

Novel insulin analogues and its mitogenic potential

Ivana Zib and Philip Raskin

Department on Internal Medicine, Division of Endocrinology and Metabolism, University of Texas Southwestern Medical Center, Dallas, TX, USA

Abstract: Insulin analogues were developed to modify the structure of the human insulin molecule in order to more accurately approximate the endogenous secretion of insulin. With the help of recombinant technology and site-directed mutagenesis, the insulin molecule can be modified to either delay or shorten absorption time, providing better insulin treatment options and facilitating the achievement of glycaemic goals. Changing the structure of the insulin molecule, however, may significantly alter both its metabolic and mitogenic activity. Multiple factors such as residence time on the receptor, dissociation rate, rate of receptor internalization and the degree of phosphorylation of signalling proteins can affect the mitogenic potencies of insulin analogues.

Changes in the structure of the insulin have raised concern about the safety of the insulin analogues. For example, questions have emerged about the relationship between the use of insulin lispro and insulin glargine and the progression of diabetic retinopathy. Two studies have shown progression of retinopathy with the use of insulin lispro. However, others have not confirmed these results, and causality could not be proven as progression of retinopathy can occur with rapid improvement in glycaemic control, and methods of assessments among studies were not consistent.

Therefore, we examine the metabolic and mitogenic characteristics of the three insulin analogues, insulin lispro, insulin aspart and insulin glargine, that are currently on the market, as well as the two insulin analogues, insulin glulisine and insulin detemir, that are soon going to be available for clinical use.

Keywords: insulin analogues, mitogenicity, metabolic potency, receptor stimulation, safety in pregnancy, retinopathy

Received 31 May 2005; returned for revision 30 September 2005; revised version accepted 3 October 2005

Introduction

After Banting and Best discovered insulin in 1921, insulin became the first major replacement therapy in the treatment of diabetes saving the lives of millions of people (figure 1). But the pharmacokinetics of current insulin therapy does not provide physiologic insulin replacement and does not lead to ideal metabolic control. One of the limitations of insulin therapy is the variability in the absorption rate of insulin from subcutaneous tissue, which largely depends on the size of molecular aggregates. Insulin has a tendency to self-associate into dimers and hexamers in subcutaneous tissue; however, it can only be absorbed through

capillary walls into the circulation when it is in a monomeric form [1,2]. This makes it difficult to accurately reproduce endogenous insulin secretion with a subcutaneous injection. In addition, long-acting insulins do not have linear absorption and lack absolutely flat profiles like endogenous insulin secretion. In contrast, short-acting human insulin is too slow to mimic the normal rapid increments of insulin in the circulation.

Development of insulin analogues

Insulin analogues were developed to modify the structure of the human insulin molecule in order to modify

Correspondence:

Philip Raskin, MD, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-8858, USA.

E-mail:

Philip.Raskin@UTSouthwestern.edu

mitogenesis is very complex. Mitogenic stimulation of insulin analogues is related to residence time on the receptor, dissociation rate [12] and other factors such as receptor internalization and the degree of phosphorylation of signalling proteins [5,13–16].

Rapid-Acting Insulin Analogues

Insulin Aspart B10

Insulin aspart B10 was one of the first analogues proposed for clinical use. The insulin aspart B10 molecule was engineered by exchanging a histidine at position B10 for an aspartic acid.

The amino acid residue at the B10 position is important for affinity to the IGF-1 receptor, and this modification leads to an insulin molecule that more closely resembles IGF-1 [17]. Receptor studies showed that, compared with insulin, the insulin aspart B10 molecule has an increased affinity for both insulin (3.5 times) and IGF-1 (nine times) receptors and increased mitogenic potency nine times (table 1) (12,16).

It was originally thought that this increase in the mitogenic effect of insulin aspart B10 was mainly attributable to its increase affinity for the IGF-1 receptor [12]. The evidence supporting this is that insulin aspart B10 stimulated IGF-1 autophosphorylation with the potency resembling IGF-1 and showed a corresponding degree of DNA synthesis and cell proliferation in aortic smooth muscle cells [17]. On the other hand, it has been found that insulin aspart B10 activates intracellular signalling pathways of both insulin and IGF-1 receptors. Insulin aspart B10 was associated with prolonged cellular processing measured by a prolonged phosphorylation state of the insulin receptor β -subunit, insulin receptor substrate (IRS)-1, IRS-2 and Shc proteins. As a consequence, there was an increase in mitogenic potency [17,18] and a two to four times increase in the metabolic

potency of insulin aspart B10 [19] when compared with human insulin.

In vitro studies showed an increase in mitogenic activity in the pancreatic β -cell lines of mice embryos [20] and in human breast cell lines [5,20]. *In vivo* studies revealed a dose-dependent increase in the incidence of adenocarcinomas and fibroepithelial tumours in female rats after 12 months of therapy with a dose of 12.5–200 U/kg of insulin aspart B10 [21]. It appears that the growth-promoting effect of insulin aspart B10 is most likely related to altered interactions with both insulin and IGF-1 receptors.

