Novel compounds for the prevention and treatment of type 2 diabetes

Author(s):
Mrosek, Nadja Evamaria

Publication Date:
2015

Permanent Link:
https://doi.org/10.3929/ethz-a-010450090

Rights / License:
In Copyright - Non-Commercial Use Permitted
Novel compounds for the prevention and treatment of type 2 diabetes

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by
NADJA EVAMARIA MROSEK
Dr. med., Ruprecht-Karls-Universität Heidelberg

born on 24.08.1984
citizen of
Germany

accepted on the recommendation of

Prof. Dr. Christian Wolfrum
Prof. Dr. Wolfgang Langhans
Prof. Dr. Gottfried Rudofsky

2015
## Table of contents

Abstract .......................................................................................................................... 1

Zusammenfassung ......................................................................................................... 3

Abbreviations ................................................................................................................ 5

1. General introduction ................................................................................................. 7
   1.1. Diabetes epidemic ......................................................................................... 7
   1.2. Current solutions to prevent and treat type 2 diabetes .................................. 11
   1.3. Trends and innovations in diabetes management in the food sector .............. 16
   1.4. Adipose tissue and diabetes treatment .......................................................... 19
   1.5. Potential novel anti-diabetic compounds targeting fat tissue ...................... 20

2. Objectives .................................................................................................................. 24

3. S100A4 for diabetes treatment ................................................................................ 25
   3.1. S100A4 is regulated in obesity ....................................................................... 25
   3.2. S100A4 affects adipocyte differentiation and function ................................. 26
   3.3. Loss of S100A4 leads to increased weight gain and deterioration of metabolic parameters in mice ............................................................................................................................... 28
   3.4. Fat depot transplants from wild type mice into S100A4 deficient mice reduces weight gain and improves insulin sensitivity ........................................................................................................ 31
   3.5. Elevation of systemic S100A4 levels improves metabolic parameters in HFD mice .......................................................................................................................... 33

4. THBA for the treatment of type 2 diabetes ............................................................. 35
   4.1. Dose-dependent effect of THBA .................................................................... 35
   4.2. Blood glucose lowering effect of THBA is maintained after end of treatment .......................................................... 36
   4.3. Safety assessment of THBA .......................................................................... 38
   4.4. Pharmacokinetic of THBA in vivo .................................................................. 39
   4.5. Toxicity profile of THBA ................................................................................ 40

5. Discussion .................................................................................................................. 42

6. Material and methods ............................................................................................. 51

7. Conclusion ................................................................................................................. 58

8. Appendix .................................................................................................................... 60

9. References .................................................................................................................. 62

Acknowledgements ...................................................................................................... 71

Curriculum Vitae .......................................................................................................... 72
Abstract

Type 2 diabetes is an increasing medical and socioeconomic problem for most countries. The International Diabetes Federation estimates that worldwide 1 out of 12 people is diagnosed with diabetes, with numbers of affected people steadily increasing. Despite a huge variety of anti-diabetic drugs, the majority of individuals with type 2 diabetes does not reach glycemic control underlining the need for novel solutions to treat type 2 diabetes. In addition, the growing aim of preventing or delaying the onset of type 2 diabetes has come into focus demanding the need of clinical interventions for pre-diabetic patients. Especially medical foods, which are primarily focused at prevention and dietary management of a disease, present an interesting alternative for targeting pre-diabetic individuals. In the recent past adipose tissue has been recognized as an important regulator of glucose homeostasis and an interesting target for anti-diabetic agents. In this study we explore the potential anti-diabetic effect of two novel compounds, S100A4 and tetrahydroxylated bile acid, THBA, as well as the efficacy and safety profile of THBA for potential clinical development.

In the first part of our investigation we demonstrate in vitro and in vivo that S100A4 stimulates adipocyte differentiation, as well as insulin sensitivity in mature adipocytes. S100A4 knockout mice fed a high fat diet gain significantly more weight than wild type mice and display decreased insulin sensitivity as well as impaired metabolic parameters, such as increased plasma insulin, glucose and triacylglyceride levels. Transplantation of S100A4 expressing wild type visceral fat depots into S100A4 deficient mice restores metabolic parameters in S100A4 knock out mice to wild type levels. In addition, adenovirus-mediated overexpression of S100A4 in visceral fat depots of obese C57BL/6 wild type mice leads to significantly increased insulin sensitivity, reduction of blood glucose and triacylglyceride levels and weight loss already 3 weeks after injection. Overall however the promising anti-diabetic profile of S100A4 is attenuated by its established, strong association with metastasis and cancer progression. Nonetheless, these studies provide further opportunity to study a new player regulating adipose tissue function and insulin sensitivity.

THBA is a naturally occurring tetrahydroxylated bile acid and endogenous ligand of retinoid-related orphan receptor γ (RORγ), which is an inhibitor of adipogenesis. It was previously shown that THBA suppresses RORγ activity and significantly increases adipocyte differentiation and insulin sensitivity in vivo. Here, we demonstrate that C57BL/6 wild type mice fed a high fat diet supplemented with THBA do not show a difference in food consumption or overall weight gain compared to control groups, but display improved glycemic control under THBA treatment. Furthermore, glucose metabolism is significantly improved after 6 weeks of treatment and is maintained for 9 weeks after end of...
treatment at week 13 indicating that insulin sensitization may be primarily mediated via increased adipogenesis. THBA displays a dose-dependent effect at different concentrations, whereby all treatment groups show a similar effect on glucose homoeostasis after 12 weeks of TBHA administration. Kinetic studies reveal that THBA accumulates in the blood after repeated daily dose, is taken up and secreted from various tissues and excreted in relatively high amounts via the urine. THBA does not raise any safety concerns as demonstrated in several *in vitro* and *in vivo* toxicity studies. In summary, THBA represents a highly promising, novel anti-diabetic medical food candidate with an auspicious preclinical efficacy and safety profile. Further pre-clinical and clinical studies will reveal the full potential of THBA as medical food product for prevention and treatment of type 2 diabetes.
Zusammenfassung


THBA, eine natürlich vorkommende tetrahydroxylierte Gallensäure, ist ein endogener Ligand von retinoid-related orphan receptor γ (RORγ), einem Inhibitor der Adipogenese. Es wurde zuvor gezeigt, dass THBA die Aktivität von RORγ hemmt und zu einer deutlichen Steigerung der
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>A-FABP</td>
<td>adipocyte fatty acid binding protein</td>
</tr>
<tr>
<td>AMES</td>
<td>biological assay to assess mutagenicity</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>cholic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CO2</td>
<td>carbon dioxide production</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EASD</td>
<td>European Association for the Study of Diabetes</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FSMP</td>
<td>Food for Special Medical Purposes</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>hERG</td>
<td>human ether-a-go-go related gene</td>
</tr>
<tr>
<td>HFD</td>
<td>high fat diet containing 60% fat</td>
</tr>
<tr>
<td>IFG</td>
<td>impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>impaired glucose tolerance</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>Mio</td>
<td>million</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MNT</td>
<td>micronucleus test</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>ob/ob mice</td>
<td>leptin deficient mice</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economics and Development</td>
</tr>
<tr>
<td>OGGT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RORγ</td>
<td>Retinoid-related orphan receptor gamma</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous (inguinal)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>THBA</td>
<td>1β, 3α, 7α, 12α - Tetrahydroxy - 5β - Cholan -24 - Oic acid</td>
</tr>
<tr>
<td>vis</td>
<td>Visceral (epididymal)</td>
</tr>
<tr>
<td>VO2</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>w/w</td>
<td>Wet weight</td>
</tr>
</tbody>
</table>
1. General introduction

1.1. Diabetes epidemic

“Diabetes is undoubtedly one of the most challenging health problems of the 21st century.”
International Diabetes Federation, 2014

The rise in diabetes presents an enormous challenge for the individual, the health care system and the society for countries at all income levels. The International Diabetes Federation (IDF) stated that despite the array of tools at our disposal to tackle the disease – effective drug therapies, advanced technology ever-improving education and preventive strategies – the battle for protecting people from diabetes and its disabling, life-threatening complications is lost [1]. It is currently estimated that worldwide 382 Mio adults have diabetes and this number is expected to rise by 55% by 2035 (Fig. 1.1).

Figure 1.1 Global diabetes numbers in 2013. Modified from IDF Diabetes Atlas, 2013 [1].

Classification of diabetes

Diabetes mellitus describes a metabolic disease of multiple etiology characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes is associated with long-term damage, dysfunction and failure of various organs [2]. Based on the suggestions of the World Health Organisation (WHO) and the American Diabetes Association (ADA) diabetes is nowadays classified according to etiological criteria [3, 4]. The general diabetes categories include type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM) and a group of rare
specific types of diabetes [5]. However, it is not always possible to assign a patient clearly to one single category as the types of diabetes can overlap and change over time.

Type 1 diabetes, formerly called insulin-dependent diabetes or juvenile-onset diabetes, is mainly caused by an autoimmune destruction of the insulin producing β-cells of the pancreas. This type of diabetes, which accounts for 5-10% of the total cases worldwide, is associated with multiple genetic predispositions as well as environmental factors [6]. The progression of β-cell destruction is variable and although type 1 diabetes is mainly seen in children or adolescents it can occur at any age [7]. In 85-90% of all patients autoantibodies are detectable, including autoantibodies to islet cells (ICA), glutamic acid decarboxylase 65 (GAD65), tyrosine phosphatases IA-2 (IA2-A) and insulin (IAA) [6]. A small minority of type 1 diabetes cases results from an idiopathic failure or destruction of β-cell [8].

Type 2 diabetes, previously referred to as non-insulin-dependent diabetes or adult-onset diabetes, is with 90-95% of all cases the most common type of diabetes. This form of diabetes usually occurs in adults, but its prevalence in childhood and adolescence is steadily increasing [9, 10]. Type 2 diabetes is a multifactorial disease and shows a strong association to genetic predisposition and especially environmental risk factors such as obesity, decreased physical activity, smoking, heavy alcohol consumption and the metabolic syndrome [11, 12]. According to the International Diabetes Federation the metabolic syndrome is diagnosed by the co-occurrence of central obesity with two further medical conditions such as elevated triglycerides, reduced HDL-cholesterol, raised blood pressure or elevated fasting blood glucose [13]. Type 2 diabetes results from insulin resistance as well as a relative insulin deficiency of non-autoimmune etiology. In the recent past, insufficient insulin secretion has been recognized as a key feature in type 2 diabetes. Insulin-resistant obese individuals can be protected from diabetes by pancreatic β-cell compensation for a certain period of time before the ability of β-cells to release sufficient insulin is compromised [14]. Of note, an increase in insulin action and concomitant reduction of β-cell secretory burden can improve β-cell function [15]. In genome-wide association studies several genes were identified which were deregulated in type 2 diabetes and are believed to be important for β-cell function, development or the regulation of β-cell mass underlining the role of genetics in the development of the disease [14, 16]. In addition, enhanced secretion of glucagon by pancreatic α-cells, leading to increased gluconeogenesis promotes hyperglycemia [17]. Insulin resistance, mainly caused by chronic overnutrition and the exposure of tissues to elevated dietary nutrients, is the prominent feature in obese patients. This leads to an overproduction and underutilization of glucose as well as increased circulation of fatty acids and ectopic lipid accumulation [18, 19]. Further mechanisms that are associated with the development of insulin resistance include the effect of inflammation, hormonal
factors such as adipokines, endoplasmic reticulum (ER) stress as well as accumulation of metabolic by-products such as long-chain acyl CoAs, diacylglycerols and ceramides in insulin sensing tissue [11, 20].

Further forms of diabetes are gestational diabetes, which occurs in approximately 2-9% of all pregnancies and mostly resolves after delivery, as well as other rare specific types of diabetes [21]. This category encompasses amongst others maturity-onset diabetes of the young (MODY) which primarily results from a monogenetic defect of the β-cell function and is characterized by impaired insulin secretion and hyperglycemia at an early age [14]. Other specific types are caused by genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, uncommon forms of immune-mediated diabetes as well as drug, chemical or infection induced diabetes [9].

As the global prevalence and burden of type 2 diabetes steadily rises, increasing efforts have been made to prevent or delay the onset of type 2 diabetes by targeting pre-diabetes. In pre-diabetes blood glucose levels are higher than normal but do not meet the criteria for diabetes. Pre-diabetic individuals show either impaired fasting glucose (IFG) of 100mg/dL (5.6mmol/L) to 125mg/dL (6.9 mmol/L), impaired glucose tolerance (IGT) of 140mg/dL (7.8mmol/L) to 199mg/dL (11.0 mmol/L) or both, and are at very high risk to develop type 2 diabetes [22, 23]. Besides being at high risk to develop type 2 diabetes pre-diabetic individuals also show a considerably increased prevalence in macro- and especially microvascular diseases [24-26]. The Centers of Disease Control estimated that 1 out of 3 Americans over the age of 20 had pre-diabetes in 2011 and the worldwide prevalence of pre-diabetes (IFG and/or IGT) was estimated to be around 800 Mio in 2014 [22].

**Diagnosis and socioeconomic burden of diabetes**

The development of clinically manifested type 1 diabetes is usually sudden while type 2 diabetes develops rather slowly and often shows a long pre-symptomatic phase, which explains why this form of diabetes often remains undiagnosed. Symptoms of diabetes mellitus include amongst others fatigue, headaches, increased hunger, frequent urination, excessive thirst, weight loss, blurred vision, frequent infections, slow-healing wounds and tingling sensation or numbness in the hands or feet [27]. Besides the presence of clinical symptoms leading to testing for diabetes, the disease is often diagnosed in high-risk individuals in the context of a medical check-up or in seemingly low-risk individuals as an incidental finding. Diabetes can be diagnosed based on several criteria including increased fasting plasma glucose (≥126mg/dL (7mmol/L)), elevated HBA1C (≥ 6.5%) or increased 2 hour plasma glucose after an oral glucose tolerance test (≥ 200mg/dL) [28, 29]. Although the oral glucose tolerance test remains the gold standard, the American Diabetes Association recommends
the use of the HbA1C test due to its greater pre-analytical stability, less day-to-day perturbations and higher practicability [30, 31]. Overweight adults and individuals with metabolic syndrome and a positive family history of diabetes, are primarily diagnosed with type 2 diabetes. However, in case of absence of the metabolic syndrome, in particular in lean patients, the possibility of latent autoimmune diabetes in adults (LADA) or maturity onset diabetes of the young (MODY) should be considered [32, 33].

