"Standing by" for Bystander Effects: Dual-Isotope Imaging of Antibody–Drug Conjugate and Payload Distribution

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See the associated article on page 1461.

Antibody–drug conjugates (ADCs) are a promising class of therapeutics for the molecular targeting of cancer. There is a significant pharmaceutical investment in this area, with more than 70 ADCs in various stages of clinical trials and 4 clinical approvals to date. Although there have been several late-stage clinical failures, encouragingly, 2 of the approvals came last year, thus brightening the prospect of additional progress. ADCs combine the specificity of antibody therapy against a tumor-associated antigen with a potent cytotoxic small-molecule payload. Despite large investments into ADC therapy, quantifying the distribution of the cytotoxic payload in the tumor with high spatiotemporal resolution has remained a major challenge. In this issue of *The Journal of Nuclear Medicine*, Ilovich et al. present a quantitative dual-isotope autoradiographic method for separately tracking the distribution of both the antibody and the payload portions of an ADC by repeated imaging after "standing by" until one of the isotopes has decayed (*1*).

A central mechanism of action for ADCs is the delivery of the cytotoxic payload to cancer cells. This delivery is a multistep process consisting of circulation in the blood, extravasation into and interstitial transport through tumor tissue, binding the target antigen, internalization into the tumor cell, payload release, and payload transport to the therapeutic target (typically microtubules or DNA). In the lysosome, the payload is released either from a cleavable linker or as a linker-payload adduct after complete protein degradation. Some payloads are capable of bystander killing by diffusing into nearby cells to exert their pharmacologic effect, whereas others cannot exit the cell in appreciable amounts. The ability of a payload to exhibit bystander effects depends on the physicochemical properties of the released payload. Bystander payloads that are small and lipophilic, such as monomethyl auristatin E (MMAE) and pyrrolobenzodiazepine, are able to permeate out of ADC-targeted cells, diffuse farther into the tumor tissue, and permeate into bystander cells untargeted by the ADC. Nonbystander payloads, such as lysine-emtansine (lysine-DM1) and monomethyl auristatin F, are often larger and more hydrophilic, preventing them from crossing cell membranes and confining their distribution to cells directly targeted by the ADC. In the clinical setting, in which tumors are a heterogeneous mixture of antigen-positive and -negative cells, bystander payloads are able to diffuse out of the ADC-targeted antigen-positive cells to reach and kill antigen-negative cells, albeit indiscriminately. To complicate delivery further, ADCs exhibit a heterogeneous, perivascular distribution in tumors, and there are limited imaging data available showing both ADC disposition and the resulting payload tumor distribution. Despite the significant investment into ADC therapeutics, there is a fundamental lack of knowledge of the relationship between the heterogeneous antibody distribution, the resulting payload distribution, and how both drive efficacy.

To fill this gap in knowledge, several in vitro, in vivo, and computational methods have been used to study bystander effects. Mosaic models, in which antigen-positive and -negative cells are mixed or cocultured, are commonly used for testing bystander efficacy both in vitro and in vivo (*2–4*). However, in these in vitro models, there are no ADC or payload transport limitations, meaning all antigen-positive cells are exposed to ADC, and antigen-negative cells are exposed to released payload in the culture medium. In vivo ADC distribution with clinically relevant doses is highly heterogeneous and often leaves significant portions of the tumor untargeted by the ADC because of limited tissue penetration (*5*). Although bystander payloads show better responses in these mosaic models, it is also unclear how far the released payload can diffuse into the tissue. When ex vivo techniques such as tumor tissue homogenization are used, the average payload concentration in the tumor can be measured through liquid chromatography-mass spectrometry (*2,6*). Although informative, this technique lacks tissue- or cellular-level data, making it difficult to discern whether cell killing is from direct targeting, bystander effects, or other mechanisms. Taken together, the in vitro and in vivo data suggest bystander effects are important for targeting antigen-negative cells; however, there is limited work quantifying the relative impact of direct payload delivery, bystander effects, and payload physicochemical properties that affect their transport and distribution in tumor tissue. Quantifying payload penetration into the tumor will allow for strategies to match payload potency and distribution, ensuring all tumor cells receive therapeutic amounts of payload (*7*).

