

# Protected Graft Copolymer (PGC) Basal Formulation of Insulin as Potentially Safer Alternative to Lantus® (Insulin-Glargine): A Streptozotocin-Induced, Diabetic Sprague Dawley Rats Study

Sandra Reichstetter · Gerardo M. Castillo · ManShun Lai · Akiko Nishimoto-Ashfield · Aryamitra Banerjee · Alexei Bogdanov · Alexander V. Lyubimov · Elijah M. Bolotin

Received: 28 July 2011 / Accepted: 5 December 2011 / Published online: 28 December 2011  
© Springer Science+Business Media, LLC 2011

## ABSTRACT

**Purpose** To develop a long-acting formulation of native human insulin with a similar pharmacodynamics (PD) profile as the insulin analogue insulin glargine (Lantus®, Sanofi-Aventis) with the expectation of retaining native human insulin's superior safety profile as insulin glargine is able to activate the insulin-like growth factor I (IGF-I) receptor and is linked to a number of malignancies at a higher rate than regular human insulin.

**Methods** Development of protected graft copolymer (PGC) excipients that bind native human insulin non-covalently and testing blood glucose control obtained with these formulations in streptozotocin-induced diabetic Sprague Dawley rats compared to equally dosed insulin glargine.

**Results** PGC-formulations of native human insulin are able to control blood glucose to the same extent and for the same amount of time after s.c. injection as the insulin analogue insulin glargine. No biochemical changes were made to the insulin that would change receptor binding and activation with their possible negative effects on the safety of the insulin.

**Conclusion** Formulation with the PGC excipient offers a viable alternative to biochemically changing insulin or other receptor binding peptides to improve PD properties.

**KEY WORDS** basal · drug delivery · excipient · human insulin · long-acting · nanocarrier · peptide · PGC · protected graft copolymer

## ABBREVIATIONS

BG blood glucose  
NPH Neutral Protamine Hagedorn insulin  
PGC Protected graft copolymer  
STZ streptozotocin

## INTRODUCTION

Insulin replacement therapy is the primary treatment for Type 1 diabetes and is also often used for Type 2 diabetes. The goal of insulin replacement therapy is to mimic the natural insulin secretion of a healthy individual's pancreas. Between meals, the pancreas of healthy people secretes a low and constant amount (basal secretion). Insulin secretion spikes after a meal to accommodate the intake in carbohydrates (prandial insulin secretion). In diabetic patients, this pattern is mimicked by the use of fast-acting (prandial) insulin with or shortly before meals, and a long-acting form of insulin once, or several times a day (1–5). The advent of short acting and fast-absorbing insulin analogues like insulin aspart and insulin lispro in the last decade has improved post-prandial glucose control (1,3,4). Basal insulin requirements are often still being addressed with the more than 50-year old protamine containing formulation Neutral Protamine Hagedorn (NPH) insulin that lasts up to 16 h in humans and has a pronounced peak of action between 4 and 6 h after injection. This profile of action increases the risk of hypoglycemia, in particular nocturnal hypoglycemia, but can also lead to hyperglycemia later in the night if NPH is injected at bed time (4). Insulin detemir, a fatylated analogue that binds to serum albumin thus extending its terminal half-life, is given

S. Reichstetter (✉) · G. M. Castillo · M. Lai · A. Nishimoto-Ashfield · E. M. Bolotin (✉)

PharmalN Corporation 720 Broadway Seattle, Washington 98122, USA  
e-mail: sreichstetter@pharmain.com  
e-mail: ebolotin@pharmain.com

A. Banerjee · A. V. Lyubimov  
Toxicology Research Laboratory, Department of Pharmacology College of Medicine, University of Illinois at Chicago 808 South Wood Street, Room 1306 Chicago, Illinois 60612, USA

