Radioiodination via \(d\)-Amino Acid Peptide Enhances Cellular Retention and Tumor Xenograft Targeting of an Internalizing Anti-Epidermal Growth Factor Receptor Variant III Monoclonal Antibody

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ABSTRACT

The mutant epidermal growth factor receptor variant III (EGFRvIII) has been found on gliomas and other tumors but not on normal tissues, including those that express the wild-type receptor. Monoclonal antibodies (mAbs) specific for EGFRvIII are rapidly internalized and degraded after binding to EGFRvIII-expressing cells. If anti-EGFRvIII mAbs are to be useful for radioimmunotherapy, then methods for trapping radioiodides in target cells after mAb processing are required. Because lysosomes are known to retain positively charged molecules, we have evaluated a new reagent for this purpose that uses a polycationine peptide composed of \(d\)-amino acids (\(d\)-Lys-\(d\)-Arg-\(d\)-Tyr-\(d\)-Arg-\(d\)-Arg; \(d\)-KRYRR). \(d\)-KRYRR was first labeled using Iodogen and then coupled to the murine anti-EGFRvIII mAb L8A4 via maleimide bond formation in 60% yield. In vitro assays with the U87EGFR cell line indicated that internalized and total cell-associated activity for the \(125\text{I}\)-labeled \(d\)-KRYRR-L8A4 conjugate were up to 4 and 5 times higher, respectively, than for L8A4 labeled with \(11\text{I}\) using Iodogen. Paired-label comparisons in athymic mice with s.c. U87EGFR xenografts demonstrated up to 5-fold higher tumor uptake for mAb labeled using \(d\)-KRYRR. Higher levels of radioiodine activity also were observed in kidney when L8A4 was labeled using \(d\)-KRYRR. Another paired-label study directly compared L8A4 labeled using radioiodinated \(d\)-KRYRR and \(L\)-KRYRR, and confirmed the role of \(d\)-amino acids in enhancing tumor uptake. These results suggest that \(d\)-KRYRR is a promising reagent for the radioiodination of internalizing mAbs, such as the anti-EGFRvIII mAb L8A4.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is overexpressed on a wide variety of human tumors, including breast, ovarian, and non-small cell lung carcinomas, as well as on gliomas (1). To exploit this characteristic, a number of wild type anti-EGFR mAbs have been developed and evaluated as potential radioimmunodiagnostic and radioimmunotherapeutic agents. Unfortunately, the clinical utility of wild type anti-EGFR mAbs is compromised by the expression of wild type EGFR on many normal tissues including the liver.

In addition to overexpression, oncogenic transformation can lead to rearrangement of the EGFR gene, potentially yielding molecular targets with greater tumor specificity than the wild-type EGFR (2). Of particular interest for targeting applications is EGFRvIII, which is characterized by a genomic deletion of amino acid residues 6–273 from the extracellular domain of the EGFR with the formation of a novel glycine at the new fusion junction (3, 4). This 145-kDa translocated receptor has been identified on gliomas and breast, lung, and ovarian carcinomas but not on normal human tissues (5, 6). Importantly, in 4 of 5 human glioma biopsies, expression of EGFRvIII was 270,000–680,000 receptors per cell, a level that should be sufficient for effective targeting (7).

We have developed a series of murine mAbs including L8A4 that bind selectively to EGFRvIII-expressing cells and tumor xenografts but not to normal tissue (6). In vitro and in vivo studies have shown that these anti-EGFRvIII mAbs are rapidly internalized after binding to cell surface receptor, resulting in degradation of the mAb inside the tumor cell (8). For this reason, the radioiodination method used to label anti-EGFRvIII mAbs could have a major impact on their potential utility for radioimmunotherapy.

Labeling the anti-EGFRvIII mAbs L8A4 and H10 by a direct iodination method (Iodogen) resulted in only modest tumor:normal tissue ratios in athymic mice bearing EGFRvIII-expressing HC2 20.2 d2 xenografts (8). Coupling radiolabeled, inert disaccharides, such as tyramine-cellobiose, to mAbs has been reported to increase the retention of radioiodine in tumor cells after intracellular mAb processing (9). With mAbs L8A4 and H10, this approach improved tumor activity levels compared with Iodogen labeling; however, low tumor localization indices were observed (8).

