Pretargeted Radioimmunotherapy Using Genetically Engineered Antibody-Streptavidin Fusion Proteins for Treatment of Non-Hodgkin Lymphoma

Steven I. Park1, Jaideep Shenoi2,3, Shani M. Frayo2, Donald K. Hamlin4, Yukang Lin2, D. Scott Wilbur4, Patrick S. Stayton5, Nural Orgun2, Mark Hylarides2, Franz Buchegger6, Aimee L. Kenoyer2, Amanda Axtman2, Ajay K. Gopal2,3, Damian J. Green2,3, John M. Page2,3, and Oliver W. Press2,3

Abstract

Purpose: Pretargeted radioimmunotherapy (PRIT) using streptavidin (SAv)-biotin technology can deliver higher therapeutic doses of radioactivity to tumors than conventional RIT. However, "endogenous" biotin can interfere with the effectiveness of this approach by blocking binding of radiolabeled biotin to SAv. We engineered a series of SAv FPs that downmodulate the affinity of SAv for biotin, while retaining high avidity for divalent DOTA-bis-biotin to circumvent this problem.

Experimental Design: The single-chain variable region gene of the murine 1F5 anti-CD20 antibody was fused to the wild-type (WT) SAv gene and to mutant SAv genes, Y43A-SAv and S45A-SAv. FPs were expressed, purified, and compared in studies using athymic mice bearing Ramos lymphoma xenografts.

Results: Biodistribution studies showed delivery of more radioactivity to tumors of mice pretargeted with mutant SAv FPs followed by $^{111}$In-DOTA-bis-biotin [6.2 ± 1.7% of the injected dose per gram (%ID/gm) of tumor 24 hours after Y43A-SAv FP and 5.6 ± 2.2%ID/g with S45A-SAv FP] than in mice on normal diets pretargeted with WT-SAv FP (2.5 ± 1.6%ID/g; $P = 0.01$). These superior biodistributions translated into superior antitumor efficacy in mice treated with mutant FPs and $^{90}$Y-DOTA-bis-biotin [tumor volumes after 11 days: 237 ± 66 mm$^3$ with Y43A-SAv, 543 ± 320 mm$^3$ with S45A-SAv, 1129 ± 322 mm$^3$ with WT-SAv, and 1435 ± 212 mm$^3$ with control FP ($P < 0.0001$)].

Conclusions: Genetically engineered mutant-SAv FPs and bis-biotin reagents provide an attractive alternative to current SAv-biotin PRIT methods in settings where endogenous biotin levels are high.

Clin Cancer Res; 17(23); 1–10. ©2011 AACR.

Introduction

Despite advances in the management of lymphoma, fewer than 60% of patients with aggressive non-Hodgkin lymphomas (NHL) and fewer than 5% of patients with indolent lymphomas can be cured with conventional chemotherapy (1–3). The remaining patients eventually relapse, and the disease becomes progressively more chemotherapy resistant. This does not preclude the therapeutic benefit of radiation therapy; however, because lymphoma cells remain highly susceptible to even low doses of radiation regardless of their inherent resistance to chemotherapeutic drugs. For this reason, radioimmunotherapy (RIT) has emerged as a promising treatment option for NHL. RIT has yielded excellent overall response rates of 50% to 80%, with complete response rates of 20% to 40% in patients with relapsed or refractory indolent lymphoma treated with iodine-131 ($^{131}$I) tositumomab (Bexar, GlaxoSmithKline) or yttrium-90 ($^{90}$Y) ibritumomab tiuxetan (Zevalin, Spectrum; refs. 4–7). Nevertheless, most patients treated with conventional doses of RIT eventually relapse even after favorable initial responses. Dose escalation of RIT to myeloablative levels with stem-cell support significantly reduces the risk of relapse by delivering potentially curative radiation doses to tumor sites. However, this approach has been associated with significant treatment-related morbidity and mortality (8–10).

One strategy for delivering higher, potentially curative, radiation doses to tumors involves a multistep "pretargeting" method. The major cause of dose-limiting toxicities in conventional RIT is due to the slow clearance of unbound radiolabeled antibodies (Ab) from the circulation with...
Translational Relevance

Conventional radioimmunotherapy (RIT) induces long-term durable remissions in more than 50% of patients with relapsed or refractory indolent non-Hodgkin lymphoma. Our research goal is to optimize RIT using a multistep pretargeted RIT scheme (PRIT) employing genetically engineered mutant-streptavidin fusion proteins and bis-biotin reagents that provide an alternative approach to current methods. This molecular engineering approach is particularly attractive in clinical settings where high endogenous biotin levels prevail due to dietary intake of this vitamin, leading to diminished efficacy of standard streptavidin/biotin reagents.