Given the fact that type 2 diabetes is often asymptomatic for a long time, many cases of diabetes remain undiagnosed. In the US, this concerns approximately one-quarter of the diabetes cases [1]. This resulted in the recommendation from various institutions such as the American Diabetes Association to screen for type 2 diabetes and pre-diabetes in asymptomatic adults at risk [5, 34]. These risk factors include age (≥ 45 years), BMI and ethnicity (e.g. Caucasians: BMI ≥ 25kg/m², Asians: BMI ≥ 23kg/m²) and certain medications such as glucocorticoids and thiazide diuretics [35-37]. In addition, people with diabetes should be regularly screened for signs of diabetes complications since the disease is strongly associated with various microvascular (retinopathy, neuropathy and nephropathy) as well as macrovascular (ischaemic heart disease, cerebrovascular disease and peripheral vascular disease) complications. While the prevalence of microvascular complication is higher, macrovascular complications in particularly ischemic heart disease account for the greatest proportion of diabetes associated mortality [38-40]. Worldwide approximately 5.1 Mio adults died from diabetes in 2013. This accounts for 8.4% of all death in adults and represents an increase of 11% compared to 2011 [40, 41]. This rise in number is mainly due to an increase of diabetes cases in low- and middle-income countries such as China, India and Brazil (Fig. 1.2).

Besides the strong impact of diabetes on morbidity and mortality, the disease is rapidly becoming a major economic burden. In 2013, worldwide 10.8% of total health expenditures were spent on diabetes amounting to at least USD 548 billion. Europe alone spent estimated USD 147 billion and North America and Caribbean region even USD 263 billion (48% of global diabetes expenditures) on
diabetes. Noteworthy, although 80% of people with diabetes live in middle-income countries only 20% of global health expenditures were spent on the disease in those countries [1].

1.2. Current solutions to prevent and treat type 2 diabetes

In diabetes treatment individualization represents a cornerstone of success. Because of the variable and progressive nature of type 2 diabetes, the huge variety of anti-diabetic agents and the lack of comparative efficacy studies in this field, management of type 2 diabetes requires a thoughtful clinical decision making [42-44]. The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) recommend a patient-centred care, an approach that focuses on the individual patient preferences, needs as well as a shared medical decision making [45, 46].

It is generally recognized that the risk of micro- and macrovascular complications is associated with hyperglycemia and that the HbA1c value, as surrogate marker for glycemic control, represents a major focus of diabetes therapy [47, 48]. In the majority of patients the aim is to lower HbA1c below 7%, which can be achieved with a mean plasma glucose of approximately 8.3-8.9 mmol/L [49]. It should be considered to target more stringent HbA1c goals (e.g. 6.0-6.5%) in young patients, and less stringent HbA1c targets (e.g. 7.5-8.0%) in patients with a history of severe hypoglycemia or a limited life expectancy [50, 51]. Several aspects such as patient attitude, disease duration, life expectancy, comorbidities, established vascular complications and the risks potentially associated with hypoglycemia influence the individual HbA1c target [46, 50].

Therapeutic lifestyle modifications such as dietary interventions, enhancing physical activity, and weight reduction are key factors during type 2 diabetes management [52, 53]. A modest weight loss of 5-10% already leads to a meaningful reduction in blood glucose levels and cardiovascular risk factors, underlining the importance of weight reduction especially in obese individuals [54, 55]. General dietary recommendations include the consumption of low-caloric foods with a high fibre content, low-fat dairy products and fresh fish, while high-caloric foods, including those rich in sugar and saturated fats should be eaten less [56, 57]. Diabetic patients should increase their physical activity, ideally targeting a minimum of 150min per week [58].

Anti-diabetic drug therapy

If lifestyle changes don’t show a sufficient impact on glycemic control after a period of three to six months, a pharmacotherapy should be initiated [34]. Antihyperglycemic pharmacotherapy follows in general a multi-stage therapy algorithm (Fig 1.3).
Figure 1.3 General recommendation from the ADA and EASD for antihyperglycemic therapy in type 2 diabetes. Modified from Inzucchi et al [59]. *Consider beginning at this stage in those with HbA1c ≥9.0. † Meglitinides may be considered in those with irregular meal schedules or who experience late postprandial hypoglycaemia when receiving SU therapy. ‡Usually basal insulin. ¥Certain noninsulin agents may be continued with insulin. Metformin (MET). Sulphonylurea (SU). Thiazolidinediones (TDZ). Glucagonlike peptide-1. Dipeptidyl peptidas-IV (DDP-4). HbA1c (A1C).

It is generally recognized that, unless no contraindications or intolerance exist, Metformin is the preferred and most cost-effective agent for initial antidiabetic drug therapy [49]. Metformin has a favourable risk/benefit profile (e.g. weight neutral, no increased risk of hypoglycemia, good safety profile) and shows an equal or higher efficacy compared to other available oral antidiabetic drugs [60]. The blood glucose-lowering effect of metformin is mainly mediated via suppression of hepatic gluconeogenesis, to a certain extent by the reduction of intestinal glucose absorption and potentially by increasing glucose uptake in peripheral tissues [61, 62]. Molecular mechanisms underlying the suppression of hepatic glucose output include the indirect activation of AMP-activated protein kinase (AMPK) via the inhibition of the mitochondrial respiratory-chain complex 1 [63, 64]. However, although Metformin is used as an antidiabetic agent for over 50 years, its mechanism of action is still not fully understood [65]. When Metformin cannot be used, another primarily oral agent such as sulfonylurea/glinide, thiazolidinedione (Pioglitozone) or DPP4-inhibitor should be considered, where patient preference, potential for hypoglycemia and weight gain, as well as susceptibilities to side effects should lead the drug selection [66, 67]. Sulphonylureas (SUs) are the oldest oral antidiabetic drugs and stimulate the insulin secretion via the closure of ATP-sensitive potassium channels on β-
cell plasma membranes [68]. While sulphonylureas have a good efficacy and a positive impact on microvascular complications, they are also associated with an increased risk of hypoglycemia, modest weight gain and exacerbation of pancreatic islet dysfunction [69]. Meglitinides/glinides, which have a similar mechanism to SUs, function as rapid-acting secretagogues and have a lower risk of hypoglycemia, but require more frequent dosing [70]. Thiazolidinediones (TZDs), which function as activators of the peroxisome proliferator-activated receptor γ (PPARγ), protect β-cell function and enhance insulin sensitivity in the liver and peripheral tissues, such as muscle and adipose tissue, leading to an increased glucose disposal and decreased gluconeogenesis [71, 72]. TZDs show a good efficacy, and may have a more sustained effect on glucose control than Metformin and SUs, but were put under strict selling restrictions in Europe and the US due to severe cardiovascular safety concerns [69, 73, 74]. More recently, dipeptidyl peptidase 4 (DPP-4) inhibitors have been introduced [75, 76]. By suppressing the DPP-4 enzyme, negative regulator of the incretin effect, DPP-4 inhibitors increase the circulating levels of the active incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinoergic peptide (GIP). GLP-1 and GIP are important for the nutrient-stimulated insulin secretion, suppression of glucagon secretion, slowdown of gastric emptying as well as reduction of appetite [76-78]. DPP-4s are well tolerated with a low risk of hypoglycemia, but only show a moderate efficacy (HbA₁c reduction 0.5-1%) compared to metformin, SUs and TZDs, which cause a HbA₁c reduction of approximately 1-1.5% [79, 80]. In occasional cases the administration of a GLP-1 receptor agonist, insulin or less commonly used drugs such as α-glucosidase inhibitors (65), Colesevelam, a bile acid sequestrant (66) or Bromocriptin, a dopamine agonist may be considered as initial drug therapy. In case the HbA₁c target cannot be achieved or maintained by monotherapy after approximately 3 months, dual combination therapy with a second oral agent, a GLP-1 receptor agonist or basal insulin should be started [44, 81]. If this therapeutic regimen is not able to achieve or maintain the glycemic goal, the next step would be a triple combination therapy, adding either a third oral antidiabetic drug or basal insulin [44, 82]. As type 2 diabetes progresses, a more complex insulin therapy combining long-acting basal insulin with rapid-acting insulin analogous might be required [83]. Of note, uniform recommendations, which diabetic patients will most likely benefit from one specific drug combination, are not well defined [59].

**Pre-diabetes**

The current ADA and EASD guidelines for the treatment of pre-diabetes focus on lifestyle modifications, including dietary interventions, weight reduction and increasing physical activity, whereas metformin may be considered in selected patients at very high risk to develop diabetes [5, 84].
Although life-style modifications enhance insulin sensitivity and improve β-cell function, these interventions and especially long term weight loss maintenance have been proven very difficult in clinical practice [85, 86]. As pre-diabetic patients have more micro- and macrovascular complications than normoglycemic peers and since microvascular complications in particular depend on the severity and duration of hyperglycemia, there is a growing consensus that normoglycemia should be targeted in pre-diabetic patients [22]. There have been various clinical trials studying the effect of pharmacological interventions on the treatment of pre-diabetes and the prevention of type 2 diabetes. These studies revealed that especially thiazolidinediones were effective to improve blood glucose levels and prevent the progression to type 2 diabetes (Fig. 1.4). It was proposed that the superiority of thiazolidinediones might result from the improvement of insulin sensitivity and the protection of β-cell function [87-89]. There is a growing consensus that for treatment of pre-diabetes and prevention or delay of type 2 diabetes novel behavioural and pharmacological interventions need to be developed [22, 89, 90].

Figure 1.4 Clinical interventional trials to reduce progression from pre-diabetes to diabetes. Modified from Perreault et al. 2014 [22]. Da Qing (Pan et al. 1997), Finish Diabetes Prevention Study (FDPS) (Tuomilehto et al. 2001), Diabetes Prevention Program (DPP) (Knowler et al. 2002), Indian Diabetes Prevention Program (IDPP) (Ramachandran et al. 2006), Stop Non-insulin-dependent diabetes mellitus (STOP-NIDDM) (Chiasson et al. 2003), Xenical in the prevention of Diabetes in Obese Subjects (XENDOS) (Torgerson et al. 2004), Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) (Holman et al. 2010), Troglitazone in the Prevention of Diabetes (TRIPOD) (Buchanan et al. 2002), Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) (Gerstein et al. 2006), ACT NOW (DeFronzo et al. 2011).
Diabetes market

As diabetes prevalence will continue to rise, it is expected that the global pharmaceutical market is going to increase from approximately $33 billion in 2010 to around 45$ billion in 2016 [91]. In the US the number of adults filing antidiabetic drug prescriptions increased by 42.9% from 13.2 Mio in 2003 to 18.8 Mio in 2012. During this period the distribution between noninsulin antidiabetic pharmaceuticals (86.7% in 2012) and insulin agents (27.1% in 2012) remained relatively constant [92]. Although the majority (> 90%) of all patients receive Metformin or Sulphonylureas worldwide, these oral antidiabetics only represent around 6% of global sales, due to their very low pricing (Fig. 1.5 left).

![Figure 1.5 Diabetes drug market](image)

Innovations in anti-diabetic drug development

Besides research going on in the main drug classes (such as long-acting GLP-analogous, DPP4-inhibitors, short-acting inhaled insulin, ultra-long acting insulin), the diabetes Research and Development (R&D) pipeline contains some development programs that focus on novel modes of actions [94]. Sodium glucose cotransporter-2 (SGLT-2) inhibitors are the most recently approved drug class in the US, EU and Japan. By inhibiting SGLT-2 protein, which is responsible for reabsorbing approximately 90% of the glucose from the primary urine in the kidneys, glucose excretion is...
enhanced and blood glucose levels are decreased [95, 96]. SGLT-2 inhibitors showed a modest efficacy (HbA1c reduction of approx. 0.5-1%) in several clinical trials, but are associated with side effects such as genital mycotic infections, osmotic diuresis, and are expensive with average costs of 310-350$ per month [94, 97]. Another novel mode of action focuses on the activation of glucokinase (GCK), which is essential for glucose metabolism. Several pharmaceutical companies have early stage programs in this glucokinase activator drug class, which is supposed to decrease hepatic glucose output and enhance insulin secretion while suppressing glucagon secretion [98, 99]. Noteworthy, regulatory guidelines were introduced by the US Food Safety Authority in 2009 to evaluate the cardiovascular risk of anti-diabetic drugs leading to a delay in several pipeline compounds. The rising diabetes numbers and the fact that only 36% of patients achieve glycemic control with current available therapies will drive the development of new agents.

