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NOVEL PHARMACEUTICAL APPROACHES TO REGULATE GLUCOSE HOMEOSTASIS

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ABSTRACT

The Western sedentary life-style and calorie excess is causing an increased incidence of obesity and type 2 diabetes. When untreated, these diseases are associated with heart- and vascular disease, stroke, cancer, neuropathic pain, renal failure and infertility. The expected lifespan of type 2 diabetic patents is reduced by ~5-10 years compared to the healthy population. Presently there are several available therapies to treat type 2 diabetic patients, but no drug can normalise the high blood glucose level characteristic of the disease. There is a need to further understand the underlying mechanisms of the type 2 diabetes and to bring more efficient drugs to the patients. Decreasing calorie intake and increasing physical exercise are valuable tools for the treatment of type 2 diabetes. Unfortunately, life-style is a challenge to change and many patients are not able to perform physical activity.

The enzyme AMP-activated protein kinase (AMPK) is activated by both fasting and muscle contraction. We have sought to illuminate the impact of chronic activation with a chemically synthesised AMPK-activator, 5-aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside (AICAR), in rodent models of type 2 diabetes (study I and II).

Type 2 diabetes is associated with increased glucocorticoid levels, which concomitantly lead to a decrease in adiponectin levels. Cortisone is transformed to its active form cortisol, via 11βhydroxysteroid dehydrogenase (HSD) type 1. In study III, we sought to ascertain if inhibition of 11βHSD1, with a new adipose-tissue-specific inhibitor, BVT116429, affects the concentration of circulating adiponectin with accompanying amelioration of glucose homeostasis in diabetic mice.

The ob/ob, KKA^y and KKA^y-CETP mice, were used to study the pharmacodynamic parameters and biomarkers of AMPK activation and 11βHSD1 inhibition. The three animal models feature severe obesity, hyperinsulinaemia and hyperglycaemia. Activation of AMPK via AICAR-treatment and inhibition of 11βHSD1 with BVT116429 resulted in improved glucose homeostasis. Clinical chemistry parameters were improved, including decreased plasma glucose.

The underlying mechanisms for the improvements in glucose homeostasis were studied. Acute AMPK activation normalised insulin-stimulated glucose uptake, which is in accordance with an observed two-fold increase in protein expression of GLUT-4, hexokinase II, and myocyte enhancer factor 2. Inhibition of 11βHSD1 with BVT116429 increased the concentration of adiponectin both *in vivo* and *in vitro*.

Pharmacodynamic studies were performed in well-validated models of type 2 diabetes, which increases the chance of succeeding in future clinical trails. This may increase the opportunity to help people with type 2 diabetes to live a longer and healthier life.

Drug discovery involves a complex series of steps, where the potential drug must pass several phases to reach the market and the patient. The chemical substances used in these studies were subjected to early drug discovery and analysis. The observed effects of these chemicals provide evidence for the usefulness of targeting AMPK and 11βHSD1 *in vivo* for the treatment of metabolic disturbances in type 2 diabetes and obesity.

LIST OF PUBLICATIONS

I. **M. Fiedler,** J.R. Zierath, G. Selén, H. Wallberg-Henriksson, Y. Liang, K.S Sakariassen. 5-aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in KKA^{y}-CETP mice. *Diabetologia* (2001) 44:2180-2186

II. X.M. Song, **M. Fiedler,** D. Galuska, J.W. Ryder, M. Fernström, A.V. Chibalin, H. Wallberg-Henriksson, J.R. Zierath. 5-aminoimidazole-4-carboxyamide ribonucleoside treatment improves homeostasis in insulin-resistant diabetic (ob/ob) mice. *Diabetologia* (2002) 45:56-65

III. **M. Sundbom**, C. Kaiser, E. Björkstrand, V. Castro, C. Larsson, G. Selén, C. Söderberg Nyhem, S.R. James. Inhibition of 11βHSD1 with the S-phenylethylaminothiazolone BVT116429 increases adiponectin concentrations and insulin sensitivity in diabetic KKA^{y} mice. *BMC Pharmacology* (2008) 8:3

CONTENTS

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotrophic hormone
ADA	American diabetes association
AICAR	5-aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside
ALAT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AS160	Akt substrate 160-kD
A^y	Agouti
BMI	Body mass index
CaMKK	Ca^{2+}/Calmodulin-dependent protein kinase kinase
CETP	Cholesteryl ester transfer protein
Compound C	6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine
CPT 1	Carnitine palmitoyltransferase
CRF	Corticotrophin-releasing factor
DEXA	Dual energy X-ray absorptimetry
DIO	Diet induced obese
DNA	Deoxyribonucleic acid
DPPIV	Dipeptidyl peptidase IV
4E-BP	Eukaryotic translation initiation factor 4E binding proteins
EDL	Extensor digitorum longus
eIF4E	Eukaryotic translation initiation factor 4E
EMEA	European medicines agency
EPI	Epitrochlearis
FDA	Food and drug administration
FOXO	Forkhead box
GK	Goto-Kakizaki rat
GLP1	Glucagon-like peptide 1
G-6-P	Glucose-6-phosphate
GPR	G-protein coupled receptor
GR	Glucocorticoid receptor
hERG	Human ether-à-go-go related Gene
HDL	High density lipoprotein
HK	Hexokinase
11βHSD	11β-hydroxysteroid dehydrogenase
IDF	International Diabetes Federation
Ip	Intraperitoneal
Iv	Intravenous
IRS	Insulin receptor substrate
KHB	Krebs-Hensleits buffer
LMW	Low molecular weight
MEF2	Myocyte enhancer factor
MIRKO	Muscle-specific insulin receptor knockout mice
MMW	Medium molecular weight
MO25α/β	Mouse protein 25

MODY	Maturity onset diabetes of young
MR	Mineralocorticoid receptors
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamid adenine dinucleotid phosphate
NCE	New chemical entity
NZO	New Zealand obese
OGTT	Oral glucose tolerance test
PI3 kinase	Phosphatidylinositol 3 kinase
PPAR	Peroxisome proliferator activated receptor
Sc	Subcutaneous
SEM	Standard error of the mean
SGT-2	Sodium-glucose cotransport
STRADα/β	Ste20-related adaptor protein
TAK1	Transforming growth factor-β-activated kinase 1
TZD	Thiazolidinediones
UDP	Uridine diphosphate
VLDL	Very low-density lipoproteins
WHO	World health organization
ZMP	5-aminoimidazole-4-carboxamide ribonucleotide

1 INTRODUCTION

1.1 TYPE 2 DIABETES, OBESITY AND INSULIN SENSITIVITY

The Western sedentary life-style and an abundance of calories are causing an increased incidence of obesity and type 2 (non-insulin-dependent) diabetes mellitus. Type 2 diabetes is a globally growing disease due to improved welfare. The International Diabetes Federation (IDF) estimates that there are 246 million people with diabetes today. In Europe, the diabetes prevalence is 8.4% according to the same organisation. Throughout evolution, the greater part of mankind has gained genes encoded for energy conservation, which allow survival during famine. Our genes have not changed much during the last thousands of years, but our life style has dramatically changed. For instance, the intake of high fructose corn syrups, used not only in soft drinks, but also in other industrially produced food, has increased 1000% between 1970 and 1990 (Bray *et al.*, 2004).

Type 2 diabetes is a heterogeneous disease leading to increased plasma glucose levels. The subtype called maturity onset diabetes of the young (MODY) is mainly due to genetic factors and has little to do with life-style. There are also many populations with increased risk for type 2 diabetes, such as the Pima Indians (Sievers *et al.*, 1992). Drugs, smoking and other toxic substances can also increase the risk of type 2 diabetes (Virmani *et al.*, 2007; Willi *et al.*, 2007). The International Diabetes Federation estimates that 80% of all type 2 diabetes cases could be prevented by adopting a healthy diet and increasing physical activity.

Untreated type 2 diabetes can result in heart and vascular disease or stroke (Asadollahi *et al.*, 2007). Increased plasma glucose and insulin levels are also associated with cancer risk (Jee *et al.*, 2005; Slattery & Fitzpatrick, 2009). There can also be problems with wound healing (Brem & Tomic-Canic, 2007), neuropathic pain, retinopathy (Crawford *et al.*, 2009), kidney disease (Alwakeel *et al.*, 2009) and fertility (Yogev & Catalano, 2009) associated with type 2 diabetes.

Diabetes is characterised by elevated plasma glucose levels, peripheral insulin resistance, impaired glucose tolerance, dyslipidaemia, increased hepatic glucose production and beta cell defects (Groop, 2000). The current diagnostic criteria for type 2 diabetes is fasting plasma glucose $\geq$ 7.0 mM (126 mg/dl) or plasma glucose $\geq$ 11.1 mM (200 mg/dl) 2 hours after an oral glucose tolerance test (OGTT; 1.75 g glucose/kg of body weight, maximum 75 g glucose) (WHO 2006). WHO has changed the criteria from $\geq$ 8.0 mM (1980) to $\geq$ 7.8 mM (1985) and now to $\geq$ 7.0 mM (1999 and 2006). One reason for these changes is that epidemiological studies provide evidence for increased diabetes complications, such as retinopathy when glucose levels are higher (Gabir *et al.*, 2000).

For many patients, the type 2 diabetes starts with visceral obesity, leading to insulin resistance and hyperinsulinaemia. At this time, the patient is often unaware of the disease and it is only when the body cannot adjust for insulin resistance by increasing insulin production that the patient will notice elevated glucose levels or hyperglycaemia. The patient will also have elevated hepatic glucose production, due to hepatic insulin resistance. In the late stage of the disease, there is severe beta cell loss and insulin levels are low, followed by a rise in glucose. At this stage, insulin therapy is one of the primary treatment options. The disease progression from pre-diabetes to type

2 diabetes is long, and it may take more then ten years before type 2 diabetes is diagnosed. As the population tends to gain weight, this time-line is compressed and today there are both adolescents and children with type 2 diabetes.

1.2 TREATMENT OF TYPE 2 DIABETES

1.2.1 Current treatments

The cure for type 2 diabetes is elusive and education about life-style changes may offer the best option for disease prevention. Most patients are treated with several drugs at the same time, since monotherapy is often inefficient. Compliance is decreased by the large number of pills and side-effects from the medication, such as hypoglycaemia, weight gain and gastrointestinal inconveniences. The current therapies are sulfonylurea, biguanides, alphaglucosidase inhibitors, peroxisome proliferator activated receptor (PPAR) γ agonist, glucagon-like peptide 1 (GLP1) analogues, dipeptidyl peptidase (DPPIV) inhibitors, and insulin.

Metformin is an inexpensive biguanide and has been on the market for several decades. The mechanism of action for metformin is still uncertain, but it inhibits gluconeogenesis and increases peripheral glucose uptake (Wiernsperger & Bailey, 1999). Metformin is metabolised via the kidneys and since many patients with type 2 diabetes present with renal disease, this can result in lactic acidosis. Phenformin and buformin, two former biguanides were withdrawn in the early 1970's due to the increased risk of lactic acidosis (Cusi *et al.*, 1996).

Another conventional treatment on the market is sulfonylurea, which increases insulin production by closing the potassium channels in the beta cell. Unfortunately, this treatment is only effective if there is sufficient beta cell mass. Since beta cell mass usually decreases with time, this treatment option is inefficient after several years. High levels of insulin not only increase the patient's weight, but an overdose of sulfonylurea can also lead to hypoglycaemia. There are two newer substances in the clinical treatmet of type 2 diabetes including Repaglinid™ and Nateglinid™, which also work through the potassium channels in pancreas. Nateglinid™ is more selective for the potassium channels in the pancreas compared to other tissues such as heart.

Alphaglucosidase inhibitors prevent the metabolism of polysaccharides to monosaccharides, which results in decreased glucose uptake from the intestine. The effect is less than that achieved with metformin or sulfonylurea. Alphaglucosidase inhibitors have gastrointestinal inconveniences as a common side effect.

The thiazolidinediones (TZD) or PPARγ agonists have been on the market for ten years and they improve glucose tolerance and whole body insulin sensitivity. Unfortunately, weight gain is one side-effect and there have been some severe problems with liver toxicity. The first TZD approved by the FDA (Food and Drug Administration) in the USA was withdrawn after reports of liver failure and deaths (Scheen, 2001). Moreover, a meta-analysis suggested that the use of rosiglitazone was associated with an increased risk for cardiovascular events (Nissen & Wolski, 2007).

Two recently approved treatments for type 2 diabetes that enhance insulin production after a meal are the long-acting GLP1 analogues Exenatide™ and Liraglutide™. Sitagliptin™, Saxagliptin™ and Vidagliptin™ are DPPIV-inhibitors, which decrease the breakdown of GLP1 and thereby prolong the half life of endogenous GLP1. The DPPIV-inhibitors have oral bioavailability, which is an advantage over the GLP1 analogues, which have to be injected. Additional data is

required to fully validate the efficacy and safety of DPPIV inhibitors and GLP1 mimetics in the long-term treatment of type 2 diabetes.

Insulin is the only current treatment for type 2 diabetes in the late stages of the disease. Injections are inconvenient and there are difficulties in finding the right dose. Buccal™ insulin (Bernstein, 2008) is a new formulation, which might be helpful for children and patients who suffer from injections.

A large systematic review of oral diabetes agents (Bolen *et al.*, 2007) has provided evidence that suggests in spite of newer treatments; metformin seems to have the best profile of benefit to risk. Metformin has similar or superior effects on glycaemia control than newer drugs. Although the number and forms of treatments for type 2 diabetes has increased in the last ten years, there are continuous difficulties in reaching the goal to normalise glycaemia. There is still a great medical need to develop novel drugs with improved efficacy and with fewer side-effects for the treatment of type 2 diabetes.

1.3 DRUG DEVELOPMENT

There are different types of drugs including small molecules, also called new chemical entities (NCE), large molecules and monoclonal antibodies. Small molecule drugs predominantly work on G protein-coupled receptors, ion channels, nuclear receptors and enzymes. The antidiabetic drug metformin is an example of a small molecule drug. The protein insulin is an example of a large molecule drug. Influximab™, used in the treatment of rheumatoid arthritis, is an example of a monoclonal antibody. Targeting the genome is possibly the most challenging approach, but new technology including antisense, RNAi, DNAzymes and ribozymes makes this a realistic possibility for the future. This thesis will emphasise on the development of small molecule drugs.

There are no governmental rules for early drug discovery. However, when the potential drug is in preclinical and clinical phases, specific guidelines must be followed. Therefore, the drug development funnel for small molecules might differ between companies and also for different targets, however most pharmaceutical companies have similarities in their pipelines.

Several years are required to move an idea to the drug product market. The time-line, can easily stretch 12 years from idea to market (Figure 1). The process starts with target discovery, followed by screening for molecules, lead generation, lead optimisation, selection of candidate drug, preclinical phase, clinical phases and ends with registration of the product. Each phase has the power to end the project. If the criteria's set for the earlier phases are not accomplished, the project cannot enter the later phase. Exactly which tests are performed in each phase varies depending on the timeline, target, strategy and economy of the company.

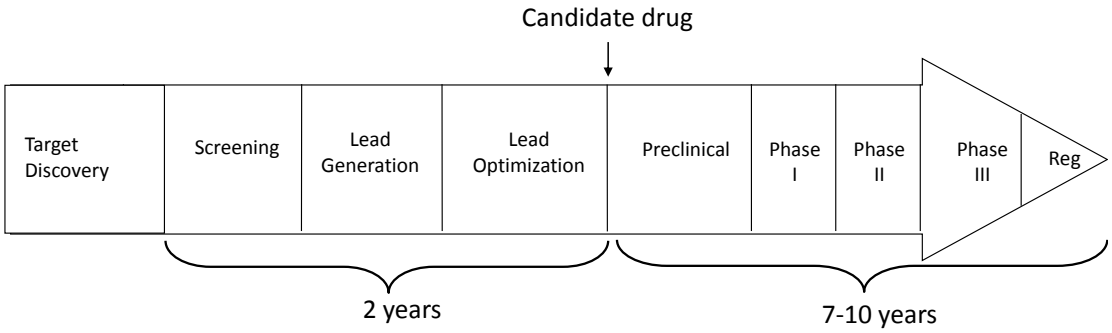

Figure 1. Timeline for drug development from target discovery to registration of drug.

1.3.1 Target discovery including identification and validation

The drug discovery pipeline starts with identification of the drug target. There are many criteria, which must be accomplished for a potential drug including an unmet medical need, the possibility to obtain a patent, the ability of the drug to reach the target, an acceptable route of administration, as well as potency and safety.

Historically, many drugs were the products of serendipity. For example phenformin, the ancestor to metformin, was first used as a drug for malaria prophylaxis, but patients with type 2 diabetes experienced improvement of their blood glucose levels and thereby an oral treatment for type 2 diabetes was discovered. The drug discovery phase is exploratory and today we have the possibility to search for information in databases and in the literature. Novel targets can be identified through gene transcription profiling, protein expression profiling, metabolic pathways, protein glycosylation, and protein-protein interactions. Underestimated target identification is to study pathophysiology and thereby draw conclusions. One historic example was when Banting and Best discovered and isolated insulin in 1921 and found that lack of insulin was the cause of diabetes mellitus.

