

# In Vivo and In Vitro Characterization of Basal Insulin Peglispro: A Novel Insulin Analog<sup>§</sup>

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## ABSTRACT

The aim of this research was to characterize the in vivo and in vitro properties of basal insulin peglispro (BIL), a new basal insulin, wherein insulin lispro was derivatized through the covalent and site-specific attachment of a 20-kDa polyethylene-glycol (PEG; specifically, methoxy-terminated) moiety to lysine B28. Addition of the PEG moiety increased the hydrodynamic size of the insulin lispro molecule. Studies show there is a prolonged duration of action and a reduction in clearance. Given the different physical properties of BIL, it was also important to assess the metabolic and mitogenic activity of the molecule. Streptozotocin (STZ)-treated diabetic rats were used to study the pharmacokinetic and pharmacodynamic characteristics of BIL. Binding affinity and functional characterization of BIL were compared with those of several therapeutic insulins, insulin AspB10, and insulin-like

growth factor 1 (IGF-1). BIL exhibited a markedly longer time to maximum concentration after subcutaneous injection, a greater area under the concentration-time curve, and a longer duration of action in the STZ-treated diabetic rat than insulin lispro. BIL exhibited reduced binding affinity and functional potency as compared with insulin lispro and demonstrated greater selectivity for the human insulin receptor (hIR) as compared with the human insulin-like growth factor 1 receptor. Furthermore, BIL showed a more rapid rate of dephosphorylation following maximal hIR stimulation, and reduced mitogenic potential in an IGF-1 receptor-dominant cellular model. PEGylation of insulin lispro with a 20-kDa PEG moiety at lysine B28 alters the absorption, clearance, distribution, and activity profile receptor, but does not alter its selectivity and full agonist receptor properties.

## Introduction

The normal human pancreas secretes approximately 1 unit of insulin (0.035 mg) per hour to maintain basal glycemic control in the fasting state (Waldhausl et al., 1979). Adequate basal insulin is critical because it regulates hepatic glucose output, which is essential for proper maintenance of glucose homeostasis during the diurnal cycle. When insulin is released from the pancreas, it flows first to the liver via the portal vein, where insulin receptor-mediated clearance results in approximately 50% reduction of the levels exiting the liver (Herring et al., 2014); therefore, under normal physiology, the exposure level of endogenously secreted insulin is greater in the liver than at peripheral tissues (Waldhausl et al., 1979). When therapeutic doses of insulin are administered subcutaneously, peripheral tissues are exposed to the exogenously delivered

insulin before extraction by the liver, which results in equal exposure of insulin in the liver and periphery (Hordern et al., 2005). This loss of the normal insulin gradient with current insulin therapeutics results in relative peripheral hyperinsulinemia with underinsulinization of the liver (Logtenberg et al., 2009).

Over the past 75 years, differing strategies have provided insulin therapies for patients with diabetes. The design of the early basal insulin analogs was directed primarily at modifying the patterns of absorption from a depot after an s.c. injection. The suspension-based basal insulin formulations—for example, neutral protamine Hagedorn (NPH) insulin, protamine zinc insulin, lente, and ultralente—were designed to delay the release of insulin from the injection site and thus prolong glucose-lowering activity (Krayenbühl and Rosenberg, 1946; Hallas-Møller, 1956; Hagedorn et al., 1984). However, these formulations were suboptimal as once-daily basal insulin replacements due to: 1) incomplete and inadequate duration of action (less than 24-hour coverage) (Bolli et al., 2011), 2) variable absorption, and 3) pronounced variation in the pharmacodynamic (PD) peaks.

Novel insulin analogs have been developed that improved basal characteristics. First-generation insulin analogs, such

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**ABBREVIATIONS:** BHI, biosynthetic human insulin; BIL, basal insulin peglispro; BSA, bovine serum albumin; hIGF-1R, human insulin-like growth factor 1 receptor; hIR, human insulin receptor; hIR-A, human insulin receptor, isoform A; hIR-B, human insulin receptor, isoform B; IGF-1, insulin-like growth factor 1;  $K_i$ , inhibitory binding constant; NPH, neutral protamine Hagedorn; PBS, phosphate-buffered saline; PD, pharmacodynamics; PEG, polyethylene-glycol; PK, pharmacokinetics; STZ, streptozotocin; T2DM, type 2 diabetes mellitus.

as insulin glargine (Wang et al., 2003) and insulin detemir (Havelund et al., 2004), extended the duration of action sufficiently to permit once-daily dosing for many patients (Lepore et al., 2000; Porcellati et al., 2007). However, the *in vivo* properties of both of these modified insulins still possessed limitations. The inpatient PD variability for insulin glargine was better than that of NPH, but still remained high, and the duration of action for insulin detemir was generally considered less than 24 hours (Lepore et al., 2000; Porcellati et al., 2007; Vora and Heise, 2013). Consequently, new generations of basal insulin analogs are in development, with the goals of prolonging the duration of action and reducing inpatient variability.