1.3. Trends and innovations in diabetes management in the food sector

Driven by the increasing socioeconomic burden of type 2 diabetes and the need for novel innovative solutions to target this disease, the food industry has started to develop healthier and more nutritious products aiming at disease prevention and personalized health care nutrition. In this process the line between the food and pharma sector is blurring and a new industry segment is rising aiming to develop disease preventing and modifying nutritional therapies [100, 101]. The health care nutrition sector can be divided in three major categories including medical food, functional food as well as supplements and ingredients; the latter are often integrated in functional foods and medical nutrition. While functional foods are supposed to provide additional health benefits for healthy individuals, medical foods are intended for the dietary management of disease in patients and are closely related to pharmaceuticals (Fig. 1.6).
Ingredients and supplements

Several ingredients and supplements are used for the management of diabetes, especially some phytonutrients have been used for centuries. Many of these substances have pharma-like properties and are associated with the modulation of metabolic pathways, which have an impact on glycemic control. These include the stimulation of insulin secretion (e.g. heartwood, gymnema sylvestre), improvement of insulin sensitivity (e.g. cinnamon, bitter lemon), reduction of intestinal glucose absorption (fenugreek, gymnema sylvestre) as well as the suppression of inflammation (omega-3 fatty acids) and oxidative stress (cinnamon). Preclinical data is available for most of these ingredients but only few have been studied in human randomized clinical trials to proof their health claims [103-106].

Chromium picolinate and Vitamin D are associated with an increased insulin secretion and insulin sensitivity and are frequently used as supplements as well as ingredients in functional and medical foods. Several clinical trials have been conducted to assess the efficacy and safety of these two compounds. Although some studies showed a positive effect on glucose metabolism, the overall scientific data remains controversial, and efficacy and safety were questioned by the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA) [107-109].

Functional foods and drinks

Functional foods are supposed to provide consumers with additional health benefits beyond adequate nutritional effects [110, 111]. To improve glycemic control various functional ingredients such as fibre, resistant starch, prebiotic fibre, vitamins, minerals and phytonutrients are currently incorporated in anti-diabetic functional foods. The main beneficial effect of these products is the reduction of the glycemic impact by increasing the fibre and reducing the glucose and carbohydrate content. Various categories of anti-diabetic functional foods such as bakery products and cereals, sweets and dairy foods and drinks are available [102, 112, 113]. According to market research by the International Food Information Council (IFIC) two thirds of consumers are interested in using functional foods and drinks to manage their blood glucose levels [102]. To respect consumer protection the European Union released a regulation in 2006 stating that health claims must be scientifically proven [114].

Medical food

Medical foods or Foods for Special Medical Purposes (FSMP), as they are named in the European Union, are intended for the dietary management of a disease or disorder. FSMPs are defined as a category of foods that is specially processed or formulated for the dietary management of a disease
and has to be used under medical supervision. They are administered enterally or parenterally and can be used for partial or exclusive feeding of patients [115]. In contrast to functional foods, which are intended for healthy people, medical foods are predominantly prescribed by medical professionals and are perceived to be more related to pharmaceuticals than to conventional food (Fig. 1.7).

Figure 1.7 Industries situated at the food-pharma interface. Modified from Weenen at al. [116]

Anti-diabetic medical foods such as diabetes-specific formulas e.g. Diabetesource AG® (Nestlé), Glucerna® (Abbott Nutrition), Diasip® (Danone) or Metanx® (Pamlab) are designed to improve glycemic control and secondary complications associated with hyperglycemia [117, 118]. Medical food companies are working on identifying the optimal formulation of protein, fat and carbohydrates and integrate functional ingredients such as chromium, vitamin D and cinnamon, which are considered to have anti-diabetic effects [119]. Of particular importance is the incorporation of fibres and particular slow release carbohydrates such as Nutrisource® (Nestlé) or Carbsteady® (Abbott Nutrition) to slow down gastric emptying and decrease glycemic impact leading to improved postprandial glucose profiles [118, 120].

Any claims stated on the label of such foods must be proven by sound scientific evidence and an in-depth safety assessment is required before market authorization [121, 122]. In addition, at least one randomized human clinical trial is required to obtain a label claim in Europe and the US. In the last few decades, controlled clinical trials showed solid evidence concerning the efficacy of medical foods increasing their credibility [118, 123]. This also explains why medical foods, in contrast to functional foods, can be reimbursed by health care providers (Fig. 1.8) [124-126]. There are no detailed market data regarding the size of the medical food market. Global sales of medical foods were estimated to
be around $7-9 billion, with approximately $1.5-2 billion in Europe and around $2-3 billion in the US in 2011. Medical foods are expected to show strong growth driven by the aging population and an increase in prevalence of chronic diseases such as diabetes and obesity [102, 127].

<table>
<thead>
<tr>
<th>Target population</th>
<th>Food</th>
<th>Functional foods</th>
<th>Medical food (FSMP)</th>
<th>Pharmaceuticals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healty individuals</td>
<td>Healty individuals</td>
<td>Patients</td>
<td>Patients</td>
</tr>
<tr>
<td>Uses</td>
<td>Satisfy nutrition requirements</td>
<td>Provide specific health benefits</td>
<td>Dietary management of a disease</td>
<td>Treatment of disease, symptom or condition</td>
</tr>
<tr>
<td>Oversight</td>
<td>No prescription or supervision</td>
<td>No prescription or supervision</td>
<td>Medical supervision, prescription possible</td>
<td>Medical supervision, prescription possible</td>
</tr>
<tr>
<td>Point of Sales</td>
<td>Supermarkets, convenience stores, online</td>
<td>Supermarkets, convenience stores, online</td>
<td>Pharmacies, hospitals, doctors</td>
<td>Pharmacies, hospitals, doctors</td>
</tr>
<tr>
<td>Regulatory</td>
<td>EFSA/FDA</td>
<td>EFSA/FDA (Health claim)</td>
<td>EFSA/FDA (Product claim)</td>
<td>EMA/FDA</td>
</tr>
<tr>
<td>Reimbursement</td>
<td>no</td>
<td>no</td>
<td>possible</td>
<td>in most cases</td>
</tr>
<tr>
<td>Example</td>
<td>Snickers (Nestlé)</td>
<td>Boost Glucose Control (Nestlé)</td>
<td>Glucerna (Abbott Nutrition)</td>
<td>Metformin (Merck)</td>
</tr>
</tbody>
</table>

Figure 1.8 Characteristics of functional and medical foods in Europe and the US [102, 111, 114, 115, 121, 122].

1.4. Adipose tissue and diabetes treatment

Adipose tissue is a complex organ, which is involved in the regulation of various metabolic functions. In general, adipose tissue is classified into two main types, white and brown adipose tissue. White adipose tissue stores energy in form of triacylglycerols, which are broken down and released as fatty acids in case of energy demand [128]. Brown adipocytes, which have a high density of mitochondria, can dissipate energy in form of heat by activating uncoupling protein-1 (UCP1) [129, 130]. While brown fat depots occur only in smaller areas of the human adult body e.g. in the cervical, suprACLavicular and paravertebral area, white adipose tissue is present in a high quantity throughout the body. White adipose tissue can be divided in the two major types of subcutaneous and visceral fat. The latter is associated with a higher risk for metabolic diseases [129, 131].

Expansion of white adipose tissue occurs via enlargement of existing mature adipocytes (hypertrophy) or via enhanced differentiation of adipocyte precursors, leading to an increased number of mature adipocytes (hyperplasia) [132]. In contrast to hyperplasia, hypertrophy is associated with adipose dysfunction and deteriorated metabolic parameters such as impaired glucose homeostasis. Adipocyte hypertrophy is accompanied with insufficient vascularization and local adipose tissue hypoxia as well as adipocyte death, infiltration of pro-inflammatory macrophages
and chronic inflammation. Hence adipose tissue expansion via hypertrophy is strongly associated with systemic insulin resistance and cytotoxic ectopic lipid accumulation. By promoting adipose tissue expansion via hyperplasia, metabolic consequences of obesity can be improved [133-135]. In the recent past adipose tissue has been recognized for its various physiological functions besides pure energy storage. It was shown that adipose tissue functions as an endocrine organ, which secretes various adipokines and is important for the regulation of energy balance and metabolic parameters like blood glucose and lipid levels [128, 136]. Therefore, it is gaining more and more attention as therapeutic target for the treatment and prevention of metabolic diseases such as obesity and type 2 diabetes [137-139].

PPARγ agonists, thiazolidinediones (TDZs), are the only drug class that primarily achieves its anti-diabetic effect by increasing insulin sensitivity. PPARγ is highly abundant in adipose tissue and stimulates fatty acid uptake and storage, glucose uptake as well as adipocyte differentiation [71]. TDZs are efficacious anti-diabetic agents in diabetes treatment and prevention. However, due to safety concerns mentioned above the use of TDZ has drastically declined leaving a gap of efficient insulin sensitizers in diabetes treatment [140]. Given the beneficial effect of hyperplasia on insulin sensitivity and glucose homeostasis the identification of a compound increasing adipogenesis might be a very promising approach to prevent and treat type 2 diabetes.

1.5. Potential novel anti-diabetic compounds targeting fat tissue

S100A4, also known as metastasin, fibroblast-specific protein (fsp), calvasculin, p9Ka, CAPL, pEL98, 18A2, or 42A, is a member of the S100 family. S100 proteins are EF-hand calcium binding proteins that generally exist as homo- or heterodimers and modulate the activity of other proteins [141, 142]. Calcium binding leads to conformational changes in S100 protein structures and influences the interaction with various target proteins in multiple cellular processes [143]. S100A4 is a small protein of 11.5kDa that occurs in the cytoplasm, nucleus and the extracellular space. The occurrence of S100A4 in human plasma and tumor interstitial fluid suggests a secretion process of the protein [144-146]. S100A4 shows increased expression in tumors, but its expression was also reported in various normal human cell types, including fibroblasts, macrophages, lymphocytes and endothelial cells. In addition, it was identified in liver, smooth muscle cells and brown adipose tissue of rats [147]. S100A4 occurs as monomer, dimer and oligomer and mainly exerts its function via calcium-dependent binding and crosslinking of homologous or heterologous target proteins [148]. S100A4 has two calcium binding EF-hands and undergoes a conformational change upon calcium binding. The resulting hydrophobic pocket is essential for the interaction with target proteins [149]. Several intracellular binding partners have been identified, including p53, liprin 1-β, actin, heavy chain of non-muscle myosin IIA and IIB, S100A1 and tropomyosin [150]. It has also been shown that S100A4
extracellularly interacts with cell surface receptors such as annexin II, receptor for advanced glycation endproducts (RAGE) and heparin sulfate proteoglycans [151-153]. S100A4 is strongly associated with cancer invasiveness and metastasis, which might be mediated by various mechanisms such as cell motility, cell proliferation, angiogenesis and remodelling of extracellular matrix [144].

**THBA, a physiological RORγ ligand**

Retinoid-related orphan receptor γ (RORγ) was identified as an important inhibitor of adipogenesis by our laboratory [154]. RORγ is a nuclear receptor with a variable N-terminal (A/B) domain which contains a ligand-independent activation function domain (AF1) that can engage coregulators, a DNA-binding domain and a C-terminal ligand-binding domain. RORγ binds as a monomer and modulates the transcription of its target genes [155, 156]. RORγ has two isoforms, RORγ (RORγ1) and RORγt (RORγ2). RORγ is expressed in various tissues predominantly in liver, kidneys, skeletal muscle and adipose tissue, while expression of RORγt is restricted to immune cells such as T cells [157, 158]. RORγt plays an important role in the differentiation program of Th17 cells, which produce interleukin-17 (IL-17) and interleukin-21 (IL-21). Due to the implication of Th17 cells in inflammatory conditions and autoimmune disorders, RORγt has been studies as potential target for the treatment of autoimmune diseases [159-161]. RORγ has been implicated in physiological processes, including regulation of metabolism and circadian rhythm. Recent studies demonstrated that circadian rhythms are linked to the regulation of energy homeostasis as well as lipid and glucose metabolism [156, 162]. It has been reported that RORγ directly increases the circadian expression of clock genes such as cryptochrome-1 (Cry1) and the heterodimeric complex consisting of brain and muscle ARNT-like-1 (Bmal1) [163]. In addition, RORγ has been proposed to regulate the diurnal expression of several lipid metabolic genes in the liver, including elongation of very long chain fatty acids-like 3 (Elov3), sterol 12α-hydroxylase (Cyp8b1) and insulin-induced gene 2a (Insig2a). In the same study global or liver-specific loss of RORγ led to a reduction in triacylglycerides, cholesterol and bile acid levels in serum and liver [164]. Global gene expression analysis of visceral adipose stromal vascular fraction (SVF) from lean and obese humans identified RORγ as the highest upregulated gene in the state of obesity [154]. Enhanced expression of RORγ is associated with increased adipocyte size and insulin resistance in obese humans. In addition, it has been shown by our laboratory that RORγ deficient mice displayed a strong increase in adipocyte differentiation and were protected against the development of insulin resistance despite obesity [154]. While several RORγt ligands have been developed into treatment against autoimmune and inflammatory diseases, no RORγ ligands have been developed into therapies so far [156, 165].
As RORγ represented a promising novel target for the treatment of type 2 diabetes, we aimed to identify the endogenous ligand of this orphan nuclear receptor. To this end, a precipitation of RORγ from pre-adipocytes followed by an extraction of lipids from this precipitation was performed (cooperation between Prof. Christian Wolfrum and Prof. Erick Carreira). The analysis of the composition of the lipid extracts with mass spectrometry led to the identification of 1β, 3α, 7α, 12α-tetrahydroxy-5β-cholan-24-oic acid, a tetrahydroxylated bile acid (THBA), as a putative ligand of RORγ in adipose tissue (Fig 1.9).

**Figure 1.9 Structure of THBA.** Chemical structure of the endogenous ligand of RORγ 1β, 3α, 7α, 12α-tetrahydroxy-5β-cholan-24-oic acid (CAS 80875-94-1).

THBA is a naturally occurring tetrahydroxylated bile acid found in humans and animal-based food products such liver and kidney. Tetrahydroxylated bile acids are usually minor components of the total bile acid pool in adults, in which di- and tri-hydroxylated bile acids represent the major constituents [166]. Interestingly, high levels of THBA were found in the urine of pregnant women and in preterm infants as the major bile acid that could be detected after birth [166, 167].

