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An immunoadsorption strategy to produce specific antisera against analogs of human proteins: development of sensitive and specific radioimmunoassays for two analogs of human leptin

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Abstract

Immunoassay technology is routinely used to measure concentrations of proteins and polypeptides in biological matrices. Increasingly, research efforts have sought to create analogs of human proteins with the aim of improving efficacy or pharmaceutical properties relative to the native protein. Pharmacokinetic assessment of these polypeptide analogs, however, can be greatly confounded by the presence of endogenous native protein. This report describes an immunization and immunoabsorption strategy that was used to create monospecific polyclonal antibodies against analogs of human leptin (LY355101 and LY396623, one and two amino acid changes relative to native human leptin, respectively). Rabbits were immunized with either LY355101 or LY396623. Antisera were screened to determine if any showed increased specificity for the analog relative to native human leptin. Antisera showing increased specificity for the leptin analog were then treated by immunoabsorption against native human leptin, thus depleting human leptin cross-reactivity. The antibodies developed in this process were used in radioimmunoassays, which were validated for use in clinical studies. Both assays proved to be highly specific for LY355101 or LY396623 in the presence of native human leptin. Use of this procedure permitted the measurement of LY355101 and LY396623 pharmacokinetics that were not confounded by the high levels of endogenous human leptin found in obese subjects. This technique has the potential for broad application in the development of assays capable of specifically measuring protein analogs without cross-reactivity to an endogenous substance.

Keywords: Immunoassay; Radioimmunoassay; Leptin; Obesity; Immunoabsorption

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1. Introduction

Leptin is a 16 kD peptidyl hormone produced in adipocytes that acts via a feedback loop to the hypothalmus in signaling nutritional status and modulating feeding and energy expenditure [1–4]. Studies in ob/ob (leptin deficient) and normal mice showed that administration of recombinant leptin resulted in weight loss [5–7]. Consequentially, there has been great interest in leptin as a potential therapeutic agent for inducing weight loss. This interest in leptin as a therapeutic comes in spite of data indicating that nearly all obese humans have high levels of circulating leptin relative to non-obese individuals [8–10].

Analogs of human leptin have been developed in an effort to improve the formulation characteristics of the native protein. Both LY355101 and LY396623 are closely related to native human leptin. In LY355101, an alanine residue was substituted for tryptophan at position 100, while LY396623 contained aspartic acid residues substituted for asparagine 72 and tryptophan 100. Both substitutions were made to increase the solubility and stability of the molecule. In developing an immunoassay for the pharmacokinetic assessment of these leptin analogs, there was the potential for assay interference by native leptin, which is elevated in the circulation of obese subjects. An antibody might not discriminate between an exogenous analog and the native endogenous form of the same protein unless some structural or chemical difference exists between the two forms. Moreover, if the structure of the exogenous protein differs slightly from the native protein, cross-reactivity of native and exogenous protein may be partial or complete depending on the epitope specificity of the antibodies in the assay [11,12]. As a result, it may be difficult to collect bioanalytical data for a protein analog that is not confounded by interference from the native protein.

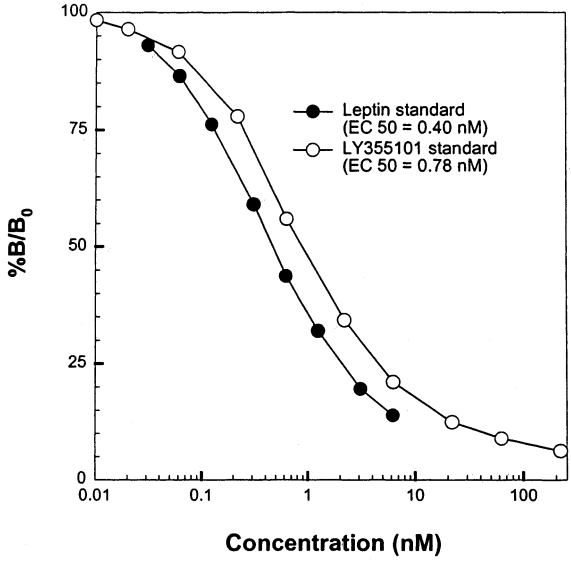

Fig. 1. Comparison of the cross-reactivity of LY355101 in the LINCO human leptin kit. Human leptin or LY355101 standards were used as calibrators in the LINCO human leptin assay kit. The assay was performed according to manufacturer's instructions utilizing [^{125}I]-human leptin as a tracer. Data are plotted as the ratio of bound cpm (B) relative to the maximum binding in the absence of unlabeled competitor (B_0).