When the target is identified, it must be biologically validated. Questions to be answered during this phase include which cell types the target is expressed in, if there are any safety implications, and if they are species specific issues, and which biomarkers can be used. It is also valuable to compare the pattern of expression between the healthy and disease state. Chemical substances, which interact with the system of interest, are also useful in target validation.

Small rodents are the first choice for both target identification and validation *in vivo*. The technique of genetically modified mice models has changed focus from rats to mice. The mouse genome was one of the first to be sequenced (Okazaki *et al.*, 2002; Waterston *et al.*, 2002). There are possibilities with transgenic mice, where new genes have been introduced and knockout mice, where genes have been removed. In a constitutive knock-out mouse, the deletion is present in all cells and during all states of development, whereas in the conditional mutation, it is only in special cell types or in a specific state of development. Caution should be taken when interpreting knock-out and transgenic models. The impact depends on which organs that are involved and the extent of the modulation.

The preferred level of validation is broad, but if the target is well-validated in the literature, the competition from other companies and groups is tougher. There is a balance between broad validation with high competition, and less validation with low competition.

1.3.2 Screening

After the target is identified and validated, the search for chemicals starts. The screen is a way of finding starting points for the construction of new chemical entities (Figure 2). First of all, an assay must be set up. This is often a binding assay, where only one concentration of the compound is tested. Large companies often do a high through-put screen using a library of thousands of chemicals. This enterprise can be outsourced to other specialised screening companies. The collaborative agreement is then exclusive for that specific target. Since high through-put screening is expensive, other alternatives have been invented, for example virtual screening by computer chemists and searching for start points from patents or other literature sources. If a high through-put screening is used, many binding substances are found in the first large screen, which is quite rough and will give both false negative and false positive values for the chemicals. The substances that are binders are then confirmed in a confirmatory assay where more concentrations of the drug are tested and thereafter in an orthogonal assay i.e. an assay which tests for the same function, but in another way. Depending on the time-line and target, several different functional assays might be used. The functional assays are biologically relevant to the target.

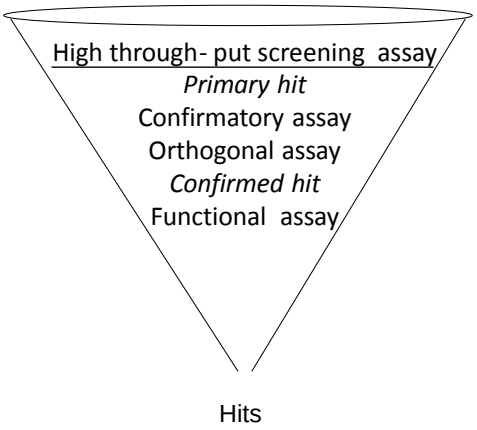

Figure 2. Screening funnel.

1.3.3 Lead generation and lead optimisation

In this phase, the starting points for the chemicals found in the initial screens are developed towards more potent molecules with the desired characteristics. The confirmed hit compounds are tested for parameters such as metabolic stability, cytochrome enzymes, solubility, permeability and predicted plasma protein binding properties. The compounds are also tested for selectivity to related targets. There is no rule for which characteristics a potential drug should have, but it is often desirable to have low protein binding, high permeability and high metabolic stability, because that will result in a lower predicted effective dose. However, the "Lipinski´s rule of 5", suggests a formula for how a chemical substance should be composed for successful oral availability (Lipinski *et al.*, 2001). High solubility is also desirable, both due to the formulation of the future drug and for easier preparations of solutions for assays. Two

to three lead series of compounds are chosen to increase the probability of success in lead optimisation (Figure 3). The border between lead generation and optimisation is often indistinct and might vary for different projects. Sometimes the first *in vivo* studies are performed already in lead generation and sometimes first in lead optimisation phase.

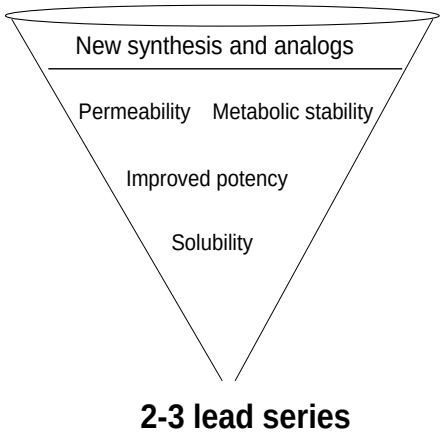

Figure 3. Lead identification funnel.

The aim with the lead optimisation phase is to find one or two candidate drugs to take into further preclinical studies (Figure 4). Several substances are chosen for studies of pharmacokinetics *in vivo*. The substances are given intravenously, orally and sometimes via some other route of administration to normal mice. A drug which can be taken orally or in the second case, subcutaneously, is desirable. The pharmacokinetic studies will reveal the degree of plasma protein binding *in vivo*, half time of the substance, clearance, distribution volume and the maximal plasma concentration. These values are used to decide if a compound is good enough to be tested *in vivo* and to predict doses for the pharmacodynamic studies. When a substance appears promising, it is tested in tolerance studies. Three doses are mostly chosen, where one is higher than the predicted dose in pharmacodynamic studies. In the tolerability study, which is similar to the regulatory core battery of safety pharmacology assays, a few animals are dosed with the substance and behaviour and health status of the animals is recorded. A neurobehavioral Irwin screen (Irwin, 1968) is often used, which is a visual scoring for parameters such as piloerection, body position and spontaneous activity. The highest tolerable dose is found and with this information, together with the pharmacokinetic information, potency and *in vitro* data, the doses for pharmacodynamic studies are chosen.

The pharmacodynamic studies can be performed in normal animal strains and in disease models, where the latter is a pathological condition resembling a human disease. The animal models can be developed spontaneously or induced with diet, surgery or toxins. The pharmacodynamic studies seldom fully mirror the human disease, and therefore the data must be interpreted with this limitation in mind. Several animal models and pharmacodynamic techniques are needed to get a complete picture of how the drug improves the disease state. Since the introduction of genetically modified mouse models, rats are seldom used for pharmacodynamic studies. The

disadvantage with using rats in drug discovery is that they are heavier than mice and therefore consume more of the potential drug.

Other safety aspects of the drug are often tested, including the impact of the drug on ion channels. Especially important is the ether-à-go-go related gene (hERG) (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995) which is coding for an ion channel in the heart. Inhibition of it may induce heart arrhythmia. This can be studied with simpler inhibition assays and more advanced techniques such as the patch clamp (Hamill *et al.*, 1981).

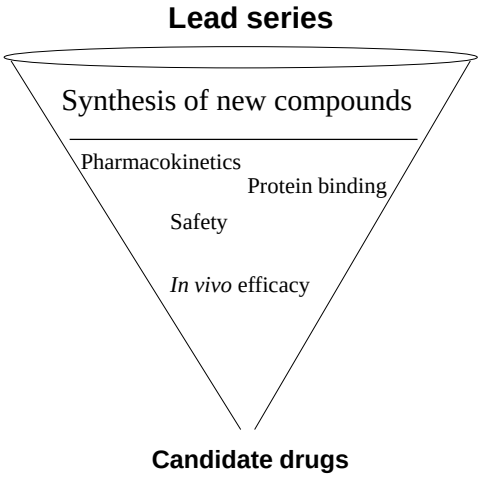

Figure 4. Lead optimisation funnel.

1.3.4 Preclinical studies

If, or when the compound has shown satisfactory effects in pharmacodynamic studies, the safety and toxicology studies follow (at least in two models, where one is a non-rodent) before the clinical program is initiated. The aim of the preclinical studies is to answer all the regulatory demands to be able to package the project for clinical trails. This is often the phase when the first human dose of the drug is predicted from animal data.

1.3.5 Clinical studies and registration

The aim of the first clinical study, called phase I is to indicate a safe dose interval for later studies in patients and to document pharmacokinetic properties of the future drug. Approximately 30 to 100 healthy men and woman receive increasing doses of the substance until some kind of effect is shown. The absorption, distribution, metabolism, excretion and tolerability are measured. The aim of the phase II study, including 300 to 1000 patients, is to show that the drug is useful for therapeutic purposes. In phase IIa, proof of concept in humans is shown i.e. that the drug has its desired effect in patients. The therapeutic dose interval is established in the phase IIb study. The aim with the phase III study, generally performed in 3000-10 000 patients, is to a) prove statistically that the drug has a favourable efficacy, b) compare the new drug with competitors and c) establish limitations in the use of the drug. Phase IV is conducted after the registration of the drug and includes a large number of patients. The aims are to show the safety profile of the drug and generalise the results to a larger population.

Depending on where the drug shall be sold, the site of registration is chosen. There are several choices; the national registration via the medical product agency for the specific country, mutual recognition or centralised registration via the European Medicines Agency (EMEA). The Food and Drug Administration is the medical product agency in the United States.

1.4 ANIMAL MODELS OF TYPE 2 DIABETES AND OBESITY

There are vast numbers of different type 2 diabetes rodent models. Since the disease presents with different pathologies, pre and post-insulin receptor, it is convenient to have models that mimic some of the different features of the diabetic phenotype. The models can be spontaneous or experimentally induced. The major concerns in the use of animal models, however, relate to their relevance with respect to human disease. The following diabetic animal models were used in the studies in this thesis: ob/ob-mice, KKA^y mice and KKA^y-CETP mice.

1.4.1 Ob/ob mice

The ob/ob mouse strain results from a single and spontaneous gene mutation. The ob/ob mouse (B6.v-lep^{ob}/J) has a mutation in the ob gene, in chromosome 6, which results in failure of the white adipose tissue to produce functional leptin (Zhang *et al.*, 1994). The hormone leptin inhibits feeding and has impact on energy expenditure. Lack of leptin will lead to hyperglycaemia, hyperinsulinaemia, insulin resistance and obesity. The ob/ob mice have all these features and the syndrome is reversed if the mice are injected with leptin (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995).

1.4.2 KKA^y-mice

The Japanese inbred KK mouse strain (Kondo *et al.*, 1957; Nakamura, 1962) develops type 2 diabetes with mild obesity, mainly due to peripheral insulin resistance. With the introduction of the agouti (A^y) allele, the KKA^y mouse was produced (Nishimura, 1969; Suto *et al.*, 1998). The agouti peptide acts as an antagonist at the melanocortin 4 receptor, to inhibit the action of melanocyte-stimulating hormone signals. Dominant mutations in the agouti gene lead to a syndrome characterised by obesity, mild hyperphagia, decreased thermogenesis, hyperinsulinaemia, peripheral insulin resistance, impaired glucose tolerance, mild hyperglycaemia in males and yellow fur. The KKA^y mouse is an obese mouse with severe hyperglycaemia. The severity of the disease often results in wounds and tumours. Daily health inspections of the animals are necessary to remove the animals with wounds and tumours from the studies.

1.4.3 KKA^y-CETP mice

One disadvantage with mice being used as diabetic disease models is that rodents with severe diabetes still have higher high-density lipoprotein (HDL) levels, which is the `good cholesterol´, in comparison with humans. The main reason for this might be that mice, rats, dogs and pigs lack cholesteryl ester transfer protein (CETP). The role of CETP is to transfer cholesteryl ester out of HDL to chylomicrons and very low-density lipoproteins (VLDL). The cholesterol is thereafter transported to the liver and eliminated. In man, about 80% of cholesteryl ester formed on HDL is carried to other

lipoproteins by this route. This redistribution of lipoproteins makes the fraction of VLDL higher compared with HDL.

To bring the lipid profile in the mouse model closer to that found in humans, simian CETP was expressed in a diabetic mouse model resulting in the KKA^y-CETP mouse whose diabetic characteristics are partly from the KK mouse (a polygenetic model of type 2 diabetes with mild obesity) and partly from C56BL/6J mice expressing the agouti gene. The procedure was as follows: the C57BL/6J mouse expressing the agouti gene (C57BL/6J -A^y) was crossed with the C57BL/6J mouse expressing simian CETP, resulting in the C57BL/6J-A^y-CETP mouse. The C57BL/6J-A^y-CETP mouse was then crossed with the KK mouse. The offspring expressing the agouti gene had yellow fur and those who also expressed the simian CETP gene were recrossed with KK mice. After six generations the offspring were defined as KKA^y-CETP mice. The transgenic KKA^y -CETP mouse is an obese and diabetic mouse with a lipid profile similar to humans (Castle *et al.*, 1998).

1.5 NOVEL TARGETS FOR THE TREATMENT OF TYPE 2 DIABETES

1.5.1 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is suggested to be a metabolic master switch, which senses the metabolic status of the organism, and regulates both anabolic and catabolic pathways (Winder & Hardie, 1999). Exercise is a natural activator of AMPK, but it is also activated by metabolic stress (Long & Zierath, 2006), glucose deprivation (de Lange *et al.*, 2007), hormones (Minokoshi *et al.*, 2002; Tomas *et al.*, 2002; Yamauchi *et al.*, 2002), fructose treatment in hepatocytes (Gillespie & Hardie, 1992), heat shock (Corton *et al.*, 1994), drugs (Zhou *et al.*, 2001; Fryer *et al.*, 2002a) and other chemical substances (Hayashi *et al.*, 1998; Cool *et al.*, 2006).

1.5.1.1 Structure of AMPK

The AMPK protein is a heterotrimer and composed of a catalytic α unit (Carling *et al.*, 1989) with the subunits α1 and α2 (Woods *et al.*, 1996), and two noncatalytic regulatory units, β (Thornton *et al.*, 1998) and γ (Davies *et al.*, 1994) with the subunits β1, β2, γ1, γ2 and γ3. Each subunit is encoded by different genes and has a unique role in the regulation and activation of AMPK. The α-subunit contains the activating phosphorylation site Thr^{172} (Mitchelhill *et al.*, 1997). The AMPK β-subunit has the function of docking the protein to membranes (Warden *et al.*, 2001) and the γ-subunit binds to AMP or ATP (Kemp, 2004). All three subunits must be expressed for full activation of the enzyme. The constitution of the heterotrimer differs between species, with the α2β2γ3 expressed predominantly in human skeletal muscle (Chen *et al.*, 1999) and α2β2γ1 in rat skeletal muscle (Cheung *et al.*, 2000).

The AMPK subunits have different subcellular distributions, where α1 is located in the cytoplasm and α2 is located in the nucleus and the cytoplasm (Salt *et al.*, 1998). The AMPK β-subunits are located in the nucleus and the cytoplasm (Warden *et al.*, 2001). The γ1-subunit is to a larger extent found in the nucleus than the other two γ-subunits (Turnley *et al.*, 1999). AMPK α2, β2 and γ1 are the subunits found in the nucleus.

1.5.1.2 AMPK signalling cascade

There are several ways for AMPK to turn from an inactive to an active form and at least one way to deactivate it (Suter *et al.*, 2006). Adenosine 5´-monophosphate (5´-AMP) can allosterically bind to AMPK via the γ-subunit (Ferrer *et al.*, 1985; Kemp, 2004). An increase in ATP inhibits the binding of AMP to the γ-subunit (Scott *et al.*, 2004). AMPK can also be activated by three AMPK kinases i.e. LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) and transforming growth factor-β-activated kinase 1 (TAK1) (Figure 5). AMPK can be dephosphorylated by protein phosphatases 2A and 2C (Suter *et al.*, 2006), and their access to AMPK is suggested to be regulated by AMP-binding (Witczak *et al.*, 2008).

Mice with muscle specific LKB1 knockdown have reduced AMPK activity and glucose uptake in skeletal muscle is impaired in response to contraction or exposure to the AMPK activator, 5-aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside (AICAR), but not after stimulation with insulin (Sakamoto *et al.*, 2005). For full AMPK activation through LKB1, additional proteins including Ste20-related adaptor protein (STRADα/β) (Baas *et al.*, 2003) and mouse protein 25 (MO25α/β) (Boudeau *et al.*, 2003) are needed.

AMPK activation by CaMKKα/β is AMP-independent (Suter *et al.*, 2006), but Ca^{2+}-dependent. When the intracellular level of Ca^{2+} is high, it forms a complex with calmodulin and this complex binds to CaMKK and AMPK is activated (Hawley *et al.*, 1995; Tokumitsu *et al.*, 2001).

The third AMPK kinase is TAK1, which *in vivo* forms a complex with TAK-1 binding protein 1 (TAB1) and TAK-1 binding protein 2 (TAB2) or TAK-1 binding protein 3 (TAB3) (Cheung *et al.*, 2004; Besse *et al.*, 2007). In mice with defective TAK1, the phosphorylation of AMPK at Thr^{172} is blocked with concomitantly less AMPK activity (Xie *et al.*, 2006). In cell cultures, TAK1 can be activated by three AMPK-activators i.e. metformin, AICAR, and the mitochondrial toxin oligomycin (Xie *et al.*, 2006). In the absence of TAK1, activation with these agents is impaired.

Liver, adipose tissue, and skeletal muscle are three important organs in the regulation of glucose homeostasis. Interestingly, skeletal muscle expresses LKB1, STRAD, MO25, CaMKKα, TAK1 and TAB1 (Shibuya *et al.*, 1996; Taylor *et al.*, 2004; Witczak *et al.*, 2007).

