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REVIEW ARTICLE

Insulin glargine and receptor-mediated signalling: clinical implications in treating type 2 diabetes

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Summary

Most patients with type 2 diabetes mellitus will eventually require insulin therapy to achieve or maintain adequate glycaemic control. The introduction of insulin analogues, with pharmacokinetics that more closely mimic endogenous insulin secretion, has made physiologic insulin replacement easier to achieve for many patients. However, there are also concerns regarding alteration of binding affinities for the insulin receptor (IR) or insulin-like growth factor-1 receptor (IGF-1R) may increase the mitogenic potential of some analogues. Therefore, this article will review the relevant preclinical and clinical data to assess the mitogenic potential of insulin glargine, a basal insulin analogue, compared with regular human insulin (RHI).

Searches of the PubMed database were performed using terms that included 'IR,' 'insulin-like growth factor-1,' 'IGF-1R,' 'type 2 diabetes mellitus,' and 'insulin glargine.' Original articles and reviews of published literature were retrieved and reviewed.

Although one study reported increased binding affinity of insulin glargine for the IGF-1R and increased mitogenic potential in cells with excess IGF-1Rs (Saos/B10 osteosarcoma cells), most *in vitro* binding-affinity and cell-culture studies have demonstrated behaviour of insulin glargine comparable to that of RHI for both IR and IGF-1R binding, insulin signalling, and metabolic and mitogenic potential.

Currently published *in vivo* carcinogenic studies and human clinical trial data have shown that insulin glargine is not associated with increased risk for either cancer or the development or progression of diabetic retinopathy.

Keywords insulin glargine; insulin analogues; IGF-1; type 2 diabetes mellitus; mitogenicity

Introduction

The benefits of early, intensive glycaemic control with insulin in patients with type 2 diabetes have been clearly demonstrated in the United Kingdom Prospective Diabetes Study (UKPDS) in two large clinical trials. In this study, benefits included a 25% risk reduction for microvascular complications in patients receiving insulin compared with those receiving conventional treatment [1]. In a further analysis of the data from UKPDS, each 1% reduction in mean glycosylated hemoglobin A1C (A1C) level was associated with a 21% reduction in risk for all diabetes-related endpoints ($p < 0.0001$), a 37% risk reduction for microvascular complications ($p < 0.0001$), and a 14% reduction in the risk of myocardial infarction ($p < 0.0001$) [2].

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Most patients with type 2 diabetes will eventually require treatment with exogenous insulin to achieve or maintain adequate glycaemic control. With the inevitable progressive decline of β-cell function, physiologic insulin regimens may be needed to successfully reach glycaemic goals [3]. The introduction of insulin analogues, which were designed to provide onset and duration of action that more closely mimic endogenous insulin secretion, have made physiologic insulin replacement easier to achieve for many patients [3,4].

Insulin glargine, a widely used basal insulin analogue, is characterized by the addition of two arginine molecules at the *C*-terminal region of the insulin B chain (position B30), and the substitution of glycine for asparagine in the A chain (position A21) [4]. These modifications to the human insulin molecule result in a less soluble analogue that precipitates in subcutaneous tissue, allowing for prolonged absorption with little peak activity [4,5]. Given once daily, insulin glargine replaces endogenous basal insulin secretion. This agent can be added to oral anti-hyperglycaemic therapy to achieve glycaemic targets in patients with type 2 diabetes experiencing inadequate glycaemic control with oral anti-diabetic agents alone [6,7]. For patients, whose A1C remains above target after the addition of basal insulin therapy, rapid-acting insulin analogues (i.e. insulin aspart, lispro, or glulisine) given at one or more meals each day can be added to the regimen to control elevations in postprandial glucose levels [4].

