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Cell killing by antibody–drug conjugates

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Abstract

Antibody–drug conjugates (ADCs) are designed to specifically bind to and kill cells expressing their target antigens. In addition to the obvious requirement of the presence of the target antigen on the cell surface, several other factors contribute to the sensitivity of target cells to the action of ADCs. These include (i) the rate of internalization of the ADC, (ii) its proteolytic degradation in late endosomes and lysosomes and the subsequent release of cytotoxic drug, and (iii) the intracellular concentration of the released drug. In addition to killing antigen-expressing cells, some ADCs were found to kill bystander cells irrespective of their antigen expression. This review summarizes the current knowledge of the mechanisms of killing of antigen-expressing and bystander cells by antibody–drug conjugates.

Keywords: Antibody–drug conjugate; Cancer

1. Introduction

Antibody–drug conjugates (ADCs) belong to the "targeted chemotherapeutics" category of anti-cancer drugs. ADCs are composed of one or several cytotoxic drug molecules covalently linked to a monoclonal antibody that binds to an antigen expressed on the surface of target cancer cells. Several ADCs containing different cytotoxic drugs are currently in development as anti-cancer treatments [1,2]. The first and only approved ADC, gemtuzumab ozogamicin, a conjugate of an anti-CD33 antibody and the DNA minor groove binder, calicheamicin, is used for the treatment of acute myeloid leukemia [3]. Despite numerous reports on the cytotoxicity of ADCs against cancer cells in culture and in xenograft tumor models, little was known about the mechanism of ADC cytotoxicity until very recently. This review attempts to summarize the current knowledge in this area with the emphasis on three aspects: (a) the modes of internalization of ADCs by cells and intracellular trafficking of the internalized conjugates; (b) the mechanisms of activation of ADCs into cytotoxic compounds; and (c) the nature and the extent of the bystander and the non-targeted cytotoxicities of ADCs.

2. Cytotoxic compounds used in ADCs

Various cytotoxic drugs that target diverse intracellular targets have been conjugated to antibodies

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to specifically kill cancer cells (Table 1). Despite this diversity of the intracellular targets of the drugs, many of them (and, presumably, their ADCs) trigger a similar chain of events: cell cycle arrest (depending on the drug either in S, or G2/M) followed by apoptosis [4–6]. These observations suggest that non-dividing cells that rest in G0 phase may be less sensitive than dividing cells, such as cancer cells, to these drugs and their respective ADCs. Indeed, non-cycling cells were found to be resistant to drugs targeting tubulin, microtubules, or DNA [7–9], suggesting that ADCs of these drugs would also preferentially kill dividing cancer cells.

3. Internalization of ADCs

The major factor that contributes to the selective cytotoxicity of ADCs is their antibody moiety that targets tumor-associated antigens. Binding of an ADC to its target antigen on the cell surface is often followed by internalization of the ADC–antigen complex [1,2]. The rates of internalization of several ADCs were compared to those of their antibodies. A conjugate of an anti-CD30 antibody with the anti-mitotic agent, auristatin [10,11], and a conjugate of an anti-MUC1 antibody with the DNA minor groove binder, calicheamicin [12], were found to internalize with rates similar to those of their respective antibodies. Interestingly, it was reported that two other auristatin conjugates, one with an anti-melanotransferrin antibody and the other with the anti-CD20 antibody rituximab, were internalized more efficiently than the respective antibodies [13,14].

With rare exceptions, proteins, such as antibodies, cannot penetrate cellular membranes, and can only get inside mammalian cells via three distinct internalization routes: clathrin-mediated endocytosis, caveolae-mediated uptake, and pinocytosis [15]. The first two types of antibody uptake are antigen-mediated, while the last one is antigen-independent. The antigen-mediated pathways typically result in accumulation of ADCs on the plasma membrane at a local concentration higher than that of the surrounding medium, in particular if the antibody is of a high affinity. On the other hand, pinocytosis occurs through sequestration of liquid which contains the ADC at the concentration added to the medium. Therefore, at equal rates of internalization, a higher concentration of the ADC in the medium would be needed to deliver the same amount of conjugate via pinocytosis inside the cell than via an antigen-mediated pathway.

