human colon carcinoma grafted into nude mice. *J Exp Med* 1983;158:413–427.
21. Rosebrough SF. Pharmacokinetics and biodistribution of radiolabeled avidin, streptavidin, and biotin. *Nucl Med Biol* 1993;20:663–668.
22. Petrelli F, Moretti P, Paparelli M. Intracellular distribution of biotin-^{14}COOH in rat liver. *Molec Biol Rep* 1978;4:247–252.
23. Eger RR, Covell DG, Carrasquillo JA, et al. Kinetic model for the biodistribution of an ^{111}In-labeled monoclonal antibody in humans. *Cancer Res* 1987;47:3328–3336.
24. Houston AS, Sampson WFD, Macleod MA. A compartmental model for the distribution of ^{113m}In-DTPA and ^{99m}Tc-(Sn)DTPA in man following intravenous injection. *Inter J Nucl Med Biol* 1979;6:85–95.
25. Brigham KL, Harris TR, Rowlett RD, Owen PJ. Comparisons of [^{14}C]urea and [^{3}H]mannitol as lung vascular permeability indicators in awake sheep: evidence against red cell urea trapping. *Microvasc Res* 1977;13:97–105.
26. Clauss MA, Jain RK. Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res* 1990;50:3487–3492.
27. Fujimori K, Covell DG, Fletcher JE, Weinstein JN. Modeling analysis of the global and microscopic distribution of immunoglobulin G, F(ab')$_2$, and Fab in tumors. *Cancer Res* 1989;49:5656–5663.
28. Green NM. Avidin: 1. The use of [^{14}C]biotin for kinetic studies and for assay. *Biochem J* 1963;89:585–591.
29. Jain RK. Transport of molecules in the tumor interstitium: a review. *Cancer Res* 1987;47:3039–3051.
30. Dedrick RL, Flessner MF, Collins JM, Schultz JS. Is the peritoneum a membrane? *ASAIO J* 1982;5:1–8.
31. Nugent LJ, Jain RK. Extravascular diffusion in normal and neoplastic tissue. *Cancer Res* 1984;44:238–244.

EDITORIAL

Tumor Pretargeting: Almost the Bottom Line

Human imaging studies have shown that while maximum human tumor concentrations of MAb are achieved in one day, the slow pharmacokinetics requires several days for the background to fall sufficiently for sensitive radioimmunoscintigraphy of tumors. With therapeutic radionuclides such as ^{90}Y, this long biological half-life imposes a high radiation burden on sensitive normal tissues from the large amount of retained radioactivity. Normal tissue toxicity, especially to the bone marrow, has been the major limiting factor in the application of radioimmunotherapy to solid tumors. The use of improved bifunctional chelating reagents and techniques reduces free yttrium, lowers liver, bone and marrow uptake and decreases the radiation dose to these normal organs. Pretargeting techniques provide an alternative way to get high selective tumor uptake of ^{90}Y with simultaneous minimization of nontarget tissue background.

Pretargeting involves administration of a long-circulating targeting macromolecule (MAb) having a high affinity noncovalent binding site for a small rapidly excreted effector molecule, which is given after the MAb has concentrated in the target tumor (T). Removal of the macromolecule-binder conjugate from the circulation with a polyvalent "chase" macromolecule before giving the effector molecule greatly improves the target-to-blood ratio (T/B). The aggregated MAb produced by cross-linking with the chase in the circulation, is rapidly endocytosed by reticuloenothelial cells (Kupffer cells), mostly in the liver (*1*). The intracellular location of the endocytosed MAb prevents the access and binding of subsequently injected effector molecules, so liver uptake of radioactivity remains low. Soon, (approximately 1 hr) after the chase, the effector molecule (radiolabeled hapten or biotin conjugate) is given, and the maximum tumor concentration and tumor- to-normal tissue ratio is achieved in 1–3 hr. Unbound radiolabel (> 90% of the injected dose) is rapidly excreted via the kidneys, leading to greatly decreased radiation exposure to normal tissues. Several targeting macromolecule-conjugate / effector small molecule pairs have been proposed (Table 1 and Fig. 1). Examples are: MAb/hapten (*2–5*), MAb-avidin/biotin (*6*), MAb-biotin/avidin (*7*), MAb-enzyme/prodrug (*8,9*) and MAb-oligonucleotide/antisense oligonucleotide (*10*). These systems give higher target-to- normal tissue ratios with less toxicity than covalent conjugates of MAbs and effector molecules.

