Review

Molecular Cancer Therapeutics

Mechanisms of Resistance to Antibody–Drug Conjugates

Frank Loganzo, Matthew Sung, and Hans-Peter Gerber

Abstract

Drug resistance limits the effectiveness of cancer therapies. Despite attempts to develop curative anticancer treatments, tumors evolve evasive mechanisms limiting durable responses. Hence, diverse therapies are used to attack cancer, including cytotoxic and targeted agents. Antibody–drug conjugates (ADC) are biotherapeutics designed to deliver potent cytotoxins to cancer cells via tumor-specific antigens. Little is known about the clinical manifestations of drug resistance to this class of therapy; however, recent preclinical studies reveal potential mechanisms of resistance. Because ADCs are a combination of antibody and small molecule cytotoxin, multifactorial modes of resistance are emerging that are inherent to the structure and function of the ADC. Decreased cell-surface antigen reduces antibody binding, whereas elevated drug transporters such as MDR1 and MRP1 reduce effectiveness of the payload. Inherent to the uniqueness of the ADC, other novel resistance mechanisms are emerging, including altered antibody trafficking, ADC processing, and intracellular drug release. Most importantly, the modular nature of the ADC allows components to be switched and replaced, enabling development of second-generation ADCs that overcome acquired resistance. This review is intended to highlight recent progress in our understanding of ADC resistance, including approaches to create preclinical ADC-refractory models and to characterize their emerging mechanisms of resistance. *Mol Cancer Ther; 15(12); 2825–34. ©2016 AACR.*

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Introduction

Cancer is an evasive disease. Genomic and epigenetic instability, deregulated signaling, sustained proliferation, replicative immortality, resistance to cell death, and immune evasion are among the "hallmarks" of cancer (1). These diverse features result in uniquely heterogeneous tumor environments that often evade therapy. As a result, the response rate to anticancer treatments varies significantly, even within a tumor type. The average 5-year cancer survival rate in the United States for all cancer types is approximately 69%, ranging from about 8% to 18% for pancreatic, lung, and liver cancers to >65% for cancers of the colon, breast, kidney, prostate, and others (2). These rates encompass all treatment regimens, including traditional cytotoxic chemotherapy (e.g., taxanes, anthracyclines, platins), molecularly targeted small-molecule inhibitors (e.g., kinase inhibitors), and biotherapeutics (e.g., therapeutic antibodies). Recent advances in antibody–drug conjugate (ADC) technology have also initiated a surge of new ADCs in clinical development and offer a targeted alternative to standard chemotherapy.

ADCs are modular biotherapeutics composed of antibody, linker, and payload (3). The antibody is designed to bind antigens that are more selectively expressed in the tumor environment. Internalization of the antibody results in release of the payload via cleavage of the linker or proteolysis of the antibody, allowing the cytotoxic payload to inhibit its target intracellularly. Two approved ADCs are trastuzumab emtansine (Kadcyla, T-DM1) targeting HER2 and delivering tubulin depolymerizing cytotoxin maytansine via a non–cleavable-linker (4), and brentuximab vedotin (Adcetris, BV) targeting CD30 and delivering tubulin depolymerizer MMAE via a protease-cleavable-linker (5). There are also currently over 50 ADCs undergoing clinical evaluation (6, 7), some with promising response rates. As with all cancer treatments, there is room to improve clinical benefit for this new generation of therapy.

There are many potential pharmacokinetic reasons for variable response rates to ADCs, including emergence of toxicity before efficacy, loss of payload, or poor absorption/distribution/metabolism/excretion (ADME) of the antibody or the released payload (8). These effects are generally independent of the cancer cell itself. Other major contributors to high drug failure rates are a combination of inherent and/or acquired resistance to the therapeutic drug due to the tumor environment and to cell-dependent mechanisms. Changes in the tumor microenvironment can limit the access of large molecules, including ADCs, to cancer cells and can significantly impact the biodistribution and efficacy of biotherapeutics (9). Such resistance based on accessibility may be most pronounced in tumors with reduced vascular permeability caused by increased formation of vascular barriers, including basement membranes and interstitial tumor matrix, therefore, limiting the diffusion of ADC from circulation to the tumor (10, 11)

Genetically unstable cancer cells evolve quickly to overcome drug efficacy. Within a few generations, tumor cells exposed to new drugs mobilize diverse responses to adapt and survive. For decades, cancer biologists have attempted to understand treatment failure and cancer relapse by studying patient tumor samples and developing preclinical models of drug resistance. As a tumor progresses, diverse genetic and epigenetic alterations can occur, including gene amplification, deletion, mutation, methylation, and many other shifts from the normal genome (12). There

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has been some progress in our understanding of resistance to antibodies and small molecule drugs, although the basis for clinical resistance is still emerging. For example, resistance to the clinically approved anti-HER2 antibody trastuzumab (Herceptin) appears to be multi-factorial, including receptor shedding, signaling pathway activation, compensatory induction of alternate surface receptors, loss of immune-mediated cytotoxicity, and other reasons (13). For kinase inhibitors, active site mutations or amplification of target enzymes as well as secondary signaling alterations frequently lead to drug failure in patients, and have been effectively modeled preclinically (14). For cytotoxic compounds, the most common payloads used for ADCs, diverse resistance mechanisms are reported. Loss of sensitivity to taxanes, *vinca* alkaloids, or peptide-site tubulin binders is mediated by overexpression of drug efflux transporters such as MDR1, acquisition of tubulin mutations or tubulin isotype changes, and chromosomal instability (15–19). Resistance to doxorubicin, etoposide, irinotecan, and related topoisomerase I or II inhibitors is mediated by mutation or altered expression of the drug target topoisomerase, drug transporter overexpression, altered apoptotic mechanisms, PTEN mutation, or other mechanisms (19–21). Although correlations are reported between patient outcome and several of these resistance markers, there are limited data proving that specific resistance mechanisms are responsible for treatment failure in patients, particularly for cytotoxic therapies. This is likely the result of both intratumor and interpatient cancer heterogeneity, limited access to sequential post-treatment tumors, and lack of validated clinical assays to detect known resistance markers such as transporter proteins. Hence, preclinical cancer cell-based models are used to extrapolate the potential mechanisms that may emerge upon chronic treatment of tumors to specific therapeutics.