3.1. Clathrin-mediated endocytosis of ADCs

The molecular mechanism of clathrin-mediated endocytosis is well characterized [15–17]. Clathrin is a protein that, together with adaptor complex proteins, forms clathrin-coated pits on the cytoplasmic side of the plasma membrane. The pits are eventually sealed forming clathrin-coated vesicles, which then traffic along the endosomal–lysosomal pathway.

Some surface antigens are constitutively concentrated in clathrin-coated pits (e.g. low density lipoprotein receptor [18]), while other antigens (e.g. epidermal growth factor receptor [19] or ErbB2 [20,21]) are randomly distributed in the plasma membrane, and become concentrated in the coated pits only upon binding of their natural ligand or an antibody. Several motifs in the cytoplasmic tails

Table 1
Cytotoxic compounds that have been used in antibody–drug conjugates

Name	Target	Mode of action	Reference
Anthracycline drugs (e.g. doxorubicin)	Topoisomerase II–DNA complexes	Inhibit DNA re-ligation, leading to double-strand breaks in DNA	[66]
Maytansinoids and dolastatin derivatives (auristatins)	β-Subunit on tubulin dimers and microtubules	Suppress microtubule dynamics at low concentrations; depolymerize microtubules at high concentrations	[5,67–69]
Taxol derivatives (taxoids)	The interior surface of microtubules	Stabilize microtubules excessively, altering microtubule dynamics	[5]
Calicheamicin derivatives	TCCT sequences on the minor groove of DNA	Cause sequence-specific double-strand DNA cleavage	[70,71]
CC-1065 and duocarmycin analogs	AT-rich regions of the minor groove of DNA	Induce adenine alkylation	[72]

of cell surface proteins, e.g. the tyrosine-based motif YGCV in CD70 and the immunoreceptor tyrosine-based inhibitory motif in CD22 and CD33, were found to contribute to the movement of these antigens into the pits and their subsequent internalization [22–24]. These motifs appear to mediate the interaction between the antigens and adaptor complex proteins that form the clathrin coat [22,23].

There is evidence that clathrin-mediated endocytosis is a major route for the intracellular uptake of some antibodies and ADCs [25]. Thus, an anti-CD70 antibody and its auristatin conjugates were reported to co-localize with clathrin [22]. Also, the intracellular uptake of several conjugates of an anti-CD30 antibody and of trastuzumab is blocked by inhibitors of clathrin-mediated endocytosis, but not by inhibitors of caveolae-mediated uptake [11,21].

3.2. Caveolae-mediated uptake of ADCs

Two examples of ADCs undergoing caveolae-mediated uptake have been reported. A conjugate of an anti-CD20 antibody with auristatin was reported to internalize via both clathrin-mediated endocytosis and the caveolin-mediated uptake, as indicated by co-localization of this conjugate with clathrin and caveolin [13]. Also, a conjugate of an anti-melanotransferrin antibody with auristatin was found to co-localize with caveolin, but only in those cells that were resistant to the conjugate [14]. However, in sensitive cell lines, the conjugate moved to lysosomes [14] presumably *via* the clathrin-mediated pathway. There data suggest that caveolae-mediated uptake may not lead to the activation of ADCs. This notion is consistent with the observation that the material endocytosed by caveolae accumulates in the endoplasmic reticulum or Golgi [26], the two compartments not known to have proteolytic activity which could release the conjugated drug.

3.3. Pinocytosis of ADCs

It has been well established that, at high concentrations, ADCs kill cells in an antigen-independent manner [9,27–32]. This non-specific killing by gemtuzumab ozogamicin (GO) was examined in detail, and the authors concluded that the cytotoxicity was derived from the ability of cells to take up conjugate dissolved in the surrounding medium via pinocytosis [9]. It should be noted that GO at lower concentrations kills only antigen-expressing cells and this antigen-dependent cytotoxicity relies on clathrin-mediated endocytosis [24]. Unlike GO internalized by the antigen-mediated uptake, pinocytosed GO induced neither S-G2/M cell cycle arrest nor apoptosis, but cells appeared to undergo necrotic lysis [9]. The authors compared the ability of resting and cycling acute myeloid leukemia cells to pinocytose a marker, FITC-dextran, and concluded that the resting cells had a lower pinocytic activity. However, a precise quantification of the amount of pinocytosed ADCs at various concentrations has not been reported.

