Disposition of Basal Insulin Peglispro Compared with 20-kDa Polyethylene Glycol in Rats Following a Single Intravenous or Subcutaneous Dose

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ABSTRACT

Basal insulin peglispro (BIL, LY2605541) is a novel, PEGylated insulin lispro that has a large hydrodynamic size (Beals et al., 2009). The compound has a covalently bonded 20-kDa polyethylene glycol (PEG) attached to insulin lispro through a urethane linkage on the lysine at position 28 in the B-chain (Moore, et al., 2012). More recently, 3H, 125I, and 14C have been used to assess tissue uptake of a 40-kDa PEG moiety attached to insulin (Elliott, et al., 2012a). NMR spectroscopy has been used to analyze the biologic fate of a PEGylated protein; however, a full mass balance could not be established with NMR spectroscopy, and these experiments could not tease apart the fates of the intact molecule and metabolites with PEG attached. There are reports of studies using immunoblotting with high-affinity antibodies, and is difficult to ionize. In addition, high-molecular-weight PEGs (>30 kDa) are refractory to mass spectrometry (MS) analyses because of their polydispersed nature (Wang et al., 2012a). NMR spectroscopy has been used to analyze the biologic fate of a PEGylated protein; however, a full mass balance could not be established with NMR spectroscopy, and these experiments could not tease apart the fates of the intact molecule and metabolites with PEG attached. There are reports of studies using immunoblotting with antibodies to PEG combined with NMR spectroscopy to assess the metabolic fate of the PEG moiety associated with PEGylated proteins. The PEG moieties used in currently available drugs range in size from a few hundred daltons to 40-kDa PEG, and some molecules have multiple PEG moieties attached (e.g., pegloticase has 40 10-kDa PEG moieties attached). The size of the PEG moiety has been reported to affect the elimination of the PEG (Webster et al., 2009); however, little information is available on how the drug or protein attached to the PEG moiety can affect the disposition of the different PEGs.

There are many PEGylated therapeutic compounds either in drug development or currently used in the pharmaceutical drug marketplace; however, little is known about the biologic fate of the PEG moiety associated with PEGylated proteins. The PEG moieties used in currently available drugs range in size from a few hundred daltons to 40-kDa PEG, and some molecules have multiple PEG moieties attached (e.g., pegloticase has 40 10-kDa PEG moieties attached). The size of the PEG moiety has been reported to affect the elimination of the PEG (Webster et al., 2009); however, little information is available on how the drug or protein attached to the PEG moiety can affect the disposition of the different PEGs.

Tracking the metabolic fate of the PEG moiety is challenging because PEG has no UV chromophore, is nonfluorescent, has few high-affinity antibodies, and is difficult to ionize. In addition, high-molecular-weight PEGs (>30 kDa) are refractory to mass spectrometry (MS) analyses because of their polydispersed nature (Wang et al., 2012a). NMR spectroscopy has been used to analyze the biologic fate of a PEGylated protein; however, a full mass balance could not be established with NMR spectroscopy, and these experiments could not tease apart the fates of the intact molecule and metabolites with PEG attached. There are reports of studies using immunoblotting with antibodies to PEG combined with NMR spectroscopy to assess the tissue uptake of a 40-kDa PEG moiety attached to insulin (Elliott et al., 2012). More recently, 3H, 125I, and 14C have been used to assess the disposition of PEGylated proteins such as 40-kDa branched

Introduction

Basal insulin peglispro (BIL, LY2605541) is a novel, PEGylated insulin lispro that has a large hydrodynamic size (Beals et al., 2009). The compound has a covalently bonded 20-kDa polyethylene glycol (PEG) attached to insulin lispro through a urethane linkage on the lysine at position 28 in the B-chain (Moore, et al., 2014). The hydrodynamic diameter is 7.8 ± 0.4 nm, a diameter 4 times larger than that of insulin lispro and analogous to the size of an ~75-kDa globular protein. In contrast, insulin lispro is a rapid-acting analog of insulin (Humalog, 1996) in which the amino acid proline at B28 has been replaced with lysine and the lysine at B29 has been replaced by proline, yielding a molecular weight of 5.8 kDa.