Despite the emergence of ADCs as an active area of cancer biotherapy over the past decade, little is known about the mechanisms that mediate resistance to these "antibody–chemotherapy" combination agents. Would resistance develop to the antibody or to the small molecule chemotherapeutic component? Would it be possible to modify antibody, linker, or payload components of an ADC and overcome these resistance mechanisms? Over the past 2 years, several reports have emerged about models of acquired ADC resistance created by chronic exposure of cancer cell lines, either *in vitro* or *in vivo*, to ADCs targeting various antigens and composed of different linkers and payloads.

Approaches to Generate Models of Acquired Resistance to ADCs

Acquired drug resistance occurs during exposure of a cancer cell to a therapeutic regimen. Patients may initially respond to the therapy, but then tumors inevitably progress even with continued cycles of treatment. Cross-resistance also may occur when a drug induces resistance mechanisms that affect the activity of other therapies. For example, drug-induced upregulation of an ATP-binding cassette (ABC) transporter, such as MDR1, will reduce the effectiveness of other MDR1-substrate drugs.

In preclinical research, there are several approaches to generate drug resistant models. The most common method is to treat cancer cell lines or tumors, either *in vitro* or *in vivo*, with a single drug and then to characterize the resistant cell progeny compared with the isogenic parental cell model. This approach has been used to generate resistant tumor models to chemotherapeutic agents commonly used in the clinic (12, 16, 18, 19, 22, 23), and the same approach can be used to induce resistance to ADCs.

To model ADC resistance, several recent studies have used either (i) continuous treatment at low ADC doses, followed by incremental dose increases, or (ii) cyclical treatment for a short duration at moderate to high doses. Continuous drug treatment is typically conducted by selecting a dose that is at or below the cytotoxic IC_{50} value, continuously maintaining cells in the presence of drug, and then increasing the dose as a sub-population of cells survive and adapt to the drug. This approach has been used for the selection of trastuzumab emtansine (T-DM1) refractory breast cancer cell lines (24), and brentuximab vedotin (BV) refractory leukemia and lymphoma cell models (25, 26).

In contrast, cyclical exposure typically involves short-term treatment at a drug concentration that is much higher than the cytotoxic IC_{50}, followed by a drug holiday to enable recovery of cell survivors, and then re-challenge with drug at the same or higher concentration as used in the initial challenge. This cyclical dosing approach may be considered more clinically relevant because it mimics standard chemotherapeutic and biotherapeutic regimens containing cytotoxic agents. Patients are typically treated for short duration at maximally tolerated drug doses, and then allowed to recover from drug-related adverse events. This process is continued over multiple cycles for several months, with monitoring of tumor size and status. Cyclical exposure to ADC has been employed to generate resistant cell models using BV (26) or T-DM1 (27).

Chen and colleagues (26) initially evaluated a continuous ADC exposure approach in two different cell lines when attempting to create BV-resistant models. Karpas-299 cells, derived from anaplastic large cell lymphoma (ALCL), were treated with 0.010 μg/mL of BV (standard 3-day cytotoxicity IC_{50} was 0.029 μg/mL), and cells eventually grew upon persistent culture in ADC at concentrations up to 0.020 μg/mL. The ADC-selected Karpas-R cells demonstrated 655-fold resistance to BV, with IC_{50} shifting to 19 μg/mL in the resistant population. The authors also tried the same approach with L428 cells, derived from Hodgkin's lymphoma, by persistent treatment in 25 μg/mL of BV (standard 3 day IC_{50} was 27 μg/mL). However, the L428 cells failed to thrive in constant exposure and the authors reverted to a pulsatile approach at 50 μg/mL of BV. The differential tolerance for constant BV exposure between the two cell lines may be due to their 1,000-fold difference in inherent sensitivity to BV, or simply due to cell-specific differences.

In our ADC resistance selection studies, we assessed five different HER2-expressing cell models for their response to long-term treatment with trastuzumab–maytansinoid conjugate (TM-ADC), a synthesized version of T-DM1 (27). We used a cyclical dosing approach, starting with approximately 100X the IC_{50} concentration of T-DM1 observed from standard 4-day proliferation assays. Cells were treated with ADC at this high concentration for 3 days, resulting in >80% cell death. Subsequently, ADC-containing media were removed, cells washed, and fresh media without drug added for approximately 4 to 14 days, depending on the rate of recovery. During initial selections, cells frequently required long (10–20 day) recovery times to ensure survival of a handful of cell colonies. Once confluence reached approximately 30% to 50%, cells were treated again at the same ADC concentration. This process was continued for several months, with regular monitoring of drug sensitivity in standard proliferation assays. In each of our five cell models, an IC_{50} shift

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was observed during cyclical T-DM1 regimens after only approximately 2 to 4 months. Other classes of ADCs may require longer times to develop resistance. In either case, long term drug treatment (either continuous, or short and cyclical) can enable selection of resistant clones, although the efficiency of the approach may depend on the cell type and the ADC.

Another approach to induce ADC resistance is by *in vivo* selection using human tumor xenograft models grown in mice. This method is resource-intensive because it involves persistent dosing of mice with drug until the tumors become refractory. One of the earliest examples of this approach using an ADC used nude mice implanted with the UCLA-P3 lung adenocarcinoma and then treated multiple times with anti–KS1/4-*vinca* ADC (28). The resulting tumors were refractory to subsequent ADC challenge. Neither altered antigen levels nor MDR1 protein were observed in these tumors, and the resistance was not maintained when cells were cultured *ex vivo*. Recently, non-Hodgkin lymphoma (NHL)–derived cell lines were made resistant to an anti–CD22-vc-MMAE ADC *in vivo*. Yu and colleagues (29) implanted two different cell lines into SCID mice and dosed with increasing concentrations of the anti–CD22-vc-MMAE ADC. Dosing was halted when the tumors failed to respond to the drug, although the treatment duration is not reported. In another example, Li and colleagues (30) report the generation of four T-DM1-resistant cells in cell culture (BT-474, NCI-N87, SK-OV-3, and MDA-MB-361) by continuous exposure to increasing concentrations of the ADC. All four cell models were refractory to at least 1 μg/mL of T-DM1 *in vitro*. When the N87-T-DM1–resistant cell line was implanted into immune-deficient mice, it is reported that *in vitro* resistance did not translate *in vivo*, wherein large variations in tumor growth was observed. As an alternative approach to develop an *in vivo* model, large (~1,000 mm^3) tumors were grown from the cell line and passed fragments into additional mice, then mice were treated intermittently with T-DM1 until the tumors were consistently refractory to 3 mg/kg T-DM1. The mechanisms of resistance to T-DM1 in these cell models are not reported. An *in vivo* approach was also used to generate NCI-N87 gastric carcinoma xenograft tumors resistant to T-DM1 in athymic mice (31). Hence, in the past few years there has been success in generating ADC-resistant models, both in cell culture and in immunodeficient mice. The mechanisms of resistance observed in each of these models are described in detail below.