Insulin Lispro

Insulin lispro is a rapid-acting insulin analogue that has been produced by reversing the proline and lysine amino acid residues that occupy position 28 and 29 of the B chain of human insulin. This change in structure modifies the spatial orientation of the C-terminus and reduces self-association in solution [22,23], resulting in an absorption and elimination rate that is two times higher compared with human insulin [24].

Insulin lispro has been shown to be safe and effective when used in multiple daily injection and insulin pump therapy [25–28]. Its primary benefit is improvement in postprandial glucose excursions. However, insulin lispro has also been associated with a reduced incidence of hypoglycaemia [29] and increased patient's satisfaction due to the convenience of being able to take the insulin immediately before or after a meal [30].

Metabolic and Mitogenic Characteristics

Insulin lispro receptor affinity and metabolic potency is slightly lower than human insulin in human osteosarcoma cells (Saos/B10) despite a higher affinity for the IGF-1 receptor (table 1) (12). Long-term safety studies on

Table 1 *In vitro* insulin receptor affinity, metabolic and mitogenic potency of insulin analogs relative to human insulin

Insulin type	Insulin receptor affinity	Metabolic potency	IGF-1 receptor affinity	Mitogenic potency (Saos/B10 cells)*
Human	100	100	100	100
Asp B10	205 \pm 20	207 \pm 14	587 \pm 50	975 \pm 173
Lispro	84 \pm 6	82 \pm 3	156 \pm 16	66 \pm 10
Aspart	92 \pm 6	101 \pm 2	81 \pm 9	58 \pm 22
Glargine	86 \pm 3	60 \pm 3	641 \pm 51	783 \pm 13
Detemir	18–46	27	16 \pm 1	11

Asp B10, insulin Aspart B10; IGF-1, insulin-like growth factor 1.

*Human osteosarcoma cells.

Copyright © 2000 American Diabetes Association from Diabetes, Vol. 49, 2000; 999-1005. Reprinted with permission from *The American Diabetes Association*.

animals have not been performed. However, insulin lispro was not carcinogenic in a battery of *in vitro* and *in vivo* genetic toxicity assays [31].

Insulin Lispro Safety during Pregnancy

Reproductive studies on animals showed no effect on fertility or teratogenic effect on the foetus after therapy with insulin lispro at four times the average dose of human insulin based on body surface area [32]. As the only analogue currently used in pregnancy, insulin lispro is the ideal treatment option to control postprandial blood glucose in pregnant women who are suffering from pregnancy-induced vomiting, because it can be injected immediately before or after a meal [33]. Insulin lispro has greater homology with IGF-1 than human insulin (51 vs. 49%) which raised concerns of a growth-promoting effect on the foetus. However, placental transfer studies did not detect insulin lispro in the umbilical cords in infants after intravenous administration of insulin lispro at low dose of 0.2 units/kg/h to mothers during labour [34]. There was only a small dose-dependent transfer across human placentas delivered at term. Insulin lispro was detected in the umbilicus when the mother was exposed to insulin lispro during four continuous hours of IV administration at a high concentration (=580 μ U/ml equivalent to administration of 75 units of insulin lispro) [35]. In another *in vitro* study, a significant amount of analogue did accumulate in the placenta but was not detected in umbilical blood [36].

Congenital anomalies in the offspring of two women taking insulin lispro through pregnancy have been reported. However, no casual relationship has been established [37].

There have been only a few small randomized trials testing insulin lispro in pregnant diabetic women. One such study was an open label multicentre study that randomized 33 well-controlled type one diabetic pregnant women to premeal insulin lispro plus neutral protamine hagedorn (NPH) insulin or premeal regular human insulin with NPH insulin beginning at 15 weeks of gestation. There were no differences in pregnancy outcome, gestational age at delivery, birth weight, neonatal complications and preeclampsia. One malformation (hypospadias) occurred in the human insulin group [38]. Similar results were seen in other studies of pregnant women who were given insulin lispro beginning at the 14th week of gestation [34,39]. A large retrospective study found that congenital abnormalities among woman taking insulin lispro occurred at a rate

comparable with the Diabetes Control and Complication Trial's (DCCT) conventional insulin therapy group [40].

Insulin Lispro and Retinopathy in Pregnancy

Current data suggest that pregnancy may be associated with a higher rate of retinopathy progression. This has been attributed to an increase in retinal blood flow and production of growth factors by the placenta. Patients with established proliferative retinopathy are at a high risk for progression and vision-threatening disease during their pregnancy. Development of new onset and progression of background diabetic retinopathy has also been reported. It usually regresses to baseline levels postpartum, and there does not seem to be a higher risk of retinopathy development in the long term [41].

