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Author: Braak, Bas ter
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Chapter 7 summary and discussion

Carcinogenicity of insulin analogues: current status and future perspectives

Highlights

o The insulin analogue carcinogenic risk assesment guidelines of the European medicine administration was used as a guidance in our research strategy

o There is no compelling evidence that any commercial available insulin analogue is inducing the risk of cancer

o Glargine has an enhanced mitogenic potential compared to regular insulin and might affect some hallmarks of cancer

o Future research on this topic should include chronic exposure in vivo experiments on humanized genetically engineered mouse models to increase the clinical relevance of these studies

◀ IN THE PICTURE

Cell culture laboratory. In vitro models depend on the culturing of living cells. To prevent the cell culture from being infected with bacteria, fungi or viruses, handling of these cells is performed in special cabinets that create a more or less sterile environment. In these cabinets cells are seeded or harvested, exposures are performed and transfections are executed.

◀ IN BEELD

Cel kweek laboratorium. In vitro modellen berusten op het kweken van levende cellen. Om infecties met bacteriën, schimmels en virussen te voorkomen, zijn de handelingen met deze cellen in speciale cabines gedaan, waarin een (bijna) steriele omgeving is gecreëerd. In deze cabines worden de cellen uitgezaaid, geoogst en worden de blootstellingen en transfecties uitgevoerd.

Biopharmaceutical drugs are medicinal products that have been manufactured or directly extracted from a biological source [209]. These compounds are used for therapeutic purposes, and should therefore be carefully assessed for potential side effects. Several regulatory national and international authorities, like the American FDA or European EMA, are responsible for legislation regarding the safety evaluation of biopharmaceuticals [1, 2].

Growth factors are an example of biopharmaceuticals. They show intrinsic mitogenic behavior and are therefore a problematic group of pharmaceuticals with regard to the carcinogenic safety evaluation, and their inherent proliferative action is a cause for concern [210]. Regulatory agencies have requested the use of specific testing strategies to ensure the safety of a sub-group of growth factors, i.e. insulin analogues [6]. This includes the use of well characterized and validated cell models, state of the art techniques to assess the mitogenic effects and the use of humanized in vivo models. In this thesis we have focused on carcinogenic risk assessment of a specific group of growth factors, the insulin analogues.

With worldwide over 380 million cases, diabetes mellitus is the most common endocrine related disease [142]. Usually, patients with type 1 diabetes depend on daily insulin (analogue) administration to control their blood glucose levels. Small alterations to the protein structure of insulin affect the pharmacokinetics and dynamics of the molecule, and consequently cause these insulin-like molecules to either act faster (rapid-acting analogues) or slower (long-acting analogues) than regular insulin. Often a combination treatment of these long- and short-acting insulin analogues is used to mimic the endogenous insulin levels in a healthy person throughout the day.

Insulin glargine (LANTUS) is the world's most commonly used long-acting insulin analogue with about 7 million users in 2011 [196]. Three amino acid replacements in the molecular structure of glargine ensure the slow release into the patient's blood and the prolonged action profile [173]. But these mutations have also altered the binding kinetics towards different receptors including the two isoforms of the insulin receptor (IRA and IRB) and the insulin-like growth factor 1 receptor (IGF1R). While activation of the IRB induces the intended metabolic effects (blood glucose drop), it is thought that activation of IRA and IGF1R will contribute to an enhanced mitogenic signaling [211].

Several epidemiological studies have suggested that the use of some of these insulin analogues is correlated with an increased incidence of cancer, especially breast cancer [76] [144] [212]. While others could not confirm these findings [19] [146] [71]. Due to the many confounding factors, the interpretation of this type of observational patient studies is often difficult. For example, diabetes mellitus is in itself thought to be a risk factor for cancer [213]. Moreover, insulin analogue dose, treatment duration, and age/gender/lifestyle of patients is often not known/taken into account while these factors are thought to have a strong effect on cancer risk [24]. A patient based random controlled clinical trial setup would overcome these problems, but

so far this type of study on the relationship between insulin analogue treatment versus cancer have been rare and highly underpowered.

It is also possible to study the direct mitogenic and anti-apoptotic effects of these insulin analogues using cell lines. Currently, over fifty in vitro studies are described in literature and sixteen of these studies have specifically focused on breast cancer, using breast (cancer) cell lines. The conclusions drawn from these studies are not always in agreement and sometimes even contradictory [118] [40] [193]. Differences in cell model and experimental procedures are in all likelihood the main cause for these conflicting observations.