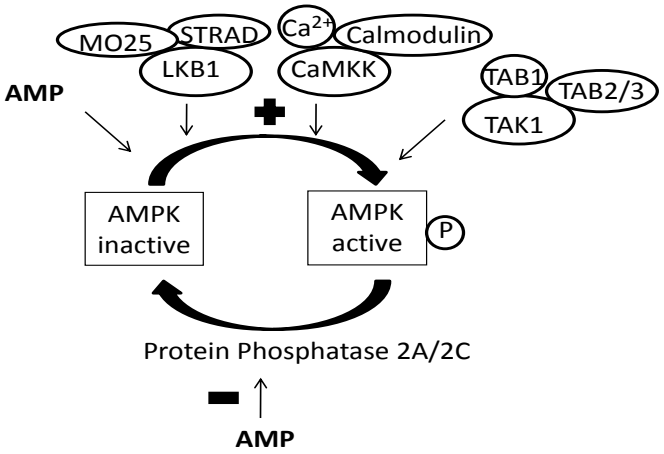

Figure 5. Regulation of AMPK. AMPK can be switched to its active form by allosteric binding of AMP, phosphorylation by LKB1 together with MO25 and STRAD, phosphorylation by CaMKK or phosphorylation by TAK1 together with TAB1 and TAB2 or TAB3. AMPK is deactivated by dephosphorylation by protein phosphatase 2A or 2C.

1.5.1.3 Physical activity and AMPK activation

Muscle contraction through physical activity is a physiological way to activate AMPK (Mu *et al.*, 2001; Musi *et al.*, 2001) and this is accomplished in several ways. Muscle contraction activity results in an increase in the intracellular Ca^{2+} levels. Calcium is released from the sarcoplastic reticulum to the muscle fibres (the myofibrils). The myofibrils are composed of actin and myosin. Calcium binds to a protein, troponin, at the actin filament and enables myosin to react with actin, which results in contraction. One ATP-molecule is used for each reaction i.e. physical activity consumes energy and it results in increased AMP:ATP levels, which can activate AMPK allosterically (Kemp, 2004). Rising AMP-levels also inhibit protein phosphatases known to dephosphorylate AMPK (Suter *et al.*, 2006). The increased intracellular Ca^{2+}-levels activate AMPK through CaMKK (Tokumitsu *et al.*, 2001).

1.5.1.4 Physiological response to AMPK activation

The activated AMPK has different effects in different organs (Long & Zierath, 2006):

- Skeletal muscle: Increased glucose uptake (Hayashi *et al.*, 1998), increased free fatty acid uptake and lipid oxidation (Merrill *et al.*, 1997; Merrill *et al.*, 1998; Osler & Zierath, 2008).
- Liver: Inhibited fatty acid synthesis, cholesterol synthesis and gluconeogenesis (Henin *et al.*, 1995; Bergeron *et al.*, 2001).
- Adipose tissue: Inhibited fatty acid synthesis and lipolysis (Sullivan *et al.*, 1994).
- Hypothalamus: Increased food intake (Andersson *et al.*, 2004)
- Pancreas: Decreased insulin secretion (Salt *et al.*, 1998)

The tissue-specific effects of AMPK constitute a physiological way to deal with enhanced physical activity. During exercise, skeletal muscle needs energy from glucose and free fatty acids. Physical activity leading to AMPK activation results in increased glucose uptake due to GLUT-4 translocation (Hayashi *et al.*, 1998). The activation of AMPK by AMPK-kinases can also lead to phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), and thereby a decrease in malonyl-CoA. The inhibition of carnitine palmitoyltransferase (CPT 1) by malonyl-CoA is relieved and fatty acid oxidation increases as exercise continues (Merrill *et al.*, 1997). Rats running on a treadmill show activation of AMPK, inhibition of ACC and decreased malonyl-CoA levels (Winder & Hardie, 1996). Furthermore, synthesis of fatty acids and cholesterol are inhibited after AMPK-activation (Henin *et al.*, 1995). Isolated muscles which were contracted for ten minutes showed increased AMPK activity with a concomitant increased glucose transport, decreased glycogen content and decreased ACC activity (Derave *et al.*, 2000). Glucose is transported into skeletal muscle by an insulin-independent mechanism in response to muscle contraction, and therefore insulin is not required. This was demonstrated using muscle-specific insulin receptor knockout mice (MIRKO), where the mice displayed normal exercise-stimulated glucose uptake, but had no response to insulin (Wojtaszewski *et al.*, 1999). After physical activity, an increase in appetite occurs to regain energy stores. Hypothalamic AMPK activity is increased during fasting and decreased during refeeding (Minokoshi *et al.*, 2004). Activation of AMPK in hypothalamus causes increased food intake in rats (Andersson *et al.*, 2004). An intake of carbohydrates will result in an increase in insulin, a decrease in leptin secretion, and inhibition of AMPK-activity. Insulin was shown to inhibit and leptin was shown to activate hypothalamic AMPK activity (Minokoshi *et al.*, 2004), (Minokoshi *et al.*, 2002).

Other hormones regulating appetite and stimulating AMPK activity are adiponectin (Yamauchi *et al.*, 2002), ghrelin, and resistin. Ghrelin stimulates hypothalamic and heart AMPK activity and inhibits adipose and liver AMPK activity (Kola *et al.*, 2005; Kola, 2008). The role of resistin is more complex. Resistin induces insulin resistance and stimulates hepatic glucose production (Banerjee *et al.*, 2004), but it also activates hypothalamic AMPK (Vazquez *et al.*, 2008).

Since AMPK-mediated glucose uptake is insulin-independent and patients with type 2 diabetes have normal AMPK α2 activity, as well as normal expression of the α1, α2 and β1 isoforms (Musi *et al.*, 2001), a new pharmaceutical compound mimicking AMPK effects in the peripheral metabolic tissues, but avoiding the CNS, would be of great value for the treatment of type 2 diabetes. Therefore a further understanding of AMPK downstream signalling and biological responses is of importance.

1.5.1.5 Validation of AMPK as target for drug development using transgenic and knock-out mice models.

There are many different models of genetically manipulated mouse strains regarding AMPK and its subunits (Long & Zierath, 2006). Glucose homoeostasis was unaltered in AMPK$\alpha 1^{-/-}$ mice, but AMPK$\alpha 2^{-/-}$ mice displayed elevated plasma glucose levels, insulin resistance, impaired glucose tolerance and reduced muscle glycogen synthesis (Viollet *et al.*, 2003). The same knockout mouse model was heavier and had increased fat mass compared with wild-type mice fed a high fat diet, despite no differences in food intake (Villena *et al.*, 2004). The α2 subunit has been studied using several models, including overexpression of AMPKα2 dominant negative subunit in skeletal and cardiac muscle (Mu *et al.*, 2001), muscle-specific overexpression of dominant negative AMPK α2 (Fujii *et al.*, 2005), liver-specific AMPK α2 knock-out (Andreelli

et al., 2006). All these models have deteriorations in glucose homeostasis. Deletion of LKB1 in either liver (Shaw *et al.*, 2005) or skeletal muscle (Sakamoto *et al.*, 2005) provides evidence for the importance of LKB1 in contraction-induced glucose uptake and glucose homeostasis. There are different knock-out and transgenic mice regarding the γ3 subunit (Barnes *et al.*, 2004). The total AMPK γ3 knock-out resulted in blunted AICAR-induced skeletal muscle glucose uptake and impaired glycogen resynthesis after two hours of swimming. Skeletal muscle specific overexpression of the wild-type form of the AMPK γ3 was without effect on the metabolic phenotype, but overexpression of a mutant form of the AMPK γ3 subunit (AMPK γ3 R225Q mutant) resulted in impaired AICAR-stimulated glucose transport and improved insulin sensitivity after high fat diet.

1.5.1.6 Chemical validation of AMPK as target for drug development

AMPK can be activated physiologically, by exercise and also pharmacologically, by chemical substances such as AICAR (Figure 6) (Sullivan *et al.*, 1994), mitochondrial toxins (Hayashi *et al.*, 2000; Fujii *et al.*, 2005), A-769662 (Cool *et al.*, 2006; Goransson *et al.*, 2007), metformin (Zhou *et al.*, 2001) and thiazolidinediones (Fryer *et al.*, 2002a). Conversely, the compound 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine (Compound C) is an inhibitor of AMPK (Fryer *et al.*, 2002b).

1.5.1.6.1 AICAR

AICAR is transported into the cell by the adenosine transporter and then transformed by the enzyme adenosine kinase to 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), which is an analogue of 5´- AMP. An increased ratio of ZMP/ATP activates AMPK (Corton *et al.*, 1995; Holmes *et al.*, 1999). Infusion of AICAR promotes GLUT-4 translocation in rat skeletal muscle (Bergeron *et al.*, 1999). Rats injected with AICAR for 5 days also have increased GLUT-4, hexokinase and glycogen content in skeletal muscle (Holmes *et al.*, 1999). AICAR is a non-selective AMPK activator, but can be used as a tool to study AMPK signalling and the role of AMPK in the metabolic balance. The limitation with AICAR is that it activates all enzymes directly activated by AMP (Fujii *et al.*, 2006) and it can also have systemic effects that are not linked to the enhancement of AMPK (Young *et al.*, 1996).

AICAR was shown to be well-tolerated in young healthy men (100 mg/kg, iv and po). AICAR has low protein binding (< 1%), poor oral bioavailability (<5 %), a half life (t½) of 2-3 hours after an intravenous injection and is metabolized in the kidneys (8%). The AMPK metabolite ZMP accumulates and therefore the t½ is prolonged (Dixon *et al.*, 1991). AICAR is acutely well-tolerated in male NMRI mice after dosing up to 125 mg/kg (ip), (Vincent *et al.*, 1996). These normoglycaemic mice became hypoglycaemic with the two higher doses (250 and 500 mg/kg) and the blood lactate level was increased more than six times at the higher dose. AICAR is in phase I study for the treatment of type 2 diabetes (Study number: 426758) and in phase I and II (expected to be completed December 2009) for the treatment of chronic lymphocyte leukaemia (Study number: NCT00559624).

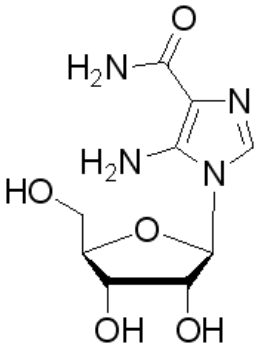

Figure 6. Chemical structure of AICAR.

1.5.2 11β-hydroxysteroid dehydrogenase type 1

1.5.2.1 Glucocorticoids

Cortisol is a glucocorticoid, produced and secreted from the adrenal cortex, which is a part of the adrenal glands. Glucocorticoids have gained this name because of their ability to stimulate gluconeogenesis. Glucocorticoids mobilise amino acids from skeletal muscle and increase liver enzymes, which convert amino acids into glucose. Elevated levels of cortisol cause a moderate decrease in the rate of glucose utilisation. Cortisol also has effects on lipid metabolism by increasing the mobilisation of fatty acids from adipose tissue, which in turn increases the concentration of free fatty acids in the plasma. High level of cortisol is needed in stress situations and starvation, when the body needs glucose for survival. The secretion of cortisol is controlled by adrenocorticotropic hormone (ACTH), which in turn is secreted from the anterior pituitary gland. ACTH release is controlled by corticotrophin-releasing factor (CRF) produced in hypothalamus. Cortisol has an inhibitory effect on CRF. This is called the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol binds to the glucocorticoid receptor (GR), which is expressed in almost all cells of the body.

The concentration of cortisol is also controlled by the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) 1, which is mainly expressed in liver, adipose and brain. 11βHSD1 regulates how inactive glucocorticoids (cortisone, 11-dehydrocorticosterone) can be transformed to the active form (cortisol, corticosterone) (Almelung, 1953). 11βHSD2 regulates the opposite reaction (i.e. inactivates cortisol) and the enzyme is mainly expressed in kidney, placenta and brain. Cortisol can also bind to mineralocorticoid receptors (MR) and therefore it is of importance that cortisol is metabolised by 11βHSD2 in tissues where aldosterone is active. Aldosterones most important function is to reabsorb sodium and water and to maintain the blood pressure.

There are differences in the structures of active and inactive glucocorticoids in humans and mice (Figure 7). The difference is only a hydroxyl-group, but this has resulted in different names: cortisol and cortisone for humans and corticosterone and 11β-dehydrocorticosterone for mice.

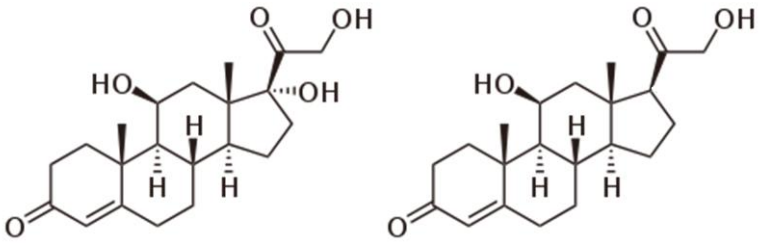

Figure 7. Human cortisol and mouse corticosterone.

1.5.2.2 Biological validation of 11βHSD1 as a drug target

Patients with obesity and type 2 diabetes have elevated intracellular levels of glucocorticoids (Rask *et al.*, 2001). Transgenic mice overexpressing 11βHSD1-selectivity in adipose tissue develop type 2 diabetes and visceral obesity (Masuzaki *et al.*, 2001), conversely 11βHSD1 knock-out mice display a favourable phenotype with normoglycaemia and normal weight (Kotelevtsev *et al.*, 1997; Morton *et al.*, 2001; Morton *et al.*, 2004). 11βHSD1 knock-out mice are resistant to diet induced obesity, have improved glucose and insulin tolerance and demonstrate favourable changes in cytokine expression, including a doubling in circulating adiponectin levels. Another transgenic mouse overexpressing HSD type 2 in adipose tissue, and thereby inhibiting corticosterone exposure intracellularly, has improved insulin tolerance and glucose tolerance compared with wild-type littermates when fed a high fat diet (Kershaw *et al.*, 2005).

1.5.2.3 Adiponectin

Adiponectin is an adipocytokine that is exclusively secreted from adipose tissue into the circulation (Scherer *et al.*, 1995). The adiponectin receptor, AdipoR1 is ubiquitously present and most abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in liver (Yamauchi *et al.*, 2003; Kadowaki & Yamauchi, 2005). There is a negative correlation between adiponectin levels and fat mass (Arita *et al.*, 1999), which is in contrast to leptin (Gil-Campos *et al.*, 2004). The same group observed a negative correlation between insulin and adiponectin in prepubertal children (Gil-Campos *et al.*, 2004). Patients with type 2 diabetes have lower adiponectin levels than age and BMI-matched nondiabetic subjects (Hotta *et al.*, 2000). Similarily, in obesity patients, plasma adiponectin levels are reduced compared to lean control subjects, and further reduced in patients with both obesity and type 2 diabetes (Yu *et al.*, 2002). An infusion of adiponectin to diet-induced obese mice ameliorated the hyperglycaemia and the hyperinsulinaemia induced by the diet (Yamauchi *et al.*, 2001). *In vitro* stimulation of muscle strips from type 2 diabetic patients with adiponectin improved insulin-stimulated glucose transport (Kuoppamaa *et al.*, 2008). High adiponectin levels are associated with a lower risk of developing type 2 diabetes, after adjusting for confounding factors, in apparently healthy individuals (Spranger *et al.*, 2003). The mRNA expression of adiponectin is lower in ob/ob mice and obese humans (Hu *et al.*, 1996). The reduction is between 50-80% for humans with a body mass index (BMI) of ~39 kg/m^2 compared with a normal BMI (Hu *et al.*, 1996). Thus adiponectin´s physiological role is apparently associated with normal and improved glucose homeostasis.

1.5.2.4 Adiponectin in mouse models of 11βHSD

Mice overexpressing 11βHSD1 in adipose tissue have increased levels of corticosterone and develop visceral obesity with concomitant low expression of adiponectin in mesenteric fat (Masuzaki *et al.*, 2001). 11βHSD1 knock-out mice have elevated levels of adiponectin in epididymal adipose tissue (Morton *et al.*, 2004). Conversely, transgenic mice over-expressing 11βHSD2 in adipose tissue have increased expression of adiponectin (Kershaw *et al.*, 2005).

1.5.2.5 Validation of 11βHSD1-inhibitors as a target for drug development using chemical substances.

Carbenoxolone is a non-selective 11βHSD-inhibitor, which increased hepatic insulin sensitivity in healthy volunteers and in nonobese type 2 diabetic patients (Walker *et al.*, 1995; Andrews *et al.*, 2003). Several selective mouse inhibitors of 11βHSD1 show multiple favourable effects on the metabolic phenotype (Alberts *et al.*, 2002; Alberts *et al.*, 2003; Hermanowski-Vosatka *et al.*, 2005; Wang *et al.*, 2006; Lloyd *et al.*, 2009). The 11βHSD1-inhibitor BVT2733 was shown to ameliorate the blood glucose level and serum insulin in KKA^y mice after 7-days infusion with an osmotic mini-pump (Alberts *et al.*, 2002), mainly through inhibition of 11βHSD1 in the liver (Alberts *et al.*, 2003). The same substance was effective in treating hyperglycaemia after oral administration in ob/ob, db/db and KKA^y mice (Alberts *et al.*, 2003; Wang *et al.*, 2006). Another 11βHSD1-inhibitor produced by Merck (Compound 544) lowered glucose and insulin levels in diet-induced obese mice (Hermanowski-Vosatka *et al.*, 2005). The 11βHSD inhibitor BVT116429 (Figure 8) was produced by Biovitrum AB (Stockholm, Sweden) in collaboration with Amgen (Ca, USA), and tested *in vivo* in study III. BVT116429 has its main effect in adipose tissue.

