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 ([Gly²¹, Arg³⁰, Arg³¹] 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 [Asp¹⁰] 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, [Asp¹⁰] 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 animals⁸,⁹ leading to the formation of 2 main metabolites [Gly²¹] human insulin (M1) and [Gly²¹, des-Thr³⁰] 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) guidance¹³ 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 16.8 mL of standard/sample was incubated with 200 µL of 125I-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 ~1500g 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]_0,24 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 analysis14 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 guidance13 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.
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 \( C_{\text{max}} \) and \( AUC_{0-24\,\text{h}} \) increased

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

| 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.
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 $C_{\text{max}}$ and $\text{AUC}_{0-24\text{ h}}$ 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 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.
The validity of those studies, however, has been questioned based on the high rate of mortality and presumed lack of adequate drug exposure. 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. 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). The highest dose of glargine (12.5 IU/kg) was found to be the maximum tolerated dose for a lifetime study. Rats injected with the supraphysiological dose of 5 IU/kg insulin glargine resulted in similar exposure over time, as measured by $C_{\text{max}}$ and AUC$_{0-24 \text{ h}}$, 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.

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 Germany GmbH. Essermeant is an employee of sanofi-aventis, Montpellier, France.

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