If in vitro data are considered insufficient and a cause for concern remains, the evaluation of the carcinogenic risk using an in vivo animal model provides added value. The few chronic exposure experiments with rats and mice that have been described have revealed an increased carcinogenic potential of insulin AspB10 [175], an insulin analogue that therefore never entered the market. All other evaluated insulin analogues, including insulin glargine, did not induce more tumors than regular human insulin.

It is unlikely that growth factors at physiological levels induce tumors, however, at pharmacological levels these compounds might be involved in tumor progression of pre-neoplastic lesions. The disadvantage of using the standard wild type rodent models is the rather low incidence of clinically relevant background tumors. Therefore, the observed carcinogenic effect of the growth factors is very mild in these models. The use of xenograft transplantation in vivo models would overcome this problem [207], but in these models only tumor progression features can be studied and no information can be retrieved about the earlier stages of tumor development.

In this chapter we will describe our view on the testing strategy of insulin analogues in terms of increased mitogenic/carcinogenic potential, bearing in mind the current guidelines on this topic, and we will provide an integrated summary of the results obtained with the models that we used throughout this thesis.

This thesis emphasizes the importance of a proper evaluation of carcinogenic side effects of insulin analogues used by diabetic patients. To ensure the design of a relevant experiment it is of course essential to evaluate the current literature about insulin analogues and cancer risk. Some review studies have summarized part of the available literature on this topic, but so far no systematic review including all in vitro, in vivo (animal) and epidemiological (human) studies is available. In **chapter 2**, we present a systematic review of current literature. We focused on studies that included breast (cancer) cell lines or evaluated the incidence of breast tumors in mice, rats or humans that have been exposed to insulin analogues. The results from the sixteen in vitro, eight animal and thirty epidemiological studies we included were highly diverse. Some studies concluded that insulin analogues, especially glargine, did have carcinogenic/mitogenic effects, while others found no effect or even milder carcinogenic/mitogenic effects compared to

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regular insulin. All in all we found no compelling evidence that any clinically available insulin analogue increases breast cancer risk. However, both in vitro and epidemiological studies suffered from methodological limitations. Besides, epidemiological studies were underpowered and animal data was scarce. Therefore, there is a strong need for well-designed studies that evaluate the carcinogenic and mitogenic potential of insulin analogues.

A comprehensive evaluation should start with in vitro studies, performed in well characterized cell lines. Since the IGF1R is likely to be the main receptor regulating the mitogenic signaling of insulin analogues, it is crucial that the used cell line expresses this receptor, and at high levels if possible. Furthermore, the concentration at which the analogues are tested is very important. Using concentrations that are too low might lead to undetectable signaling events whereas the use of overtly high concentrations will overstimulate the receptor. In both cases one is unable to discriminate between the mitogenic effects of the different compounds. Thus, a broad concentration range is preferred. In addition, the use of positive and negative controls is essential. Without including regular insulin and IGF1 as a reference compounds, it is difficult to put the obtained results in perspective and provide a quantitative mitogenic score for individual analogues. **Chapter 3** describes an in vitro study in which we tested all commercially available insulin analogues using a human breast cancer cell line panel that expresses either one of the isoforms of the insulin receptor (IRA/IRB) or the IGF1R at high levels. The activation of downstream signaling was evaluated upon insulin analogue treatment. We found that treatment with insulin glargine or AspB10 induced a significantly stronger activation of the MAPK and the PI3K signaling pathways, compared to regular insulin, especially via the IGF1R. With a functional assay we tested the direct proliferative effects of these compounds using a concentration range from physiological to supra-physiological levels. We found that AspB10, though not glargine, had a significant higher proliferative potential compared to regular insulin. We demonstrated that the glargine was rapidly degraded, into metabolites with a low mitogenic potency, by enzymes present in the plasma. This conversion could also explain the difference we observed between the results of the SRB proliferation assay (in which serum was present and thus the glargine was converted) and the WB experiments (in which no serum was added and most of the glargine was still present). Since this bioconversion of glargine is also relevant for the human in vivo situation we tested the mitogenic potential of the two main metabolites of glargine and found that both metabolites (M1 and M2) have a low mitogenic potential similar to that of regular insulin. In conclusion, glargine had a strong intrinsic mitogenic potential but due to the rapid degradation into metabolites with a low proliferative behavior there was no sustained activation of the IGF1R signaling pathway. All other tested commercially available insulin analogues showed a mitogenic potential that was comparable to regular insulin.