To investigate the activity and the effect of THBA, THBA was synthesized by a 12 step chemical synthesis route developed by Dr. Riccardo Cribiu and several *in vitro and in vivo* studies were performed by Dr. Susanne Wolfrum. A reporter gene assay, in which luciferase was expressed under the promoter of the RORγ target genes matrix metallopeptidase 3 (MMP3) or Interleukin 17 (IL-17), confirmed that THBA strongly inhibits RORγ. Also, treatment of 3T3 L1 cells with THBA led to increased adipocyte differentiation (data not shown). In a prevention study wild type mice were challenged for 6 weeks with a HFD alone or supplemented with 0.1% THBA (w/w), 0.01% THBA (w/w), or cholic acid as control. Mice treated with THBA showed no significant difference in weight gain, but had significantly smaller adipocytes as well as a higher percentage of lean mass relative to their body weight. No differences were detected in energy expenditure (such as oxygen consumption, CO2 production and respiratory exchange rates) and pro-inflammatory cytokines amongst test animals on HFD (Appendix 1). THBA-mediated suppression of RORγ led to strong improvement in glucose metabolism with a reduction of 33% in fasted blood glucose levels, 44% in insulin levels as well as increased insulin sensitivity. The proposed mechanism of THBA is outlined in figure 1.10.
Figure 1.10 Schematic representation of proposed mode of action of THBA. The anti-diabetic effect of THBA is mediated via suppression of RORγ leading to an increase in adipogenesis.

Besides prevention of the diabetic phenotype THBA was also successfully used for the treatment of manifested insulin resistance in wild type mice. After 16 weeks of treatment obese mice, fed a high fat diet, displayed a reduction of 49% in fasted blood glucose levels and approx. 72% in insulin levels. Given the natural occurrence of THBA in food products and due to above demonstrated promising efficacy data and an expected good safety profile, we considered to exploit THBA as a medical food.
2. Objectives

Because of the increasing individual and socioeconomic burden of pre-diabetes and type 2 diabetes, and limited success of current treatment options, novel solutions are needed to combat this epidemic. With regards to currently available pharmaceutical agents, the development of safe and efficacious insulin sensitizers is of particular interest. In the recent past adipose tissue has been recognized as an important regulator of glucose homeostasis and an interesting target for anti-diabetic agents.

Alterations of S100A4 expression in subcutaneous adipose tissue of obese humans raised the question if S100A4 may influence adipose tissue function and thereby metabolic parameters, such as glucose homeostasis. Therefore, the first aim of this work was to evaluate the effect of S100A4 on adipose tissue function and its therapeutic potential for the treatment of 2 diabetes and the metabolic syndrome.

In addition, the endogenous ligand of RORγ, THBA (tetrahydroxylated bile acid), was shown to significantly promote adipogenesis and insulin sensitivity in vivo. Because of the high efficacy, the favourable risk-benefit profile and the natural occurrence of THBA in food products, we hypothesized that THBA might be a promising novel medical food candidate for the prevention and treatment of type 2 diabetes. Therefore, the second aim of this work was to clarify the potential of THBA to enter human clinical trials and to be marketed as anti-diabetic medical food. To this end, several preclinical in vitro and in vivo studies were performed to evaluate the efficacy, kinetics, and in particular safety of THBA.
3. S100A4 for diabetes treatment

3.1. S100A4 is regulated in obesity

S100A4 was initially identified in an Agilent gene array analysis as a gene that was upregulated (9-fold) in human subcutaneous whole fat of obese patients vs. lean subjects. To investigate the potential function of S100A4, we first examined mRNA expression levels in various tissues from C57BL/6 wild type mice. S100A4 mRNA was expressed in several tissues with the highest occurrence in thymus and fat tissue, in particular, in the stromal vascular fraction (Fig. 3.1a). Next, we investigated S100A4 protein levels in environmentally obese (diet-induced obesity, DIO) and genetically obese (ob/ob) mouse models. In contrast to our data from human subjects, S100A4 levels were decreased in adipocytes, stromal vascular fraction and plasma of diet-induced (DIO) or genetically obese (ob/ob) mice (Fig. 3.1b-c).

Figure 3.1 S100A4 expression profile in various tissues and S100A4 protein levels in tissue and plasma of different mouse models. a) Relative mRNA expression levels of S100A4 in various wild type C57BL/6 mouse tissues. b) S100A4 protein levels in stromal vascular fraction (SVF) and adipocytes of wild type (WT), ob/ob and mice fed a high fat diet (DIO). c) S100A4 plasma levels in wild type (WT), ob/ob and mice fed a high fat diet (DIO). Mean +SD of n=3. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

1 Figure 3.1b and c were performed by Eva Röder, a previous member of the lab who also studied S100A4.
3.2. S100A4 affects adipocyte differentiation and function

Given the high abundance of S100A4 in adipose tissue and its down-regulation in obesity we moved on to analyse the role of S100A4 in adipogenesis and for mature adipocyte function. To examine a potential function of S100A4 in adipogenesis, we first analyzed the expression of S100A4 during differentiation of 3T3-L1 cells (Fig.3.2a). Expression of S100A4 increased while cells became confluent and a strong peak was detectable one day after induction of differentiation (day 0). To further investigate the impact of S100A4 on adipocyte differentiation, we designed lentiviruses mediating overexpression or knockdown of S100A4 and infected 3T3-L1 pre-adipocytes 3 days prior induction of differentiation. Overexpression of S100A4 strongly increased 3T3-L1 differentiation, while shRNA-mediated knockdown of S100A4 led to reduction of adipogenesis (Fig.3.2b).

Figure 3.2 Impact of S100A4 on adipocyte differentiation. a) Relative S100A4 mRNA expression during differentiation of 3T3-L1 cells. b) Relative A-FABP levels as marker for adipocyte differentiation after CMV promotor mediated overexpression (CMV-S100A4) and shRNA mediated knockdown of S100A4 (sh) in 3T3-L1 pre-adipocytes. Mean ±SD of n=3-4. *p ≤ 0.05.

Since S100A4 promoted adipogenesis in vitro, we evaluated the adipogenic potential in S100A4 loss-of-function primary derived stromal vascular fraction. To this end, ex vivo differentiation of visceral and subcutaneous primary SVF of wild type and S100A4 knock mice was measured. In line with results in 3T3-L1 cells, SVF derived from mice with a null-mutation for S100A4 displayed decreased differentiation, whereby the effect was higher in subcutaneous SVF (Fig. 3.3a). Next, we analyzed adipogenesis of wild type and S100A4 knockout mice challenged with a HFD for 18 weeks by quantifying the relative distribution of adipocyte size in visceral and subcutaneous fat tissue. Adipose tissue sections of S100A4 deficient animals displayed a lower number of small adipocytes (Fig. 3.3b-d).
Figure 3.3 S100A4 knockdown decreases adipogenesis in vivo. a) Ex vivo differentiation of subcutaneous (sc) and visceral (vis) stromal vascular fraction of wild type (WT) and S100A4 knockout mice (S100A4−/−). Relative A-FABP levels served as maker for adipocyte differentiation. b) Relative distribution of adipocyte size in subcutaneous fat of wild type and S100A4 knockout condition. c) Relative distribution of adipocyte size in visceral fat of wild type and S100A4 knockout condition. d) Representative images of tissue sections of visceral fat of wild type (WT) and S100A4 knockout mice (S100A4−/−). Mean ±SEM of n=5-6. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Besides the effect on adipocyte differentiation we also investigated the function of S100A4 in mature adipocytes. To do so, the ability of adipocytes derived from young wild type mice or S100A4 knockout mice to take up glucose in response to insulin was measured. Adipocytes with a null mutation for S100A4 showed a significantly reduced uptake of 14C-deoxyglucose after insulin stimulation in visceral and subcutaneous adipocytes (Fig. 3.4a). In addition, we quantified the lipolytic activity of subcutaneous and visceral wild type and S100A4-deficient adipocytes. Insulin-mediated inhibition of lipolysis of the latter was lower compared to S100A4 expressing, wild type adipocytes (Fig. 3.4b). However, it should be noted that a potential difference in size of the tested adipocytes might influence experimental outcome.
3.3. Loss of S100A4 leads to increased weight gain and deterioration of metabolic parameters in mice

Because of the pro-adipogenic effect of S100A4 in vitro and in vivo as well as the observation that its ablation led to decreased insulin sensitivity in mature adipocyte, we investigated its potential effect on the metabolic phenotype. To this end, wild type mice and S100A4 knockout mice were challenged with a high fat diet (HFD) for 18 weeks. Mice lacking S100A4 gained significantly more weight during this period than wild type animals (Fig. 3.5a-b). Analysis of the body fat composition with computer tomography revealed that S100A4 knockout mice had 2.2-fold more total fat than wild type mice (Fig. 3.5c). The increase in overall subcutaneous fat was slightly higher compared to the changes in visceral adipose tissue.
Figure 3.5 Increased weight gain and fat mass in S100A4 knockout mice. Wild type (WT) and S100A4 knockout mice (S100A4−/−) were fed a high fat diet for 18 weeks starting at 6-7 weeks of age. a) Body weight of (n=6-7). b) Weight gain (n=6-7). c) Relative total, subcutaneous (sc), and visceral (vis) fat mass of wild type and S100A4 knockout mice after 16 weeks of high fed diet (n=4). Mean ±SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Next to body weight gain we measured several metabolic parameters such as blood glucose, insulin, free fatty acids, cholesterol and triacylglycerides over the course of the high fat diet regimen. S100A4 knockout mice demonstrated a steady and stronger increase of blood glucose, insulin and triacylglyceride levels compared to wild type mice (Fig.3.6a-c), whereas no significant changes were detectable in free cholesterol and free fatty acid levels (data not shown). To assess insulin sensitivity of the test animals in more detail, an insulin tolerance test was performed. In consistency with increased blood glucose and insulin levels S100A4 deficient mice showed a significantly reduced response to insulin in an insulin tolerance test (Fig.3.6d).

Figure 3.6 Impairment of metabolic parameters in S100A4 knockout mice. Wild type (WT) and S100A4 knockout mice (S100A4−/−) were fed a high fat diet for 18 weeks starting at 6-7 weeks of age.
Fasting blood glucose, insulin and triacylglyceride levels were measured at indicated days. a) Fasting blood glucose levels. b) Fasting insulin levels. c) Fasting triacylglyceride levels. d) Insulin tolerance test was performed after 18 weeks of high fat diet. Mean ±SD of n=6-7. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

To further characterize the impact of S100A4 ablation on the metabolic phenotype, wild type and S100A4 knockout mice were transferred to metabolic cages after 10 weeks of high fat diet. Total oxygen consumption (VO2) and oxygen consumption at rest (VO2res) as well as a total carbon dioxide production (CO2) and carbon dioxide reduction at rest were reduced in S100A4 knockout mice compared to wild types (Fig.3.7a). Both cohorts showed a similar respiratory exchange ratio, namely 0.76 in wild type and 0.74 in S100A4 knockout mice suggesting that lipids were the main source for catabolic metabolism. In line with the decreased overall energy expenditure as well as the energy expenditure at rest, S100A4 deficient mice moved less and showed a trend towards reduced food and water consumption after 10 weeks of high fat diet (Fig. 3.7b-c).

Figure 3.7 Metabolic cage data. Metabolic parameters of wild type (WT) and S100A4 knockout mice (S100A4−/−) were measured after 10 weeks of high fat diet. a) Oxygen consumption and carbon dioxide production. b) Locomotor activity. c) Food and water consumption. Mice were analyzed in metabolic cages for 24h. VO2 = total oxygen consumption, VO2res = oxygen consumption at rest, CO2 = total carbon dioxide production, CO2res = total carbon dioxide production at rest. MovX = Movements in X axis, MZ = Movements in Z axis. Mean ±SD of n=4. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
3.4. Fat depot transplants from wild type mice into S100A4 deficient mice reduces weight gain and improves insulin sensitivity

Given the high expression of S100A4 in fat tissue and the observation that its ablation leads to impairment of metabolic parameters, we evaluated whether S100A4 protein is secreted by the fat tissue and can improve metabolic parameters. To this end we performed cross transplantations of the two epididymal visceral fat depots between S100A4 knockout mice and wild type mice. Transplantations within S100A4 deficient mice and between wild type mice served as control (Fig. 3.8a). 6 weeks after surgery mice were put on a high fat diet for a period of 12 weeks and metabolic parameters were measured. S100A4 knockout mice, which received the visceral fat depots of S100A4 expressing wild type mice (WT->S), had a lower weight gain than the control group of S100A4 deficient mice that received fat pads of S100A4 knockout mice (S->S). In contrast, the transplantation of S100A4 deficient fat pads into wild type mice (S->WT) did not cause a significant change in weight gain (Fig.3.8b). In line with the S100A4 knockout and wild type mice that did not undergo surgery, S100A4 knockout mice receiving S100A4 deficient fat pads (S->S) gained around twice as much weight than wild type mice receiving wild type transplants (WT->WT).