A. Bogdanov  
University of Massachusetts Medical School  
Worcester, Massachusetts, USA

twice a day, while insulin glargine, an analogue that is less soluble than regular human insulin at physiologic pH, builds a depot in the injection site and allows once daily s.c. dosing. Both insulin detemir and insulin glargine show a more even activity profile over the course of the day for basal insulin replacement (6). The ability of insulin glargine to activate the insulin-like growth factor 1 receptor (IGF-1R) more strongly than regular human insulin (7–12) together with recent controversial reports that glargine use is associated with higher rates of breast cancer and other cancers (13–28), highlight the need for an insulin formulation that provides comparable efficacy as insulin glargine, but does not pose an increased cancer risk compared to regular human insulin. Here, we report new nanocarrier formulations of regular human insulin that show comparable blood glucose control to insulin glargine in streptozotocin (STZ)-induced diabetic rats.

The nanocarriers used in these formulations are the next generation of the Protected Graft Copolymer (PGC) initially developed by Bogdanov *et al.* for imaging (29). The PGC carriers used to formulate insulin contain a polylysine backbone, protective polyethylene-glycol side chains, and side chains that facilitate non-covalent insulin binding to the carrier. In the case of the PGC-insulin formulations presented here these side chains consist of polyethylenimine (PEI) and a chelating moiety to allow insulin binding through a Zn bridge. These side chains can, however, be modified to allow binding of a wide variety of therapeutic payloads from peptides and proteins to nucleic acids.

## MATERIALS AND METHODS

### PGC Carrier Synthesis

#### Carrier 605c

For carrier synthesis, 6 g 20 kDa Polylysine (20PL, SAFC, St. Louis MO) was dissolved in 135 ml 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Fisher Scientific, Waltham, MA). This was followed by addition of 9 mmol Methoxy-PEG-carboxymethyl (MPEG-CM, Mw=5000; Laysan Bio, Arab, AL) dissolved in 80% ethanol with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, Pierce, Rockford, IL) pH=4.7. 91.6 mmol N-hydroxysuccinimide (NHS, Acros, Morris Plains, NJ). Next, 23.5 mmol 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide HCl (EDC, Sigma, St. Louis MO) were added. After 20 min incubation, this solution was added to the 20PL solution and adjusted to pH 7.1 with NaOH. The amount of free amino groups of the PL was measured by TNBS assay (2,4,6-trinitrobenzenesulfonic acid, Fluka, St. Louis MO) as previously described (30) as a measure of saturation of the PL with PEG-side chains. When

a free amino concentration indicating 54% PEG saturation was reached, 20 mmol succinic anhydride (Sigma, St. Louis MO) was added, followed by 600ul of triethylamine (TEA, Sigma, St. Louis MO). The pH was re-adjusted to 7.1 with NaOH and the mixture was stirred at room temperature until free amino groups were undetectable by TNBS assay (4 h). The reaction mixture was concentrated and washed with 10 changes of water in a 100,000 MWCO ultrafiltration cartridge (UFP-100-E-5A, GE Healthcare, Waukesha, WI). The cartridge was washed with 2×50 ml water to collect the remaining sample. After filtration using a 0.2 µm filter, the sample was lyophilized; 7.7 g of the lyophilized PEG-ylated 20PL was dissolved in 30 ml MES and 260 mg NHS was added. After addition of 1200 mg EDC the pH was adjusted to pH 4.7 with HCl. The mixture was incubated for 20 min and 50 ml Polyethyleneimine MW 1200 Da (PEI12) was dissolved in 20 ml of 1 M HEPES and adjusted to pH7.4 with HCl, added to the PEG-ylated PL and incubated for 2 h. The pH was adjusted to pH 5.0 with HCl and 1200 mg EDC was added. After 20 min incubation the pH was adjusted to 7.2 with NaOH. The reaction mixture was concentrated and washed with 10 changes of water in a 100,000 MWCO ultrafiltration cartridge (UFP-100-E-5A, GE Healthcare, Waukesha, WI). The cartridge was washed with 2×50 ml water to collect the remaining sample. After filtration using a 0.2 µm filter, the sample was lyophilized. 2 g of the samples were dissolved in 30 ml 1 M HEPES and 2 g succinic anhydride was added. The reaction was slowly titrated with 10 N NaOH to pH 7.1 and stirred for 2 h. The reaction mixture was washed with 10 changes of water in 100,000 MWCO ultrafiltration cartridge (UFP-100-E-5A). Cartridge was washed with 50 ml water to collect the remaining sample. 2 g of this sample was dissolved in 20 ml 2 mM MES and 160 mg NHS was added, followed by 650 mg EDC. The pH was maintained at 5.4 during a 20 min incubation. 10 ml of a 15µM N-alpha,N-alpha,-Bis(carboxymethyl)-L-Lysine (NTA, Sigma, St. Louis MO) solution in 1 M HEPES containing 0.32 M ZnCl was added and during the 2 h incubation the pH was maintained at 7.1 with NaOH. After 2 h the pH was adjusted to 5 with HCl, 650 mg EDC was added and incubated for 20 min. The pH was re-adjusted to 7.1 and the reaction was incubated for another hour. The sample was filtered using 0.2 µm filter and lyophilized.