The initial suggestion that use of insulin lispro was associated with retinal disease came from the reports of Kitzmiller et al. [42]. Fourteen pregnant type one or type two diabetic mothers were started on insulin lispro before or during early pregnancy because of difficulties in controlling plasma glucose with human insulin. Ten of the patients had a negative dilated retinal examination before pregnancy. Three of the patients who started insulin lispro after 7 weeks of pregnancy progressed from no disease at all to bilateral proliferative retinopathy, and two developed vitreous haemorrhages.

However, two of these patients had poorly controlled diabetes at baseline with HbA1c levels that were 1, 3.7 and 3.5% above normal and experienced a 2.2–3.6% decline in HbA1C by the third trimester. It was thought that retinopathy development was related to this relatively rapid improvement in glucose control rather than to insulin lispro therapy alone.

One prospective randomized clinical trial as well as multiple retrospective studies tested the safety and efficacy of insulin lispro in pregnant women. None of them have confirmed the above-mentioned findings [38,43,44]. Larger studies and longer follow-up of patients are probably needed to be certain that insulin lispro is not going to cause and exacerbate retinopathy in pregnancy.

Insulin Aspart

Insulin aspart is a rapid-acting analogue that was designed by changing a proline in position B28 to an aspartic acid [45]. This insulin is similar in several aspects to insulin lispro. It has comparable receptor affinity, but in contrast to insulin lispro, its affinity to the IGF-1 receptor is the same as human insulin (table 1) [12]. Insulin aspart has similar pharmacokinetics and

pharmacodynamics to insulin lispro [46] and has been similarly approved for use in multiple daily injection regimens [47,48] and insulin pumps [49].

Metabolic and Mitogenic Characteristics

A standard 2-year carcinogenicity study of insulin aspart has not yet been performed. In a 52-week study, there was increased incidence of mammary gland tumours, preimplantation and postimplantation foetal losses and visceral/skeletal abnormalities in female rats given insulin aspart at a dose 32 times the average human subcutaneous dose of 1.0 U/kg/day. Human insulin had a similar effect and may have been the result of severe hypoglycaemia caused by very large doses of insulin. Insulin aspart was not genotoxic in a standard battery of genetic tests. Because there are no studies in pregnant women, insulin aspart remains a category C medication for pregnancy [45].

Insulin Glulisine

Insulin glulisine has been manufactured by substituting lysine in position B29 for glutamine and aspartic acid in position B3 for lysine. This structural change decreases the rate of self-association into dimers and hexamers when injected into the subcutaneous tissue [50]. Insulin glulisine is a rapid-acting insulin that is absorbed twice as rapidly as is human insulin but has a similar total glucose disposal rate. In phase three studies, insulin glulisine has been shown to be effective when used in multiple dose injection regimens [51,52] and insulin pumps [53].

Metabolic and Mitogenic Properties

Insulin glulisine is equivalent to human insulin in its affinity to the insulin receptor, rapidity of phosphorylation and subsequent signalling [54]. However, multiple other studies showed that it only marginally activates IRS-1 protein. In contrast, activation of IRS-2 with insulin glulisine is the same or higher than with human insulin [55,56]. It has also been shown to have a much lower activation of secondary messenger (mitogen-activated protein kinase) and DNA synthesis than human insulin. Due to its unique properties of preferentially activating IRS-2, insulin glulisine exerted anti-apoptotic effects against fatty acid and cytokine-induced apoptosis of pancreatic cell lines (30–35% inhibition of apoptosis in comparison with human insulin which has 10–20% inhibition) [57]. The clinical significance of this finding is unclear.

In contrast to previous studies, *in vivo* testing did not show any differences between insulin glulisine and human insulin in activating the insulin receptor and secondary messengers [58]. There were no increases in incidence of mammary gland tumours in female rats treated for 12 months with different doses of insulin glulisine (2×20 U/kg and 2×50 U/kg) [56].

Long-Lasting Insulin Analogues

Insulin Glargine

Insulin glargine is produced by recombinant DNA techniques by adding two arginine residues to the B chain at position 31 and 32 and substituting asparagine with glycine in the A chain at position 21 [59]. These changes cause a shift of the isoelectric point to a neutral pH, and precipitation of the analogue at a physiologic pH by forming hexamers, remaining soluble (monomeric) at an acidic pH in the vial [60]. The slow dissolution of hexamers into blood results in a flatter prolonged time–action profile as compared with insulin NPH. Insulin glargine has been found to last 24 h after subcutaneous injection and has been effective in once daily dosing regimens [61].

Metabolic and Mitogenic Properties

It is important to realize that changes in the C-terminus of the insulin molecule (an area important for insulin affinity towards the IGF-1 receptor) can change the way the insulin molecule interacts with the IGF-1 receptor, giving rise to the possibility of increased mitogenic action [8]. Receptor-binding studies demonstrated that insulin glargine has a 60% lower binding affinity to the insulin receptor and 1.5 times faster dissociation than does human insulin. However, it has a 6.5 times increased binding affinity for the IGF-1 receptor and an eightfold increased potency for stimulating DNA synthesis in human osteosarcoma cells [12] (table 1).