Qualitative comparison of the pharmacokinetics of directly labeled MAb, two-step and three-step pretargeting is depicted in Figures 2, 3 and 4. For simplicity, only the blood and tumor concentrations are illustrated over 4 days. Directly labeled MAb circulates for days with maximum tumor concentration occurring at 1–2 days with continuing high blood concentration for several more days (Fig. 2). Reducing the circulating half-time by decreasing the molecular size [F(Ab), F(v) fragments, peptides] improves the tumor-to-blood ratio, but decreases the time integral in the blood (blood concentration × time). This shortens the period during which a high concentration gradient exists between the blood and the tumor, which is the driving force for diffusion into the tumor. In addition, high concentrations in nontarget normal organs such as the kidney [Fab] (*11*) and lung [VIP] (*12*) can be problematic with labeled fragments and peptides (*13*). Thus, with directly labeled low molecular weight fragments, a low blood concentration giving high T/B1 ratios is achieved only at the cost of lower tumor uptake.

Pretargeting combines the pharmacokinetics of long circulating MAb with rapidly excreted small effector molecules to give both high tumor concentration and high tumor-to-normal tissue ratios (Figs. 3 and 4). The two-step method eliminates radiation during the MAb localizing phase, which can take several days. Nonspecific localization at this stage in liver, spleen and bone marrow, due to damaged or heavily labeled molecules and aggregates, does not contribute to normal tissue radiation since radioactivity is only injected later. Previous at-

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TABLE 1
Targeting Macromolecule-Binding Site Conjugate: Effector Molecule Pairs for Pretargeting

Targeting macromolecule	Effector binding site	Effector molecule
Monoclonal antibody	Anti-hapten MAb	Hapten – DTPA, DOTA + radionuclide
Monoclonal antibody	Streptavidin (Step 2 or 3)	Biotin conjugate – DTPA, DOTA + radionuclide
Monoclonal antibody	Oligonucleotide	Anti-sense oligonucleotide + radionuclide
Monoclonal antibody	Enzyme	Prodrug

tempts to overcome this problem have required the administration of large amounts of "cold" MAb along with directly labeled MAb to saturate the nonspecific binding sites. Two-step pretargeting requires a long waiting period for the blood concentration to fall, since any MAb still circulating must be saturated before any activity can reach the tumor.

Blood activity can be efficiently lowered by the addition of a chase step (Fig. 4). The chase quickly reduces blood MAb to low concentrations so that radioactivity can be administered within 1 hr. The relative exposure:
[RE = TR: therapeutic ratio: area under curve tumor (AUC T)/area under curve blood (AUC BL)]

is greatly increased as a result of the chase, which is highly desirable for radioimmunotherapy. The essence of pretargeted tumor localization or therapy deals not only with the absolute concentration but also with the rate of

