Preclinical Characterization of LY3209590, a Novel Weekly Basal Insulin Fc-Fusion Protein

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ABSTRACT

The benefit of once-weekly basal insulin is less frequent dosing, which has the potential to reduce the barrier to injection therapy and impact patient activation, adherence and compliance, quality of life, and outcomes. Basal Insulin Fc (BIF, LY3209590, or insulin efsitora alfa) is a once-weekly basal insulin in clinical testing for type 1 and type 2 diabetes mellitus. BIF is comprised of a novel single-chain variant of insulin fused to a human IgG2 fragment crystallizable region of an antibody domain using a peptide linker. The in vitro binding affinity of BIF for the human insulin receptor (IR) was two orders of magnitude weaker relative to human insulin. BIF stimulated IR phosphorylation in cells with reduced potency, yet full agonism, and exhibited a significantly faster dephosphorylation kinetic profile than human insulin or AspB10 insulin. BIF stimulated de novo lipogenesis in 3T3-L1 adipocytes and cell proliferation in SAOS-2 and H4IIE cells with ≥70-fold reduction in in vitro potency compared with human insulin. BIF possessed markedly reduced binding to hIGF-1R, making definitive measurements unattainable. In vivo pharmacology studies using streptozotocin-treated diabetic rats demonstrated a significant decrease in blood glucose compared with vehicle-treated animals 24 hours post-injection, persisting through 336 hours following subcutaneous administration. In streptozotocin-treated rats, BIF reached time at maximum concentration at 48 hours and possessed a clearance rate of ~0.85 ml/h per kg, with a terminal half-life of ~120 hours following subcutaneous administration. These results demonstrate BIF has an in vitro pharmacological profile similar to native insulin, with significantly reduced potency and an extended time-action profile in vivo that supports once-weekly dosing in humans.

SIGNIFICANCE STATEMENT

BIF is a novel basal insulin Fc-fusion protein designed for once-weekly dosing. In this study, we demonstrate that BIF has an in vitro pharmacological profile similar to human insulin, but with weaker potency across assays for IR binding and activity. BIF has a PD and PK profile in STZ-treated rats supportive of weekly dosing in humans.

Introduction

Diabetes mellitus is increasing in prevalence and is estimated to affect >400 million people worldwide (World Health Organization: https://www.who.int/publications/i/item/9789241565257). Moreover, insulin remains a critical therapeutic for many of these patients. Patients with type 1 diabetes mellitus (T1DM) are insulin-dependent, whereas patients with type 2 diabetes mellitus (T2DM) often transition to insulin as the disease progresses. However, studies show T2DM patients exceed glycated hemoglobin (HbA1c) targets for years before treatment intensification with insulin (Khunti and Millar-Jones, 2017) because of clinical or therapeutic inertia attributed to both the health care professional and the patient (Russell-Jones et al., 2018).

Real-world studies repeatedly show that adherence to insulin is suboptimal with a medication possession ratio <80%, a level generally accepted as the conventional threshold for medical adherence (Davies et al., 2013). The reasons for poor adherence or omission of insulin therapy include perceived burden, embarrassment, and prior experience with painful injections (Peyrot et al., 2010; Russell-Jones et al., 2018). Complexity of the dosing regimen, including daily dose adjustment because of diet and exercise, or the need to split dosing, is linked with poor adherence and high HbA1c levels (Russell-Jones et al., 2018; Stephenson et al., 2018). A quantitative study of basal insulin users who failed to achieve recommended HbA1c targets noted concerns with weight gain,

ABBREVIATIONS: BIF, basal insulin Fc; Fc, fragment crystallizable region of an antibody; HbA1c, glycated hemoglobin; HEK293, human embryonic kidney-293 cells; IGF-1R, insulin-like growth factor-1 receptor; IR-A, insulin receptor isoform A; IR-B, insulin receptor isoform B; Ki, inhibitory constant; PD, pharmacodynamics; PK, pharmacokinetics; QD, once-daily; QW, once-weekly; RME, receptor-mediated endocytosis; SCI, single-chain insulin; STZ, streptozotocin; $t_{1/2}$, terminal half-life; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; T_{max}, time at maximum concentration.

perception of worsening of disease with increasing dose, fear of hypoglycemia, and frustration with the time to reach HbA1c goal (Berard et al., 2018). Many of these factors also affect T1DM (Ramchandani et al., 2019), where despite advances in insulin therapies, a paucity of patients are achieving HbA1c goals across all age groups (Foster et al., 2019).

Strategies to increase adherence and patient confidence with existing insulins (Arvanitis et al., 2020) include devices and technology-enabled self-management solutions (Peyrot et al., 2010). This research focuses on design of a once-weekly (QW) basal insulin to lessen the injection barrier and provide convenience to lessen the burden of treating diabetes while maintaining both efficacy and safety. This hypothesis is grounded in studies showing that a reduction in dosing frequency from twice-a-day to once-daily (QD) and from QD to QW associates with improved adherence or persistence across multiple disease states (Bae et al., 2012). Notably, adherence to once-weekly glucagon-like peptide-1 (GLP-1; exenatide) by patients with T2D was significantly increased compared with patients initiating once daily GLP-1 (liraglutide) (Qiao et al., 2016). Thus, in combination with the device and technology-enabled solutions, a therapeutic opportunity exists to create notable advances in outcomes with insulin-based therapy.

The obvious design goal for QW basal insulin is the creation of a long-lived agonist capable of providing insulin exposure over an entire week in a single injection while preserving efficacy outcomes, i.e., HbA1c, and maintaining a similar or improved safety profile, i.e., hypoglycemia. To achieve a QW basal insulin, the extended pharmacokinetic (PK) profile must be balanced with the receptor engagement; thus, the strategy requires attenuating IR binding to decrease clearance by receptor mediated endocytosis (RME) (Tokarz et al., 2018), as well as mitigating renal filtration (Ferrannini et al., 1983; Henriksen et al., 1987). Importantly, the development of a basal insulin with a stable steady state insulin concentration, wherein the peak-to-trough ratio associated with insulin exposure is close to 1.0, requires the use of therapeutic accumulation to minimize pharmacodynamic variations over time (Heise, 2021). A basal insulin with a longer half-life will have a flatter peak-to-trough ratio at steady state compared with a basal insulin with a shorter half-life (Heise and Meneghini, 2014). For once daily basal insulins, dosed appropriately, a flatter profile results in a reduction in hypoglycemic episodes compared with short-acting insulins (Heise and Meneghini, 2014; Heise and Mathieu, 2017). Compared with insulin glargine, which has a peak-to-trough ratio of 1.8, the longer acting insulin degludec has a flatter profile and provides equivalent glycemic control with significantly lower nocturnal hypoglycemia rates (Vora et al., 2014). Thus, a QW insulin with an ultra-long half-life and a low peak-to-trough fluctuation has the potential to achieve consistent glucose-lowering with a favorable hypoglycemia profile, yet with less frequent dosing (Heise and Mathieu, 2017).