We have been investigating an alternative approach for labeling internalizing mAbs that involves the conjugation of a labeled prosthetic group to the protein that is positively charged at lysosomal pH. Because positively charged molecules are often used as lysosomal markers (10), we hypothesized that positively charged catabolites generated during mAb proteolysis should be retained in lysosomes. The feasibility of this strategy was demonstrated in a series of experiments in which the anti-EGFRvIII mAb L8A4 was radioiodinated using SIPC (11, 12). HPLC analyses of cell lysates demonstrated that radioiodine activity was retained in these EGFRvIII-expressing cells in the form of iodonicotinic acid-lysine, a positively charged species. Although SIPC labeling enhanced the retention of radioiodine activity in EGFRvIII-expressing cells in vitro and yielded higher activity levels in tumor xenografts compared with mAb labeled using Iodogen, these advantages were of short duration.

The current study was performed to determine whether coupling the mAb to a labeled peptide prosthetic group containing multiple positively charged amino acids would result in prolonged enhancement of tumor uptake. Prototype pentapeptides with the sequence Lys-Arg-Tyr-Arg-Arg (KRYRR) composed entirely of both \(d\)- and \(L\)-amino acids were evaluated.

MATERIALS AND METHODS

Materials. The \(d\)-KRYRR peptide was purchased from Peptide Technologies Corp. (Gaithersburg, MD), and \(L\)-KRYRR was obtained from Louisiana State University Medical Center (New Orleans, LA). s-SMCC and streptavidin-coated magnetic beads were obtained from Pierce (Rockford, IL). 2-Iminothiolane, iodoacetamide, and Iodo-Gen were purchased from Sigma Chemical Co. (St. Louis, MO). Na\(^{125}\text{I}\) and Na\(^{131}\text{I}\) iodide were obtained from NEN LifeScience (Boston, MA). Trifunctional reversed-phase cartridges (iC18, 145 mg adsorbent) were purchased from Waters (Milford,
MA). ITLC silica gel plates were obtained from Gelman Science (Ann Arbor, MI). Bakerbond solid phase extraction columns (3 ml) were used as spin columns, and were provided by VWR (South Plainfield, NJ). Sephadex G-25 (medium) and PD10 columns were provided by Amersham Pharmacia Biotech (Piscataway, NJ).

**General Chemical Methods.** Analytical HPLC analyses were conducted on a Beckman system (model 126 pump, model 168 UV detector, and model 170 radioactivity detector) connected to a model 406 analogue interface module. The chromatographic system consisted of a reverse-phase C18-Basic column (5 μm, 4.6 × 250 mm), obtained from YMC (Wilmington, NC), eluted in gradient mode at a flow rate of 1 ml/min. The gradient used 0.1% trifluoroacetic acid in water as solvent A and 0.1% trifluoroacetic acid in acetonitrile as solvent B. Starting with 100% A for 1 min, the gradient was decreased to 35% A over 25 min, held for 5 min, changed to 100% B for 15 min, and finally reequilibrated at 100% A for 30 min. Radioactivity was quantified using a LKB 1282 Compugamma (Wallingford, NC), eluted in gradient mode at a flow rate of 1 ml/min. Radioactivity was measured with a LKB 1282 Compugamma (Wallingford, NC), eluted in gradient mode at a flow rate of 1 ml/min. Radioactivity was quantified using a Capintec-7R radioisotope calibrator.

**mAbs and Cell Lines.** The characterization and purification of murine L8A4, an IgG1 that binds specifically to EGFRvIII with an affinity of 2 × 10⁴ M⁻¹, have been detailed in previous publications (6, 13). The EGFRvIII-expressing U87 MGΔEGFR cell line has been described by Nishikawa et al. (14) and was generated by transfecting the U87 MG human glioma cell line with a vector containing the cDNA for EGFRvIII. Cells were grown in zinc acetate–containing 10% fetal bovine serum and 600 μg/ml Geneticin sulfate. U87 MGΔEGFR cells express approximately 4–13 × 10⁶ EGFRvIII receptors per cell in culture (12).

**Radioiodination of KRYRR Peptides.** Nat[¹²⁵]I-iodide or Nat[¹³¹]I-iodide (1 mCi) was added to ω-KRYRR (10 μl, 1 mg/ml PBS) diluted with PBS (60 μl) in a glass vial coated with Iodogen (100 μg). The vial was placed on an orbital shaker for 40 min, and then the mixture was diluted with 3 ml of water. The solution was eluted through a tC18 cartridge, which was rinsed with an orbital shaker for 40 min, and then the mixture was diluted with 3 ml of water. The radioiodinated peptide was eluted in 400 μl of acetonitrile containing 1% acetic acid. The solvent was evaporated under a stream of nitrogen prior to reaction with the mAb.