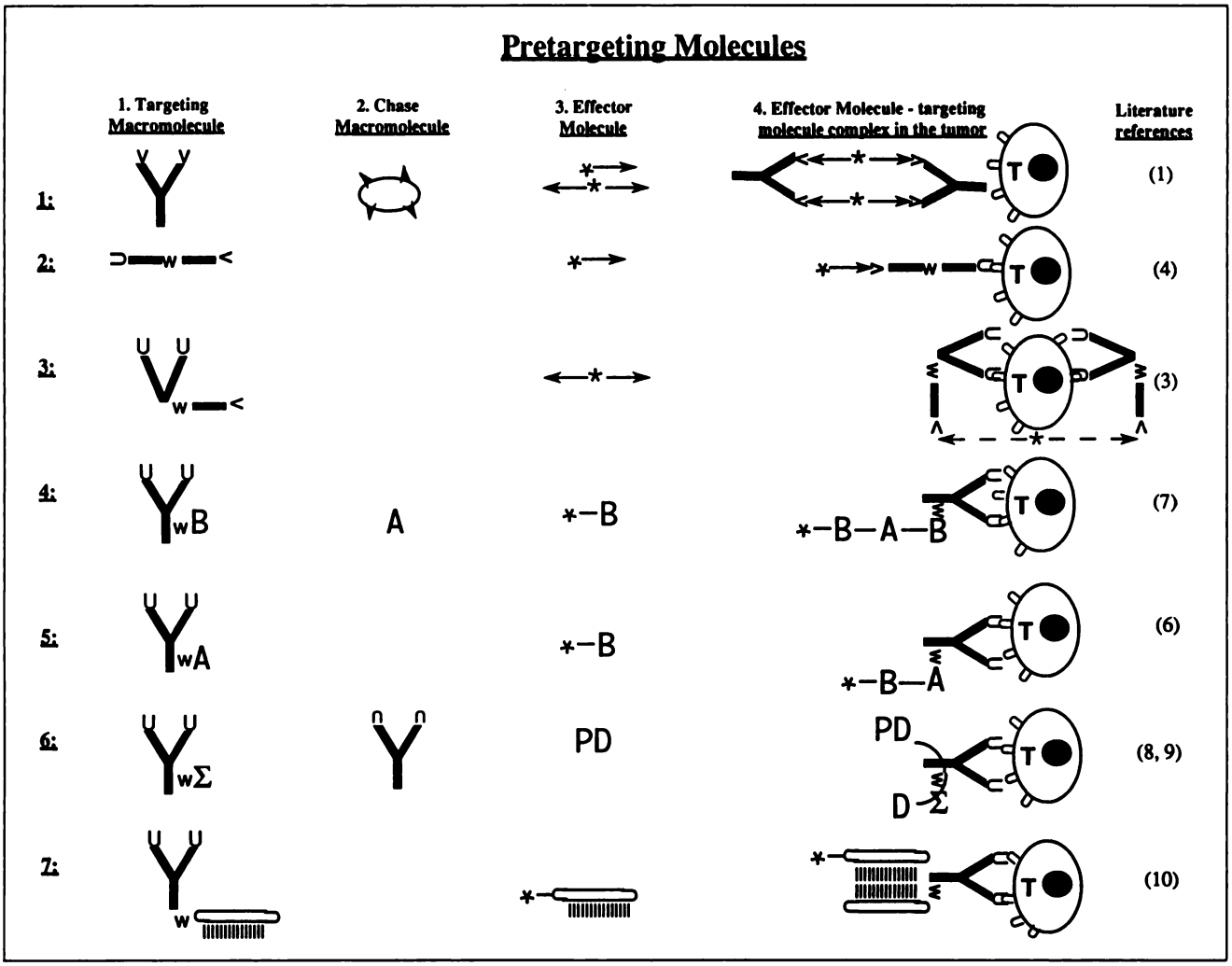

FIGURE 1. Pretargeting molecules based on MAbs. Seven pairs are shown: three hapten/anti hapten, two biotin/avidin, one enzyme/prodrug ADEPT (antibody dependent prodrug therapy) and one antibody-oligonucleotide/antisense. Three include a chase step. The targeting molecule is large and long-circulating, whereas the effector molecule is small, rapidly diffusable, short-circulating and quantitatively excreted by the kidneys without concentrating in any normal organs other than the kidney. ⟷ is hapten radiolabeled, bivalent; ⟷*⟷ is hapten radiolabeled, monovalent; w is chemical linker; < is Anti-hapten CDR; ⊃ is Anti-tumor CDR; ≺ is whole antibody (MAb); ≺ is F(ab')$_2$, MAb; – is Fab, MAb; A is avidin (streptavidin); *–B is biotin radiolabeled conjugate; Σ is enzyme (MAb conjugate); PD is prodrug; D is drug; *– is radiolabeled antisense DNA; ⟲ is polyvalent hapten (protein conjugate).