#### Carriers 421a and 421b

Syntheses were identical to that of carrier 605c except that 10 kDa PEG was used instead of 5 kDa PEG, and 400 Da PEI (421a), or 800 Da PEI (421b), respectively, were used instead of 1200 Da PEI. No zinc was added to 421b.

## Loading of Carriers with Insulin and Binding to PGC-Carriers

Carriers were loaded with insulin at 10% w/w ratio by mixing 1 mg of insulin with 10 mg of carrier in the presence of 500 nmol ZnCl in 0.1 M ammonium acetate (Sigma, St. Louis MO). The reactions were incubated for 120 min at room temperature. The formulations were subsequently frozen at  $-80^{\circ}\text{C}$ , lyophilized, and stored as a dry powder at  $-20^{\circ}\text{C}$  until use.

For binding experiments, insulin was dissolved at 2.5 mg/ml in 100 mM glycine HCl at pH 7.0 in quadruplicates. Carriers were dissolved in the same solution at 50 mg/ml according to a loading percentage of 10%. The mixtures were incubated for 2 h at room temperature. A 12 min centrifugation at a relative centrifugal force of  $18000 \times g$  did not produce any visible precipitate. The mixtures were filtered using 100 kDa MWCO cellulose membrane centrifuge filter (Amicon Ultra Ultracel 100 k, Millipore, Billerica, MA) by centrifuging at  $18,000 \times g$  for 12 min. This filter retains the carrier and insulin that is bound to the carrier. Free insulin in the filtrate was analyzed using HPLC (Waters, Milford, MA) and expressed as percentage of total insulin. The following HPLC protocol was used: Solvent A: 5% Acetonitrile (Fisher Scientific, Waltham, MA) in water with 0.1% Tetrafluoroacetic acid (TFA Fisher Scientific, Waltham, MA); Solvent B: 100% Acetonitrile with 0.1% TFA. Column: Synergi 2.5  $\mu\text{m}$  Max-RP (20 $\times$ 4 mm) 100A beads; Mercury (Phenomenex, Torrance, CA), flow: 1.5 ml/min; 10  $\mu\text{l}$  injection. Gradient: 0.0–0.5 min: 0.0%B–0.0%B; 0.5–1.0 min: 0.0%B–10%B, 1.0–5.0 min: 10%B–50%B; 5.0–5.5 min: 50%B–99%B, 5.5–6.0 min: 99%B–0.0%B. Monitoring and quantification at 220 nm, retention time: 3.0 min. A standard curve was used for quantification of peak area under the curve (AUC).

## Animal Experiments

### Animals

Male Sprague Dawley rats, 8–9 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in a SPF facility at 12 h/12 h light/dark cycle and had access to food and water *ad libitum*.