Notably, exogenous insulin clearance is very rapid with a terminal half-life ($t_{1/2}$) ~5 minutes (Home et al., 1982); consequently, QW basal strategies require modifying the insulin and/or formulation to extend exposure for the desired time action. To date, insulin time extension strategies focus on controlling payout from the subcutaneous depot, introducing plasma retention strategies to avoid renal filtration (Ferrannini et al., 1983; Henriksen et al., 1987), and/or weakening receptor engagement to attenuate receptor-mediated clearance (Tokarz et al., 2018). The creation of a QW basal insulin requires safe and efficacious exposure of an IR agonist having a $t_{1/2} > 2000\times$ longer than the native molecule. Current strategies being researched for QW insulin include acylation for albumin binding (Pridal et al., 2015; Hovelmann et al., 2020; Rosenstock et al., 2020; Nishimura et al., 2021), PEGylated insulin encapsulated in microspheres (Hinds et al., 2015), and fragment crystallizable region of an antibody (Fc) -fusions (Baldwin et al., 2018; Song et al., 2018; Tagmose et al., 2020). The characteristics of a novel QW basal insulin comprised of a single-chain variant of insulin fused to a Fc domain of an IgG2 molecule, BIF, are presented in this work.

Materials and Methods

Materials. BIF, human insulin, and AspB10 insulin were prepared at Eli Lilly and Company (Indianapolis, IN). BIF was expressed from a CHO cell line and purified using a two-column purification process that included Protein A and size exclusion chromatography. Purified fractions were pooled, sterile filtered, and stored at 4°C in either citrate at pH 6.5 or phosphate-buffered saline at pH 7.4. Human insulin solutions were sourced from Lilly manufacturing and formulated at 100 IU containing water, 16 mg of glycerin, 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. Insulin-like growth factor-1 (IGF-1, PeproTech, Rocky Hill, NJ) and AspB10 insulin were prepared at 1 mg/ml in PBS, pH 7.4.

Binding to Human Insulin Receptor, Rat Insulin Receptor, and Human Insulin-Like Growth Factor-1 Receptor. The binding affinities of BIF for human insulin receptor isoform A (hIR-A), human insulin receptor isoform B (hIR-B), and human IGF-1 receptor (hIGF-1R) were determined using competitive radioligand binding assays using membrane-bound receptors prepared from human embryonic kidney-293 (HEK293) cells overexpressing either hIR-A, hIR-B, or hIGF-1R, as previously described (Owens et al., 2016). Rat insulin receptor (rIR) binding affinity was determined using membranes from HEK293 cells expressing rat IR-A using the same methodology (Owens et al., 2016). Inhibitory constant (Ki) values were determined from a four-parameter logistic nonlinear regression analysis. All results are reported as the geometric mean with the S.E.M. and indicated number (n) of independent replicates (Table 1). Curves were fit using GraphPad Prism Software (v8.4.3).

TABLE 1
Inhibitory constants (Ki) for BIF determined in competitive ligand binding assays for the receptors hIR-A, hIR-B, hIGF-1R, and rIR-A

	Receptor Binding Affinity, Ki, nM (S.E.M., n)			
Ligand	hIR-A	hIR-B	hIGF-1R	rIR-A
BIF	25 (4, $n = 10$)	26 (4, $n = 10$)	>7190 ($n = 3$)	78 (14, $n = 3$)
Human insulin	0.17 (0.01, $n = 10$)	0.20 (0.01, $n = 10$)	64 (21, $n = 3$)	0.82 (0.18, $n = 3$)
AspB10 insulin	0.10 (0.01, $n = 10$)	0.12 (0.01, $n = 10$)	50 (7, $n = 2$)	0.32 (0.07, $n = 3$)
Human IGF-1	4.4 (0.4, $n = 10$)	51 (4, $n = 10$)	0.07 (0.02, $n = 3$)	21 (5, $n = 3$)

Ki values are geometric means. n, number of independent experiments.

Insulin Receptor and IGF-1 Receptor Phosphorylation Assay. The functional activity of BIF for stimulation of IR tyrosine phosphorylation was determined using HEK293 cells overexpressing either hIR-A or hIR-B, both containing a C9 epitope as previously described (Owens et al., 2016). IGF-1R auto-phosphorylation was performed in a similar manner using HEK293 cells overexpressing human IGF-1R containing a C9 epitope. Tyrosine phosphorylation was determined using a sandwich ELISA format where IR-A, IR-B, or IGF-1R were captured with an anti-C9 monoclonal antibody (Oprian et al., 1987) followed by detection with the antiphosphotyrosine monoclonal antibody 4G10–horseradish peroxidase conjugate (EMD Millipore, Billerica, MA) with the addition of 3,3,5,5-tetramethylbenzidine substrate (Thermo Scientific, Rockford, IL). The absorbance values for each assay were normalized to the response to a maximally efficacious concentration of human insulin (100 nmol/l, for IR-A and IR-B) or IGF-1 (10 nmol/l, for IGF-1R). EC_{50} values were determined from a four-parameter logistic nonlinear regression analysis. Curves were fit using four parameter nonlinear logistic equations using GraphPad Prism Software (v8.4.3).

Insulin Receptor Dephosphorylation Assay. To follow the dephosphorylation kinetics of hIR, the same cell lines and ELISA protocol used in the hIR-A and hIR-B phosphorylation assays were used, except that the cells were stimulated for 30 minutes with maximally efficacious concentrations of the insulin analogs (100 nM human insulin, 30 nM AspB10 insulin, and 20 μM BIF). Dephosphorylation was started after a wash with low pH MBB buffer (100 mM MES, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM Glucose, 15 mM Na Acetate and 1% BSA, pH 5.6) followed by two media exchanges into serum-free growth medium supplemented with 0.1% BSA. At the indicated time points, cells were rinsed with ice-cold PBS and lysed with NP40 lysis buffer, and ELISA analysis for IR phosphorylation was performed as described earlier. Values were normalized to an insulin lysate titration curve and calculated as a percentage of the mean starting (prewash) values for all control wells using a maximally efficacious concentration of each ligand. Treatments were compared at each time point by t tests.

Lipogenesis Assay. The metabolic potency of BIF was determined by de novo lipogenesis using differentiated murine 3T3-L1 adipocytes (Owens et al., 2016).

Mitogenesis Assays. The mitogenic potential of BIF was determined by measuring [^{3}H]-thymidine incorporation into the newly synthesized cellular DNA of proliferating SAOS-2 human osteosarcoma cells using the previously described method (Owens et al., 2016). The results were reported as the concentration that elicited an EC_{50} relative to a maximally efficacious concentration of human insulin (1000 nM). H4IIE rat hepatoma cells, (CRL-1548, ATCC), were maintained in complete media, [DMEM (SH30024.02, Hyclone), 10% FBS (10082, Gibco)], 10% calf serum (16170-078, Gibco), 2 mM glutamine (Gibco35050), 1 mM sodium pyruvate (HyClone SH30239.01), 0.1 mM nonessential amino acids (HyClone SH30238.01), and 1% penicillin or streptomycin (HyClone SV30010). Cells were harvested with 0.05% trypsin/EDTA then plated at 2×10^4 cells per well in 96-well black (35460, Corning) microplates for 3 hours in assay buffer after washing once with assay buffer [DMEM (17-305-CV, Corning), 0.1% BSA (15260, Gibco), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1% penicillin or streptomycin]. Cells were treated with test samples for 23 hours followed by the addition of 10 μl of 100 μM stock of BrdU (cell proliferation ELISA, BrdU, 11669915001, Roche) in 100 μl per well of assay buffer for 3 hours. BrdU was detected by immunoassay according to manufacturer's protocol and the chemiluminescence quantified on an Envision instrument (PerkinElmer). Dose response values were normalized to the response of a maximally efficacious concentration of human insulin (100 nM) and calculated as percent of maximal response.