4. Intracellular trafficking of ADCs internalized via clathrin-mediated endocytosis

Following uptake via clathrin-coated pits, internalized antibody–cell surface antigen complexes traffic along the endosomal–lysosomal pathway. First, clathrin-coated vesicles are uncoated by the Hsp70 protein complex [33] and then the uncoated vesicles fuse with ''early'' endosomes (also known as ''sorting'' endosomes) [17]. Conjugates of an anti-CD70 antibody co-localize with markers of early endosomes, such as transferrin and early endosomes-associated protein EEA-1, after incubation with cells for 3–4 h [20,22]. No data are available on the timing of the transfer of other ADCs from the cell surface to early endosomes.

Endosomes and lysosomes contain the vacuolar H^+-ATPase, also known as the ATP-dependent proton pump, that acidifies the organelles to pH 6.0–6.2 (early endosomes), pH 5.5 (late endosomes), and pH 4.5–5.0 (lysosomes) [34]. We found that Bafilomycin A1, an inhibitor of the vacuolar H^+-ATPase, prevented trafficking of anti-CanAg-maytansinoid conjugates to lysosomes [35]. Similarly, neutralization of the acidic environment of the endosomal and lysosomal compartments with a lysosomotropic agent, such as ammonium chloride or chloroquine, delayed internalization of anti-CD30 and trastuzumab–drug conjugates [11,21]. These observations suggest that acidification of endosomes is required for intracellular trafficking of ADCs. Also, the acidic environment of late endosomes and lysosomes is optimal for functioning of the proteolytic enzymes [34] that hydrolyze antibodies and release conjugated drug molecules (see the next section).

Upon acidification of endosomes, some physiological ligands, such as insulin, epidermal growth factor, and low density lipoprotein, dissociate from their receptors in early endosomes, and the empty receptors recycle back to the cell surface through a so called "recycling compartment", which is composed of narrow tubules [17]. Unlike natural ligands, antibodies, in general, do not dissociate from their antigens unless the pH drops to 3 or lower [36,37], and, therefore, are unlikely to dissociate from their antigens in early endosomes. Depending on the antigen and the antibody, the intact antigen–antibody complexes either recycle to the cell surface (ErbB2-trastuzumab, GD3-antibody complexes [20,38]), or remain in early endosomes (anti-CD20, anti-CD30, anti-CD70, and anti-p97 antibodies [11,13,14,22]).

Eventually, early endosomes fuse with other endosomes and trans-Golgi-network vesicles that deliver proteases and hydrolases [17], thus becoming late endosomes. The content of late endosomes is then delivered to lysosomes through the fusion of these organelles. It takes at least several hours to transfer the endocytosed material from the early compartment to late endosomes and lysosomes, where the endocytosed material is proteolytically degraded. For example, after three hours of incubation of cells with trastuzumab, the antibody was found in sorting endosomes, but not yet in late endosomes or lysosomes, while after 24 h, about half of the initially bound trastuzumab was catabolized [20]. Similarly, we found that CanAg-positive COLO 205 cells hydrolyzed approximately twenty percent of bound anti-CanAg-maytansinoid conjugate in 5 h and it took 22 h to process most (73%) of the initially bound conjugate [35].

5. Intracellular release of the drug moiety

The large size and the hydrophilic nature of intact ADCs prevent them from crossing cellular membranes, such as plasma or endosomal/lysosomal membranes. Therefore, drugs conjugated to antibodies can exert their cytotoxic action only after dissociation from the antibody moiety and subsequent diffusion to their targets in the cytoplasm.

With this in mind, various linkers, that are expected to be relatively stable outside the cells (in the blood), but labile once the ADC is inside the cells, were designed to connect the drug and the antibody moiety. Doxorubicin and calicheamicin were conjugated to antibodies via hydrazone-based linkers that are relatively stable at neutral pH of the blood, but hydrolyze in the acidic environment of lysosomes [2]. Taxoids were coupled to antibodies via pH sensitive ester bonds [39–42]. Maytansinoids were attached via disulfide bond-containing linkers that can be cleaved by disulfide exchange with thiols, such as reduced glutathione which is at about 1000-fold higher concentration inside cells than in blood [1,43,44]. Doxorubicin, dolastatins, and cyclopropylindole were conjugated via peptide linkers containing valine–lysine sequence or β-glucuronic acid [11,45–47]. These linkers are also stable in blood, but get degraded in lysosomes, presumably by cathepsin B or β-glucuronidase.