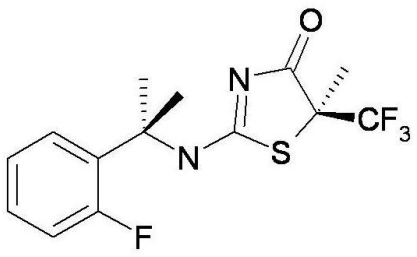

Figure 8. BVT116429 (also named compound 2922 in some publications).

2 AIMS

The major aims of this thesis are:

1. To investigate the acute and chronic effects of AICAR on glucose uptake in skeletal muscle, as well as whole body peripheral insulin sensitivity and glucose homeostasis in KKA^{y}-CETP mice (STUDY I).

2. To investigate the acute and chronic effects of AICAR on glucose uptake in skeletal muscle and glucose homeostasis, with emphasis on biomarkers in ob/ob mice (STUDY II).

3. To investigate the association between adiponectin and insulin sensitivity after inhibition of the enzyme 11βHSD 1 (STUDY III).

3 EXPERIMENTAL PROCEDURES

3.1 ANIMALS

The regional animal ethical committee in Stockholm or Uppsala approved all experimental procedures. Upon arrival at the animal facilities, the animals were placed in individual cages under 12-h light/12-h dark cycle. Room temperature was maintained at 21 ± 1ºC or 22 ± 1ºC and humidity at 50%, depending on the different animal facilities. Individual cages are stressful for the animals, but this form of housing was chosen because individual food consumption was measured. Individual cages were also needed for the animals with permanent catheters in vena cava since the littermates tend to chew on the outer part of the catheter. The animals were provided nesting material and chewing sticks. Animals had free access to food and water except during fasting. Lean (C57BL) and ob/ob mice (male, 8-10 wks old, B&K Universal, Sollentuna, Sweden) received normal rodent diet R34 (Lactamin, Vadstena, Sweden). KKA^y (male, 10 weeks, Charls River, Japan) and KKA^y-CETP mice (female, 4-6 month old, Pharmacia Corporation, Kalamazoo, MI, USA) received high fat diet (32.5 kcal% fat, D12451, Research diets, NJ, USA) since high fat food accentuates the progress of type 2 diabetes.

In accordance with the tradition in pharmacological studies, male mice were used in study II and III. Female mice were used in study I due to accessibility at the time. With experience from both genders, females might be preferable, since their complications from type 2 diabetes are less severe then for male KKA^y-CETP mice. They have less wounds and tumours, probably due to slightly less aggravated illness. Female KKA^y-CETP mice have lower basal blood glucose and basal insulin values than males, although both parameters are significantly elevated. In the studies with KKA^y and KKA^y-CETP mice, animals were grouped based on blood glucose. This was done to minimise the variation between the groups.

3.2 TREATMENTS

3.2.1 AICAR (Study I and II)

3.2.1.1 Acute treatment

Non-fasted KKA^y-CETP mice received an intraperitoneal (ip) injection of either AICAR (500 or 1000 mg/kg, 10 ml/kg, Sigma) or vehicle (saline). Tail blood samples were collected at 0, 30, 60 120 and 240 minutes after ip injection to measure blood glucose (Accutrend glucometer, Accutrend, Roche). Non-fasted lean (C5BL) and ob/ob mice were dosed by subcutaneous (sc) injection, with AICAR (1000 mg/kg, 10 ml/kg, Sigma) or vehicle (saline) and blood glucose was measured after 1, 2, 3 and four hours (tail blood, One-Touch glucose monitor, Lifescan, Milpitas, Calif, USA).

3.2.1.2 Long term treatment

KKA^y-CETP mice were dosed with intraperitoneal injections with AICAR (100, 300, or 500 mg/kg/day) or saline at 0900 for 7 days. Blood glucose was monitored by Accutrend glucometer using tail blood sample every day before dosing and 24 hours after the last dose. The lean (C57BL) and ob/ob mice were untreated, or dosed by subcutaneous injection, with AICAR (1000 mg/g body, 10 ml/kg) or saline,

administered at 09:00 for 7 days. Physiological testing of animals assigned to the long-term dosing study was performed 24 hours after last treatment.

3.2.2 11βHSD1-inhibitors (Study III)

One set of KKA^y mice were administered the 11βHSD-inhibitor BVT116429 (3, 10 or 30 mg/kg), or rosiglitazone (5 mg/kg) or vehicle by oral gavage (po) once daily at 17:00 for 10 days. The other set of KKA^y mice were administered the 11βHSD-inhibitor BVT2733 (30, 100 or 300 mg/kg), rosiglitazone (5 mg/kg) or vehicle by oral gavage twice daily at 07:00 and 17:00 for eight days. BVT116429 and BVT2733 were produced by chemists at Biovitrum AB (Stockholm, Sweden) or Amgen (Ca, USA).

3.3 CLINICAL CHEMISTRY

Blood samples were kept on ice for 30 minutes before centrifugation at 2000-3000 g for 10 minutes and subsequently stored in tubes at -70ºC until analysis. Serum glucose concentrations were measured spectrophotometrically (Cobas Mira, Roche, Montclair, N. J., USA) using a commercially available Glucose GHD Unimate 7 kit (Roche, Basel, Switzerland) for study I. For study III, plasma glucose was analysed with a UV method (Hitachi 912 Multianalyzer, Roche Diagnostics, Switzerland). Plasma triglycerides and plasma FFA were analysed with an enzymatic fluorometric method NEFAC (WAKO, Neuss, Germany) in study I and II and in study III with Cobas Mira using reagent kit of Triglycerides/GB (Boehringer Mannheim, Indianapolis, Ind., USA) was used. HbA1c, HDL and LDL cholesterol, triglycerides and free fatty acids were analysed with an enzymatic colorimetric method (Hitachi 912 Multianalyser) in study III. Serum adiponectin and leptin were analysed with radioimmuno assays (Linco Research, St.Louis, MO, USA). Serum insulin was analysed using an enzyme-linked immunoassay (ELISA, Mercodia, Uppsala, Sweden). A 24 hour urine sample was collected from the mice that had received either AICAR (500 mg/kg/day) or vehicle treatment using metabolic cages after the last dosing. The urine glucose content was measured by Cobas Mira and Glucose GHD Unimate 7. The lipid profile was analysed by column chromatography using fast protein liquid chromatography on superpose-6B column (Pharmacia, Uppsala, Sweden) (Castle *et al.*, 1998). Plasma alanine aminotransferase (ALAT) was determined spectrophotometrically (Cobas Mira) using a commercially available ALT Unimate 3 kit (Roche).

3.3.1 Clinical chemistry (Study I and II)

Blood glucose was monitored before the daily dosing in non-fasted mice. Twenty-four hours after the last treatment, animals were anaesthetised (Isoflurane, Baxter, IL, USA) and blood samples for plasma analysis were taken from orbital sinus.

3.3.2 Clinical chemistry (Study III)

Blood samples were taken from anesthetised (Forene, Abbot, Chicago, USA) mice via heart puncture or orbital plexus, after a four hour fast on the final day of the study.

3.4 BODY WEIGHT, FOOD INTAKE AND BODY COMPOSITION

Body weight was measured on the first and last day of the study and the total food intake was recorded. Fat pads were dissected at the end of the studies to compare fat content between the groups. For the KKA^y mice, body composition was analysed by

dual energy X-ray absorptimetry (DEXA, Lunar Piximus densitometer) (Study III). In ob/ob mice, epididimus fat pad weight and body fat as measured by DEXA analysis is positively correlated (unpublished data).

3.5 TISSUES

The epididymus fat pad and liver were dissected, weights were measured, and tissue samples were snap frozen in liquid nitrogen and stored at -70ºC. Lipid extracts were prepared by homogenizing frozen liver in Heptan:Isopropanol 3:2, Tween 1% (1:10) and then analysed with an enzymatic colorimetric test (Roche).

3.6 BASAL AND INSULIN-STIMULATED GLUCOSE UPTAKE IN SKELETAL MUSCLE

3.6.1 Muscle incubation in non-treated animals

Mice were anaesthetised by an injection (ip, 0.02 ml/g body weight)of 2.5 % Avertin (tribromoethanol, tertamyl alcohol, Aldrich, Milwaukee, Wis., USA), and isolated soleus and extensor digitum longus (EDL) muscles were excised and utilised for *in vitro* incubations. All incubation media were prepared from stock solution of Krebs-Hensleits buffer (KHB) that contained hydroxyethylpiperazineethanesulphoric acid (HEPES; 5 mM; pH 7.2, 30°C) and 0.1% BSA (RIA Grade, Sigma, St. Louis, Mo., USA). The gas phase throughout all incubations was maintained at 95% O_2/5% CO_2. Soleus and EDL muscles were incubated for 30 minutes at 30°C in KHB supplemented with 20 mM mannitol (pre-incubation media). Muscles were incubated in the absence or presence of 120 nM insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), 2 mM AICAR, or a combination of insulin (120 nM) and AICAR (2 mM).

3.6.2 Muscle incubation in pre-treated animals

One day after the final dosing of the 7-day AICAR treatment regime, mice were anaesthetised with 2.5 % Avertin (ip injection, 0.02 ml/g body weight). Soleus and EDL muscles were dissected and transferred to vials containing 1 ml of incubation media prepared from a pre-gassed (95% O_2/5% CO_2) Krebs Henseleit buffer supplemented with HEPES (5 mM; pH 7.2, 30°C) and 0.1% BSA in a shaking water bath (30°C). Muscles were incubated for 30 minutes in KHB containing 5 mM glucose and 15 mM mannitol, with or without added insulin (Actrapid, 120 nM).

3.6.3 Assessment of glucose transport activity.

Following pre-incubation, muscles were incubated for 10 minutes at 30°C, in KHB containing 20 mM mannitol and the specific additions as described for each experiment. This incubation step was included to rinse glucose from the extracellular space of the muscle. Glucose transport was assessed at 30°C using 2-deoxyglucose (Hansen *et al.*, 1995). Muscles were incubated for 20 minutes in KHB containing 1 mM 2-deoxy-[1, 2,^{3}H] glucose (2.5 μCi/mmol), and 19 mM mannitol (26.3 μCi/mmol). 2-deoxyglucose uptake reflects glucose transport and not metabolism in mouse skeletal muscle when used under these conditions (Hansen *et al.*, 1995). Muscles were processed as previously described (Wallberg-Henriksson *et al.*, 1987). Glucose transport activity is expressed as micromoles of glucose analogue accumulated per ml of intracellular water per hour.

3.7 PHOSPHOTYROSINE-ASSOCIATED PHOSPHATIDYLINOSITOL 3-KINASE

Soleus or EDL muscles were incubated for 30 minutes in basal KHB media (no insulin or AICAR present). Thereafter, muscles were incubated for 6 minutes in the absence or presence of insulin (120 nM). Muscles were homogenised in 500 μl lysis buffer as described (Saad *et al.*, 1993), solubilised by continuous stirring for 1 hour at 4ºC and thereafter centrifugation (12 000 g for 10 minutes). Protein was determined in the supernatant using a commercial kit (Bio-Rad, Richmond, Calif., USA). The supernatant (500μg) was immunoprecipitated overnight (4ºC) with an anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, Ky., USA) coupled to protein A-sepharose (Pharmacia). The PI 3-kinase activity was assessed directly on the protein A-sepharose beads as described (Krook *et al.*, 1997). The bands corresponding to PI 3-phosphate were quantified using a PhosphoImager (Bio-Rad).

3.8 GLYCOGEN AND TRIGLYCERIDE ANALYSIS

Gastrocnemicus muscle and liver were removed from anaesthetised mice and immediately frozen in liquid nitrogen. Glycogen content was measured fluorometrically on extracts of muscle or liver as described (Wallberg-Henriksson *et al.*, 1987). Results are expressed as μmol glucose per g wet weight. Muscle and liver triglycerides were extracted using chloroform-methanol. Free glycerol was measured using Sigma triglyceride reagent (Sigma) and the reaction was read using a spectrophotometer (540 nm). Results are expressed as μmol per g wet weight.

3.9 PROTEIN EXPRESSION STUDIES

Portions of the gastrocnemius muscle were polytron homogenized in 1.5 ml ice-cold HES buffer (10 mM HEPES, 1 mM EDTA, sucrose 250 mM, pH7.4) after which the muscle homogenates were subjected to centrifugation for 10 minutes at 12 000 g (4ºC). The supernatant was used to assess GLUT4, hexokinase II or glycogen synthase protein content. Aliquots of muscle homogenates containing 20 μg of protein were suspended in Leammli buffer. Proteins were separated by SDS-PAGE (10% resolving gel), transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, Mass., USA), and blocked with 5% non-fat milk. Membranes were incubated in primary antibody over night at 4ºC. The polyclonal antibodies used for the detection of hexokinase II and glycogen synthase were generous gifts from Dr. O. Pedersen (Steno Memorial Hospital, Gentofte, Denmark). Each polyclonal antibody was generated by immunising rabbits with a synthetic peptide homologous to the first nine amino acids in the COOH-terminus of the respective protein (Vestergaard *et al.*, 1993; Vestergaard *et al.*, 1995). GLUT4 protein expression was assessed using a polyclonal antibody raised against the COOH-terminal peptide of GLUT4 (Biogenesis, Poole, UK), diluted 1:1000 in phosphate-buffered saline (pH 7.4) containing 1% milk. Membranes were washed in TBST (10 mM TRIS, 140 mM NaCl, 0.02% Tween 20, pH 7.6), incubated with the appropriate secondary antibody and washed in TBST. Protein bands were visualised by enhanced chemiluminescence and quantified by densitometry.

3.10 NUCLEAR EXTRACTS PREPARATION

Nuclear extracts from gastrocnemius skeletal muscle were prepared as previously described (Mora & Pessin, 2000) with minor modifications. Tissues were pulverised in liquid nitrogen and homogenised in 10 volumes (wt/v) of buffer A (250 mM sucrose, 10 mM HEPES, ph 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.015 mM spermine, 0.1 mM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 6 μg/ml each L-1-tosylamido-2-phenylethyl chloromethyl ketone and 1-chloro-3-tosylamido-7-amino-2-heptanone) with 20 strokes of a pellet pestle (Kebo lab, Sweden) in eppendorf tubes and filtered through a gauze. The homogenate was centrifuged 10 minutes at 3900 g at 4ºC. The pellet was resuspended in 1 ml of buffer A and homogenised 10 seconds by a pellet pestle with motor. The homogenate was layered over one-half volume of buffer B, 81 M sucrose, 10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 mM spermine, 0.1 mM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 6 μg/ml each L-1-tosylamido-2-phenylethyl chloromrthyl ketone and 1-chloro-3-tosylamido-7-amino-2-heptanone) and centrifuged at 3900 g for 10 minutes at 4ºC. The pellet was resuspended in buffer A/glycerol (9:1, w/w) and layered over one-third volume of buffer B/glycerol (9:1, w/w). The gradient was centrifuged at 48 000 g for 30 minutes at 4ºC. The semi-purified nuclear pellet was resuspended in 100 μl of nuclear extraction buffer (10 mM HEPES, pH 7.6, 400 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF). Nuclear proteins were extracted on ice for 30 minutes and the samples were centrifuged at 13 000 g for 10 minutes at 4ºC. The supernatant was diluted four times in nuclear extraction buffer omitting KCl, assayed for total protein using Bradford assay kit (Bio-Rad) and stored at -70ºC.

3.11 ELECTOPHORETIC MOBILITY SHIFT ASSAY

The myocyte enhancer factor 2 (MEF2) DNA-binding site oligonucleotide was commercially available (Santa Cruz biotechnology, Santa Cruz, Calif., USA). The oliogonucleotide was end-labelled with T4 polynucleotide kinase. The probes (0.5 ng) were incubated with 2 μg of nuclear extracts in a 20-μl reaction containing 1μg poly (dI–dC), 40 mM KCl, 5 mM $MgCl_2$, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.05 mM DTT, and 5% glycerol for 20 minutes at room temperature. For competition studies, the extract was pre-incubated with a 10-fold molar excess of unlabeled oligonucleotide for 5 minutes before addition of radiolabelled probe. The samples were processed (200 V for 2 h 4ºC) on non-denaturation 6% polyacrylamide (38:1 acrylamide/bisacrylamide) gels buffered with TRIS borate/EDTA (TBE, 22 mM TRIS, 22 mM boric acid, and 0.5 mM EDTA). The dried gels were analysed in PhosphoImager.