PK and Pharmacodynamics (PD) Studies in Streptozotocin (STZ)-Treated Diabetic Rats. The in vivo effects of BIF were investigated in the streptozotocin (STZ)-treated rat diabetes model. Male Sprague-Dawley rats, 400 to 425 g body weight, were obtained from Harlan Laboratories (Indianapolis, IN). After acclimation for approximately 1 week, the rats were anesthetized with isoflurane and administered a single injection of Zanosar (item # 89256, Teva Parenteral Medicines, 40 mg/kg, IV). Only animals with nonfasted blood glucose between 400 to 550 mg/dl were used in studies 3 days after injection of Zanosar. The mean body weights across groups before STZ treatment ranged from 421–427 g and the mean body weights across groups after STZ treatment ranged from 394–398 g, with similar variability in each group. The rats were distributed into groups (n = 6 per group) to provide comparable variance in blood glucose and body weight. The STZ-treated rats were administered a single subcutaneous injection of vehicle (saline) or BIF (3, 10, or 30 nmol/kg). Blood samples for glucose measurements were collected by tail bleed conducted under brief restraint. Blood glucose was measured preinjection, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72, 96, 120, 144, 168, 240, and 336 hours postdose. The blood glucose was measured using an Accucheck Aviva glucometer (Roche). The animals had free access to food and water throughout the experiment. Statistical comparisons between groups were done using JMP9 to perform a Dunnett's multiple comparison test with Box-Cox transformation at each time point.

Circulating BIF was measured using an insulin receptor ELISA that required the presence of insulin that could bind the insulin receptor. In this ELISA, a mouse anti-5x HisTag antibody (Novagen 70796) was bound to an Immulon 4 HBX plate and used to capture the human insulin receptor (R&D Systems1544-IR/CF). BIF standard curve and samples were diluted in 100% rat K3 EDTA plasma and detected by mouse antihuman IgG Fc horseradish peroxidase (SouthernBiotech 9040-05). Pharmacokinetic parameters for BIF were computed using standard noncompartmental analyses, using Phoenix WinNonlin 6.3 software.

Results

Molecular Design

BIF, possessing the international nonproprietary name of insulin efsitora alfa, is an Fc-fusion protein that is expressed from CHO cells as a disulfide-linked homodimer with molecular weight of 64.1 kDa. Each monomer of the homodimer is comprised of a single-chain variant of insulin with B-chain linked to A-chain by a short linker (Linker 1), an interdomain linker (Linker 2) connecting the A-chain of insulin to the Fc, and the Fc domain from IgG2. The protein sequence for the monomer is provided in Fig. 1. The sequence was designed to minimize insulin self-association under locally high concentration generated by the homodimer state of BIF and contributes to the manufacturability properties (e.g., expression, chemical stability, physical stability), and the weak agonism of the molecule. The single-chain variant of insulin has the following modifications from human insulin TyrB16Glu, PheB25His, ThrB27Gly, ProB28Gly, LysB29Gly, ThrB30Gly, IleA10Thr, TyrA14Asp, and AsnA21Gly. Linker 1 is comprised of a seven amino acid, flexible linker, Gly-Gly-Ser-Gly-Gly-Gly-Gly.

In Vitro Characterization. *Competitive Binding Assay.* BIF binding affinities for hIR and hIGF-1R were determined from competitive radio-ligand binding assays using membranes from HEK293 cells expressing either hIR-A, hIR-B, rat IR-A (rIR-A), or IGF-1R. The Ki values of BIF for each receptor are shown in Table 1 and assay curves are shown in Fig. 2. The Ki values determined for BIF on hIR-A and hIR-B were 25 nM (S.E.M. = 4) and 26 nM (S.E.M. = 4), respectively (Table 1). Compared with human insulin, BIF showed ~150-fold reduced binding affinity to the hIR-A and ~130-fold reduced binding affinity for hIR-B (Table 1). BIF
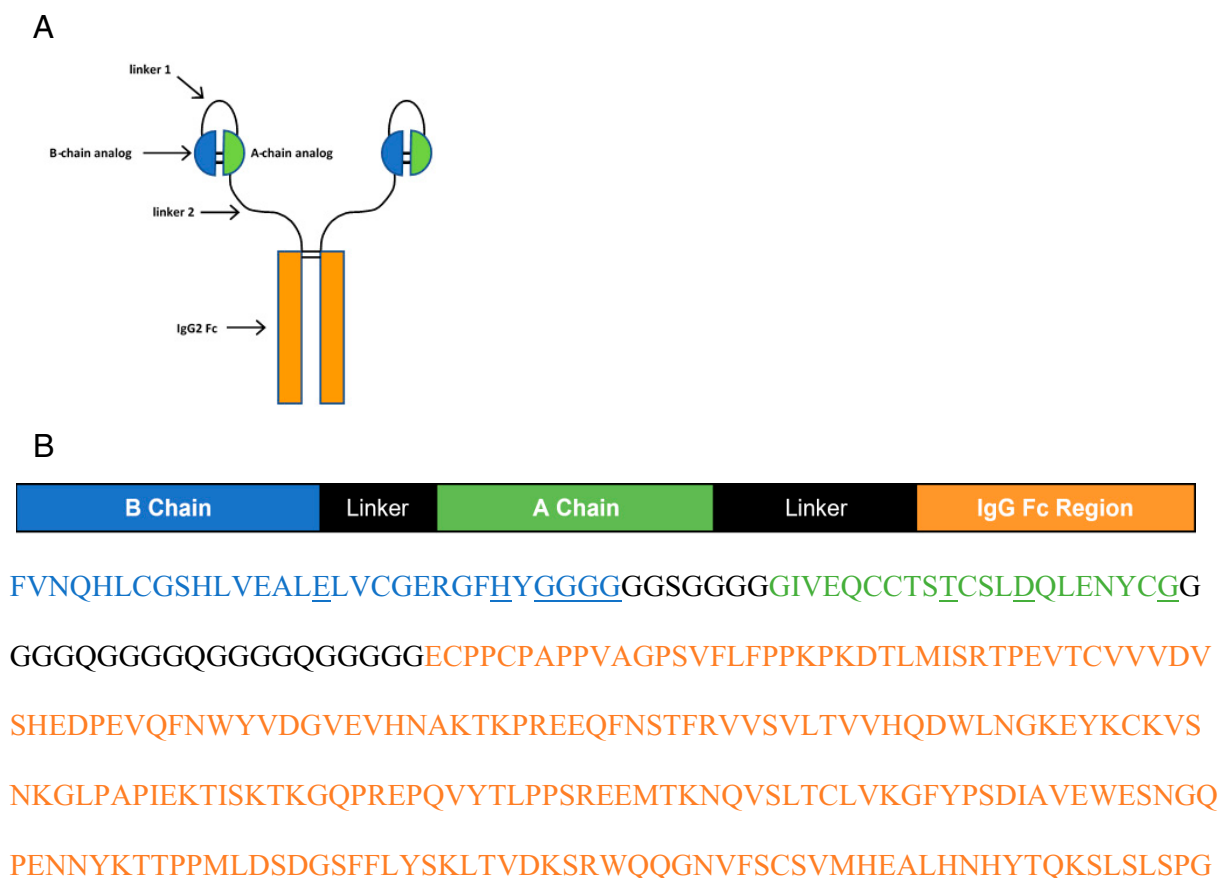
Fig. 1. Structure of the homodimer (A) and primary sequence of a monomeric component in single-letter amino acid designations (B) of BIF. The single-chain variant of insulin is designated in blue (B-chain), black (Linker 1), and green (A-chain). The underlined residues identify sites in insulin wherein amino acid changes were made. The interdomain linker 2 is in black and the IgG2 Fc domain is in orange.

