Chapter 1

Aim and scope of thesis

Highlights

- Insulin analogues are widely used by diabetic patients to control blood glucose levels
- The use of some of these compounds is correlated with an increased cancer risk
- The medicine administration agencies propose new carcinogenic risk assessment strategies for insulin analogues

This chapter is adapted from:

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Carcinogenicity of Biophamaceuticals

In press, March 2015, "Genotoxicity and Carcinogenicity Testing of Pharmaceuticals", Springer Press

And

B. ter Braak

Anti-diabetic insulin analogue drugs and breast cancer development

Februari 2015, Guest Blog on biomedcentral.com

◀ IN THE PICTURE

Insulin analogue pens and refills. Diabetic patients will certainly recognise these user friendly injection pens. Using the rotating cap the patient can adjust the required dose. The needle can be mounted on top of the pen. Typically, the thigh or belly is used as suitable injection site. All analogue flasks (as well as those of regular insulin, IGF1 and glargine metabolites) in the picture have been used in this research. Recently it appeared that IGF1 was misused to enhance sport performances by bodybuilders and cyclist.

◀ IN BEELD

Insuline analogen pennen met navulsysteem. Diabetes patiënten zullen deze gebruiksvriendelijke pennen zeker herkennen. Met de roterende dop kan de dosis ingesteld worden. De naald wordt op de voorkant geschroefd. Normaal gesproken wordt de dij of buik gebruikt als injectieplaats. Alle analogen flesjes (evenals die van normaal insuline, IGF1 en de metabolieten van glargine) op de foto zijn gebruikt in dit onderzoek. Recent is gebleken dat IGF1 is misbruikt door bodybuilders en wielrenners om hun sportprestaties te verbeteren.

Every year hundreds of new chemical entities are produced intended for the human pharmaceutical market. Many of these compounds will never reach the market or will eventually be redrawn from it because of serious side effects. The process to get safe medicines on the market is regulated by national authorities of each individual member state in Europe, and a centralized organization called the European Medicines Agency (EMA).

The demands for the development of a medicinal product are laid out in numerous guidelines, drawn up by the regulatory authorities. A number of these guidelines is aimed at providing guidance on the non-clinical development and risk assessment of new products, mainly of the small chemical type. However, because of their unique biological and physiochemical characteristics, a specific guideline was written for biotechnology-derived compounds which is based on a scientific case-by-case approach [1] [2].

Although biopharmaceuticals are not genotoxic and therefore are not expected to be 'complete carcinogens', chronic administration could potentially lead to tumor promotion or progression of specific neoplasm(s) based on their expected pharmacologic activity [3]. In several scenarios evaluation of the carcinogenic potential should be considered for non-genotoxic biopharmaceuticals:

1. In case different biological effects are observed between the recombinant product and the endogenous protein.
2. When there are structural differences between the recombinant product and natural product.
3. If the recombinant products are administered at pharmacologic doses greater than expected endogenous levels.

But emphasizing a 2-year rodent study (rat or mouse), and thus referring to rodents as a golden standard is neglecting the high number of false positives in these species as compared to humans. Half of all long- term used pharmacotherapies induces rodent cancer, due to the high sensitivity of rodents versus humans, human irrelevant age-related tumors arise in these animals [4] [5]. The purpose of the S6 guideline with the recent addendum was to offer alternative strategies rather than to default to the rodent bioassay to provide an appropriate carcinogenic risk assessment [2]. Alternative strategies include in vitro approaches and if necessary a more relevant rodent in vivo model, besides a review of available literature data, information from similar targets or class effects, and clinical data.

The molecules discussed in this thesis are the insulin analogues, these molecules are very similar to regular insulin but have improved pharmaco-kinetic and -dynamic parameters. Insulin analogues are used by diabetic patients to regulate their blood glucose levels. Long- and short-acting insulin analogues have been developed so that plasma levels can be tuned accurately during the day reflecting the physiological activity of endogenous insulin without much fluctuation after physical activity or food intake.

Insulin analogues (as well as regular insulin) are growth factors and have besides the intended metabolic effects also an intrinsic mitogenic behavior. While the pharmacological action of insulin is mediated through the insulin receptor, the mitogenic potential of insulin and insulin analogues are mainly related to their affinity for and downstream effect via the insulin like growth factor-1 receptor (IGF1R) (Figure 1). The natural ligand of the IGF1R, IGF1, is structurally very similar to insulin which results in cross-reactivity of the different ligands for the above mentioned receptors. An increased binding affinity towards the IGF1R compared to regular insulin could be the result, which is indeed the case for some insulin analogues. Therefore a major concern with respect to safety aspects for insulin analogues is a disproportional increased mitogenic activity.