3.12 ORAL GLUCOSE TOLERANCE TEST

3.12.1 Study I and II

After four hours fasting, glucose (3 g/kg, 10 ml/kg, Fresenius Kabi, Uppsala, Sweden) was administered orally by gavage to saline- or AICAR-treated conscious mice. Blood was sampled via the tail vein at 0, 30, 60 and 120 minutes after insulin or glucose administration. Blood glucose was analysed using a One Touch glucose monitor or Accutrend glucometer.

3.12.2 Study III

An oral glucose tolerance test was performed after an overnight fast and two hours post-dosing on day seven of the 10-day experiment with 11βHSD-inhibitor BVT116429 (3, 10 or 30 mg/kg), or rosiglitazone (5 mg/kg). Mice were given an oral dose of glucose (2 g/kg; 10 ml/kg Fresenius Kabi, Uppsala, Sweden) and blood samples (tail cut, 25 μl) for glucose (Accu-check) and insulin (ELISA, Mercodia) were taken immediately before the glucose load at 0, 15, 30, 60 and 120 minutes.

3.13 INSULIN TOLERANCE TEST

3.13.1 Study I and II

Insulin tolerance tests were performed in KKA^{y}-CETP mice after 7 days treatment with AICAR (500 mg/kg) or saline. Mice were injected with insulin (ip, 10 U/kg) 24 hours after the last dosing. Blood glucose was measured from the tail using a Accutrend glucometer at 0, 30, 60 and 120 minutes after insulin or glucose administration.

3.13.2 Study III

An insulin tolerance test was performed after a four hour fast on day seven. Two hours after the daily dose of substance or vehicle, mice were given an intraperitoneal dose of insulin (1 U/kg) and blood samples (tail cut, 5 μl) were obtained after 0, 15, 30, 60 and 120 minutes for glucose analysis (Accu-check). For data analysis, glucose concentrations were normalised to the concentration at time zero and the rate of glucose elimination was calculated by fitting the data to a single exponential equation ($y = y_o e^{-kt}$).

3.14 EUGLYCAEMIC CLAMP TECHNIQUES

3.14.1 Clamp techniques after treatment with AICAR

The acute effect of AICAR on glucose clearance and endogenous glucose production was examined by an euglycaemic clamp using KKA^{y}-CETP mice not exposed to AICAR. The chronic effect of AICAR was evaluated by the hyperinsulinaemic euglycaemic clamp in mice after the 7-day treatment with AICAR or vehicle. Two days before the clamp study, a catheter was inserted into the left jugular vein of the mouse during isoflurane anesthetization. Euglycaemic- or hyperinsulinaemic-euglycaemic clamps were then performed using conscious, unrestrained mice fasted for 6 hours. After adapting the clamp conditions, KKA^{y}-CETP mice received a constant infusion of D-[3-^{3}H]-glucose (Am Radiolabeled Chem, Inc.) at a dose of 0.5 μCi/100 g/min for 150 minutes. Sixty minutes after starting the tracer infusion, AICAR (0.2 mg/100 g/min) or insulin (5 mU/100 g/min) was infused intravenously for 90 minutes and the blood glucose was maintained at the same level as basal by adjusting the glucose infusion rate. Blood samples of 50 μl were collected from the tail at 60 and 150 minutes of tracer infusion to determine blood glucose, plasma insulin and D-[3-^{3}H]-glucose radioactivity.

3.14.2 Clamp after treatment with 11βHSD-inhibitor

Catheters were placed into the right jugular vein of male KKA^{y} mice under anaesthesia, and the animals were allowed to recover for three days. After dosing vehicle or BVT116429 (30 mg/kg, p.o.) once daily for eight days, animals were

fasted for 6 hours. At the start of the clamp experiment, all animals were infused with radiolabelled glucose tracer (2.5 μCi/kg/min) and 70 minutes later, the insulin infusion was started at a rate of 12.5 mU/kg/min. Blood glucose concentrations were monitored thereafter and glucose was infused from a 30% solution as required to maintain euglycaemia. The experiment was terminated after 160 minutes (90 minutes after the insulin infusion was commenced) and animals were euthanized. Data from the animals was included in the final analysis if there was no more than 20% variation in the basal and clamped glucose levels, if there was at least a two-fold increase in the blood insulin concentration upon insulin infusion, if the specific activity of the tracer glucose was at least 40 000 cpm per mg glucose in whole blood and if the catheter was shown to be correctly inserted when the experiment was terminated.

3.14.3 Calculation of endogenous glucose production

Glucose disposal rate (GDR) was expressed as the ratio between infusion rate (cpm/min) and the activity concentration in blood (cpm/mg). Under basal conditions prior to clamp, endogenous glucose production (EGP) equals GDR. Under clamped conditions, EGP was calculated as the difference between GDR and glucose infusion rate (GIR).

3.15 11βHSD1 EX VIVO ASSAY

DMEM (GIBCO BRL, 11965-092) media (37°C) was supplemented with NADPH (Sigma, Cat# N1630) and cortisone (Sigma, Cat# C2755) to a final concentration of 100 μM and 1 μM, respectively. Supplemented media (500 μl) was dispensed to each well of a 24-well plate (Falcon, BD, Cat# 353047). Frozen tissue was dissected into 30-40 mg aliquots (weights recorded) and directly placed in the pre-warmed media. Plates were incubated at 37°C for 3 hours with substrate, and media subsequently collected (without the tissue pieces) by transferring the supernatant to a fresh 24-well plate (samples stored at –80°C until ready for assay). A cortisol ELISA (Correlate-EIA kit, Assay Designs Inc. Cat# 901-071) was performed as suggested by the manufacturer with appropriate sample dilution (usually 1:10 for mouse adipose tissue).

3.16 VELOCITY SEDIMENTATION OF SERUM SAMPLES

Sucrose gradients (5-40%) in 10 mM HEPES, pH 8.0, 125 mM NaCl were poured stepwise in 2 ml thin walled ultracentrifuge tubes (Becton-Dickinson) and allowed to equilibrate overnight at 4ºC. Following layering of the sample on the top of the tube (diluted 1:10 with 10 mM HEPES, pH 8.0, 125 mM NaCl), gradients were centrifuged at 55 000 rpm for 4 hours at 4ºC in a Beckman Optima XL-80K ultracentrifuge (SW55TI). Gradient fractions (200 μl) were sequentially retrieved and subjected to Western blot analysis.

3.17 IMMUNOBLOTTING

Proteins were separated by SDS-PAGE (non-reducing, non-heat denaturing) and subsequently transferred to PVDF-membranes (BioRad). Membranes were blocked with 5% non-fat dry milk in TBST (Tris-buffered saline, 0.1% Tween20). As primary antibody, a rabbit polyclonal antibody against adiponectin was used (Santa Cruz #sc-17044-R). As secondary antibody, a horseradish peroxidase (HRP) conjugated goat antirabbit antibody was used (Upstate #12-348). Blots were visualised using chemoluminescense, (enhanced chemoluminescense (ECL) Plus detection kit, GE Healthcare).

3.18 ADIPONECTIN ANALYSES IN HUMAN PRIMARY ADIPOCYTES

Pre-adipocytes from human donors (BMI < 25 kg/m^2) were purchased from ZenBio (SP-F-1). Culture medium (PM-1), differentiation medium (DM-2) and adipocyte medium (AM-1) were also from ZenBio. One ampoule of human primary pre-adipocytes was thawed in a 37ºC water bath and subsequently suspended in 10 ml culture medium. An aliquot of cell suspension (30 µl) was transferred to an Eppendorf tube and 15 µl Trypan blue was added. Cells were counted and the viability was calculated. The remaining cell suspension was diluted to a final volume of 78 ml culture medium. Cell suspension aliquots, containing 8-9000 cells, were added per well in 60 wells of a 96-well plate. Cells were differentiated according to the ZenBio-protocol. On the 11th day from the start of differentiation (before the peak of adiponectin secretion), dexamethasone was removed from the medium. The cells were subjected to the following treatments 24 hours later: medium only, 100 nM cortisone, 0.1 µM BVT116429 + 100 nM cortisone, 10 µM BVT116429 + 100 nM cortisone. Cells were incubated for 48 hours, whereafter the medium was transferred to a fresh 96-well plate and stored at -20ºC until analysis. The adiponectin levels were analysed with a human adiponectin ELISA kit (CYT350, Chemicon Int.) according to the manufacturer's instructions.

3.19 STATISTICS

Data are expressed as means ± SEM in all three studies. The *post hoc* analysis is described in the following paragraphs. Significance was accepted at $p < 0.05$.

3.19.1 Study I

Data were analysed using one-way analysis of variance (ANOVA) followed by Neuman-Keules test when comparing more than two groups. A paired or non-paired student´s *t*-test was used to distinguish differences between two groups.

3.19.2 Study II

Statistical differences were determined by two-way ANOVA for the treatment-effect on blood glucose and glucose tolerance, and by a one-way ANOVA for glucose transport, biochemical analysis and protein expression studies. When the ANOVA resulted in a significant F-ratio ($p < 0.05$), the location of the significance was determined with the Fisher-LSD test.

3.19.3 Study III

Results were subjected to a one-way ANOVA followed by Dunett´s multiple comparison test, or the non parametric Kruskal-Wallis test followed by Dunn´s test. Linear regression analysis was used to determine the relationship between the plasma exposure of BVT116429 and the different functional parameters.

4 RESULTS AND DISCUSSION

4.1 POTENCY AND PHARMACODYNAMIC ACTIVITY OF BVT116429

BVT116429 is > 250 times more selective for 11βHSD1 over HSD2 and the inhibition constants for 11βHSD in human and mouse were found to be 29 mM and 36 mM, respectively in the *in vitro* inhibition assay. Human primary adipose cells were treated with BVT116429 to test the ability to convert cortisone to cortisol and the IC50 was found to be 26 nM. The compound was also found to be more effective in adipose tissue, than in liver tissue (Jean *et al.*, 2007). This could possibly be due to P-glycoprotein transporters, which prevent adequate uptake of BVT116429 into the liver cell.

4.2 GLUCOSE HOMEOSTASIS

A normal fasting plasma glucose concentration is difficult to define, but it is set to < 6.1 mM by WHO. The American Diabetes Association (ADA) changed their statement from ≤ 6.1 mM (1999) to ≤ 5.6 mM in 2003. Another way of measuring glucose homeostasis is by measuring HbA_{1c}, which reflects the average plasma glucose level over the previous 2–3 months in a single measure. A blood sample for HbA_{1c} analysis can be obtained at any time of the day and does not require any special preparation by the patient, such as fasting. The goal for any treatment of diabetes is to normalise the elevated plasma glucose level and thereby decrease the risk of diabetes complications.

4.2.1 Study I and II

4.2.1.1 Effect on blood glucose after acute treatment with AICAR

Acute AICAR-treatment was followed by blood glucose measurements for up to four hours in lean, ob/ob and KKA^y-CETP mice. Blood glucose was normalised in both diabetic mouse strains and the effect was maintained during the study. The lean mice treated with 1000 mg/kg (sc) of AICAR were hypoglycaemic (2.8 mM) the first hour and blood glucose was normalised after two hours. The highest dose (1000 mg/kg, ip) in the KKA^y-CETP mice was considered to be too high for the long term study. Intraperitoneal injections were used in study I and subcutaneous injections were used in study II where the latter form of administration results in a lower C_{max}. Intraperitoneal injections were tested with both 500 mg/kg and 1000 mg/kg in ob/ob mice but both doses were too effective and resulted in hypoglycaemia.

The glucose lowering effect of AICAR has been studied and confirmed in earlier studies. Normoglucaemic mice were injected with AICAR (250 mg/kg, ip) and the plasma glucose levels was decreased within 15 minutes and remained low for three hours (Vincent *et al.*, 1996). In another study using rats, AICAR was infuced during a euglycaemic clamp. To maintain the normal plasma glucose of 5.3 mM a glucose infusion was needed, indicating an acute plasma glucose lowering effect of AICAR (Bergeron *et al.*, 1999).

4.2.1.2 Effect on plasma parameters after 7-days treatment with AICAR

KKA^{y}-CETP mice were treated with 100, 300 and 500 mg/kg AICAR (ip) for seven days and blood glucose was monitored once daily. Only the highest dose (500 mg/kg) had a blood glucose lowering effect, which persisted during the whole study (Figure 9). Ob/ob mice treated with 1000 mg/kg AICAR normalised their blood glucose after 7-days treatment. Thus, AICAR-treatment improves the blood glucose level in rodent models of type 2 diabetes.

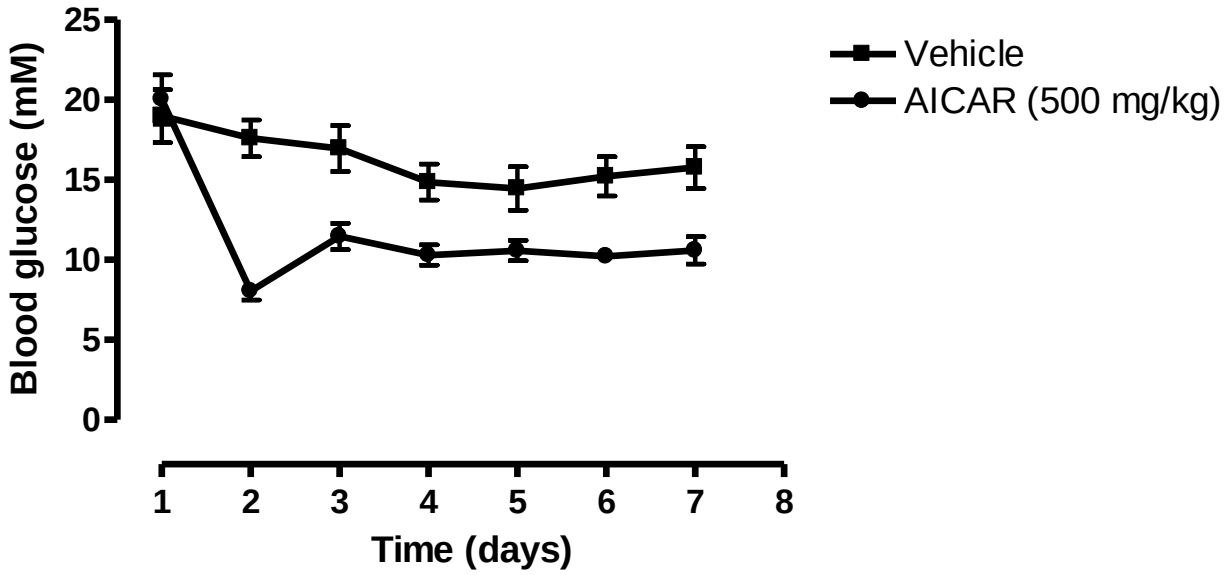

Figure 9. Chronic effect of AICAR (500 mg/kg) (circles) or vehicle (squares) on blood glucose in KKA^{y}-CETP mice. Values are given as means ± SEM, n = 24. All values except day 1 are lower than the corresponding control with at least $p < 0.05$.

The plasma insulin level was decreased in KKA^{y}-CETP mice treated with AICAR at doses of either 300 or 500 mg/kg. The 25% reduction in plasma insulin seen in ob/ob mice was not significant. This might be due to the fact that blood glucose was measured in fed mice, since ob/ob mice have meals during the light phase of the day (Ho & Chin, 1988). Lean mice normally consume the main part of their calories in the dark phase (Kurokawa *et al.*, 2000).

KKA^{y}-CETP mice were put in metabolic cages after 7-days treatment with AICAR and urine volume and glucose concentration were measured. Both the urine volume and the urine glucose concentration were improved. These animals drink extreme amounts of water due to the hyperglycaemia and therefore the untreated animals excreted 4.5 ml in 24 hours, which is approximately ten times more than in normal C57BL mice (Rao *et al.*, 2005).

Alanine aminotransferase (ALAT) is a liver enzyme often used for toxicology measures and provides an indication of liver failure. Interestingly, in humans there is a linear relationship between hip-waste-ratio and ALAT (Illouz *et al.*, 2008). Plasma ALAT was measured in the ob/ob mice in study II and there was no difference between the groups, possibly indicating AICAR did not have any toxic effect on the liver.

Triglycerides, free fatty acids and cholesterol were measured in all mice and surprisingly AICAR-treatment had a detrimental effect on dyslipidaemia. This was also

shown in a later study where a 2.5-3-fold increase in serum triglyceride levels was recorded in ob/ob and db/db mice after treatment with AICAR (Halseth *et al.*, 2002). The same group also showed that cholesterol concentration ($p < 0.0001$) was significantly increased in db/db mice following AICAR treatment. This is not in accordance with the theory of AMPK activation and is in disagreement with several published data. For example, obese Zucker fa/fa rats were infused with AICAR and an acute lowering of both plasma free fatty acids and triglycerides were observed (Bergeron *et al.*, 2001). Streptozotocin-induced type 1 diabetic mice with a defective LDL-receptor were treated with polyphenol, which stimulated AMPK 200 times more than metformin and both serum cholesterol and serum triglycerides were reduced by approximately 50%. (Zang *et al.*, 2006). Moreover, AICAR (0.75 mg/kg) was infused in ten male type 2 diabetic patients and both plasma glucose and free fatty acids were reduced (Boon *et al.*, 2008). Obese Zucker (fa/fa) rats were treated with AICAR for seven weeks and the plasma triglycerides, free fatty acids and HDL were improved (Buhl *et al.*, 2002). Fatty acid oxidation was studied after stimulation with ACIAR in muscles from fed and fasted rats. AICAR increased fatty acid oxidation and decreased malonyl-CoA in fed, but not in overnight-fasted rats (Kaushik *et al.*, 2001).