Insulin degludec (Jonassen et al., 2012), which contains a fatty-acid modification similar to that in insulin detemir, offers both reduced inpatient variability (Heise et al., 2012) and a longer duration of action (Heise et al., 2011) than that of insulin glargine. The noncovalent association of insulin detemir and insulin degludec with serum albumin alters the activity, disposition, and clearance of these insulins as compared with other exogenously administered insulins (Rabkin et al., 1970; Ferrannini et al., 1983; Henriksen et al., 1987). However, the low-affinity agonism of insulin detemir and insulin degludec, coupled with albumin binding (Nishimura et al., 2010; Sorensen et al., 2010), necessitates higher plasma concentrations to achieve *in vivo* potency comparable to that of NPH (Brunner et al., 2000). Insulin detemir has shown a slightly increased hepatic versus peripheral activity (Hordern et al., 2005); however, no data on hepato-preferential activity with insulin degludec have been reported.

Basal insulin peglispro (BIL), an insulin lispro molecule having a single 20-kDa polyethylene-glycol (PEG) moiety covalently attached to the epsilon amine of lysine at position B<sub>28</sub> of the insulin lispro molecule, represents a new generation of basal insulin (Hansen et al., 2012b). BIL showed lower inpatient PD variability, a lower PD peak profile at steady state, and an increased duration of action compared to insulin glargine (Bergental et al., 2014; Sinha et al., 2014a,b). Recent analysis has shown that the addition of the 20-kDa PEG moiety makes the hydrodynamic diameter of BIL similar to that observed for human serum albumin, a molecule with a molecular weight too large to be readily cleared by the kidneys (Hansen et al., 2012b). Recent studies demonstrated that BIL has limited diffusion across the continuous capillary endothelial cells of the periphery, decreased renal clearance, and displays a hepato-preferential action (Henry et al., 2014; Moore et al., 2014). This hepato-preferential activity may account, in part, for the weight loss observed in patients previously treated with alternative insulin therapies that create hyperinsulinemia in the periphery (Jacoher et al., 2014).

This report describes the pharmacokinetic (PK) and PD characteristics of BIL and compares them with those of insulin lispro in the insulin-deficient streptozotocin (STZ)-treated diabetic rat model. In addition, an assessment of the *in vitro* pharmacological properties of BIL was compared with the properties of insulin-like growth factor 1 (IGF-1), biosynthetic human insulin (BHI), and other insulin analogs (insulin lispro, insulin AspB10, insulin glargine, and insulin detemir).

## Materials and Methods

**Materials.** BIL, BHI, insulin lispro, and insulin AspB10 were prepared at Eli Lilly and Company (Indianapolis, IN). BHI, insulin lispro, and BIL were prepared at 100 IU containing water, 16 mg of glycerine, 1.88 mg of dibasic sodium phosphate, 3.15 mg of m-cresol, and 0.0197 mg of zinc ion per 1 ml of pH 7.4 solution and stored at 4°C. IGF-1 (PeproTech, Rocky Hill, NJ) and AspB10 were prepared at 1 mg/ml in phosphate-buffered saline (PBS), pH 7.4. Insulin detemir (Levemir; Novo Nordisk A/S, Copenhagen, Denmark; Novo Nordisk, 2013) and insulin glargine (Lantus; Sanofi-Aventis, Paris, France; Sanofi-Aventis, 2013) were 100 IU/ml in their commercial formulation.

**PK and PD Studies in STZ-Treated Diabetic Rats.** *In vivo* data represent data obtained across multiple studies in male Sprague-Dawley rats. Rats were rendered diabetic by a single intravenous injection of 40–45 mg/kg STZ (Sigma-Aldrich, St. Louis, MO or Teva Parenteral Medicines, Irvine, CA). After approximately 3 days, fed blood glucose levels were determined, and rats were randomized by body weight and blood glucose. All rats had predose blood glucose levels >345 mg/dl. Compounds or placebos used in the studies were administered subcutaneously, with animals receiving water and food *ad libitum*. Blood samples were collected for measurement of compound by immunoassay and blood glucose by glucometer or Hitachi Clinical Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN). PK parameters were calculated using standard noncompartmental analysis techniques. Animals were maintained in accordance with the Institutional Animal Care and Use Committee of Eli Lilly and Company and the National Institutes of Health Guide for the Use and Care of Laboratory Animals.

**Binding to Human Insulin Receptor and Human Insulin-Like Growth Factor 1 Receptor.** The inhibitory binding constant ( $K_i$ ) was determined using crude plasma membranes prepared from stably transfected human embryonic kidney-293 cells overexpressing human insulin receptor (hIR) isoform A (hIR-A), hIR isoform B (hIR-B) containing a C-terminal C9 epitope (Hodges et al., 1988), or human IGF-1 receptor (hIGF-1R). The scintillation proximity assay uses either (3-[<sup>125</sup>I]iodotyrosyl-A14)-insulin or [<sup>125</sup>I]IGF-1 and is configured as previously published (Kohn et al., 2007). Receptor-bound radioactivity was quantified using a MicroBeta TriLux scintillation counter (PerkinElmer, Waltham, MA).