**Conjugation of Radioiodinated Peptide to mAb.** As shown in Fig. 1, peptide activation was achieved by addition of s-SMCC (25 μl, 2 mg/ml PBS), and 0.1 M borate buffer (200 μl) to the vial containing the labeled peptide and reacting for 30 min at room temperature. Meanwhile, to add free sulphydryl groups to L8A4, the mAb (69 μl, 2.88 mg/ml PBS) and 2-iminiothiolane (6 μl, 3 mg/ml) were incubated in PBS (100 μl) for 30 min at room temperature. The thiolated mAb was purified using a 3-ml Sephadex G-25 spin column. Thiolated mAb was added directly to the vial containing the maleimido-activated, radioiodinated KRYRR, and the coupling was allowed to proceed at room temperature for 45 min, at which point the reaction was quenched by the addition of an excess of iodoacetamide (10 μl, 100 mg/ml PBS). After 20 min, the mixture was purified over a PD-10 column eluted with PBS.

**Radiiodination of L8A4 by the Iodo-Gen Method.** For some experiments, L8A4 was also labeled directly with ¹²¹I using the Iodogen method as described (11). The mAb (100 μl, 4.17 mg/ml PBS) and 1 mCi of Nat[¹²⁵]I-iodide were added to a glass vial coated with 100 μg of Iodogen. The reaction was allowed to proceed for 20 min at room temperature, and the labeled mAb was purified over a PD10 column eluted with PBS.

**Evaluation of Immunoreactivity.** Streptavidin-coated magnetic beads (1 ml) were mixed with the biotinylated recombinant extracellular domain of EGFRvIII (100–150 μl, 1 mg/ml) for 3 h at room temperature and rinsed with 115 mM phosphate buffer, pH 7.4, containing 0.05% Brij 35 and 0.05% BSA to remove unbound proteins. The beads were resuspended in 1 ml of this buffer and stored at 4°C. Control beads were fabricated as described above except that biotinylated BSA was conjugated to the beads. The immunoreactive fraction was determined by incubating the labeled L8A4 mAb preparations (approximately 20,000 cpm in 20 μl) in triplicate with increasing volumes of beads (10, 20, and 40 μl) in 500 μl of 115 mM PBS. After a 45-min agitation at room temperature, the beads were isolated from the supernatant using a magnetic separator. Beads and supernatants were counted for radioactivity, and specific binding was determined by subtracting nonspecific binding to BSA beads from the binding to EGFRvIII beads. The immunoreactive fraction was calculated according the method of Lindmo et al. (15) by plotting the inverse of the percentage of specific binding versus the inverse of the bead volume.

**Assay for mAb Internalization in Vitro.** The internalization and processing of ¹²⁵I-labeled ω-KRYRR-L8A4 and L8A4 labeled with ¹³¹I using the Iodogen method were compared in a paired-label experiment as described in Fig. 1. Scheme for the synthesis of radioiodinated KRYRR peptide-mAb L8A4 conjugates. The peptide is first radioiodinated on its tyrosine residue using Iodo-Gen and then activated with s-SMCC. The mAb is thiolated by reaction with 2-iminothiolane and then reacted with the activated peptide.
previous publications (8, 13). Approximately 1 μg of each mAb was incubated with 1 × 10^6 U87 MGΔEGFR cells for 1 h at 4°C and then washed with cold zinc option culture media containing 10% ECS. The cells were then resuspended in media/10% ECS to a density of 3 × 10^6 cells/ml, and the culture temperature was raised to 37°C. Aliquots of cells in triplicate were removed at 2, 6, and 24 h and centrifuged, and the supernatants were saved for counting. The cell pellet was washed twice at 4°C for 15 min with zinc option culture media containing 10% ECS (adjusted to pH 2.0 with HCI) to remove cell surface-associated activity. The acid washes were combined and counted along with the culture supernatants and cell pellets in the dual-channel gamma counter. The supernatants were incubated with 12.5% trichloroacetic acid to determine protein-associated activity. The counts in each compartment were expressed as a percentage of the activity bound to the cells immediately after the 1-h incubation at 4°C.