![Figure 3.8 Reduced weight gain after transplantation of visceral fat pads from wild type mice into S100A4 deficient mice.](image)

*Figure 3.8 Reduced weight gain after transplantation of visceral fat pads from wild type mice into S100A4 deficient mice. a) Transplantation scheme. b) Weight gain of wild type mice, which received S100A4 knockout transplants (S->WT); S100A4 knockout mice, which received wild type transplants (WT->S); S100A4 knockout mice, which received S100A4 knockout transplants (S->S); S100A4 knockout mice, which received S100A4 knockout transplants (S->S), and wild type mice, which received wild type transplants (WT->WT). Mice were subjected to a high fat diet 6 weeks after surgery for 12 weeks. Mean ± SD of n=3-5. *p ≤ 0.05.*
Blood glucose levels in fasted and refed state of S100A4 knockout mice with transplanted wild type fat pads (WT->S) were lower than in S100A4 knockout mice with S100A4 deficient transplants (S->S) after 4 weeks of HFD (Fig3.9a). This effect was significantly increased after 12 weeks of HFD. S100A4 knockout mice with wild type transplants (WT->S) showed similar blood glucose levels as wild type mice with wild type transplants (WT->WT) (Fig.3.9b). No significant difference could be observed between wild type mice which received S100A4 deficient (S->WT) or wild type fat depots (WT->WT). Fasted and refed insulin levels of S100A4 knockout mice with wild type transplants (WT->S) were around 60% lower compared to insulin levels of S100A4 knockout mice with S100A4 deficient fat pads (S->S) (Fig.3.9c). In addition, the transplantation of wild type fat depots into S100A4 deficient mice led to a reduction of triglyceride levels in refed state (Fig.3.9d), whereas no significant difference could be detected in free fatty acid and cholesterol levels (data not shown). Taken together, these data show that transplantation of S100A4 expressing visceral fat depots improves the metabolic impairment of obese S100A4 knockout mice.

**Figure 3.9 Improved metabolic parameters after transplantation of visceral fat depots from wild type mice into S100A4 deficient mice.** Metabolic parameters of wild type mice, which received wild type transplants (WT->WT); wild type mice, which received S100A4 knockout transplants (S->WT); S100A4 knockout mice, which received S100A4 knockout transplants (S->S) and S100A4 knockout mice, which received wild type transplants (WT->S). Mice were subjected to a high fat diet 6 weeks after surgery for 12 weeks. a) Blood glucose levels after 4 weeks. b) Blood glucose levels after 12 weeks. c) Insulin levels after 12 weeks. d) Triacylglyceride levels after 12 weeks. Mean ±SD of n=3-5. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
3.5. Elevation of systemic S100A4 levels improves metabolic parameters in HFD mice

To evaluate the effect of S100A4 secretion in visceral fat depots of S100A4 knockout mice in a different way, adenovirus mediating overexpression of S100A4 or Ad-GFP were injected into the two complementary visceral fat depots of S100A4 knockout mice fed a HFD for 6 weeks. 3 weeks after injection, a reduction in refed blood glucose levels of 28%, as well as a significant improvement in insulin sensitivity in an insulin tolerance test was detectable. No significant difference in body weight was observed (data not shown).

Given the positive impact of S100A4 on metabolic parameters in S100A4 deficient mice, we next asked the question whether S100A4 protein can be used as anti-diabetic treatment in obese mice. To this end, we injected Ad-S100A4 or Ad-GFP as control into the two complementary visceral fat depots of C57BL/6 mice fed a HFD for 6 weeks. After surgery mice received a HFD for another period of 3 weeks before metabolic parameters were measured. Adenovirus-mediated overexpression of S100A4 led to improved glucose metabolism in the treatment group. These mice showed lower blood glucose levels (Fig.3.10a) as well as increased insulin sensitivity in an insulin tolerance test (Fig.3.10b). In addition, a reduction in refed triacylglyceride levels and weight gain was observed in Ad-S100A4 injected animals compared to the control group (Fig.3.10c-d). In summary, above data demonstrate the ability of S100A4 to improve metabolic parameters in S100A4 deficient as well as in obese wild type mice suggesting a pharmacological potential for treatment of type 2 diabetics.

![Graphs](image-url)

**Figure 3.10** Adenovirus induced expression of S100A4 improves metabolic parameters in wild type mice fed a HFD. Visceral fat pads of C57BL/6 mice, fed a high fat diet for 6 weeks starting at 6 weeks
of age, were injected with either Ad-S100A4 (S100A4) or control Ad-GFP (GFP). After 3 weeks metabolic parameters were measured. a) Blood glucose levels. b) Insulin tolerance test. c) Triacylglyceride levels. d) Body weight. Mean ±SD of n=7. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
4. THBA for the treatment of type 2 diabetes

4.1. Dose-dependent effect of THBA

As THBA was shown to act as efficient insulin sensitizer, we elucidated next the dose response of THBA as well as the sustainability of the anti-diabetic effect after treatment. To this end young, adult, male wild type mice were fed a high fat diet (HFD) supplemented with 0.01% THBA (w/w), 0.0033% or 0.001% of THBA, or pure HFD as control for a period of 13 weeks. With a food consumption of 3g per day these concentrations amounted to a daily dose of approximately 0.3mg, 0.1mg and 0.03mg THBA. No significant difference in weight gain and food consumption was detectable amongst the groups, whereby the medium dose group (0.0033% w/w) and the control group showed the strongest weight gain (Fig.4.1).

Figure 4.1 Impact of THBA on food consumption and weight gain. C57BL/6 mice were fed a high fat diet for 13 weeks starting at 10 weeks of age to induce insulin resistance. Mice received HFD, supplemented with different concentration of THBA (0.01%, 0.0033%, 0.001%) or pure HFD as control. a) Body weight. b) Food consumption at week 1 and week 13. Mean ±SD of n=14-18.

To evaluate a potential dose-dependent effect of THBA on glucose metabolism, we measured blood glucose levels after 6 and 12 weeks and performed an oral glucose tolerance test (OGTT) after 13 weeks of treatment. After 6 weeks of THBA supplementation, blood glucose levels were decreased in mice that received the high (0.01%) or medium (0.0033%) dose of THBA by 12 and 15%, whereas no effect was observed in the low dose group (Fig.4.2a). Interestingly, all treatment groups showed a similar reduction in blood glucose levels of around 22% after 12 weeks of THBA administration (Fig.4.2b). To assess the glucose tolerance of the test animals we performed an oral glucose tolerance test after 13 weeks of treatment. All treatment groups displayed a significant decrease in fasting blood glucose of 42-50% compared to control and glucose tolerance was strongly improved in the animals receiving THBA. The low dose was slightly less efficacious than the two other dosages (Fig.4.2c).
Figure 4.2 Dose-dependent effect of THBA on glucose metabolism. C57BL/6 mice were fed a high fat diet (HFD) for 13 weeks starting at 10 weeks of age to induce insulin resistance. Mice received HFD, supplemented with different concentration of THBA (0.01%, 0.0033%, 0.001%) or pure HFD as control. a) Blood glucose levels in fed state after 6 weeks. b) Blood glucose levels in fed state after 12 weeks. c) Oral glucose tolerance test after 13 weeks. Mean +SD of n=14-18. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

4.2. Blood glucose lowering effect of THBA is maintained after end of treatment

As THBA acts via stimulation of adipogenesis and given the fact that adipose tissue has a relatively low turn-over rate, we hypothesized that the anti-diabetic effect of THBA may be maintained after the end of treatment. To this end, we put mice after a 13-week treatment phase on a chow diet for another 13 weeks and monitored their body weight and blood glucose levels (Fig. 4.3). 9 weeks after the end of THBA administration all treatment groups still showed reduced blood glucose levels compared to the control group (Fig.4.3a). However, after 13 weeks no effect was observed anymore (data not shown). In line with the higher body weight of the medium dose group, the blood glucose levels of these animals were slightly higher than in the high and low dose group. The maximum overall change in weight gain was within the range of 2.2% after 9 weeks of chow diet (Fig.4.3c).
Taken together, above data demonstrate a dose-dependent efficacy of THBA and the maintenance of its anti-diabetic effect after end of treatment.

**Figure 4.3** Improvement of glucose metabolism by THBA can be maintained after treatment end. After C57BL/6 mice were fed a high fat diet (HFD), supplemented with different concentration of THBA (0.01%, 0.0033%, 0.001%) or pure HFD as control for 13 weeks, mice were put on a chow diet for 13 weeks. a) Blood glucose levels in fed state after 9 weeks of chow diet. b) Body weight after 9 weeks of chow diet. c) Relative weight loss within 9 weeks after end of THBA treatment. Mean ±SD of n=14-18. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
4.3. Safety assessment of THBA

To evaluate the safety and pharmacology of THBA, several toxicity, kinetic and stability studies were performed in vitro as well as in vivo (Fig. 4.4). With respect to the requirements of medical food development some of the studies were conducted externally by clinical research organizations (CROs) following OECD guidelines.

Figure 4.4 Safety assessment of THBA. To evaluate the safety profile of THBA various in-vitro and in-vivo toxicity and kinetic studies were performed. The studies were conducted at the ETH (in-house) or externally at qualified clinical research organizations following OECD guidelines. Micronucleus test (MNT). Human ether-a-go-go related gene (hERG). Absorption, Distribution, Metabolisation and Elimination (ADME).

As THBA is administered orally we analysed the stability of THBA at parameters mimicking the gastric milieu. THBA was chemically stable at pH 2 at 37°C for 2h (data not shown). To investigate the first pass effect in vitro, a microsomal stability assay, a test that primarily focuses on phase I metabolism in the liver, was performed. The intrinsic clearance and respective turnover rate of THBA was very low in rat (1.06 µl/min/mg protein) as well as in human (1.51 µl/min/mg protein) microsomes, suggesting that only minor amounts of THBA are subject to first pass metabolism in the liver. To assess the absorption of THBA across the human gut wall in vitro, a CaCo-2 permeability assay was performed.
The assay showed a permeability coefficient of 0.38 ±0.09 x 10^{-6} cm/second indicating that approx. 50% may be absorbed at the gut wall.

4.4. Pharmacokinetic of THBA in vivo

After completion of the first kinetic studies in vitro we moved on to investigate the absorption and excretion of THBA in vivo. To this end, 3H-labeled THBA was designed with the label positioned such that it cannot be cleaved off by common P450 enzymes. 0.3mg and 0.03mg THBA spiked with the radiolabeled version was then orally administered daily for 5 consecutive days to mice on a high fat diet. Radioactivity was measured in whole blood, urine and feces during gavage period (11h - day 4) and after last gavage of THBA (day 1 - day 28 post gavage). THBA entered the circulation in a dose-dependent manner and showed a half-life of approx. 5 days. Consecutive oral administration of THBA led to continuous increase of THBA in blood with the maximal concentration 1 day upon last gavage. THBA was almost completely cleared from the blood 2 weeks after last gavage and was not detectable after 4 weeks (Fig.4.5a). A similar pattern was seen in urine (Fig.4.5b). The excretion of THBA via the feces also declined steadily and was not detectable 6 days upon last gavage (Fig.4.5c).

![Figure 4.5 THBA blood levels and excretion profile after repeated THBA administration. C57BL/6 mice were fed a high fat diet for 4 weeks starting at 7 weeks of age to induce insulin resistance. At 11 weeks of age the mice were divided in two groups (n=25-35) and gavaged with 0.3 or 0.03mg of radiolabeled THBA for 5 consecutive days (t0= first gavage). Radioactivity was measured at 11h, 24h, 2 days, 4 days following the first gavage of THBA and 1, 3, 6, 9, 16 and 28 days after the last gavage. Mice were sacrificed 1 day, 2 and 4 weeks following last gavage. a) THBA levels in blood. b) THBA excretion via urine. c) THBA excretion via feces. Mean +SD of n=8-35.](image-url)
To determine the distribution and potential accumulation of THBA in various metabolically active tissues, we measured the radioactivity in these tissues 1 day, 2 and 4 weeks after the last administration of 0.03mg (Fig.4.6a) or 0.3mg (Fig.4.6b) spiked THBA. THBA was dose-dependently taken up in all measured tissues. One day after the last gavage high amounts of THBA were found in muscle, liver, brain, kidney and spleen. Lower amounts of THBA were found in subcutaneous, visceral and brown fat tissue with the highest being detected in brown adipose tissue (BAT). After 2 weeks the amount of THBA decreased in all tissues and was nearly undetectable after 4 weeks.

![Figure 4.6 Tissue distribution of THBA](image)

Figure 4.6 Tissue distribution of THBA. C57BL/6 mice were fed a high fat diet for 4 weeks starting at 7 weeks of age to induce insulin resistance. At 11 weeks of age the mice were divided in 2 groups (n=25-35) and gavaged with 0.3 or 0.03mg of radiolabeled THBA for 5 consecutive days (t0= first gavage). The uptake of THBA was measured 1 day, 2 and 4 weeks following the last gavage and normalized to tissue weight (n=8-11 for each time point). Kidney, liver, muscle, brain, spleen, brown fat, subcutaneous fat (sc fat) and epididymal fat (vis fat) were analyzed. a) THBA concentration in the indicated tissues after oral administration of 0.03mg THBA per day. b) THBA concentration in the indicated tissues after oral administration of 0.3mg THBA per day. Mean +SD of n= 8-11.

Interestingly, in the tissues that showed high uptake of THBA, such as kidney, liver, muscle, brain and spleen, the THBA concentration decreased to around 5% after 2 weeks and less than 1 % after 4 weeks. In contrast, all fat tissues showed a lower reduction of THBA over time. After 2 weeks, THBA concentration was reduced to 40% in visceral fat, 20% in subcutaneous and 10% in BAT. After 4 weeks the values decreased further to 20% in visceral fat, 10% in subcutaneous and 1.5% in BAT.

### 4.5. Toxicity profile of THBA

To evaluate a potential genotoxicity of THBA an Ames test and a micronucleus test (MNT) were performed. No signs of genotoxicity were observed for TBHA. In addition, THBA was analyzed for potential cardiotoxic effects by measuring the inhibition of the human ether-a-go-go related gene (hERG), which encodes a potassium ion channel that contributes to the electrical stimulus conduction of the heart. Several concentrations (0.008–25µM) of THBA were tested. No concentration-dependent inhibition, hence no signs of cardiotoxicity were observed.
Having confirmed that THBA is not genotoxic or cardiotoxic \textit{in vitro}, we moved on to investigate the oral toxicity of THBA \textit{in vivo}. First, THBA was tested in an acute oral toxicity study at the limit dose of 2000mg/kg in female Wistar rats. No signs of clinical impairment or marks of toxicity in the necropsy of test animals and in the histological analysis of liver and kidney were found. Thus, the maximal tolerated dose of THBA by oral route was classified to be higher than the tested limit dose of 2000mg/kg. To assess the toxicity of THBA during chronical oral administration, we performed an escalating toxicity study in female wild type mice. Test animals received an increasing dose of THBA via oral gavage 6 times per week for a period of 4 to 8 weeks. The daily dosages of 3mg to 6mg corresponded to 150mg to 260mg/kg body weight. These dosages were selected to determine whether a 100-fold of the effective medium dose (approx. 2.5mg/kg body weight) causes toxic effects. In line with the acute toxicity study, we did not detect signs of impairment or clinical disease (Appendix 2) and all test animals showed a similar weight gain as well as food and water consumption (Fig.4.7).

