Relapsed or treatment refractory B-cell lymphomas are currently incurable with conventional chemotherapy and radiation treatments. High-dose chemoradiotherapy and stem cell transplantation can cure some patients with relapsed or refractory lymphoma, but the majority of such patients die of progressive disease. We have investigated the potential utility of pretargeted radioimmunotherapy using monoclonal antibody-streptavidin, immunoconjugates, and fusion proteins in combination with N-acetylglucosamine dendrimeric clearing agent and radiometal-labeled 1,4,7,10-tetraazacyclododecane-<i>N</i>,<i>N</i>’,<i>N</i>”<i>N</i>”-tetraacetic acid biotin for treatment of lymphomas using mouse and primate models. We have targeted a variety of cell surface antigens, including CD20, CD22, CD45, and HLA-DR, using conventional and pretargeted radioimmunotherapy. These studies showed the marked superiority of pretargeted radioimmunotherapy for each of the antigenic targets in terms of superior biodistributions, more complete tumor regressions, and longer survival. We are optimistic that this novel approach will provide a meaningful prolongation of survival for patients with relapsed or refractory lymphomas.

**Major Points**

**Pretargeting.** One method to overcome the dose limitation imposed by nonspecific radiation damage to normal tissue is through dissociation of the slow antibody distribution phase from delivery of the therapeutic radionuclide. Through a multistep pretargeting process, antibody localization on tumor targets is allowed to progress independent of the radiation delivery phase (7–10). Once the target cells have achieved maximum uptake of antibody, radioactivity can be delivered by a small ligand possessing high affinity for the engineered pretargeted antibody (11, 12).

Our group has favored the use of a high-affinity streptavidin-biotin system for this ligand-fusion protein interaction. Our initial studies involved the use of a synthetic chemical conjugate that can be produced by attaching the intact antibody to streptavidin using the heterobifunctional cross-linker succinimidyl 4-[<i>N</i>-maleimidomethyl]cyclohexane-1-carboxylate (Pierce Chemical; ref. 13). A major advance in pretargeted radioimmunotherapy, developed and characterized by Schultz et al. (14), has resulted in the capacity to produce genetically engineered antibody-streptavidin fusion proteins. These fusion proteins can be expressed at high levels in the periplasm of <i>Escherichia coli</i>. This homogenous fusion protein construct is both economical to produce and scaleable to quantities

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**Patients with indolent B-cell lymphoma experience significantly better disease response rates when treated with anti-CD20 antibodies conjugated to <sup>131</sup>I or <sup>90</sup>Y compared with those treated with unlabeled anti-CD20 antibodies (1, 2). Single-agent radioimmunotherapy with <sup>131</sup>I-tositumomab has produced response rates as high as 96% in previously untreated patients (3). In more difficult to treat patients who have been refractory to, or relapsed after, multiple prior therapies, radioimmunotherapy has produced remission rates of 50% to 80% and complete response rates of 25% to 40% (1, 4, 5). The principles underlying targeted delivery of radionuclides to lymphoma cells have been repeatedly validated; however, patients treated with conventional radioimmunotherapy continue to experience high rates of relapse.

The high recurrence rates following conventional nonmyeloablative radioimmunotherapy are thought to be a function of suboptimal levels of radiation delivered to the tumor. With conventional radioimmunotherapy, the tumor-to-normal organ ratios of absorbed radioactivity are low (1.5-10 times that of critical normal organs) because normal tissues experience obligate radiation exposure from radiolabeled antibodies in the bloodstream (6). The accumulation of this nonspecific radiation over time results in dose-limiting toxicities. Myeloablative doses of radioimmunotherapy followed by stem cell rescue offers the potential for cure in some patients. Yet, the morbidity and mortality associated with this aggressive approach limits its utility. Furthermore, whereas the absolute risk of developing indolent B-cell lymphoma increases with advancing age, the capacity to tolerate aggressive therapy regimens seems to decrease.
sufficient for clinical trials (14–16). The tetravalent structure of the streptavidin fusion protein allows for amplification of the radioactivity delivered to target (see Fig. 1). A further refinement to optimize the pretargeting process involves introduction of a clearing agent designed to enhance hepatic removal of unbound fusion protein before infusion of the small-molecule radionuclide. The clearing agent we have used is a dendrimeric compound composed of 16 N-acetylgalactosamine carbohydrate moieties and a single modified biotin (Aletheon Corp.). The clearing agent complexes with excess conjugate in the circulation and is cleared by the liver with first-pass kinetics. The small-molecule radionuclide delivery ligand, possessing a 1,4,7,10-tetraazacyclododecane-\(\text{N},\text{N},\text{N}^\circ,\text{N}^\circ\)tetraacetic acid (DOTA) chelate capable of complexing with several radiometals (\(\text{\(^{177}\text{Lu}\)}, \text{\(^{90}\text{Y}\)}, \text{and \(\text{\(^{111}\text{In}\)}\)) is injected following the clearing agent (4 h in our studies; Fig. 2). The biotin molecule on the clearing agent does not affect the binding kinetics of this small-molecule radionuclide, as the clearing agent biotin has an affinity for streptavidin that is one-one thousandth that of the radiobiotin in the treatment step (17). Clearing agent therefore neither blocks the binding of the DOTA-conjugated radionuclide nor is capable of stripping antibody bound to tumor from its target (13, 18, 19).