binding is more selective for hIR-A and hIR-B compared with hIGF-1R, wherein three independent assay runs for hIGF-1R binding failed to reach 50% of the IGF-1 control. These results inform that the relative IC_{50} of BIF for hIGF-1R binding is exceedingly weak and unattainable at the maximum concentration that could be tested; consequently, the calculated Ki value is designated with a ">" description (Table 1). Hence, BIF exhibits selectivity for hIR binding versus hIGF-1R. The binding affinity, based on Ki, of BIF for the rIR-A was 78 nM (S.E.M. = 14) (Table 1). Compared with human insulin receptor, BIF showed a threefold reduction in binding affinity for rIR-A.

Functional Analysis. The insulin receptor contains an intracellular tyrosine kinase domain that upon ligand binding autophosphorylates to allow recruitment of adaptor proteins that induce the insulin signaling pathways. Functional activity, i.e., receptor autophosphorylation on tyrosine residues, was determined after ligand treatment of HEK293 cells overexpressing hIR-A, hIR-B, or hIGF-1R. BIF showed a dose-dependent phosphorylation response on hIR-A and hIR-B (Fig. 3, A and B, respectively). Relative to human insulin, there was a pronounced reduction in the relative potency of BIF for activation of hIR-A and hIR-B phosphorylation; however, BIF exhibited full agonism. The measured EC_{50} for BIF activation was

4241 nM (S.E.M. = 1427) and 391 nM (S.E.M. = 22) for hIR-A and hIR-B, respectively (Table 2); therefore, BIF appears to exhibit a degree of selectivity for phosphorylation of hIR-B over hIR-A in these assays. Notably, the receptor binding assays for BIF did not show selectivity for hIR-A and hIR-B (Table 1). BIF was highly selective for phosphorylation of hIR relative to hIGF-1R where the EC_{50} for phosphorylation was >10,000 nM (Table 2, Fig. 3C). This value is derived from data showing <50% activation of IGF-1R phosphorylation at all concentrations of BIF tested up to 10 μM, the highest concentration tested, which is in stark contrast to the maximal concentration of IGF-1 comparator.

Prolonged insulin binding to the hIR results in prolonged activation of the tyrosine kinase domain, leading to extended tyrosine phosphorylation and downstream signaling (Kurtzhals et al., 2000; Hansen et al., 2011). Prolonged hIR phosphorylation by AspB10 insulin is linked to increased mitogenic activity (Hansen et al., 2012). To gain insight into the BIF/hIR residence time, a time course of hIR dephosphorylation following washout of a maximally efficacious concentration of ligand was measured. Compared with human insulin, tested at 100 nM, the time course of hIR-A and hIR-B dephosphorylation was more rapid with BIF, tested at 20 μM (Fig. 4). In contrast, treatment with AspB10 insulin, tested at 30 nM,
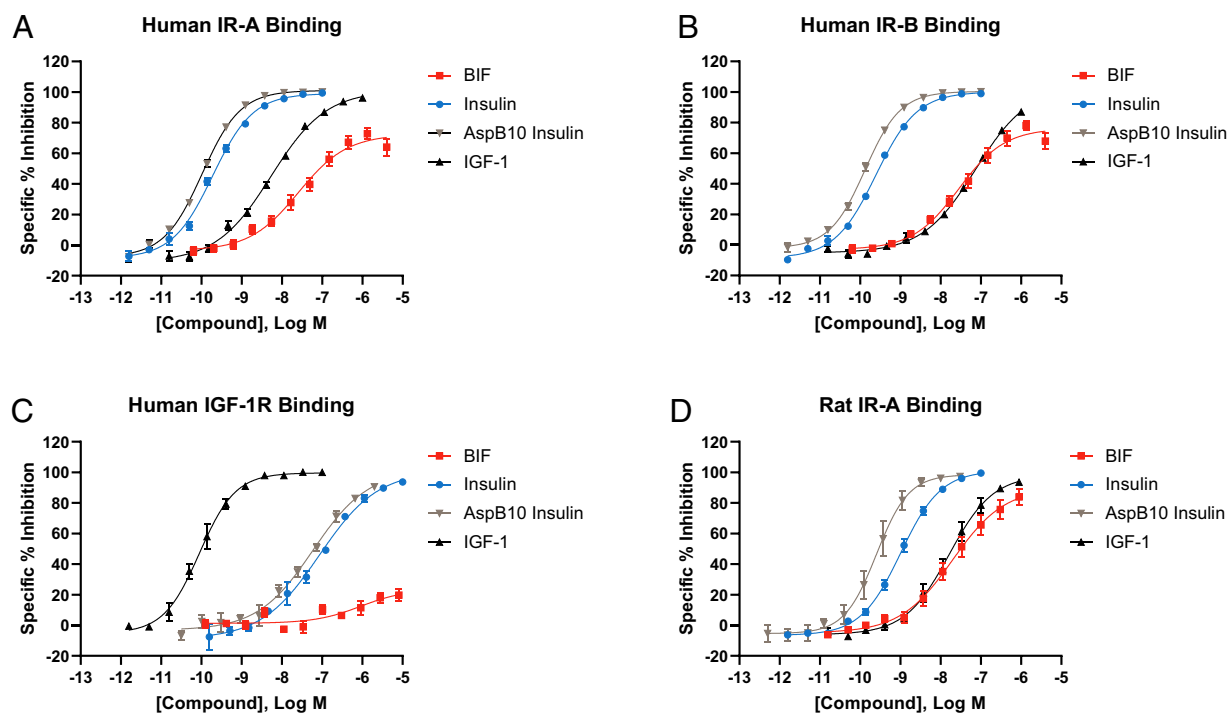
Fig. 2. Radioligand binding competition for IR and IGF-1R. Receptor binding was determined using membranes from HEK293 cells expressing hIR-A (A), hIR-B (B), hIGF-1R (C), and rat IR-A (D). SPA binding assays were performed in the presence of threefold serial dilutions of BIF (squares), human insulin (circles), AspB10 insulin (inverted triangles), or IGF-1 (triangles). Percent specific inhibition (y-axis) was plotted against log concentration of inhibitor (x-axis). Curves are representative. Data points are the average of three to seven independent experiments with error bars represented as S.E.M.

resulted in prolonged hIR phosphorylation compared with human insulin, consistent with published data (Hansen et al., 2012). Thus, BIF demonstrates a preferred rapid hIR dephosphorylation profile compared with the more mitogenic AspB10 insulin and exhibits a more rapid dephosphorylation profile compared with human insulin, suggestive of a faster off-rate from the receptor at endosomal pH levels.