We anticipate that this approach will result in superior efficacy compared with both conventional RIT and current PRIT approaches. If the results are promising, we anticipate eventually translating this approach into phase I/II clinical studies of PRIT for patients with relapsed B-cell lymphomas.

resultant high levels of background radioactivity in normal tissues (11–13). Pretargeted radioimmunotherapy (PRIT) dissociates the slow distribution phase of the Ab molecule from administration of the therapeutic radionuclide. This approach permits the tumor-reactive Ab to localize and accumulate at tumor sites without subjecting the rest of the body to nonspecific irradiation from circulating radiolabeled Ab (14–17). After maximal accumulation of Ab in the tumor, a small molecular weight radioactive moiety, which has high affinity for the tumor-reactive Ab is administered. This second reagent penetrates tumors rapidly due to its small size. Excess unbound molecules of the second radioactive reagent are also rapidly cleared from the blood and excreted in the urine due to their small size. To facilitate this process, a “clearing agent” (CA) may be injected before the radiolabeled small molecule to enhance elimination of the unbound Ab from the bloodstream thus preventing it from complexing with the radiolabeled small molecule in the circulation.

Preclinical data have shown that streptavidin (SAv)-biotin pretargeting strategies can dramatically increase the tumor-to-normal organ ratio of delivered radioactivity by 10- to 100-fold (16–18). Pilot clinical trials have also shown promising results in both solid tumor and lymphoma models, though the outcomes were not as dramatic as those observed in preclinical studies (19–22). Suboptimal responses observed in the pilot clinical trials may be partially attributable to the presence of competing endogenous biotin in patient serum, derived from dietary sources. Biotin is present in blood and tissues at sufficient concentrations to irreversibly block the biotin-binding sites of SAv and may impair its efficacy in pretargeting applications (21, 23). In preclinical studies, mice may be fed a biotin-deficient diet for several days prior to the therapy to reduce the endogenous biotin concentration in serum. In contrast, biotin depletion in human clinical trials is more problematic due to the ubiquitous presence of this vitamin in all common foods, patient noncompliance, and concerns over complications attributable to biotin deficiency. Although the serum concentration of biotin present in humans is somewhat lower than in mice, endogenous biotin remains a significant concern, particularly because the liver acts as a storage and release reservoir for biotin (24). Strategies that avoid the blocking of the biotin-binding sites of SAv by endogenous biotin may therefore offer therapeutic advantages. To address this issue, Stayton and colleagues produced a large library of SAv variants by site-directed mutagenesis directed at the SAv-binding pocket (Supplementary Table S1), covering a wide range of $K_a$ and $K_{off}$ properties (25–26). Along with the engineering of SAv mutants, divalent bis-biotin ligands were designed to effectively engage 2 adjacent biotin-binding sites of SAv mutants, thus overcoming the competitive binding by endogenous biotin (27–29). The lower affinities and faster biotin off-rates of the SAv mutants allow exchange of prebound endogenous biotin, although the dual binding of the bis-biotin ligand results in a striking avidity effect and essentially irreversible capture of the modified divalent ligand (27, 30–32).

Materials and Methods

Cell culture

The human Ramos Burkitt lymphoma cell line was obtained from the American Type Culture Collection (ATCC). Cell lines were maintained in log phase growth in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO$_2$ incubator. Cell viability exceeded 95% by trypan blue exclusion.