Mechanisms of ADC Resistance

There are several emerging mechanisms of ADC resistance based on preclinical acquired resistance models, and these appear to be converging into four general bins: (i) antigen downregulation, (ii) drug transporter protein over-expression, (iii) defects in ADC trafficking pathways, and/or (iv) alterations in receptor, apoptotic, or other signaling pathways. Table 1 summarizes reported models of ADC resistance.

Antigen downregulation or drug transporter protein overexpression

ADCs are highly dependent upon antigen expression to exert their targeted cytotoxic effects. Hence, one predicted mechanism of ADC resistance would be antigen downregulation in cancer cells chronically exposed to the drug. We conducted studies to generate acquired resistance to T-DM1 in high HER2 (NCI-N87, HCC1954, BT-474) and moderate HER2 (MDA-MB-361-DYT2, JIMT1)-expressing models. T-DM1 doses were approximately 10 to 100 nmol/mL (payload concentration), corresponding to approximately 0.3 to 4 μg/mL antibody concentration, and were conducted in a cyclical dosing scheme as described above, with 3 days exposure followed by washout into drug-free media. There appear to be cell-dependent differences in T-DM1 resistance mechanisms observed in these studies. For example, JIMT1 cells were originally derived from a patient whose tumor failed trastuzumab antibody therapy (32), yet small xenograft tumors responded *in vivo* to T-DM1 ADC, albeit at moderately high concentrations (33). We treated JIMT1 cells in a cyclical fashion with T-DM1 to generate JIMT1-TM cells *in vitro*, which became 16-fold resistant to T-DM1 and showed cross-resistance to essentially all other trastuzumab-based ADCs (27). This cross-resistance drug profile was consistent with a possible decrease of antigen, and JIMT1-TM cells showed a marked decrease in HER2 protein levels within several months of initial T-DM1 treatments. HCC1954 and BT-474 cell lines were treated with the same cyclical dosing approach at high T-DM1 concentrations and, after only approximately 2 months, the HCC1954-TM cells became >1000-fold resistant to T-DM1 and other ADCs, while BT-474-TM cells were approximately 11-fold resistant. Immunoblots confirmed dramatic reductions of HER2 levels in both cell models, consistent with the magnitude of the resistance (34). Antigen downregulation in ADC-resistant cells reduces overall binding of the antibody portion of the ADC to the cell surface. In many cases, long-term maintenance of the cells in the absence of ADC selection pressure caused reversal of the resistance phenotype, concomitant with recovery of HER2 expression and/or outgrowth of cells with high HER2 protein levels.

Another possible mechanism of resistance that may arise to ADCs is dependent upon the payload. ABC transporters are known to reduce the effectiveness of structurally diverse small molecule chemotherapeutic agents (35). It is not surprising that these drug efflux pumps might also contribute to resistance associated with the conjugated cytotoxin because many of these payloads are themselves substrates of ABC transporters (27, 29, 36).

Clinical and preclinical data suggest a role for MDR1 (ABCB1) in reduced effectiveness of Mylotarg (gemtuzumab ozogamicin; anti–CD33-AcBut-NAc-calicheamicin) in acute myeloid leukemia (AML; ref. 37). Calicheamicin is a good substrate of MDR1, expression of this drug transporter is consistently associated with reduced survival in AML in general, and patients with lower levels of MDR1 have improved responses to Mylotarg (38). Moreover, Mylotarg shows impressive activity in acute promyelocytic leukemia (APL), which in contrast to AML is typically low or negative for MDR1 (39). Hence, although Mylotarg shows reduced efficacy against MDR1-expressing AML (40), it is not clear if Mylotarg itself induces increased expression of MDR1 in these cancers.

Preclinical data also support a role for ABC transporters in other ADC-refractory cancer cell models. In one of the five cell lines that we made resistant to T-DM1 via cyclical dosing *in vitro*, we observed increased expression of the drug efflux pump ABCC1, also known as MRP1 (27). MDA-MB-361-DYT2 breast carcinoma cells were treated in a cyclical fashion with approximately 4 μg/mL of T-DM1. Within approximately 3 months, these cells became 256-fold resistant to T-DM1 and >200- to >2000-fold cross-resistant to auristatin-based trastuzumab-ADCs conjugated via non-cleavable linkers. Resistance to T-DM1 was also observed when 361-TM cells were implanted into NSG mice. Proteomic

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Table 1. Preclinical models of acquired ADC resistance