To link the functional activation of IR signaling with an intrinsic metabolic response, insulin-stimulated de novo lipogenesis of newly synthesized triglycerides from $[^{14}C]$-glucose was determined in differentiated 3T3-L1 murine adipocytes. The lipogenic potency values, EC_{50}, were calculated as the concentration eliciting the half-maximal response relative to the maximally efficacious concentration (100 nM) of the positive control, human insulin. The EC_{50} of BIF, 19 nM (S.E.M. = 4) was ~70-fold weaker compared with the EC_{50} for human insulin, 0.27 nM (S.E.M. = 0.08) (Table 3). Maximal efficacy of BIF was similar to that of insulin, consistent with full agonism of IR activation by BIF (Fig. 5A).

Mitogenic Analysis. The mitogenic potential of BIF was assessed by measuring methyl-$[^3H]$thymidine incorporation into the newly synthesized cellular DNA of proliferating human SAOS-2 osteosarcoma cells following treatment. The SAOS-2 cell line was previously reported to express >10× more hIGF-1R than hIR; therefore, the cell proliferation response in the SAOS-2 cell line is more dependent on the IGF-1 signaling pathway than the insulin signaling pathway (Liefvendahl and Arnqvist, 2008). The EC_{50} values were reported relative to a maximally efficacious concentration (10 nM) of hIGF-1, used as the reference control. Consistent with greater responsiveness of SAOS-2 cells to IGF-1 signaling, hIGF-1 was the most

potent molecule tested, with an EC_{50} of 0.16 nM (S.E.M. = 0.02), whereas the EC_{50} for human insulin showed reduced potency, 1.8 nM (S.E.M. = 0.1) (Table 3 and Fig. 5B). Consistent with data reported in the literature (Liefvendahl and Arnqvist, 2008), AspB10 insulin was more potent than human insulin with an EC_{50} 0.50 nM (S.E.M. = 0.05) and BIF demonstrated the weakest potency with an EC_{50} of 134 nM (S.E.M. = 27) (Table 3). BIF potency was ~70-fold weaker relative to human insulin.

The potency of BIF in a cell proliferation assay was assessed by measuring BrdU incorporation into newly synthesized cellular DNA of proliferating rat H4IIE hepatoma cells. The H4IIE cell line expresses endogenous levels of rIR and lacks functional rIGF-1R (Yau et al., 1999); therefore, the mitogenic response in the H4IIE cell line reflects the contribution of the rIR signaling pathway rather than the rIGF-1R signaling pathway. The EC_{50} values were reported relative to a maximally efficacious concentration (100 nM) of human insulin as the reference control. The ligand AspB10 insulin was the most potent reference molecule tested with an EC_{50} of 0.13 nM (S.E.M. = 0.03) and hIGF-1 was the least potent reference molecule tested with an EC_{50} of 14 nM (S.E.M. = 1) (Table 3 and Fig. 5C), consistent with greater responsiveness of the H4IIE cells to insulin signaling. The EC_{50} for BIF was weakest at 20 nM (S.E.M. = 2), which was ~80-fold reduced in the H4IIE cell proliferation assay compared with human insulin.

In Vivo Efficacy. *Dose Response Profiles in STZ-Treated Diabetic Rats.* The PD and PK profiles of BIF were characterized following subcutaneous dosing in STZ-treated diabetic rats (Fig. 6, A and B, respectively). BIF exhibited a dose-proportional exposure profile and concomitant glucose-lowering effects with prolonged duration of action, with an
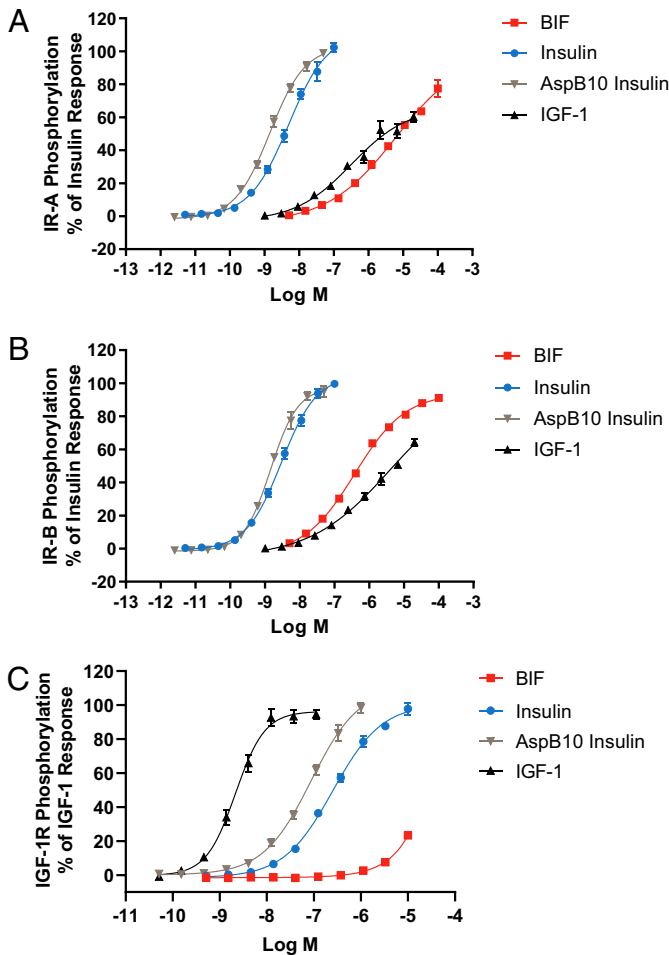
Fig. 3. Functional activity of BIF for phosphorylation of hIR-A (A), hIR-B (B), and IGF-1R (C). Receptor phosphorylation was determined using HEK293 cells expressing hIR-A, hIR-B, or hIGF-1R using an ELISA assay after stimulation of cells with BIF (squares) or human insulin (circles), AspB10 insulin (inverted triangles), or IGF-1 (triangles). Results are shown as the percent response relative to controls treated with a maximum concentration of human insulin (100 nM for IR-A and IR-B) or IGF-1 (10 nM for IGF-1R). Curves are representative; data points are the average of four independent assays for IR-A and IR-B or three independent assays for IGF-1R, with error bars represented as S.E.M.

initial transient reduction in blood glucose was observed in all dose groups at timepoints between 1 hour to 12 hours post-dose. There was no statistically significant difference between the vehicle control and BIF groups at these time points, indicating that the effect was related to initial dosing and handling across groups, rather than BIF-specific effects on glucose lowering at these time points. The PK parameters from the dose-response study in STZ-treated diabetic rats are shown in Table 4, with BIF possessing a very low apparent clearance and a long half-life consistent with the potential for weekly dosing.