**Tissue Distribution of Radioiodinated KRYRR Peptides.** The biodistribution of the labeled peptides was investigated in BALB/c mice weighing 20–25 g. In the first experiment, groups of five animals were injected i.v. with 5 μCi of 125I-labeled t-KRYRR and 5 μCi of 131I-labeled d-KRYRR and killed by halothane overdose 1, 2, 4, and 24 h later. A second study was performed to determine the effect of co-administration of t-lysine on radioiodine distribution. Groups of five mice received 5 μCi of 125I-labeled t-KRYRR and 7 μCi of 131I-labeled t-KRYRR i.v. and simultaneously were given 0, 30, or 60 mg of t-lysine via the i.p. route. All groups were killed 2 h after injection by halothane overdose. Organs were harvested, weighed, and counted in the dual-channel gamma counter. The % ID/g was calculated by comparison to injection standards of appropriate count rate.

**HPLC Analysis of Peptide Catabolites in Urine.** Urine samples obtained during the first experiment were pooled at each time point, diluted with 500 μl of PBS, and passed through a 45 μm acetate membrane filter. The samples were analyzed on the C18-Basic HPLC column. Fractions were collected every 15 s using a fraction collector and assayed for 125I and 131I activity in the dual-channel gamma counter. The fraction eluting at 15 min was further analyzed by ITLC. The plate was eluted in saline:ethanol (95:5), cut in half, and counted for radioactivity.

**Tissue Distribution of Labeled L8A4 mAb.** Xenografts of the U87 MGΔEGFR cell line were established in athymic mice by s.c. injection of 50 μl of tumor homogenate in the flank of each mouse. Previous flow cytometric studies have shown that disaggregated U87 MGΔEGFR xenografts have a mean of 2.5 × 10^6 EGFR/vIII receptors per cell (12). Mice weighing 22–26 g were injected with 1 μCi of 125I-labeled d-KRYRR-L8A4 and 2 μCi of 131I-labeled L8A4 prepared using the Iodogen method. Groups of five animals were killed by halothane overdose 12, 24, 36, 48, and 72 h later. In the second experiment, mice weighing 14–22 g were injected with 1.5 μCi (6 μg) each of 125I-labeled t-KRYRR-L8A4 and 131I-labeled t-KRYRR-L8A4, and groups of five animals were necropsied at 6 and 12 h and 1, 2, 3, 5, and 6 days.

**RESULTS**

**Radioiodination and Immunoreactivity.** Radioiodination of the peptide was performed prior to reaction with s-SMCC to maximize peptide concentration during the labeling reaction and thereby optimize radioiodination yield. After purification with a tC18 cartridge, 125/131I-labeled d- or t-KRYRR was obtained in 80% yield. The labeled peptide eluted on the C18-Basic HPLC column with a retention time of 15 min, similar to that of authentic KRYRR peptide (14.25 min). The activation of each radioiodinated peptide with s-SMCC was shown to be nearly quantitative on HPLC: the labeled peptide peak at 15 min was absent, and a new peak was observed at 19.6 min, consistent with the more lipophilic nature of the activated peptide. Coupling of this maleimido-derivatized radiolabeled peptide to the thiolated L8A4 mAb proceeded in a 60% radiochemical yield. The specific activity of the preparations used in these experiments ranged between 0.3 and 1.9 mCi/mg; no attempt was made to obtain higher specific activities. The yield for direct iodination of L8A4 by the Iodogen method was 94%. For all preparations, 99% of the activity remained at the baseline on ITLC, and no evidence of aggregation was observed on size-exclusion HPLC. Immunoreactive fractions for L8A4 labeled using Iodo-Gen, t-KRYRR, and d-KRYRR, measured using EGFRvIII-coated magnetic beads, were 95, 92, and 95%, respectively.