Although there are advantages to using insulin analogues rather than regular human insulin (RHI) to provide more physiological insulin replacement, there are also potential concerns. Insulin analogues have often altered receptor-binding affinities for the insulin receptor (IR), and the insulin-like growth factor-1 receptor (IGF-1R). Although composed of a single chain, the insulin-like growth factor-1 (IGF-1) molecule has both A and B domains, with 45–50% homology to the A and B chains of the insulin molecule; the two receptors (IR and IGF-1R) also show substantial structural homology to each other [8]. The receptor-signalling pathways activated by insulin/IGF-1 binding to their receptors are responsible for both metabolic regulation and mitogenic responses (i.e. cell growth and differentiation) [9,10].

Insulin analogues may therefore have increased mitogenic potential, either through altered affinity (as measured by dissociation rates) for the IGF-1R and/or increased half-life of the insulin-IR complex [8,9]. For example, the insulin analogue AspB10 (not in clinical use) has a three-fold higher affinity for IR and IGF-1R and threefold higher *in vitro* metabolic activity, compared with RHI [11]. AspB10 has a ten- to twenty-fold higher *in vitro* mitogenic effect, with demonstrated dose-dependent mitogenic activity in female rats treated for 1 year with supraphysiologic doses (range: 12.5–200 U/kg/d) [11]. For these reasons, it is important to evaluate the mitogenic potential of any insulin analogue because of the likelihood of its long-term use in the treatment of diabetes. This article will review the relevant pre-clinical and clinical data to assess the mitogenic potential of insulin glargine compared with RHI.

A brief review of insulin and IGF-1R signalling

Insulin signalling is characterized by a series of complex, highly interconnected pathways that form a network regulating important metabolic and mitogenic processes (Figure 1) [10]. The IR and IGF-1R are both tetrameric proteins with two extracellular α-subunits and two intracellular β-subunits. Each tetramer is formed from two $\alpha-\beta$ dimers linked with disulfide bonds, and hybrid IR/IGF-1R complexes can also be formed [10]. However, in human coronary artery endothelial cells expressing the hybrid IR/IGF-1R, only IGF-1 appears to phosphorylate the hybrid receptor [12]. In rat vascular smooth muscle cells expressing IR, IGF-1R, and hybrid receptors, only IGF-1 stimulated glucose metabolism and DNA synthesis at physiologic concentrations [13]. Importantly, it is also possible for insulin to bind to the IGF-1R and IGF-1 to bind to the IR, albeit with significantly lower affinities (indicated by the dashed arrows in Figure 1). Both the IR and IGF-1R are receptor tyrosine kinases, which are activated by ligand binding and phosphorylate downstream signalling components [10].

Most of the metabolic effects of insulin are mediated via phosphorylation of insulin receptor substrate (IRS) proteins. In turn, IRS proteins activate the phosphatidylinositol 3-kinase-Akt/protein-kinase B pathway, leading ultimately to glucose uptake, glycogen synthesis, and gluconeogenesis blockade. The other major effect is activation of the Ras-mitogen–activated protein-kinase pathway, either through Src-homology-2-containing proteins (Shc) or IRS proteins (phosphorylated by either IGF-1R or IR). This Ras pathway regulates gene expression to control cell growth and differentiation [10].

Although these pathways are interconnected, *in vitro* evidence suggests that IRS-1 is primarily responsible for the metabolic effects of insulin, while Shc largely mediates the mitogenic effects. These differences between the two pathways may explain the increased ratio of mitogenic to metabolic potency seen with some insulin analogues that bind to the IR with a longer half-life or slower dissociation rates. This pathway differentiation results in sustained phosphorylation of Shc, but not of IRS-1, which is dephosphorylated in a selective, time-dependent manner by protein tyrosine phosphatases [9].