**hIR Phosphorylation Assay.** Stably transfected human embryonic kidney-293 cells, overexpressing either hIR-A or hIR-B, both containing a C9 epitope (Hodges et al., 1988), were plated in growth medium (see Supplemental Material) on poly-D-lysine-coated 96-well plates. After 24 hours, the cells were treated overnight in serum-free growth medium supplemented with 0.1% fraction V–fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich), and then test samples were added. Stimulation was stopped by rinsing with ice-cold PBS and lysing with ice-cold NP40 buffer (see Supplemental Material). Tyrosine phosphorylation was determined using a sandwich enzyme-linked immunosorbent assay format capturing with an anti-C9 monoclonal antibody (Oprian et al., 1987) and detecting with the antiphosphotyrosine monoclonal 4G10–horseradish peroxidase conjugate (EMD Millipore, Billerica, MA) with the addition of 3,3',5,5'-tetramethylbenzidine (Thermo Scientific, Rockford, IL). The absorbance values were normalized by the response to a maximally efficacious dose of BHI (100 nM).

**Metabolic Potential Assay.** Murine 3T3-L1 fibroblasts (CL-173; American Type Culture Collection, Manassas, VA; American Type Culture Collection, 2011) were differentiated into adipocytes as described by the supplier (by using modified concentrations of 5 µg/ml insulin and 0.5 µM dexamethasone). Differentiated murine 3T3-L1 adipocytes were serum-starved overnight at 37°C. Cells were treated with test samples in Krebs-Ringer buffer supplemented with 10 µM glucose and 0.05% BSA in the presence of 0.1 µCi/well D-(U-<sup>14</sup>C)-glucose (PerkinElmer, Waltham, MA). After 4 hours, the adipocytes were rinsed and lysed in 0.05 ml of 0.5% Triton X-100 and shaken at

600 rpm for 5 minutes. The newly synthesized triglycerides were extracted by adding 0.05 ml of *sec*-butanol and 0.1 ml of MicroScint-E (PerkinElmer, Waltham, MA), followed by overnight incubation. The radioactivity incorporated into the triglycerides was quantified using a MicroBeta TriLux scintillation counter and expressed as counts per minute. Values were normalized by the response of a maximally efficacious dose of BHI (100 nM).

**Mitogenic Potential Assay.** The human osteosarcoma cell line, SAOS-2, (HTB-85; American Type Culture Collection) was plated at 40,000 cells/well in CytoStar T microplates (PerkinElmer) for 72 hours in growth medium (see Supplemental Material). The following morning, cells were rinsed and incubated with serum-free growth medium supplemented with 0.1% BSA for 6 hours before the test samples were added. After 20 hours, 1  $\mu$ Ci/well methyl- $^{3}$ H]thymidine (PerkinElmer) was added, and the amount of radioactivity incorporated into the cellular DNA was determined 4 hours later using a MicroBeta TriLux scintillation counter and expressed as counts per minute. Values were normalized by the response to a maximally efficacious dose of BHI (1000 nM).

**hIR Dephosphorylation Assay.** To follow the dephosphorylation kinetics of hIR, the same cell lines and protocol used in the hIR phosphorylation assay were followed, except that the cells were stimulated for 30 minutes with maximally efficacious doses of the insulin analogs (100 nM insulin lispro, 100 nM insulin AspB10, 1000 nM insulin glargine, and 1000 nM BIL). Dephosphorylation was started after two media exchanges into serum-free growth medium supplemented with 0.1% BSA. At defined time points, cells were rinsed with ice-cold PBS and lysed with NP40 lysis buffer, and enzyme-linked immunosorbent assay analysis was performed as described earlier. Values were normalized as a percentage of the mean starting (pre-wash) values for all control wells using a maximally efficacious dose of insulin (100 nM).

**Data Analysis for In Vitro Activity.** IC<sub>50</sub> or EC<sub>50</sub> values were determined from a four-parameter logistic nonlinear regression analysis (see Supplemental Material). Functional assay results were reported as EC<sub>50</sub> values. All results are reported as the geometric mean  $\pm$  S.E.M.