In addition, several linkers that lack an obvious cleavage site were used to construct ADCs. As expected, these "non-cleavable" ADCs were stable in human serum [48]. Surprisingly, these conjugates were specifically cytotoxic to antigen-expressing cells [29,35]. The conjugate cytotoxicity was abolished by inhibitors of trafficking to lysosomes, Bafilomycin A1 or chloroquine, and by inhibitors of lysosomal serine proteases [21,35]. Metabolic analysis of the antigen-expressing cells revealed that, upon internalization, the conjugates were delivered to late endosomes and lysosomes, where their antibody moiety was completely digested releasing protein-free derivatives of the cytotoxic drug. The released drug of the "non-cleavable" conjugates remains covalently linked to a single amino acid, either lysine for maytansinoid conjugates or cysteine for auristatin conjugates [29,35]. It appears that the lysine and cysteine adducts of these two drugs are as active at interfering with microtubule dynamics resulting in cell death as the respective non-conjugated drugs.

6. Intracellular concentrations of drugs released from ADCs

Many factors affect the concentration of a cytotoxic drug inside cells. Some of them are (i) the rate of internalization of the conjugate, (ii) the rate of degradation of its protein moiety and release of the protein-free cytotoxic drug derivative(s), (iii) the rate of efflux of these derivatives from endosomal/lysosomal compartment into the cytoplasm, (iv) the rate of their efflux outside the cell, and (v) the rate of conversion of these drug derivatives into non-active species (if such a process takes place). If the combination of these factors allows an active drug to accumulate inside a cell at a concentration above the cell-killing threshold, then the antibody–drug conjugate will kill this cell.

The dynamics of intracellular accumulation of protein-free drug molecules released from ADCs was reported only for maytansinoid conjugates [35]. The authors quantified the rate of internalization of ADC molecules into a compartment capable of releasing protein-free active maytansinoids, the levels of accumulation of protein-free maytansinoids derivatives inside the cell, and the rates of their efflux outside the cell [35].

Little is known about the nature of the efflux of low-molecular weight compounds from the endosomal–lysosomal compartment into the cytoplasm and from the cytoplasm to the extracellular space. Several mechanisms, such as the transport of lysine-derivatives of maytansine or cysteine-derivatives of auristatin via an amino acid transporter, passive transmembrane diffusion, or the transport mediated by an ATP-binding cassette (ABC) transporter may contribute to the efflux [35].

The human genome encodes 48 genes for ABC transporters that are predicted to mediate transmembrane transport of various molecules [49]. Three ABC proteins, P-glycoprotein (also known as MDR1), multidrug resistance associated-protein 1 (MRP1, also known as ABCC1), and ABCG2 (also known as MXR or BCRP) were found to be expressed on certain cancer cells rendering them resistant to various hydrophobic drugs [50] including those used in ADCs. For example, all three transporters effectively pump out antracyclines, e.g. doxorubicin [50], and immunoconjugates of this drug are not effective in killing MDR cell lines [51]. P-glycoprotein [4,52,53], and to a lesser degree MRP1 [54] can mediate efflux of the enediyne antibiotic calicheamicin used in gemtuzumab ozogamicin. The P-glycoprotein also transports taxanes and maytansinoids out of cancer cells [55,56].

To overcome the resistance of cells expressing ABC proteins to antibody–drug conjugates, drugs or linkers of ADCs were modified. The substitution of a hydrazide group in the linker by an amide rendered the conjugate of an anti-MUC1 antibody with calicheamicin more efficacious against MDR cells [51]. Also, conjugates of antibodies with the charged molecule, auristatin MMAF, but not with the neutral analog, MMAE, were reported to be potent against several MDR cell lines [29], possibly due to preferential interaction of ABC proteins with neutral hydrophobic molecules [49].

7. Bystander effect of ADCs

Solid tumors often express the target antigen in a heterogeneous fashion [57–59]. ADCs that selectively kill only antigen-positive cells, and spare neighboring antigen-negative cancer cells may be ineffective in eradicating such tumors. Our recent studies revealed that some ADCs kill not only antigen-positive cells, but also other cells in the vicinity, irrespective of the antigen expression on their surface. We observed this ability to kill bystander cells in conjugates of the anti-CanAg antibody, huC242, with maytansinoids, and with a CC1065 analogue [35,60]. These conjugates, at concentrations that are normally non-toxic to antigen-negative cells, completely eradicated them if antigen-positive COLO 205 or SNU 16 cells were in vicinity. Only conjugates linked via a reducible disulfide bond demonstrated this bystander cytotoxicity, while conjugates linked via a non-reducible thioether link exhibited no bystander effect [60].