PEGylation of protein compounds in development as pharmaceutical agents has resulted in novel therapeutic agents with modified pharmacokinetic (PK) profiles that may improve their therapeutic potential (Webster et al., 2007, 2009; Fishburn, 2008). BIL has a prolonged duration of action compared with insulin lispro that is related to a delay in insulin absorption and a reduction in clearance. BIL is currently in phase 3 studies for the treatment of patients with type 1 or type 2 diabetes mellitus.

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ABBREVIATIONS: AUC, area under the curve; BIL, basal insulin peglispro; CL, clearance; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; ISF, in-source fragmentation; LC, liquid chromatography; LY2605541, basal insulin lispro; MS, mass spectrometry; PEG, polyethylene glycol; PK, pharmacokinetic; t1/2, half-life; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.
PEGylated [125I]BIL (Wang et al., 2012a), 2 × 20-kDa PEGylated [14C]peгинесastide (Woodburn et al., 2012, 2013), 46-kDa PEGylated [3H]neuraminidase-U receptor agonist (Zou et al., 2013), and 30-kDa PEGylated [125I]erythropoietin (Wang et al., 2012b).

For this report, we sought to characterize the disposition of BIL with a focus on the PEG component and compare those findings to the fate of unconjugated [14C]20-kDa PEG. The fates of the PEG and lispro portions of BIL were examined by analyzing the PK and elimination profiles of [14C]20-kDa PEG–labeled BIL and [125I]lispro-labeled BIL after subcutaneous or intravenous administration in rats. For comparison, the fate of unconjugated [14C]20-kDa PEG was similarly analyzed for PK and elimination profiles in rats in a separate study.

Materials and Methods

Materials. The [14C]-radiolabeled PEG test articles ([14C]20-kDa PEG and [14C]BIL) were synthesized by Analytical Bio-Chemistry Laboratories, Inc. (Columbia, MO); the BIL was labeled with 125I at Eli Lilly and Company (Lilly Research Laboratories, Indianapolis, IN). The 14C label was placed on carbons 5 through 8 on the 20-kDa PEG from the urethane bond in the BIL. Lactoperoxidase was used to iodinate the tyrosines in the lispro portion of BIL, and free iodine was removed from the preparation. The specific activity of the 14C-labeled BIL was 7.89 mCi/mg, the radioactivity was 97%, and the chemical purity was 95%–99% by high-performance liquid chromatography (HPLC). The specific activity of the 125I-labeled BIL was 0.23 μCi/mg, and the radioactivity was 99%. The 14C in the unconjugated PEG was on carbons 4 and 5. The specific activity of the 14C-unconjugated PEG was 4.8 mCi/mg, and the radioactivity was 99.5%. 14C-labeled BIL, two groups received unconjugated [14C]BIL, two groups received intravenous [14C]BIL, and two groups received subcutaneous [14C]BIL. For each dose paradigm, one of the two groups was used for PK sampling and one was used for mass balance determination.

In the study evaluating the biologic fate of unconjugated PEG, male rats were assigned to eight groups: two groups received intravenous [14C]BIL, two groups received subcutaneous [14C]BIL, two groups received intravenous [125I]BIL, and two groups received subcutaneous [125I]BIL. For each dose paradigm, one of the two groups was used for PK sampling and one was used for mass balance determination.

In the study evaluating the biologic fate of unconjugated PEG, male rats were assigned to four groups: two groups received the intravenous dose, and two groups received the subcutaneous dose. For each dose paradigm, one of the two groups was for PK sampling and one was for mass balance.

Dosing and Sample Collection. For both studies, the volume of radiola- beled dose formulation to be administered to each rat was calculated by using the rat’s body weight on the day of dose administration. The intravenous dose was administered via a tail vein, and the subcutaneous dose was administered in the intrascapular region. Rats were not fasted and had immediate access to food after dosing.

BIL doses were 0.5 mg/kg (19.4 nmol/kg) for the intravenous dose and 2 mg/kg (77 nmol/kg) for the subcutaneous dose. The vehicle for the BIL study was 16.5 mM Tris, 29 mM NaCl, 0.39 mM zinc oxide at pH 7.3 in sterile water for injection. The 14C radioactive doses were 4.35 mCi/kg for the intravenous dose and 18.1 μCi/kg for the subcutaneous dose. The 125I radioactive doses were 118 μCi/kg for the intravenous dose and 464 μCi/kg for the subcutaneous dose. Starting ~48 hours before dose administration through the day of dose administration, the rats in the [125I]BIL groups received water containing 20 mM sodium iodide ad libitum. In the unconjugated PEG study, the dose was 10 mg/kg (500 nmol/kg) i.v. and s.c. and the vehicle was phosphate-buffered saline. The dose was ~51 μCi/kg.