### Diabetes Induction

The rats were fasted for 4 h and then treated with 100 mg/kg STZ in saline at a concentration of 100 mg/ml, given *i.p.* Two hours after STZ treatment the rats were provided with food *ad libitum*. Blood glucose (BG) was measured starting 3 days after STZ treatment using 2  $\mu\text{l}$  blood from a tail prick and a

Bayer Contour handheld glucometer (Bayer HealthCare, Mishawaka, IN). Rats that were not diabetic (BG > 16 mmol/l on two consecutive days) 5 days after STZ treatment were euthanized.

### Insulin Dosing and Blood Glucose Measurement

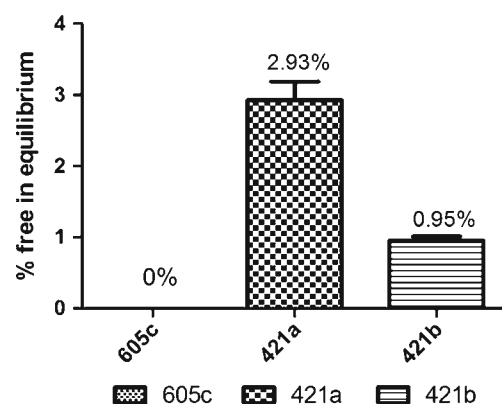
Diabetic rats ( $n=12$ ) were treated with 1 mg/kg (21.98 U/kg) insulin glargine by *s.c.* injection. BG was measured at  $t=0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20,$  and 24 h. After a 2 day washout period the animals were dosed again with 0.5 mg/kg (10.99 U/kg) insulin glargine.

To decrease the stress from blood draws for BG measurements the animals were divided into two subsets of 6 animals each to test two PGC-insulin formulations consecutively after a 48 h washout period after each dosing at doses of 0.5 (10.99 U/kg) and 1 mg/kg (21.98 U/kg). BG was measured at time points  $t=0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20,$  and 24 h. After the dosing with the two PGC formulations per subset, all 12 animals were dosed once again with insulin glargine at 1 mg/kg by *s.c.* injection. BG was measured at  $t=0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20,$  and 24 h. After a 2 day washout period the animals were dosed again with 0.5 mg/kg insulin glargine. The BG values for the insulin glargine measurement series before and after the PGC-insulin formulations were averaged.

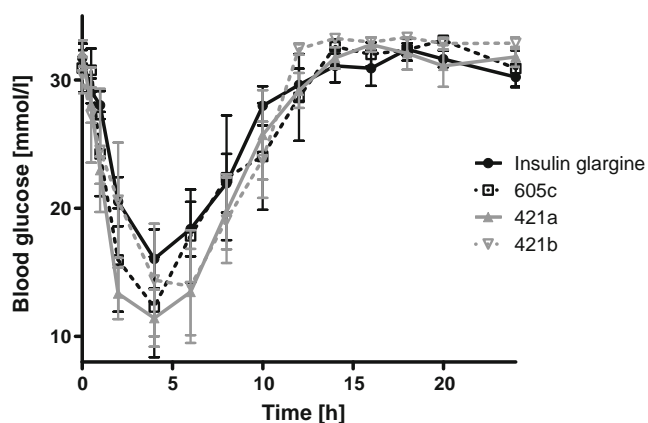
## RESULTS

### Binding of Insulin to PGC-Carriers

All three carriers are off-white powders that are completely soluble in an aqueous medium and form a slightly



**Fig. 1** Amount of free insulin in equilibrium in a solution of PGC-formulation 605c, 421a, or 421b with 10% loading at 2.5 mg/ml insulin. Free insulin was detected by HPLC in the filtrate after filtering through a 100 kDa molecular weight filter which retains PGC carrier. Insulin was below the detection limit in the formulation with carrier 605c. The experiment was performed in quadruplicates.



