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Insulin Glargine: A Reevaluation of Rodent Carcinogenicity Findings

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Abstract

The 1995 to 1997 lifetime carcinogenicity studies of insulin glargine in rats and mice were reanalyzed and reassessed for their validity according to current guidelines. In 2-year studies, 50 animals per sex and per group were used. Survival rates between weeks 80 and 90 in female mice and rats were greater than 20 animals in all groups, fulfilling current Food and Drug Administration requirements that enough animals lived long enough to provide adequate exposure to glargine and to be at risk of forming late-developing tumors. Exposure to 5 or 12.5 IU/kg glargine was similar to or 2 to 3 times greater than 5 IU/kg neutral protamine Hagedorn insulin, respectively. Using statistical methods recommended by current guidelines, no significant effect of glargine on mammary gland neoplastic lesions in female rodents was found, confirming earlier results. Thus, both studies can be considered valid according to contemporary standards. Insulin glargine does not present a carcinogenic risk.

Keywords

carcinogenicity studies, insulin glargine

Sequence or secondary structural modifications were introduced into insulin analogues to alter their time–action profile.[1] Insulin glargine ([Gly^{A21}, Arg^{B30}, Arg^{B31}] insulin) is a long-acting insulin that differs from human insulin by substitution of asparagine by glycine in position 21 of the A-chain and by carboxy-terminal extension of the B-chain by 2 arginine residues. These alterations shift the isoelectric point from pH 5.4 to 6.7. Because of its low solubility at physiological pH, the analogue precipitates at the injection site and its subsequent slow dissolution is the basis for its long-acting profile.

However, structural modifications of insulin may also change its metabolic or mitogenic responses. The long-acting analogue, insulin detemir, which has myristic acid attached to lysine at position 29 of the B-chain, induced a modest proliferative effect in the mammary gland of young female rats during a 26-week toxicity study.[2] Clinical development of [Asp^{B10}] insulin was stopped due to a higher incidence of mammary tumors in rats in a 12-month toxicity study.[3] Compared to regular human insulin, [Asp^{B10}] insulin displays higher affinity toward both the insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R) in vitro, a prolonged occupancy time at the IR, and a higher proliferation rate in mammalian cell lines.[4–7] Together, these results have led to the generally held belief that insulin analogs with increased IGF-1R affinity in vitro have increased growth-promoting activity in vivo.

Insulin glargine has an in vitro IR signaling and metabolic profile comparable to that of human insulin while displaying slightly greater affinity toward IGF-1R.[4,5,7] Glargine undergoes rapid and significant metabolism in humans and animals[8,9] leading to the formation of 2 main metabolites [Gly^{A21}] human insulin (M1) and [Gly^{A21}, des-Thr^{B30}]-human insulin (M2); these have in vitro metabolic and mitogenic profiles comparable with human insulin.[7] Glargine was extensively studied in 1995 to 1997 in lifetime carcinogenicity studies in rats and mice, targeting the incidence of spontaneously occurring tumors and development of rare tumors.[10] There were no neoplastic findings to indicate that insulin glargine had a systemic carcinogenic potential in rodents. More recently, the validity of the studies has been questioned due to the high mortality and lack of adequate exposure during the study.[11,12]

The lifetime carcinogenicity studies were carried out in compliance with the testing guidelines that were in effect at the time when the studies were conducted, that is, 1995 to 1997 (European Community Note for Guidance of October 1983 [Council recommendation, EE83/571], Ministry of Health and Welfare Japan, September 11, 1989, and the USA Federal Regulation 50, March 4, 1985). Overall, these guidelines described on a very general level the standards of the study design of lifetime carcinogenicity studies.

In the meantime, a Food and Drug Administration (FDA) guidance[13] is available which allows for approaches to high dose selection based on toxicity end points, pharmacokinetic

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end points (multiple of maximum human exposure), pharmacodynamic end points, and maximal feasible dose. It specifies in detail the standards regarding statistical aspects of the design and analysis and interpretation of chronic rodent carcinogenicity studies of pharmaceuticals; standards are given for the appropriate statistical analysis of tumor rates and for the adjustment of tumor rates for intercurrent mortality. For example, details are given for the appropriate survival rates: "As a rule of thumb, a 50% survival rate of the 50 initial animals in any treatment group between weeks 80 and 90 of a 2-year study would be considered to yield a sufficient number of animals with adequate exposure. The percentage can be lower or higher if the number of animals used in each treatment/sex group is larger or smaller than 50, but between 20 and 30 animals should be still alive during these weeks."

