical efficacy of an acyl-lysine-MMAD ADC targeting the antigen M1S1 was impressive and comparable to that of a stochastically drugged ADC containing nearly twice as much MMAD. Site-dependent differences in pharmacokinetics, particularly in rat, were again noted.

Dennler et al. (2014) demonstrated an additional method involving mTG catalyzed crosslinking, but lacking the requirement for any engineering of the antibody cDNA sequence. The approach takes advantage of conserved heavy chain residue glutamine-295, which is the only site recognized by mTG in deglycosylated IgG. Although direct conjugation of auristatins prederivatized with linker containing the amine donor was demonstrated, the yields were poor and required a 40-fold excess of linker-drug per GLN-295 site. Better yields were realized, at much lower linker-drug to GLN-295 site ratios, when bifunctional linkers containing the amine donor at one end, and either an S-protected thiol or azide at the other, were first coupled to GLN-295.
This was followed by reacting the appropriately derivatized auristatin linker-drug construct with the pre-existing GLN-295 adduct. One potential liability is the lack of carbohydrate on the antibody, although it remains unclear whether this would affect the pharmacokinetics or activity of an ADC in humans.

Direct chemical modification of antibody carbohydrate to a site reactive for conjugation was described by Zuberbuhler et al. (2012). Here, sodium periodate was used to selectively oxidize the fucose moiety in the N-linked glycan to an aldehyde, thereby making it reactive with a hydrazide derivative of a dolastatin. The resulting hydrazone trigger in the ADC is not particularly stable, though, exhibiting a half-life of about 18 hours at physiologic pH in phosphate-buffered saline. Carbohydrate as a site of antibody conjugation was also described by Okeley et al. (2013), but in this scheme the reactive site was generated by metabolic incorporation of derivatized fucose into the N-linked glycan. The antibody is produced by Chinese hamster ovary cells in media containing 1 mM of a N-linked glycan. The antibody is produced by Chinese hamster ovary cells in media containing 1 mM of a fucose analog substituting for native fucose as a substrate for fucosyltranseferase VIII. Thus, incorporation of 6-thiofucose into the glycan presented a unique free thiol that facilitated conjugation by Michael addition with a maleimide containing linker drug. The ADC drugged with MMAE in this fashion was quite stable, losing only 15% of its drug over 4 days in plasma. The average DAR was only 1.3, largely owing to incomplete incorporation of the fucose analog into the glycan.

V. Targets

Assessing both the relative expression level of a target on tumor and normal tissues and its representation across a large panel of tumors is a critical first step in selecting a target for ADC therapy. This was facilitated markedly by the advent of high-throughput technologies enabling the measurement of thousands of mRNA transcripts across thousands of tissue samples. One could readily identify putative cell surface proteins overexpressed in a significant portion of tumors while lacking expression in particularly vital or regenerative normal tissues. Subsequent validation steps included verifying the abundance and cell surface localization of the target protein as well as its ability to internalize a bound antibody. However, the paradigmatic target—highly expressed on cancer cells and altogether absent on normal cells—is extremely rare, if not nonexistent. As some clinical failures attest, the target does matter, both for efficacy and safety. It is far too expansive to cover all targets entertained for ADC therapy, but some select examples can help convey lessons learned from the clinical experience.