The assays described in this paper were developed to support clinical investigation of the pharmacokinetics of LY355101 and LY396623. Immunization with the leptin analog followed by use of an immunoabsorption strategy was employed to produce monospecific antisera. Using these monospecific antisera, assays were then developed and validated for use in clinical trials of the leptin analogs. Based on these efforts, it was determined that it was possible to increase the specificity of antisera demonstrating partial cross-reactivity between human leptin and leptin analogs by utilizing immunoabsorption against native human leptin. The antisera depleted of native human leptin cross-reactivity were highly specific for the leptin analogs, and permitted measurement of these proteins in human clinical trials utilizing obese individuals. This technique is widely applicable for increasing the specificity of polyclonal antisera against recombinant proteins that may differ only slightly from the native form of the protein.

2. Materials and methods

Recombinant human leptin, LY355101 (ala 100 hOB), and LY396623 (asp 72, 100 hOB) were

Table 1
Cross-reactivity of LY355101 antibodies and human leptin

Antibody	Conjugate[a]	ED_{50} nM[b]		% Cross-reactivity[c]
		LY355101 standard	Human leptin standard	
1127	None	6.39	7.04	91
1128	None	0.81	7.23	11
1129	None	3.45	4.59	75
1130	None	2.32	3.33	70
1131	None	4.26	3.58	119
1132	None	2.44	4.21	58
1133	BSA	0.25	6.91	4
1134	BSA	0.44	2.70	16
1136	BSA	0.31	0.25	125
1138	BTG	1.38	1.51	91
1140	BTG	1.25	1.76	71
1141	PLL	0.63	0.63	100
1142	PLL	0.81	0.63	130
1143	PLL	1.25	1.19	105
1144	PLL	0.75	1.63	46

[a] Carrier protein conjugated to LY355101 for immunization purposes. BSA, bovine serum albumin; BTG, bovine thyroglobulin; PLL, poly-L-lysine.
[b] Competitions were performed using [^{125}I]-LY355101 as a tracer. Assays were performed as described in Section 2 for antibody screening.
[c] ED_{50} of LY355101 divided by the ED_{50} of human leptin.

prepared at Eli Lilly via *Escherichia coli* expression. Bovine serum albumin (BSA), bovine thyroglobulin (BTG), poly-L-lysine (PLL), disuccinimidyl glutarate (DSG), and DMSO for preparation of conjugates were obtained from Pierce (Rockford, IL). RIA-grade BSA and polyethylene glycol (Mw 8000) were purchased from Sigma (St. Louis, MO). Normal rabbit serum, bovine gamma globulin (BGG) and goat anti-rabbit gamma globulin (GARGG) were from Calbiochem (San Diego, CA). Affigel-15 was obtained from BioRad Laboratories (Hercules, CA). Human EDTA plasma was from Western States Plasma Co. (Oceanside, CA). All other reagents were of analytical grade. The human leptin RIA kit (Catalog No. HL-81K) was purchased from LINCO Research Inc. (St. Charles, MO). [^{125}I]-labeled leptin, LY355101, and LY396623 were prepared by LINCO Research Inc.

2.1. Production of immunogens

Conjugates were prepared by linking purified LY355101 or LY396623 at a 10:1 molar ratio to carrier molecules (BSA, BTG or PLL) using DSG as a linker. Briefly, LY396623 or LY355101 was suspended in phosphate buffer (pH 7.5; 20 mM). DSG was dissolved in dry DMSO and was added dropwise with stirring to achieve a 10-fold molar excess over the leptin analog. Following 10 min of gentle stirring at room temperature, carrier protein was added and the reaction was allowed to continue overnight at room temperature. The conjugates were then dialyzed extensively versus phosphate buffered saline with a 12 000 kDa cut-off membrane.