**Cell Retention and Internalization in Vitro.** The internalization and cellular processing of 125I-labeled t-KRYRR-L8A4 after incubation at 37°C with EGFRvIII-expressing U87 MGΔEGFR cells was compared with that of L8A4 labeled with 131I using Iodogen. As shown on Fig. 2, internalized and total cell-associated (internalized + cell surface) radioiodine activity levels were significantly higher for 125I-labeled t-KRYRR-L8A4 at all time points, with the differences between the two labeling methods increasing with time. At the 2-h time point, differences in cellular retention corresponded to differences in the release of intact mAb from the cells. After a 24-h incubation at 37°C, the total cell-associated activity was 5.3 times higher for 125I-labeled t-KRYRR-L8A4 at this time (iodogen, 11.1 ± 1.0%; d-KRYRR, 58.3 ± 12.4%). Likewise, the percentage of activity retained as internalized counts was nearly 4 times higher for the t-KRYRR-L8A4 conjugate (iodogen, 5.0 ± 1.2%; d-KRYRR, 18.8 ± 5.4%). With 125I-labeled t-KRYRR-L8A4, there was a corresponding decrease in the fraction of mAb initially bound to U87 MGΔEGFR cells released into the cell culture supernatant as labeled degradation products (trichloroacetic acid-soluble counts) into the cell culture supernatant. For example, degraded counts in the supernatant at 24 h accounted for 19.7 ± 6.8 and 51.1 ± 6.3% of total counts for L8A4 labeled via t-KRYRR and Iodogen, respectively.

**Tissue Distribution and Catabolism of Radioiodinated KRYRR Peptides.** Because of the potential influence of peptide tissue distribution on the in vivo behavior of its corresponding mAb conjugate, the biodistribution of 131I-labeled t-KRYRR and 125I-labeled t-KRYRR were evaluated in normal mice. The d- and l-peptides exhibited markedly different radioiodine distribution patterns (Fig. 3). With the exception of the kidneys, radioactivity levels generally were significantly lower for the d-amino acid peptide. Approximately 30% ID of 125I-labeled n-KRYRR was retained in the kidneys during the first 4 h after injection, declining to about half this level by 24 h. In contrast, kidney uptake of 125I-labeled t-KRYRR was 1.30 ± 0.27% ID at 1 h and declined rapidly thereafter. On the other hand, retention of 131I-labeled n-KRYRR in lungs, blood, thyroid, and stomach, as well as intestines, muscle, heart, and brain (data not shown), was significantly
lower \((P < 0.05, \text{paired } t\text{ test})\) than retention of \(^{125}\text{I}\)-labeled \(\text{L-KRYRR}\) from 1 to 4 h, and in some cases, at 24 h after injection. Because of the proclivity of free iodide for the thyroid, accumulation of radioiodine activity in the thyroid can reflect dehalogenation. The \% ID of \(^{131}\text{I}\) in the thyroid 0.2\% ID or less at all time points, levels significantly lower than those observed for \(^{125}\text{I}\) (2.65 ± 0.50 to 5.24 ± 1.26\%).

Another tissue distribution study was performed to determine the effect of co-administration of \(\text{D-lysine}\) on the retention of radioiodine activity 2 h after injection of radiolabeled \(\text{D- and L-KRYRR}\). Kidney retention of \(^{125}\text{I}\)-labeled \(\text{D-KRYRR}\) was reduced from 19.13 ± 3.46\% to 7.96 ± 3.21 and 6.12 ± 1.49\% by the co-administration of 30 and 60 mg of \(\text{D-lysine}\), respectively, whereas kidney accumulation of \(^{131}\text{I}\)-labeled \(\text{L-KRYRR}\) was unaffected (Fig. 4). Liver activity levels were somewhat higher in animals receiving \(\text{D-lysine}\), but these differences were not statistically significant.

Urine samples were analyzed to determine the nature of the labeled catabolites excreted from mice receiving \(^{131}\text{I}\)-labeled \(\text{D-KRYRR}\) and \(^{125}\text{I}\)-labeled \(\text{L-KRYRR}\). In this HPLC system, free iodide eluted at \(\approx 5\) min. As shown in Fig. 5, there was little evidence for free iodide in the urine from the \(\text{D-KRYRR}\) (0.8\% at 1 h to 4.2\% at 24 h), whereas free iodide generated from \(\text{L-KRYRR}\) ranged from 25 to 69\%. Because both iodotyrosine and the radiiodinated KRYRR peptide eluted at \(\approx 13\) min (Fig. 4), ITLC was used to differentiate between these species. ITLC analysis revealed that 18–22\% of the labeled \(\text{L-KRYRR}\) was excreted as iodotyrosine, and 82–98\% of the urine activity from \(^{131}\text{I}\)-labeled \(\text{D-KRYRR}\) was present as intact peptide.