**Figure 4.7 THBA shows no signs of toxicity in chronic toxicity study.** THBA was administered daily to female C57BL/6 mice (6 days per week) for 8 weeks starting at 8 weeks of age. The following doses of THBA were administered: week 1-4 (3mg), week 5 (3.75mg), week 6 (4.5mg), week 7 (5.25mg), week 8 (6mg). Mice were sacrificed after 4 weeks and 8 weeks for histopathological examination. a) Body weight (n=20 at week 4, n=10 at week 8). b) Food and water consumption (n=10). Mean +SD.

Furthermore, no toxicity was observed in the necropsy of test animals and the histological analysis on liver, brain, kidney and spleen, neither in the intermediate evaluation after 4 weeks nor at the end of the study after 8 weeks (data not shown). In conclusion, THBA displayed no signs of toxicity \textit{in vitro} and \textit{in vivo} at the concentrations tested in above studies. Hence, no safety concerns for THBA were raised.
5. Discussion

Here presented investigations on S100A4 and RORγ ligand, THBA, indicate and substantiate their potential as promising novel compounds for prevention and treatment of type 2 diabetes. As S100A4 significantly improves insulin sensitivity in adipose tissue and leads to weight loss, it might be an interesting pharmaceutical agent for treatment of the metabolic syndrome. But its established association with cancer progression and metastasis may eventually represent an obstacle to drug development.

THBA, the endogenous ligand of RORγ, acts as insulin sensitizer by increasing adipogenesis. Because of its natural occurrence in food and favorable risk-benefit profile during pre-clinical development as shown in the second study, it appears to become a highly promising medical food, in particular for the treatment of pre-diabetes.

S100A4

Studies on S100A4 are mainly associated with cancer metastasis, tumor progression, and fibrosis [147]. However, our previous studies indicated a potential role in adipose tissue function as S100A4 mRNA was highly abundant in obese human subcutaneous whole fat. In contrast S100A4 expression was decreased in stromal vascular fraction and adipocytes of diet induced and genetically obese (ob/ob), insulin-resistant mice, most likely due to differences in processing of raw material samples. S100A4 is highly abundant in monocytes, macrophages, granulocytes and lymphocytes [144]. These cells are mostly removed in murine SVF and adipocyte samples during processing, while these are still present in human whole fat tissue. Consistent with the tissue profile, S100A4 plasma levels are reduced in diet induced and especially genetically obese, insulin-resistant mice confirming the secretory ability of S100A4 into plasma. As S100A4 protein is also detectable in human plasma [146], it would be interesting to evaluate in future studies whether there is a correlation between S100A4 levels and obesity or insulin resistance in humans.

An important mechanism for white adipose tissue expansion and function is differentiation of adipocyte precursors. S100A4 mRNA levels are upregulated during early differentiation and overexpression of S100A4 increases adipogenesis in 3T3 cells in vitro. In addition, S100A4 ablation leads to a reduction in adipogenesis in S100A4 knockout mice ex vivo as well as in vivo. These findings are in line with the association of S100A4 with cell motility, proliferation, differentiation, angiogenesis and remodeling of extracellular matrix (ECM) [144]. ECM remodeling is an important event during adipogenesis, enabling the morphological and cytoskeletal reorganizations in pre-adipocytes, which are required for the transition into mature adipocytes. In this context the degradation of fibronectin-rich matrix by proteases such as matrix metalloproteases (MMPs) is
essential [168]. It was proposed that transcriptional activity of MMP2 and MMP9 might be induced by S100A4-mediated activation [147, 169] leading to ECM remodeling, which may then increase adipogenesis. In addition, S100A4 is associated with the activation of SMADs, which are associated with stimulating adipocyte differentiation. For example, S100A4 directly binds SMAD3 and increases its transcriptional activity [170, 171]. Further adipogenesis-promoting mechanisms influenced by S100A4 might include cytoskeletal dysregulation, for example by direct interactions of S100A4 with non-muscle myosin and non-muscle tropomyosin [172, 173] as well as by stimulation of angiogenesis via the interaction with annexin II [135, 146, 153]. Despite the wide variety of proposed functions and targets of S100A4, the exact mechanism of action in cancer progression and especially adipogenesis remains elusive. Furthermore, increased adipocyte size in S100A4 knockout animals may not be mainly due to decreased adipogenesis in absence of S100A4 as suggested by a 33% higher body weight in S100A4 knockout mice compared to wild type mice, but due to a much higher lipid load and overall adipose tissue expansion.

Our analysis also suggests that S100A4 plays a role in mature adipocyte function. Adipocytes derived from S100A4-deficient, lean mice fed a chow diet exhibit decreased response to insulin compared to wild type mice as shown in glucose uptake and lipolysis assays. It should be noted that the difference in insulin sensitivity and adipocyte function might also be influenced by a potential difference in analyzed adipocyte size, which, however, is difficult to estimate. To date, no connection between S100A4 and insulin sensitivity has been reported. Kriaievska et al. showed by immunoprecipitation analysis that S100A4 directly binds liprin-β1 and suppresses phosphorylation of liprin-β1, potentially by inhibiting recruitment or activity of protein kinases (PKC and CK2) [174]. Liprin-β1 and α-liprins are members of the liprin family of transmembrane tyrosin-phosphatase LAR-interacting proteins. Leukocyte common antigen related (LAR) phosphatase is highly expressed in insulin responsive tissue and was shown to inhibit insulin signaling via dephosphorylation of the insulin receptor [175]. It has been reported that β-liprins do not bind to LAR but form heterodimers with α-liprins, which in turn were shown to bind to the cytosolic membrane domain of LAR and influence its localization and substrate specificity [176, 177]. Hence it was hypothesized that S100A4 might affect LAR phosphatase function by modulating liprin α1-liprin β1 complex formation [174, 177]. However, the underlying mechanism how S100A4 might alter insulin signaling in insulin sensitive tissues remains unclear and is subject to further studies.

S100A4 knockout mice - when fed a chow diet - demonstrated no alterations of body weight and metabolic phenotype. In addition, no abnormalities have been detected in histological analysis of liver, skin, brain, thymus and spleen obtained from 12-24 weeks old S100A4-deficient mice [178].
However, our study shows that S100A4 knockout mice challenged with a high fat diet (HFD) gained significantly more weight than wild type animals due to increased subcutaneous and visceral white adipose tissue expansion. Analysis of metabolic parameters using a metabolic cage system reveals that this weight gain is not caused by hyperphagia or significantly reduced energy expenditure. It is generally recognized that an overabundance of adipose tissue is strongly linked with the development of insulin resistance, type 2 diabetes and the metabolic syndrome [131, 179]. Consistently, S100A4 deficient mice increasingly develop insulin resistance leading to elevated insulin, blood glucose and triacylglyceride levels under HFD regimen. However, S100A4 treatment studies reveal that the positive effect of S100A4 on insulin sensitivity and metabolic parameters is also detectable in absence of a difference in body weight.

Transplantation of S100A4 expressing wild type epididymal fat depots into S100A4 knockout mice (Wt->S) leads to decreased weight gain and improves metabolic parameters during high fat diet regimen. Although a significant difference in body weight is already detectable after 2 weeks on HFD, the effect progressively increases after 8 weeks, reaching a weight difference of 23% after 12 weeks. This lagged effect is likely due to the time needed for vascularization, which is crucial to provide an adequate supply of nutrients and oxygen needed for normal adipocyte function [135]. S100A4-deficient mice with wild type transplants (Wt->S) show already 4 weeks after transplantation a significant improvement in fasting and fed blood glucose levels compared to S100A4 knockout mice with S100A4-deficient transplants (S->S). This effect is significantly enhanced after another 8 weeks and is paralleled by decreased insulin levels. While blood glucose levels of S100A4-deficient mice with wild type transplants (Wt->S) are rescued to wild type levels (Wt->Wt) at 12 weeks post-surgery, insulin levels are still slightly higher compared to wild type levels (Wt->Wt) indicating a lower insulin sensitivity. It is well established that the enhanced insulin secretion in obese, insulin-resistant subjects, is particularly pronounced postprandial and can be explained by compensatory larger functional β-cell mass [180]. This postprandial insulin effect is also seen in refeed S100A4-deficient mice with wild type transplants (Wt->S) suggesting an enhanced compensatory β-cell function in these animals.

There is a growing consensus that S100A4 is secreted to the extracellular space, as the protein is detectable in murine and human plasma and is released into supernatant from tumor cells, macrophages and fibroblasts in vitro [145, 146, 181]. Our S100A4 transplant studies provide further in vivo indication that S100A4 is secreted by epididymal fat tissue. In fact studies in our lab have revealed that S100A4 was detectable in plasma of S100A4 knockout mice with wild type transplants (data not shown). Due to the relatively low abundance of macrophages and other S100A4-releasing
immune cells present in adipose tissue, it is reasonable to assume that S100A4 is secreted mainly by pre-adipocytes and adipocytes. This would also be in line with the mRNA expression profile of S100A4, revealing high expression of S100A4 in adipose tissue. Moreover, the secretion capacity is corroborated by the sufficiency of the two wild type epididymal fat transplants to rescue impaired metabolic parameters of S100A4-deficient mice (after 12 weeks of treatment). It is likely to hypothesize that S100A4 is secreted in an endocrine fashion, thus mediating a systemic insulin sensitizing effect. On the other hand, epididymal fat depots from S100A4-deficient mice did not lead to impairment of metabolic parameters in wild type mice demonstrating that other visceral or subcutaneous fat depots, or other tissues respectively cell types can compensate for the loss.

Also adenovirus-mediated overexpression of S100A4 in epididymal fat depots improves metabolic parameters in overweight, wild type mice fed a HFD. Already after 3 weeks significantly enhanced insulin sensitivity, decreased refed blood glucose and triglyceride levels and a mild effect on weight gain are detectable. In addition, preliminary findings from our lab indicated an improvement of glucose homeostasis upon repeated intraperitoneal injections of S100A4 (500ng/kg body weight) (data not shown). Whether it is also possible to increase S100A4 plasma levels by administering the protein subcutaneously given its small molecule size of 11.5kDa is subject to further studies. In summary, S100A4 reduces weight gain and improves insulin sensitivity in mice fed a high fat diet. The insulin sensitizing effect might be mediated via a dual mechanism: the enhancement of adipogenesis and the stimulation of insulin sensitivity in mature adipocytes, whereas the relatively fast onset of action underlines the importance of the latter. However, further studies will be required to evaluate kinetics, efficacy and safety of S100A4 in long term use and to gain a better understanding of the underlying mode of action.

As S100A4 promotes insulin sensitivity and weight loss, the protein might be an interesting novel compound for the treatment of type 2 diabetes and the metabolic syndrome. For this reason we filed an international patent application for the use of S100A4 for the treatment of insulin resistance as well as the treatment and prevention of type 2 diabetes and the metabolic syndrome (international publication number: WO 2012/095313 A1). Besides the insulin sensitizing effect, the reduction in body weight in particular is a beneficial feature of S100A4. Hence S100A4 might be an interesting novel treatment of the metabolic syndrome, which is often accompanied by type 2 diabetes [13, 182]. To date GLP-1 analogues, which function via stimulation of insulin secretion, are the only anti-diabetic drug class that lead to a modest, but significant weight loss [79]. As S100A4 leads to significant weight reduction and functions as insulin sensitizer, it represents a novel pharmaceutical agent with an interesting therapeutic profile. An unfavorable feature of S100A4 is the fact that it has
to be administered parenterally. In this context it should be commented that GLP-1 agonist, which have to be injected, had global sales of USD 625 Mio in 2010 and their market share is expected to grow in the future [91]. However, the most problematic aspect regarding the development of S100A4 as a pharmaceutical agent is its strong association with metastasis and cancer progression [147]. Expression levels of S100A4 have been negatively associated with patient outcome and survival and even the use of S100A4 as prognostic marker in clinical practice has been suggested [183, 184]. Noteworthy, transgenic mouse models overexpressing S100A4 did not show any phenotypical alterations or increased frequency of neoplastic transformations [146, 185, 186]. In addition, it has been reported that S100A4 knockout mice develop more tumors than wild type mice, which might be due to loss of p53 tumor suppressor activity [178]. Taken together, although there is a general consensus that S100A4 itself does not induce initiation and expansion of the primary tumor, it is widely accepted that it promotes metastasis [144]. The in vivo efficacy data of S100A4 in mice are very promising. But due to the massive safety concerns that would emerge for this anti-diabetic agent, we decided not to pursue the pre-clinical development of S100A4. Nevertheless, S100A4 remains an interesting molecule providing insight into adipocyte function. The mode of action underlying the favorable effects of S100A4 needs to be evaluated further.