2.2. Production of antisera

Antibody production was performed in rabbits at Covance (Denver, PA). In initial immunizations, a total of 0.4 mg of protein was emulsified in Freund's complete adjuvant and was administered as an intramuscular injection. Subsequent booster injections were performed intramuscularly at 2, 4, 12, 16, 20, 24, 28 and 32 weeks and intravenously at 8 weeks. Booster injections consisted of 0.1 mg of immunogen emulsified in Fre-

und's incomplete adjuvant. Sera were collected at 2-week intervals, beginning 6 weeks after initial immunization.

2.3. Radioimmunoassay

2.3.1. Solutions

The assay buffer used to dilute tracers and antibodies consisted of sodium phosphate (pH 7.5; 100 mM), 20 g/l EDTA (dipotassium), 1 g/l sodium azide, 0.5 ml/l Tween-20 and 1 g/l BSA. Normal rabbit serum was prepared by reconstituting one vial of lyophilized serum with 5 ml Alpha-Q water and diluting to a final concentration of 0.5% by adding 5 ml to 1 l of assay buffer. In the final RIA procedure, we employed a secondary antibody-PEG precipitating reagent consisting of sodium phosphate (pH 7.5; 50 mM), 1 g/l EDTA (dipotassium), 5 ml/l Tween-20, 1 g/l sodium azide, 60 g/l PEG-8000, and 15 ml/l goat anti-rabbit gamma globulin (GARGG). All solutions, except the stock solutions of antiserum and peptide calibrators, were stored at 4°C. Antisera and calibrators were stored at −20°C or colder.

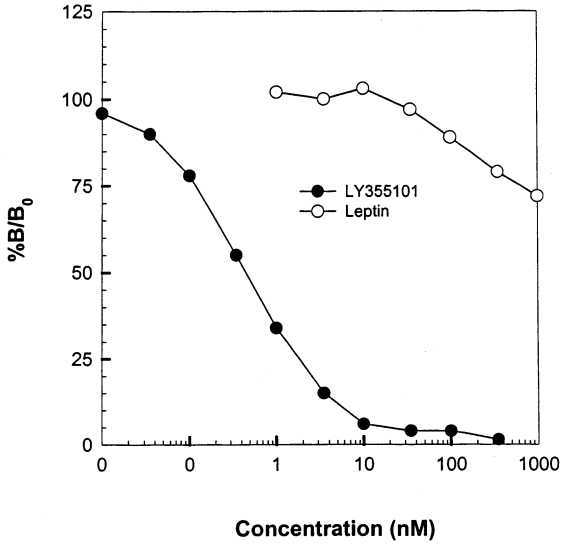

Fig. 2. Competition of antiserum 1133-A binding to [^{125}I]-LY355101 by human leptin. Unlabeled LY355101 or human leptin were used to compete antiserum 1133-A binding to [^{125}I]-labeled LY355101. Antiserum 1133-A had been immunoabsorbed with immobilized leptin to deplete leptin cross-reactivity. Data are expressed as the ratio of bound cpm (B) relative to the maximum binding in the absence of unlabeled competitor (B_0). The assay was performed as described in Section 2 for the RIA procedure.