Receptor binding and intracellular signalling of insulin glargine *versus* RHI

Insulin receptor

Insulin glargine and RHI have similar time courses for IR binding and associated intracellular signalling events. In a

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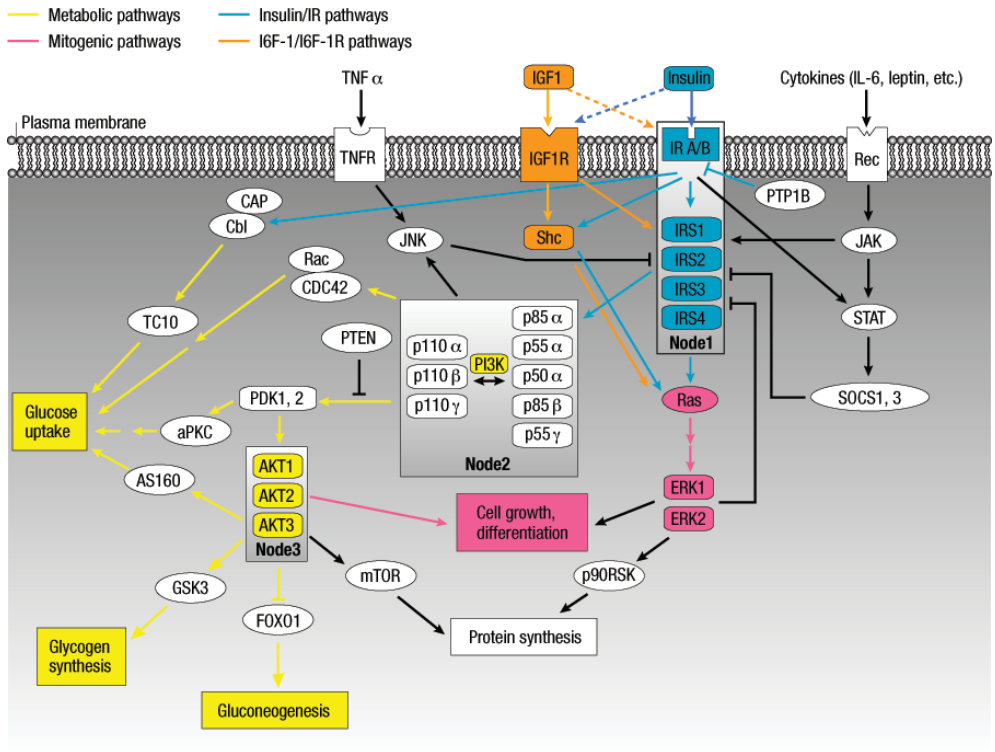

Figure 1. An overview of the insulin signalling network. Insulin (blue arrows) and insulin-like growth factor-1 (IGF-1) (orange arrows) bind to their respective receptors (IR and IGF-1R) and initiate a series of interconnected signalling pathways that lead to both metabolic (yellow pathways) and mitogenic (pink pathways) effects. The first critical node where these pathways connect is through the group of IR substrate proteins (IRS-1 through IRS-4); from this point, most of the metabolic effects of insulin/IGF-1 occur through activation of the phosphatidylinositol 3-kinase (PI3K) and Akt/protein-kinase B (Akt1 through Akt3) pathways. Modulators or downstream effectors of these pathways include Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl); Cbl-associated protein (CAP); ras homologue gene family member Q (ARHQ, also called TC10); atypical protein kinase C (aPKC); Akt substrate of 160 kDa (AS160); cell-division cycle 42 (CDC42); Rac; phosphatase and tensin homologue (PTEN); phosphoinositide-dependent kinase 1 and 2 (PDK1 and 2); glycogen synthase kinase 3 (GSK3); forkhead box O1 (FOXO1); and mammalian target of rapamycin (mTOR). Activation of the Ras pathway [through the IRS proteins or through the Src-homology-2-containing protein (Shc)] regulates genes that control cell growth and differentiation via extracellular signal-regulated kinase 1 and 2 (ERK1 and 2). Signalling pathways that are activated by cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and leptin interfere with insulin signalling through crosstalk, mediated via c-Jun-N-terminal kinase (JNK), Janus kinase (JAK), signal transducer and activator of transcription (STAT), and suppressor of cytokine signalling (SOCS) [10]. Reprinted with permission from Taniguchi *et al*. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 2006; 7: 85–95

study of rat-1 fibroblasts overexpressing human IR isoform A or isoform B, insulin glargine was similar to RHI with respect to receptor association and dissociation, while the analogue AspB10 showed delayed receptor dissociation [14]. Insulin glargine and RHI also showed similar rates of receptor autophosphorylation, phosphorylation of IRS-1/IRS-2 and Shc, and incorporation of [^{3}H]thymidine into DNA (a measure of mitogenicity). In contrast, AspB10 induced prolonged phosphorylation of IRS and Shc molecules and increased the rate of [^{3}H]thymidine incorporation [14].