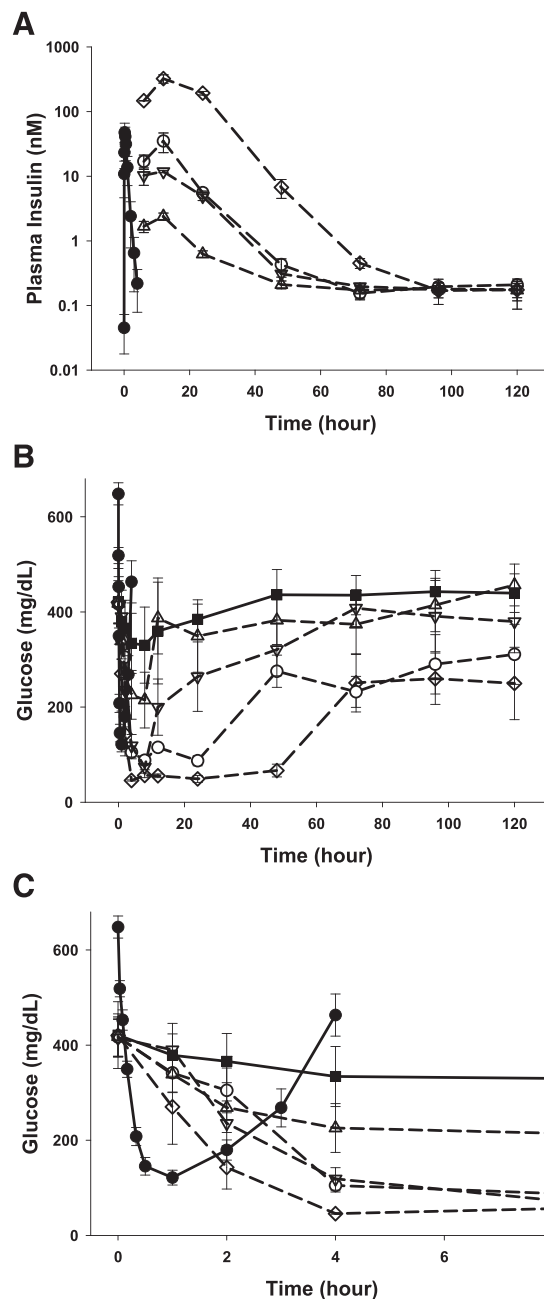
Analysis of the dephosphorylation results was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC), using a mixed model including fixed treatment group, time, and treatment group-by-time interaction effects and random plate effects. Treatments were compared at each time point by model-based *t* tests.

## Results

### PK and PD Profiles in STZ-Treated Diabetic Rats.

BIL and insulin lispro were compared for PK (Fig. 1A) and PD (Fig. 1, B and C) characteristics in rats rendered diabetic by treatment with STZ. BIL exhibited a clear, protracted time-action profile relative to insulin lispro, and the response was dose-dependent, as exemplified in the PK and PD profiles. In fact, the insulin lispro PK profile was nearly complete at the first time point recorded for BIL (Fig. 1A). Analysis of the PK data reveals that BIL demonstrated a 13-fold decrease in clearance and a 10-fold increase in area under the concentration-time curve, as well as a substantial increase (24.5-fold) in the time to maximum concentration, suggesting that the rate of absorption as well as clearance was reduced relative to insulin lispro (Table 1).

**Binding Affinity for hIR and hIGF-1R Selectivity.** All insulin test articles demonstrated comparable *K<sub>i</sub>* for hIR-A, the ubiquitously expressed isoform, and hIR-B, the isoform predominantly expressed in the liver (Table 2). Unlike the insulins, hIGF-1 showed an approximately 15-fold greater binding affinity (lower *K<sub>i</sub>* value) for hIR-A than for hIR-B.



**Fig. 1.** The protracted time-action profiles of BIL relative to insulin lispro. (A) Pharmacokinetic profiles of insulin lispro (black circles, *n* = 15) dosed at 50 nmol/kg and BIL (white triangles, *n* = 4 per group) dosed at 9.4 nmol/kg, 56.8 nmol/kg (white inverted triangles), 94 nmol/kg (white circles), and 568 nmol/kg (white diamonds) for STZ-treated diabetic rats given a single s.c. dose of the test articles. Data represent the mean  $\pm$  S.D. (B) Glucose-lowering profiles from the same experiment as in (A) for vehicle control (black squares, *n* = 4), insulin lispro, and BIL. (C) Glucose-lowering profiles for the first 8 hours of the glucose profiles from (B). Glucose data are presented as the mean  $\pm$  S.E.M.

Compared with insulin lispro, the binding affinities of insulin detemir for hIR-A and hIR-B were reduced approximately 8-fold and 7-fold, respectively, whereas the binding affinities of BIL were reduced approximately 15-fold and 17-fold for hIR-A and hIR-B, respectively (see Supplemental Fig. 1, A and B for binding curves).

Results in Table 2 show that each of the insulin test samples exhibited lower binding affinity for hIGF-1R than for hIR.

TABLE 1

Pharmacokinetic parameters of BIL and insulin lispro after s.c. injection in streptozotocin-treated rats

Test Sample	$T_{max}$ <i>h</i>	$AUC_{0-\infty}$ <i>nM<sup>a</sup>h</i>	CL/F <i>l/h/kg</i>
Insulin lispro <sup>a</sup>	0.4 (0.2)	25 (15)	3.0 (1.8)
BIL <sup>a</sup>	9.8 (1.1)	255 (37)	0.23 (0.04)
BIL/insulin lispro, mean	24.5	10.2	0.08

$AUC_{0-\infty}$ , area under the concentration-time curve from time zero to infinity; CL/F, apparent clearance;  $T_{max}$ , maximum concentration.

<sup>a</sup>Values for insulin lispro and BIL represent the mean (S.D.) across multiple studies that used similar dose levels (approximately 50 nmol/kg).