**Biodistribution studies.** Through studies involving an athymic murine xenograft lymphoma model with Raji (Burkitt lymphoma), Ramos (Burkitt lymphoma), and FL-18 (transformed follicular lymphoma) flank tumors, we have shown dramatic differences in blood clearance kinetics between clearing agent–treated and non-clearing agent–treated animals (13). In the absence of clearing agent, fusion protein conjugate undergoes relatively slow bloodstream clearance. In contrast, when clearing agent is used, 95% of the circulating conjugate is removed within 15 min of injection (Fig. 3). A small rebound effect is present after clearing agent delivery; however, the area under the curve comparison versus control reveals very little activity remaining in the circulation of the treated animal.

Our group has conducted a series of biodistribution studies comparing directly conjugated \(\text{\(^{111}\text{In}\)-DOTA-1F5}\) (anti-CD20 antibody) with \(\text{\(^{111}\text{In}\)-DOTA-biotin}\) pretargeted with 1F5 fusion protein in the athymic murine Ramos tumor model (15). As expected, directly conjugated antibody resulted in increased tumor activity when compared with normal tissue; however, overall tumor-to-normal organ ratios of absorbed radiation were low, and there was no time point at which the tumor-to-blood ratios of antibody (measured in percentage injected dose per gram) were favorable (Fig. 4). By contrast, the pretargeted animals experienced a significant increase in the radioactivity delivered to tumor and a dramatic reduction in the amount of radionuclide remaining in the circulation, resulting in enhanced tumor-to-normal organ ratios for absorbed radiation.

**Therapy studies.** Groups of 10 animals were injected s.c. with Ramos cell tumor suspensions and, following the formation of palpable flank tumors (10–14 days after injection), received the 1F5-streptavidin conjugate. After a 24-h period to allow for optimal antibody-streptavidin conjugate distribution, clearing agent was infused. Then, 2 to 4 h after clearing agent, each group received \(\text{\(^{90}\text{Y}\)-DOTA-biotin}\) in doses escalating from 200 to 800 \(\mu\text{Ci}\).

Groups treated with \(\text{\(^{90}\text{Y}\)-DOTA-biotin}\) alone, and those treated with a nonbinding control antibody-streptavidin (\(\text{\(^{90}\text{Y}\)-DOTA-biotin}\)) conjugate followed by \(\text{\(^{90}\text{Y}\)-DOTA-biotin}\), experienced exponential tumor growth at even the highest (800 \(\mu\text{Ci}\)) concentrations, as determined through biweekly tumor measurement. In animals that received conventional directly labeled \(\text{\(^{90}\text{Y}\)-anti-CD20}\) antibody, a transient response was noted (consistent with responses seen in patients receiving this therapy); however, all mice in these groups experienced relatively rapid and eventually fatal recurrence. In contrast, following determination of optimal dosing, animals in the multistep pretargeting groups experienced almost universal rates of cure (Fig. 5; ref. 13).

Additional studies designed to compare conventional one-step radioimmunotherapy with antibody-streptavidin pretargeting using \(\text{\(^{90}\text{Y}\)-Lym-1}\) and \(\text{\(^{90}\text{Y}\)-HD39}\) were done. Conventionally treated animals again experienced transient partial tumor

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**Fig. 1.** The structure of tetrameric (scFv)\(_4\)-streptavidin (SA) fusion proteins.