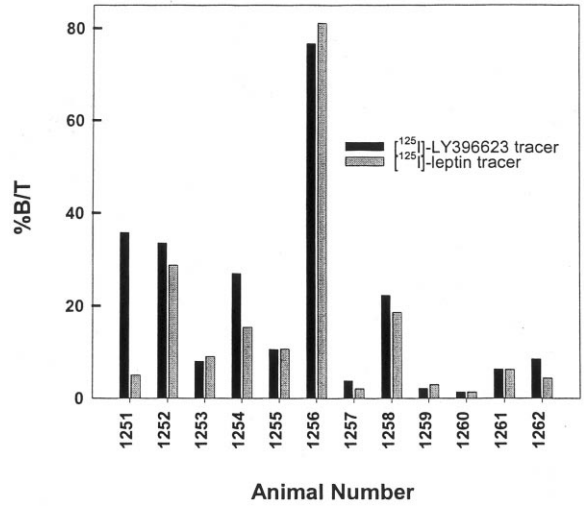

Fig. 3. Binding of LY396623 antisera to [^{125}I]-LY396623 or [^{125}I]-leptin. Antisera were diluted 1:5000 with assay buffer, and binding of the antibodies to either [^{125}I]-LY396623 or [^{125}I]-leptin was compared (see antibody screening in Section 2). Animals 1251–1258 were immunized with LY396623 conjugated to BSA; animals 1259–1262 were immunized with LY396623 conjugated to bovine thyroglobulin. Data are expressed as the ratio of bound cpm (B) to the total input cpm (T).

2.3.2. Antibody screening

Assays to screen and titer antibodies were performed in 12×75 mm polypropylene tubes as follows. Antisera were diluted in assay buffer and a 100 μl volume was mixed with 200 μl [^{125}I]-labeled tracer ($\approx$ 20 000 cpm). The reaction mixture was incubated overnight at 4°C. Antibody:antigen complexes were precipitated by adding 100 μl of 1% BGG followed by 1 ml of cold 20% PEG. Tubes were vortexed, incubated at 4°C for $\approx$ 15 min and precipitates were collected by centrifugation at $3000 \times g$ for 20 min. Supernatants were decanted and radioactivity remaining in pellets was determined using a gamma counter.

2.3.3. RIA procedure

RIAs for the quantification of plasma LY355101 or LY396623 were performed in a 12 × 75 mm polypropylene tube using the following procedure. Binding reactions (400 μl total volume) consisted of 100 μl antisera (anti-LY396623 diluted 1:3000, anti-LY355101 diluted 1:300), 200 μl [^{125}I]-labeled tracer (LY396623 or LY355101) and 100 μl of sample or calibration standard prepared in human EDTA plasma. Calibration standard concentrations ranged between 0.0035 and 350 nM for the LY355101 assay and 0.0035 and 100 nM for the LY396623 assay. Nonspecific binding was determined by replacing antiserum with assay buffer. Samples were mixed and incubated at 4°C overnight. Antigen:antibody complexes were precipitated by the addition of 100 μl 0.5% normal rabbit serum and 1 ml cold secondary antibody-PEG precipitating reagent.

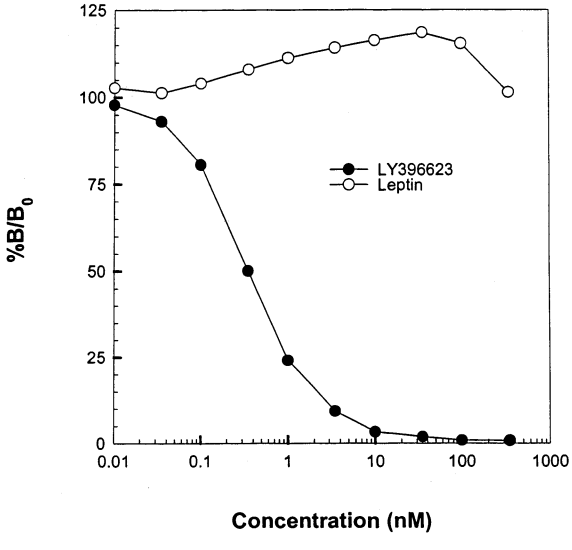

Fig. 4. Competition of antiserum 1251-A binding to [^{125}I]-LY396623 by human leptin. Unlabeled LY396623 or human leptin were used to compete antiserum 1251-A binding to [125-I]-labeled LY396623. Antiserum 1251-A had been immunoabsorbed with immobilized leptin to deplete leptin cross-reactivity. Data are expressed as the ratio of bound cpm (B) relative to the maximum binding in the absence of unlabeled competitor (B_0). The assay was performed as described in Section 2 for the RIA procedure.