To characterize the mechanism of this phenomenon, we analyzed the intracellular processing of disulfide- and thioether-containing conjugates of maytansinoids, and found that antigen-positive cells released the attached maytansinoids inside the cell at the same rate, producing a similar cytotoxic effect [35]. However, the nature of the drug metabolites differed. Thioether-linked conjugates were processed into maytansinoid-linker-lysine derivatives. When such derivatives were tested as pure compounds *in vitro*, they were found to be only poorly cytotoxic, probably due to their hydrophilicity and charge, which likely prevented their diffusion across the plasma membrane into the cell. The disulfide linked conjugate was also metabolized to a maitansinoid-linker-lysine. However, additional processing of this disulfide metabolite resulted in a maytansinoid thiol and an *S*-methyl maytansinoid [35]. These neutral lipophilic compounds are highly cytotoxic, and, upon release into the medium, can enter the neighboring cells.

The charge of the drug derivatives released from ADCs may determine the bystander potency of not only maytansinoids, but conjugates of other drugs. Thus, a conjugate of the anti-CD30 antibody with auristatin MMAE is metabolized to a neutral MMAE drug [30] and demonstrates a potent bystander killing [61], whereas the conjugate of the same antibody with auristatin MMAF is metabolized to a charged relatively non-toxic molecule [29] that is, most likely, incapable of bystander killing. Similarly, the disulfide linked conjugate of the CC1065 analogue, DC1, demonstrates a prominent bystander effect [60], because, most likely, it is metabolized to neutral drug molecules. On the other hand, the "non-cleavable" conjugate of DC1 does not kill bystander cells [60], presumably because it is processed to a charged lysine derivative.

In addition to the ability of target cells to process ADCs to membrane-permeable molecules, other factors may contribute to the bystander cytotoxicity of conjugates. For example, a conjugate of an anti-Le^{y} antibody with doxorubicin does not display significant bystander effect in human tumor xenografts [62,63] even though it is likely processed to neutral doxorubicin derivatives by target cells. The cytotoxic potency of doxorubicin against cancer cells is lower that those of maytansinoids and auristatins [64], and the lack of bystander activity of this ADC might be due to the inability of bystander cells to accumulate a cell-killing threshold level of the drug. Thus, the bystander potency of ADCs may also depend on the cytotoxic potency of molecules released by target cells.

The bystander effect adds a degree of non-selective killing activity to the target-cell-restricted cytotoxicity of ADCs. Potentially, this could be a drawback if normal cells in tissues surrounding the cancer are affected. However, this collateral toxicity might be well tolerated if it is limited only to a small number of cells in the immediate proximity to the tumor tissues. Furthermore, the potential toxicities contributed by the bystander effect to normal tissues might be mitigated by the inherent resistance of non-dividing cells to cytotoxic drugs [7,8]. As a potential advantage, the bystander cytotoxicity may enhance the potency of ADCs against solid tumors that express the antigen in a heterogeneous fashion, or when there are barriers to ADC access to all cells of a solid tumor [57,65]. Furthermore, bystander killing may damage tissues intricately involved in supporting tumor growth, such as endothelial cells and pericytes of the tumor neovascula-