A. Human Epidermal Growth Factor Receptor II—Is Level of Expression Important?

It is tempting to trumpet the success of Kadcyla (Trastuzumab-SMCC-DM1) as a harbinger of future triumphs of ADCs in solid cancers. However, with its multimillion copies of oncogenic receptor per tumor cell driven by DNA amplification and a drug conjugated to an already functionally neutralizing antibody, HER2 is hardly a representative ADC target (Kallioniemi et al., 1992; Burris et al., 2011b). Therefore it is difficult to gauge the general applicability of the SMCC-DM1 linker-drug used in Kadcyla based solely on the HER2 experience. The SMCC linker yields a released maytansinoid drug with little bystander cell killing (Erickson et al., 2010), yet performs quite well in the context of Kadcyla. One could conclude that the exceptionally high and relatively homogenous target expression of HER2 may enable use of the SMCC linker. However, the results of additional clinical investigations with SMCC-DM1 ADCs, such as AMG 172 and AMG 595, targeting CD70 and EGFR (Table 1), respectively, should help determine whether this linker-drug configuration has broad utility. We have learned from Kadcyla that the level of target expression may correlate with outcome. For patients with tumors diagnostically HER2 positive by fluorescent in situ hybridization, the overall response rate was 40% compared with 20% for those scored as HER2 normal (Krop et al., 2012). In this study, an exploratory analysis in which only HER2-positive tumors were further ranked by reverse-transcription polymerase chain reaction or fluorescent in situ hybridization revealed a trend for improved outcome for patients whose tumor biopsy samples scored above the median. Additional positive correlations between HER2 mRNA transcript levels, as measured by reverse-transcription polymerase chain reaction, and patient outcomes have been reported in other studies (Burris et al., 2011a; Perez et al., 2014).

B. CD30—Does Antibody Effector Function Contribute to Efficacy?

To what extent does the recruitment of effector cells by the antibody portion of the ADC contribute to its activity? To address this, Adcetris (brentuximab vedotin), which targets CD30 and produces excellent response rates in refractory Hodgkin’s lymphoma (Younes, 2014), provides an example less encumbered than Kadcyla, because unlike HER2, it is not expressed at unusually high levels nor is it oncogenic. It is a member of the tumor necrosis factor receptor family, and although its function remains unclear, a variety of mouse models implicate it in the regulation of autoimmune responses. Normal tissue expression of CD30 is primarily restricted to activated immune cells, whereas positive lymphoid cancers express considerably higher
levels. Also dissimilar to HER2, naked antibodies to CD30, including SGN-30 used in Adcetris, have not generated compelling responses in the clinic, particularly in Hodgkin's lymphoma (reviewed by Kumar and Younes, 2014). Moreover, a variety of clinical investigations were initiated, including radioactive and proteinaceous toxins appended to anti-CD30, as well as bispecific antibodies, but none have progressed beyond early stage clinical testing (Kumar and Younes, 2014). Together, these clinical failures, especially that of SGN-30, contrasted with the remarkable success of Adcetris, suggest that anti-CD30 itself contributes little in the way of effector function but rather acts primarily as a means for specifying the delivery of MMAE. Nevertheless, one could credit some of the success of Adcetris to its application in hematologic cancers, where approved antibody therapeutics are disproportionately represented (Niwa and Satoh, 2015). Numerous disparate clinical ADC trials (Table 1), wherein the same VC-MMAE linker-drug configuration in Adcetris is aimed at other targets, will help define the specific attributes that contribute to success.

C. CD79 versus CD22—How Important Is the Specific Target?

It also remains possible that CD30 is simply a spectacular target for ADC therapy. Although we have learned from clinical correlations with Kadcyla that high target expression is beneficial, the rules that determine the utility of a potential ADC target remain murky. The degree to which the specific target per se influences safety and efficacy of an ADC can be glimpsed from a recent Phase II trial in which antibodies to CD22 and CD79b, both conjugated to VC-MMAE, were tested side by side in relapsed/refractory non-Hodgkin's lymphoma (Morschhauser et al., 2014). CD79b is a positive signaling component of the B-cell receptor, whereas CD22 is a B-cell specific transmembrane glycoprotein that negatively regulates antigen receptor signaling (Chu and Arber, 2001; Sullivan-Chang et al., 2013). Both targets are detected on normal adult B-cells and amply expressed on the vast majority of NHL. The two corresponding ADCs equipped with VC-MMAE produced similar findings in preclinical efficacy and safety studies, including the target-dependent killing of normal B-cells in nonhuman primates (Dornan et al., 2009; Li et al., 2013). In the Phase II trial, the two ADCs, combined with Rituxan jointly by Biogen-Idec (Cambridge, Mass) and Genentech (South San Francisco, CA), were well-tolerated with similar primary toxicities, consisting of neutropenia, peripheral neuropathy, and diarrhea, and very comparable overall response rates in diffuse large B-cell lymphoma- 22/39 (6 CR+16 PR) and 24/42 (10 CR+14 PR) for CD79b and CD22, respectively. So, despite their different structures and opposing functions, the two targets are nearly indistinguishable with respect to MMAE ADC therapy. This is not to say that target selection is irrelevant—CD22 and CD79b satisfied a battery of preclinical qualifications, including efficacy and safety, before consideration for further development in the first place. But, having qualified, they seem equally competent despite their differing functional and biochemical backgrounds.