In **chapter 4,** we performed a stimulation experiment using the same cell line panel as described in chapter 3. This time mRNA was extracted and a full transcriptomic analysis was performed

using Affymetrix micro-arrays. This allowed us to study the insulin analogue induced mitogenic signaling cascades in more detail. In general, activation of the IRA resulted in a transcriptomic response similar to that of IGF1R. Only AspB10 stimulation resulted in a very distinct gene expression profile via the IRA, which is in line with an older study that showed a prolonged IRA occupancy time for AspB10, therefore it was suggested that AspB10 induces its mitogenic signaling especially via the IRA [149]. We identified a set of genes that was significantly upregulated upon IGF1 and AspB10 stimulation but to a much milder extent after regular insulin stimulation, suggesting that these genes could act as transcriptomic markers for mitogenic signaling. Examples of these markers are the early growth response (EGR) genes, all four EGR genes were picked up by our assay. Although these genes are well known to play a role in proliferation, so far they have not been linked to insulin receptor (ISNR)/IGF1R or insulin analogue signaling. Next we evaluated the expression of these genes after stimulation with other commercially available insulin analogues. The mitogenic score, or absolute expression value of these genes, showed a clear correlation with the mitogenic indexes as calculated from functional bioassays as well as receptor kinetic studies in literature [114] [113]. Furthermore, we were able to confirm the robustness of some of these classifiers in mammary gland tissue of an insulin injected mice as well as insulin analogue stimulated human primary (non-cancer) mammary cells. Although more research is needed to determine the clinical relevance of these mitogenic classifiers, we think that this straightforward RT-Q-PCR based analysis has the potential to become a quick alternative for a mitogenic screen of compounds that act on the IGF1R.

As mentioned before, glargine itself has a strong mitogenic potential, but due to the rapid enzymatic conversion to low mitogenic compounds, this might not be relevant in vivo. Due to uncertainties about the exact rate of conversion and therefore possible exposure to parental and mitogenic glargine, an in vivo experiment would provide further insight into glargine induced carcinogenicity. In **chapter 5** a chronic exposure experiment is described, in which we injected mice every other day with a high dose of insulin, glargine, AspB10, IGF1 or a vehicle solution. For this the p53$^{R270H/+}$WAPCre mouse model was used. In this model, mice will develop spontaneous human relevant mammary gland tumors. We found that frequent AspB10 and IGF1 injections significantly decreased the latency time for breast tumor development, while chronic glargine exposure showed a slight non-significant decrease on tumor latency time. The only other chronic in vivo experiment that included glargine as one of the test compounds did not find any effect in terms of tumor latency time [175]. The time to develop a mammary gland tumor was not affected by regular insulin injections compared to the tumors in the vehicle treatment group. Insulin analogue treatment in general did not affect the tumor type in this mouse model and cell lines isolated from these tumors did not reveal an enhanced aggressiveness or invasiveness. This was in agreement with a study on diabetic breast cancer patients in which clinical and

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histopathological screening of the tumors revealed that glargine treatment did not affect the tumor stage compared to regular insulin treatment [93]. However, we found that the protein expression profiles of AspB10 and IGF1 treated tumors showed an enhanced/sustained activation of the PI3K and MAPK signaling cascades, which are thought to be the main driver for tumor development. These pathways were also upregulated in glargine induced tumors. A metabolite analysis revealed that similar to the human situation, the glargine was rapidly converted by enzymes in the blood. Nevertheless, in contrast to the human situation this enzymatic conversion seemed to be somewhat slower in mice as we were still able to detect low concentrations of glargine in the blood one hour after glargine injection [83] [140]. Furthermore, besides very high concentrations of M1 we were able to detect also some M2, which was not observed in the human studies. These observations suggest that glargine dynamics is slightly different from mice to man and highlight the need for studies that examine the differences between these species.

The altered protein expression in the glargine induced tumors together with the slight decrease in tumor latency time suggests that frequent glargine injections might affect tumorigenesis in this mouse model to some degree. To further investigate this, mRNA from fifty mammary gland tumors was isolated and analyzed using next generation sequencing. This whole transcriptome analysis is described in **chapter 6**. This is the first study in which such an experiment was performed on the most clinically relevant target tissue (and not just the standard liver tissue). Several "Hallmarks of Cancer" appeared to be affected by chronic insulin analogue treatment [214] [197]. AspB10 and IGF1 treated tumors showed an increased and sustained proliferative and invasive potential. These tumors as well as the tumors treated with glargine also relied more on aerobic glycolysis than on oxidative phosphorylation for their ATP generation, which is the so-called Warburg effect. To our knowledge, such a link between insulin analogue signaling and the Warburg effect has never been described. Mutational analysis indicated that chronic insulin analogue stimulation did not affect the genetic instability of the tumors, since no additional mutations were found in the treatment groups. However enrichment of *Ezh1* and *Hras* were found in tumors of mice treated with glargine, X10 or IGF1. Furthermore we found that chronic AspB10 and IGF1 treatment resulted in tumors with highly induced metabolic processes that are involved in biomass production. This confirms the hypothesis that chronic AspB10 and IGF1 treatments decrease tumor latency time in the $p53^{R270H/+}$WAPCre mouse model by inducing tumor progression rather than tumor initiation.