The effects of insulin glargine and RHI were also studied in cultured human skeletal muscle cells (HSMCs), because this tissue is a major site of glucose disposal and a key site of insulin resistance in patients with type 2 diabetes [15,16]. This study reported that insulin glargine was equivalent to RHI in its ability to compete for [^{125}I]insulin binding to the IR, and induced comparable stimulation of glucose uptake in cultured HSMCs from patients with and without type 2 diabetes [15].

IGF-1R

While insulin glargine behaved comparably to RHI with regard to IR binding in the studies described previously, differences in binding to the IGF-1 receptor have been reported (Table 1). Notably, insulin glargine showed increased affinity (approximately 6.5-fold) for soluble IGF-1Rs *in vitro* (using purified, solubilized human receptors) compared with RHI [17]. However, binding affinities were equivalent and very low compared with IGF-1 when cell-based systems were used [15]. In the study of cultured HSMCs, insulin glargine did have a slightly higher affinity for IGF-1R than RHI, but only at supraphysiologic concentrations (i.e. the highest concentration level tested, 200 nM; insulin glargine's physiologic concentration is 0.9–1.2 nM) [18]; furthermore, the affinity of insulin glargine for IGF-1R was only 0.1% compared to 0.25% of that of IGF-1 [15].

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Table 1. Binding affinities of insulin glargine *versus* regular human insulin (RHI) for the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) [15,17]

Receptor	Source of receptor	Binding affinity		Difference, GLAR *versus* human insulin
		RHI	Insulin glargine	
IR [17]	Solubilized human IR	100%[a]	86 ± 3%[a]	Similar
IGF-1R [17]	Solubilized human IR	100%[b]	641 ± 51%[b]	~6.5-fold ↑
IR [15]	Cell-based system[c]	0.71 ± 0.16 nM[d]	1.59 ± 0.62 nM[d]	Similar (p = NS)
IGF-1R [15]	Cell-based system[c]	ND	431 ± 131 nM[d] (IGF-1: 0.34 ± 0.03 nM[d])	Similar[e] ($p < 0.05$ (*vs*) RHI)

RHI, regular human insulin; GLAR, insulin glargine; ND, not determined, displacement did not exceed 50%; NS, not significant.
[a]RHI bound to solubilized human IR with an affinity of ~0.01 nmol/L. The affinity of insulin glargine was reported as a percentage relative to RHI (set at 100%), mean ± SE.
[b]RHI bound to solubilized human IGF-1R with an affinity of ~200 nmol/L. Again, the affinity of insulin glargine was reported as a percentage relative to RHI (100%), mean ± SE.
[c]Cultured human skeletal muscle cells from individuals with diabetes, maintained in the culture system with normal glucose and insulin levels.
[d]ED_{50} = concentration required to attain 50% displacement of specific ^{125}I-insulin bound, mean ± SEM.
[e]Binding curves showed that affinity of IGF-1R for insulin glargine was significantly higher than that for RHI only at the highest concentration level tested [200 nM ($p < 0.05$)].

Metabolic and mitogenic potential of insulin glargine

Insulin glargine has demonstrated metabolic potency (measured as lipogenesis or glucose transport) comparable to that of RHI in several studies using different cell lines [15,17,19]. Freshly cultured HSMCs have the unique advantage of mirroring the relative expression of IR and IGF-1R observed in skeletal muscle *in vivo* [15]. In cultured HSMCs from individuals both with and without type 2 diabetes, stimulation of glucose uptake for RHI and insulin glargine was similar [15].