Compared with insulin lispro, BIL showed an 86-fold reduced binding affinity for hIGF-1R (see Supplemental Fig. 1C for binding curves). Insulin lispro and BIL were 238-fold and 1351-fold more selective for hIR-A binding than for hIGF-1R binding, respectively, as assessed by the ratio of the  $K_i$  values for each receptor. Overall, the hIR-A selectivity of BIL was approximately 6-fold, 37-fold, and 9-fold greater than that of insulin lispro, insulin glargine, and insulin detemir, respectively. Since equivalent binding to hIR-A and hIR-B was observed for all insulin molecules tested, only the selectivity ratios for hIR-A are shown in Table 2.

To place the data for in vitro binding to hIR-A and hIGF-1R in clinical context, the competitive displacement profiles of BIL for the cognate ligands were overlaid with the steady-state plasma concentration range observed in a clinical trial in type 2 diabetes mellitus (T2DM) patients (Sinha et al., 2014b) (Fig. 2). In the 14-day clinical trial, patients achieved steady-state levels of BIL; daily doses of BIL ranged from 0.33 to 1.0 U/kg. The shaded box within Fig. 2 delineates the lowest (3.2 nM) and the highest (16.0 nM) plasma concentrations of BIL observed in the clinical trial. This range of plasma concentrations encompasses the steady-state levels achieved in clinical testing in T1DM (3.2 nM) and T2DM (4.3 nM) patients (Henry et al., 2014). Because the range of clinical exposures for BIL spanned the binding affinity for hIR-A, BIL would be expected to engage the receptor. Given the lower binding affinity for hIGF-1R, even at the highest exposures, BIL would not be expected to engage hIGF-1R.

**Functional Activity by hIR Autophosphorylation and De Novo Lipogenesis in Differentiated Mouse 3T3-L1 Adipocytes.** As expected from the reduced binding affinities,

BIL also showed approximately 10-fold lower functional activity for stimulating tyrosine autophosphorylation of both hIR-A and hIR-B compared to that of insulin lispro (Table 3). BIL induced a maximal level of phosphorylation similar to that of insulin lispro (Supplemental Fig. 2, A and B), which suggests that BIL is a full agonist for both hIR-A and hIR-B autophosphorylation. All other test samples also reached maximal responses similar to that of insulin lispro (data not shown).

To link the functional activation of hIR with an intrinsic metabolic response, insulin-stimulated de novo lipogenesis of triglycerides from [<sup>14</sup>C]-labeled glucose was measured by using mouse 3T3-L1 adipocytes (Table 3). BIL achieved a maximal response comparable to that of insulin lispro (Supplemental Fig. 2C), as did the other insulin test samples (data not shown). The relative lipogenic potency ( $EC_{50}$ ) of BIL was approximately 10-fold lower than the potency observed for insulin lispro, which correlates with the observed lower hIR binding affinity and hIR autophosphorylation potency. The comparable lipogenic potencies of BHI and IGF-1 in the 3T3-L1 adipocytes correlate with the comparable expression levels of the mouse IGF-1R and mouse insulin receptor. Because of albumin binding of the acyl moiety caused by the presence of BSA in these functional assays, these results should be considered an underestimate of the functional and lipogenic potency of insulin detemir, which prevents a conclusive comparison with BIL (Sorensen et al., 2010).

**hIR Tyrosine Dephosphorylation.** Prolonged binding of an insulin analog to hIR can result in prolonged activation of the tyrosine kinase domain of hIR, leading to extended tyrosine phosphorylation and downstream signaling (Kurtzhals et al., 2000; Hansen et al., 2011). To gain insight into an insulin agonist/hIR interaction or residence time, a time course of hIR dephosphorylation following a washout of maximally efficacious doses of insulin test samples was measured (Fig. 3). A similar time course of hIR dephosphorylation was observed after treatment with either insulin lispro or insulin glargine for hIR-A (Fig. 3A) or hIR-B (Fig. 3B). The time course of hIR dephosphorylation was more rapid after treatment with BIL (tested at 1000 nM because of its reduced affinity compared with the other insulins). In contrast, treatment with insulin AspB10 resulted in notably longer hIR phosphorylation treatment with BIL, insulin glargine, or insulin lispro, consistent with published data (Hansen et al., 2011, 2012b).

TABLE 2

Binding affinities for BIL compared with those of various insulin analogs in membranes prepared from stably transfected human embryonic kidney-293 cells overexpressing hIR-A and hIR-B or hIGF-1R