ADC	Cell model	Dosing approach	Fold resistance vs. Parental	Mechanism of resistance proposed	Reference
T-DM1	KPL-4-T-DM1-R	Continuous, with increasing dose	~1,000X	HER2 reduction; MDR1 induction; EGFR and IGF1Rβ induction, altered signaling	Lewis Phillips (24)
T-DM1	BT-474-M1-T-DM1-R	Continuous, with increasing dose	~100X	PTEN and IGF1Rβ reduction; DARPP32 induction; MRP4 RNA induction	Lewis Phillips (24)
T-DM1	361-TM	Cyclical, with constant high dose	256X	MRP1 induction, trafficking protein modulation	Loganzo, et al. (27)
T-DM1	JIMT1-TM	Cyclical, with constant high dose	16X	HER2 reduction; trafficking protein modulation	Loganzo et al. (27)
T-DM1	HCC1954-TM, BT-474-TM	Cyclical, with constant high dose	>1,000X, ~11X	HER2 reduction	Loganzo et al. (34)
T-DM1	N87-TM	Cyclical, with constant high dose	~100X	Trafficking or lysosomal defects; CAV1 overexpressed	Sung et al. (49)
T-DM1	BT-474, NCI-N87, SK-OV-3, MDA-MB-361	Continuous, with increasing dose	>100 to >1,000X	Not reported	Li et al. (30)
BV	Karpas-R	Continuous, at high dose	655X	CD30 downregulation	Chen et al. (26)
BV	L428-R	Cyclical, with constant high dose	8.7X	MDR1 induction	Chen et al. (26)
BV	Karpas-299-35R	Continuous, with increasing dose	>1,000X	CD30 downregulation	Lewis et al. (25)
BV	L540cy-35R	Continuous, with increasing dose	>100X	Low-level MDR1 induction	Lewis et al. (25)
BV	DEL-35R	Continuous, with increasing dose	>1,000 to >10,000X	MDR1 induction	Lewis et al. (25)
Anti–CD22-vc-MMAE	BJAB.Luc-22R1; WSU-DLCL2-22R1	*In vivo* xenograft; cyclical increasing dose	No response to 8–12 mg/kg αCD22-vc-MMAE *in vivo*	MDR1 induction	Yu et al. (29)
DM1/maytansine-containing ADCs	786-0 with SLC46A3 shRNA	shRNA transfection (no drug induction)	n/a	SLC46A3 knockdown	Hamblett et al. (57)

Abbreviations: T-DM1, trastuzumab emtansine [Kadcyla; anti–HER2-trastuzumab-MCC-DM1]; BV, brentuximab vedotin [Adcetris; anti–CD30-cAC10-vc-MMAE].

profiling of the membrane fraction of the parental (361) and resistant (361-TM) cell lines was conducted, and MRP1 was one of the most highly induced proteins. Immunoblots, flow cytometry, and rhodamine-efflux studies confirmed that MRP1 was functionally induced in the 361-TM model. No change in HER2 antigen expression was observed. Interestingly, 361-TM cells retained sensitivity to unconjugated DM1-SMe, even though DM1-SMe showed a high IC_{50} in MRP1-expressing H69-AR cells (27). It is possible that the released species of T-DM1, Lys-MCC-DM1, may be a better substrate than free DM1. Addition of an MRP1-reversal agent or siRNA-mediated knockdown of MRP1 restored sensitivity to non-cleavable linked ADCs, further confirming the role of MRP1 in ADC resistance. Remarkably, the 361-TM cell line retained sensitivity to MC-Val-Cit-linked trastuzumab-ADCs, further suggesting that the released species, which are uncapped auristatins (lacking amino acid Cys plus the linker-payload), are able to overcome MRP1 expression. Hence, this cell model suggests that MRP1-induced resistance to T-DM1 can be overcome by switching therapy to a cleavable-linked auristatin ADC while retaining the same antigen-targeting antibody.

Similar observations of acquired resistance to T-DM1 were reported by Lewis Phillips (24). Two HER2-positive cell lines were continuously incubated with increasing doses of T-DM1 up to 4 μg/mL. KPL-4 cells became approximately 1,000-fold resistant to T-DM1, and were approximately 100-fold cross-resistant to the tubulin inhibitor, docetaxel. Levels of HER2 decreased in these cells, as evidenced by DNA, RNA, and protein analyses. In addition to decreased antigen, KPL-4-T-DM1-R cells displayed markedly increased MDR1 (ABCB1) expression, as well as slightly increased expression of BCRP (ABCG2). Studies with the reversal agents verapamil and Ko143 support a role for MDR1 in resistance but not BCRP, respectively. No cross-resistance data for other ADCs was provided to understand whether the decreased expression of receptor or MDR1 expression were key mediators of the resistance phenotype. In the same study, BT-474-M1 cells were continuously treated with T-DM1. The resulting BT-474-M1-T-DM1-R cells were approximately 100-fold resistant to T-DM1 but retained sensitivity to docetaxel. HER2 levels were unchanged, yet there was a 3- to 10-fold increase of MRP4 RNA levels via RT-PCR or microarray which was yet to be verified at the protein level. Changes in several signaling proteins were observed in both the KPL-4 and BT-474-M1 T-DM1–resistant cell lines, which will be discussed below; however, it is not clear whether these alterations mediated resistance.

The drug transporter MDR1 is known to be induced in cancer cells during chronic exposure to other standard-of-care (unconjugated) tubulin inhibitors (41); hence, a role for resistance to the maytansine analog DM1 was conceivable, and is observed for T-DM1 in KPL-4-T-DM1-R cells. A role for MDR1 in T-DM1 resistance was further confirmed by overexpressing MDR1 in SKBR3 cells (42). Expression of MRP1 upon exposure to T-DM1 in the 361-TM cell model was unanticipated; however, DM1 itself can be a substrate of MRP1 in cells with high expression of the transporter (27). In contrast, MRP1 was not observed to mediate

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resistance to maytansinoid-containing ADCs in other cell models (36, 42, 43). Hence, acquired resistance to T-DM1 was mediated by decreased levels of HER2 antigen and/or expression of MRP1 or MDR1 among a panel of seven T-DM1–selected cell lines evaluated in two independent laboratories (24, 27). In the clinic, T-DM1 response rates are higher in patients previously untreated with trastuzumab and those with high HER2 expressing cancers, suggesting that inherently low antigen expression or acquired resistance from chronic anti-HER2 therapy may contribute to tumor progression (44).

Decreased antigen expression and increased drug transporter protein expression upon chronic ADC exposure are not unique to HER2. Brentuximab vedotin (BV) was used to select several CD30-expressing cell models for drug resistance. The ALCL cell model Karpas-299 was treated persistently with 0.010 μg/mL BV (about a third of the IC_{50} dose) and then incrementally increased to 0.020 μg/mL (26). After several months of exposure to ADC, Karpas-R cells were derived and showed approximately 655-fold resistance to BV. Flow cytometry revealed a >38% reduction in the percentage of CD30-positive cells and a 79% reduction of signal intensity, which likely contributed to the strong resistance phenotype. When the cells were removed from drug for about 6 months, CD30 levels recovered and the level of resistance dropped to 11-fold. Hence, loss of drug selection pressure either caused resistant cells to regain CD30 expression, or more likely allowed a sub-population of cells with normal CD30 levels to grow out in the absence of chronic ADC exposure.