Blood for serum samples for the BIL study was collected via a jugular vein at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168, 192, 240, and 336 hours after dosing from four rats per time point for [125I]BIL and from six rats per time point for [14C]BIL. Each rat was sampled at three or four time points. Terminal blood samples were collected via cardiac puncture under isoflurane anesthesia. For [125I]BIL, serum from two rats from each time point was pooled to yield a total of three samples. In the unconjugated PEG study, blood for plasma was collected via a jugular vein before dosing and at 6, 24, 48, 72, 96, 120, 144, 168, 192, 240, 288, and 336 hours after dosing from all animals at each time point in the PK groups (n = 4 samples per time point). Urine was collected from 0 to 12 and 12 to 24 hours after dosing (BIL study only) and 24-hour intervals through 336 hours after dosing from four animals in each group. Feces were collected at 24-hour intervals through 336 hours after dosing from four animals in each group. Carcasses were collected at the end of 2 weeks.

Determination of Radioactivity. Concentrations of radioactivity in plasma, serum, urine, feces, cage rinse, cage wash, cage wipe, and residual carcass were determined using liquid scintillation spectrometry for 14C. Plasma, serum, and urine were counted directly by using Ultima Gold XR scintillation cocktail. Feces were homogenized with a 1:1 ethanol/water solvent, and aliquots of the homogenates were combusted using a Packard Instrument Company (Downers Grove, IL) Model 307 Sample Oxidizer; the resulting [14CO2 was trapped with a mixture of Permafluor and Carbo-Sorb. Carcasses were digested in 1 N NaOH with ethanol before counting.

Concentrations of 125I were determined in serum, urine, feces, cage rinse, cage wash, cage wipe, and residual carcass using solid scintillation spectrometry with a PerkinElmer Wallac 1480 Wizard. Urine samples from [125I]BIL-dosed rats were analyzed by solid scintillation spectrometry before and after trichloroacetic acid (TCA) precipitation of proteins. To precipitate the proteins in urine, a 1% solution of bovine serum albumin and then cold 50% TCA were added. Samples were mixed gently and then allowed to sit for ~30 minutes at 4°C. Cold 10% TCA was added, samples were centrifuged, and the supernatants were discarded. The pellets were analyzed by solid scintillation spectrometry. Feces were homogenized with 1:1 ethanol/water, and an aliquot was counted. The carcass was digested with 1 N NaOH until dissolved, and ethanol was added for homogenization.

Determination of BIL in Serum and Urine. The concentrations of immunoreactive BIL in serum and urine were determined by using a validated enzyme-linked immunosorbent assay (ELISA) at Charles River Laboratories (Senneville, QC, Canada). A purified guinea pig anti-human insulin antibody (Eli Lilly and Co.) was coated onto a black Nunc MaxiSorp plate (Thermo Fisher, Pittsburgh, PA), and I-Block (Life Technologies, Grand Island, NY) solution was used to block free sites. Samples were loaded into the wells of the plate, and BIL was allowed to bind to the immobilized insulin antibody. The bound complex was detected by addition of rabbit monoclonal anti–PEG–biotin antibody (Abcam, Cambridge, MA). Following a wash step, peroxidase-conjugated streptavidin was added to the plate and signal was produced by adding QuantaBlu substrate (Thermo Scientific, Waltham, MA). The fluorescence signal was then measured at an excitation wavelength of 320 nm and an emission wavelength of 420 nm. The concentration of the samples was extrapolated from a standard curve fitted with a 5-PL equation, with a weighting factor of 1/Y2. The concentrations of BIL in the calibration standards, quality control samples, and study samples were directly proportional to the fluorescence measured in the wells of the plate.

The quantitative range for the serum assay was 75–500 pM, and for urine it was 30–500 pM. Samples with higher concentrations were analyzed after dilution of the sample. The percentage relative error, a measure of accuracy, was <13% for serum and <5% for urine. The percentage relative S.D., a measure of precision, was <25% for both serum and urine assays.