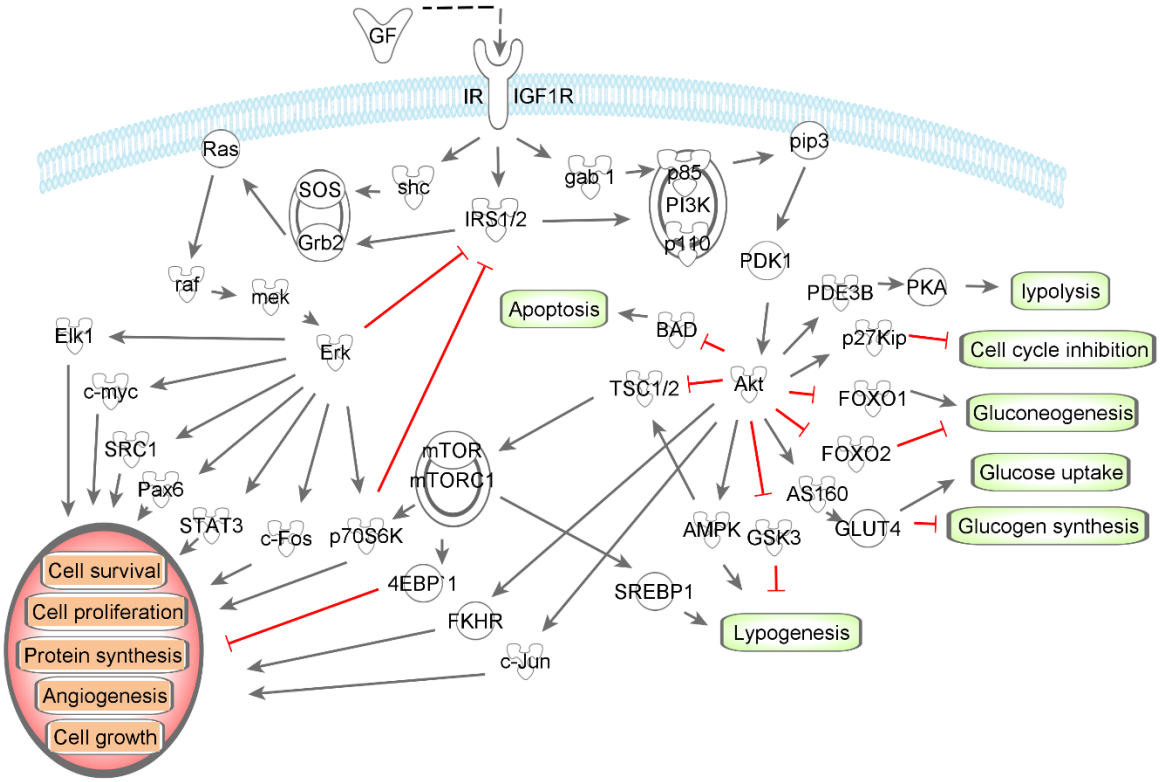


Figure 1. The insulin receptor (IR) and insulin like growth factor-1 receptor (IGF1R) signalling pathway. Activation of these receptors by a growth factor (e.g. insulin or insulin analogues) triggers its auto-phosphorylation. Several substrates (e.g. shc and gab1) can bind the membrane bound receptor and get phosphorylated, which in turn will activate two distinct signalling cascades, the PI3K/AKT and MAPK/ERK. The PI3K is thought to play a major role in metabolism (e.g. glucose uptake, glucogen synthesis), whereas the MAPK leads to the more mitogenic effects (e.g. cell proliferation and survival). But as is clear from the pathway many cross links can be made between these different cascades, making the IR/IGF1R signalling pathway highly complex.

In 2002, the EMA proposed testing strategies for insulin analogues specifically in a guidance document [6], in which it was stated that the preclinical safety evaluation of these compounds should focus on the mechanisms of action of the expected carcinogenic effect. Besides receptor kinetics and binding affinity, characterization of the different intracellular pathways is needed, not only for the IGF1R both also for the different isoforms of the insulin receptor. There is

evidence that these receptors might react differently in neoplastic tissue compared to normal tissue, therefore in vitro studies should be performed in both cancer cell lines and primary cells to make a comparison of insulin analogue induced pathway activation. The use of adapted animal models is encouraged to increase the human clinical relevance of these chronic rodent experiments. Furthermore, the importance of including the right reference compounds was highlighted. Native insulin, IGF1 and AspB10 (an insulin analogue with a known increased mitogenic activity) should be included in the mitogenic assays to put the obtained results in perspective.

In this thesis we have used the recommendations from the EMA as a guideline for the carcinogenic risk assessment of all commercial available insulin analogues.

In **chapter 2**, an introduction is presented that included a literature search of all available experimental data on this topic. For this systematic review we have focused on the link between insulin analogue exposure and breast cancer development by including only in vitro studies that have used breast cancer cell lines or animal and patient derived studies that have focused on mammary gland tumor development specifically.