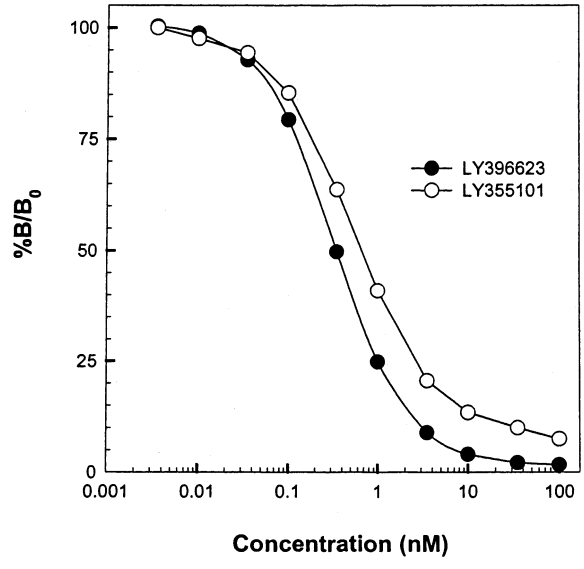

Fig. 5. Representative calibration curves for LY355101 and LY396623 in human EDTA plasma. RIAs were performed as described in Section 2 for the RIA procedure. Antibodies used in the RIAs were depleted of leptin binding with immobilized leptin (1133-A and 1251-A); iodinated LY355101 or LY396623 were used as tracers. Data are plotted as the ratio of bound cpm (B) relative to the maximum binding (B_0) in the absence of competitor.

Tubes were then vortexed, incubated at 4°C for 1 h and the precipitates were pelleted by centrifugation at 3000 × *g* for 20 min at 4°C. After decanting the aqueous phase, radioactivity in the precipitates was measured using a gamma counter. A VAX computer was used to analyze the RIA data by a weighted four-parameter logistic model algorithm [13].

2.4. Immunoabsorption

Antibodies that cross-reacted with native leptin were removed from pools of anti-LY396623 or anti-LY355101 antisera by immunoabsorption with native leptin coupled to Affigel-15. Leptin-Affigel-15 affinity resin was prepared according to the manufacturer's instructions. Leptin (29 mg) was dissolved in 5 ml HEPES buffer (pH 7.5; 5 mM). Ten millilitres of a 50% slurry of Affigel-15 resin was prepared by washing over a scintered

glass funnel with cold alpha-Q water. The gel was dried to a moist cake and transferred immediately to a polypropylene tube containing the leptin solution. After mixing, the conjugation reaction was allowed to proceed overnight at 4°C. The supernatant from the conjugation reaction was collected and any remaining reactive groups on the Affigel-15 were blocked by the addition of 1.5 ml of glycine (pH 2.2; 1 M). Analysis of the supernatant from the conjugation reaction demonstrated that the reaction proceeded with 97% efficiency. Prior to use in immunoabsorption, the leptin-affinity resin was washed extensively with assay buffer.

The amount of leptin-Affigel-15 affinity resin necessary to absorb leptin cross-reactive antibodies in the anti-LY355101 and anti-LY396623 sera was determined empirically. For preparation of the LY396623 specific sera, bleeds 4–11 from animal 1251 were pooled and 12 ml of this pool was diluted by the addition of 11 ml of assay buffer. The diluted LY396623 antiserum was mixed overnight at 4°C with leptin-Affigel-15 corresponding to 3.5 mg of leptin. The affinity resin was pelleted by centrifugation at $3000 \times g$ for 15 min and the specificity of the resulting antisera was determined by titering binding to [^{125}I]-leptin and [^{125}I]-LY396623. For preparation of the LY355101 specific sera, bleeds 7–11 from animal 1133 were pooled and 5 ml of the pool was diluted with 4 ml of assay buffer. The diluted antiserum was mixed with leptin-Affigel-15 corresponding to 3.5 mg of leptin and processed as described for LY396623. Specificity was determined by titering binding of the absorbed LY355101 antiserum to [^{125}I]-leptin and [^{125}I]-LY355101.