Table 2 summarizes the available data regarding mitogenicity of insulin glargine compared with RHI in a variety of cell-culture systems. As shown, the mitogenic potential of insulin glargine was low relative to AspB10 or IGF-1 and comparable to RHI in four of the five cell lines tested [14,15,19,20]. The only exception was the cell line Saos/B10, a human osteosarcoma cell line that overexpresses IGF-1R [17]. In this cell line, insulin glargine resulted in an approximately eight-fold increase in ^{3}H-thymidine incorporation relative to RHI; however, this response occurred at supraphysiologic concentrations of insulin glargine. Moreover, because of the 1000-fold weaker affinity of insulin glargine *versus* IGF-1 for the IGF-1R, it would be unlikely that insulin glargine would add significantly to the endogenous activity of IGF-1.

Given the parallel increase in IGF-1R affinity and mitogenic potential of insulin glargine in the Saos/B10 osteosarcoma cell line, the data reviewed in this section appear to support the importance of the IGF-1R in mediating mitogenic responses to RHI and/or insulin glargine [17]. This study reported a lack of association between IR dissociation rates and mitogenicity. In addition, while it has been reported that insulin-induced activation of the Ras-mitogen-activated protein-kinase pathway can induce vascular smooth muscle cell proliferation and migration, animal and clinical studies of type 2 diabetes indicate that insulin is anti-atherogenic and induces increased myocardial blood flow *in vivo* [21–24]. There is, however, evidence that mitogenic responses can be elicited through the IR. As discussed previously, insulin analogues with slower IR dissociation rates (e.g. AspB10) show greater mitogenic potency than RHI in Chinese hamster ovary cells overexpressing human IR, suggesting that the duration of IR occupancy may be a primary component of IR–mediated mitogenesis [9]. In the light of this possibility, it is important to note that insulin glargine, while similar to RHI in its IR binding and dissociation rates, does have a slightly lower IR affinity and slightly faster dissociation rate compared with RHI [14,15,17].

In vivo animal and human clinical data on mitogenicity of insulin glargine

The overall similarity of insulin glargine to RHI in receptor–binding studies, as well as the general lack of increased mitogenic potential in most cell lines tested, argue against any increased cancer risk associated with insulin glargine treatment in humans. Despite increased IGF-1R binding and mitogenic potential of insulin glargine in the cell line B10, prior reports have indicated that IGF-1R affinity and/or *in vitro* mitogenicity (induction of cell cycle progression) do not necessarily correlate with increased *in vivo* tumour incidence (carcinogenicity) [8]. Indeed, the *in vivo* safety of insulin glargine was confirmed in the 2-year carcinogenicity studies in mice and rats (Table 3) [25].

In these studies, 100 mice (50 males and 50 females, Table 3A) or 100 rats (again, 50 males and 50 females, Table 3B) were given once-daily subcutaneous injections of NaCl control, vehicle control, increasing dosages of insulin glargine (2, 5, or 12.5 U/kg), or neutral protamine Hagedorn (NPH) insulin (12.5 U/kg in mice or 5 U/kg in rats) for 2 years. As shown in the Tables, neither the

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Table 2. Mitogenicity of insulin glargine *versus* regular human insulin (RHI) in cell culture [14,15,17,19,20]