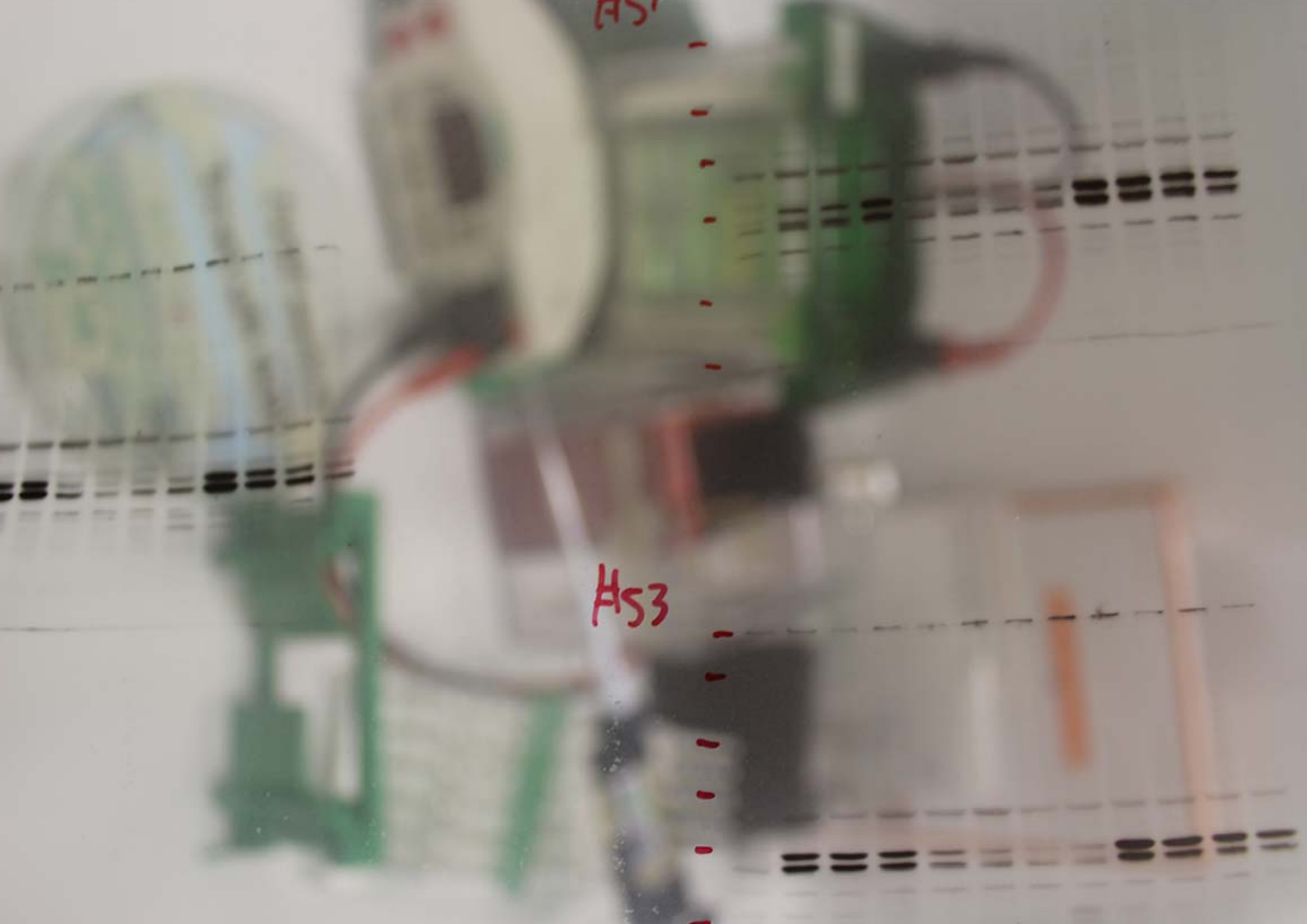
An in vitro study is described in **chapter 3**, in which a new cell model was used to determine the mitogenic activities of insulin analogues. A stable knockdown was combined with a retroviral overexpression system by which the MCF7 human breast cancer derived cell model only expressed one of the involved receptors (IRA, IRB or IGF1R). Exposure experiments have been performed including all commercial insulin analogues as well as regular human insulin, AspB10 and IGF1 as reference compounds. Several exposure times as well as concentrations have been included. Both a mechanistic information was gathered using the protein quantification methods as a read out as well as functional assays determining the direct proliferative effects of these compounds.

In **chapter 4**, the same cell model was used as in chapter 3. This time a full transcriptomic analysis was performed using micro-arrays. The genes involved in insulin analogue mitogenic signaling were identified. The predictive potential of these mitogenic gene classifiers were tested using all commercially available analogues. Furthermore validation and possible clinical relevance was assessed by testing the gene expression levels of these classifiers in different models, including primary human mammary cells and mouse mammary glands.

An in vivo experiment was carried out using the $p53^{R270H/+}$WAPCre mouse model. This model has a human relevant mutation in the tumor suppressor p53 gene by which it will develop spontaneous mammary gland tumors. In **chapter 5**, we determined if chronic insulin analogue treatment would affect the tumor latency time. Furthermore a phospo-proteomic analysis on the tumors was performed to detect treatment specific differences between the insulin analogue induced tumors.

These tumors were further characterized in **chapter 6** using next generation sequencing. We anticipated that the full transcriptomic analysis would shed light on how these tumors have developed and to pick up treatment specific tumor-related effects. Furthermore a mutational analysis was performed on these tumors.

Finally, **chapter 7** provides a general discussion on the results obtained in our studies and on the implications for future research.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