There are computational methods of estimating small-molecule delivery in tumor tissue (*8*), and these principles can be incorporated into ADC tissue models to provide precise predictions of tissue, cellular, and subcellular payload distribution. Theoretically, bystander payloads with optimal physicochemical properties can

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distribute homogeneously throughout the tumor (*9*). However, there are currently a lack of available experimental payload distribution data to validate or refute these computational predictions. The high potency of ADC payloads makes their distribution challenging to study, because they are often present in minute quantities within the tissue. The experimental work presented here (*1*) provides a critical step in this direction.

In their study, Ilovich et al. present a dual-isotope cryoimaging quantitative autoradiography (CIQA) methodology to independently track the tumoral distribution of both antibody and payload of an ADC (*1*). To our knowledge, this study is the first to visualize both ADC delivery and payload bystander effects at tissue-scale spatial resolution. Alley et al. used a similar strategy to track payload and ADC; although, their study focused on total-organ uptake rather than intratumor distribution (*10*). The CIQA technique consists of labeling the antibody with a residualizing, short half-life, γ-emitting ^{111}In label and a tritiated MMAE payload. The ^{3}H β-decay signal is shielded with foil, whereas the ^{111}In signal of the antibody is imaged through autoradiography. After greater than ten ^{111}In half-lives, when ^{111}In radioactivity is diminished, the shielding is removed, and ^{3}H β-decay signal from the payload is imaged. Once imaged, the autoradiographs are aligned, and colocalization between antibody and payload is measured. ^{111}In is a residualizing label, meaning it remains trapped within the cells in which the antibody is degraded, whereas the ^{3}H-labeled MMAE payload can diffuse between cells. At early times (1 h after injection), the tumor sections showed colocalization between the antibody and payload. By 24 h, the payload distribution started to deviate from the antibody, indicating that he released MMAE payload diffused into neighboring bystander cells. By 96 h after ADC administration, the payload diffused even farther into the tumor and diverged from the antibody distribution, showing only 0.8% colocalization between the payload and antibody signal versus 15% in antigen-negative tumors. The images showing the diverging distribution of ADC and payload provide the first direct visualization of the bystander effect. Although the images showing the distribution of ADC and payload are compelling, the authors did not include the colocalization analysis for the early times, so it is difficult to determine the impact of the processing/alignment steps on the maximum expected colocalization with intact ADC. However, the higher colocalization in antigen-negative tumors is consistent with the lack of antigen-mediated cleavage of the payload or more diffuse uptake of ADC throughout the antigen-negative tumors through nonspecific macropinocytosis. These results are also an important reminder that macroscopic imaging in the clinical setting, due to practical PET scanner resolution and fundamental positron diffusion distances, does not elucidate the microscopic heterogeneity within these lesions.

Although the CIQA methodology appears promising, there are several considerations for future work and additional questions surrounding payload distribution that remain to be answered. It will be interesting to see how sensitive the technique is to capturing ADC and payload distribution in antigen-positive tumors with lower expression, or when the ADC dose is low. This may be relevant for higher-potency payloads administered at small doses. The distance the ADC traverses into the tumor (a dynamic saturation front often called the binding-site barrier) is in part a function of antigen expression and antibody internalization rate. For example, the divergence of the payload signal from ADC signal in tumors with lower antigen expression may be reduced due to better antibody penetration. Staining interleaving histology sections may provide more detailed tumor structure and help quantify uptake in the immune infiltrate and other noncancer cells. These and other adaptations of the CIQA method should help elucidate (or rule out) the impact of heterogeneous payload delivery on efficacy for various ADC carriers and payloads to improve ADC design.

In summary, the CIQA methodology has the potential to significantly improve our understanding of the link between antibody/payload distribution and overall efficacy of ADCs. This technique provides tissue-scale visualization of the distribution of both the ADC and the payload in a relevant tumor microenvironment. Despite the promise of this approach, much work remains to be done. We anticipate this method will provide critical data to optimize payload physicochemical properties and improve tumor distribution. Additionally, Ilovich et al. have outlined methods for improving the CIQA method by using a less time-consuming and more cost-effective ^{67}Ga isotope on the antibody. We await the insights the CIQA methodology will provide on the distribution of both bystander and nonbystander payloads to help bridge the knowledge gap between tumor payload distribution and ADC efficacy.

DISCLOSURE

Greg M. Thurber has advising/consulting relationships with Abbvie, Advanced Proteome Therapeutics, Bristol-Myers Squibb, Crescendo Biologics, Eli Lilly and Company, Immunogen, Nodus Therapeutics, Roche/Genentech, and Takeda Pharmaceuticals. No other potential conflicts of interest relevant to this article exist.

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