**Fig. 2** Blood glucose curves after s.c. injection of 1 mg/kg regular human insulin ( $t=0$  h) formulated with PGC-nanocarrier 605c ( $n=5$ , open square), 421a ( $n=6$ , gray filled triangle), and 421b ( $n=5$ , open triangle), and insulin glargine ( $n=23$ , filled circle). Error bars show standard error mean.

viscous, clear solution in water or saline. To determine the amount of free insulin in a solution of the PGC-formulations with 10% loading, the formulations were dissolved at an insulin concentration of 2.5 mg/ml and filtered through a molecular weight filter that retains the PGC-carrier. Insulin was detected in the filtrate by HPLC and revealed less than 3% free insulin in all formulations: undetectable amounts in carrier 605c, 2.93% free in the filtrate of 421a, and 0.95% free insulin in the filtrate of 421b. These results indicate strong binding to the carriers for all formulations (Fig. 1).

### Blood Glucose After Insulin Glargine and PGC-Insulin

Diabetes was induced in Sprague Dawley rats by one time s.c. injection of 100 mg/kg STZ. One week after STZ treatment, 12 diabetic rats (rats with non-fasting blood glucose of  $>16$  mmol/l 2 days in a row) were treated with 1 mg/kg and 0.5 mg/kg insulin glargine, and blood glucose was monitored for 24 h. After a 2 day washout period each time, the animals were consecutively dosed with 1 mg/kg and 0.5 mg/kg insulin formulated with each carrier 605c, 421a, 421b, and one more time with 1 mg/kg and

0.5 mg/kg insulin glargine. The non-fasting blood glucose values averaged slightly above 30 mmol/l at the start of all glucose curves immediately before dosing with insulin (Fig. 2 and Table I). After the s.c. bolus dose, either as insulin glargine or regular human insulin in a PGC formulation, blood glucose dropped sharply. At the insulin dose of 1 mg/kg the lowest point of blood glucose was reached 4 h after dosing and averaged between 10 and 16 mmol/l (Fig. 2). After 4 h the blood glucose levels began to rise slowly, reaching pre-dosing levels between 12 and 14 h after dosing. Formulation 421a seemed to act slightly faster than insulin glargine with significantly lower blood glucose levels 1 and 2 h after dosing (Fig. 2 and Table I). The blood glucose lowering effect of this formulation lasted as long as the other formulations or insulin glargine and there was no significant difference between the action of insulin glargine and any of the three long-acting formulations of regular human insulin at any other time points (Table I). The blood glucose curves with the lower 0.5 mg/kg dose are very similar to the 1 mg/kg dose. Again, formulation 421a showed a slightly faster profile: the maximum insulin action of the formulation with carrier 421a is reached after 2 h with a significantly lower blood glucose level compared to insulin glargine (Fig. 3 and Table II). At this dose the insulin lowering action of formulation 421a is slightly shorter than that of insulin glargine, which shows significantly lower blood glucose levels at 10 h compared to 421a (Fig. 3 and Table II). Glucose levels reach a later minimum when formulation 421b is used at the 0.5 mg/kg dose (Fig. 3), but the difference compared to insulin glargine is not statistically significant (Table II). Insulin action again lasts 12–14 h in insulin glargine, and formulations 605c, and 421b, before blood glucose levels return to before-dosing levels and, other than the 2 and 10 h time points with formulation 421a, no statistically significant differences compared to insulin glargine were detected at the 0.5 mg/kg dose.

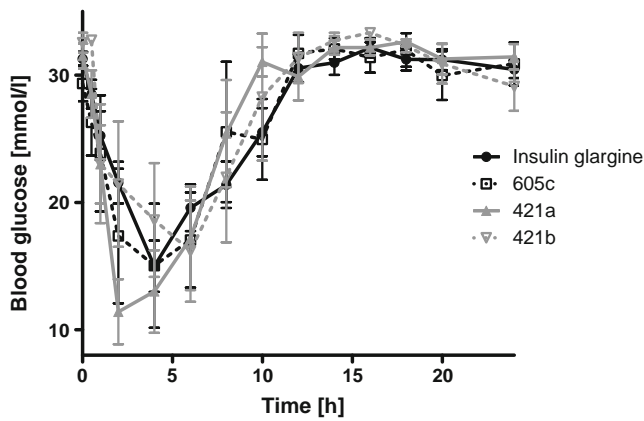
### DISCUSSION

Development of a basal, long-acting alternative to currently available long-acting insulin analogues, with identical