This increase in mitogenic potency has not been confirmed in other studies using human diabetic muscle cells [62], rat fibroblasts overexpressing human insulin receptor [63] or myoblasts overexpressing the IGF-1 receptor [64]. An *in vivo* study performed on mice and rats indicates that there was no increase in the incidence of mammary gland tumours using insulin glargine at doses of 2–12.5 μ /kg [65].

The reproductive toxicity of the analogue was evaluated in several animal studies. There was no direct effect on reproduction or embryo foetal development. Pregnant rats and rabbits, however, did show an increased chance of abortion, intrauterine deaths and

single anomalies when treated with moderate to high doses of insulin glargine and insulin NPH. This effect was probably related to hypoglycaemic episodes caused by high doses of insulin [66]. There is no data on pregnant women apart from anecdotal reports. Insulin glargine remains a category C medication for pregnancy.

Insulin Glargine and Retinopathy

An incidence in the progression of retinopathy has been addressed in conjunction with the use of the rapid-acting insulin, insulin lispro. Because of this concern, extra effort has been directed towards eliminating retinopathy risk with all new insulin analogues. The higher affinity of insulin glargine for the IGF-1 receptor raised concern; this is why the FDA has required that the effect of insulin glargine on the progression of diabetic retinopathy be evaluated.

Four of the randomized multinational glargine trials of 28–52-week duration were reviewed for development of retinopathy [67]. During the treatment period, retinal examinations were done by funduscopic clinical examination and also with fundus photographs. Multiple parameters were tested. In one of the four studies, more patients in the insulin glargine group had three-step or greater (not one or two-step) progression on the standard scale (early treatment diabetic retinopathy study) for evaluation of retinopathy (7.5 vs. 2.7%, $p < 0.05$) [68]. In another study, a higher incidence of new onset macular oedema (11.2 vs. 6.5%) was observed [69]. These were isolated findings of these two measures in only two of four studies. There was lack of consistency between measurement of different parameters by clinical examination and retinal photography, and the most common ocular adverse effect of IGF-1 treatment, optic disc swelling, was not present (table 2).

In relationship to this finding, a small randomized prospective study of 28-week duration was performed [70]. Twenty type 1 well-controlled diabetic patients on regular insulin were randomized to insulin NPH or glargine combined with premeal regular insulin. Patients were evaluated by dilated fundus examination and seven standard field retinal coloured photographs. There was no difference in mean retinopathy grades between the two groups at baseline or at the end of the study.

As a safety measure, the pharmaceutical company is performing a 5-year randomized multicentre study with type two diabetic patients to determine whether patients treated with insulin NPH or glargine are at similar risk for three or more step progression of diabetic retinopathy. More than 1000 type two diabetic patients with HbA1c > 6.7 have been randomized to either insulin

NPH or glargine daily combined with premeal regular human insulin. The goal is a HbA1c $< 7.0\%$. Retinal examinations are performed by seven standard field retinal colour photographs at 3, 6 and 12 months and then yearly for 5 years. The study is still in progress, and the data are pending.

Insulin Detemir

Another long-lasting insulin analogue is insulin detemir, in which the 14 carbon myristic acid is acetylated to the B29 lysine position. This change in the structure of the insulin molecule delays the absorption of insulin detemir by three mechanisms [71,72]: (i) the insulin remains liquid in the subcutaneous tissue, providing greater surface area with reduced variability in absorption, (ii) there is strong binding to albumin at the injection site, in the plasma (98% remains bound to albumin) and in the target tissue (96% remains bound to albumin). This protects insulin detemir from liver clearance which is only about 1/500 or 8% of the single pass extraction of human insulin [73]. In the interstitial fluid, albumin reduces insulin receptor affinity and therefore results in the relatively low biological efficacy of the insulin analogue compared with human insulin [74] and (iii) the side chain fatty acid interacts with neighbouring insulin molecules and further prolongs the rate of absorption.

Metabolic and Mitogenic Properties

In vitro experiments showed that insulin detemir's affinity for the insulin receptor is 50% lower and it dissociates from the receptor two times faster than native insulin, which results in a 50-fold lower metabolic potency (27% if adjusted for albumin binding only). The affinity for the IGF-1 receptor was 15 times lower than that of human insulin, and growth-promoting effects using human Saos/B10 were >250 -fold lower than that of human insulin (11% if corrected for albumin binding) [12].