**Tissue Distribution of Radioiodinated Anti-EGFRvIII L8A4 mAb.** Two paired-label biodistribution studies were performed in athymic mice bearing s.c. U87 MG\(\text{A}3\)EGFR xenografts to determine the effect of labeling method on tumor and normal tissue accumulation of radioiodine activity. Direct comparison of \(^{125}\text{I}\)-labeled \(\text{D-KRYRR-L8A4}\) and \(\text{L8A4 labeled with }^{131}\text{I}\) using Iodogen demonstrated that use of the \(\text{D-amino acid prosthetic group}\) resulted in significantly higher \((P < 0.05)\) tumor levels at all time points (Fig. 6).

The peptide labeling method increased the tumor retention of radioiodine activity by factors of approximately 2 at 12 h, 3 at 24 h, and 5 at 36 h, 48 h, and 72 h. Maximum tumor accumulation occurred at 24 h for \(^{125}\text{I}\)-labeled \(\text{D-KRYRR-L8A4}\) (21.5 ± 3.9\% ID/g) and at 12 h for \(\text{L8A4 labeled with }^{131}\text{I}\) using Iodogen (7.99 ± 0.84\% ID/g). The \% ID/g in tumor for the peptide-mAb conjugate declined about 2-fold
for both conjugates was observed at 24 h (D-KRYRR, 55.6 ± 0.01% ID/g; L-KRYRR, 18.4 ± 0.18% ID/g) and declined by a factor of about 2.7 for the D-KRYRR-L8A4 conjugate by day 6 (Table 2). Again, rapid xenograft growth (day 1, 0.12 ± 0.04 g; day 6, 1.58 ± 1.07 g) probably contributed to the decline in % ID/g radioiodine activity in tumor with time. Normal tissue levels for the D-KRYRR-L8A4 conjugate generally were significantly higher than for the L-KRYRR-L8A4 conjugate except in the stomach and the thyroid. Uptake of radioiodine in the thyroid ranged from 0.01% ID to 0.17% ID/g for D-KRYRR-L8A4 and 0.01% ID to 0.12% ID/g for L-KRYRR-L8A4 conjugates.

A second experiment in tumor-bearing mice was performed to determine the effect of peptide chirality on the tissue distribution of their mAb conjugates (Table 2). Compared with L-KRYRR, use of radioiodinated D-KRYRR increased the tumor retention of radioactivity by 301 ± 14, 418 ± 167, 657 ± 80, 956 ± 222, and 1525 ± 663% on days 1, 2, 3, 5, and 6, respectively. Maximum tumor accumulation for both conjugates was observed at 24 h (D-KRYRR, 55.6 ± 8.7% ID/g; L-KRYRR, 18.4 ± 2.5% ID/g) and declined by a factor of about 2.7 for the D-KRYRR-L8A4 conjugate by day 6 (Table 2).

**DISCUSSION**

EGFRvIII is an attractive target for radioimmunotherapy because it is found on gliomas and breast, lung, and ovarian carcinomas but not on normal tissues (5, 6). EGFRvIII is the most frequently detected EGFR deletion mutant (2) and is expressed at receptor densities that should be compatible with radioimmunoconjugate applications (7). Moreover, mAbs, such as L8A4, that bind with high affinity to
EGFRvIII but not wild-type EGFR are available (6). However, to initiate clinical evaluation of L8A4, a labeling procedure appropriate for internalizing mAbs is needed; radioiodination of internalizing mAbs by conventional methods results in rapid loss of radioiodine from tumor cells as iodotyrosine because of proteolytic degradation of mAb in lysosomes (11, 16, 17).

Herein, we have investigated whether radioiodination of L8A4 using a D-amino acid peptide containing several positively charged amino acids (D-KRYRR) leads to internalization of mAbs and initiates clinical evaluation of L8A4, a labeling procedure appropriate for internalizing mAbs by conventional methods results in rapid loss of radioiodine from tumor cells as iodotyrosine because of proteolytic degradation of mAb in lysosomes (11, 16, 17).

**Table 2** Paired-label tissue distribution of radioiodine activity in athymic mice bearing subcutaneous 898 xenografts, after injection of L8A4 mAb labeled with 125I using L-KRYRR and 131I using D-KRYRR

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<th>Tissues</th>
<th>% ID/g&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>L8A4 labeled using L-KRYRR</td>
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<td>Liver</td>
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<td>Spleen</td>
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<td>Blood</td>
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<sup>a</sup> i.v. injection, mean ± SD of five animals.
<sup>b</sup> % ID per organ for thyroid.