WT-SA

The development of the 1F5-WT-SA FP has been previously described (33–35). Briefly, the process involved fusion of the single-chain variable regions (scFv) of the murine anti-CD20 1F5 Ab to the full length genomic SAv of Streptomyces avidinii WT gene to obtain the 1F5(scFv)$_2$SAv fusion gene. This gene construct was modified by PCR-based site-directed mutagenesis to produce mutant genes.
carrying either the S45A or the Y43A mutations, with an SSGSGA peptide linker between the SAv and the scFv genes in each construct. The residue changes in fusion genes were determined by DNA sequencing analysis, and the gene products were analyzed by mass spectrometry, which indicated all FPs differed only at the deliberately engineered positions without any extraneous mutations (data not shown). E. coli XL1 Blue (Stratagene) transformants of the gene constructs, WT-SAv, S45A-SAv, or Y43A-SAv, were grown in shaker flasks under control of an IPTG inducible lac promoter for qualitative expression of the FPs. A 4 L fermentor (BioFlo 3000; New Brunswick Scientific) was used for bulk production of FPs. The FPs were purified by inimobiotin chromatography as described (18), except that the loading pH was raised from 9.2 to 11 due to the reduced affinities of the mutant FPs for inimobiotin (33–35). Aggregates were reduced to approximately 3% by treatment with 20% dimethyl sulfoxide (DMSO). The eluted FPs were dialyzed against PBS at 4°C overnight and concentrated to 2.0 to 2.3 mg/mL using a YM30 membrane. The final FPs were filter-sterilized and stored in 5% sorbitol at −80°C. A negative control FP (CC49-WT-SAv) that recognizes the TAG 72 antigen expressed on human adenocarcinomas, but not on lymphomas, was prepared similarly.

**Pretargeting reagents**

A synthetic, dendrimeric CA containing 16 N-acetylgalactosamine residues and a single biotin residue per molecule (NAGB) was obtained from Aletheon Pharmaceuticals for use with 1F5-WT-SAv FP, as described previously (35). A bis-biotin-trigalactose (BBTG) CA conjugate containing 16 N-acetylgalactosamine residues and 3 galactose residues (C12H218N19O51S6) was used for pretargeting reagents. The BBTG CA conjugate was obtained from Aletheon Pharmaceuticals for use with 1F5-WT-SAv FP, as described elsewhere (35). A bis-biotin–trigalactose (BBTG) CA conjugate containing 16 N-acetylgalactosamine residues and 3 galactose residues (C12H218N19O51S6) was used for the clearance of mutant-SAv FPs. The syntheses of BBTG CA and DOTA-bis-biotin have been recently described (28).

**Radiolabeling of biotin compounds**

111In labeling of DOTA-biotin and DOTA-bis-biotin were conducted as published (28). 90Y labeling for therapy was done similarly, using 2 mg/mL DOTA-bis-biotin, 500 mmol/L ammonium acetate pH 5.3 and 90Y heated for 30 minutes at 85°C. After cooling to room temperature, 100 mmol/L DTPA was added.

**In vitro characterization**

The FPs were analyzed by SDS-PAGE on 4% to 12% Tris-glycine gels (Invitrogen) under nonreducing conditions. The gels were stained with 0.2% Coomassie blue solution and destained in acetic acid-methanol solution. The gels were stained with 0.2% Coomassie blue solution and destained in acetic acid-methanol solution. The gels were stained with 0.2% Coomassie blue solution and destained in acetic acid-methanol solution. The gels were stained with 0.2% Coomassie blue solution and destained in acetic acid-methanol solution. The gels were stained with 0.2% Coomassie blue solution and destained in acetic acid-methanol solution.

**Biodistribution studies**

Biodistribution experiments were done using the "double label" method of Pressman by trace labeling the 1F5-SAv FPs with 125I and the biotin moiety with 111In to allow independent assessment of tumor targeting of the FPs and the biotin ligands (36). Groups of 5 mice with similar-sized tumors were injected i.v. with 2.8 nmol (400 μg) of 1F5-WT-SAv, 1F5-Y43A-SAv, or 1F5-S45A-SAv FPs labeled with 20 to 40 μCi (0.74–1.48 MBq) of 125I. Twenty hours later, mice were injected with either 5.8 nmol of NAGB CA (50 μg) followed 4 hours later by 1.2 nmol DOTA-biotin labeled with 20 to 40 μCi (0.74–1.48 MBq) of 111In or with 5.8 nmol of BBTG CA (20 μg) followed 4 hours later by 1.2 nmol of either NAGB or BBTG CA. Venous sampling was done via the tail vein at serial time points up to 68 hours. 125I was counted on a gamma counter and the percent of the injected dose per gram (%ID/g) of blood was calculated.