Conclusion

Despite the fact that the biological behaviour of most insulin analogues is predictable based on receptor kinetics, some discrepancies exist. Insulin aspart B10 showed an increased association with insulin and IGF-1 receptors, and these were linked to an increased mitogenic activity *in vitro* and an increase in cancerogenesis *in vivo*. The relationship between these three components is only speculative, because two analogues (insulin lispro and insulin glargine) that have a higher affinity for the IGF-1 receptor than does human insulin

Table 2 Incidence of \geq three-step retinopathy progression, new onset of PDR, new onset of MO, new AEs and disc swelling in four multicentre international trials comparing insulin glargine with insulin NPH

Finding	Type 1 diabetes				Type 2 diabetes			
	3001 (%)		3004 (%)		3002 (%)		3006 (%)	
	Glargine	NPH	Glargine	NPH	Glargine	NPH	Glargine	NPH
Progression								
Clinical exam	4.8	5.7	9.5	7	8.4	13	9.2	10.7
Photography	5.3	3.4	3.2	3.9	5.9	9.1	7.5*	2.7*
New PDR								
Clinical exam	1.9	1.1	1.3	1.7	0.7	0	2.7	1.3
Photography	2.2	2.6	1.8	3.8	2.1	1.8	4.1	2.2
New MO								
Clinical exam	3.7	1.9	0.9	1.3	1.8	2.4	3.1	3
Photography	6.9	7.9	0.9	1.3	11.2*	6.5*	2.8	2.2
Retinal AEs	18	12	9.8	10.4	3.1	2.5	22	24.7
Disc swelling	0	0	0	0	0	0	0	0

AE, aneurysm; MO, macular oedema; NPH, neutral protamine hagedorn; PDR, proliferative diabetic retinopathy.

*Statistically significant increased incidence in new onset macular oedema and three steps and more progression of retinopathy in insulin glargine groups

With kind permission of Springer Science and Business Media.

Glargine and NPH are insulin types.

Retina was assessed by clinical ophthalmologic examination and seven standard retinal fields coloured photography.

did not show an increase in mitogenic activity *in vitro* or *in vivo*. However, there are concerns about the ability of insulin lispro and insulin glargine to promote retinopathy progression. In the case of insulin lispro, causality has not been proven. It is thought that the cause of severe retinopathy progression in three pregnant patients was due to rapid improvement in glucose control and not due to insulin lispro. In the case of insulin glargine, four studies were examined for development of retinopathy. More than a three-step progression of retinopathy was found in one study and new onset macular oedema in another one. There was a lack of correlation between the methods of assessment (clinical examination and retinal photography), and findings were isolated to two of five parameters in two of four studies. Two small prospective trials did not confirm these findings with either insulin lispro or insulin glargine. These important safety issues can only be resolved with long-term prospective trials.

In the future, conclusion regarding the safety of novel insulin analogues should be based not only on *in vitro* studies, animal models or isolated cases but mainly on long-term human studies.

References

- Kang S, Brange J, Burch A, Volund A, Owens DR. Subcutaneous insulin absorption explained by insulin's physicochemical properties. Evidence from absorption studies of soluble human insulin and insulin analogues in humans. *Diabetes care* 1991; **14**: 942–948.
- Hildebrandt P, Sejrsen P, Nielsen SL, Birch K, Sestoft L. Diffusion and polymerization determines the insulin absorption from subcutaneous tissue in diabetic patients. *Scand J Clin Lab Invest* 1985; **45**: 685–690.
- Shaw LC, Grant MB. Insulin like growth factor-1 and insulin-like growth factor binding proteins: their possible roles in both maintaining normal retinal vascular function and in promoting retinal pathology. *Rev Endocr Metab Disord* 2004; **5**: 199–207.
- Stewart AJ, Johnson MD, Mys FEB, Westley BR. Role of insulin-like growth factor and the type I Insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. *J Biol Chem* 1990; **265**: 21172–21178.
- Milazzo G, Sciacca L, Papa V, Goldfine ID, Vigneri R. ASPB10 insulin induction of increased mitogenic responses and phenotypic changes in human breast epithelial cells: evidence for enhanced interactions with the insulin-like growth factor-I receptor. *Mol Carcinog* 1997; **18**: 19–25.
- Milazzo G, Giorgino F, Damante G *et al.* Insulin receptor expression and function in human breast cancer cell lines. *Cancer Res* 1992; **52**: 3924–3930.
- Mynarcik DC, Williams PF, Schaffer L, Yu GQ, Whittaker J. Identification of common ligand binding determinants of the insulin and insulin-like growth factor 1 receptors. Insights into mechanisms of ligand binding. *J Biol Chem* 1997; **272**: 18650–18655.