D. gpNMB and CD44—Is There Dose Limiting Target-dependent Toxicity?

These two targets have very little in common biologically, structurally or otherwise, and the clinical studies targeting them with ADCs used different antimitotic drugs. Nevertheless, they share a common side effect not common to other ADCs containing their respective antimitotic drugs. Glycoprotein nonmetastatic melanoma protein B (gpNMB), the melanoma-related glycoprotein homolog of the pigment forming protein premelanosome protein 17 (PMEL-17), is expressed on the surface of epidermal melanocytes in situ (Tomihari et al., 2009; Naumovski and Junutula, 2010). Glembatumumab vedotin (anti-gpNMB-VC-MMAE) has been investigated in advanced melanoma, where skin rash was identified as the most common adverse event (Ott et al., 2014). At the MTD of 1.88 mg/kg administered every 3 weeks, 30% of patients experienced grade 3 or higher skin rash. Nevertheless, partial responses were observed in 13% (5/40) of this cohort, and the MTD, although attained for different reasons, is similar to that reached for the approved VC-MMAE ADC Adcetris (Younes et al., 2012b). A separate study involving anti-CD44v6 conjugated to DM1 also evoked skin toxicities, again not typically observed with maytansinoid conjugates. In this Phase I study of bivatuzumab mertansine, 24/31 patients experienced dose-dependent skin events involving rash, blister formation, skin desquamation, which included a fatal case of toxic epidermolysis, ultimately prompting discontinuation of the investigation. The expression of CD44v6 on normal keratinocytes likely accounted for the skin toxicities. It seems that the lesson learned from these two ADC studies is to avoid targets expressed in normal skin tissue.

E. NaPi2b and Mesothelin—Will Expression on Normal Tissue Always Result in Target-dependent Toxicities?

Although it is clear that high normal tissue expression can present a liability, it is not universally the case. The target sodium phosphate transporter 2b (NaPi2b), a sodium phosphate transporter and product of the SLC34A2 gene, is well expressed in normal lung tissue and is clearly critical for normal function there, as evidenced by the linkage of SLC34A2 germ line mutations to heritable pulmonary alveolar microlithiasis (Traebert et al., 1999; Corut et al., 2006). Yet in a Phase I study in which anti-NaPi2b-VC-MMAE was administered to 30 ovarian cancer patients, including 18 treated...
at 1.8–2.8 mg/kg, pulmonary toxicity was not reported (Gordon et al., 2013). Most toxicities were constitutional, whereas those resulting in dose limitations, such as peripheral neuropathy, were generally consistent with VC-MMAE ADCs aimed at orthogonal targets. Thus the propensity for target-dependent toxicity may depend on the particular normal tissue expressing the target. The reasons for this remain unclear, but could relate to the selectivity of antimitotic drugs, including MMAE and DM1, for proliferating or regenerative tissues, such as skin and bone marrow. By contrast, lung tissue, in the absence of injury, is not highly regenerative. Secondly, NaPi2b expression is confined to the apical surface of large cuboidal type II pneumocytes (Traebert et al., 1999), which could potentially hinder the access of the ADC to the target in normal tissue.