Discussion

To date, commercial basal insulin analogs and derivatives developed for the treatment of diabetes focused on trying to achieve a flat QD profile through insulin derivatization or pI-precipitation. These basal insulin products include insulin glargine, insulin detemir, and insulin degludec (Hirsch et al., 2020). Consequently, the development of a safe, QW basal insulin analog with a flat profile would be a significant protein engineering feat, providing patients with diabetes an attractive and convenient option. To achieve a QW basal insulin requires balancing time extension for PK with receptor engagement for PD; thus, the strategy requires attenuating IR binding to decrease clearance by receptor mediated endocytosis (RME) while also mitigating renal clearance (Ferrannini et al., 1983; Henriksen et al., 1987). Moreover, to be optimally used for the treatment of both T1DM and T2DM, the peak-to-trough ratio should be as small as possible. A potential safety concern with long-acting insulins, as with any current insulin, is the possibility of hypoglycemia. For a basal insulin with extended half-life sufficient to support once weekly dosing, a potential risk is the possibility of protracted or repetitive hypoglycemia in comparison with once daily basal insulins. Analogous to daily basal insulins, low peak-to-trough fluctuations spread over the course of a week, rather than one day, coupled with attenuated receptor potency, could lead to a more stable glycemic control, thus decreasing glycemic variability and lowering risk of hypoglycemia (Sinha et al., 2014; Heise and Mathieu, 2017). Moreover, a QW basal insulin may provide a significant opportunity to help T1DM patients reduce diabetic ketoacidosis events (Schmitt and Scott, 2019).

One element to achieve these goals is to leverage FcRn binding to protect and extend the exposure of BIF by exploiting an Fc-fusion strategy (Cavaco et al., 2017; Liu, 2018). Insulin circulating in the blood is susceptible to cellular uptake through either fluid endocytosis, known as pinocytosis, or RME. In antibodies, the Fc domain of the IgG binds to FcRn in the acidic endosomal environment after pinocytosis (pH ~5.8) and protects the antibody from degradation; thus, allowing it to be

apparent steep exposure-response relationship between BIF concentrations and glucose-lowering efficacy, as was seen previously with a daily basal insulin in this model (Owens et al., 2016). A statistically significant decrease in blood glucose compared with vehicle-treated animals was seen 24 hours postinjection and maintained through 336 hours postinjection following a single 30 nmol/kg subcutaneous dose of BIF. An

TABLE 2

Functional activity of BIF as determined by phosphorylation of hIR-A, hIR-B, and hIGF-1R expressed in 293 cells

| | Receptor Phosphorylation EC_{50}, nM (S.E.M., n) | | |
Ligand	hIR-A	hIR-B	hIGF-1R
BIF	4241 (1427, $n = 5$)	391 (22, $n = 5$)	>10,000 ($n = 3$)
Human insulin	4.4 (0.5, $n = 5$)	2.8 (0.4, $n = 5$)	254 (29, $n = 3$)
AspB10 insulin	1.4 (0.2, $n = 5$)	1.4 (0.2, $n = 5$)	87 (13, $n = 3$)
IGF-1	393 (52, $n = 5$)	3034 (715, $n = 5$)	2.1 (0.2, $n = 3$)

EC_{50} values are geometric means. n, number of independent experiments.
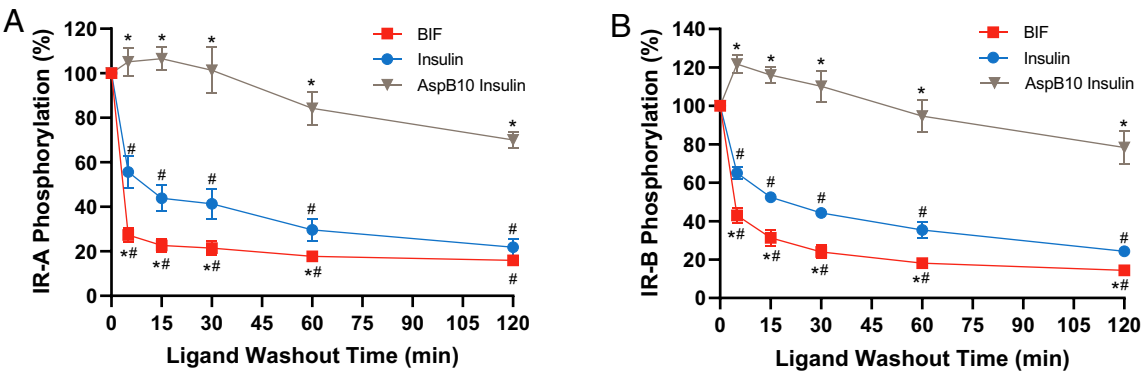
Fig. 4. The time course of hIR-A and hIR-B dephosphorylation. IR phosphorylation was determined in HEK293 cells expressing either hIR-A (A) or hIR-B (B) after treatment followed by ligand wash-out for the indicated times and quantitation of hIR tyrosine phosphorylation by ELISA. Results are graphed as percent remaining hIR phosphorylation relative to the initial phosphorylation for each ligand (without washout, set at 100% for each ligand) after treatment of cells for 30 minutes with a maximal concentration of BIF (20 μM, squares), human insulin (100 nM, circles), or AspB10 insulin (30 nM, inverted triangles). Data points are the average of four independent runs, except $n = 3$ for IR-A with AspB10 insulin. Error bars show the S.E.M. *$P < 0.05$ vs. insulin; **$P < 0.05$ vs. AspB10 insulin.

recycled back to the surface. As the endosome migrates back to the cell surface, the Fc dissociates from the FcRn/Fc complex as it encounters the extracellular neutral pH environment (pH ~7.2); thus, slowing clearance and increasing time action. Fusion proteins are composed of a biologically active protein or peptide and an Fc domain of IgG to achieve a time extension amenable for QW dosing, which has been applied successfully for dulaglutide (Trulicity) (Glaesner et al., 2010), a QW GLP-1 receptor agonist for the treatment of T2DM and in an analogous engineering fashion, but not identical, on BIF.