Chronic treatment of cultured cell lines with brentuximab vedotin can also induce MDR1. In the same study as above, the authors also treated the Hodgkin's lymphoma (HL) cell model L428 with BV (26). Constant exposure of the cells to ADC was attempted first, however, the L428 cells failed to acquire resistance. As a result, L428 cells were treated by a cyclical approach of high concentration of 50 μg/mL (about twice the IC_{50}) followed by recovery until the cells continued to thrive through the drug. The L428-R cell line became only about 8.7-fold resistant after cyclical exposure to BV. Because CD30 levels were maintained in this population and cross-resistance was observed to the payload MMAE, which is a known MDR1 substrate, the authors evaluated expression of drug efflux proteins. RT-PCR and immunoblot analyses identified induction of MDR1 mRNA and protein, respectively, but no changes in MRP1 or MRP3. After 5 months in the absence of drug exposure, the levels of MDR1 decreased, although the level of resistance was not reported. Tumor tissue was evaluated from patients who had responded then relapsed upon treatment with BV and persistent CD30 expression was observed among the 5 ALCL and 12 HL samples (26, 45). Among four patients whose tumors became refractory to BV, positive signals were detected by immunohistochemistry for MDR1 (1 sample), MRP1 (1 sample), and MRP3 (2 samples; ref. 26). A recent independent case study reports loss of CD30 expression in nodules of an ALCL patient treated with 7 cycles of BV (46).

A parallel study was conducted with brentuximab vedotin exposure to ALCL and HL cultured cell lines (25). Cells were continuously exposed to increasing concentrations of BV, and then clones were selected for characterization. Karpas-299-35R cells developed >1,000-fold resistance to the ADC but remained sensitive to MMAE payload. Like in the previously described study using a Karpas-299 cell population resistant to BV (26), three of these Karpas-299-35R clones also showed significant downregulation of CD30 antigen (25). In this case, no full length CD30 mRNA or protein were detectable by flow cytometry, immunoblot, or RNA sequencing, but low levels of a 5′-truncated CD30 transcript lacking exons 1–7 was detected. In contrast to the Karpas cell models, the L540cy-35R resistant cells retained CD30 but showed approximately 2- to 3-fold increased MDR1 by flow cytometry, as well as a small increase of drug-efflux pump ABCC7 RNA. The high level of resistance to BV (>300-fold) but general sensitivity to MMAE (5-fold) suggests that the low level of MDR1 induction may be a contributor to ADC resistance, but that other factors may be involved. In the same report, DEL-35R cells were generated to be >1000 to 10,000-fold resistant to BV, about 10- to 40-fold cross-resistant to MMAE, and displayed 5- to 15-fold increased MDR1 protein levels. These cells remained sensitive to other anti-CD30 ADCs with alternate payloads which are not MDR1 substrates. These complementary studies with resistant models to brentuximab vedotin demonstrate that the same ADC can induce either antigen downregulation or drug transporter protein upregulation, depending upon the cell model.

Chronic treatment with another vc-MMAE ADC can also induce MDR1 *in vivo*. Yu and colleagues (29) treated mice harboring two different NHL tumor xenografts, either BJAB.Luc or WSU-DLCL2, with increasing doses of anti–CD22-vc-MMAE. Replicate models were made for each of the two tumor lines for a total of four models evaluated. Mice with BJAB.Luc tumors were intermittently treated with 1.5 to 20 mg/kg of ADC until the tumor no longer responded to the ADC. Re-challenge of these BJAB.Luc-22R1.2 tumors with anti-CD22-vc-MMAE up to 8 mg/kg caused no change in tumor growth compared with vehicle or a negative control vc–MMAE conjugate. The same observation was made with the WSU-22R1.1 tumor model, induced with 12 to 30 mg/kg anti–CD22-vc-MMAE over time. Re-challenge with 12 mg/kg of the ADC failed to cause growth inhibition; hence, the tumors were made refractory to the ADC *in vivo*. The tumors were also cross-resistant to another vc-MMAE ADC delivered via anti-CD79b antibody, indicating that the mode of resistance was common to the linker-payload. Oligonucleotide microarray analyses of the tumor models detected elevated ABCB1 (MDR1) gene expression in all four models, ranging from 8- to 263-fold induction. No other genes were modulated by more than 2-fold. Flow cytometry confirmed 100-fold overexpression of MDR1 protein in the BJAB-Luc-22R1.2–and WSU-22R1.1–resistant models compared with parental cells. The authors demonstrate that treatment with an alternate payload (an ultra-potent metabolite of the anthracycline nemorubicin) overcomes resistance to the microtubule inhibitor MMAE likely because the nemorubicin-related payload itself is not a substrate of MDR1.

Therefore, these several examples suggest that chronic exposure of tumor cell lines, either *in vitro* or *in vivo*, with ADCs delivering known substrates of MDR1 or MRP1, including microtubule inhibitors DM1 and MMAE, are capable of inducing acquired resistance by upregulation of either MDR1 or MRP1 protein.

Defects in ADC trafficking pathways or receptor/apoptotic signaling pathways

ADCs require additional events compared with small-molecular inhibitors to induce their intended effects. Effective biological activity of a cell-internalizing ADC typically depends upon antibody binding, internalization, and catabolism, as well as linker-payload processing and release which generally occurs in the endosomal and lysosomal vesicles (47, 48). Defects in any of

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these processes in a cancer cell could affect ADC efficacy and lead to drug resistance. As described above, downregulation of surface antigen has been verified to reduce ADC binding and significantly reduce ADC activity. There are no reported ADC-resistant models where conjugate internalization rates mediate loss of activity. However, in two cell lines made refractory to T-DM1, proteomic profiling demonstrated alterations in multiple proteins involved in vesicle transport, lysosome/endosome biogenesis, actin/microtubule cytoskeleton, or antibody trafficking (27). JIMT1-TM cells had reduced HER2 but also showed significant changes in the level of proteins associated with ADC transport and processing. T-DM1-resistant 361-TM cells retained HER2 expression but increased MRP1, and yet also showed trafficking and cytoskeletal protein changes as well as altered ADC localization. Hence, broad proteomic characterization of ADC resistant cells suggested many diverse approaches by tumor cells to overcome killing by cytotoxic immunoconjugates before a primary mechanism emerged in a specific cancer cell population.