Characterization of Serum and Urine PEG-Derived Products Using Liquid Chromatography–MS. The serum and urine samples of [14C]PEG-derived products after subcutaneous administration of BIL were assessed by using a modified liquid chromatography (LC)-MS method described by Liu et al. (2013) for characterizing PEG and its related products in biologic matrices. This method provided information regarding the identity of the products but was not used to quantitate the products. Serum samples and urine samples from rats in each group were each pooled by time point, and a 50-μl aliquot was mixed with 75 μl of 0.1% trifluoroacetic acid (TFA) in acetonitrile. After centrifugation, a 50-μl aliquot of the supernatant was mixed with 25 μl of...
Metabolic Fate of Basal Insulin Peglispro

0.1% formic acid in water. The sample was centrifuged at 4000 rpm for 20 minutes at 4°C, and the supernatant was injected onto the chromatographic system.

A combination of intact and in-source fragmentation (ISF) techniques was applied to the MS analyses. Intact mass of the PEGylated products was obtained using a PLRP-S column (100 x 0.3 mm) at 85°C with a binary gradient consisting of mobile phase A (0.1% TFA/water) and mobile phase B (0.1% TFA/water). The binary gradient was set up for a programmed elution profile of time, %B (0.00, 40) (2.00, 40) (2.05, 50) (16.0, 65) (16.05, 95) (17.00, 95) (17.05, 40) (22.00, 40) at a flow of 15 µl/min. The effluent from the chromatographic system was mixed with a delivery stream containing a secondary amine/water/acetonitrile to provide charge reduction. The neutralized effluent was connected to a Micromass Synapt Q-TOF mass spectrometer (Waters, Inc., Milford, MA) for detection in the positive ion mode. Post-column neutralization was used for analyses of intact PEGylated products.

ISF MS/MS confirmation of the PEGylated products was obtained by using a similar chromatographic system described above without the post-column neutralization. The chromatographic system was connected directly to the Micromass Synapt Q-TOF mass spectrometer for mass spectral detection in the positive-ion mode. The eluent from the LC was introduced into the Q-TOF system using an electrospray source.

For the post-column neutralization analyses and the ISF, data from the Q-TOF mass spectrometer were collected using MassLynx (version 4.1; Waters, Inc.) and MaxEnt1 (Waters, Inc.) deconvolution software.

Data Analyses. The key PK parameters reported for radioactivity in serum or plasma and for immunoreactive BIL in serum included maximum concentration (C_max), the time to reach maximum concentration (T_max), extrapolated concentration at time zero (C_0), half-life (t_1/2), area under the concentration-time curve from 0 to the last measurable time point (AUC_0-τ), area under the concentration-time curve from 0 to infinity (AUC_0-∞), volume of distribution (V_d), total body clearance (CL), and bioavailability ([AUC_0-∞,sys] / [AUC_0-∞,circ]). Noncompartmental PK parameters were calculated using WinNonlin Professional Edition, version 5.2 (Pharsight Corporation, Princeton, NJ). A composite concentration-time curve was used to calculate the PK parameters due to the sparse sampling paradigm, where there were samples from 3–4 animals per time point. S.D. values were calculated for the individual time points and are shown in the figures, but no further statistical analyses were conducted. Dose tables were compiled with mean and S.D. values calculated with Excel, version 11.0 (Microsoft Corporation, Redmond, WA). Radioanalysis data tables were generated by Debra, version 5.7.8.124 (LabLogic Systems Ltd., Sheffield, UK).

Results

Pharmacokinetics. Following intravenous administration, the serum CL of immunoreactive BIL was ~2.4-fold greater than the CL of 14C radioequivalents and 1.6-fold greater than the CL of 125I radioequivalents (Table 1). The CLs of immunoreactive BIL after 14C dosing and 125I dosing were similar, although the t_1/2 values appeared to differ. Immunoreactive BIL was quantifiable longer after 125I administration and so appears to have a longer elimination phase. The mean V_d of immunoreactive BIL was ~2.4-fold lower than the V_d of total 125I radioactivity in serum and 47-fold lower than that of 14C radioactivity in serum.