The aim of the current report was to reassess the 1995 to 1997 studies for their validity with regard to design, survival rates, incidence of neoplastic mammary lesions in female animals, statistics, and toxicokinetics according to contemporary standards.

Methods

Study Design

The design and conduct of the 2-year studies have been fully described, including the selection of doses, by Stammberger et al.[10] A total of 50 animals per sex and per group were used. Three dose levels of insulin glargine were used (2, 5, and 12.5 IU/kg) along with saline control, vehicle control, and neutral protamine Hagedorn (NPH) insulin (12.5 in mice or 5 IU/kg in rats) groups. The expected in-life parameters (body weight, food consumption, survival, and behavior) were regularly monitored. Animals were palpated for nodules monthly until 6 months of age and then every 2 weeks to study end. Rats found dead were autopsied the same day.

Toxicokinetic Analyses

Blood samples for the determination of insulin were collected from the retrobulbar venous plexus from 3 female, nonstarved rats each at 1, 2, 3, 4, 7, and 24 hours after dose 27, 188, and 370. The vehicle control group served as the control group for both the insulin glargine and NPH insulin groups. The blood was centrifuged and the serum concentration of insulin was determined by radioimmunoassay using a commercial human insulin RIA kit (RIA-gnost Insulin; Behringwerke, Marburg, Germany). For the insulin glargine-treated groups, a total of 100 µL of standard/sample was incubated with 200 µL of ^{125}I-insulin tracer and 200 µL of anti-insulin serum for 21 to 23 hours at room temperature. Antibody-bound and free radiolabeled ligand were separated by adding 1 mL of a 17.5% polyethylene glycol solution to each tube and vortexing until a homogenous solution was achieved. After centrifugation at ~1500*g* for 15 minutes at room temperature, the supernatant was decanted and radioactivity in the precipitate counted. An insulin glargine standard curve was prepared by serial dilutions of a stock solution in human insulin-free serum. The limit of quantification (LOQ) was 0.5 ng/mL and the measuring range was 0.5 to 100 ng/mL. Samples >50 ng/mL were diluted. The serum concentration of insulin in the NPH insulin-treated and vehicle control groups was determined using the commercial human insulin RIA kit (RIA-gnost Insulin; Behringwerke) as described by the manufacturer. The LOQ was 7.5 µIU/mL. Samples >165 µIU/mL were diluted. A total of 16.8 µIU/mL corresponded to 1 ng/mL of human insulin.

Statistical Analyses

For toxicokinetic analysis, the maximum concentration (C_{max}) of insulin glargine was obtained directly from measured data. Area under the serum insulin glargine concentration–time curve (area under the curve $[AUC]_{0\text{-}24\ h}$) was calculated using the linear trapezoidal rule where values below the LOQ were entered as 0.25 ng/mL. The 2 pharmacokinetic parameters were summarized using descriptive statistics.

For each type of tumor, statistical analyses were performed using a modified Peto lifetime-adjusted analysis[14] and the Bieler-Williams Poly-3 test,[15,16] 2 of the recommended methods.[13] In the modified Peto lifetime adjustment, time strata were defined, in weeks, as 0 to 50, 51 to 80, 81 to terminal sacrifice, and terminal sacrifice. When less than 20 findings were present, exact permutation tests were used.[17] Three separate tests were performed, a 1-tailed test for increasing monotonic trend in tumor rate for the saline control group versus the treated groups, a 1-tailed test for increasing monotonic trend in tumor rate for the vehicle control group 2 versus the treated groups, and a 1-tailed test for increasing monotonic trend in tumor rate for the pooled control groups versus the treated groups. A test for differences between the control groups was also performed at the 5% level. Pairwise comparisons between the controls (separately or pooled) and the high-dose group were performed.

A Bieler-Williams Poly-3 test approach does not depend on the classification of tumors (fatal or incidental). It is a survival-adjusted quantal-response procedure that modifies the denominator in the quantal estimate of lesion incidence of the Cochran-Armitage linear trend test to approximate more closely the total number of animal-years at risk. The thresholds considered were those given in the FDA guidance[13] for trend tests; 0.025 for rare tumors and 0.005 for common tumors and, for control-high pairwise comparisons, 0.05 for rare tumors and 0.01 for common tumors. All tumor analyses were performed using the MULTTEST and LIFETEST procedures in version 9.1 of the SAS system on Windows XP.