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**Fig. 7.** Tumor: normal tissue ratio of radioiodine activity in athymic mice bearing s.c. U87-MGEGFR xenografts after paired injection of 131I-labeled D-KRYRR-L8A4 (●) and 125I-labeled L-KRYRR-L8A4 (○). Data points, average of five mice per time point; bars, SD.
amino acids would improve the retention of radioiodine activity in EGFRvIII-expressing tumor cells. Lysosomal proteases readily degrade proteins composed of D-amino acids but D-amino acids are not substrates for endogenous proteases (18, 19). The prototypical peptide selected for investigation was D-KRYRR. A tyrosine was included for facile radioiodination, and a lysine was added at the N-terminal to permit convenient coupling of the peptide to the mAb. To add three positive charges to the peptide, three arginine residues were chosen to add positive charges because the arginine side chain is the most basic (pK_a = 13.2) of the naturally occurring amino acids.

Our rationale for using a polycationic peptide is based on several observations. First, chloroquine and other dyes with multiple positive charges are retained in lysosomes to such a degree that they are used to stain this organelle (10). Second, when a series of D-dipeptides were introduced into lysosomes by endocytosis, a positively charged dipeptide was trapped, whereas six neutral dipeptides were not (19). Finally, radioiodine was retained in tumor cells as positively charged, lower molecular weight molecules when mAb L8A4 was labeled using SIPC (11).

The in vivo behavior of radioiodinated D-KRYRR and L-KRYRR were compared in normal mice to facilitate interpretation of KRYRR-mAb conjugate biodistributions. Moreover, only limited information is available concerning the tissue distribution of D-amino acid peptides. Pappenheimer et al. (20, 21) evaluated the excretion of five octapeptides composed of D-amino acids of varying charge and lipophilicity. Peptides containing serine or leucine exhibited slower whole body clearances compared to more hydrophilic peptides, suggesting that exclusion of lipophilic amino acids should be a design criteria for labeled D-amino acid peptide acylation agents.

A potential concern with labeling peptides by direct iodination is that previous studies have shown that L-amino acid peptides labeled in this manner are rapidly dehalogenated in vivo, as reflected by high radioiodine levels in the thyroid and stomach (22, 23). Our results revealed high radioiodine levels in the thyroid and stomach after injection of 125I-labeled L-KRYRR, reaching maximum values of 5.24 ± 1.26 and 8.51 ± 1.45%, respectively. The blood and normal organ distribution of 125I activity were in excellent agreement with those reported for free iodide (24), suggesting that rapid dehalogenation of 125I-labeled L-KRYRR (or its intermediate catabolite) had taken place. Thyroid and stomach levels from co-administered 131I-labeled D-KRYRR were 1–2 orders of magnitude lower, suggesting low recognition of its D-iodotyrosine residue by endogenous deiodinases. This is in agreement with previous studies demonstrating that the deiodination of D-tyrosine was considerably slower than that of its L-enantiomer (25, 26).

The in vivo results also are consistent with differential sensitivity of L- and D-KRYRR to proteolysis. Chromatographic analysis of urine from mice injected with the two peptides suggests that 131I-labeled D-KRYRR was largely excreted intact, whereas radioiodinated L-KRYRR was eliminated as iodide, iodotyrosine, and other unidentified catabolites that probably correspond to proteolytically degraded peptide fragments. These results are in agreement with those reported for the renal excretion of other D-amino acid peptides (20, 21).

Normal tissue clearance of both peptides generally was rapid except for 125I-labeled D-KRYRR in the kidneys. The kidney plays an active role in the metabolism of peptides. These molecules can be reabsorbed by the proximal tubular cells, internalized via endocytosis, and routed to renal lysosomes. Positively charged molecules, such as D-KRYRR, are much more likely to undergo tubular absorption because the kidney luminal membrane is anionic (27). High concentrations of cationic amino acids can inhibit this process by competing for the anionic sites on the luminal cell surfaces (28). Indeed, basic amino acids have been used to reduce kidney uptake of radioactivity after the injection of radiolabeled mAb fragments and peptides (29, 30). To determine whether this approach could lower 131I-labeled D-KRYRR kidney uptake, co-administration of D-lysine was investigated. One injection of 60 mg D-lysine yielded a 3-fold reduction in kidney levels, an effect similar to that reported for a single dose of D-lysine on the renal uptake of labeled octreotide analogues (29). Further reductions in kidney levels have been achieved through the use of optimized D-lysine administration protocols (30).