In the KKA^{y}-CETP mice, any effect on the lipid profile generally mimics the situation in humans better than other mice strains. However in KKA^{y}-CETP mice we also noted that AICAR-treatment had a detrimental effect on the lipid profile, with increased VLDL and reduced HDL-cholesterol. The detrimental effect on the lipid profile in our studies might be due to that AICAR is a non-specific AMPK-activator and that it might possess qualities that affect plasma lipid metabolism. Alternativerly this could also be a compensatory effect due to starvation signals, possibly induced by long-term AICAR-treatment. Detrimental effects of AICAR on the blood lipids have been shown after at least 7-days treatment in several models of type 2 diabetes i.e. ob/ob, db/db and KKA^{y}-CETP mice (Fiedler *et al.*, 2001; Halseth *et al.*, 2002; Song *et al.*, 2002).

4.2.1.3 Effect on oral glucose tolerance after 7-days treatment with AICAR

Glucose tolerance was improved in ob/ob and KKA^{y}-CETP mice after 7-days treatment with AICAR (Figure 10). This was consistent with an earlier study in obese Zucker (fa/fa) rats with slightly elevated plasma glucose levels. After seven weeks of subcutaneous AICAR injections (500 mg/kg), glucose tolerance was improved (Buhl *et al.*, 2002).

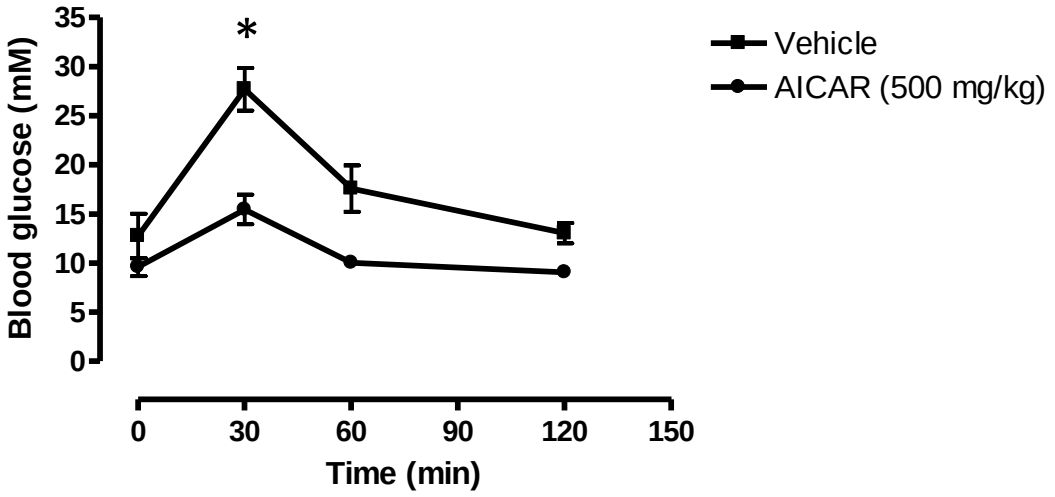

Figure 10. Oral glucose tolerance test (3 mg/kg) after 7-days treatment with vehicle (squares) or AICAR (circles) in KKA^{y}-CETP mice. Values are means ± SEM, n = 8, *$p < 0.05$.

4.2.1.4 Effect on insulin tolerance after 7-days treatment with AICAR

Insulin sensitivity was improved in KKA^{y}-CETP mice after 7-days treatment with AICAR. This was consistent with reductions in the basal insulin concentration seen in the same model.

4.2.1.5 Effect on endogenous glucose production after acute and 7-days treatment with AICAR in KKA^{y}-CETP mice

A catheter was inserted into the left jugular vein of the animal two days prior to the study. After six hours of fasting, an acute euglycaemic clamp was performed in non-treated animals. AICAR or vehicle was infused for 90 minutes. The glucose disposal rate was not altered, but the endogenous glucose production was decreased to approximately 50% after AICAR infusion. This is in agreement with a study in ten male type 2 diabetic patients infused with AICAR (0.75 mg/kg), where hepatic glucose production was inhibited (Boon *et al.*, 2008). Similar findings were also shown in obese Zucker fa/fa rats, where endogenous glucose production was suppressed more than 50% after AICAR (10 mg/kg) infusion (Bergeron *et al.*, 2001).

An euglycaemic hyperinsulinaemic clamp was performed after 7-days treatment with AICAR. There was no significant difference in the basal glucose disposal rate, but the endogenous glucose production was decreased compared to vehicle-treated animals (85% vs. 35% inhibition of basal values, $p < 0.05$) during insulin infusion, indicating that insulin sensitivity is increased in AICAR-treated mice (Figure 11).

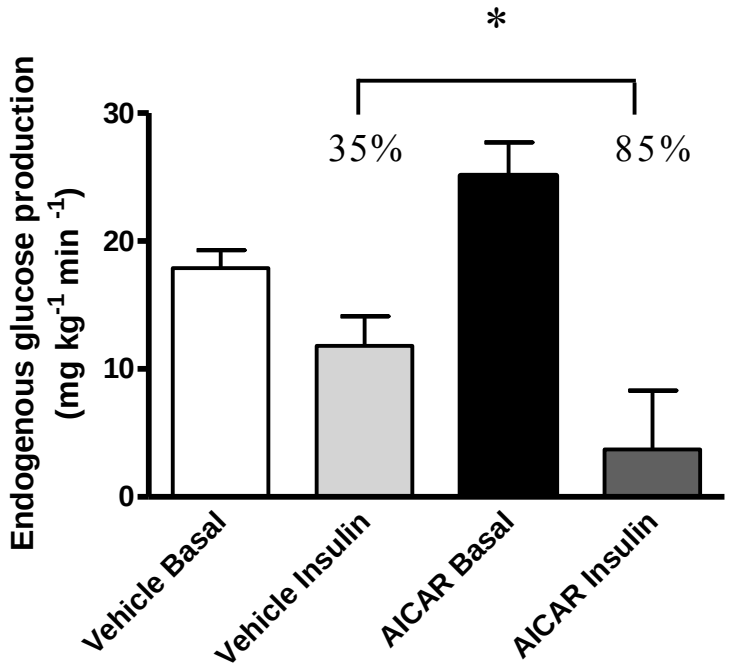

Figure 11. The endogenous glucose production after 7 days treatment with AICAR in KKA^{y}-CETP mice. Values are means ± SEM, n = 6,*p < 0.05 compared with vehicle.

4.2.2 Study III

4.2.2.1 Effect on plasma glucose after treatment with BVT116429

Mice were treated with BVT116429 (3, 10 or 30 mg/kg), rosiglitazone or vehicle once daily for ten days. Fasting blood glucose was reduced after treatment with 30 mg/kg of BVT116429 to the same extent as the positive control rosiglitazone (Figure 12). BVT116429 has also been tested in the Ldlr 3 knockout mouse, a model of diabetes, dyslipedaemia and atherosclerosis, where the metabolic syndrome was improved when the substance was given along with food (Lloyd *et al.*, 2009).

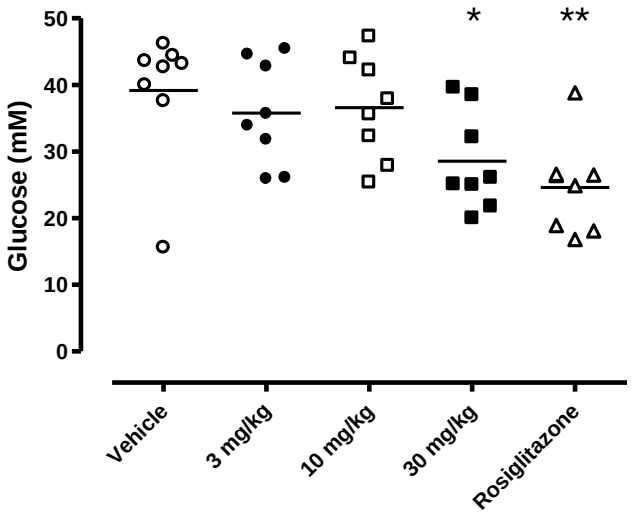

Figure 12. The effect after 10-days treatment with vehicle (white circles), BVT116429 3 mg/kg (black circles), BVT116429 10 mg/kg (white squares), BVT116429 30 mg/kg (black squares) or rosiglitazone 5 mg/kg (white triangles) on plasma glucose. * p < 0.05 and** p < 0.01 when compared with vehicle.

4.2.2.2 Effect on glucose tolerance after seven days treatment with BVT116429

Animals were orally dosed with 2 g/kg of glucose solution after 12 hours of fasting and two hours after the last dose with BVT116429, plasma glucose and insulin were followed for two hours. Glucose tolerance was unaffected by either BVT116429 or rosiglitazone. Rosiglitazone usually has positive effects on glucose tolerance (Hu *et al.*, 2006). In contrast with our results, BVT116429 has been shown to improve glucose tolerance in Ldlr 3 knockout mice (Hu *et al.*, 2006).

4.2.2.3 Effect on insulin sensitivity after treatment with BVT116429

Treatment with either BVT116429 or rosiglitazone decreased basal insulin levels at day 7, after 12 hours fast, compared with vehicle, indicating increased insulin sensitivity. There was no effect on the basal insulin level after ten days of treatment with either of the substances, which might be due to the shorter time of fasting (four hours instead of twelve). There was a trend of an improvement in insulin sensitivity, as measured by an insulin tolerance test, although this was not statistically ($p = 0.07$).

4.2.2.4 Effect on endogenous glucose production after 8-days treatment with BVT116429

There was no effect of BVT116429-treatment on hepatic glucose production, which is in line with earlier evidence that the plasma glucose lowering effect of this substance originates from its inhibitory effect in adipose tissue and not in liver. BVT2733, which has another profile and inhibits 11βHSD1 in the liver, decreases hepatic glucose production in both KKA^y and ob/ob mice (Alberts *et al.*, 2003).

4.2.2.5 Effect on adiponectin after treatment with BVT116429 and BVT2733

Different mouse models with altered 11βHSD1 or 11βHSD2 activity demonstrate elevated adiponectin concentrations (Morton *et al.*, 2004; Kershaw *et al.*, 2005). Adiponectin is a hormone secreted by adipcytes (Scherer *et al.*, 1995) and the circulatory levels are reduced in both obese mice and humans. Ob/ob mice had a 70-90% reduction in adiponectin levels compared with controls and samples from obese humans (BMI = 39 ± 1.4) had a reduction of 50-80% compared with normal lean humans (BMI = 21 ± 0.3) (Hu *et al.*, 1996). The negative correlation between adiponectin and obesity (Hu *et al.*, 1996; Arita *et al.*, 1999) has also been observed in type 2 diabetes patients (Hotta *et al.*, 2000). Moreover, adiponectin was also decreased in diet-induced obese mice, db/db mice and in KKA^y-mice compared with their own controls (Yamauchi *et al.*, 2001). Infusions with adiponectin in these animal models ameliorated hyperglycaemia and hyperinsulinaemia (Yamauchi *et al.*, 2001). Therefore it was an interesting task to evaluate the effect of treatments with BVT2733 and BVT116429 in the respect of adiponectin level. As positive control, rosiglitazone was used. This control was not only used for its positive effect on circulating glucose levels, but also for its reported effect on adiponectin. Treatment with thiazolidinediones increases the level of adiponectin in both humans and rodents (Maeda *et al.*, 2001; Yu *et al.*, 2002; Pajvani *et al.*, 2004). Troglitazone increases the plasma adiponectin levels in lean, obese and obese patients with type 2 diabetes (Yu *et al.*, 2002). Another study using troglitazone also showed elevated plasma adiponectin concentrations in humans (Maeda *et al.*, 2001). The same study improved or normalised mRNA level and plasma

adiponectin concentration in lean and db/db mice after treatment with troglitazone (Maeda *et al.*, 2001). Total adiponectin levels were elevated after treatment with BVT116429 and rosiglitazone compared with control. This is interesting since patients and mice treated with thiazolidinediones often gain weight and this is normally associated with a lowering of adiponectin levels (Arita *et al.*, 1999). BVT2733 had no effect on serum adiponectin levels after eight days of treatment, despite the effect on plasma HbA_{1c} percentage. This is in accordance with its primary effect in liver. Interestingly, the expression of 11βHSD and adiponectin has been reported to be similar between healthy subjects and type 2 diabetic patients. Thus, obesity *per se* and not insulin sensitivity may be the major regulator of 11βHSD expression in adipose tissue (Koistinen *et al.*, 2004).

Adiponectin can exist in several different forms, such as high molecular weight (HMW), medium molecular weight (MMW) and low molecular weight (LMW) forms (Waki *et al.*, 2003). The reduced adiponectin concentration in insulin resistant obese people and type 2 diabetic patients is mostly due to a decrease in the high molecular weight form (Lara-Castro *et al.*, 2006). In our study, both the MMW and the HMW forms of adiponectin were increased after treatment with BVT116429 and rosiglitazone.

To understand the relationship between 11βHSD1 inhibition and an increase in the circulating adiponectin level, primary adipocytes were studied. Adiponectin released from the primary adipocytes into the culture medium increased 1.5-fold, from 13.6 ng/ml in the absence of compound to 20.1 ng/ml in its presence. Thus, inhibition of 11βHSD activity in adipocytes can increase adiponectin production, suggesting that the effect seen in intact mice is a direct effect on the adipose tissue

Plasma adiponectin is not only decreased in obese people and type 2 diabetic patients, but also in patients with clinical manifestations of coronary artery disease (Ouchi *et al.*, 1999; Kumada *et al.*, 2003). Adiponectin inhibits monocyte adhesion to endothelial cells and reduced their phagocytic activity. This suppresses the accumulation of lipoproteins in the vascular wall, and thereby has an anti-atherogenic and anti-inflammatory effect. Adiponectin also seems to be protective against cardiovascular disease development (Ouchi *et al.*, 1999; Ekmekci & Ekmekci, 2006; Ouchi *et al.*, 2006b, a). Interestingly, women have more plasma adiponectin compared with men (Masuzaki *et al.*, 2001), probably due to differences in fat distribution, with more subcutaneous and less visceral fat. This could be one of the reasons why women have reduced risk for cardiovascular disease. The increase in adiponectin concentration may be an integral component in the mechanism of action of 11βHSD1-inhibitors, which are active in the adipose tissue. Study III guided the investigators to add serum adiponectin as biomarker in clinical trials of 11βHSD1-inhibitors.

4.3 BODY WEIGHT, BODY COMPOSITION, FOOD INTAKE AND LEPTIN

4.3.1 Study I and II

AICAR-treatment had no effect on body weight or fat pad weight in lean mice and ob/ob mice, but body weight was reduced in KKA^{y}-CETP mice. All the animals in the study lost some weight, but the group treated with the highest dose of AICAR (500 mg/kg) lost more weight than the vehicle group (-2.2 ± 0.3 g vs. -1.1 ± 0.3 g, $p < 0.001$). Weight loss was moderate and was probably due to a decrease in food intake (38.3 ± 2.0 g vs. 43.9 ± 1.3 g, $p<0.05$). The decreased food intake and weight loss was probably beneficial for these overeating and obese mice. Ob/ob and db/db mice treated

with 375-500 mg/kg AICAR for eight days did not alter body weight or fat pad weight (Halseth *et al.*, 2002). AICAR injection into the hypothalamus of non-diabetic rats increases food intake (Andersson *et al.*, 2004). The discrepancy in food intake between these studies may possibly be due to the different animal models or to the route of administration of the drug. Activation of AMPK in the hypothalamus has been suggested to enhance orexigenic signals, with concomitant increased food intake and body weight, where as low AMPK activity suppresses these signals under *ad libitum* fed conditions (Minokoshi *et al.*, 2004).

The normal relationship between nutrient intake and protein translation has been descried (Teleman *et al.*, 2008). At the cellular level, after carbohydrate intake, the beta cells in pancreas produce insulin and the increase in insulin secretion results in inhibition of the Forkhead box (FOXO). FOXO promotes the eukaryotic translation initiation factor 4E binding proteins (4E-PB) and the kinase Lk6. Reduction in FOXO will result in a reduction of 4E-PB and LK6. When 4E-PB and LK6 are inhibited, they in turn cannot inhibit eukaryotic translation initiation factor 4E (eIF4E) and therefore protein translation continues i.e. food intake will lead to the production of protein (Teleman *et al.*, 2008). Activation of AMPK has the opposite effect and enhances FOXO transcription and thereby inhibits protein translation (Nakashima & Yakabe, 2007; Greer *et al.*, 2009). Activation of AMPK with AICAR inactivates mammalian target of rapamycin (mTOR) *in vitro* (Bolster *et al.*, 2002). mTOR has the opposite effects as FOXO on 4E-BP (Teleman *et al.*, 2008). Inhibition of protein synthesis during exercise or fasting is a logical physiological response since it is catabolic. The mechanism for body weight loss in KKA^{y}-CETP mice could possibly be the result of reduced protein synthesis.