Table 2
Precision and recovery of LY355101 RIA

Concentration nM[a]	Intraassay[c]			Interassay[d]		
	Mean ± S.D. (nM)	% CV	% Recovery[b]	Mean ± S.D. (nM)	% CV	% Recovery[b]
0.1	0.10 ± 0.02	17.9	100	0.12 ± 0.01	8.3	120
0.25	0.23 ± 0.01	4.3	92	0.25 ± 0.02	8	100
1.0	0.91 ± 0.05	5.5	91	0.91 ± 0.02	2.2	91
10.0	8.02 ± 0.66	8.2	80	8.40 ± 0.58	6.9	84

[a] Control samples were prepared by spiking LY355101 into human EDTA plasma at the indicated concentration.
[b] Percent recovery is the measured concentration as a percentage of the theoretical concentration.
[c] $N = 6$ determinations.
[d] $N = 4$ determinations.

Table 3
Precision and recovery of LY396623 RIA

Concentration (nM)[a]	Intraassay[d]			Interassay (N)[c]		
	Mean ± S.D. (nM)	% CV	% Recovery[b]	Mean ± S.D. (nM)	% CV	% Recovery[b]
0.1	0.09 ± 0.01	9.1	91	0.09 ± 0.01 (6)	9.9	90
0.2	0.21 ± 0.01	3.9	104	0.19 ± 0.01 (10)	6.9	96
3.5	3.25 ± 0.39	11.9	93	3.3 ± 0.3 (5)	8.2	94
5.0	4.7 ± 0.5	10.3	93	5.0 ± 0.4 (5)	8.1	100
10.0	8.5 ± 0.7	8.3	85	8.9 ± 0.3 (4)	3.2	89

[a] Control samples were prepared by spiking LY3596623 into human EDTA plasma at the indicated concentration.
[b] Percent recovery is the measured concentration as a percentage of the theoretical concentration.
[c] (N) = number of assay runs.
[d] $N = 6$ determinations.

Table 4
Plasma concentrations of human leptin immunoreactivity in placebo subjects[a]

Subject ID	Leptin immunoreactivity	
	LINCO assay[a] (nM)	LY355101 assay[b] (nM)
620	0.41	<0.1
631	0.56	<0.1
702	0.96	<0.1
707	0.91	<0.1
1812	0.15	<0.1
1967	0.54	<0.1
1986	1.97	<0.1
2204	0.69	<0.1
2209	3.00	<0.1
2217	2.25	<0.1
2221	1.85	<0.1

[a] Concentrations of human leptin immunoreactivity were measured using the LINCO human leptin kit. The lower limit of quantitation of the LINCO kit (as stated by the manufacturer) is 0.03 nM.

[b] Concentrations of immunoreactivity were measured using the LY355101 specific assay (using antisera 1133A). The lower limit of quantitation with this assay was 0.1 nM.

2.5. Assay validation

RIAs specific for LY355101 and LY396623 were validated to support pharmacokinetic studies in man [14]. Validation included assessment of intra- and interassay imprecision (CVs), recovery and serum stability. These assays were performed using controls consisting of LY355101 or LY396623 reference standard spiked into pooled EDTA plasma from normal healthy adults. Plasma controls were stored at approximately −70°C.

2.6. Clinical sample analysis

In order to verify the lack of cross-reactivity of the LY355101 antibody with human leptin, placebo control samples from a clinical study designed to evaluate the safety of LY355101 were analyzed for the presence of leptin and LY355101 immunoreactivity. This study was conduced in accordance with the ethics principles stated in the latest version of the Declaration of Helsinki and the applicable guidelines for good clinical practice. The protocol was approved by the local Institutional Review Board and each participant gave informed consent. Each subject received a single subcutaneous vehicle injection and venous blood samples were collected over a 24-h interval into EDTA vacutainer tubes. Samples were processed as plasma and concentrations of immunoreactive leptin or LY355101 were determined. Native human leptin levels were measured using the LINCO human leptin RIA kit as instructed by the manufacturer, while LY355101 levels were measured using the LY355101 specific assay.