**Mouse xenograft model**

FoxN1 H-2d athymic female mice (6–8 weeks old) were obtained from Harlan Sprague-Dawley and housed in the FHRC animal facility after approval of the experimental protocol by the Institutional Animal Care and Use Committee. Some groups of mice were switched to a biotin-deficient diet (Purina Mills) 5 to 6 days prior to PRIT studies. Ramos cells (10 × 10^6) were injected subcutaneously in the right flank 10 days prior to experiments to produce lymphoma xenografts measuring 6 to 10 mm in diameter. Anti-asialoGM1 antiserum (30 μL, WAKO) was injected 9 days and 6 days prior to FP injection to abrogate natural killer cell activity and prevent spontaneous tumor regressions.
nmol of $^{111}$In -DOTA-bis-biotin. Blood samples, tumors, and body organs were procured and $^{125}$I and $^{111}$In activities measured in a dual channel gamma counter, adjusting for crossover between channels (17).

**Therapy studies**

The therapeutic efficacy of $^{90}$Y using various pretargeted approaches was evaluated in groups of 5 to 10 mice. Groups of mice with similar sized, palpable tumors were selected and randomized for the studies. Mice were given 2.8 nmol (400 µg) of 1F5-WT-SA, 1F5-Y43A-SA, 1F5-S45A-SA, or the negative control CC49-WT-SA followed by 5.8 nmol of either NAGB or BBTG CA 20 hours later. A single dose of 1.2 nmol of either DOTA-biotin or DOTA-bis-biotin labeled with 500 µCi (18.5 MBq) $^{90}$Y was administered 4 hours after the CA. Mice were assessed every few days for tumor volume measurements, weight change, and general appearance. Mice were euthanized if xenografts exceeded 10% of total body weight, caused obvious discomfort or impaired ambulation, or if mice lost more than 30% of their baseline body weight.

**Toxicity studies**

Toxicity studies were done in parallel cohort groups, corresponding to the therapy groups. Mice were monitored for lethargy, poor grooming, weight loss and other behaviors consistent with debility. Blood testing was done 14 days after injection of $^{90}$Y, and at the time of euthanasia. Laboratory tests done included the leukocyte and platelet counts, hemoglobin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and blood urea nitrogen (BUN) levels. Age-matched, untreated athymic mice were used for comparisons of hematology and chemistry data.

**Results**

**Expression and purification of WT-SA and mutant-SA FPs**

All FPs were successfully expressed in the periplasmic space of *E. coli* as soluble stable tetramers with molecular weights of approximately 174 KDa (Fig. 1). Expression levels for the FPs varied, with the S45A-SA construct.
producing the highest yield (358 mg/L), followed by Y43A-SA v (144 mg/L) and WT-SA v (100 mg/L; ref. 18). SDS-PAGE analysis confirmed purities of approximately 95% for the WT-SA v and mutant-SA v FPs after iminobiotin chromatography. All tetrameric FP bands resolved into a single species of MW ~ 44 kDa when the FPs were denatured by boiling before electrophoresis, consistent with a single protein entity dissociable into a homogeneous, monomeric subunit (Fig. 1).

**In vitro characterizations of mutant-SA v FPs**

Flow cytometry showed similar binding of the WT-SA v, S45A-SA v, and Y43A-SA v FPs and of the parental 1F5 antibody to CD20-expressing Ramos cells (Fig. 2). In vitro cell-binding assays were then done to compare the capture and retention of radiolabeled biotin moieties by FPs bound to CD20 on the surface of lymphoma cells (Fig. 3). As expected, the engineered low-affinity mutant FPs, 1F5-S45A-SA v and 1F5-Y43A-SA v, bound significantly less radiolabeled monovalent biotin (2.9 ± 0.003% and 4.9 ± 0.010% of the total 90Y activity presented to the cells, respectively) than did the WT-SA v FP (12.1 ± 0.02% of the 90Y activity added to the cultures). S45A-SA v and Y43A-SA v FPs were much more successful, as designed, in binding and retaining radiolabeled divalent bis-biotin (18.1 ± 0.005% and 17.4 ± 0.001% of applied 90Y activity, respectively). By comparison, WT-SA v FP bound 25.6 ± 0.02% of applied 90Y-labeled divalent bis-biotin. These results show that the divalent binding of the bis-biotin ligand compensated for the lower affinity of the Y43A and S45A mutant SA v molecules, yielding binding pairs at least comparable with the standard WT-SA v and monovalent DOTA-biotin reagents.