In this work, BIF is characterized with regards to receptor binding, in vitro potency, signaling properties, and in vivo time extension of glucose-lowering action in STZ-treated rats. BIF showed selective binding and activation of hIR with full agonistic properties, albeit with markedly reduced potency and binding affinity compared with human insulin. The binding to human IR, either hIR-A or hIR-B, is reduced by approximately two-orders of magnitude compared with human insulin (Table 1). The discovery that this relative reduction in binding of BIF, achieved through the introduction of mutational changes in the insulin backbone and the utilization of a single chain insulin (SCI) format, was a key component to the time extension strategy by modulating receptor engagement and subsequently RME (Flier et al., 1982), which enables the safe exploitation of the Fc-fusion time extension strategy. Results from an alternate QW insulin strategy used by insulin icodec, i.e., strengthening the acyl diacid binding of derivatized insulin receptor agonist to human serum albumin, provides support that an attenuated agonist can reduce the risk of hypoglycemia (Rosenstock et al., 2020). The observed incidence of level 1 (blood glucose level ≥54 and <70 mg per deciliter)

hypoglycemia was 53.6% in the icodec group and 37.7% in the glargine group [estimated rate ratio, 2.42; 95% confidence interval (CI), 1.50 to 3.88]. The observed incidence of combined level 2 (clinically significant, <54 mg per deciliter) or level 3 (severe) hypoglycemia was 16.0% in the icodec group and 9.8% in the glargine group (estimated rate ratio, 1.09; 95% CI, 0.45 to 2.65) (Hovelmann et al., 2020; Nishimura et al., 2021). These results may be attributable to a terminal half-life for icodec of only 196 hours, which is only slightly longer than a week and produces a peak-to-trough profile similar to insulin glargine (Heise, 2021). The PK and PD properties of BIF in T2DM have been examined, but not yet reported (Clinical-Trials.gov Identifier: NCT03367377). The initial results of a head-to-head clinical trial with insulin degludec (Clinical-Trials.gov Identifier: NCT03736785) have been reported in abstract form but are not yet available in peer reviewed manuscript form (Frias et al., 2021; Heise et al., 2021). BIF had a mean half-life of approximately 17 days in patients with T2DM and demonstrated a nearly peak-less PK profile over a one-week dosing interval with a peak-to-trough ratio of ~1.1 at steady state (Heise et al., 2021). This profile is notably flatter than insulin glargine, where following once-daily dosing, insulin glargine has a daily peak-to-trough ratio of ~2 (Heise, 2021). BIF, when administered weekly, was noninferior to insulin degludec for glycemic control (Frias et al., 2021). The results of additional clinical trials will demonstrate whether BIF, using an Fc-fusion time extension strategy coupled with weak IR agonism, can produce a time action profile which has sufficiently low peak-to-trough to minimize the risk of hypoglycemia similar to the flattest peak-to-trough QD basal insulins on the market.

TABLE 3
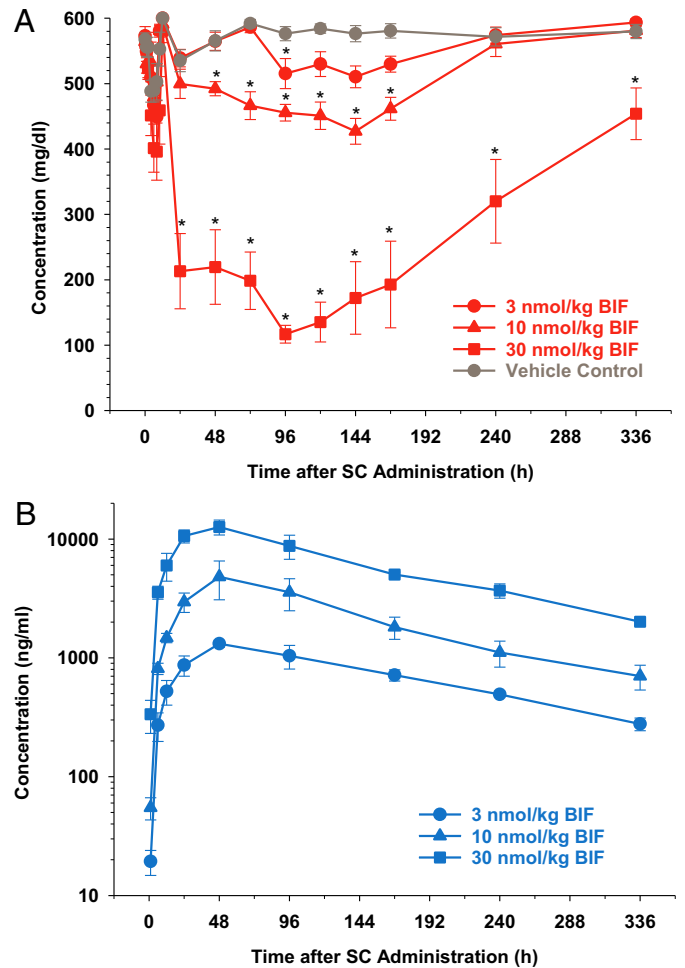Functional activity of BIF as assessed by lipogenesis and cellular proliferation

Ligand	Lipogenesis in 3T3-L1 Adipocytes, EC$_{50}$ nM (S.E.M., n)	Proliferation in Saos-2 Cells, EC$_{50}$ nM (S.E.M., n)	Proliferation in H4IIE Cells, EC$_{50}$ nM (S.E.M., n)
BIF	19 (4, $n = 4$)	134 (27, $n = 8$)	20 (2, $n = 6$)
Human insulin	0.27 (0.08, $n = 4$)	1.8 (0.1, $n = 19$)	0.26 (0.06, $n = 5$)
AspB10 insulin	0.15 (0.04, $n = 4$)	0.50 (0.05, $n = 15$)	0.13 (0.03, $n = 5$)
IGF-1	0.13 (0.01, $n = 4$)	0.16 (0.02, $n = 18$)	14 (1, $n = 5$)

EC$_{50}$ values are geometric means. n, number of independent experiments.
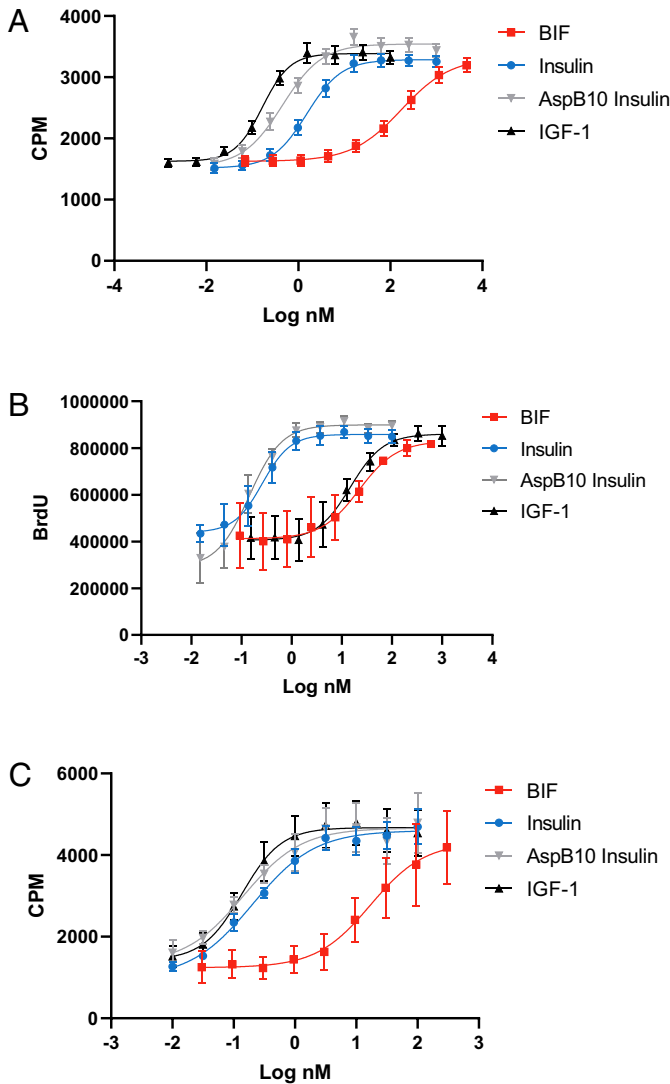
Fig. 5. Functional activity of BIF demonstrating reduced potency in mitogenicity and lipogenesis assays. The mitogenic potential of BIF was determined by measuring [³H]-thymidine or BrdU incorporation into the newly synthesized cellular DNA of proliferating SAOS-2 human osteosarcoma cells (A) or H4IIE rat hepatoma cells (B). Lipogenesis was determined by incorporation of D-(U-^{14}C)-glucose into triglycerides in 3T3-L1 murine adipocytes (C). Curves are representative and were fit using four parameter nonlinear logistic equations using GraphPad Prism Software (v8.4.3). Data points are the average of independent assay runs with error bars represented as the S.E.M. Data from the following number of replicates per assay are shown: SAOS-2 assay (BIF, $n = 8$; human insulin, $n = 8$; AspB10 insulin, $n = 8$; IGF-1, $n = 8$); H4IIE cell assay (BIF, $n = 3$, human insulin, $n = 5$, AspB10 insulin, $n = 5$, IGF-1, $n = 5$); and 3T3-L1 cell assay (BIF, $n = 2$; human insulin, $n = 4$; AspB10 insulin, $n = 4$; IGF-1, $n = 4$). BIF, squares; human insulin, circles; AspB10 insulin, inverted triangles; and IGF-1, triangles.