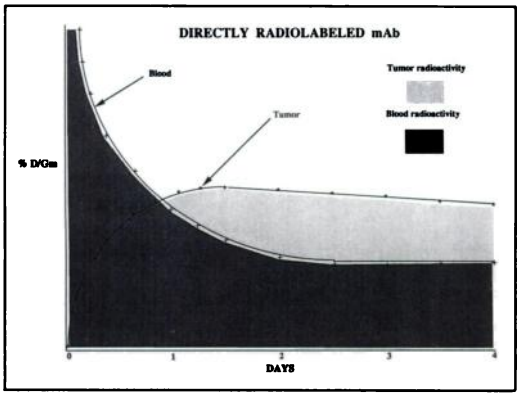

FIGURE 2. Time-concentration curves in tumor mice of covalent conjugates of directly labeled MAb in blood and tumor. The high tumor concentration is offset by high blood levels, giving a TR ≈ 3/1 compared to ≈24/1 for pretargeting.

clearance of radiolabel from the body (which should be fast) relative to the rate of clearance from the tumor (which should be as slow as possible). These rates have been measured directly and were shown to be potentially adequate for ^{90}Y therapy in a mouse tumor model using a MAb hapten system (*14*).

Many questions regarding the optimal dosing schedule, timing, molecular weight, valency, affinity constants, specific activity, rates of metabolism and antigen modulation in pretargeting still need to be answered. The effect of varying each parameter can be studied by a very large number of well-designed and carefully controlled experiments, but much insight into the pharmacokinetics can be obtained from an appropriate mathematical model. A set of nonlinear ordinary differential equations has been developed by Jain et al. for this purpose (*15*). In this issue, Sung and van Osdol (*16*) have compared a directly labeled MAb with two variations of the avidin-biotin system. They pay special attention to the problems of temporal uptake and spatial distribution of radioactivity within the tumor, factors important for the timing of the steps and for homogeneous delivery of therapeutic radionuclides.

Some insight into the complexity of the pharmacodynamics is gained from a comparison of the results from tumor mouse experiments with the results predicted by the Sung and van Osdol model. The size of the tumor used in this model was a sphere with a radius equaling 150 microns; this corresponds to a tumor weight of ≈14.14 μg. This is a very small tumor compared to the usual 100–350 mg range. The following calculations were carried out for the low dose 50 nM plasma concentration of biotin. By using the 3000 ml plasma volume specified by the authors for a 70-kg man, the amount of free plasma biotin = 75 nM × 3 liter = 225 nmole − 15 nmole (saturation of 10% circulating MAb) = 210 nanomoles. The total amount of MAb in the small tumor is calculated from the molar concentration × wt [325 nM: Fig. 3 in Ref. (*16*) = 325 × 10^{-3} pmole/μg] × 14.14 μg = 4.6 pmole. Assuming the MAb in the tumor is saturated with biotin at a 1:1 molar ratio, the maximum % ID in tumor = 4.6 × 10^{-12}/2.1 × 10^{-7} × 100 = 2.2 × 10^{-3} % (or 0.156 %/g). This suggests that for higher uptake in the tumor, we should inject less biotin of higher specific activity. High specific activity ≥ 1000 Ci/mM becomes necessary for receptor (Ag) targeting at nanomolar concentrations. These values are easily obtained by labeling hapten or biotin chelate conjugates with no-carrier-added radionuclides such as ^{99m}Tc, ^{111}In, ^{90}Y or ^{68}Ga.

A MAb tumor concentration of 325 nM is rather high, ≈33% of the theoretical maximum. For example a 1-g tumor contains ≈10^9 cells, each with ≈10^6 epitopes (a high number of Ag) = 10^{15} molecules [≈1 nmole/g (ml) or 1 μM (1000 nM)]. Thus, it would take one-third saturation with MAb at a 1:1 molar ratio under ideal conditions to reach 325 nM. It would be interesting to model the chase (three-step), where the circulating MAb is ≈0.1% rather than 10%, for tumors in the more usual milligram range.