4.3.2 Study III

Food intake was unaltered between the different treatment groups. Rosiglitazone was the only treatment to promote weight gain during the study. This weight gain was expected and it has been reported in other rodent and human studies (Leiter *et al.*, 2006; Yilmaz *et al.*, 2007). The body composition of the animals was determined by DEXA and adiposity was increased only in the rosiglitazone-treated animals.

Leptin is increased in obese subjects (Arita *et al.*, 1999) and in type 2 diabetic patients (Hotta *et al.*, 2000). Interestingly, leptin is also increased after rosiglitazone treatment because of the weight gain (Leiter *et al.*, 2006). A similar increase in leptin levels was also seen in study III. Thus rosiglitazone paradoxically increases body weight and leptin levels, whilst simultaneously enhancing insulin sensitivity. The leptin concentration for animals treated with BVT116429 (30 mg/kg) was apparently higher than for the control animals, although this may reflect a maintenance of the leptin concentration, rather than an increase, since body weight was maintained in this group, whereas the control group lost 4% body weight (Table 1, study III). This could indicate that the degree of adiposity of the animals changes with BVT116429 treatment, although DEXA results indicated that body composition was unchanged between groups. The biological effects of an increased serum leptin concentration could include reduced food intake, of which we observed a tendency (Table 2 in study III), and increased energy expenditure, which would contribute to the compound's beneficial effects. Indeed, work with BVT2733 has provided evidence that energy expenditure is increased in dietary-induced obese C57Bl/6J mice after treatment with the compound

for 16 days (Wang *et al.*, 2006). In contrast to our study, leptin levels were unaltered in diabetic mice treated with compound 544 (Hermanowski-Vosatka *et al.*, 2005). Moreover, mice with 11βHSD1 deficiency have reduced mRNA expression and plasma levels of leptin (Morton *et al.*, 2004). Diet-induced obese rats were injected with leptin once daily and glucose tolerance was improved (Yaspelkis *et al.*, 2001). Thus, the changes in leptin concentration in the BVT116429-treated mice may contribute to the metabolic advantages seen in compound-treated animals.

4.3.2.1 Effect on plasma and liver lipids after treatment with BVT116429

Plasma and liver lipids were unaltered in animals treated with BVT116429. Total plasma cholesterol was elevated in animals treated with rosiglitazone, compared with the control group, and this was accompanied by elevations in plasma HDL-cholesterol levels and reductions in liver cholesterol levels.

4.4 EFFECT ON BIOMARKERS AFTER TREATMENT WITH AICAR

4.4.1 Phosphatidylinositol 3-Kinase

Insulin stimulation results in phosphorylation of the insulin receptor, insulin receptor substrate proteins (IRS) and activation of PI3-kinase in insulin sensitivity tissue such as skeletal muscle. The activation of PI3-kinase is essential for insulin-stimulated GLUT4 translocation. Wortmannin is a chemical substance which can block insulin-activated PI3-kinase and thereby also reduce GLUT 4 translocation. However, wortmannin does not affect exercise-induced GLUT4 translocation (Lee *et al.*, 1995).

The effect on long-term AICAR-treatment on PI3-kinase was studied in normal C57/BL and ob/ob mice. Muscle strips were incubated with a dose of insulin designed to give a sub-maxmal activation of PI3-kinase. The obese ob/ob mice had a marked reduction in insulin-stimualted PI3-kinase activity compared with lean mice, which indicates that ob/ob mice have severe insulin resistance. AICAR treatment did not normalise insulin-stimulated PI3-kinase activity after insulin stimulation in ob/ob mice. Thus, the effect of chronic AICAR treatment on fasting glucose levels and glucose tolerance is unlikely to be caused by improvements in insulin action on PI3-kinase and glucose transport in skeletal muscle.

4.4.2 GLUT4

There are 13 different glucose transporter proteins in humans. GLUT4 is highly expressed in adipose tissue and skeletal muscle, but these tissues also express many of the other transporters. In the skeletal muscle GLUT1, GLUT5 and GLUT12 might also contribute to sugar uptake (Stuart *et al.*, 2000; Stuart *et al.*, 2006). Both insulin and exercise stimulate GLUT4 recruitment to the cell surfaces of muscle and adipose cells (Rose & Richter, 2005; Herman & Kahn, 2006), but in two different ways. After intake of nutrients containing carbohydrates, insulin is produced, the insulin receptor is activated, insulin receptor substrate proteins are phosphorylated and PI3-kinase is activated. PI3-kinase catalyses the conversion of phosphatidylinositol (4,5)P2 to phosphatidylinositol (3,4,5)P3, which in turn activates the protein kinase Akt through PDK1 and Rictor/mTor. (Vanhaesebroeck & Alessi, 2000; Sarbassov *et al.*, 2005). Phosphorylation and deactivation of the Akt substrate 160-kD (AS160) by Akt, lead to GLUT4 translocation to the cell membrane and enables glucose uptake (Sakamoto &

Holman, 2008). Muscle contraction also increases AS160 phosphorylation, but by a PI3-kinase independent pathway (Bruss *et al.*, 2005). This AS160 phosphorylation, with concomitant GLUT4 translocation is mediated through the AMPK pathway (Jessen & Goodyear, 2005).

Protein content of GLUT4 was unaltered between lean and ob/ob mice. Regardless of genotype, AICAR-treatment was associated with a 2-fold increase in GLUT4 protein expression ($p<0.01$). Since AICAR treatment did not affect the activation of PI3-kinase, this indicates that the increase in GLUT4 protein may account for the increase in non-insulin dependent glucose uptake. Similar findings have been reported in rats. In rats dosed with AICAR (1 mg/g, sc) for 5 days, cell-surface GLUT4 content in epitrochlearis muscle is increased 68% after insulin stimulation, and GLUT4 mRNA expression is increased 123% in the white part of the gastrocnemius muscle, compared with control (Buhl *et al.*, 2001). Another group treated rats with AICAR for 5 days and GLUT4 was increased 100% in epitrochlearis muscle and 60% in gastrocnemius (Holmes *et al.*, 1999).

4.4.3 Myocyte enhancer factor 2

Vertebrate skeletal muscle differentiation is regulated by the cooperative interactions of myogenic transcription factors, together with the myocyte enhancer factor 2 (MEF2) (Molkentin *et al.*, 1995; Wang *et al.*, 2001). The myocyte enhancer factor 2 is necessary to increase GLUT4 mRNA expression in skeletal muscle (Thai *et al.*, 1998; Santalucia *et al.*, 2001). In study II, the myocyte enhancer factor 2 was enhanced in tissue from AICAR-treated animals, compared with control (1.9 fold and 2.4-fold for lean and ob/ob, $p<0.05$). The enhanced MEF2 in the AICAR-treated animals is in accordance with the increased GLUT4 protein content.

4.4.4 Hexokinase II

The phosphorylation of glucose to glucose-6-phosphate (G-6-P) is the first committed step in glucose uptake in skeletal muscle. This reaction is catalyzed by hexokinase (HK). Two hexokinase isoforms, HKI and HKII, are expressed in human skeletal muscle, but only HKII is regulated by insulin. Protein expression of HKII was similar between ob/ob and lean mice. AICAR treatment increased the HKII 2-fold ($p<0.001$) regardless of genotype. The increase in HKII could imply an increase in glucose uptake; however this was neither seen in study I or study II after 7-days treatment with AICAR. The increase in HKII might be too low to have impact on glucose transport.

4.4.5 Glycogen synthase protein content

Glycogen synthase is the enzyme responsible for adding uridine diphosphate (UDP)-glucose to a growing chain of glycogen and is also the rate-determining enzyme for glycogen synthesis. Glycogen synthase protein content was unaltered in skeletal muscle from AICAR-treated mice. This implies that it is not newly formed glycogen, but rather a lower output of glycogen which results in the higher level of glycogen in AICAR-treated animals. This is consistent with the acute clamp test, where a decrease in hepatic glucose production was observed (Study I) and the hyperinsulinaemic euglycaemic clamp performed after 7-days treatment with AICAR. This is also in accordance with another study in which the impact of AICAR on glycogen synthesis in basal and

insulin-stimulated rat soleus muscle was measured and no changes were seen (Young *et al.*, 1996).

4.5 GLYCOGEN CONTENT IN LIVER AND GASTROCNEMICUS MUSCLE

AICAR-treatment increased the liver glycogen content 50% under fed ($p < 0.001$) and 70% ($p = 0.01$) under fasted conditions, in ob/ob mice but not in lean mice. The lack of any effect in lean mice could possibly be explained by the fact that AICAR only has an effect when liver metabolism is impaired. Under fed conditions, AICAR had no effect on glycogen content in gastrocnemicus muscle in either genotype, but under fasted conditions, AICAR treatment increased glycogen content ($p < 0.001$). This was in accordance with a previous study performed in normal Wistar rats, where 5 days of AICAR treatment (1 mg/kg, sc) increased the glycogen content 105% in red muscle fibers from gastrocnemicus and 24 % in white muscle fibers from the same sample (Buhl *et al.*, 2001). AICAR-treatment seems to restore liver glycogen content when the animals are fasted. The increased liver glycogen content in AICAR-treated ob/ob mice in study II could be an effect of either inhibition of the hepatic glycogen breakdown or increased glycogen formation, or both. The protein content of glycogen synthase was not increased in the same study, indicating that the increased glycogen content in liver and muscle was not a result of increased glycogen formation. On the contrary, the clamp studies from study I provide evidence that the hepatic glucose output is lowered, at least in the acute model or when insulin is infused.

4.6 TRIGLYCERIDE CONTENT IN LIVER AND MUSCLE

The triglyceride content data from gastrocnemius muscle are puzzling. They show that fasting increases the skeletal muscle triglyceride content even in vehicle-treated mice. Triglycerides should normally decrease with fasting (Jaromowska & Gorski, 1985). However, there seems to be difficulties with various biochemical methods to analyze triglyceride content in skeletal muscle tissue and the technique used in our analysis was not a standard assay at the time. It could for example be the blank sample that caused the increased values. In the fed state, AICAR treatment normalised liver triglycerides in ob/ob mice. This has also been reported for db/db mice treated eight days with AICAR where a reduction in liver triglycerides could be seen (Halseth *et al.*, 2002).

4.7 GLUCOSE UPTAKE IN ISOLATED MUSCLES

Glucose uptake was measured after an acute AICAR injection and after 7-days ACIAR-treatment (24 hours after the last dose). Two different types of muscle specimens were chosen. Soleus represented red muscle fibres, which are slow-twitch oxidative fibres, also called type I. These fibres have a smaller diameter and contain more myoglobin, mitochondria and little glycogen compared to glycolytic muscle fibres. Extensor digitorum longus (EDL) represents white muscle fibres, fast-twitch glycolytic fibres and also called type 2. There are two types of 2 fibres, 2a with fast-twitch oxidative glycolytic fibres and type 2b, which are fast-twitch glycolytic. Extensor digitorum longus is a mixture of type 2a and b.

4.7.1 Glucose uptake after acute stimulation

Muscles from non-treated animals were incubated in the absence or presence of insulin (120 nM) or AICAR (2 mM) or a combination of insulin and AICAR. Basal glucose uptake was similar between lean and ob/ob mice, but insulin-stimulated glucose uptake was reduced in ob/ob mice compared to lean mice, for both muscle types studied. AICAR-treatment normalised the glucose uptake in ob/ob mice.

4.7.2 Glucose uptake after 7-days AICAR-treatment

As in the acute AICAR-treatment study, insulin-stimulated glucose uptake was impaired in ob/ob compared with lean mice. Seven days of AICAR-treatment did not improve either basal or insulin-stimulated glucose uptake in either KKA^y-CETP (Study I), lean (Study II) or ob/ob (Study II) mice. There is a discrepancy between the results from the acute and chronic treatment regarding glucose uptake. This could possibly be an effect of the time-frame for the dosing of AICAR and the measurement of glucose uptake. Each injection of AICAR could have had a transient, short-term insulin-independent effect on skeletal muscle glucose transport, which collectively resulted in a blood glucose lowering effect. Twenty-four hours is a long time between the injection and the glucose transport measurement, especially since the half-life of AICAR is 2-3 hours (Dixon *et al.*, 1991) in humans, and probably even faster in mice, since they normally have higher metabolic rates. The exact values for glucose transport obtained in different studies are difficulat to compare. Nevertheless, it is interesting to note that the rate of basal and insulin-induced glucose uptake in the KKA^y-CETP mice is quite normal compared to lean mice in our studies. There are a few studies where KKA^y-CETP mice have been used, but our report is the first to provide data for glucose transport in this diabetic mouse model. The implication is that glucose transport was similar between KKA^y and KKA^y-CETP mice. KKA^y mice are reported to have approximately 50 % less GLUT4 mRNA in fat tissue (Hofmann *et al.*, 1991) and 34% less hexokinase II mRNA in quadriceps muscle than lean mice (Braithwaite *et al.*, 1995).

Male Wistar rats were treated 5 days with AICAR or control and thereafter basal and insulin-stimulated 3-O-metylglucose uptake was measured. The rate of 3-O-metylglucose uptake was increased 63% in fast-twitch epitrochlearis (EPI) muscle, 26% in EDL muscle (fast-twitch with both oxidative and glycolytic properties) and unchanged in the slow-twitch soleus muscle (Buhl *et al.*, 2001). Obese Zucker (fa/fa) rats were treated for seven weeks with AICAR and insulin-stimulated glucose transport was improved in EDL and EPI muscle, but not in soleus muscle (Buhl *et al.*, 2002). We cannot exclude the possibility of a tissue-specific AICAR response on glucose transport, which may be present only in EPI muscle.

4.8 ADDITIONAL AMPK ACTIVATORS AND INHIBITORS

The two mitochondrial toxins dinitrophenol and rotenone were shown to activate AMPK by decreasing the ATP production in the respiratory chain (Hayashi *et al.*, 2000; Fujii *et al.*, 2005). The newly developed chemical A-769662 was found to activate AMPK and ameliorate glucose homeostasis in ob/ob mice by a so far unknown mechanism (Cool *et al.*, 2006; Goransson *et al.*, 2007). Metformin (Zhou *et al.*, 2001) and rosiglitazone also (Fryer *et al.*, 2002a) activate AMPK by unknown mechanisms.

Possibly, rosiglitazone activates AMPK via adiponectin. Compound C is a non-specific, reversible and ATP-competitive inhibitor of AMPK (Fryer *et al.*, 2002b; Bain *et al.*, 2007). Specific AMPK activators or inhibitors are not yet available. More specific compounds would be useful tools to understand the underlying mechanisms of AMPK regulation. Another way of approaching this target could be to delay the inactivation of naturally activated AMPK.

4.9 THE RELATIONSHIP BETWEEN AMPK AND 11βHSD

The expression of 11βHSD1 in preadipocytes has been reported to be increased in a dose-dependent manner in AICAR-treated cells and this effect is blocked by the AMPK inhibitor, compound C (Arai *et al.*, 2007). Both AMPK and 11βHSD1 are induced by cellular stress. It is of importance for proper health to have balance or homeostasis of biological systems, which should be considered when choosing the level of activation or inhibition with a future drug. Adiponectin stimulates glucose utilisation and fatty-acid oxidation by activating AMPK (Fruebis *et al.*, 2001; Yamauchi *et al.*, 2002; Yamauchi *et al.*, 2003). Interestingly, inhibition of 11βHSD1 in study III resulted in increased levels of adiponectin, which is an AMPK activator. Thus, this could represent a feed-back loop to restore homeostasis.

4.10 TREATMENTS IN THE PIPELINE

Finding new targets for treatment of type 2 diabetes and obesity is a goal for many academic groups and pharmaceutical companies. There is still an unmet medical need and there are plenty of intelligent ideas to test. The treatment list in this section is not exhausted, but rather a representative of major activities ongoing.

A second-generation of TZDs is under development. Metaglidasen™ (Zhang *et al.*, 2007), (formerly MBX-102), is currently being evaluated in phase II/III clinical trials at Metabolex for the treatment of type 2 diabetes. Balaglitazone is a partial PPARγ agonist, which in rats induced comparable anithyperglycaemic effects as rosiglitazone, but with fewer side effects (Larsen *et al.*, 2008). A third example of a second-generation TZD is Rivoglitazone™, now in a phase III study (Schimke & Davis, 2007).

New DPPIV-inhibitors and GLP-1 analogues are in late clinical phases. For example, the DPPIV inhibitors Alogliptin™ (Nauck *et al.*, 2009) and BI-1356 (Thomas *et al.*, 2009) and the GLP-1-analouge Albiglutide™ (Bush *et al.*, 2009) are currently under clinical trial. Another approach of enhancing the positive effect of GLP-1 is through PSN-821, a G-protein-coupled receptor (GPR)119 agonist currently in phase I clinical trials for the treatment of obesity and type 2 diabetes (Fyfe *et al.*, 2008).