Results

Study Design

The current guidelines call for 2-year carcinogenicity studies in rats and mice with 50 animals per sex and per group, 3 dose

Table 1. Survival Rates in Female Mice and Female Rats From Weeks 80 to 90 and at Scheduled Termination (Weeks 105-107)

	Survival Rates in Female Mice, n (%)					
Week	SC	VC	GLA 2	GLA 5	GLA 12.5	NPH 12.5
Week 0	50 (100)	50 (100)	50 (100)	50 (100)	50 (100)	50 (100)
Week 80	32 (64)	26 (52)	25 (50)	31 (62)	36 (72)	28 (56)
Week 81	32 (64)	25 (50)	25 (50)	31 (62)	36 (72)	28 (56)
Week 82	28 (56)	25 (50)	22 (44)	30 (60)	35 (70)	28 (56)
Week 83	27 (54)	24 (48)	21 (42)	27 (54)	34 (68)	27 (54)
Week 84	26 (52)	23 (46)	21 (42)	26 (52)	32 (64)	26 (52)
Week 85	25 (50)	23 (46)	19 (38)	24 (48)	30 (60)	25 (50)
Week 86	23 (46)	22 (44)	18 (36)	24 (48)	29 (58)	24 (48)
Week 87	19 (38)	20 (40)	18 (36)	24 (48)	28 (56)	23 (46)
Week 88	19 (38)	19 (38)	17 (34)	24 (48)	28 (56)	22 (44)
Week 89	17 (34)	17 (34)	16 (32)	21 (42)	28 (56)	22 (44)
Week 90	16 (32)	17 (34)	15 (30)	21 (42)	27 (54)	21 (42)
Terminal sacrifice	3 (6)	5 (10)	5 (10)	9 (18)	11 (22)	11 (22)
Survival rates in female rats, n (%)						
Week	SC	VC	GLA 2	GLA 5	GLA 12.5	NPH 5
Week 0	50 (100)	50 (100)	50 (100)	50 (100)	50 (100)	50 (100)
Week 80	35 (70)	37 (74)	36 (72)	37 (74)	30 (60)	36 (72)
Week 81	35 (70)	37 (74)	36 (72)	37 (74)	29 (58)	36 (72)
Week 82	35 (70)	37 (74)	35 (70)	37 (74)	27 (54)	36 (72)
Week 83	34 (68)	36 (72)	33 (66)	36 (72)	26 (52)	35 (70)
Week 84	34 (68)	36 (72)	33 (66)	34 (68)	26 (52)	35 (70)
Week 85	34 (68)	35 (70)	32 (64)	34 (68)	24 (48)	34 (68)
Week 86	33 (66)	34 (68)	31 (62)	33 (66)	23 (46)	34 (68)
Week 87	31 (62)	32 (64)	29 (58)	33 (66)	23 (46)	34 (68)
Week 88	30 (60)	32 (64)	29 (58)	30 (60)	22 (44)	33 (66)
Week 89	30 (60)	32 (64)	28 (56)	30 (60)	21 (42)	33 (66)
Week 90	30 (60)	30 (60)	28 (56)	30 (60)	18 (36)	33 (66)
Terminal sacrifice	21 (42)	19 (38)	18 (36)	16 (32)	7 (14)	9 (18)

Abbreviations: GLA, insulin glargine; SC, saline control; VC, vehicle control.

levels of experimental drug plus saline and vehicle control groups and, if appropriate, a comparator group. The design and conduct of both studies meet the practice guidelines of the present time.

Survival Rates

More than 20 female mice were still alive at week 80 in all groups but not at week 90 (Table 1). In the saline control group, 23 mice were alive at the start of week 86; in the vehicle control group, 20 were alive at the start of week 87; in the glargine low-dose group, 21 were alive at the start of week 84, while 21 animals remained at week 90 in the middle-dose group and 27 in the high-dose group. There were 21 animals alive at week 90 in the NPH group.