Mesothelin (MSLN), a 40-kDa glycosphatidylinositol-anchored protein, is expressed normally on the surface of mesothelial cells lining the pleura, peritoneum, and pericardium and is overexpressed on a variety of solid tumors (Hassan and Ho, 2008). An ADC targeting MSLN and armed with VC-MMAE was evaluated in a Phase I clinical trial for the treatment of pancreatic cancer (Weekes et al., 2014). Some grade 3/4 toxicities, including neutropenia, AST/ALT elevation, and fatigue were reported but were not consistent with disruption of mesothelial linings. By contrast, the targeting of MSLN with the recombinant immunotoxin SS1P [SS1(dsFv) PE38] provoked a dose-limiting toxicity of grade 3 pleuritis, characterized by fever, hypoxia, pleural effusion, and pain (Hassan et al., 2007). Grade 1/2 pericardial effusion was also noted. These are quite likely target-dependent events but were not observed with anti-MSLN-VC-MMAE. A critical distinction here is the payload in SS1P, Pseudomonas exotoxin A, which kills cells by inhibiting protein synthesis, as opposed to the antimitotic MMAE. This implies that target-dependent toxicity might also depend on the mechanism of action of the cytotoxin. Additionally, SS1P is somewhat smaller than an ADC, consisting of an IgG Fv fragment fused to a 38-kDa portion of the Pseudomonas exotoxin A, which could facilitate better access to the mesothelium.

**F. Eph Family Receptor Tyrosine Kinase A2—Is Target-dependent Toxicity Predictable?**

EphA2 is an Eph family receptor tyrosine kinase expressed in a variety of cancers that was recently targeted with MEDI-547, an anti-epha2 antibody conjugated to MMAF using the noncleavable MC linker (Annunziata et al., 2013). The clinical experience here helps exemplify the difficulties of target evaluation. Clinical studies evaluating SGN-75 and SGN-CD19a, both of which also contain MC-MMAF but target CD70 and CD19, respectively, provide a background for evaluating adverse events driven specifically by the antibody portion of MEDI-547 (Borate et al., 2013; Tannir et al., 2014). In striking contrast to SGN-75 and SGN-CD19a, which were tolerated at doses as high as 3 and 6 mg/kg, respectively, MEDI-547 was discontinued because of drug-related adverse events after dosing the first cohort at 0.08 mg/kg (Annunziata et al., 2013). Bleeding and coagulation events reported for five of the six patients, three of which were recorded as severe adverse events, were responsible for termination of the study. These pathologies were not entirely surprising because they were observed preclinically in cross-reactive rats and primates, albeit at much higher doses than that attained in humans. The toxicities are probably target dependent, but the specific tissue(s) involved in eliciting the adverse events were not identified. Although GLP compliant tissue cross-reactivity studies were performed, this practice involves the immunohistochemical application of the drug itself, in this case MEDI-547, to frozen tissue sections as a means of detecting potential sites of reactivity (Leach et al., 2010). Accordingly, some weak staining restricted to tonsilar and esophageal epithelium was noted for MEDI-547. However, a more rigorous analysis using an antibody exhibiting demonstrable attributes as an immunohistological reagent would be more compelling. The authors concluded that a reassessment of tissue cross-reactivity was warranted. It also remains possible that the specific antibody per se, either on or off target, was the driver of toxicity.

**G. MUC1 and MUC16—What are Consequences of Shed Target Antigen?**

These two examples address the consequences of circulating shed target antigen on the performance of the ADC. Muc16 is an extremely large cell surface antigen containing multiple tandem mucin domains, which bind the antibody in DMUC5754A, an ADC containing the VC-MMAE linker-drug (Chen et al., 2007). Preclinically, two distinct antibodies were tested as ADCs against Muc16-11D10, which bind a non-repeating epitope, and 3A5, which recognized multiple mucin repeats. Despite having lower average affinity, the 3A5 ADC was vastly superior to that of 11D10, supporting the notion that higher antibody target density correlates with greater efficacy. However, the presence of these multiple mucin repeats on CA125—the extracellular portion of Muc16 that is shed into circulation—evoked fear of toxic ADC immune complexes as well as pharmacokinetic interference. None of this came to bear in the clinic, though, because there was no impact on efficacy, safety, or pharmacokinetic parameters that could be attributed to CA125 levels, ranging from very low (100 U/ml) to very high (7177 U/ml), in patients administered the recommended Phase II dose of 2.4 mg/kg (Liu et al., 2013).