Following subcutaneous administration of 14C-BIL, radioactivity was rapidly absorbed, with the same mean T_max values for both total radioactivity and immunoreactive BIL. AUCs for immunoreactive BIL were 41% and 16% of those for circulating 14C after intravenous and subcutaneous dosing, respectively (Fig. 1; Table 1). AUCs for immunoreactive BIL were 63% and 31% of those for circulating 125I after intravenous and subcutaneous dosing, respectively. The subcutaneous bioavailability of BIL was 23%–29%, and it was determined on the basis of comparison of the AUC of 14C radioactivity after intravenous and subcutaneous administration that ~75% of the dose was absorbed. After subcutaneous dosing, the AUC of 14C was 1.75-fold greater than the AUC of 125I, indicating that during catabolism the protein portion with the 125I- and the 14C-containing PEG moiety was cleaved. After subcutaneous administration of both 14C[BIL] and 125I[BIL], the molecule appeared to be extensively catabolized; some of the catabolism was most likely to have occurred before reaching the systemic circulation. The t_1/2 of 14C was 4.4-fold longer than that of 125I, whereas the t_1/2 of immunoreactive BIL was longer for the 125I group than for the 14C group, as observed after intravenous administration.

In the unconjugated PK study, following the subcutaneous dose, the highest mean concentrations of radioactivity in plasma were at 10.5 hours after dosing (Fig. 2; Table 1). Of note is that the first sample was not collected until 6 hours after dosing in this study. After subcutaneous and intravenous administration, radioactivity was eliminated from plasma with a mean t_1/2 of 202 ± 27 hours and 165 ± 8 hours, respectively, which was similar to that for 14C after administration of either intravenous or subcutaneous [14C]BIL. The CLs of 14C radioactivity for unconjugated PEG and PEG from BIL were similar following intravenous dosing. The relative subcutaneous bioavailability of [14C]20-kDa PEG in rats was calculated to be ~78%, which was higher than the bioavailability of immunoreactive BIL but similar to the percentage of the [14C]BIL dose absorbed.

Excretion. Excretion of radioactivity was generally similar after subcutaneous or intravenous dosing of [14C]BIL, although a slightly

TABLE 1

<table>
<thead>
<tr>
<th>Route</th>
<th>14C[BIL] SC</th>
<th>125I[BIL] SC</th>
<th>14C[20-kDa PEG] SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>2 mg/kg (77 nmol/kg)</td>
<td>0.5 mg/kg (19.4 nmol/kg)</td>
<td>2 mg/kg (77 nmol/kg)</td>
</tr>
<tr>
<td>Analyte</td>
<td>14C BIL</td>
<td>14C BIL</td>
<td>125I BIL</td>
</tr>
<tr>
<td>C_0 (nM or nM eq)</td>
<td>102</td>
<td>95</td>
<td>550</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>1</td>
<td>1</td>
<td>N.A.</td>
</tr>
<tr>
<td>t_1/2 (h)</td>
<td>197</td>
<td>6</td>
<td>191</td>
</tr>
<tr>
<td>MEC_{C_{0}} (nM eq-h or nM-h)</td>
<td>4530</td>
<td>930</td>
<td>1680</td>
</tr>
<tr>
<td>CL (l/h per kg)</td>
<td>N.A.</td>
<td>0.00978</td>
<td>0.0239</td>
</tr>
<tr>
<td>V_d (l/kg)</td>
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<td>1.43</td>
<td>0.030</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>75</td>
<td>29</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Route</th>
<th>14C[BIL] IV</th>
<th>125I[BIL] IV</th>
<th>14C[20-kDa PEG] IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>2 mg/kg (77 nmol/kg)</td>
<td>0.5 mg/kg (19.4 nmol/kg)</td>
<td>10 mg/kg (500 nmol/kg)</td>
</tr>
<tr>
<td>Analyte</td>
<td>14C BIL</td>
<td>125I BIL</td>
<td>14C BIL</td>
</tr>
<tr>
<td>1/2s</td>
<td>0.5</td>
<td>0.0117</td>
<td>0.0127</td>
</tr>
<tr>
<td>1/2s</td>
<td>1.14</td>
<td>0.073</td>
<td>0.073</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>78</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

eq. equivalents of [14C]BIL or [125I]BIL: representing any molecules containing the radiolabeled 14C and 125I, although the exact identity is unknown; IV, intravenous; N.A., not applicable; SC, subcutaneous.