The work described in this thesis provides novel insights into the role of insulin analogues in cancer and deepens the recommendations of "the points to consider document" of the EMA [6]. Our in vitro studies included pathway activation studies, functional proliferation studies as well as an in depth transcriptomic analysis. This in combination with the use of a well-characterized MCF7-based cell model provided new information regarding the mitogenic mode of action of

the insulin analogues and contributed to the inter-species-translatory aspects of these assays. To further follow the EMA guidelines we were able to validate some of the transcriptomic results in human primary mammary cells. Future in vitro research on this topic should contain other functional read-outs (e.g. MTT or BrdU proliferation assays), as well as FACS-assays to determine a shift in cell cycle upon insulin analogue stimulation. It would be ideal to have these additional tests to be performed in human primary mammary cells as such a model is clinically more relevant and therefore the true carcinogenesis (the development of a cancerous cell) can be better assessed.

In the EMA guidelines concerning carcinogenic risk assessment of insulin analogues the use of in vivo experiments is encouraged. It is thought that in carcinogenicity testing the rat represents the human situation better as a model compared to mice [4]. However, there are hardly any humanized breast cancer rat models available. For this reason we used the $p53^{R270H/+}$WAPCre mouse model. All mice in this model develop spontaneous mammary tumors, so we could test if the insulin analogues affect tumorigenesis either by decreasing the tumor latency time, increasing the tumor multiplicity or affecting tumor type. The main mammary gland tumors that developed in our mouse model were characterized as EMT tumors. These tumors make up only a small fraction of the human tumors. Future chronic in vivo studies should use humanized breast cancer mouse models with e.g. a PI3K mutation predispositioned to develop carcinomas to increase the clinical relevance of their study [215] [190].

Although more research is needed, the available data suggests that chronic glargine treatment is not involved in tumor initiation but might influence several features of tumorigenesis, and thus it might enhance tumor progression [193]. We suggest that future epidemiological studies (preferably case control studies) should focus on these potentially tumor promoting effects rather than on the tumor initiating effects of glargine. Until this research is performed we advise people with a high risk of cancer (e.g. patients that have already been diagnosed with cancer, survivors of oncogenic diseases or people with a familial genetic predisposition to develop this disease) to reconsider their glargine prescription. Alternative diabetic treatments, like the use of long acting insulin determir or the bioglycolin metformin, are in fact sometimes correlated with a decreased incidence of cancer [216] [58].

For the future it is essential that regulatory agencies react quickly, yet not overhasty to indications from scientific studies that a specific compound might possess an increased carcinogenic risk. In 2009, concerns about the carcinogenic potential of glargine were raised by an epidemiological study by Hemkens and colleagues [16]. Instead of directly publishing this article and thereby risking the possibly of setting of an unwarranted alarm, the European Association for the Study of Diabetes (EASD) asked several other epidemiological research groups to study the same topic on different patient groups. Within a couple of months three other epidemiological studies were performed and published together with the study of

Hemkens [17-19]. The other studies found no correlation or only a correlation with breast cancer and the use of glargine treatment. A response from the European regulatory agencies was slightly delayed, but in 2011 a new FP7 "Cancer risk and insulin analogues" (CARING) program was initiated. This international collaboration aims to quantify the risk of cancer associated with the long-term use of insulin and insulin analogues, with the main focus on epidemiological research. The review in chapter 2 is a direct product of this program. In my opinion, also an additional guideline should have been proposed by these ministries directly after the appearance of the epidemiological studies regarding follow up in vitro and in vivo studies on this topic. Yet, to this day no addendum has been proposed on "The points to consider document" of the EMA. I expect that such a recommendation from these organizations would have led to more and higher quality studies in a shorter timeframe.

In this thesis the recommendations of the ICHS6 guideline and "The points to consider document" of EMA were followed for the carcinogenic risk assessment of all commercial insulin analogues [1, 2, 6]. Although we could propose some improvements for the used models (see suggestions as discussed above), we think that the research described in this thesis provides the most accurate, complete and in-depth carcinogenic safety evaluation for insulin analogues to date. We hope that with this research we have contributed to the improvement of the quality and consistency of the preclinical safety data supporting the development of insulin analogues.

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