Altered ADC trafficking was observed in NCI-N87 gastric carcinoma cells made resistant to T-DM1 by cyclical exposure over several months. These N87-TM cells were >100-fold resistant to T-DM1 and highly cross-resistant to other non–cleavable-linked ADCs, but retained full sensitivity to anti-HER2 ADCs conjugated to a protease cleavable linker (i.e., MC-Val-Cit-PAB; ref. 49). There was no major reduction of HER2 antigen, nor any detectable changes in ABC family drug transporter proteins as characterized by proteomics and immunoblots. Proteomics detected several upregulated proteins in the N87-TM cells, including caveolin-1 (CAV1) and polymerase I and transcript release factor (PTRF or Cavin1), proteins reportedly associated with caveolae. Immunoblots confirmed strong over-expression of CAV1 and moderate increases in PTRF in the N87-TM resistant cells compared with parental NCI-N87. In addition, fluorescence microscopy showed that trastuzumab-ADCs with non-cleavable linkers such as T-DM1 colocalize with caveolae and not lysosomes. These data are also consistent with the altered localization of an ADC targeting p97 (melanotransferrin) that was observed in cancer cells which were inherently less sensitive to the ADC (50). Relatively high antigen–expressing melanoma cell models, H3677 and SK-MEL-8, were >30 to 10,000-fold resistant to anti–p97-L49-vc-MMAF ADC compared with other melanoma cells. Immunofluorescence microcopy indicated that the ADCs in these resistant cell lines colocalized with caveolin-1 (a protein marker of caveolae) rather than with lysosomes (50). Caveolar endocytosis is a clathrin-independent event and is mediated by the oligomerization of the structural coat caveolin proteins and stabilized by a multicavin protein complex (51). The intracellular trafficking fate of caveolae is not well understood; however, they have been shown to fuse with early endosomes to form a unique endosomal compartment called the caveosome that are characterized by neutral pH and distinct from clathrin-coated endosomes (52). Recently, Chung and colleagues (53) suggest the opposite relationship wherein caveolin-1 expression may contribute to T-DM1 sensitivity. They observe that SK-BR-3 cells express higher levels of CAV1 protein than BT-474 cells, and suggest that this correlates with the higher sensitivity of SK-BR-3 cells to T-DM1. When BT-474 cells were transfected with GFP-CAV1, sensitivity to T-DM1 improved by 2-fold. Conversely, when CAV1 levels were reduced with siRNA in SK-BR-3 cells, a 2-fold loss of sensitivity was observed. However, only T-DM1 ADC was tested and only at one concentration, and the difference in sensitivity to T-DM1 between these high HER2-expressing cells was only approximately 5-fold. Moreover, SK-BR-3 cells are reportedly approximately 4 to 5X more sensitive to unconjugated DM1 than BT-474 cells, which may also contribute to the ADC potency differential (54). In the absence of full dose–response curves or the evaluation of other ADCs, it is not possible to understand the impact of CAV1 expression on ADC resistance in these two inherently T-DM1–sensitive high HER2 cell models. The observation that cancer cells with either inherent (50) or acquired (49) ADC resistance may mis-localize ADCs to caveolae suggests a novel role for this trafficking pathway in ADC resistance.

Another unique feature of ADCs is that the cytotoxic payload must be released from the antibody to exert cell death. Therefore, a potential resistance mechanism that cancer cells may acquire upon persistent treatment with an ADC is defective linker-payload processing or transport to the drug target (i.e., the nucleus for DNA inhibitor payloads or the cytoplasm for tubulin inhibitors). Non–cleavable-linked ADCs require nearly complete catabolism of the antibody in the endosome and lysosome, releasing the amino acid capped linker-payload (55). In the context of lysine and cysteine conjugations, these metabolites are highly impermeable. For example, Lys-MCC-DM1 is significantly less potent than free DM1 in cells (56). One longstanding enigma in the biology of ADCs is the specific mechanism whereby these poorly permeable Lys- or Cys-capped linker-payloads are released from the lysosome into the cytoplasm and/or nucleus to exert their cytotoxic effects by tubulin or DNA binding. A predominate hypothesis is that amino acid transporters expressed on the lysosomal membrane could contribute to metabolite efflux into the cytoplasm.

In the N87-TM and 361-TM models of T-DM1 resistance developed in our group, we observed that non–cleavable-linked trastuzumab ADCs are less effective at cell killing, whereas protease-cleavable–linked (e.g., MC-Val-Cit-PAB) ADCs retain high potency (27, 49). In addition to other mechanisms of resistance, which we identified in these cell models, the possibility of loss of a transporter on the lysosomal membrane is currently under investigation. In search of putative transporters that may actively efflux ADC metabolites from the lysosome, Hamblett and colleagues (57) conducted an elegant shRNA library screen using anti–CD70-MCC-DM1 as the ADC model. The renal carcinoma cell line 786-0 was transduced with lentiviral shRNA from two libraries, targeting over 2,500 genes in total. Infected cells that survived 4 days of ADC treatment were expanded and shRNA hits identified. In addition to antigen CD70, the lysosomal solute carrier family protein SLC46A3 was the only other highly significant hit common to both libraries. Functional assays confirmed that siRNA-mediated knockdown of SLC46A3 made 786-0 cells less sensitive to anti-CD70-MCC-DM1 treatment. Strikingly, SLC46A3 knockdown in other cell lines also protected from treatment with alternate MCC-DM1-based ADCs, including those targeting EGFR and HER2. Further studies suggested that the payload component (DM1/maytansine) was likely recognized by the solute carrier protein, since ADCs with an alternate linker or payload (e.g., mcMMAF) retained potency in the SLC46A3-attenuated cell lines. Hence, SLC46A3 likely mediates the active transport of the released species of non–cleavable-linked maytansine from the lysosome into the cytoplasm. The expression of SLC46A3 on tumors of patients who fail DM1-based ADC therapy will need to be evaluated to determine a possible role for this solute carrier protein in clinical ADC resistance.