**Blood clearance studies**

The blood clearances of 125I-labeled mutant-SA FPs were evaluated after administration of CAs to athymic mice fed biotin-replete normal diets. Groups of 4 mice each were given 2.8 nmol of S45A-SA v or Y43A-SA v FP followed 20 hours later by divalent BBTG CA, monovalent NAGB CA, or PBS (negative control). A single injection of 5.8 nmol BBTG CA resulted in an approximately 80% decrease of circulating 125I-1F5-S45A-SA v within 30 minutes (shown logarithmically in Fig. 4) and an approximately 60% decrease in 125I-1F5-Y43A-SA v (not shown). In contrast, the monovalent biotin CA, NAGB, was ineffective at clearing the low affinity 125I-1F5-S45A-SA v or 125I-1F5-Y43A-SA v FPs from the blood, with clearances not significantly different from those seen in negative control mice treated with PBS (Fig. 4). These results show that the bis-biotin BBTG CA we designed works effectively as planned for the low affinity FPs, but that the standard NAGB CA, which possesses only a single biotin moiety, is ineffective for these constructs.

**Biodistribution studies**

Biodistribution experiments were conducted in mice with 1F5-Y43A-SA v, S45A-SA v, and 1F5-WT-SA v FPs and biotin compounds using the "dual label" method described previously. Groups of 5 athymic mice-bearing Ramos xenografts were injected i.v. with 400 µg (2.8 nmol) of 125I-labeled WT-SA v, Y43A-SA v, or S45A-SA v FPs followed 20 hours later by 5.8 nmol of BBTG or NAGB CA. Four hours later, mice were injected i.v. with 1.2 nmol of 111In-DOTA-bis-biotin or 111In-DOTA-biotin. Mice were euthanized 24 hours (Fig. 5A) or 48 hours (Fig. 5B) later and 125I and 111In were measured in excised tumors and normal organs using a dual channel gamma counter, adjusting for crossover of the 2 isotopes between channels. Biodistributions using the mutant-SA FPs did not include groups of mice on biotin-deficient diets because previous studies showed no differences in tumor uptake between mice on biotin-deficient diets.
diets and normal diets with mutant sAv conjugates (29). There were no significant differences in tumor or normal organ biodistributions of $^{125}$I in groups given mutant-sAv or WT-sAv FPs at either 24 or 48 hours, indicating that all the anti-CD20 FPs have similar tumor targeting and tissue biodistributions (Supplementary Fig. S1). Analyses of the biodistributions of $^{111}$In-labeled biotin compounds indicated that $^{111}$In-DOTA-bis-biotin was effectively targeted to tumors in mice pretargeted with mutant-SAv FPs even when mice were fed normal biotin-containing diets, whereas monovalent $^{111}$In-DOTA–biotin was not effectively targeted to lymphoma xenografts with the standard WT-SAv FP unless mice were fed rigorously restricted biotin-free diets (Fig. 5). Tumors from mice pretargeted with Y43A-SA FP followed by $^{111}$In-DOTA-bis-biotin contained 6.2 ± 1.7% of the injected $^{111}$In per gram of tumor (%ID/gm) after 24 hours compared with 5.6 ± 2.2%ID/g with S45A-SA but only 2.5 ± 1.6%ID/g with WT-SA FP ($P = 0.01$; Fig. 5A). The amount of radioactivity in tumors of mice treated with the mutant FP/bis-biotin systems were similar to the radioactivity in tumors excised from mice fed a biotin-deficient diet and treated with the standard WT-SA FP and DOTA-biotin PRIT system ($7.2 ± 3.2%ID/g$ after 24 hours, $P > 0.3$). Similar conclusions were derived from biodistribution studies conducted 48 hours after injection of $^{111}$In-labeled biotin compounds (Fig. 5B). Tumors excised from mice on normal diets treated with the mutant-SAv FPs followed by $^{111}$In-DOTA-bis-biotin contained 4.6 ± 1.0%ID/g of the injected $^{111}$In-DOTA-bis-biotin with Y43A-SA FP and 3.6 ± 1.1%ID/g with S45A-SA FP after 48 hours compared with 1.1 ± 0.3%ID/g with WT-SA FP (Fig. 6B; $P < 0.0001$). Tumors from mice fed a biotin-deficient diet and treated with WT-SA FP and monovalent $^{111}$In-DOTA-biotin contained 5.2 ± 0.4%ID/g after 48 hours. The biodistributions of $^{111}$In-DOTA-bis-biotin and $^{111}$In-DOTA-biotin in normal organs were similar in mice treated with either mutant-SAv FPs or WT-SA FP, except that radioactivity in the blood and liver were slightly higher with mutant-SAv FPs, especially for Y43A-SA (Fig. 5). The amount of radioactivity measured in tumors of mice fed normal diets treated with WT-SA FP did not vary significantly whether $^{111}$In-DOTA-biotin or $^{111}$In-DOTA-bis-biotin was used, indicating that even the bis-biotin ligand was not able to displace prebound endogenous biotin from the biotin-binding sites of WT-SAv. Overall, the combination of mutant-SAv FPs, BBTG CA, and DOTA-bis-biotin resulted in superior biodistributions of radioactivity compared with conventional WT-SA FP, NAGB CA, and DOTA-biotin in mice fed normal diets, where endogenous biotin levels are known to be high.