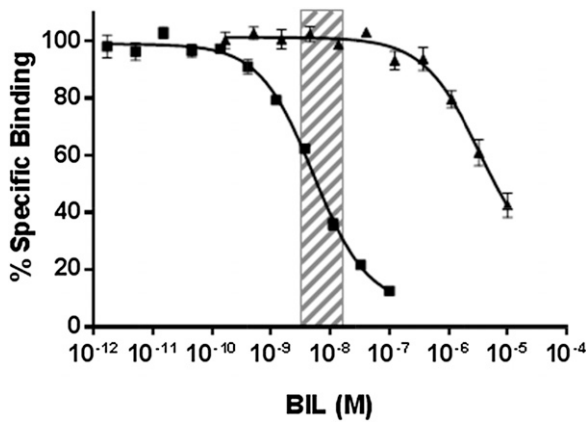
Test Sample	hIR-A $K_i$ , Mean (S.E.M.), Number of replicates <sup>a,b</sup>	hIR-B $K_i$ , Mean (S.E.M.), Number of Replicates <sup>a,b</sup>	hIGF-1R $K_i$ , Mean (S.E.M.), Number of Replicates <sup>a,b</sup>	Selectivity hIR-A <sup>c</sup>
	<i>nM</i>	<i>nM</i>	<i>nM</i>	
BIL	4.51 (0.33, 3)	5.59 (1.49, 3)	6090 (673, 4)	1351
Insulin lispro	0.31 (0.02, 6)***	0.32 (0.01, 6)***	73.6 (13.0, 7)***	238
BHI	0.29 (0.02, 10)***	0.36 (0.04, 10)***	101 (8, 12)***	347
Insulin AspB10	0.10 (0.02, 3)***	0.10 (0.01, 3)***	34.9 (3.4, 3)***	366
Insulin glargine	0.32 (0.03, 3)***	0.40 (0.04, 3)***	12.0 (0.5, 3)***	37
Insulin detemir	2.45 (0.25, 6)**	2.22 (0.26, 6)***	372 (34, 5)***	152
IGF-1	7.17 (0.61, 4)*	111 (3, 4)***	0.18 (0.02, 18)***	0.02

<sup>a</sup>Binding affinities are expressed as the inhibitory binding constant ( $K_i$ ) calculated from the experimental  $IC_{50}$ , radioligand concentration, and the ligand dissociation constant ( $K_d$ ; hIR-A = 0.212 nM, hIR-B = 0.191 nM, or hIGF-1R = 0.107 nM).

<sup>b</sup>Means are reported as the geometric mean using the log-transformed potency values (S.E.M., number of replicates). Larger  $K_i$  values represent lower binding affinity.

<sup>c</sup>Selectivity expressed as a multiple from the ratio of  $K_i$  for hIGF-1R over the  $K_i$  for hIR-A.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by Dunnett's analysis in  $K_i$  relative to BIL.



**Fig. 2.** Relationship between BIL plasma concentrations in patients with T2DM and observed binding affinities for BIL against hIR-A and hIGF-1R. Plasma concentrations measured from a 14-day, steady-state clinical trial in patients with T2DM who were exposed to daily doses of BIL ranging from 0.33 to 1.0 U/kg were compared with the concentrations of BIL required to competitively displace either  $^{125}$ I-human insulin from human insulin receptor (solid squares) or  $^{125}$ I-human insulin-like growth factor 1 from hIGF-1R (solid triangles). The shaded box shows the observed range in plasma concentrations from the lowest (3.2 nM) to the highest (16.0 nM). It should be noted that the plasma concentration of BIL is assumed to reflect the hepatic concentration (Moore et al., 2014). Error bars represent the S.E.M. for three independent determinations.

**Mitogenic Potential.** The mitogenic potentials of BIL and the comparator insulins were evaluated by using the human osteosarcoma cell line SAOS-2, a cell line that expresses 10 times more hIGF-1R than hIR (Kurtzhals et al., 2000) and is commonly used for determining the mitogenic potential of insulin analogs (Kurtzhals et al., 2000; Liefvendahl and Arnqvist, 2008). As expected, IGF-1 was more potent than insulin lispro for increasing DNA synthesis, as measured by methyl- $^3$ H]thymidine incorporation (Table 4). Insulin glargine and insulin AspB10 had the greatest mitogenic potentials of the insulins tested, which correlated with their observed higher binding affinities for hIGF-1R and was consistent with previously reported results (Kurtzhals et al., 2000). BIL showed lower mitogenic potential than insulin lispro (by 25-fold) and insulin glargine (by 49-fold). The presence of BSA in this assay contributes to an underestimation of the mitogenic potential of insulin detemir because of albumin binding, and therefore prevents a conclusive comparison with BIL.

## Discussion

Compared with BHI and the analogs lispro, detemir, and glargine, the unique in vitro and in vivo activity profiles emerging for BIL support the therapeutic usefulness of this novel basal insulin molecule. BIL exhibited stronger selectivity for hIR than for hIGF-1R and showed full agonistic activity in all of the functional in vitro assays (albeit with weaker receptor binding affinity and, consequently, higher  $EC_{50}$  values), lower mitogenic potential, and more rapid dephosphorylation of hIR following activation by BIL.

