The Direct Route May Not Be the Best Way to Home

. . . life was like a box a chocolates, never know what you're gonna get.

Forest Gump
Paramount Pictures

Pretargeting is a good example of this statement, because it involves various alternative strategies and ingredients of the basic principle of separating the targeting agent from the compound that secondarily delivers a diagnostic or therapeutic modality. Pretargeting was first conceptualized as a means to reduce the amount of radioactivity in the blood to better visualize tumors, since, at the time, the alternative was directly radiolabeled antibodies, primarily IgG. The early pretargeting procedures were shown to provide excellent tumor-to-nontumor ratios (*1*,*2*), and early clinical experience with a pretargeting procedure was also encouraging, providing better visualization of tumors than a directly radiolabeled IgG (*3*). However, the level of enthusiasm for using a multistep pretargeting procedure that required several days for tumor visualization (e.g., allowing time first for the bispecific antibody to localize before the radiolabeled compound could be given) diminished when similar localization ratios were achieved with directly radiolabeled antibody fragments in just one step (*4*). Although there was still a measure of optimism that pretargeting, because of its high tumor-to-background ratios, could be used more successfully than a directly radiolabeled antibody for therapy, there was still a concern that the amount of radioactivity delivered to a tumor would be far less than that of a directly radiolabeled IgG. After all, it had been repeatedly shown by many groups that fragmentation of IgG accelerated its blood clearance, which inevitably reduced the fraction of the injected product localized to the tumor. So it was logical to assume that a compound as small as a peptide would accrete at a lower level than that seen with an IgG. However, this view soon changed. Studies in a human ovarian cancer/nude mouse model were one of the earliest reports of a pretargeting procedure based on the avidin-biotin approach (pretargeted biotinylated antibody followed by radiolabeled streptavidin) that achieved higher tumor uptake than a directly radiolabeled antibody (*5*). This was performed in an intraperitoneal model and with the agents also given intraperitoneally; therefore, while important, this finding may have been a consequence of the model rather than the method. Additional encouragement that pretargeting could be a viable therapeutic alternative came from evidence obtained by Le Doussal et al. (*6*), using a bispecific antibody pretargeting procedure, who showed clinically that tumor uptake taken from biopsies 48 h after a pretargeted ^{111}In-DTPA-hapten (DTPA is diethylenetriaminepentaacetic acid) were similar to those found with an ^{111}In-anti-carcinoembyonic antigen (CEA) $F(ab')_2$. This suggested that pretargeting could localize an appreciable fraction of the injected dose to the tumor and still deliver higher localization ratios. Axworthy et al. (*7*), working with an avidin-biotin pretargeting procedure, showed that, with appropriate adjustments, uptake (i.e., percentage injected dose per gram [%ID/g]) of radiolabeled biotin in a subcutaneous tumor xenograft could rival that of a directly radiolabeled IgG, achieving tumor uptake in excess of 20 %ID/g, while maintaining significantly higher tumor-to-nontumor ratios, which led to the first report of improved therapy. There now have been several reports showing that pretargeting procedures improved therapy, not just because of a higher therapeutic index but also because the procedures allow for a substantial fraction of the injected activity to be targeted to the tumor (*8*–*17*). Despite these findings, there continues to be an enigma: How can a small molecule (e.g., <2 kDa) that clears so quickly from the blood achieve the same uptake in a tumor as a radiolabeled IgG that is able to accumulate to a relatively high level seemingly because of its slow blood clearance which allows multiple passes of the antibody through the tumor? Indeed, there have been several peptides and other small molecules used as direct targeting agents in various animal models that do not achieve the level of uptake obtained by most pretargeting procedures (*18*–*23*).

Perhaps one of the most remarkable pretargeting findings was that reported in 1998 by Boerman et al. (*24*) at the Society of Nuclear Medicine's Annual Meeting, with the full report appearing in 1999 (*25*). They showed that >80 %ID/g of a bivalent hapten could be targeted to a renal cancer xenograft in nude mice within just 1 h, using an anti-G250 bispecific antibody pretargeting system, and that this level persisted over several days, resulting in some extraordinary tumor-to-nontumor ratios. Others had previously shown relatively high uptake in renal

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cell cancer xenografts using whole IgG (*26,27*) in comparison with the values typically reported for other tumors, but nothing compared with the level achieved with a pretargeted peptide in a subcutaneous renal tumor xenograft.