Therapy and toxicity studies
Therapy studies compared the efficacy of $^{90}$Y-labeled biotin compounds for treating lymphoma xenografts in mice pretargeted with the WT and mutant SAv FPs or with a nonbinding negative control, CC49-WT-SA FP (Fig. 6). Mice were assigned into 6 different groups and fed either a biotin-deficient diet or a regular biotin-containing diet. The median tumor size was $86 ± 42 \text{ mm}^3$ at the initiation of the study with no significant differences between groups. Mice were treated with WT-SA, S45A-SA, Y43A-SA, or CC49-WT-SA FPs followed 20 hours later by either NAGB or BBTG CA. A single injection of 500 $\mu$Ci [$^{90}$Y]-labeled DOTA-bis-biotin or monovalent DOTA-biotin was given 4 hours after the CA injection. Animals on a regular diet that received Y43A-SA and S45A-SA FPs had mean tumor volumes of $236 ± 66 \text{ mm}^3$ and $543 ± 320 \text{ mm}^3$ 11 days after therapy, respectively, compared with $1435 ± 212 \text{ mm}^3$ for mice that received a nonbinding...
control CC49-SAv FP ($P < 0.0001$) and 1129 ± 322 mm³ for animals that received WT-SAv FP ($P < 0.03$). Administration of WT-SAv FP to mice fed a biotin-deficient diet was associated with the most significant delay in tumor growth (11 ± 22 mm³ at day 11). Treatment was well tolerated with no evidence of acute toxicity. Complete blood counts, creatinine, and transaminase levels 14 days after therapy were similar in all groups of mice except that mildly lower leukocyte counts were observed in mice in all of the treatment groups compared with control, untreated mice (Supplementary Table S2).

Discussion

The efficacy of RIT in patients with NHL has been established by a multitude of clinical trials, but the majority of patients treated with nonmyeloablative doses of RIT eventually relapse (37–38). PRIT using SAv-biotin methodology has emerged as one promising approach to deliver potentially curative radiation doses to tumors while minimizing toxicities arising from the nonspecific radiation delivered to normal organs. Although the high affinity of SAv for biotin is essential for this pretargeting system, this tight interaction...
Figure 6. Growth of Ramos xenografts in athymic mice pretargeted with CC49-SA8 FP (nonbinding negative control, ◆), 1F5-SA5-SA8 FP (◆), 1F5-Y43A-SA8 FP (▲), 1F5-WT-SA8 (●), or 1F5-WT-SA8 with a biotin-deficient diet (●) followed by the indicated CA and 99mTc-DOTA-bisbiotin or 90Y-DOTA-biotin. Mice were fed a normal, biotin-replete diet unless otherwise specified.

can paradoxically present a significant limitation when naturally occurring endogenous biotin irreversibly blocks the SA8-binding sites, decreases the capacity of SA8 to bind radiolabeled DOTA-biotin, and reduces therapeutic efficacy. Although short-term biotin-depletion can be routinely achieved in mouse models, rigorous biotin-depletion is difficult in clinical settings due to its ubiquitous presence in all common foods. Even if endogenous biotin is successfully depleted, suboptimal targeting may still occur because the extremely slow “off rate” of radiobiotin hinders its diffusion to the center of tumor masses, due to “trapping” of radiobiotin in perivascular or peripheral locations by irreversible, high-affinity binding to the most accessible tumor cells. Computer models and experimental data have verified that such a “binding-site barrier” does exist and may compromise therapeutic efficacy of radioimmunoconjugates of extremely high-binding affinities, though this problem can be at least partially overcome by administration of a large excess of Ab-SA8 conjugate and radiobiotin (39–40).