**Table I** P-values of Comparison of Blood Glucose Measurements Between Insulin Glargine and PGC-Formulations

	Time [h]													
	0	0.5	1	2	4	6	8	10	12	14	16	18	20	24
Insulin glargine														
vs 605c	0.71 <sup>a</sup>	0.44	0.32	0.35	0.44	0.90	0.94	0.41	0.80	0.29	0.52	0.97	0.31	0.70
vs 421a	0.77	0.52	0.004	0.02	0.17	0.25	0.56	0.57	0.81	0.69	0.22	0.82	0.77	0.18
vs 421b	0.43	0.64	0.51	0.98	0.75	0.39	0.50	0.25	0.32	0.11	0.16	0.37	0.15	0.15

<sup>a</sup>p-values of two-tailed t-test of average blood glucose values after insulin glargine vs. formulation 605c, 421a, and 421b at a dose of 1 mg/kg



**Fig. 3** Blood glucose curves after s.c. injection of 0.5 mg/kg regular human insulin (t=0 h) formulated with PGC-nanocarrier 605c (n=5, open square), 421a (n=6, gray filled triangle), and 421b (n=5, open triangle), and insulin glargine (n=23, filled circle). Error bars show standard error mean.

receptor activation properties as regular human insulin that allows once daily dosing, continues to be a challenge (4). Similar problems hamper the development of many biologically active peptides and proteins as therapeutics for human diseases. While covalent peptide PEGylation can increase the half-life of the peptide *in vivo*, it often comes at the expense of bioactivity (31,32). Another approach is to derivatize the peptide or protein with a hydrophobic side chain to enable albumin-binding as has been done in the case of insulin detemir (4,6). This can lead to significant improvement of the half-life of the peptide or protein *in vivo*, however, bioactivity can be reduced, e.g. affinity of insulin detemir for the insulin receptor is reduced by about 50% compared to regular human insulin (7). To avoid loss of bioactivity incurred by associating insulin with bulky molecules, efforts have been made to improve the pharmacokinetic properties by targeted changes in the amino acid sequence of insulin. These approaches, as illustrated by insulin glargine, solve the issue of lower bioactivity (2,3,33–36), but come with their own risks. Changing the biochemical properties of the peptide or protein by changing or adding specific target amino acids can sometimes

significantly impact pharmacokinetics. Insulin glargine, the longest-acting FDA-approved insulin analogue, is a good example of this technology. Compared to regular human insulin it has two additional arginine residues at the C-terminus that shifts the isoelectric point from 5.4 to 6.7 resulting in glargine being insoluble at physiologic pH so that it forms a precipitate that acts as depot at the injection site upon s.c. injection (33). Insulin glargine also contains a modification at the asparagine residue 21 in the alpha-chain to prevent dimerization due to the acidic pH of the formulation. This residue is replaced by glycine in insulin glargine. While these modifications allow a once daily dosing to provide basal insulin in humans they also change binding of insulin glargine to the human insulin receptor as well as its close relative the IGF-1 receptor which has been shown to increase the growth of a tumor cell line *in vitro* (7–12). While reports about increased cancer incidence on insulin glargine therapy are controversial, safety concerns remain (13–28).

Here, we report the development of three different long-acting formulations of regular human insulin using PGC nanocarriers that use a completely different strategy. The native protein, in this case regular human insulin, is not modified in any way. Instead, a polymer-based nanocarrier is synthesized that allows non-covalent binding of the peptide or protein payload. The PGC carriers consist of a backbone (e.g. polylysine) that is grafted with protective PEG side chains, as well as other side chains that facilitate the binding of the payload peptide or protein. In the case of regular human insulin as a payload, the binding is facilitated through the zinc-binding properties of insulin. Other carriers create a hydrophobic environment in their core for the binding of hydrophobic peptides or can contain cationic or anionic charges for the binding of charged peptides. The PGC formulations of regular human insulin contain a low level of free insulin *in vitro* indicating strong binding. *In vivo*, the formulations form a reservoir of bound insulin in the blood stream establishing a thermodynamic equilibrium with the free insulin. If free insulin is removed from the bloodstream (mostly by