**Fig. 2.** Schematic depiction of three-step pretargeting process in which (scFv)\(_4\)-streptavidin fusion protein is injected followed 24 h later by the dendrimeric N-acetylgalactosamine–containing clearing agent and, 4 h thereafter, by small-molecule radiolabeled DOTA-biotin.
remissions (~50% of initial volume) at a dose of 200 μCi. However, tumors regrew in all of these mice, mandating euthanasia before day 20. The animals receiving 200 μCi of direct 90Y-antibodies experienced short-term reversible toxicity. Those receiving 400 μCi of conventional 90Y-antibodies experienced more striking tumor regressions, with xenografts shrinking to 10% of their initial volumes by day 10 after therapy. However, in mice receiving 400 μCi of conventional 90Y-antibodies, there was universal lethal toxicity by day 10 that resulted from complications associated with marrow suppression and infection. Doses of direct conjugates >400 μCi were therefore not explored. Ramos-bearing mice pretargeted with 1F5-streptavidin fared better than other groups in terms of toxicity, tumor responses, and survival. As noted above, all mice receiving optimal dosing (pretargeted 1F5-streptavidin + 800 μCi 90Y-DOTA-biotin) achieved complete remissions by day 10 and 80% to 100% were cured in multiple experiments with follow-up up to 1 year. Pretargeted radioimmunotherapy of Ramos xenografts was less successful with Lym-1-streptavidin or HD39-streptavidin than was seen with IF5. Even with 800 μCi 90Y-DOTA-biotin, only partial remissions were achieved. Combinations of all three conjugates followed by 800 μCi 90Y-DOTA-biotin resulted in more toxicity and less efficacy than therapy with 1F5-streptavidin alone, apparently due to saturation of the ability of the liver to clear complexes of clearing agent-antibody-streptavidin. Therapy experiments with FL-18 and Raji xenografts are currently under way.

These findings have identified dramatic differences in cumulative survival for animals receiving conventional radioimmunotherapy versus those receiving pretargeted therapy. Animals in the conventional radioimmunootherapy group experienced irreversible bone marrow failure at doses >400 μCi of 90Y; however, animals in the pretargeted radioimmunotherapy group were able to tolerate the maximum dose of 800 μCi without evidence of toxicity. All animals in the control and conventional radioimmunotherapy groups died within 20 days of initiating treatment, whereas 90% of the animals in the 1F5-streptavidin pretargeted radioimmunotherapy group receiving the 800 μCi dose remained alive for >80 days (Fig. 6; ref. 13).

The ultimate goal of pretargeting is to improve the therapeutic index of radioimmunotherapy, thereby reducing toxicity to normal tissues. In our studies, a significant difference in toxicity has been noted between conventional and pretargeted groups. In animals receiving conventional therapy, doses of 200 μCi of radionuclide resulted in profound thrombocytopenia; no animal survived >10 days after treatment in the groups receiving radionuclide doses of ≥400 μCi. In contrast, pretargeted radioimmunotherapy groups treated with 800 μCi experienced platelet reductions that were less severe than the conventional radioimmunotherapy animals treated at the 200 μCi dose. Similar differences with respect to absolute neutrophil count recovery and weight loss have been identified. Overall, these murine studies have clearly identified the superiority of

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**Fig. 3.** The effect of N-acetylgalactosamine – containing clearing agent (arrow) on blood clearance of circulating 125I-1F5-(scFv)4-streptavidin when compared with control (no clearing agent) BALB/c athymic mice. Serial blood samples were obtained at times indicated and measured with dual-channel gamma counting. Reproduced with permission from Press et al. (13).

**Fig. 4.** Biodistributions of radioactivity in blood and tumors of athymic mice bearing Ramos xenografts that were injected with either 1F5 (scFv)4-streptavidin fusion protein, B9E9 (scFv)4-streptavidin fusion protein, 1F5 antibody-streptavidin chemical conjugate, CC49 (scFv)4-streptavidin fusion protein, or directly labeled conventional 111In-DOTA-1F5 antibody. Mice in pretargeted groups (A) were injected with 1.4 nmol/L of each unlabeled construct followed 20 h later by 5.8 nmol/L clearing agent and 4 h after that by 1.2 nmol/L 111In-DOTA-biotin. In the directly labeled group (B), mice were injected with 1.4 nmol/L of conventional trace-labeled 111In-DOTA-1F5 antibody at time 0 h. Groups of five mice were euthanized 24, 48, 96, and 144 h after injection of radiobiotin or 111In-DOTA-1F5 antibody. The radioactivity in blood and tumors was quantified by gamma counting, corrected for decay, and expressed as the percentage injected dose per gram (% ID/g) of tissue. 1F5 (scFv)4-streptavidin fusion protein ( ●, tumor; ○, blood), B9E9 (scFv)4-streptavidin fusion protein ( ■, tumor; □, blood), 1F5 antibody-streptavidin chemical conjugate ( △, tumor; ◊, blood), CC49 (scFv)4-streptavidin fusion protein ( ▲, tumor; □, blood), and directly labeled conventional 111In-DOTA-1F5 antibody ( ●, tumor; ○, blood). RIT, radioimmunotherapy. Reproduced in modified form with permission from Pegel et al. (15).
pretargeting with respect to antitumor effects and reduction in toxicity compared with conventional radioimmunotherapy.