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Changes in kinase signaling pathways may also be cancer cell attempts to overcome persistent exposure to some ADCs, in particular to those targeting HER2 receptor kinase. In the T-DM1–resistant cell model, KPL-4-T-DM1-R, levels of HER2 and HER3 decreased concomitantly with an increase of EGFR, IGF1Rβ, and c-Met receptor kinases (24). In contrast, BT-474-T-DM1-R cells, which were created in the same study, retained HER2 and HER3 but showed a reduction of IGFR1β and PTEN protein levels. Further characterization of the BT-474-T-DM1-R model showed increased DARPP32 (dopamine and cAMP regulated phosphoprotein of 32 kDa) and DUSP6 (dual specificity phosphatase 6), which have been associated with resistance to HER2 therapy (58, 59). Similarly, changes in growth factor signaling, such as the HER3 ligand neuregulin-1 (NRG1β), may modulate cytotoxic drug and ADC sensitivity. Addition of NRG1β to cultured cells reduced the potency of T-DM1 in HER2-expressing cells with wild type PI3KCA (60). This effect translated to the unconjugated payload, DM1, as well as to other anti-microtubule agents such as docetaxel and vinorelbine, suggesting that neuregulin may mediate resistance to the payload as well as to ADCs carrying such inhibitors. Apoptotic dysregulation in cancer cells may also shift sensitivity to the payload. For example, inherent sensitivity to anti–CD79b-vc-MMAE ADC in a large NHL cell line panel was shown to correlate with BCL-XL gene expression via microarray, as well as with antigen expression, but not with cell doubling time (61). Evaluation of such apoptotic markers in acquired ADC resistance (isogenic) cell models is warranted.

Next-Generation ADCs Overcome Resistance

Theoretically, ADCs offer the opportunity that modification of any one of their components may improve their efficacy or safety, and extensive drug development efforts focusing on optimizing each of these components are being deployed. There are already sufficient data suggesting that modification of the antibody, or the linker, or the payload, can improve activity in tumor models with acquired ADC resistance.

Linker modification has been shown to overcome MDR1-mediated liabilities. Kovtun and colleagues (36) replaced the MCC-linker of T-DM1 with a more hydrophilic PEG_4Mal linker and demonstrated improved potency *in vitro* and *in vivo* in MDR1-expressing tumor models. Our group has demonstrated that switching out a non-cleavable linker with a protease cleavable linker, MC-Val-Cit-PAB, can effectively overcome acquired T-DM1 resistance in at least two cell models (27, 49). For example, N87-

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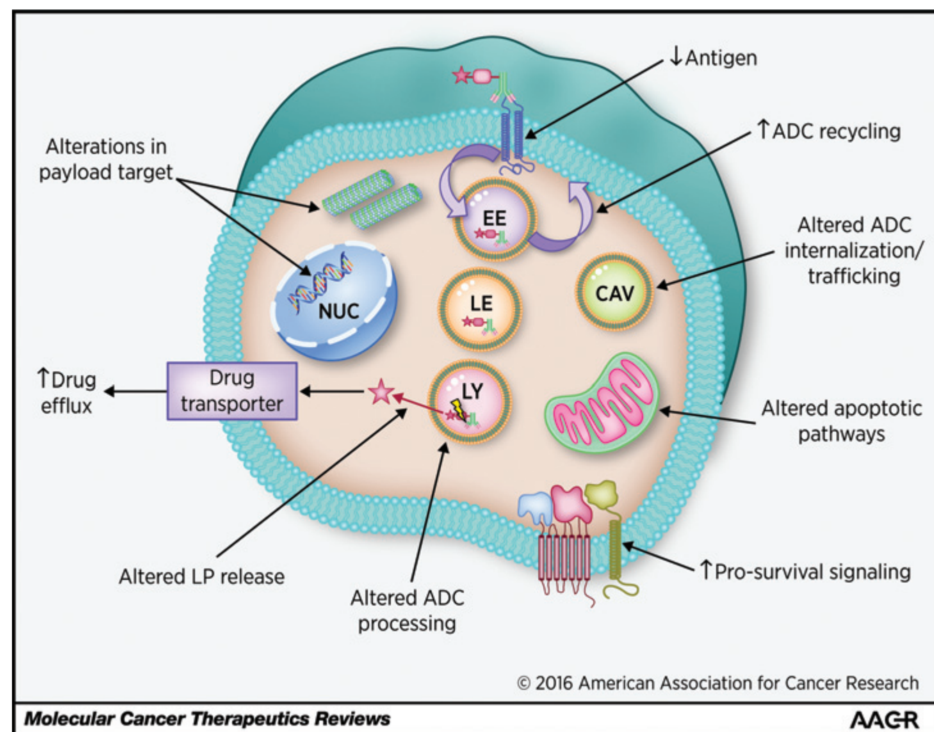

Figure 1.
Emerging mechanisms of ADC resistance. ADCs are complex biomolecules whose mechanism-of-action requires a coordinated series of events, including binding to a cell-surface antigen, internalization, catabolism, and transport of the released payload from the endo-lysosomal lumen to the cytoplasm. Cancer cells, under the selective pressure of ADC treatment, may evolve to become ADC resistant by altering any one of these necessary events. First, target antigen downregulation can prevent proper binding and/or internalization into cells. Following internalization, cells may evolve to divert the lysosomal delivery of the ADC by increasing recycling of the ADC-bound antigen complex to the cell surface or use alternative endocytic compartments for ADC trafficking (e.g., caveolae). Impairment of the lysosomal milieu that is responsible for ADC catabolism may lead to decreased ADC processing and payload liberation from the antibody. If the released species from the ADC requires a lysosomal membrane transporter to efficiently enter the cytoplasm, then loss-of-function of a putative transporter may prevent cytoplasmic accumulation of payload. Alterations in drug efflux transporters (e.g., MDR1, MRP1), the drug target (e.g., tubulin mutations), or any pro-survival downstream signaling pathways (e.g., PI3K/Akt) are potential features of ADC-resistant cells. EE, early endosome; LE, late endosome; LY, lysosome; NUC, nucleus; CAV, caveolae.