*Represents immunoreactive BIL.

aThe last plasma sampling time was <2 t_1/2; therefore, the estimated mean elimination t_1/2 should be interpreted with caution.
greater percentage of the dose was recovered in feces after intravenous administration than after subcutaneous administration of the [14C]BIL (Fig. 3). 14C radioactivity was eliminated slowly in both urine and feces and was still being eliminated in both urine and feces at the end of the 2-week sample collection period after the intravenous and subcutaneous doses. Because sample collection was terminated after 2 weeks (<5 elimination t1/2 for 14C in serum), between 32% and 40% of the 14C dose was found either in the carcass or in the cage rinses/washes. 125I radioactivity was eliminated rapidly; after intravenous and subcutaneous administration of 2 mg/kg [14C]BIL or [125I]BIL (A) and intravenous administration of 0.5 mg/kg [14C]BIL or [125I]BIL (B) to male rats. Radioactivity was determined by solid (125I) or liquid (14C) scintillation spectrometry, and BIL was determined by ELISA.

Immunoreactive BIL in urine accounted for 11% of the intravenous dose of [14C]BIL and 2.5% of the subcutaneous dose of [14C]BIL. The mean percentages of TCA-precipitable radioactivity in urine after dosing with [125I]BIL ranged from 7.24% to 29.8%, indicating that radioactivity in urine was primarily associated with small peptide fragments or free iodine, rather than with labeled intact BIL or tyrosine-containing peptides attached to the 20-kDa PEG moiety.
For the unconjugated PEG study, urinary excretion was the primary route of elimination for $^{14}$C (Fig. 4). Some radioactivity was still being excreted 2 weeks after dosing, although the majority of the elimination occurred within the first 72 hours after dosing. Sample collection was terminated after 2 weeks, which was <5 plasma elimination $t_1/2S$. At the end of the sample collection period, 62%–86% of the $^{12}$C dose of unconjugated PEG had been recovered in urine and feces.

A summary of intravenous and subcutaneous excretion after dosing of $^{[14C]}$20-kDa PEG, $^{[14C]}$PEG-BIL, or $^{[125I]}$lispro-labeled BIL is shown in Fig. 5.

**Catabolism Products.** The catabolism products of BIL in serum and urine were characterized following subcutaneous administration by using MS. BIL was identified using the specific ion trace of 1821.53 amu corresponding to the A-chain + the B-chain B27 ion in the ISF MS/MS spectra. PEG-lysine is 20-kDa PEG covalently linked to lysine and was a potential catabolism product of BIL. Standards of BIL and PEG-Lys were used to establish retention times and spectral differentiation. Urine sample analyses showed that BIL was detectable only in the 0- to 12-hour urine samples even though it was quantifiable at additional time points using the ELISA. This difference was because of the higher detection limit in the MS analyses (15.5 nM) than in the ELISA (30 pM).

In addition to BIL, a second peak at 12.5 minutes was detected in the urine samples through 240 hours. Figure 6 shows zoomed spectra for the 12.5-minute PEGylated peak compared with BIL, PEG-Lys, and 20-kDa PEG. The spectra show that the peak corresponds to PEG-Lys. The intact spectra confirmed the assignment, although the MS/MS spectra of the product were insufficient. No other catabolism product was observed in the urine samples.

Serum samples analyzed by intact analyses showed that BIL was detected at 9–10 minutes but only through 12 hours. BIL was detectable in serum using the ELISA for much longer times. The lower limit of quantitation for the serum ELISA was 75 pM and that for the ISF/MS spectra. PEG-lysine was 20-kDa PEG covalently linked to lysine and was a potential catabolism product of BIL. Standards of BIL and PEG-Lys were used to establish retention times and spectral differentiation. Urine sample analyses showed that BIL was detectable only in the 0- to 12-hour urine samples even though it was quantifiable at additional time points using the ELISA. This difference was because of the higher detection limit in the MS analyses (15.5 nM) than in the ELISA (30 pM).

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Exploratory size-exclusion chromatography of urine from the rats receiving unconjugated $^{[14C]}$PEG indicated that the 20-kDa PEG remained intact, similarly to after BIL dosing (data on file, Eli Lilly and Company).