In the article by van Schaijk et al. (*28*) on pages 495–501 of this issue of *The Journal of Nuclear Medicine*, this group has now examined a variety of properties of 3 different human renal cancer cell lines in an attempt to explain the high delivery capability. Antigen density varied among the 3 cell lines grown in vitro from as low as only 4,000 copies to as many as 600,000 per cell. Importantly, the investigators were also careful in making an assessment of the relative antigen content in transplanted tumors isolated from the animals. They also determined the relative number of antigen-expressing cells from these in vivo tumors by flow cytometry and used immunohistology to reveal the architecture of the tumor in relation to the presence of the antigen, in addition to an assessment of the blood vessel number and distribution within the tumors. These results confirmed that the antigen content, as defined by in vitro analysis, was reflected in the transplanted tumors but also revealed that not all cells expressed the antigen in the transplants. However, the uptake of the pretargeted peptide was not related to antigen content at all, with the tumor cell line having the least antigen achieving the highest uptake. This cell line also had the highest density of blood vessels, but these vessels were distributed primarily on the outer rim of the tumor, with the xenografts from this cell line showing more central necrosis than the other 2 cell lines. Although the uptake in this tumor cell line achieved a remarkable 300 %ID/g within 1–4 h after injection, this line also had a significant washout, decreasing to 100 %ID/g within 24 h, but then remaining at this level through 3 d. Unfortunately, autoradiography of the radiolabeled peptide was not performed to assess whether pretargeting had allowed for a peptide to distribute uniformly in these tumors or whether the activity was still mostly restricted to the areas near the blood vessels. This assessment would have enhanced our understanding of how tissue architecture, antigen location, and necrosis (e.g., increased interstitial pressure, "binding-site" barrier (*29,30*)) influence the distribution of the bispecific antibody and the radiolabeled peptide within the tumor in a pretargeting setting. Since the distribution of radioactivity within the tumor would also depend on the bispecific antibody protein dose and the degree of antigen saturation, it is likely that the pretargeting conditions used in this study would need to be adjusted for each cell line to provide favorable conditions that could lead to a uniform distribution of radioactivity. In addition, though the investigators clearly used conditions that favor a high fractional uptake of the radioactivity, it is uncertain whether these conditions could be duplicated had the amount of peptide been adjusted to a level suitable to deliver therapeutic doses of radioactivity to these tumors. Our experience with a different bispecific antibody pretargeting system has indicated that the optimization of this type of pretargeting method is simplified when a good estimate of the amount of peptide that may be required at therapeutically active doses is known (*31*). This then can be used to define the minimum amount of bispecific antibody necessary to capture the highest fraction of this amount of administered peptide and an appropriate interval that will optimize tumor-to-nontumor ratios. It should also be appreciated that uptake expressed as %ID/g can be misleading. Clearly, it is impossible for tissue uptake to exceed the amount given (i.e., >100%) and, in fact, based on the actual weights of the tumors, about 15% of the total injected peptide was captured. We have found that as much as 80% of the injected radioactivity is cleared from the body within a few hours and also achieve 10%–15% of the total injected activity delivered to tumors. This is truly incredible when one considers how quickly the peptide is cleared and the small fractional amount of the administered activity that could possibly pass through the tumor in this short period of time. This suggests that pretargeting procedures are highly efficient capturing systems, possibly capturing a high fraction of the total activity encountered by the tumor.

As was found earlier with the targeting of radiolabeled antibodies to renal cancer xenografts (*32*), vascular permeability was the only measured property that was directly attributed to tumor uptake and efflux of the peptide for each tumor cell line. Although vascular permeability may have been a deciding factor in their defining the level of pretargeted peptide uptake in these renal cancer xenografts, it is unlikely that this is the primary factor that defines the level of peptide targeting to set these renal cell lines in contrast to other solid tumor models. For example, the vascular permeability of 2 of the renal cancer cell lines was similar to the vascular permeability of the 2 colorectal cancer cell lines. If vascular permeability were *the* deciding factor in determining uptake, one would suspect that a relevant pretargeting system for these colorectal cell lines could achieve the same level of targeting. Although our group has not studied pretargeting of radiolabeled peptides using anti-CEA bispecific antibodies in these particular colorectal cancer cell lines, we have observed a maximum peptide uptake of about 30 %ID/g in the signet-ring-cell, GW-39 human colonic adenocarcinoma weighing 0.3–1.0 g (*33*). Although the vascular permeability in this cell line grown subcutaneously in nude mice was determined to be 43.8 ± 9.2 μL/g/h (*34*), which is ~2-fold less than that measured in the colorectal cancer cell lines that they tested, it would seem doubtful that this factor alone would lead to such an impressive uptake. Indeed, van Schaijk et al. (*28*) have shown uptake of ~100 %ID/g can be obtained in one of the other renal cancer cell lines that has a vascular permeability similar to that of the colorectal cell lines. The vascular volume of the renal cancer cell lines is also another parameter that could con-

tribute to the overall success of targeting in this model, since it was nearly 2–4 times that of the colorectal cancer cell lines. Vascular volume could be of particular importance for a targeting system in which the effector is cleared so rapidly from the blood. In this situation, a tumor with a high permeability and larger vascular volume would be expected to have a larger throughput of the peptide and, thereby, a greater likelihood for capture. Indeed, having the blood vessels concentrated in the perimeter of the tumor would not only provide ease of access (i.e., would not have to compete against higher central interstitial pressure in necrotic zones), but they may be present in a larger surface area because the vessels are on the outer circumference of the tumor. Thus, with less influence of interstitial pressure, a large surface area, a large vascular volume, and a highly permeable blood vessel, enhanced targeting for this one cell line could be achieved.