In this study, we show the potential advantage of a novel, engineered PRIT system utilizing mutant-SA8 FPs and bivalent radiolabeled DOTA-bis-biotin for treatment of lymphomas in situations where endogenous biotin may limit the effectiveness of standard SA8-biotin PRIT. Our group has previously shown that specific delivery of high doses of radiation to tumor sites is achievable even in the presence of endogenous biotin using chemical conjugates of 1F5 Ab and mutant-SA8 protein produced using heterobifunctional cross-linkers in combination with bivalent biotin ligands (28–29). Here, we describe the genetic engineering, expression, in vitro characterization, and in vivo testing of novel 1F5-mutant-SA8 FPs, which are more homogenous, more amenable to scale-up, and less costly to manufacture than the previously described Ab-mutant-SA8 chemical conjugates (33).

We have produced anti-CD20 mutant-SA8 FPs that maintain the full antigen-binding capacity of the parent monoclonal Ab, but have a reduced avidity for endogenous biotin compared with WT-SA8 FP. We selected the Y43A and S45A mutants for incorporation into scFv-SA8 FPs from a panel of available mutants produced by site-directed mutagenesis based on prior in vitro assays, which suggested that these particular mutations would be most advantageous (27, 29). The Y43A-SA8 and S45A-SA8 mutant molecules have 67- and 907-fold lower affinity for biotin, respectively, compared with WT-SA8, but retain robust binding to divalent biotin compounds, as shown by their significantly slower off-rates for bis-biotin than biotin (27). Mutant-SA8 FPs were directly compared with WT-SA8 FP using a cell-binding assay (Fig. 3), which showed that both Y43A-SA8 and S45A-SA8 FPs have significantly lower affinity for monovalent biotin compared with WT-SA8 FP, but have sufficiently high affinity to efficiently capture bis-biotin ligands. These in vitro results suggest that the lower affinity of the mutant-SA8 FPs for monovalent biotin may allow prebound endogenous monovalent biotin to quickly dissociate, yet permit the durable capture and retention of radiolabeled divalent bis-biotin ligands by mutant-SA8 FPs. To utilize this mutant-SA8 FP and bis-biotin system effectively, a synthetic trigalactose-containing bis-biotin CA was conceptualized and generated by our group to facilitate blood clearance of the mutant-SA8 FPs (28). Pharmacokinetic experiments showed that treatment of mice with BBTG CA resulted in efficient clearance of mutant-SA8 FPs from the blood. As expected, the standard CA containing a single biotin moiety (NAGB) had a negligible effect on the blood clearance of the mutant-SA8 FPs.

Biodistribution experiments confirmed that the uptake and retention of radiolabeled DOTA-biotin by WT-SA8 FP was significantly compromised at tumor sites when animals were fed a regular diet, presumably because of the deleterious impact of endogenous biotin blocking the biotin-binding sites of SA8. In marked contrast, the mutant-SA8 FPs exhibited excellent tumor retention of radiolabeled DOTA-bis-biotin even in animals fed a regular biotin-rich diet. The amounts of radioactivity in normal tissues were similar in mice treated with mutant-SA8 and WT-SA8 FPs, except for the blood and liver, which contained slightly more radioactivity in groups treated with mutant-SA8 than in mice treated with WT-SA8 and NAGB CA. We presume these differences emerged because the BBTG CA was less efficient at clearing mutant-SA8 FPs from the blood than the NAGB CA was at clearing WT-SA8 (17, 41). Therefore, further optimization of the bis-biotin CA is planned using variations in the galactose content of the molecule.

In therapy experiments, mutant-SA8 FPs in combination with 99mTc-DOTA-bis-biotin produced significant tumor growth delays in mice bearing lymphoma xenografts fed biotin-replete normal diets while the standard regimen, utilizing WT-SA8 and 99mTc-DOTA-biotin, had no significant effect on tumor growth in the presence of endogenous biotin. These data support the hypothesis that mutant-SA8 FPs with optimized biotin-binding affinities can improve the amount of radiation delivered specifically to tumor sites.
compared with WT-SA FP in the presence of competing endogenous biotin.

Although it must be acknowledged that the current gold standard combination of WT-SA FP and radiolabeled DOTA-biotin remains superior in situations where rigorous biotin depletion can be achieved, these studies show the promise of such a genetically engineered PRIT system for situations where endogenous biotin may limit its efficacy through competitive inhibition at the SAv-binding site. In addition, it is conceivable that the SAv mutants used in these experiments may not possess the optimal off-rates for biotin and bis-biotin to maximize tumor retention. Therefore, additional mutant-SAv FPs will be engineered and tested to further optimize the affinity and off-rate for monovalent biotin and bivalent biotin ligands.