Fig. 6. The PD (A) and PK (B) of BIF observed in STZ-treated rats after administration of 3, 10, and 30 nmol/kg of BIF via subcutaneous administration. Pharmacokinetic data represent mean and S.D. of $n = 3$. Glucose data represent mean and S.E. of $n = 6$. *$P < 0.05$ for BIF treatment versus vehicle control.

An important consideration in the development of a novel insulin receptor agonist is the characterization of the biologic properties of the molecule on hIR and hIGF-1R. To assess metabolic and mitogenic potential, BIF was characterized in a battery of cellular assays for functional metabolic and mitogenic activity. Notably, BIF exhibited selective phosphorylation activity, favoring hIR-B over hIR-A. Literature suggests that hIR-A plays a predominant role in prenatal growth and development; whereas hIR-B plays a predominant role in metabolic regulation, with the latter being the dominate activity

required of a basal insulin, i.e., fasting conditions (Belfiore et al., 2017). Thus, the differential signaling exhibited by BIF on the IR-B isoform may support a preferred metabolic profile apropos for exogenously administered basal insulin. With regards to mitogenic activity mediated through the IGF-1R, the mutations introduced into the SCI of BIF concomitantly weaken IGF-1R engagement to a point that precluded attaining accurate binding and activity measurements, suggesting a bias for IR signaling (Table 1) under physiologic concentrations. Notably, the rapid dephosphorylation of the IR (Fig. 4) alleviates concerns of sustained IR signaling contributions to mitogenicity.

The in vivo characterization demonstrated that the fusion of a SCI to the Fc-domain of an IgG2 delays the time at max concentration (T_{max}) after subcutaneous injection compared with insulin lispro (48 ± 0 hours for BIF in the present study versus 0.4 ± 0.2 hours for insulin lispro in a previous study (Owens et al., 2016), consistent with a shift from a predominantly capillary absorption process observed with rapid-acting insulins (Charman et al., 2001) to a slower, lymphatic absorption process observed with antibodies and Fc-fusion proteins (Liu, 2018). In addition to the effects on absorption rate, the presence of the Fc leads to a dramatic decrease in the clearance of

TABLE 4
PK parameters of BIF in STZ-treated diabetic Sprague-Dawley rats in a dose-response study

	STZ-Treated Diabetic Sprague-Dawley Rats, N = 3, Mean ± S.D.		
	Insulin Receptor ELISA		
Route	SC	SC	SC
Dose (nmol/kg)	3	10	30
$AUC_{0-\infty}$ (µg*hr/ml)	286 ± 30	808 ± 208	2331 ± 115
C_{max} (µg/ml)	1.31 ± 0.08	4.81 ± 1.73	12.6 ± 1.81
T_{max} (hr)	48 ± 0	48 ± 0	48 ± 0
CL or CL/F (ml/hr per kg)	0.70 ± 0.08	0.85 ± 0.20	0.85 ± 0.04
$t_{1/2}$ (hr)	128 ± 10	104 ± 4	120 ± 21

AUC_{0-inf}, area under the curve from 0 to infinity; CL/F, apparent clearance; C_{max}, maximal concentration (for IV administration C_{max} is extrapolated concentration at time 0); SC, subcutaneous; ; $t_{1/2}$, half-life; T_{max}, time at maximum concentration.

BIF relative to insulin lispro in rats, with the apparent CL of BIF being ~0.8 ml/h per kg, compared with ~3000 ml/h per kg for insulin lispro (Owens et al., 2016). This >3000-fold difference is attributed to the ability of BIF to exploit the FcRn recycling system, minimize renal clearance, and attenuate RME. It should be noted that the BIF measured in the plasma was functionally active, based on the receptor-based ELISA assay employed. Collectively, these PK alterations imbue BIF with a markedly longer duration of action in rats, compared with insulin lispro, with BIF effects spanning 14 days following a single subcutaneous dose, compared with a few hours following a single subcutaneous dose of insulin lispro. This sustained time action supported QW dosing in humans (Frias et al., 2021; Heise et al., 2021).

Based on antibody research, hydrodynamically large and polar molecules like BIF transit very slowly across the vascular endothelium, from the blood to the interstitial space of tissues, via a process referred to as convection (Richter and Jacobsen, 2014); thus, it is plausible that BIF also exploits this mechanism. Furthermore, with antibodies, the concentration in the interstitial fluid of tissues can be substantially reduced relative to plasma concentrations because of the slow rate of convective uptake and faster target-mediated elimination from the tissue. Biodistribution studies with nonbinding IgG demonstrate a tissue-to-blood ratio in the range of 0.04 to 0.68 (Lobo et al., 2004; Shah and Betts, 2013). Moreover, studies with nonbinding antibodies across multiple species have been used to establish antibody biodistribution coefficients for tissues relative to plasma (Shah and Betts, 2013). In tissues relevant to glucose control, nonbinding antibody concentrations relative to plasma in gluconeogenic organs, e.g., kidney and liver, were 14% and 12%, respectively, and in critical glucose disposal tissues, e.g., adipose and muscle, 5% and 4%, respectively. This work has been expanded to include antibody fragments with different molecular size (Li et al., 2016), and molecules of ~60 kDa demonstrate similar biodistribution to the kidney and liver. Further data would be needed to determine if BIF shows a similar profile, but this suggests that the vascular compartment may act as a circulating reservoir for BIF, wherein BIF exploits a slow convection process to regulate diffusion across the vascular endothelium to tissues, which, together with the attenuated insulin receptor engagement, appears to provide appropriate control of peripheral basal activity to safely control glucose with QW administration.

This work demonstrates that BIF's Fc-fusion time extension strategy and insulin engineering can be exploited for extending the PK/PD profile needed for the creation of QW basal insulin with a viable activity profile.

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