TM and 361-TM cells displaying 100- to 200-fold resistance to T-DM1 are completely sensitive to other trastuzumab-ADCs conjugated to MC-Val-Cit-PAB with auristatins (27, 49). Auristatins are also peptide-binding site tubulin inhibitors, yet converting to a cleavable linker overcomes resistance. Furthermore, a next generation cysteine site-specific HER2 ADC delivering an MC-Val-Cit-PAB-auristatin linker-payload shows improved activity in lower HER2-expressing models and overcomes T-DM1 resistance *in vitro* and *in vivo* (31).

Modification of the payload to an alternate mechanism of action can also overcome drug resistance. For example, the *in vivo* NHL tumor models with acquired resistance to anti–CD22-vc-MMAE were challenged with a different ADC delivering a DNA inhibitor-based payload (29). A metabolic product of the anthracycline nemorubicin, PNU-159682, was conjugated via the same cleavable linker MC-Val-Cit-PAB as previously used with payload MMAE, but via site-specific conjugation to A114C to produce a product with two linker-payloads (delivering half the amount of payload as used for the MMAE ADC). The resulting ADC, anti–CD22-NMS249, overcame resistance in both NHL tumor models *in vitro* and *in vivo*. Single-administration of 1 mg/kg of anti–CD22-vc-NMS249 produced initial tumor stasis of the resistant BJAB.Luc-22R1.2 tumors for about 15 days followed by progression, while 2 mg/kg caused prolonged stasis of the WSU_22R1.1 model up to 28 days.

Li and colleagues (30) introduced a novel biparatropic HER2 ADC, which is constructed from the single-chain variable fragment of trastuzumab plus the heavy chain of another HER2 antibody 39S, conjugated at site-mutants S239C and S442C to tubulysin, a microtubule depolymerizing agent. This new anti-HER2 ADC inhibits the growth (at <1 nmol/mL ADC) of their four T-DM1-resistant *in vitro* cell models as well as the N87-T-DM1 resistant *in vivo* model (at 3 mg/kg administered weekly x 4). Because the mechanisms of resistance in these models were not characterized, it is not clear whether the ability of their new anti-HER2-tubulysin ADC to overcome T-DM1 resistance is related to the novel targeting modality, the apparent cleavable linker, or the tubulysin payload. Such a "cassette" approach to ADC construction allows retention of an antigen-mediated strategy of drug delivery in patients whose tumors still express the target but have reduced response due to non–antigen-related mechanisms of resistance.

Conclusions

There are multiple theoretical mechanisms of resistance to ADCs based on their complex structure and function (Fig. 1). Emerging preclinical data with different ADCs and multiple cell line models now suggest that ADC resistance mechanisms may be binned into four general categories: decreased antigen expression, induction of drug transporter proteins, trafficking defects, and/or altered signaling/apoptotic pathways. It does not appear that the method of generating resistance models impacts the mode of resistance; however, there may be cell-dependent differences. In some cases, the emerging resistant pool may result from selection of a small subset of pre-existing refractory clones in the population (12). At this time, there are insufficient data on the mechanisms of resistance mediating clinical failure from ADC therapy due to the paucity of patient pre- and posttreatment biopsy samples and the limited number of approved immunoconjugates. Both inherent and acquired resistance likely contributes to the varied response rates to ADCs in patients. Of the ADCs in clinical use, limited clinical resistance data exist with Mylotarg, Kadcyla, or Adcetris. Patients with AML tend to have higher MDR1 expression (38), but it is not clear if calicheamicin-containing ADCs induce MDR1 expression in patients, or whether such differences in MDR1 expression are caused by prior treatment with chemotherapy. For HER2-directed therapies, PIK3CA mutations and related pathway alterations are associated with poor clinical responses to trastuzumab, pertuzumab, lapatinib, and lapatinib/capecitabine (62, 63). However, recent clinical results indicate that PIK3CA mutations do not correlate with progression-free survival of patients treated with Kadcyla (T-DM1; ref. 63), suggesting some divergence of resistance mechanisms for anti-HER2 antibody and ADC. For Adcetris (BV), initial immunohistochemistry with a small subset of patient samples suggests either retention or reduction of CD30 antigen expression and the potential for drug transport protein overexpression (26, 45, 46).

The mainstay of oncology drug development is to understand the underlying biology for drug success and failure and to develop second- and third-generation therapies based on these data. For next-generation ADCs, there is an opportunity to modify structural ADC components that can address evolving knowledge of cancer biology while retaining the antigen targeting or cytotoxic features of the drug. Bystander activity of released payload in a heterogeneous tumor environment can inhibit antigen-negative cancer cells (64), and is likely an effective approach to enable appropriately designed ADCs to overcome inherent or acquired resistance mediated by various mechanisms. In some T-DM1 ADC-resistant models, enabling a bystander mechanism by converting a non-cleavable linker-payload to a cleavable linker with a permeable cytotoxin effectively overcomes resistance, even when delivered by the same antibody (27, 31, 49). Likewise, rational redesign of the payload to overcome known resistance mechanisms can also improve efficacy in such refractory models (29). Target antigen also remains a key determinant in ADC efficacy, and the targeting of tumor-initiating cells (TIC) provides an opportunity for new ADCs (65). Another promising approach that is being explored to promote durable responses in patients is the combination of ADCs with immunotherapeutics (66). By eliciting the immune system to contribute to tumor detection, it may be possible to overcome the resistance caused by cancer cell autonomous drug resistance mechanisms.

Inherent and acquired drug resistance remains a major barrier to successful cancer therapy. Cellular progression from normal to neoplastic to malignant is a microevolution where genetically unstable cells attempt to bypass the finely tuned regulatory checkpoints which inherently prevent errors. When chronically exposed to drugs, cancer cells use the same elegant mechanisms of diversion to attempt to survive. Cancer cells leave "fingerprints" of these pleiotropic attempts to overcome the drug, allowing us to interrogate their biology with sophisticated tools. By understanding the complex contributors of this evasion, it is possible to identify markers of resistance and to develop impactful new therapies for cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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