**Discussion**

BIL is a novel, PEGylated insulin lispro that has a large hydrodynamic size. It has a prolonged duration of action that is related to a delay in insulin lispro absorption and a reduction in CL (Sinha et al., 2014). The fate of therapeutic proteins is generally thought to be via catabolism to small peptides and amino acids, but the biologic fate of PEGylated proteins has not been well studied because of the difficulties in quantifying PEG. The fate of a 40-kDa PEG attached to insulin has been determined by using immunoblotting with antibodies to PEG combined with NMR spectroscopy (Elliott et al., 2012), and the tissue uptake of 40-kDa branched PEG on Adnectin was determined by using a $^{14}$C-labeled cross-linker on 40-kDa branched PEGylated Adnectin (Wang et al., 2012a). The disposition of $^{[14C]}$PEGylinesatisse, a 2 x 20-kDa PEGylated erythropoietin, after intravenous administration to rats and monkeys has been reported (Woodburn et al., 2012, 2013); renal excretion of the parent molecule was the primary route of elimination. Zou et al. (2013) reported that for a 46-kDa PEGylated $[^{3}H]$neuromedin-U receptor agonist the primary route of elimination in both rats and dogs was urinary excretion of the parent molecule, although recovery of radiolabeled PEG ranged from 38%–80%.

In our studies, we administered $^{[14C]}$PEGG-labeled BIL and $^{[125I]}$lispro-labeled BIL to assess the biologic fate of both the protein and 20-kDa PEG portions of BIL. We also determined the PK of the intact molecule (immunoreactive BIL) with a validated ELISA. By tracking the intact molecule using ELISA, the PEG moiety and its products using $^{14}$C, and the protein moiety and its products using $^{125I}$, in addition to MS for PEG products, we were able to determine the disposition of BIL and to determine its catabolism products. Finally, we compared the biologic fate of $^{14}$C-labeled unconjugated 20-kDa PEG with $^{[14C]}$PEG-labeled BIL to assess how the disposition of 20-kDa PEG is influenced by the attached protein.

Following administration of labeled BIL, greater systemic exposure and longer $t_{1/2}$ were observed for $^{14}$C than for $^{125I}$, which indicates that the $^{125I}$ or the protein portion (lispro) of BIL (containing the labeled tyrosine residues) was cleaved from the PEG moiety. MS indicated that the 20-kDa PEG primarily retained a lysine. The resulting PEG-lysine product was cleaved more slowly than the protein portion of BIL. The $t_{1/2}$ of $^{14}$C after BIL dosing was similar to the $t_{1/2}$ of $^{125I}$ after unconjugated PEG dosing. The 23%–29% bioavailability of radiolabeled BIL and the >75% absorption of $^{14}$C indicate extensive catabolism of BIL. This is also shown by the low percentage of the circulating $^{125I}$ and $^{14}$C that is present as intact BIL. Therapeutic biologic drugs are generally eliminated via catabolism to smaller peptides and amino acids, but little information is available on whether this occurs presystemically or after absorption. In the case of BIL, it appears that catabolism is at least partially presystemic in rats; similar results have been reported for PEGylated erythropoietin in rats (Wang et al., 2012b). The presystemic catabolism in rats appears to be higher than in humans; the subcutaneous bioavailability of BIL in humans was reported to be 75% (Sinha et al., 2014).

After administration of $^{[14C]}$BIL, the radioactivity recovered in the urine was primarily PEG-lysine, indicating that the 20-kDa PEG moiety did not undergo catabolism and that the catabolism was primarily via proteolysis on the protein moiety in BIL. Through a comparison of circulating BIL, $^{125I}$, and $^{14}$C, PEG-lysine also appeared to be a major contributor to the circulating radioactivity, although this could not be confirmed with MS because of the high MS detection level of PEGylated products in serum. Unconjugated 20-kDa PEG also did not appear to undergo catabolism but was excreted in urine primarily unchanged (data on file, Eli Lilly and Company). These data are

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**Fig. 4.** Cumulative excretion as percentage of dosed radioactivity (mean ± S.D.; n = 4 samples per group) after subcutaneous and intravenous administration of 10 mg/kg $^{[14C]}$20-kDa PEG. Radioactivity was determined using liquid scintillation spectrometry.
consistent with the current literature showing that the metabolic CL of PEGs with a molecular weight of >5000 is insignificant (Fishburn, 2008). This appears to be the case whether the PEG is unconjugated or conjugated to a protein such as lispro. Thus, the $^{14}$C can be considered to represent the “total PEG” and is the intact BIL and all of the PEG-related products that retain the $^{14}$C label, including PEG-lysine.