For female rats, more than 20 animals were still alive at week 80 in all groups and at week 90 in all but the high-dose group (Table 1). Only in the high-dose group the number of live animals was below 20 at week 90; and in this group, 21 animals were still alive at the start of week 89. Thus, comparing these survival rates with the FDA "rule of thumb" that 20 to 30 animals should still be alive between weeks 80 and 90, it is considered that there were enough animals living long enough to provide adequate exposure to the drug and to be at risk of forming late-developing tumors in both studies.

Statistical Analyses

Malignant tumors were found in mammary glands from 2 female mice in the saline control group and in 2 from the glargine high-dose group. Analysis of tumor incidence revealed no statistical significant difference between the control groups and the glargine high-dose group using either the Peto analysis at the 5% level (Table 2) or the Bieler-Williams Poly-3 test at the 2.5% level. In the latter analysis, *P* values for treated versus saline control, vehicle control, or dual control groups were 0.5934, 0.0334, and 0.3738, respectively. Similar results were obtained with rats using either the Peto analysis of individual tumor (Table 2) or combined tumor (Table 3) incidences, or using the Bieler-Williams Poly-3 test (Table 4). Raw mammary tumor data are available in Supplemental Tables 1 and 2.

Exposure to Drug

The toxicokinetic parameters of insulin glargine in female rats are summarized in Table 5. Both C_{max} and $AUC_{0\text{-}24\ h}$ increased

Table 2. Female Mice and Rats—Mammary Gland Tumor Incidence Peto Analysis

Tumor	Group	SC[a]	VC[a]	GLA 2	GLA 5	GLA 12.5[b]
Mice						
Adenocarcinoma	Examined tissues	38	41	46	45	46
	Nonlethal tumors	0	0	0	0	1
	Lethal tumors	2	0	0	0	1
	Treated versus Dual	0.4891				0.5157
	Treated versus SC	0.6932				0.7290
	Treated versus VC		0.0790			0.0790
Rats						
Adenocarcinoma	Examined tissues	50	47	49	49	49
	Nonlethal tumors	6	8	6	7	5
	Lethal tumors	3	1	1	1	2
	Treated versus Dual	0.7826				0.8189
	Treated versus SC	0.6579				0.7127
	Treated versus VC		0.7442			0.7912
Adenoma	Lethal tumors	0	1	3	0	0
	Treated versus Dual	0.8174				0.6315
	Treated versus SC	0.8629				0.6718
	Treated versus VC		0.9310			0.8275
Carcinoma arising in fibroadenoma	Nonlethal tumors	1	2	1	1	1
	Lethal tumors	2	0	0	0	1
	Treated versus Dual	0.5224				0.5654
	Treated versus SC	0.5787				0.6292
	Treated versus VC		0.3548			0.3914
Fibroadenoma	Nonlethal tumors	21	20	22	18	15
	Lethal tumors	5	1	4	4	0
	Treated versus Dual	0.8679				0.8304
	Treated versus SC	0.9577				0.9428
	Treated versus VC		0.7422			0.6738
Mixed tumor malignant	Lethal tumors	0	2	0	0	0
	Treated versus Dual	1.000				1.000
	Treated versus SC	1.000				1.000
	Treated versus VC		1.000			1.000

Abbreviations: Dual, SC plus VC; GLA, insulin glargine; SC, saline control; VC, vehicle control.
[a] *P* values from upper-tailed Peto trend tests.
[b] *P* values from upper-tailed Peto pairwise comparisons to the control.

Table 3. Female Rats—Mammary Gland Combined Tumor Incidence Peto Analysis

Tumor	Group	SC[a]	VC[a]	GLA 2	GLA 5	GLA 12.5[b]
Benign combined	Examined tissues	50	47	49	49	49
	Nonlethal tumors	21	20	22	18	15
	Lethal tumors	5	1	4	4	0
	Treated versus Dual	0.8679				0.8304
	Treated versus SC	0.9577				0.9428
	Treated versus VC		0.7422			0.6738
Malignant combined	Nonlethal tumors	7	8	7	8	6
	Lethal tumors	5	3	1	1	3
	Treated versus Dual	0.7623				0.7988
	Treated versus SC	0.6684				0.7209
	Treated versus VC		0.6448			0.6931

Abbreviations: Dual, SC plus VC; GLA, insulin glargine; SC, saline control; VC, vehicle control.
[a] *P* values from upper-tailed Peto trend tests.
[b] *P* values from upper-tailed Peto pairwise comparisons to the control.

with repeated dosing and with increasing dosing for insulin glargine and with repeated dosing for the single dose of NPH insulin. Mean values of both C_{max} and $AUC_{0\text{-}24\ h}$ at 5 U/kg insulin glargine were similar to or greater than those with 5 U/kg NPH insulin and were approximately 2- to 3-fold greater at 12.5 U/kg insulin glargine compared with 5 U/kg NPH insulin.