We have reported previously that accessibility of the antigen plays a significant role in defining the level of targeting obtained with a directly radiolabeled antibody (*34*). Accessibility is a very convenient, but complex, term that encompasses multiple factors, both on a physiologic and an anatomic level. Thus, though this article may not have exhaustively explored pretargeting in these renal cell xenografts with a side-by-side comparison with another pretargeting system with other tumors, it is clear there is something unique within the these renal cancer xenografts that sets the accessibility of the antigen apart from other systems. The fact that the cell line with the highest targeting ability only had 4,000 copies of antigen per cell is also a testament of the importance of accessibility to any targeting procedure, since, though there is a paucity of antigen, the level of uptake behaves as if there were larger numbers of targets. Indeed, accessibility can encompass issues of how the antigen is presented (most likely on the cell surface) and even whether they are in close proximity to one another. This group had previously shown the importance of a divalent hapten in this pretargeting procedure, not only from the perspective of magnitude of uptake but also its retention (*25*). With just 4,000 copies per cell, perhaps proximity becomes important to encourage the binding of the hapten in a divalent manner, thereby engendering the visual concept of the affinity enhancement system as it was first proposed, with the divalent hapten bridging 2 monovalently bound bispecific antibodies on the cell surface to create a more avidly bound complex (*35*).

Of interest, too, was the finding in this study that there may be appreciable differences in how the bispecific antibody is processed within the tumor, since comparisons of ^{125}I- versus ^{111}In-labeled anti-G250 IgG in these tumors showed a significantly higher uptake with the ^{111}In-labeled IgG than with the ^{125}I-IgG. This result suggests that the bispecific antibody may have a higher tendency to internalize more with some cell lines compared with others. Although variability in bispecific antibody accessibility on the cell surface will likely occur, this finding should be viewed cautiously, since the anti-G250 IgG, rather than the anti-G250xDTIn-1 bispecific antibody, was used for this assessment. Since cellular processing of macromolecules could be influenced by the manner in which they are bound to the cells (e.g., monovalent, as in the case of the anti-G250xDTIn-1 bispecific antibody vs. potentially divalent with the anti-G250 IgG), it is possible that these differences might not be as pronounced as that represented by the whole IgG.

Thus, tumor physiology does play a role in defining the level of targeting and, although antigen content did not appear to be relevant, care must be taken not to overstate the importance or lack of importance of one factor over another in defining the targeting of a given system. Targeting is a complex process that undoubtedly is controlled by several factors. Some of these can be identified, whereas others are more obtuse. Controlling all of these factors (including the quality of the targeting agents, and host and tumor physiology) in a manner to selectively increase the targeted fraction remains a worthy goal and still a challenge. There have been numerous reports of improved tumor targeting by altering tumor physiology by several means (*36*), but these approaches, at least at this time, have not translated clinically to improved therapeutic outcome.

Despite the fact that the exquisite targeting afforded by this renal cancer model has not been duplicated in other tumor systems, pretargeting procedures have evolved considerably, and there is now a growing consensus that these methods can substantially increase the therapeutic prospects for targeted radionuclides. Though pretargeting may remain an enigma to many, it is clear that the rapid movement of the radionuclide from the vascular to the extravascular space places the radionuclide where it can interact with the tumor, whereas a substantial fraction of a directly radiolabeled antibody spends a large part of its time in the vascular space, outside the reach of the tumor cells. However, pretargeting appears to go beyond this simple truth, with its surrogate receptors (e.g., whether an antihapten antibody or streptavidin) seemingly more accessible or amenable (e.g., enhanced affinity or avidity) to capturing the small effector molecules, thereby providing very rapid uptake of the radionuclide with equally rapid removal from untargeted tissues. These properties, together with it ability to concentrate an appreciable amount of the radionuclide in the tumor, will hopefully lead to the successful application of targeted radionuclides for solid tumors, as well as potentially improving the therapeutic outcome in the approved indication of non-Hodgkin's lymphoma for directly radiolabeled anti-CD20 antibodies (*37,38*).

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