We acknowledge that one potential limitation of SAv-biotin pretargeting systems is the immunogenicity of SAv (and of murine Ab). Although human anti-mouse Ab (HAMA) and human anti-SAv Ab (HASA) may be major limitations for applications in immunocompetent patients and for experimental regimens requiring repetitive rounds of therapy, we do not anticipate that immunogenicity will be a major limitation for this approach in patients with advanced leukemia and lymphoma, who are the focus of our investigations. We have administered RIT using murine Ab to 476 patients with non-Hodgkin lymphoma or acute myeloid leukemia at our center since 1987. Only 14 of these 476 patients (2.9%) formed HAMA between the dosimetric and therapeutic infusions of radiolabeled Ab. Furthermore, we recently opened a clinical trial of PRIT using a murine anti-CD45 Ab with SAv and radiolabeled biotin for patients with acute myeloid leukemia (ClinicalTrials.gov identifier NCT00998715; IND # 104683). No immune responses to either Ab or SAv have yet been detected in the 5 patients treated, presumably due to the compromised immune system of these patients (unpublished data). HASA have also not been major impediments in published studies of lymphoma patients treated with streptavidin-biotin PRIT by others (19, 22). We believe that our ongoing trial, and previous PRIT studies by others, show that Ab-SA PRIT approaches are translatable to the clinic.

In summary, we have shown the potential of rationally engineered mutant-SAv FPs to be used in conjunction with divalent bis-biotin CA and bis-biotin radioisotope carriers to circumvent the potential blocking effects of endogenous biotin. This approach may allow the effective delivery of higher doses of radiation to tumor sites than is possible with standard SAv-biotin PRIT systems in settings where rigorous biotin depletion is not feasible.

Disclosure of Potential Conflicts of Interest

O.W. Press has received honoraria for consultation from Algeta, Roche/Genentech, Spectrum Pharmaceuticals, and Seattle Genetics and research support from Roche/Genentech. A.K. Gopal has received honoraria for lectures from Seattle Genetics and Millennium and research support from Glaxo Smith Kline, Merck, Cephalon, Piramal, Pfizer, Abbott, BioMarin, Seattle Genetics, Eli Lilly, and Spectrum Pharmaceuticals. J.M. Pagel has research support from Glaxo Smith Kline. The other authors disclosed no potential conflicts of interest.

Authors’ Contributions

S.I. Park designed and performed research, analyzed data, and drafted the manuscript. J. Shenoi designed and performed research, analyzed data, and drafted the manuscript. S. Frayo performed research, collected data, and analyzed data. D.K. Hamlin contributed vital reagents and performed research. Y. Lin engineered, expressed, purified, and tested the fusion proteins. P.S. Stayton produced the mutant streptavidin gene constructs by directed mutagenesis. D.S. Wilbur contributed to the conception, design, analysis, and interpretation of research. N. Orgun, A. Kenoyer, and A. Axman performed research and collected data. M. Hyladakes contributed vital reagents, design and interpretation of research. F. Bucheger performed research and analyzed data. A.K. Gopal and D.J. Green contributed to the interpretation of data. J.M. Pagel contributed to the conception, design, analysis, and interpretation of research and edited the manuscript. O.W. Press contributed to the conception, design, analysis and interpretation of research, revised the manuscript, and funded the experiments.

Grant Support

This work was supported by grants from the NIH: RO1 CA076287, PO1 CA44991, and the Lymphoma Research Foundation and gifts from David and Patricia Giulian, Mary and Geary Britton-Simmons, James and Sherry Raisbeck, the Wyner-Stokes Foundation, and the Hext Family Foundation. A.K. Gopal is a scholar in clinical research of the Leukemia and Lymphoma Society. S.I. Park is a recipient of a Lymphoma Research Foundation Fellowship Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 10, 2011; revised July 23, 2011; accepted September 21, 2011; published OnlineFirst October 5, 2011.

References

Park et al.

Pretargeted Radioimmunotherapy Using Genetically Engineered Antibody-Streptavidin Fusion Proteins for Treatment of Non-Hodgkin Lymphoma

Steven I. Park, Jaideep Shenoi, Shani M. Frayo, et al.

Clin Cancer Res  Published OnlineFirst October 5, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-1204

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/10/05/1078-0432.CCR-11-1204.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.