- 8 Slieker LJ, Brooke GS, Dimarchi RD *et al.* Modifications in the B10 and B26-30 regions of the B chain of human insulin alter affinity for the human IGF-I receptor more than for the insulin receptor. *Diabetologia* 1997; **40** (Suppl. 2): S54–S61.
- 9 Chisalita SI, Arnqvist HJ. Insulin-like growth factor I receptors are more abundant than insulin receptors in human micro- and macrovascular endothelial cells. *Am J Physiol Endocrinol Metab* 2004; **286**: E896–E901.
- 10 Rechler MM, Nissley SP. The nature and regulation of the receptors for insulin-like growth factors. *Annu Rev Physiol* 1985; **47**: 425–442.
- 11 Brange J, Ribbel U, Hansen JF *et al.* Monomeric insulins obtained by protein engineering and their medical implications. *Nature* 1988; **333**: 679–682.
- 12 Kurtzhals P, Schaffer L, Sorensen A. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000; **49**: 999–1005.
- 13 Shymko RM, Dumont E, De Meyts P, Dumont JE. Timing-dependence of insulin-receptor mitogenic versus metabolic signalling: a plausible model based on coincidence of hormone and effector binding. *Biochem J* 1999; **339**: 675–683.
- 14 Hansen BF, Danielsen GM, Drejer K *et al.* Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. *Biochem J* 1996; **315**: 271–279.
- 15 Schwartz GP, Burke GT, Chanley JD, Katsoyannis PG. An insulin analogue possessing higher *in vitro* biological activity than receptor binding affinity. [21-Proline-B]Insulin. *Biochemistry* 1983; **22**: 4561–4567.
- 16 Drejer K, Kruse V, Larsen UD *et al.* Receptor binding and tyrosine kinase activation by insulin analogues with extreme affinities studied in human hepatoma HepG2 cells. *Diabetes* 1991; **40**: 1488–1495.
- 17 Bornfeldt KE, Gidlöf RA, Wasteson A *et al.* Binding and biological effects of insulin, insulin analogues and insulin-like growth factors in rat aortic smooth muscle cells. Comparison of maximal growth promoting activities. *Diabetologia* 1991; **34**: 307–313.
- 18 Drejer K. The bioactivity of insulin analogues from *in vitro* receptor binding to *in vivo* glucose uptake. *Diabetes Metab Rev* 1992; **8**: 259–285.
- 19 Schwartz GP, Burke GT, Katsoyannis PG. A superactive insulin: [B10-aspartic acid]insulin (human). *Proc Natl Acad Sci USA* 1987; **84**: 6408–6411.
- 20 Vincent MT, Carroll RJ, Hammer RE *et al.* A transgene coding for a human insulin analog has a mitogenic effect on murine embryonic beta cells. *Proc Natl Acad Sci USA* 1995; **92**: 6239–6243.
- 21 Dideriksen LH, Jorgensen LN, Drejer K. Carcinogenic effect on female rats after 12 months administration of the insulin analogue B10 Asp. *Diabetes* 1992; **41**: 143A.
- 22 Holleman F, Hoekstra JB. Insulin lispro. *N Engl J Med* 1997; **337**: 176–183.
- 23 Brems DN, Alter LA, Chance RE *et al.* Altering the association properties of insulin by amino acid replacement. *Protein Eng* 1992; **5**: 527–533.
- 24 Howey DC, Bowsher RR, Brunelle RL, Woodworth JR. [Lys (B28), Pro (B29)]-human insulin. A rapidly absorbed analogue of human insulin. *Diabetes* 1994; **43**: 396–402.
- 25 Melki V, Renard E, Lassmann-Vague V *et al.* Improvement of HbA1c and blood glucose stability in IDDM patients treated with lispro insulin analog in external pumps. *Diabetes Care* 1998; **21**: 977–982.
- 26 Zinman B, Tildesley H, Chiasson JL, Tsui E, Strack T. Insulin lispro in CSII: results of a double-blind crossover study. *Diabetes* 1997; **46**: 440–443.
- 27 Renner R, Pftzner A, Trautmann M, Harzer O, Sauter K, Landgraf R. Use of insulin lispro in continuous subcutaneous insulin infusion treatment. Results of a multicenter trial. German Humalog-CSII Study Group. *Diabetes Care* 1999; **22**: 784–788.
- 28 Raskin P, Holcombe JH, Tamborlane WV *et al.* A comparison of insulin lispro and buffered regular human insulin administered via continuous subcutaneous insulin infusion pump. *J Diabetes Complications* 2001; **15**: 295–300.
- 29 Anderson JH Jr, Brunelle RL, Koivisto VA *et al.* Reduction of postprandial hyperglycemia and frequency of hypoglycemia in IDDM patients on insulin-analog treatment. Multicenter Insulin Lispro Study Group. *Diabetes* 1997; **46**: 265–270.
- 30 Scherthaner G, Wein W, Sandholzer K, Equiluz-Bruck S, Bates PC, Birkett MA. Postprandial insulin lispro. A new therapeutic option for type 1 diabetic patients. *Diabetes Care* 1998; **21**: 570–573.
- 31 Eli Lilly C. Humalog Insulin Lispro injection (rDNA origin) 100 units per ml (U-100) Description. 2004: 1–11.
- 32 Buelke-Sam J, Byrd RA, Hoyt JA, Zimmerman JL. A reproductive and developmental toxicity study in CD rats of LY275585, [Lys(B28), Pro(B29)]-human insulin. *J Am Coll Toxicol* 1994; **13**: 247–260.
- 33 Scherthaner G, Wein W, Sandholzer K *et al.* Postprandial insulin lispro. A new therapeutic option for type 1 diabetic patients. *Diabetes care* 1998; **21**: 570–573.
- 34 Jovanovic L, Ilic S, Pettitt DJ *et al.* Metabolic and immunologic effects of insulin lispro in gestational diabetes. *Diabetes care* 1999; **22**: 1422–1427.
- 35 Boskovic R, Feig DS, Derewlany L *et al.* Transfer of insulin lispro across the human placenta: *in vitro* perfusion studies. *Diabetes care* 2003; **26**: 1390–1394.
- 36 Holcberg G, Tsadkin-Tamir M, Sapir O *et al.* Transfer of insulin lispro across the human placenta. *Eur J Obstet Gynecol Reprod Biol* 2004; **115**: 117–118.
- 37 Diamond T, Kormas N. Possible adverse fetal effect of insulin lispro. *N Engl J Med* 1997; **337**: 1009.