Cell line	Source of cells/receptor expression	Rate of thymidine incorporation		Difference,GLAR *versus* RHI
		RHI	Insulin glargine	
Rat-1 [14]	Rat fibroblast/Overexpress hIR-B	EC_{50}: ~3 nM	EC_{50}: ~3 nM (**AspB10:** ~1 nM)	Comparable
H9c2 [19]	Rat heart muscle/IGF-1R	~100% ↑[a] (5×10^{-7} M)	~100% ↑[a] (5×10^{-7} M) **IGF-1:** ~150% ↑ (1×10^{-7} M)	Comparable
Saos/B10 [17]	Human osteosarcoma/Overexpress IGF-1R	100%[b] (EC_{50}: ~4 nM)	783 ± 132%[b] (**IGF-1:** EC_{50} ~ 50 pM)	~8-fold ↑
Not applicable [15]	Freshly cultured HSMCs[c]	51 ± 14 nM[d]	63 ± 18 nM[d] (**IGF-1:** 0.57 ± 0.20 nM)	Comparable (**IGF-1:** $p < 0.05$ (*vs*) RHI)
HMVECs [20]	Human dermal microvascular endothelial cells/IR and IGF-1R	~100%[e] (1×10^{-7} M)	~125%[e] (1×10^{-7} M)	Comparable
			IGF-1: ~200% (1×10^{-7} M)	**IGF-1:** $p < 0.002$

EC_{50}, concentration at which there is 50% of the maximal ^{3}H-thymidine incorporation response seen in the presence of 10% fetal calf serum; GLAR, insulin glargine; hIR-B, human insulin receptor, isoform B; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; IR, insulin receptor.
[a]Rate of ^{3}H-thymidine incorporation is expressed as the percentage increase over basal levels of incorporation in serum-starved cells, at the given concentration of insulin or insulin glargine (i.e. maximal concentration, 5×10^{-7} M).
[b]The rate of thymidine incorporation for insulin glargine was reported as a percentage relative to human insulin (set at 100%), mean ± SE.
[c]Cultured human skeletal muscles cells (HSMCs) were isolated from patients with type 2 diabetes ($n = 7$).
[d]EC_{50}, concentration required to achieve 50% maximal stimulation in each subject ± SEM.
[e]Rate of ^{3}H-thymidine incorporation is expressed as the percentage increase over control cells at the given concentration of RHI, insulin glargine, or IGF-1 (e.g. 1×10^{-7} M).

mice nor the rats demonstrated any significant increase in the incidence of mammary tumours in those injected with insulin glargine *versus* vehicle or NPH insulin [25]. While male mice injected with low doses of insulin glargine did show slightly increased incidence of hepatocellular tumours and malignant fibrous histiocytoma (MFH) at injection sites compared with NaCl control animals (Table 3A), these increases were not dose-dependent, did not occur in female mice, and were also seen in the vehicle control group. Similarly, a higher incidence of MFH at injection sites was seen in male rats at all dosages of insulin glargine, as well as in the vehicle control group (Table 3B); this increase was not observed in female rats. Hepatocellular tumours (in mice) and MFH (in rats or mice) are commonly seen in laboratory rodents and are not considered relevant to humans [25]. Thus, these studies clearly demonstrated that insulin glargine did not cause *in vivo* mammary tumours or other neoplastic changes relevant to humans.

IGF-1 has been implicated in the development and progression of retinopathy via promotion of neovascularization; in addition, patients with rapidly progressing retinopathy have elevated levels of serum IGF-1 [26]. Therefore, a safety study also analysed the risk for diabetic retinopathy in patients with type 1 or type 2 diabetes treated with insulin glargine in 4 phase 3 clinical trials [27]. This analysis revealed no increased risk for either the development or progression of diabetic retinopathy in patients treated with insulin glargine *versus* NPH insulin. In addition, the UKPDS demonstrated a relationship between elevated blood glucose levels and developing retinopathy in patients with type 2 diabetes, and that intensive glycaemic control with insulin decreased the risk for retinopathy [1].

IGF-1 synthesis is induced by growth hormone from the pituitary gland with negative feedback from serum levels of IGF-1 [28]. To test whether insulin glargine could regulate serum levels of IGF-1, a small, 3-week crossover study ($n = 42$) examined IGF-1 levels in patients with type 1 or type 2 diabetes using insulin glargine or NPH insulin [29]. Insulin glargine treatment was not associated with a decrease in serum IGF-1 levels, suggesting that insulin glargine did not have biologically relevant IGF-1–like activity at the pituitary level.