Cantuzumab mertansine, an early iteration of a maytansinoid ADC targeting Muc1, or CanAg, was...
investigated in cancer patients ranging widely in plasma levels of shed CanAg (Tolcher et al., 2003). Of the 30 patients with detectable plasma CanAg levels, 25 experienced rapid reductions to undetectable levels after the first dose of the ADC, which was maintained for up to 21 days in the majority of the patients. Again, shed antigen levels did not impact pharmacokinetics nor exhibit any relation to toxicity. However, an impact of shed CanAg was called out in a Phase II study of huC242-DM4, a subsequent iteration of a maytasinoid ADC targeting Muc1 (Goff et al., 2009). In this study, exposure of huC242-DM4 in patients with gastric cancer was inversely correlated to plasma CanAg levels. Interestingly, those with low levels of CanAg, and thus higher circulating levels of huC242-DM4, appeared more susceptible to the ocular toxicity associated with this class of ADC than those with high CanAg. Accordingly, the study was amended to administer a higher dose of huC242-DM4 to patients with high plasma CanAg. Although it was apparent from this study that shed antigen was likely binding to and promoting the clearance of ADC, the presumed formation of ADC-target immune complexes did not exacerbate toxicity.

VI. Conclusions and Perspectives

The expanding register of ADCs under clinical investigation certainly attests to the interests and hopes we harbor for ADC therapeutics in the oncology clinic. However, there have been a fair number of failures, many of which have occurred quite recently. Although some were grounded by insufficient activity, it is apparent that even a marginal gain in tolerability could have prevented their failure. Such untoward safety signals can arise from at least four potential sources (Fig. 4). Normal cells expressing target are a proven liability, although not a common one, so far. Systemic deconjugation of ADCs certainly occurs and can be monitored, but the consequences for safety depend on the nature and amount of released drug or drug adduct. Nevertheless, toxicity abounds even when highly stable linkers are used or when the released free drug is relatively impotent.

On balance, toxicity unrelated to target expression is probably the most significant obstacle hampering the progression of ADCs. The catabolism of ADCs resulting from their pharmacological clearance yields intracellular amounts of the very drug intended to kill the cancer cell. Furthermore, once liberated, the drug can diffuse into neighboring tissues or escape into circulation and, depending on its nature, kill additional cells through the bystander effect. Even the successes, of which formally there are just two, would provide better benefit if not for this target-independent toxicity. HER2 is not expressed on megakaryocytes nor is CD30 on peripheral neurons, yet therein lie the reasons for dose limitations and reductions of Kadcyla and Adcetris, respectively. Thus it is likely that these toxicities emanate, either directly or indirectly, from uptake and conversion of the prodrug, i.e., the ADC, to the active small molecule drug by cells lacking target. This underscores the need to exploit the differences between nonspecific, pinocytotic uptake and target driven uptake of the ADC, as well any differences by which normal and cancer cells process the prodrug to drug.

Altering the antibody in ways that reduce either the amount or rate of uptake by cells lacking target might enhance the safety margin of ADCs. Obviously, appending drugs that exhibit greater selectivity toward cancer cells would also be beneficial. In principal, highly potent “targeted” therapies that exploit clearly defined genetic differences in cancer cells could be made more effective and safer if conjugated to antibodies. Similarly, drugs designed to take advantage of more generalized attributes of cancer that distinguish them from normal cells, such as hypoxia or endoplasmic reticulum (ER) stress, could benefit from conjugation to antibodies. Although such drugs are designed to be more selective to cancer, conjugating them to antibodies could further reduce toxicities, improve pharmacokinetic exposure, and facilitate entry of impermeable molecules into targeted cells. Some of these efforts, and other potential improvements, are underway.

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