**Table II** P-values of Comparison of Blood Glucose Measurements Between Insulin Glargine and PGC-Formulations

Insulin glargine	Time [h]													
	0	0.5	1	2	4	6	8	10	12	14	16	18	20	24
vs 605c	0.25 <sup>a</sup>	0.47	0.79	0.48	0.99	0.57	0.51	0.88	0.54	0.40	0.60	0.65	0.53	0.82
vs 421a	0.85	1.00	0.549	0.01	0.61	0.61	0.41	0.02	0.76	0.29	0.98	0.16	0.97	0.43
vs 421b	0.22	0.10	0.68	0.98	0.49	0.47	0.92	0.63	0.75	0.14	0.13	0.58	0.86	0.44

<sup>a</sup>p-values of two-tailed t-test of average blood glucose values after insulin glargine vs. formulation 605c, 421a, and 421b at a dose of 0.5 mg/kg



receptor binding (1–4)), additional insulin is released from the nanocarrier reservoir. This way, the PGC formulations of regular human insulin provide blood glucose lowering action similar to the long-acting insulin analogue insulin glargine in STZ-induced diabetic Sprague Dawley rats, making it reasonable to assume similar activity in humans.

## CONCLUSION

PGC nanocarriers provide a new way to prolong the *in vivo* bioactivity of peptide and protein drugs such as regular human insulin. Binding to the nanocarrier is accomplished by the zinc-binding properties of insulin and leads to insulin formulations that provide blood glucose control in diabetic rats similar to a equivalent dose of the long-acting insulin analogue, insulin glargine without the need for modification of the insulin load molecule. Future goals will be to determine the toxicological properties of the PGC-insulin formulations and to do trials in humans.

## ACKNOWLEDGEMENTS & DISCLOSURES

The work described in this publication has been supported by the NIH/NIDDK SBIR grant 5R44 DK069727-04 and 3R44DK069727-04S1. We also thank Ms. Cynthia C. Jones for expert help in editing and proof reading the manuscript.

Sandra Reichstetter, Man Shun Lai, Akiko Nishimoto-Ashfield, Gerardo Castillo, and Elijah Bolotin are paid employees and stock option holders of PharmaIN Corporation. Alexei Bogdanov is a shareholder of PharmaIN Corporation.