Table 4. Female Rats—Mammary Gland Bieler-Williams Poly-3 Test

Tumor	Treated Versus		
	Dual	SC	VC
Adenocarcinoma	0.5535	0.5099	0.5313
Adenoma	0.6964	0.7070	0.8705
Carcinoma arising in fibrinoma	0.6498	0.6514	0.4589
Fibrinoma	0.8760	0.9352	0.8541
Tumor mixed malignant	0.9203	1.0000	0.9738
Benign combined	0.8760	0.9352	0.8541
Malignant combined	0.6182	0.5891	0.5100

Abbreviations: Dual, SC plus VC; SC, saline control; VC, vehicle control.

Table 5. Pharmacokinetics of Insulin Glargine and NPH Insulin in Female Rats[a]

		Insulin Glargine, IU/kg				NPH, IU/kg	
Dose	Parameter	0	2	5	12.5	0	5
28	C_{max}[b]	113	151	901	1495	71	520
	AUC[c]	2352	2280	2802	4234	1505	1919
189	C_{max}	154	533	1440	3407	85	1162
	AUC	2876	2956	4387	6764	1669	3558
371	C_{max}	228	778	2330	4662	112	2249
	AUC	4104	4184	5783	12148	2165	5453

Abbreviations: NPH, neutral protamine Hagedorn; AUC, area under the curve.
[a] Values are means of n = 3.
[b] μIU/mL.
[c] μIU × h/mL.

Discussion

In 2-year studies conducted in 1995 to 1997, insulin glargine was shown to not have carcinogenic potential in mice or rats.[3] The validity of those studies, however, has been questioned based on the high rate of mortality and presumed lack of adequate drug exposure.[11,12] Because revised guidelines have been published since the studies were completed, the design of the studies, the survival rates, and the incidence of neoplastic mammary lesions in female animals were reassessed for their validity according to current guidelines. The design of these studies and the survival rates in both the mouse and rat carcinogenicity studies were found to be adequate to assess the carcinogenic potential of insulin glargine; there were sufficient numbers of animals who lived long enough to provide adequate exposure to the drug and to be at risk of forming late-developing tumors. Using methods recommended by the current guidelines, no statistically significant effect of glargine on mammary gland neoplastic lesions in either female mice or rats was found, confirming the earlier results.[10] Thus, both studies can be considered valid according to modern-day standards.

The lowest dose of insulin glargine used in the carcinogenicity studies (2 IU/kg) is approximately 2 to 4 times the mean daily human dose (0.5-1.0 IU/kg).[18,19] The highest dose of glargine (12.5 IU/kg) was found to be the maximum tolerated dose for a lifetime study.[10] Rats injected with the supraphysiological dose of 5 IU/kg insulin glargine resulted in similar exposure over time, as measured by C_{max} and $AUC_{0\text{-}24\ h}$, as the same dose of NPH insulin, while the highest dose of insulin glargine resulted in exposure that was 2 to 3 times greater over time than 5 IU/kg NPH insulin. These results supported the conclusion that there was adequate exposure in the carcinogenicity studies for the animals to be at risk of developing late-forming tumors. Yet the risk was found to be no greater for animals treated with insulin glargine than for the control-treated animals. Insulin glargine remains the only insulin analog that has undergone such extensive toxicological and carcinogenicity testing.[20]

In conclusion, the present reassessment of the 2-year insulin glargine carcinogenicity studies confirms the earlier findings that this basal insulin analog does not present a carcinogenic risk. Together with a metabolic and mitogenic profile in vitro that is similar to human insulin, these results indicate that insulin glargine is not likely to pose a cancer risk in humans. These findings may be confirmed by ongoing clinical studies.

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Declaration of Conflicting Interests

Stammberger is an employee of sanofi-aventis Deutschland Gmbh. Essermeant is an employee of sanofi-aventis, Montpellier, France.

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