THBA
In this study we provide further evidence that the naturally occurring tetrahydroxylated bile acid THBA is a promising new compound for the prevention and treatment of type 2 diabetes and pre-diabetes. Besides the need for novel solutions in diabetes management, treatment of pre-diabetes has come into focus [89]. On the one hand this development is driven by the intention to prevent or delay the onset of type 2 diabetes and on the other hand by the fact that pre-diabetes itself is associated with severe secondary complications such as microvascular diseases [22]. In particular thiazolidinediones have been identified as efficacious pharmacotherapy in pre-diabetes treatment and diabetes prevention, which has been linked to the stimulation of insulin sensitivity [89, 90]. Unfortunately, due to safety concerns, thiazolidinediones have been put under selling constriction leaving an unmet need for insulin sensitizers in diabetes treatment and prevention [93, 140]. Our group has previously shown that THBA acts as an insulin sensitizer and promotes adipogenesis via the suppression of the nuclear receptor RORγ (unpublished data). There is general consensus that white fat tissue expansion by hyperplasia, in contrast to hypertrophy, increases insulin sensitivity of the body [133, 135]. C57BL/6 mice fed a high fat diet supplemented with THBA show no significant difference in weight gain compared to control mice. Hence the observed THBA-mediated increase in adipocyte number and decrease in adipocyte size can be attributed to stimulation of adipocyte differentiation. Further aspects arguing for adipogenesis-mediated improvement of insulin...
sensitivity, are unchanged energy intake and expenditure during THBA treatment as well as the long
time until an effect on glucose homeostasis was detected. The latter effect would be expected to
occur earlier if mediated by increased insulin secretion or suppressed gluconeogenesis.

The dosing study reveals that the highest dose of 0.01% w/w is not more efficacious than 1/3 of this
dose. After 6 weeks of treatment blood glucose levels of mice receiving the medium dose (0.0033% w/w; equivalent to around 2.5mg/kg body weight for a 35g mouse with food consumption of
2.6g/day) are even slightly lower than in the high dose group and after 12 weeks of treatment no
difference in blood glucose levels or insulin sensitivity is observed among these groups. These data
indicate that a daily dose of around 0.1mg (calculated with food intake of 3g/day) either reached the
maximum absorption capacity of THBA or that THBA plasma levels were already sufficient to fully
suppress RORγ. Interestingly, while no significant improvement in glucose homeostasis is detectable
in the low dose group (0.001% w/w) after 6 weeks, almost an equal reduction in blood glucose levels
and improvement in insulin sensitivity is observed after 12 weeks of treatment. This suggests that
THBA might accumulate in the blood with repeated dose. The beneficial blood glucose lowering
effect of THBA is maintained even after end of treatment for a certain period of time in treated mice
while keeping a stable body weight during this period. The effect is not detectable anymore 13 weeks
after treatment end indicating that the missing adipogenic effect of THBA leads to a presumable
restructuring of adipose tissue over time. This is in line with the observation that adipose tissue
undergoes a constant turnover of pre-adipocyte and adipocyte [187, 188]. However, due to the
divergence in weight gain and THBA uptake via food intake, these data can only reveal an
approximate dose-response for THBA in C57BL/6 but provide useful information for future human
clinical trials.

It has been reported that bile acid levels and composition are altered in type 2 diabetes and that
modulation of the bile acid pool might be a therapeutic approach for the treatment of type 2
diabetes and obesity [189]. As naturally occurring bile acid it can be assumed that THBA undergoes
enterohepatic circulation. The size of the circulating enterohepatic bile acid pool amounts to
approximately 4mg in mice and 2-4g in humans with a daily approximate reabsorption of 20mg in
mice and 30g in humans. [190]. Bile acids are reabsorbed by active transport in the terminal ileum as
well as by passive transport in the proximal ileum and the colon. Active transport is mainly mediated
by the apical sodium bile acid transporter (ASBT) as well as by organic solute transporter OStα-Ostβ
and is supposed to amount for the majority of bile acid reabsorption [191, 192]. A CaCo2
permeability assay indicated THBA absorption of around 50% across the human gut wall. However,
the exact transport mechanism of THBA remains unknown and needs to be subject to further
investigations.
THBA naturally occurs in small amounts in human plasma and amounts to approximately 1-3% of the total bile acid pool in adult women [166]. Repeated oral administration of THBA in C57BL/6 mice led to a dose-dependent uptake of THBA and a steady increase in THBA blood levels. This finding is in line with the results from the dosing study which indicated an accumulation of circulating THBA levels. Kinetic studies in C57BL/6 mice reveal a half-life of around 5 days, which is comparable to ursodeoxycholic acid (UCDA), a bile acid that is increasingly used for the treatment of cholestatic liver diseases. UCDA normally accounts for 3% of the total bile acid pool but increases to 40% to 60% at a daily dose of 10-15mg/kg whereby the fraction cannot be increased by a higher dose [193]. Given the similar long term effect of the low dose treatment (0.03mg/day) compared to medium and high dose group it might be feasible to administer a higher dose at the beginning of the treatment followed by a lower maintenance dose.

Depending on the bile acid species a fraction of approximately 10-50% escape hepatic clearance, enters systemic circulation and is filtered by the kidneys. Glomerular filtration is reduced by binding to plasma proteins [190]. Bile acids are also actively reabsorbed from renal proximal tubule cells by ASBT [194]. For UCDA it has been reported that less than 5% of an administered dose are found as conjugates and metabolites in the urine [195]. In contrast, besides fecal excretion, THBA is significantly eliminated via urine, which may be due to low elimination by the liver or low affinity to ASBT. It has been reported that hydroxylation of bile acids and excretion of tetrahydroxylated bile acids via urine is increased in patients with cholestasis. This finding suggested that tetrahydroxylation might be a compensatory alternative pathway for bile acid elimination [196, 197], which might also support increased urinary excretion of THBA.

THBA is taken up in a dose-dependent manner by various tissues but is also efficiently secreted after end of administration. As THBA demonstrates efficient excretion, it may be predicted that extensive accumulation and therefore an increased risk of toxic effects may not occur under long-term treatment with THBA. In line with proposed binding of THBA to RORγ in adipocytes, THBA levels decrease more slowly in adipose tissue after THBA administration was terminated. Microsomal stability assays suggest that minor amounts of THBA are subject to first pass metabolism in the liver. However, to date the pathway of THBA synthesis, its regulation or THBA metabolism have not been thoroughly investigated and will require further analysis. Further studies will also be necessary to obtain a better understanding of the absorption distribution, metabolization and excretion of THBA. Besides additional pharmacokinetic experiments it will also be of interest to assess if THBA is subject to bacterial biotransformation in the gut, which might induce toxic metabolites.
A profound safety assessment is crucial for authorization of a novel food product especially for Foods for Special Medical Purposes [198]. No safety concerns were raised for THBA during the assessment of genotoxicity and cardiotoxicity in vitro or acute and subchronic toxicity in vivo. The maximal tolerated dose of THBA at single oral administration is classified to be higher than the limit dose of 2000mg/kg body weight. The European Food Safety Authority requires that novel foods and novel food ingredients are not allowed to cause toxicity at chronic administration at 100-fold dose of the target dose [199]. No signs of toxicity or clinical impairment are observed for THBA at 100-fold dose during chronic administration. However, additional safety studies will be required to receive novel food approval for THBA. An important study will include testing THBA in a repeated dose 90 days oral toxicity study in rats (according to OECD guideline 408). Due to the positive preliminary safety profile it might be reasonable to test THBA at a limit dose of 1000mg/kg body weight/day in this future study. Further potential studies may include the assessment of THBA with respect to chronic toxicity, reproduction and developmental toxicity and carcinogenicity.

THBA represents a promising compound to control glucose homeostasis in humans as well as in animals. For this reason we submitted an international patent application that protects the use of THBA for the treatment of pre-diabetes as well as the prevention and treatment of type 2 diabetes (WO 2013/041519 A1). Given the natural occurrence in food, good efficacy and excellent safety profile of THBA in pre-clinical testing, we decided to develop THBA into a medical food/ Food for Special Medical Purposes (Appendix 3). Personalized health care nutrition aiming at disease prevention and management represents a new emerging industry located between food and pharma industry. In this food-pharma interphase medical foods have a science driven focus and are positioned close to pharmaceuticals [101, 102]. Due to its pharma-like properties and its intended use in diabetes prevention and management, THBA fits well into the medical food category.

Besides efficacy, tolerability, convenient administration and dosing frequency, low risk for hypoglycemia and positive impact on weight control are important factors for patient compliance and market success of an anti-diabetic drug or medical food [94]. Thiazolidinediones are the only current anti-diabetic drug class that primarily acts by increasing insulin sensitivity. Insulin sensitization has been associated with several beneficial effects on glucose homeostasis, including protection of β-cell function and durable glycemic control. However, due to side effects and adverse events clinical use of thiazolidinediones has declined in recent past (Appendix 4) [140]. Metformin, the first-line drug in diabetes treatment, is often described as an insulin sensitizer, but it primarily functions by suppressing hepatic gluconeogenesis. Insulin sensitization in peripheral tissue is quite small, varies across studies and could not be confirmed in meta-analysis [61]. In addition, novel and
emerging drugs mainly focus on mechanisms which primarily are not aimed at increasing insulin sensitivity [91]. THBA might fill this gap providing a safe and efficacious insulin sensitizer. In addition, unlike thiazolidinediones, which are associated with weight gain, THBA is weight neutral in our pre-clinical animal studies. Given the mode of action and safety profile of TBHA, a combination therapy with current anti-diabetic drugs might be beneficial. In further in vivo studies the synergistic effect and the safety profile of THBA in combination therapy with metformin will be assessed. To date, the beneficial effect of medical foods on glucose homeostasis is mainly mediated by the low glycemic impact of these products. Functional ingredients are associated with various anti-diabetic effects such as insulin sensitization, increased insulin secretion and reduction of intestinal glucose uptake, but they generally lack solid clinical efficacy data [102, 118]. THBA with its pharma like properties and good efficacy might be a highly promising novel anti-diabetic medical food.

THBA is a promising compound for management of type 2 diabetes, and in particular, for treatment of pre-diabetes and prevention of type 2 diabetes. Because of the lack of efficacious agents for pre-diabetes treatment and the favorable risk-benefit balance of THBA, pre-diabetic patients represent an interesting first target group for THBA treatment. In the recent past treatment of pre-diabetes has come into focus due to the high risk of pre-diabetic individuals to develop type 2 diabetes and the observation that pre-diabetes itself is associated with severe secondary complications such as microvascular diseases [22]. Early detection of pre-diabetes and effective interventions are expected to have enormous beneficial socioeconomic impact. Screening of the general population was recommended by Diabetes Associations and has already been implemented within screening programs [89, 200]. According to the American Diabetes Association all adults above 45 years, and individuals of any age, who are overweight (BMI >25 kg/m², BMI>23kg/m² Asian Americans) and have one additional risk factor (e.g. family history of diabetes, physical inactivity and high blood pressure), should be screened for pre-diabetes [9]. Insulin sensitization has been reported to be the most efficacious mode of action in pre-diabetes treatment and in prevention or delay of type 2 diabetes underlining the potential benefit of THBA in this indication [90]. Taken together, these data suggest that THBA is a safe and efficacious insulin sensitizer which primarily acts via stimulation of adipogenesis. As medical food it could be a novel promising approach to combat type 2 diabetes in an emerging health care nutrition industry.
6. Material and methods

Chemicals
All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Cell culture
All cells were cultured in high-glucose (4.5g/l) Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all Invitrogen). 3T3-L1 cells and stromal vascular fraction were plated on collagen-coated plates prior to differentiation. Adipogenesis was induced by treating 2 days post-confluent 3T3-L1 pre-adipocytes or 80% confluent stromal-vascular cells with an induction cocktail (1µg/ml insulin, 1µM dexamethasone, 115µg/ml isobutylmethylxanthine) for 2 days, followed by 2 days insulin (1µg/ml insulin) containing medium and 2 to 3 days of un-supplemented medium. Adipocyte differentiation was measured by quantifying adipocyte fatty acid binding protein (A-FABP).

Western blot and antibodies
Cells and tissue were lysed in hypo- and hypertonic buffer. Protein concentration was determined using the DC protein assay (BioRad). Proteins were separated with 12% SDS-PAGE system (Bio-Rad) and transferred to nitrocellulose membranes using a wet transfer system. Western Blotting was performed according to standard producers and chemoluminescence was measured with LAS 4000 mini ImageQuant system (Ge Healthcare).

The following primary antibodies were used: S100A4 (Abcam, ab27957, 1:100) and γ-tubulin (Sigma Aldrich, 1:6000) as loading control. For detection a horseradish peroxidase coupled mouse (Santa Cruz, 1:5000) and rabbit IgG antibody (Abcam, 1:2000) were used.

Quantitative PCR
mRNA was isolated and reverse transcribed using the Multi-MACS cDNA module (Miltenyi). mRNA levels were quantified by real-time PCR using SYBR Green (Invitrogen) according to the manufacturer’s protocol. Expression levels were normalized to GAPDH or 36B4. Primer sequences are listed below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A4</td>
<td>GAGGCCCTGGAATGTATTGT</td>
<td>TGACACAGTACTCCTGGAAGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTGACGTGGCCGCTGGAGAAA</td>
<td>CCGGCATGGAAGGTGGAAGAGT</td>
</tr>
<tr>
<td>36B4</td>
<td>GCCGTGATGCCCAGGGAAGA</td>
<td>CATCGCTTGGAGCCACGTT</td>
</tr>
</tbody>
</table>
Plasmids
For lentivirus production MISSION shRNAs (Sigma Aldrich) against S100A4 were cloned into the pLKO1 puro vector (addgene). For overexpression S100A4 cDNA was amplified from murine adipocyte cDNA and cloned into the pLKO1cmv vector (addgene). The sequences for shRNA constructs are shown below:
Sh1: forward: CCGGAACCACAAATACTCAGGCAAATCTCGAGTTTGCCTGAGTATTTGTGGTTTTTTTG, reverse: AATTCAAAAAACGATATACCTAAGAAGATCTCGAGTTTGCCTGAGTATTTGTGGTT;
Sh2: forward: CCGGAATCCAGAAGGTGATGAGCAACTCGAGTTGCTCATACCTCTCGAGTTTTTTTG, reverse: AATTCAAAAATCCAGAAGGTGATGAGCAACTCGAGTTGCTCATACCTCTCGAGTTTTTTTG

Virus production and transfection
For lentivirus preparation HEK293T cells were transfected using Lipofectamine 2000 with the overexpression, shRNA containing or control vector, and the packaging vectors pMD2.G and psPAX2. 24h after transfection medium was changed to high BSA (11mg/ml), DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. Virus containing supernatant was harvested after 24h on high BSA medium. For functional assays 3T3-L1 cells were infected for 24h with lentivirus containing medium supplemented with polybrene (8µg/ml) 3 days before induction of differentiation. Differentiation was continued until day 8. Purified S100A4 adenovirus (VQAd S100A4-GFP, here referred to as Ad-S100A4) and control GFP adenovirus (Ad-GFP) were purchased from ViraQuest, Inc.