## REFERENCES

- Bolli GB, et al. Insulin analogues and their potential in the management of diabetes mellitus. *Diabetologia*. 1999;42(10):1151–67.
- Campbell RK, et al. Insulin glargine. *Clin Ther*. 2001;23(12):1938–57. discussion 1923.
- Heise T, Heinemann L. Rapid and long-acting analogues as an approach to improve insulin therapy: an evidence-based medicine assessment. *Curr Pharm Des*. 2001;7(14):1303–25.
- Hirsch IB. Insulin analogues. *N Engl J Med*. 2005;352(2):174–83.
- McKeage K, Goa KL. Insulin glargine: a review of its therapeutic use as a long-acting agent for the management of type 1 and 2 diabetes mellitus. *Drugs*. 2001;61(11):1599–624.
- Porcellati F, et al. Comparison of pharmacokinetics and dynamics of the long-acting insulin analogs glargine and detemir at steady state in type 1 diabetes: a double-blind, randomized, crossover study. *Diabetes Care*. 2007;30(10):2447–52.
- Kurtzhals P, et al. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes*. 2000;49(6):999–1005.
- Le Roith D. Insulin glargine and receptor-mediated signalling: clinical implications in treating type 2 diabetes. *Diabetes Metab Res Rev*. 2007;23(8):593–9.
- Staiger K, et al. Comparison of the mitogenic potency of regular human insulin and its analogue glargine in normal and transformed human breast epithelial cells. *Horm Metab Res*. 2007;39(1):65–7.
- Liefvendahl E, Arnqvist HJ. Mitogenic effect of the insulin analogue glargine in malignant cells in comparison with insulin and IGF-I. *Horm Metab Res*. 2008;40(6):369–74.
- Mayer D, Shukla A, Enzmann H. Proliferative effects of insulin analogues on mammary epithelial cells. *Arch Physiol Biochem*. 2008;114(1):38–44.
- Weinstein D, et al. Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells. *Diabetes Metab Res Rev*. 2009;25(1):41–9.
- Bloomgarden Z, Handelsman Y. Cancer mortality and insulin glargine. *J Diabetes*. 2009;1(3):131–3.
- Colhoun HM. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia*. 2009;52(9):1755–65.
- Currie CJ, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia*. 2009;52(9):1766–77.
- Dejgaard A, et al. No evidence of increased risk of malignancies in patients with diabetes treated with insulin detemir: a meta-analysis. *Diabetologia*. 2009;52(12):2507–12.
- Gale EA. Insulin glargine and cancer: another side to the story? *Lancet*. 2009;374(9689):521.
- Garg SK, Hirsch IB, Skyler JS. Insulin glargine and cancer—an unsubstantiated allegation. *Diabetes Technol Ther*. 2009;11(8):473–6.
- Hemkens LG, et al. Insulin glargine and cancer. *Lancet*. 2009;374(9703):1743–4. author reply 1744.
- Hemkens LG, et al. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia*. 2009;52(9):1732–44.
- Home PD, Lagarenne P. Combined randomised controlled trial experience of malignancies in studies using insulin glargine. *Diabetologia*. 2009;52(12):2499–506.
- Idris I. Observational registry database studies link insulin glargine with cancer risk. *Diabetes Obes Metab*. 2009;11(9):910–2.
- Jonasson JM, et al. Insulin glargine use and short-term incidence of malignancies—a population-based follow-up study in Sweden. *Diabetologia*. 2009;52(9):1745–54.
- Pocock SJ, Smeeth L. Insulin glargine and malignancy: an unwarranted alarm. *Lancet*. 2009;374(9689):511–3.
- Rosenstock J, et al. Similar risk of malignancy with insulin glargine and neutral protamine Hagedorn (NPH) insulin in patients with type 2 diabetes: findings from a 5 year randomised, open-label study. *Diabetologia*. 2009;52(9):1971–3.
- Dawson LK, Hamilton LA. Risk of cancer in patients receiving insulin glargine. *Am J Health Syst Pharm*. 2010;67(23):2025–31.
- Hernandez-Diaz S, Adami HO. Diabetes therapy and cancer risk: causal effects and other plausible explanations. *Diabetologia*. 2010;53(5):802–8.
- Nagel JM, et al. Insulin resistance and increased risk for malignant neoplasms: confounding of the data on insulin glargine. *Diabetologia*. 2010;53(1):206–8.
- Bogdanov Jr AA, et al. A new macromolecule as a contrast agent for MR angiography: preparation, properties, and animal studies. *Radiology*. 1993;187(3):701–6.
- Habeeb AFSA. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem*. 1966;14(3):328–36.

31. Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv Drug Deliv Rev.* 2003;55(10):1261–77.
32. Fishburn CS. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. *J Pharm Sci.* 2008;97(10):4167–83.
33. Bolli GB, Owens DR. Insulin glargine. *Lancet.* 2000;356(9228):443–5.
34. Gillies PS, Figgitt DP, Lamb HM. Insulin glargine. *Drugs.* 2000;59(2):253–60. discussion 261–2.
35. Levien TL, *et al.* Insulin glargine: a new basal insulin. *Ann Pharmacother.* 2002;36(6):1019–27.
36. Reinhart L, Panning CA. Insulin glargine: a new long-acting insulin product. *Am J Health Syst Pharm.* 2002;59(7):643–9.