- 38 Persson B, Swahn ML, Hjertberg R *et al.* Insulin lispro therapy in pregnancies complicated by type 1 diabetes mellitus. *Diabetes Res Clin Prac* 2002; **58**: 115–121.
- 39 Mecacci F, Carignani L, Cioni R *et al.* Maternal metabolic control and perinatal outcome in women with gestational diabetes treated with regular or lispro insulin: comparison with non-diabetic pregnant women. *Eur J Obstet Gynecol Reprod Biol* 2003; **111**: 19–24.
- 40 Masson EA, Patmore JE, Brash PD *et al.* Pregnancy outcome in type 1 diabetes mellitus treated with insulin lispro (Humalog). *Diabet Med* 2003; **20**: 46–50.
- 41 Star J, Carpenter MW. The effect of pregnancy on the natural history of diabetic retinopathy and nephropathy. *Clin Perinatol* 1998; **25**: 887–916.
- 42 Kitzmiller JL, Main E, Ward B, Theiss T, Peterson DL. Insulin lispro and the development of proliferative diabetic retinopathy during pregnancy. *Diabetes care* 1999; **22**: 874–876.
- 43 Buchbinder A, Miodovnik M, Mcelvy S *et al.* Is insulin lispro associated with the development or progression of diabetic retinopathy during pregnancy? *Am J Obstet Gynecol* 2000; **183**: 1162–1165.
- 44 Bhattacharyya A, Vice PA. Insulin lispro, pregnancy, and retinopathy. *Diabetes care* 1999; **22**: 2101–2104.
- 45 Novo, Nordisk, Pharmaceutical. NovoLog Insulin aspart (rDNA origin) Injection, 2004.
- 46 Plank J, Wutte A, Brunner G *et al.* A direct comparison of insulin aspart and insulin lispro in patients with type 1 diabetes. *Diabetes care* 2002; **25**: 2053–2057.
- 47 Home PD, Lindholm A, Riis A. Insulin aspart vs. human insulin in the management of long-term blood glucose control in type 1 diabetes mellitus: a randomized controlled trial. *Diabet Med* 2000; **17**: 762–770.
- 48 Raskin P, Guthrie RA, Leiter L, Riis A, Jovanovic L. Use of insulin aspart, a fast-acting insulin analog, as the mealtime insulin in the management of patients with type 1 diabetes. *Diabetes Care* 2000; **23**: 583–588.
- 49 Bode B, Weinstein R, Bell D *et al.* Comparison of insulin aspart with buffered regular insulin and insulin lispro in continuous subcutaneous insulin infusion: a randomized study in type 1 diabetes. *Diabetes Care* 2002; **25**: 439–444.
- 50 Barlocco D. Insulin glulisine. Aventis Pharma. *Current Opin Investig Drugs* 2003; **4**: 1240–1244.
- 51 Dailey G, Rosenstock J, Moses R, Ways K. Glycemic control with insulin glulisine vs regular human insulin in a basal-bolus regimen in patients with type 2 diabetes. American Diabetes Association 64th Scientific Session. *Diabetes* 2004; **53** (Suppl. 2): A121–P.
- 52 Dreyer M, Prager R, Robinson A, Busch K, Souhami E. Efficacy and safety of insulin glulisine and insulin lispro combined with insulin glargine in patients with type 1 diabetes. American Diabetes Association 64th Scientific Session. *Diabetes* 2004; **53** (Suppl. 2): A123–P.
- 53 Hanaire-Broutin H, Schumicki DM, Hoogme RPLM, Souhami E. Safety of insulin glulisine compared with insulin aspart administered by continuous subcutaneous insulin infusion (CSII). American Diabetes Association 64th Scientific Session. *Diabetes* 2004; **53** (Suppl. 2): A4–OR.
- 54 Hennige AM, Kellerer M, Strack V, Metzinger E, Seipke E. New human insulin analogs: characteristics of insulin signaling in comparison to Asp B10 and regular insulin. *Diabetologia* 1999; **42** (Suppl. 1): A665.
- 55 Rakatzi I, Seipke G, Eckel J. The novel insulin analog [LysB3, GluB29] insulin (HMR1964) preferentially activates IRS-2 signaling in K6-myoblasts. *Diabetes care* 2000 **49** (Suppl. 1): A9.
- 56 Rakatzi I, Ramrath S, Ledwig D *et al.* A novel insulin analog with unique properties: LysB3,GluB29 insulin induces prominent activation of insulin receptor substrate 2, but marginal phosphorylation of insulin receptor substrate 1. *Diabetes* 2003; **52**: 2227–2238.
- 57 Rakatzi I, Seipke G, Eckel J. [LysB3, GluB29] insulin: a novel insulin analog with enhanced beta-cell protective action. *Biochem Biophys Res Commun* 2003; **310**: 852–859.
- 58 Hennige AM, Lehmann R, Weigert C *et al.* Insulin glulisine: insulin receptor signaling characteristics in vivo. *Diabetes* 2005; **54**: 361–366.
- 59 Gillies PS, Figgitt DP, Lamb HM. Insulin glargine. *Drugs* 2000; **59**: 253–260.
- 60 Bolli GB, Di Marchi RD, Park GD, Pramming S, Koivisto VA. Insulin analogues and their potential in the management of diabetes mellitus. *Diabetologia* 1999; **42**: 1151–1167.
- 61 Lepore M, Pampanelli S, Fanelli C *et al.* Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting human insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro. *Diabetes* 2000; **49**: 2142–2148.
- 62 Ciaraldi TP, Carter L, Seipke G *et al.* Effects of the long-acting insulin analog insulin glargine on cultured human skeletal muscle cells: comparisons to insulin and IGF-I. *J Clin Endocrinol Metab* 2001; **86**: 5838–5847.
- 63 Berti L, Kellerer M, Bossenmaier B *et al.* The long acting human insulin analog HOE 901: characteristics of insulin signalling in comparison to Asp (B10) and regular insulin. *Horm Metab Res* 1998; **30**: 123–129.
- 64 Bèahr M, Kolter T, Seipke G, Eckel J. Growth promoting and metabolic activity of the human insulin analogue [GlyA21,ArgB31,ArgB32]insulin (HOE 901) in muscle cells. *Eur J Pharmacol* 1997; **320**: 259–265.
- 65 Stammberger I, Bube A, Durchfeld-Meyer B, Donaubaue H, Troschau G. Evaluation of the carcinogenic potential of insulin glargine (LANTUS) in rats and mice. *Int J Toxicol* 2002; **21**: 171–179.