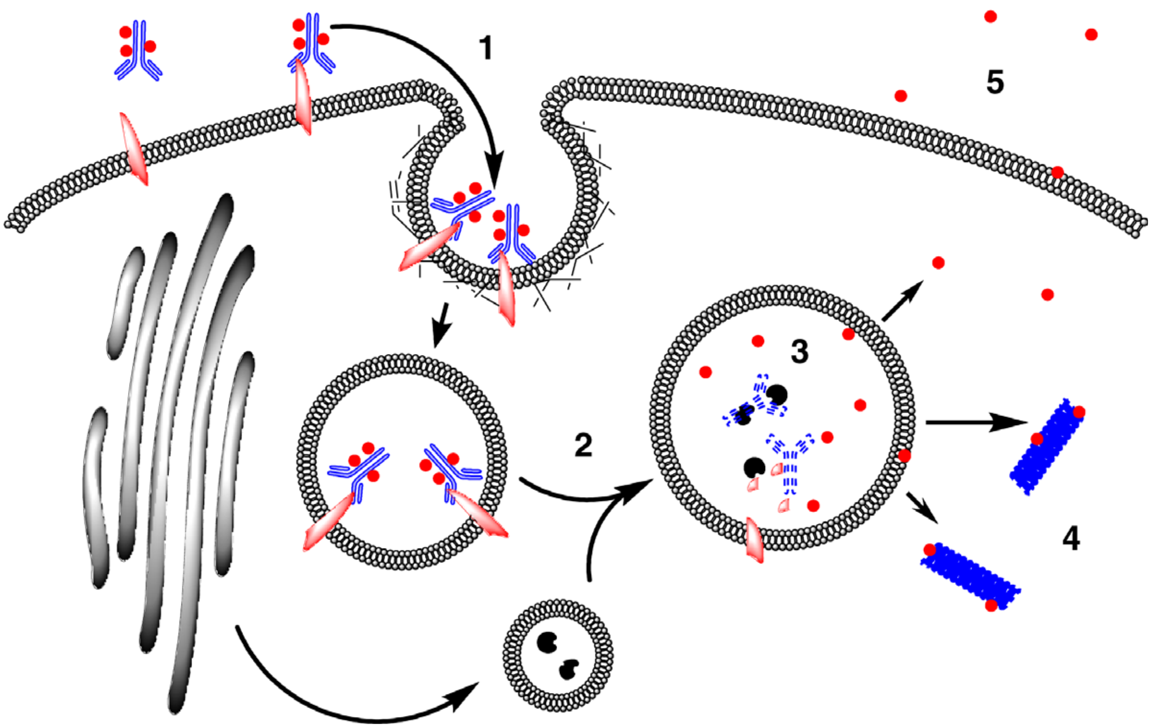

Fig. 1. A model for the internalization and activation of antibody–drug conjugates. The antibody moiety of an ADC binds to the target cell surface antigen, and then the conjugate–antigen complex moves into a clathrin-coated pit (1). The clathrin-coated pits form clathrin-coated vesicles, which internalize, shed the clathrin coat, and fuse with endosomes, lysosomes, and vesicles formed by Golgi (2). The fusion enriches the organelles with proteases and hydrolases, which are most active at the acidic pH of the late endosomes and lysosomes. There, the enzymes digest the antibody and/or the linker and release the drug (3). The drug molecules then cross vesicle membranes and enter the cytoplasm, where they interact with their intracellular target (4) causing cell cycle arrest and subsequent apoptosis. A fraction of the intracellular pool of the released drug leaves the cell, either by diffusion across the plasma membrane, by active transport via ABC pump proteins, or, by loss of plasma membrane integrity in dying cells (5). If these extracellular drug molecules retain their cytotoxic activity and are non-charged molecules, they can cross membranes of neighboring viable cells, killing them via a bystander effect.

ture, or tumor stromal cells, resulting in an enhanced anti-tumor effect.

8. Conclusion

Antibody–drug conjugates (ADCs) combine the potency of cytotoxic drugs and the specificities of antibodies. After more than 20 years of research, ADCs are poised to become a new line of targeted treatments for clinical management of many cancers. The current hypothesis on the mechanism of cytotoxicity of ADCs is summarized in Fig. 1.

Even though a rather comprehensive picture of the intoxication of cells by ADCs is starting to emerge, many questions remain unanswered. For example, it is still not clear if and to what degree non-clathrin-internalization pathways contribute to ADC cytotoxicity, and if various Fc receptors expressed in a number of cell types mediate internalization and activation of ADCs. Also, little is known about the importance of antigen recycling to the cell surface following the delivery of ADCs to endosomes. It might be of interest to find out how soon newly synthesized antigen molecules appear on the surface of target cells after depletion of the initial antigen pool. Regarding the properties of the released drug, some unanswered questions are how long the released drug remains in the cancer cell and how many layers of cells adjacent to the antigen-positive cells can be affected by the bystander cytotoxicity. Answers to these questions would not only explain ADC behavior in culture and in mouse tumor models, but also may help in understanding the relationship between activity seen in preclinical models and in patients, and ultimately allow for the design of more effective antibody–drug conjugates.

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