Amylin is co-secreted with insulin from pancreatic beta cells (Cooper *et al.*, 1987) and acts centrally to slow gastric emptying (Young *et al.*, 1995), thereby suppressing postprandial glucagon secretion (Silvestre *et al.*, 2001), and decreasing food intake (Rushing, 2003). These actions complement those of insulin to regulate blood glucose concentrations. Amylin is relatively deficient in type 2 diabetic patients (Makimattila *et al.*, 2000), depending on the severity of beta-cell secretory failure, and is essentially absent in type 1 diabetic patients (Cooper *et al.*, 1989). Pramlintide acetate, an analogue of the pancreatic hormone amylin was launched in 2005 in USA for the treatment of both type 1 and 2 diabetes (subcutaneous injections), but is still not approved in any other country. Phase III studies were initiated in 2002 in the European

Union and in 2004 in Switzerland, but no further developments have been reported. In clinical trials, pramlintide added to insulin treatment in type 2 diabetic patients was shown to reduce postprandial glucose levels, improve glycaemic control, and promote weight loss (Weyer *et al.*, 2001; Fineman *et al.*, 2002; Hollander *et al.*, 2003).

Aldose reductase acts on the first step of the polyol metabolic pathway to catalyze the reduction of glucose to sorbitol with nicotinamid adenine dinucleotid phosphate (NADPH) as a coenzyme. Hyperactivity of the pathway in individuals with high blood glucose levels is closely related to the onset or progression of diabetic complications. Tolrestat, an aldose reductase inhibitor, slowed the progression of diabetic autonomic neuropathy compared with placebo (Didangelos *et al.*, 1998).

The kidneys play an important role in glucose homeostasis, reabsorbing 99% of the plasma glucose that filters trough the glomeruli. The glucose transporter, SGLT2 is important in this process, accounting for 90% of the glucose reabsorbation (Kanai *et al.*, 1994). Sergliflozin^{TM} (Katsuno *et al.*, 2007) and Dapagliflozin^{TM} (Washburn, 2009) are sodium-glucose cotransport (SGT-2) inhibitors, which act to increase the renal metabolism of glucose and thereby decreasing the plasma glucose level.

4.11 OTHER ANIMAL MODELS OF TYPE 2 DIABETES

Almost any mouse strain can be used for the diet-induced obese (DIO) model, but some are more sensitive to high-fat diet (West *et al.*, 1992). The strains and diets vary and the phenotype can transition between a pre-diabetic state to severe type 2 diabetes. One advantage with the DIO mouse is that it does not result from a single mutation and therefore it might be one of the most suitable models available that mimics human type 2 diabetes. Other mouse models used for studies of type 2 diabetes are the KK mice (Iwatsuka *et al.*, 1970), New Zealand Obese (NZO) mice (Bielschowsky & Bielschowsky, 1956; Subrahmanyam, 1960) and the db/db-mice with a non-functional leptin receptor (Hummel *et al.*, 1966). There are also rat models of type 2 diabetes, such as the Berry Lewin-rat (Levin *et al.*, 1989), Goto-Kakizaki rat (GK) (Goto & Kakizaki, 1981) or the Zucker fa/ fa rat (Zucker & Antoniades, 1972).

4.12 THE CLINICAL RELEVANCE

4.12.1 The clinical relevance of diabetic animal models

Lean, ob/ob, KKA^{y} and KKA^{y}-CETP mice were used as animal models in this thesis. They are all practicable models in the study of type 2 diabetes. Another model of type 2 diabetes is the high-fat diet-induced C57BL mouse described above in DIO mouse (West *et al.*, 1992). Blood glucose levels are not elevated to the same degree compared with models used in this study, however it might also be to an advantage, since the profile of these mice is more similar to type 2 diabetic patients. Very few humans are leptin deficient (associated with the ob/ob mice) and few patients have the extreme profiles of the KKA^{y} and KKA^{y}-CETP mice. But the main advantage of studying the KKA^{y}-CETP mouse over the DIO mouse is the human-like lipid profile (Castle *et al.*, 1998). Rosiglitazone lowers the serum glucose from 40 mM to 25 mM in KKA^{y} mice (Study III). Ob/ob mice were treated with rosiglitazone (4 mg/kg) and a decrease in blood glucose of 35% was measured already after four days (Hu *et al.*, 2006). In humans, rosiglitazone is less potent and lowers plasma glucose from 9.0 ± 0.9 mM to 7.9 ± 0.9 mM (Mayerson *et al.*, 2002). Another study in humans showed similar

findings, with reduction in plasma glucose from 8.8 ± 0.8 mM to 7.3 ± 0.4 mM (Tiikkainen *et al.*, 2004). Diet-induced obese mice were treated with rosiglitazone (10 mg/kg) and plasma glucose was reduced from approximately 9 mM to7 mM (Minge *et al.*, 2008). The same study included AICAR-treatment, but no effect on plasma glucose was seen probably since the dose used (30 mg/kg, ip) may be too low to have any effect. In KKA^{y}-CETP mice (Study I) 500 mg/kg was the lowest AICAR dose to reduce plasma glucose.

Rosiglitazone improves plasma glucose in both rodents (Hu *et al.*, 2006) and humans (Mayerson *et al.*, 2002), but consistent results with metformin are more difficult to find. Ob/ob mice were treated for 12 days with metformin without any effect on plasma glucose (Green *et al.*, 2006). Diet-induced obese rats were treated for one month with metformin (300mg/kg) and fasting plasma glucose decreased from 3.53 ± 0.26 mM to 3.28 ± 00.19 mM, which is a reduction of approximately 7% (Liu *et al.*, 2006). Metformin has also been used in our laboratory with varying effects and establishing a tolerable dose that gives the desired effect has been a challenge. If a too high dose is used, lacto acidosis can occur. In our experience, 250 mg/kg twice daily is often a functional and tolerable dose in ob/ob, db/db, DIO, KKA^{y} (Figure 12) and KKA^{y}-CETP mice.

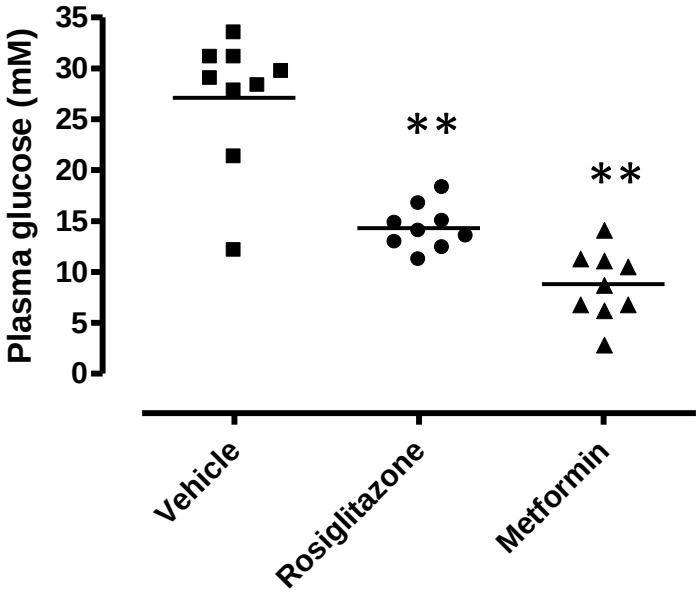

Figure 12. KKA^{y} mice treated for seven days with vehicle (squares), rosiglitazone (5 mg/kg, circles) or metformin (500 mg/kg, triangles). ** $p < 0.01$ compared with vehicle.

When an extreme diabetic animal model is used, there is a higher risk of false positives or too aggravated answers. Large improvements of blood glucose levels observed in rodents might not occur to the same extent in humans, and thus, new drugs may fail in the clinic. For studies regarding glucose homeostasis, it could be an advantage to use extreme models of obesity and type 2 diabetes, but it might be wise to include a mild diabetic model like the DIO mouse before going into the clinic. Rosiglitazone treatment improves glucose homeostasis in in ob/ob and KKA^{y} mice, which is in accordance with type 2 diabetic humans. The extent of the blood glucose

lowering effect of rosiglitazone is more similar between type 2 diabetic patients and DIO mice, than in the more extreme models of type 2 diabetes.

4.12.2 The clinical relevance of substances and diabetes targets

If it is possible to mimic physical activity with an AMPK-activating substance, it may prove to be clinically relevant for the treatment of type 2 diabetes. AMPK is a well-validated diabetes prevention target. Although AICAR has been under evaluation for the treatment of type 2 diabetes, the clinical phase I study was conducted several years ago and no further clinical trialss for the treatment of type 2 diabetes have been started. AICAR, together with metformin and rosiglitazone, are probably the best chemical tools today to study AMPK activation, but unfortunately none of these substances are specific AMPK activators.

Since there are differences in the structures of glucocorticoids in humans and rodents there is a need to develop species specific drugs for chemical validation. Mouse models were chosen as a surrogate model for study III, and thus, specific mouse 11βHSD1-inhibitors were developed. It was regarded too risky not to have a mouse model, since the potential human drug could otherwise not be tested *in vivo* before going into primates, which is both an ethical issue and can lead to a higher risk of failure in the clinic. The liver specific 11βHSD inhibitor BVT2733 had positive effects on glucose homeostasis in several mouse models of type 2 diabetes (Alberts *et al.*, 2002; Alberts *et al.*, 2003), however the human equivalent to this drug failed in the clinical trail. The clinical relevance was insufficient with BVT2733. This could be due to species differences between mice and humans. Glucose homeostasis in mice is probably more dependent on liver than in humans. But it is also possible that inhibition of 11βHSD1 has no clinical relevance, since there was no effect on glucose homeostasis noted in humans after treatment with BVT2733. The clinical relevance of 11βHSD1 inhibition in adipose tissue is still to be determined. However, Incyte recently showed proof of concept in humans with the 11βHSD1-inhibitor INCB13739 (Rosenstock *et al.*, 2009).

4.13 ETHICAL ISSUES

Working with animal models is an ethical dilemma. The persons responsible for the *in vivo* parts in this thesis are well educated regarding animal welfare and have treated the animals with respect. The animal studies were classified as moderate by the local ethical committee. It is stressful for the mice to be taken out of the cage for weighing or blood sampling, but all major interventions were done under anaesthesia. Another question is the human suffering versus the animal suffering regarding the diseases, in this case type 2 diabetes and obesity. There are other, maybe better, ways of losing weight and treating high blood glucose than taking drugs. For example moderate walking has shown reduced risk of mortality for patients with type 2 diabetes (Caspersen & Fulton, 2008). Consuming fewer calories and exercising more could be one inexpensive alternative. However for many it seems to be extremely difficult to change life style to a more healthy way of life. There are also patients with reduced capability to exercise and patients who have extra difficulties in changing their life style. Overeating can sometimes be compared with an abuse of alcohol. It is of importance to understand the underlying mechanisms of the disease and different treatments. More knowledge can possibly encourage some patients to change their life style and new drugs are needed to help the patients who are unable to change.

Therefore it is necessary to perform animal studies, however the studies shall always take the three “R”s into account i.e. refinement, reduction and replacement.

5 SUMMARY

5.1 STUDY I AND II

AMPK-activation had several positive effects in the pharmacodynamic studies in rodent models of type 2 diabetes. Plasma glucose was normalised after AICAR treatment in ob/ob mice and ameliorated in KKA^{y}-CETP mice. The volume of urine and the concentration of glucose in urine were measured in KKA^{y}-CETP mice and both were reduced. The reduction in these three parameters could possibly be due to increased glucose uptake measured by improved glucose tolerance after 7-days treatment with AICAR, and increased glucose uptake in isolated muscles after acute exposure to AICAR. This was supported by increased protein expression of GLUT4 and hexokinase II, and increased MEF2 DNA binding, all biomarkers of glucose uptake. The activity of PI3-kinase was not enhanced, indicating that the decreased plasma glucose and improved glucose transport were not likely to be linked to improved insulin action on PI3-kinase. Basal plasma insulin was decreased and insulin sensitivity measured by an insulin tolerance test and using a hyperinsulinaemic euglycaemic clamp technique was improved in KKA^{y}-CETP mice.

The increased liver glycogen content in AICAR-treated ob/ob mice in study II could be due to either inhibition of the hepatic glycogen breakdown or to an increased glycogen formation, or both. The protein content of glycogen synthase was not increased indicating that the increased glycogen content in liver and skeletal muscle is not a result of increased glycogen formation. On the contrary, the clamp studies from study I showed that the hepatic glucose output is reduced after AICAR treatment, both acutely and after 7-days treatment (during infusion of insulin).

All the above described changes are in line with the hypothesis of AMPK-activation. In theory, activation of AMPK should also increase the fatty acid oxidation (Merrill *et al.*, 1997; Merrill *et al.*, 1998). An increased fatty acid oxidation should possibly result in an ameliorated lipid profile. However, this was not the case in study I and II. Instead the plasma triglycerides, free fatty acids and cholesterol were increased in both rodent models. In the KKA^{y}-CETP mice the HDL-cholesterol were reduced and the VLDL-cholesterol increased, but liver triglycerides were decreased in ob/ob mice. The deteriorative effect on the lipid profile could possibly arise from side-effects from the non-specific AMPK activator or as a compensatory mechanism triggered by starvation signals.

Activation of AMPK in hypothalamus should increase food intake (Andersson *et al.*, 2004), instead food intake was reduced in the KKA^{y}-CETP mice and lost weight occured. The ob/ob and the lean mice did not alter body weight or food intake in response to AICAR treatment. This could possibly be explained by the different routes of administration i.e. subcutaneous for the ob/ob and lean mice or intraperitoneal for the KKA^{y}-CETP mice.

5.2 STUDY III

Inhibition of 11βHSD1 resulted in a decrease in the plasma glucose concentration, which could partly be caused by the increased adiponectin concentration. Low levels of

the high molecular form of adiponectin is especially associated with type 2 diabetes and obesity (Lara-Castro *et al.*, 2006). Both the medium and the high molecular forms of adiponectin were elevated after 11βHSD1inhibition. This was in line with *in vitro* results, where primary human adipocytes also secreted more adiponectin in response to *in vitro* BVT116429 exposure. Glucose tolerance was not improved in study III, but the same substance improved the glucose tolerance in Ldlr knockout mice (Hu *et al.*, 1996). Basal plasma insulin concentration was improved. The leptin concentration was elevated, which was not due to increased food intake or weight gain. BVT2733, with its 11βHSD1 inhibitory effect primarily in the liver, had no effect on adiponectin, but decreased hepatic glucose production (Alberts *et al.*, 2003). BVT116429, a compound with its primary effect in adipose tissue, was without effect on endogenous glucose production. The positive effect of 11βHSD1 inhibition in adipose tissue on glucose levels could be partly caused by effects downstream of adiponectin.

6 CONCLUSION AND FUTURE PERSPECTIVES

In this thesis, two novel targets regarding the treatment of type 2 diabetes have been investigated and discussed. Both targets have characteristics which could be of use in the clinic, if an approved drug is produced. AICAR is in phase I study for the treatment of type 2 diabetes, but newer substances might be more specific, targeting the subunits of AMPK, and therefore more effective and safer. However, screening for subunit-specific AMPK activators is extremely difficult and so far no company has found a more specific substance. Unfortunately, it is not sufficient to identify and validate targets. In drug development, all phases need to deliver positive results. In this case, no starting points for new chemical entities were found in the screening. However, there are opportunities for others to be more successful in the search for AMPK activators and there might be more innovative screens in the future.

11βHSD1 is a well-validated target and starting points for chemistry were found in the high-throughput screen. Substances were approved and improved in lead generation and in lead optimisation. The pharmacodynamic studies showed efficacy *in vivo* and no issues were found in the preclinical studies. The human specific equivalent to BVT116429 was tested in a clinical phase I study and adiponectin was used as a biomarker. However, additional information has not been further released from Amgen, the owner of the drug. Inhibition of 11βHSD1 is now validated for the treatment of type 2 diabetes in humans. Results from Incyte´s clinical phase II study were recently revealed (Rosenstock *et al.*, 2009). Delivery of the 11βHSD1-inhibitor INCB13739 to type 2 diabetic patients inadequately controlled by metformin resulted in statistically significant reduction in HbA_{1C}, fasting plasma glucose, homeostasis model assessment-insulin resistance and total cholesterol after 12 weeks treatment.

Both AMPK and the glucocorticoids are influenced by stress. Adipocytes treated *in vitro* with AICAR showed increased expression of 11βHSD1 (Arai *et al.*, 2007). In all physiological systems, homeostasis is preferable and it is of great importance to find the right level of inactivation or activation. Cortisol does not only have impact on metabolism, but it has other important roles for example in the immune system. The safety risks with long-term treatment with 11βHSD-inhibitors will now have to be determined, especially for patients with reduced immune response.

The features of type 2 diabetes can also be viewed as a defence against obesity. By decreasing insulin sensitivity and glucose tolerance, energy is stored to a less extent. By increasing blood glucose, more will be excreted in the urine. This decrease in glucose uptake is not sufficient as a defence mechanism. Thus, type 2 diabetes is associated with complications such as a higher risk of heart disease. Therefore it is innovative to produce drugs which raise the energy output by increasing fat oxidation, which an AMPK-activator should do. New strategies are needed for drug development, to find novel therapies for the treatment of type 2 diabetes. Inhibition of 11βHSD1 and activation of AMPK might help people achieve a better quality of life, but this will need to be corroborated by more studies and a greater understanding before addressing whether these targets can result in drugs and benefit to type 2 diabetic patients.

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