The in vivo PK and PD characterization of BIL revealed a markedly delayed  $T_{max}$  after s.c. injection, an increased area under the concentration-time curve, and a prolonged duration of action compared with insulin lispro in the STZ-treated diabetic rat. PEGylation of insulin lispro delays or alters s.c. absorption, reduces renal clearance (Hansen et al., 2012b), and probably alters receptor-mediated clearance via weaker binding affinity (Flier et al., 1982). It is possible that the slower absorption of BIL, because of its larger hydrodynamic size, may reflect a shift from a predominantly more rapid capillary absorption (Charman et al., 2001) to a slower lymphatic absorption. Moreover, the therapeutic advantages of BIL over insulin glargine observed in the phase 2 clinical trials (Bergental et al., 2012; Rosenstock et al., 2013) may be attributable, in part, to the increased hepato-preferential activity (Henry et al., 2014; Moore et al., 2014). These advantages include weight loss, reduced mealtime insulin dosage, reduced nocturnal hypoglycemia, and improved glycemic control in patients with type 1 diabetes (Rosenstock et al., 2013), and weight loss, reduced nocturnal hypoglycemia, and reduced intraday glucose variability in patients with type 2 diabetes (Bergental et al., 2012).

The hIR binding affinity of BIL, although significantly lower than that of insulin lispro and BHI, was still comparable with the binding affinity observed for insulin detemir. The higher concentrations of BIL needed to activate the hIR in the current preclinical studies have also been observed in phase 1 dose-response studies that achieved steady-state plasma levels ranging from 3.2 to 16 nM (Sinha et al., 2014a), consistent with the in vitro  $K_i$  (hIR) for BIL of approximately 5 nM. This lower binding affinity also provides a means to modulate receptor-mediated clearance of BIL with protracted PK profiles. Thus, the weaker binding affinity to hIGF-1R for BIL relative to BHI and insulin lispro coupled with the comparatively lower

TABLE 3

Reduction of the functional activity of BIL for hIR-A and hIR-B receptor autophosphorylation and de novo lipogenesis in mouse 3T3-L1 adipocytes

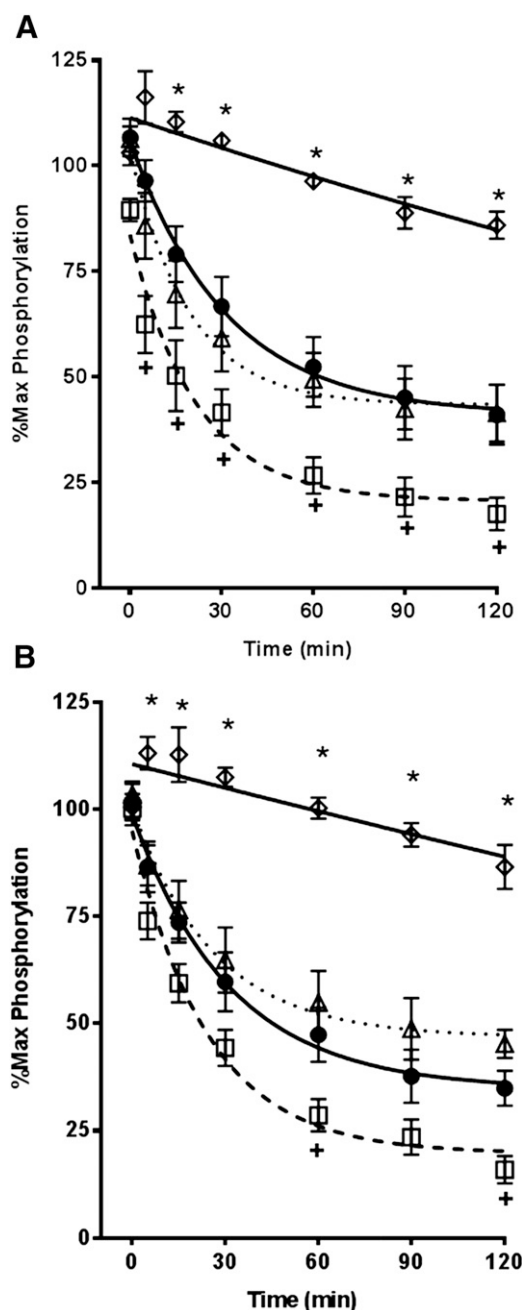
Test Sample	hIR-A Phosphorylation, $EC_{50}$ (S.E.M., Number of Replicates) <sup>a</sup>	hIR-B Phosphorylation, $EC_{50}$ (S.E.M., Number of Replicates) <sup>a</sup>	De Novo Lipogenesis, $EC_{50}$ (S.E.M., Number of Replicates) <sup>a</sup>
	nM	nM	nM
BIL	35.4 (1.5, 7)	29.4 (3.8, 7)	21.4 (5.8, 3)
Insulin lispro	3.69 (0.68, 12)***	2.66 (0.36, 11)***	2.56 (0.13, 4)***
BHI	4.42 (0.31, 79)***	3.06 (0.06, 58)***	2.70 (0.39, 22)***
Insulin AspB10	1.38 (0.57, 10)***	1.00 (0.31, 8)***	0.77 (0.09, 7)***
Insulin glargine	5.24 (0.33, 16)***	3.32 (0.20, 16)***	8.30 (0.10, 12)**
Insulin detemir	344 (137, 4)***	313 (188, 3) <sup>b</sup> ***	ND
IGF-1	143 (39, 5)***	>20,000 (n=2)***	1.99 (0.72, 8)***

ND, not determined.