A surprising result given by the model is the very long time it takes for MAb (low dose) to reach the center of the tumor (r = 150 microns): 3 days for MAb, 9 days for MAb-avidin conjugate. Simple linear extrapolation reveals that over 1 yr (372 days) would be required for MAb-avidin conjugate to reach the center of a 1-g tumor (r = 6.2 mm). These data suggest a long interval is necessary before giving the radiolabel in tumors 1 g or larger. The model gives a T/B1 ratio of 10:1 at only 18 hr after biotin compared to 90 hr after directly labeled MAb. It should be noted that with rapidly diffusable radiolabeled biotin or hapten conjugates maximum tumor concentrations are obtained much sooner, usually within 3 hr (see Fig. 4). Clearly, more input is needed from microscopic radioautographic distribution data to get a range of values for diffusion times in different tumors of various sizes.

Consideration of the pharmacoki-

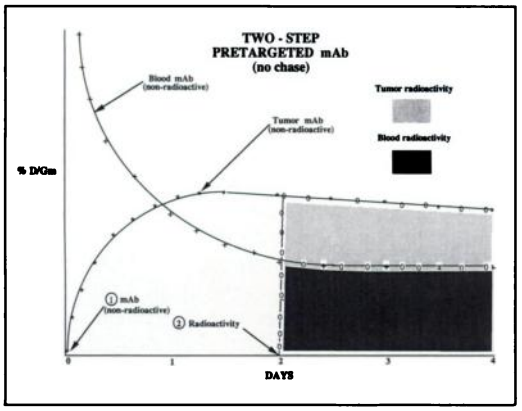

FIGURE 3. Pharmacokinetics of two-step pretargeting. A long waiting period is needed for blood MAb levels to fall. There is no radioactivity present during the MAb localization phase.

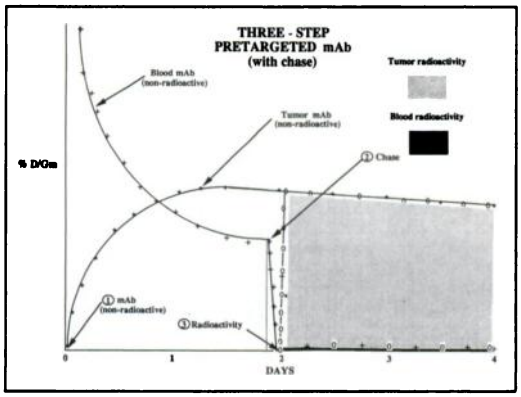

FIGURE 4. Pharmacokinetics of three-step pretargeting. Rapid uptake at 3 hr and slow release of hapten from the tumor is shown over 4 days with pretargeted MAb. Note the large difference between the rates of diffusion into and out of the tumor: very rapid uptake (hours) compared to very slow loss (days) from the tumor. Blood levels are low at all times.

netics illustrated in Figures. 2–4 reveals three key features of effector molecules (biotin, hapten):

1. They must be small, hydrophilic and rapidly diffusable.
2. They must be quickly excreted solely by the kidney.
3. They must have little or no concentration in any normal tissues.

Since streptavidin lacks many of these features, it did not perform as well as the MAb-streptavidin conjugate/biotin system in the model. There are several reasons for not using it in the radiolabeled form in pretargeting. Distribution data have shown considerable kidney uptake, which probably eliminates it as a viable therapeutic strategy when given intravenously. Also, the administration of labeled streptavidin in the presence of circulating MAb-biotin conjugate has been shown by Paganelli to crosslink the MAb efficiently and deposit it in the liver (7). This worsens at higher doses where more MAb-biotin conjugate will be circulating at the time radiolabeled streptavidin is injected, adding to the liver dose. Unfortunately, the kidney and liver, probable normal target organs, have not been included in the model.

The relative exposure values (RE = TR), a measure of rad-to-tumor/ rad-to-blood, calculated from the model for directly labeled MAb = 60/1, are at least an order of magnitude (10×) higher than those actually measured in tumor mouse models. Indeed, if this value were correct, we would need to look no further than directly labeled MAbs for an effective therapeutic agent. The authors point out that these RE values are overestimated and they direct our attention to the relative value for biotin which is 5 to 6 times higher than directly labeled MAb. This, in fact, may be a conservative estimate.