3. Results

The initial strategy for determination of LY355101 and LY396623 pharmacokinetics was to measure total concentrations of 'leptin-like immunoreactivity' with a standard leptin radioimmunoassay. However, using a commercial kit developed for the measurement of human leptin (LINCO Research Inc.) it was found that LY355101 cross-reacted 50% as well as native leptin (Fig. 1). This difference in cross-reactivity was despite the fact that LY355101 differed from native human leptin by only a single amino acid. The LINCO kit was therefore deemed unsuitable for use in the assessment of LY355101 pharmacokinetics. The observation that human leptin and LY355101 were detected with different sensitivities by the same antibody suggested that it was possible to develop an antiserum specific for LY355101.

Rabbit antisera that had been generated against LY355101 were titered using [^{125}I]-LY355101 as a tracer to determine a dilution that yielded suitable percent binding. The selectivity of these antisera for LY355101 versus leptin was then compared by competing [^{125}I]-LY355101 binding with unlabeled human leptin or LY355101. The percentage of cross-reactivity was determined as the ratio of the ED_{50} of LY355101 (the ED_{50} is the concentration of unlabeled peptide necessary to produce 50% displacement of radiolabeled LY355101) to the ED_{50} of human leptin. Table 1 compares the ratio

of ED_{50} values LY355101 and leptin. Using this screen, it was determined that sera from animal 1133 had the greatest selectivity for LY355101 compared to human leptin, displaying only 4% cross-reactivity as determined by the ratio of ED_{50} values for the two proteins. Bleeds 7–11 were pooled and the amount of leptin-Affigel 15 required to remove leptin cross-reactivity was determined experimentally with small aliquots of the pooled serum. The scaled-up absorption technique required a total of 3.5 mg of leptin linked to the Affigel-15 to treat the entire antibody pool. Fig. 2 shows displacement of [^{125}I]-LY355101 binding to the absorbed 1133 sera (designated antiserum 1133-A) by LY355101 or human leptin. As demonstrated by these curves, 1133-A displayed minimal cross-cross reactivity to human leptin.

In an effort to facilitate antibody screening, a slightly different approach was used to evaluate antisera for differential selectivity for LY396623 versus human leptin. All antisera were diluted 1:500, 1:1000, 1:5000 and 1:10 000, and percent of total [^{125}I]-LY396623 or [^{125}I]-human leptin bound was compared at these dilutions using the screening RIA technique. Fig. 3 shows the results from bleed 8 at a dilution of 1:5000. Animal 1251 demonstrated the greatest binding to LY396623 with the least cross-reactivity to leptin. A comparison of the percent bound versus total counts (B/T) for bleeds 4 through 11 from animal 1251 were consistent with the results from bleed 8 (data not shown). Antisera from bleeds 4–11 obtained from rabbit 1251 were, therefore, pooled and treated by immunoabsorption with the leptin-Affigel 15. The amount of leptin-Affigel 15 required to remove leptin cross-reactivity was determined with small aliquots of the pooled serum and the technique was then scaled up to treat the entire pool. Fig. 4 shows LY396623 and leptin displacement curves generated with the absorbed 1251 pool (designated 1251-A) using [^{125}I]-labeled LY396623 as a tracer. While the antiserum remained highly sensitive to LY396623, essentially no cross-reactivity to leptin was seen after the leptin immunoabsorption.

3.1. Assay performance

3.1.1. Calibration curve parameters

Typical standard curves for LY396623 and LY355101 calibrators in human plasma are shown in Fig. 5. Values represent mean $\pm$ S.D. over all validation runs. Over four LY355101 assays, the percentage of non-specific binding was 1.4 ± 0.2, the maximum binding was 27.1 ± 4.3, the slope was 0.93 ± 0.03 and the EC50 was 0.58 ± 0.08 nM. Standard curve parameters for the LY396623 assay ($n = 9$) were $1.3 \pm 0.6\%$ non-specific binding, $25.9 \pm 2.5\%$ maximum binding. The slope and EC50 values were 1.02 ± 0.04 and 0.32 ± 0.05 nM, respectively. Tables 2 and 3 list assay validation results for the LY396623 and LY355101 specific assays.