**Streptavidin fusion proteins.** Our initial pretargeting work involved direct chemical conjugates consisting of anti-CD20 antibodies linked to streptavidin with the SMPT heterobifunctional cross-linker; however, several salient factors caused us to transition to the use of fusion proteins [i.e., 1F5 (scFv)4-streptavidin]. These advantages included superior reagent homogeneity, improved ease of generation, and lower production costs. The fusion protein homogeneity derives from the spontaneous formation of the 174-kDa fusion proteins in the periplasm of *E. coli*. Between 250 and 300 mg of fusion protein are produced in each liter of fermentor culture. Binding specificity of both anti-CD20 1F5 (Fred Hutchinson Cancer Research Center) and B9E9 (NeoRx) fusion proteins is equivalent or superior to chemical conjugates, whereas a nonbinding control fusion protein CC49 (anti-TAG72) reveals negligible uptake. Fusion proteins in our therapy studies reveal a dose response that correlates directly with the amount of radionuclide delivered. Moreover, through fusion protein pretargeting, we have been able to reliably cure 100% of animals receiving up to 1,200 μCi of activity without significant toxicity to the animals. By comparison, negative control CC49 fusion protein–treated animals receiving the same dose uniformly experience fatal tumor progression, revealing that the responses are specific (Fig. 7; ref. 15).

**Comparative assessment of CD20, CD22, and HLA-DR antigens for pretargeting.** For the purpose of comparison, and to evaluate potential additive benefit, our group has explored targeting antigens other than CD20 that are expressed on the B-cell surface (20, 21). The relative density of the antigens CD22 and HLA-DR is closely associated with the tumor cell line studied. The Ramos cell line exhibits high expression of CD20; however, both CD22 and DR are expressed at significantly lower levels. By comparison, the Raji line maintains a high level of DR expression (>CD20) and a low level of CD22. DR is recognized by the Lym-1 antibody and CD22 is recognized by the antibody HD39. A third cell line, FL-18, possesses high CD20 and DR with low CD22 expression.

To evaluate the pretargeting model, antibody-streptavidin chemical conjugates were produced for anti-CD22 (HD39) and anti-HLA-DR (Lym-1). Blood clearance rates for all three conjugates [including anti-CD20 (1F5)] were equivalent. A series of studies to compare the antibody-streptavidin conjugates with directly radiolabeled antibodies were then done. Pretargeting was superior to directly labeled antibody for all three antibodies in all three cell lines (Fig. 8). 1F5-streptavidin in the Ramos, Raji, and FL-18 lines generated
excellent target-to-nontarget ratios despite the lower expression of CD20 in the Raji line. In the Lym-1 model, Raji and FL-18 had excellent uptake, whereas Ramos (possessing significantly lower constitutive DR antigen expression) revealed reduced uptake (lower ratios). HD39 tumor targeting was present but at lower levels for all cell lines. This finding is potentially a function of lower antigen expression; however, the more rapid rate of CD22 receptor internalization may play a role. A nonbinding negative control antibody-streptavidin hybridoma expressing a nonspecific IgG2a (HB8181) revealed only background levels of uptake.

To evaluate the potential for improving the therapeutic ratio further through combined therapy targeting multiple antigenic targets, additional studies were done in which all three antibodies (1F5, HD39, and Lym-1) were administered in combination. The results revealed additive uptake in Ramos tumors; however, the uptake in normal organs, specifically in the liver, showed a proportional increase in activity, thus obviating any improvement in the tumor-to-normal organ ratio of absorbed radiation. The increased uptake in the liver is potentially a function of clearing agent-conjugate complexes saturating the hepatic capacity for removal when such high levels of antibody are present.

**Conclusion**

Our group has shown that pretargeted radioimmunotherapy is superior to conventional radioimmunotherapy in CD20-expressing, CD22-expressing, and HLA-DR–expressing murine xenograft tumors. Determining which antigen is the most appropriate target may depend on the antigen expression levels on a patient’s lymphoma, a factor that can be easily determined in lymphoma patients. The current findings do not suggest that combined therapy targeted to multiple receptors will be superior; however, this is an extrapolation based solely on biodistribution data, and therapy studies are ongoing. We are encouraged by the efficacy of pretargeting overall. We are currently in the process of studying anti-CD20 fusion proteins in a nonhuman primate model and are producing current Good Manufacturing Practice (cGMP) reagents for patient trials. This treatment approach offers the potential to overcome previously defined maximum doses of radionuclide deliverable to patients, thereby affording more complete and durable remissions, and the possibility of cures for patients with relapsed B-cell lymphomas through a therapeutic approach other than stem cell transplantation.

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Pretargeted Radioimmunotherapy for B-Cell Lymphomas

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