Some of the apparent anomalies between the model and experience may be the result of using assumed parameters in the model which are not truly representative. The authors have listed these in their Table 1: MAb antigen binding, interstitial volume fraction of tumor nodule, valence of biotin binding and antigen turnover rate. Another factor that might confound the results is the large variety of sources for the input parameters: rats, mice, guinea pigs, rabbits, sheep and humans. With the acquisition of more data from experiments, the computer results will become correspondingly more informative and yield new insights and undoubtedly some surprises.

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REFERENCES

1. Goodwin DA, Meares CF, McCall MJ, McTigue M, Chaovapong W. Pretargeted immunoscintigraphy of murine tumors with indium-111 labeled bifunctional haptens. *J Nucl Med* 1988; 29:226–234.
2. Reardon DT, Meares CF, Goodwin DA, et al. Antibodies against metal chelates. *Nature* 1985; 316:265–268.
3. Le Doussal J-M, Martin M, Gautherot E, Delaage M, Barbet J. In vitro and in-vivo targeting of radiolabeled monovalent and divalent haptens with dual specificity monoclonal antibody conjugates: enhanced divalent hapten affinity for cell-bound antibody conjugate. *J Nucl Med* 1989;30:1358–1366.
4. Stickney DR, Anderson LD, Slater JB, Ahlem CN, Kirk GA, Schweighardt SA, Frincke JM. Bifunctional antibody: a binary radiopharmaceutical delivery system for imaging colorectal carcinoma. *Cancer Res* 1991;51:6650–6655.
5. Schuhmacher J, Klivényi G, Matys R, et al. Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chelate suitable for immunoscintigraphy with positron emission tomography. *Cancer Res* 1995;55:115–123.
6. Hnatowitch DJ, Virzi F, Rusckowski M. Investigations of avidin and biotin for imaging applications. *J Nucl Med* 1987;28:1294–1302.
7. Paganelli G, Magnani P, Zito F, et al. Three-step monoclonal antibody tumor targeting in carcinoembryonic antigen-positive patients. *Cancer Res* 1991;51:5960–5966.
8. Bagshawe KD. The First Bagshawe lecture. Towards generating cytotoxic agents at cancer sites. *Br J Cancer* 1989;60:275–281.
9. Senter PD. Activation of prodrugs by antibody-enzyme conjugates: a new approach to cancer therapy. *FASEB J* 1990;4:188–193.
10. Bos ES, Kuijpers WHA, Meesters-Winters M, et al. In vitro evaluation of DNA-DNA hybridization as a two-step approach in radioimmunotherapy of cancer. *Cancer Res* 1994;54:3479–3486.
11. Pimm MV, Gribben SJ. Prevention of renal tubule re-absorption of radiometal (indium-111) labeled Fab fragment of a monoclonal antibody in mice by systemic administration of lysine. *Eur J Nucl Med* 1994;21:663–664.
12. Virgolini I, Raderer M, Kurtaran A, et al. Vasoactive intestinal peptide-receptor imaging for the localization of intestinal adenocarcinomas and endocrine tumors. *N Engl J Med* 1994;331: 1116–1121.
13. Fischman AJ, Babich JW, Strauss HW. A ticket to ride: peptide radiopharmaceuticals. *J Nucl Med* 1993;34:2253–2263.
14. Goodwin DA, Meares CF, Watanabe N, et al. Pharmacokinetics of pretargeted MAb 2D12.5 and Y-88-JANUS-DOTA in BALB/c mice with KHJJ mouse adenocarcinoma: a model for Y-90 radioimmunotherapy. *Cancer Res* 1994;54: 5937–5946.
15. Yuan F, Baxter LT, Jain RK. Pharmacokinetic analysis of two-step approaches using bifunctional and enzyme-conjugated antibodies. *Cancer Res* 1991;51:3119–3130.
16. Sung S, van Osdol WW. Pharmacokinetic comparison of direct antibody targeting with pretargeting protocols based on streptavidin-biotin binding. *J Nucl Med* 1995;36:867–875.