An important objective in developing labeling strategies for L8A4 and other internalizing mAbs is to maximize radioiodine retention in the tumor cell after cellular processing of the mAb. For this reason, the internalization and cellular processing of 125I-labeled D-KRYRR-L8A4 by EGFRvIII-expressing U87ME2EGFR cells was compared to L8A4 labeled with 125I using Iodo-Gen. Both internalized and total cell-associated counts were significantly higher for the D-KRYRR-mAb conjugate at all time points. After 24 h, the percentage of activity retained as intracellular counts was nearly 4 times higher, and total cell-associated activity was 5.3 times higher for 125I-labeled D-KRYRR-L8A4. These results are superior to those obtained using other residualizing methods for labeling L8A4. When the same assay was used to compare the cellular retention of L8A4 labeled using tyramine-cellobiose and iodogen on the EGFRvIII-expressing HC2 20 d2 line, the maximum advantage seen for TCB was a factor of 2.8 at 20 h (8). Using the same cell line and mAb, the maximum advantage seen for SIPC compared with Iodo-Gen was only a factor of 1.6 at 2 h, and the advantage declined rapidly thereafter (11). We speculate that the excellent cellular retention observed with the D-KRYRR-L8A4 conjugate reflects the proteolytic generation of 125I-labeled D-KRYRR, which, having multiple positive charges, exhibits restricted passage through lysosome and cell membranes. Detailed studies of the labeled catabolites generated from mAbs labeled using D-KRYRR will be done to determine the validity of this assumption.

The cellular processing of mAbs labeled using another acylation agent containing D-amino acids has been described recently (31). A D-amino acid peptide was used to couple DTPA to the mAb with the goal of exploiting the fact that mAb-DTPA-radiometal conjugates frequently exhibit effective retention of the radiometal in tumor cells. When n-Gly-Tyr-Lys-DTPA was radioiodinated and coupled to internalizing mAbs, retention of radioiodine activity by tumor cells was up to 3 times higher than for the same mAb labeled using chloramine-T. The magnitude of these gains was lower than that of the gains seen with D-KRYRR; however, comparing results obtained with mAbs binding to different internalizing antigens must be done with care because of potential differences in variables such as dynamics of mAb internalization and intracellular processing. With that caution in mind, it should be noted that similar results to those described herein were obtained by labeling internalizing mAbs using dilactitol-tyramine-T (32). Unfortunately, poor mAb coupling yields (3–6%) detract from the utility of this method.

Tissue distribution studies were performed in athymic mice to determine whether the increased retention of radioiodine observed in EGFRvIII-expressing tumor cells with D-KRYRR-L8A4 in vitro provided a similar advantage in vivo. Consistent with the results of the cellular processing and internalization assays, the tumor delivery enhancement achieved with D-KRYRR (relative to iodogen labeling) was more than twice that obtained with either TCB or SIPC. When L8A4 was labeled using TCB, the tumor delivery advantage ranged between 2 and 3 between 1 and 7 days after injection (8), whereas SIPC yielded a maximum enhancement of 1.28 at 1 day, with no improvement in tumor levels seen after 48 h (11). In contrast, D-KRYRR imparted a 3-fold advantage at 1 day, increasing to a 5.5-fold enhancement 3 days after injection of L8A4.

In summary, the practical objective of this study was to increase the
retention of radioiodine in tumor after injection of radioiodinated anti-EGFRvIII mAbs, and this was achieved. The mechanisms responsible for this effect will be confirmed in subsequent experiments. The tumor uptake of radioiodinated D-KRYRR-L8A4 was significantly higher than that of co-administered l-KRYRR-L8A4, suggesting that the inertness to proteolytic degradation of radioiodinated D-KRYRR played a critical role. To optimize this general strategy, we are investigating the effect of amino acid charge, lipophilicity, and peptide length on the in vitro and in vivo behavior of radioiodinated D-amino acid-internalizing mAb conjugates.

REFERENCES

Radioiodination via d-Amino Acid Peptide Enhances Cellular Retention and Tumor Xenograft Targeting of an Internalizing Anti-Epidermal Growth Factor Receptor Variant III Monoclonal Antibody

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