3.1.2. Specificity

Analysis of pools of blank human plasma (0 controls) showed that there was no interference from native leptin in either assay (data not shown). We further verified the performance of the LY355101 assay by analyzing samples from placebo dosed subjects for both native human leptin and LY355101. While measurable concentrations of leptin were found using the LINCO assay (Table 4), results from analysis of the same samples with the LY355101 specific assay were all below the limit of quantitation (< 0.1 nM).

4. Discussion

Advances in biotechnology have fueled tremendous growth in the discovery and development of new therapeutic proteins. To date, recombinant DNA technology has yielded both novel recombinant proteins and recombinant versions of 'native' protein therapeutics [15–17]. Additionally, biotechnology has stimulated the growth in the development of analogs, second generation therapeutic proteins that have been modified systematically to optimize either their pharmacokinetic and pharmacodynamic behavior [18]. Analogs of human proteins can present a unique challenge for the validation and application of GLP compliant immunoassays to support clinical pharmacokinetic

and bioequivalence studies. Polyclonal antisera may be capable of detecting the structural differences between an analog and its corresponding native human protein. As a consequence, the analog can cross-react differently and show non-parallelism relative to the native human protein [11,12]. The analytical challenge arises when the endogenous protein is present in test samples at pharmacologically relevant concentrations, such as in the case of insulin. Thus, one is faced with the task of attempting to validate an immunoassay for an analog when the closely related native protein is present in the same biological specimen.

A recent report by Bowsher et al. described the use of immunization with an analog to a human protein followed by immunoabsorption to develop an immunoassay that was specific for insulin lispro in the presence of native insulin [19]. This technique has been extended in the present experiments to generate antisera against two analogs of human leptin. LY396623 differed from native human leptin by the substitution of two amino acids, while LY355101 differed by only a single amino acid. Despite differences of only one or two amino acids among 146 in the human leptin sequence, it was found that immunization with the leptin analog resulted in some polyclonal antisera with greater specificity to the analog than native human leptin.

Following initial antibody generation, leptin cross-reactivity was further decreased by immunoabsorbing the sera with leptin linked to Affigel-15. The antisera depleted of leptin cross-reactivity were suitable for the development of sensitive assays for the measurement of LY355101 and LY396623 in clinical samples. Two lines of evidence demonstrated the specificity of these assays for LY355101 and LY396623. In competition experiments, super-physiological amounts of leptin were spiked in human plasma and caused only minimal displacement of [^{125}I]-LY396623 or [^{125}I]-LY355101 binding to their respective specific antibodies (Figs. 2 and 4). Second, to demonstrate that the addition of exogenous leptin mimicked the in vivo cross-reactivity, placebo dosed samples from a clinical trial were analyzed for levels of human leptin and LY355101. Despite concentrations of human leptin as high as 3 nM, the LY355101 assay did not show measurable concentrations of LY355101 immunoreactivity. Placebo samples were not assayed for LY396623 and leptin immunoreactivity, as the experiment with LY355101 was taken as verification that the in vitro studies was reflective of the in vivo cross-reactivity.

Development of these analog-specific assays was significant because it permitted a direct assessment of the pharmacokinetics of these two polypeptides that was not confounded by the measurement of endogenous human leptin. This technique was first described in the development of assays specific to insulin analogs in the presence of native human insulin [19]. Successful use of the same immunization/immunoabsorption procedure in the development of assays specific for leptin analogs indicated the general usefulness of the approach. In summary, a technique has been described that can be used to optimize the specificity of a polyclonal antiserum. This technique has the potential for broad application in the development of assays capable of specifically measuring protein analogs without cross-reactivity to an endogenous substance.

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