<sup>a</sup>Means are reported as the geometric mean using the log-transformed  $EC_{50}$  potency values (S.E.M., number of replicates) for each test material using log10 transformation of each value.

<sup>b</sup>Potency values,  $EC_{50}$ , reported for insulin detemir may not reflect the actual potency because of the presence of serum albumin in the assay buffers.

\*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by Dunnett's analysis in  $EC_{50}$  relative to BIL.



**Fig. 3.** BIL shows lower residence time at the insulin receptor as reflected by a faster rate of receptor tyrosine dephosphorylation. Rate of hIR-tyrosine dephosphorylation on hIR-A (A) and hIR-B (B) after a 30-minute stimulation with a maximally efficacious dose of insulin AspB10 (100 nM; white diamonds/solid line), insulin lispro (100 nM; black solid circles/solid line), insulin glargine (1000 nM; white triangle/dotted line), or BIL (1000 nM; white squares/dashed line). Note that the insulin AspB10 phosphorylation response continues to increase to greater than 100% during the 30 minutes after ligand washout in both hIR-A and hIR-B cell models. Data shown represent the results from three independent time courses. \* $P < 0.001$  for differences between BIL and insulin lispro; \* $P < 0.001$  for differences between insulin lispro and insulin AspB10 for phosphorylation changes from maximum phosphorylation level.

mitogenic potential for BIL in vitro suggests that BIL will exhibit an overall mitogenic safety profile equal to or better than those of other insulin analogs. Moreover, as shown in Fig. 2, the relationship between the observed plasma concentrations in T2DM patients treated with BIL and the  $K_i$  values

**TABLE 4**

The reduced mitogenic potential of BIL in the human SAOS-1 osteosarcoma cell line

Insulin Test Sample	SAOS-2 Mitogenesis, EC <sub>50</sub> (S.E.M., Number of Replicates) <sup>a</sup>
	<i>nM</i>
BIL	29.4 (8.1, 4)
Insulin lispro	1.18 (0.24, 8)***
BHI	2.69 (0.63, 30)***
Insulin AspB10	0.37 (0.06, 22)***
Insulin glargine	0.61 (0.002, 17)***
Insulin detemir <sup>b</sup>	17.3 (3.7, 3)***
IGF-1	0.04 (0.01, 3)***

<sup>a</sup>Means are reported as the geometric mean using the log-transformed potency values (S.E.M., number of replicates).

<sup>b</sup>Potency values, EC<sub>50</sub>, reported for detemir may not reflect the actual potency because of the presence of serum albumin in the assay buffers.

\*\*\* $P < 0.001$  by Dunnett's analysis in EC<sub>50</sub> relative to BIL.

required for BIL to displace either human insulin from hIR or hIGF-1 from the hIGF-1R suggests the concentrations would be sufficient to engage the hIR, but incapable of competing with hIGF-1 for the hIGF-1R.

Data generated with insulin AspB10 suggest that activity through the hIR and hIGF-1R can stimulate cell proliferation at appropriate concentrations; thus, mitogenicity can be a risk with any insulin therapy. During chronic administration in rats, insulin AspB10 induced mammary tumors (Jorgensen et al., 1992). This tumorigenic action is hypothesized to involve both the activation of the IGF-1R and the prolongation of the residence time on the insulin receptor (Jorgensen et al., 1992; Hansen et al., 1996, 2011, 2012a). The hIR dephosphorylation assay performed in this research is in agreement with previous studies, i.e., insulin AspB10 showed a markedly longer duration of hIR phosphorylation, whereas insulin lispro, insulin glargine, and BIL all showed significantly faster rates of hIR dephosphorylation, with BIL displaying a more rapid rate of dephosphorylation. This rapid dephosphorylation, coupled with increased receptor selectivity for the hIR, supports a mitogenicity profile for BIL that is equal to or better than that of BHI or insulin lispro.

In conclusion, BIL demonstrated in vivo PK and PD properties and in vitro metabolic and mitogenic properties that are appropriate for a once-daily basal insulin analog. The longer PD profile, which is related to delayed absorption and altered clearance, seems suitable for a basal insulin. Thus, BIL provides a mechanism to alter absorption, clearance, distribution, and, consequently, activity profile, while maintaining full agonist receptor signaling properties. The in vitro metabolic and mitogenic characteristics of BIL were similar or improved compared to those of BHI, insulin lispro, and insulin glargine, all well-established therapeutic agents, with the added benefit of improved receptor selectivity against hIGF-1R. These results provide additional support for a favorable mitogenic profile, with an effective once-daily basal insulin therapy as demonstrated in clinical studies (Bergental et al., 2012; Rosenstock et al., 2013).

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# Authorship Contributions

**Participated in research design:** Owens, Moyers, Cutler, Michael, Beals.

**Conducted experiments:** Hansen, Vick, Koester, Zhang, Ruan, Kahl, Li, Michael.

**Contributed new reagents or analytic tools:** Li, Ruan, Kahl.

**Performed data analysis:** Owens, Farmen, Qian.

**Wrote or contributed to the writing of the manuscript:** Owens, Michael, Moyers, Beals.

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