Following subcutaneous or intravenous dosing of $[^{14}$C]$20$-kDa PEG or $[^{14}$C]BIL, radioactivity declined over time but was still measurable for $\sim 40$ days to obtain full recovery in excreta based on 5 elimination $t_{1/2}$s.

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**Fig. 5.** Comparison of the elimination of radioactivity following administration of $[^{14}$C]$20$-kDa PEG, $[^{14}$C]BIL, or $[^{125}$I]BIL. “Other” includes the residual radioactivity in the carcass at the end of the 2 weeks. Samples were collected for 2 weeks, but urine and feces would need to be collected for $\sim 40$ days to obtain full recovery in excreta based on 5 elimination $t_{1/2}$s.

**Fig. 6.** Summed ($\sim 40$ scans) and aligned (vertical lines) positive-ion full scan spectra for 24- to 48-hour urine sample (A), 20-kDa PEG-Lys standard (B), 20-kDa PEG standard (C), and BIL standard (D). The LC eluent was introduced into the Q-TOF using an electrospray source. The region of $m/z = 5190–5280$ captures the most abundant charge states for BIL.
were important routes of elimination for [14C]BIL-related radioactivity as not catabolized (Kiselyov et al., 2014). Fecal and urinary excretion shown that the 20-kDa PEG in BIL is recycled out of the cells and is not expected to show that it had been catabolized. In vitro experiments have also reported recovery of 35%–47% of the PEG dose in urine after 28 days. These studies and ours demonstrate the difficulty of obtaining complete recovery of the dose when PEGylated molecules are administered.

The dose of the unconjugated 20-kDa PEG was higher than the PEG dose in BIL due to the differences in specific activity of the dose and the difficulty in measuring unconjugated PEG. The use of a higher dose of unconjugated PEG did not appear to alter the elimination of the unconjugated 20-kDa PEG from being primarily urinary. As indicated in the literature, urinary excretion has been reported to be the major elimination route for PEG moieties of <50 kDa (Yamaoka et al., 1994; Webster et al., 2007). In contrast, when the 20-kDa PEG was covalently attached to lispro in BIL, the PEG appeared to be excreted about equally in urine and feces, even though examination of circulating and urinary products did not seem to show that it had been catabolized. In vitro experiments have also shown that the 20-kDa PEG in BIL is recycled out of the cells and is not catabolized (Kiselyov et al., 2014). Fecal and urinary excretion were important routes of elimination for [14C]BIL-related radioactivity after a single subcutaneous or single intravenous dose in rats. These differences between the excretion of PEG administered alone or as part of BIL indicate that the biologic fate of the PEG is directed, at least in part, by the insulin-receptor interactions and subsequent intracellular protein degradation that occur with BIL but not with unconjugated PEG.

Mass balance studies in humans with [14C]-radiolabeled BIL are not feasible because only low doses (typically <9 nmol/kg) of BIL could be administered without hypoglycemia occurring and because high specific activity would be necessary for detection of radioactivity at such low doses. The dose of 2 mg/kg (77 nmol/kg or 8.6 U/kg) BIL s. c. chosen for the rats was the high dose that was used in toxicology studies and was much higher than the doses that are administered therapeutically. The higher dose in the rats was a dose used in the 6-month toxicology studies demonstrating dose linearity in the exposure of basal insulin peglispro (data on file, Eli Lilly and Company). The excretion observed in the rats is expected to be predictive of the excretion in humans; therefore, in humans, the PEG in BIL would be expected to be eliminated via urine and feces. There are limited data in the literature regarding the excretion profiles of PEG molecules in humans; however, it has been hypothesized that the size of the PEG moiety determines the elimination route, i.e., via urine and/or feces (Webster, et al., 2007). This study in rats demonstrates that the size of the PEG is the only determinant of the excretion and that the protein attached to the PEG moiety may play a more important role in the elimination. Thus, to understand the biologic fate/elimination of PEG in a given PEGylated biopharmaceutical agent, studies specific to the molecule are needed because the polypeptide component may influence the balance between the routes of excretion.

Together, the data suggest that elimination of PEG derived from BIL in rats is by both biliary and renal routes. Although studies with radiolabeled BIL are not feasible in humans, we would anticipate a similar observation: that PEG in BIL has two routes of elimination, nonrenal and renal, and that the lispro portion of BIL would be eliminated renally as small peptides and/or amino acids.

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