- 66 Hofmann T, Horstmann G, Stammberger I. Evaluation of the reproductive toxicity and embryotoxicity of insulin glargine (LANTUS) in rats and rabbits. *Int J Toxicol* 2002; **21**: 181–189.
- 67 Forjanic-Klapproth PDH. Progression of retinopathy with insulin glargine or NPH – a multi-trial analysis. *Diabetologia* 2001; **44** (Suppl.): 1103.
- 68 Rosenstock J, Schwartz SL, Clark CM, Park GD, Donley DW, Edwards MB. Basal insulin therapy in type 2 diabetes: 28-week comparison of insulin glargine (HOE 901) and NPH insulin. *Diabetes Care* 2001; **24**: 631–636.
- 69 Yki-Jarvinen H, Dressler A, Ziemer M. Less nocturnal hypoglycemia and better post-dinner glucose control with bedtime insulin glargine compared with bedtime NPH insulin during insulin combination therapy in type 2 diabetes. HOE 901/3002 Study Group. *Diabetes Care* 2000; **23**: 1130–1136.
- 70 Peters K, Garg SK, Jackson WE *et al.* Diabetic retinopathy in patients with type 1 diabetes treated with insulin glargine or NPH. *Diabetes* 2001; **50**: A443.
- 71 Whittingham JL, Havelund S, Jonassen I. Crystal structure of prolonged – acting insulin with albumin-binding properties. *Biochemistry* 1997; **36**: 2826–2831.
- 72 Kurtzhals P, Havelund S, Jonassen I *et al.* Albumin binding of insulins acylated with fatty acids: characterization of the ligand–protein interaction and correlation between binding affinity and timing of the insulin effect *in vivo*. *Biochem J* 1995; **312**: 725–731.
- 73 Dea MK, Hamilton-Wessler M, Ader M *et al.* Albumin binding of acylated insulin (NN304) does not deter action to stimulate glucose uptake. *Diabetes* 2002; **51**: 762–769.
- 74 Hamilton-Wessler M, Ader M, Dea M *et al.* Mechanism of protracted metabolic effects of fatty acid acylated insulin, NN304, in dogs: retention of NN304 by albumin. *Diabetologia* 1999; **42**: 1254–1263.