Conclusions

As a result of improved pharmacokinetic and pharmacodynamic profiles, insulin analogues provide clinical benefits in the treatment of type 2 diabetes, allowing them to more closely mirror the normal physiologic pattern of endogenous insulin secretion compared with subcutaneous injection of RHI. However, alterations of the human insulin molecule can result in possible altered binding kinetics to the IR and IGF-1R. For example, Asp(B10) has been shown to produce sustained activation of the IR via prolonged IRS phosphorylation and Shc molecules [14]. Therefore, even years after their initial availability, it is important to remain vigilant in evaluating the mitogenic potential of all insulin analogues.

In receptor–binding studies, insulin glargine generally behaved comparably to RHI with respect to IR and IGF-1R affinity. Mitogenicity of insulin glargine was also similar to that of RHI in four of five cell lines tested, including *ex vivo* HSMCs. The only exception was a sixfold to eightfold increase in IGF-1R binding and mitogenicity in Saos/B10 osteosarcoma cells when insulin glargine was used, but

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Table 3A. *In Vivo* animal studies of insulin glargine (GLAR) carcinogenic potential [25] incidence of specific tumours in a 2-year carcinogenicity study in mice ($n = 100$)[a]

Treatment	Hepatocellular adenoma	Hepatocellular carcinoma	Malignant fibrous histiocytoma	Mammary gland adenocarcinomas	Mammary gland adenocanthoma malignant
Males					
NaCl control	0	6	0	0	0
Vehicle control	6[b]	7	4	0	0
GLAR 2 U/kg	5[b]	3	8[c]	0	0
GLAR 5 U/kg	5[b]	3	3	0	0
GLAR 12.5 U/kg	1	1	2	0	0
NPH insulin 12.5 U/kg	1	3	2	0	0
Females					
NaCl control	0	0	0	2	0
Vehicle control	0	1	0	0	0
GLAR 2 U/kg	0	2	2	0	0
GLAR 5 U/kg	0	0	0	0	0
GLAR 12.5 U/kg	0	0	0	2	0
NPH insulin 12.5 U/kg	0	0	0	2	1

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Table 3B. Incidence of specific tumours in a 2-year carcinogenicity study in rats ($n = 100$)[a]

Treatment	Pancreatic islet cell adenoma	Malignant lymphoma	Malignant fibrous histiocytoma	MG Adenoma	MG Adeno-carcinoma	MG Fibro-adenoma	MG Carcinoma in fibro-adenoma	MG Tumour mixed malignant
Males								
NaCl control	9	0	0	0	0	1	0	0
Vehicle control	3	6[c]	9[c]	0	0	2	0	0
GLAR 2 U/kg	1[c]	1	13[c]	0	1	2	0	0
GLAR 5 U/kg	5	4	12[c]	0	0	0	0	0
GLAR 12.5 U/kg	0	1	6[b]	0	1	1	0	0
NPH insulin 5 U/kg	7	1	0	0	0	1	0	0
Females								
NaCl control	3	1	0	0	9	26	3	0
Vehicle control	1	3	1	1	9	21	2	2
GLAR 2 U/kg	2	2	2	3	7	26	1	0
GLAR 5 U/kg	3	2	1	0	8	22	1	0
GLAR 12.5 U/kg	7	0	0	0	7	15[b]	2	0
NPH insulin 5 U/kg	3	0	0	1	7	28	2	0

MG, mammary gland; NPH, neutral protamine Hagedorn.
[a]Values are numbers of tumours observed in each group.
[b]$p < 0.05$ *versus* NaCl control.
[c]$p < 0.01$ *versus* NaCl control.

at doses that were well above the concentrations typically achieved *in vivo* by insulin glargine [30]. However, this finding of altered *in vitro* IGF-1R binding was not translated into any *in vivo* effects, as determined in rodent carcinogenic studies of up to 2 years duration. Currently available studies have indicated no increased risk for cancer, and no changes in the risk for development or progression of diabetic retinopathy have been reported in patients treated with insulin glargine, further confirming the safety of this basal insulin analogue.

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Conflict of interest

None declared.

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