Glucose uptake and lipolysis
Glucose uptake and lipolysis was measured as described previously [201]. Briefly, to measure glucose uptake, primary adipocytes were pre-incubated with 0-20nM insulin for 10min. Cells were incubated for 1h with 1mM glucose spiked with D-[1-14C]glucose in Krebs-Ringer buffer supplemented with 1% BSA at 37°C. Primary adipocytes were spun through dinonylphthalate oil. The oil phase with adipocytes and the aqueous phase were transferred. Cells were washed, lysed and intracellular radioactivity and total protein content was measured with a BCA Assay (Sigma). Osmium tetroxide fixed adipocytes were counted for normalization using a Coulter Cell Counter. To assess lipolysis, glycerol release was measured in the aqueous phase using glycerol reagent (Sigma) according to the manufacturer’s protocol.

SVF and adipocyte isolation
Visceral (epididymal) and subcutaneous (inguinal) adipose tissue were minced and incubated in 0.2% collagenase type II buffer at 37°C for 1h. (25mM KHCO3, 12mM KH2PO4, 1.2mM MgSO4, 4.8mM KCl,
120mM NaCl, 1.4mM CaCl₂, 5 mM glucose, 2.5% BSA, 1% Pen/Ste, pH 7.4). The collagenase digest was resuspended in an equal volume of regular growth medium (DMEM, 10% FBS, 1% Pen/Strep) and centrifuged 10min at 1000rpm. The adipocyte fraction was removed from the top, filtered through 100μm cell strainers (BD), centrifuged for 1min at 1000rpm and resuspended in regular growth medium. The stromal vascular fraction containing pellet from the initial centrifugation was filtered through 40μm cell strainers and centrifuged for 4min at 1000 rpm. The SVF was incubated in erythrocyte lysis buffer (154mM NH₄Cl, 10mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 5 minutes, centrifuged at 1000rpm for 4min and resuspended in regular growth medium.

**Adipocyte and fat pad size**
Visceral (epididymal) and subcutaneous (inguinal) adipose tissue was fixated in 5% paraformaldehyde solution for 24h at RT before paraffin embedding and sectioning to 10μm thin slices. Sections were stained with haematoxylin and eosin. Microscopic pictures were taken and cell size was analyzed using Cell Profiler software (Massachusetts Institute of Technology). Per animal a minimum of 10,000 adipocytes were measured to determine adipocyte size. To quantify the fat depots size, mice were anesthetized with isoflurane (induction: 4-5% isoflurane, maintenance: 1-3%) and placed in an animal CT-scanner (LaTheta).

**Animals**
CS7BL/6N wild type and ob/ob mice were obtained from Charles River. All wild type and transgenic S100A4 knockout mice were kindly provided by Noona Ambartsumian (Department of Molecular Cancer Biology, Danish Cancer Society, Copenhagen). Animals were housed on an inverted 12h light cycle with free access to water and food. To induce obesity mice were fed a high fat diet (Provimi Kliba AG) containing 60% kcal from fat. For tissue sampling mice were euthanized with carbon dioxide. Animal studies were approved by national and institutional guidelines.

Metabolic parameters such as food and water intake, locomotor activity, oxygen consumption (VO2), carbon dioxide production (VCO2), and respiratory exchange ratio (VCO2/VO2) were measured using an Oxymax metabolic cage system (Columbus Instruments). A respiratory exchange ratio (RER) of 1 indicated the exclusive use of carbohydrates, a RER of 0.707 the exclusive use of lipids as fuel source for catabolic metabolism. To quantify the energy expenditure at rest only time intervals (10min) with less than 15 movements (along X-axis) were measured. Movements along the X axis were only counted when a series of motion sensors were activated to exclude stereotype such as grooming, scratching, etc. Mice were transferred 24h prior to measurement to the metabolic cage for acclimatization.
Glucose metabolism analysis

To perform an insulin tolerance test (ITT) mice were injected intraperitoneally with insulin (0.75 Units/kg body weight, Actrapid, Novo Nordisk) containing PBS (100 µl/10 g mouse). For an oral glucose tolerance test (OGTT) mice were gavaged with a glucose solution (2 g/kg body weight, 100 µl/10 g mouse). Prior (T0) and 15, 30, 60, 90 and 120 min after administration of insulin or glucose respectively, blood glucose levels of blood received from the tail vein were measured (Contour, Bayer).

Fat pad transplantation

6-week-old male wild type mice and S100A4 deficient mice were used as recipients and donors of epididymal fat depots. Both epididymal fat pads of wild type mice were transplanted in S100A4 deficient mice, and vice versa. To control these cross transplants, the epididymal fat pads were also transplanted within the respective mouse model. To remove the epididymal fat pads the intra-abdominal cavity was opened by midline laparotomy (incision length 0.5-1 cm, ending approx. 1 cm rostral to the urethral orifice) and the fat pads were carefully pulled out of the intra-abdominal perigonadal area. The proximal end of the fat depots was ligated (Vicryl 6-0) and the pads were separated distal from the ligation. The epididymal fat pads were transplanted in the intra-abdominal cavity of the recipient mice and fixed with 2-3 stitches using absorbable suturing material (Vicryl 6-0, Ethicon). The transplants were positioned at the peritoneum parietale close to their physiological position (approx. 1 cm lateral to the center of the midline laparotomy). The abdominal wall was sutured in two layers (peritoneum parietale, skin), using absorbable suturing material (Vicryl 6-0, Ethicon). Anaesthesia was induced with 4-5% and maintained at 1-3% isoflurane. For analgesia mice received Meloxicam (5 mg/kg, sc) 30 min prior to surgery and post-surgery Meloxicam via drinking water (1.7 µg/ml) for 3-4 days. As prophylaxis against infections, a broad spectrum antibiotic (Trimethoprim 5 mg/kg and Sulfadoxin 20 mg/kg) was administered 3-6 h prior and 2 days post-surgery. After surgery mice were maintained on a high fat diet and a standard 12 h light/dark cycle.

Adenoviral injection into fat pad

S100A4-GFP adenovirus was injected (40 µl, 4.0 x 10^{10} PFU/ml per fat pad) into the two epididymal fat depots of 9-10-weeks-old S100A4 knockout and C57BL/6 wild type mice, being fed a high fat diet for 6 weeks. As control another cohort was injected with GFP adenovirus (40 µl, 4.0 x 10^{10} PFU/ml per fat pad). To place the injections exclusively into the epididymal fat pads, the intra-abdominal cavity was opened by midline laparotomy (incision length 0.5-1 cm, ending approx. 1 cm rostral to the urethral orifice) and the epididymal fat depots were carefully pulled out of the intra-abdominal perigonadal area. Adenovirus was slowly injected into the epididymal fat depots using a 30G needle (BD
Diabetes). The fat pads were carefully repositioned into the intra-abdominal perigonadal area and the abdominal wall was sutured in two layers (peritoneum parietale, skin), using absorbable suturing material (Vicryl 6-0, Ethicon). Anaesthesia was induced with 4-5% and maintained at 1-3% isoflurane. For analgesia mice received Meloxicam (5mg/kg, sc) 30min prior to surgery and post-surgery Meloxicam via drinking water (1.7 µg/ml) for 3-4 days. As prophylaxis against infections a broad spectrum antibiotic (Trimethoprim 5mg/kg and Sulfadoxin 20mg/kg) was administered 3-6h prior and 2 days post-surgery. After surgery animals were maintained on a high fat diet and a standard 12h light/dark cycle.

Serum measurements
Blood glucose was measured with a glycometer (Contour, Bayer). Plasma insulin levels were determined using the Rat/Mouse Insulin ELISA kit (Crystal Chem). Free fatty acids were measured with the NEAFA-HR kit (Wako, Germany), Triglycerides were quantified with the triglyceride/GB kit and cholesterols with the Chol kit (both Roche, Switzerland) according to the manufacturer’s protocol.

Chemical and metabolic stability assay
THBA was tested in a chemical stability assay that was combined with an assessment of non-specific binding to polypropylene. Chemical stability was tested in hydrochloric acid buffer at pH 2 at 37°C, over a period of 2h. For analysis samples were taken immediately after mixing (T0) and after 5, 15, 30, 45 and 120min. For testing non-specific binding samples were removed at 0 and 120min. Chemical degradation was stopped by adding 2 volumes of methanol and TBHA was quantified by LC-MS/MS. For chemical stability the percentage of THBA relative to the 0min sample and for non-specific binding the percentage of THBA bound to polypropylene was determined.

The metabolic stability of THBA was evaluated in a microsomal stability assay. Briefly, liver microsomes from human and rat origin were incubated with 1µM THBA at 37°C in the presence of the co-factor, NADPH. For analysis five time points (0, 5, 15, 30 and 45min) were taken. To discern any chemical instability or non-NADPH dependent enzymatic degradation of THBA a control incubation with phosphate butter (pH 7.4) instead of NADPH was included. Dextromethorphan and Verapamil were used as positive controls for human liver microsomes and Diazepam and Diphenhydramine as positive control for rat liver microsomes. The reactions were stopped by the addition of methanol. Following protein precipitation, samples were analyzed by LC-MS/MS for the presence of THBA. As read-out an intrinsic clearance value (CLint) in µL/min/mg protein, indicating
the turnover rate and were determined. (Both studies were performed in collaboration with Cyprotex Discovery Ltd.)

CaCo-2 cell permeability
To predict the absorption of THBA across the human gut wall a Caco-2 permeability assay was performed and the permeability coefficient (Papp) of THBA was measured. Two reference compounds, atenolol (paracellular transport) with a known absorption of 50% and propranolol (passive transcellular transport) with a known absorption of 90%, were screened alongside THBA (The study was performed in collaboration with Cyprotex Discovery Ltd.).

Kinetic studies
C57BL/6 wild type mice were fed a high fat diet for 4 weeks starting at 7 weeks of age to induce insulin resistance. At 11 weeks of age mice were divided in two groups (n=25-35 each) and gavaged with 100µl coffee cream/water (1:2) solution supplemented with 0.3 or 0.03mg of spiked THBA (³H-labeled THBA) for 5 consecutive days (T0= first gavage). The tritium label was positioned at THBA so that it could not be cleaved off by common P450 enzymes. Radioactivity was measured in whole blood, urine and feces samples at the following time points: 11h, 1 day, 2 days, 4 days following the first gavage and on days 1, 3, 6, 9, 16 and 28 after the last gavage. Feces samples were dissolved in NaOH supplemented with 2.5% Triton at 70°C by vortexing at 1000rpm for 1.5h.

To determine THBA uptake and elimination in various tissues (kidney, liver, muscle, brain, spleen, brown fat, subcutaneous fat, visceral fat) mice were sacrificed 1 day, 2 weeks and 4 weeks after the last day of THBA administration. Prior to tissue sampling, mice were perfused with 20ml of PBS supplemented with 1mM EDTA. Tissue samples were dissolved in NaOH added with 2.5% Triton at 70°C by vortexing at 1000rpm for 45min and THBA concentration was normalized to tissue weight. Whole blood (3µl), urine (20µl), feces homogenate (80µl) and tissue homogenate were transferred to 4 ml of scintillation liquid and incubated for 12 h before measurement. The concentration of THBA was calculated based on the amount of ³H-THBA in the solution that was orally administered.

In-vitro toxicity studies
To assess the genotoxic potential of THBA an AMES test as well as a micronucleus test were performed based on OECD guidelines. Briefly, the Ames mutagenicity potential of THBA was tested in Salmonella typhimurium TA98 (hisD3052, rfa, uvrB / pKM101) to detect frame-shift mutations and S. typhimurium TA100 (hisG45, rfa, uvrB / pKM101) to detect base-pair substitutions. The following test concentrations of THBA were used: 0.063, 0.125, 0.25, 0.5, 1 and 2mg/ml. The in vitro micronucleus test (OECD Guidelines for the Testing of Chemicals Section 4, Test No. 487) was used to assess
cytotoxicity by quantification of micronuclei. CHO-K1 cells derived from Chinese hamster ovaries were incubated with THBA over a ten point concentration range (0.02, 0.05, 0.2, 0.5, 2.0, 5.0, 20, 50, 200, 500µM) in duplicates to determine the potential of THBA to induce micronuclei in the absence as well as in the presence of metabolic activation with Aroclor-1254 induced rat liver S9 fractions. The potential of THBA to cause cardiotoxic effects was evaluated by analyzing its inhibition of the human ether-a-go-go related gene (hERG), encoding a potassium ion channel that contributes to the electrical stimulus conduction of the heart. THBA was tested at concentrations of 0.008, 0.04, 0.2, 1, 5, and 25µM with 4 replicates per condition (The studies were performed in collaboration with Cyprotex Discovery Ltd.).

**Escalating toxicity study**

Female C57BL/6 wild type mice were gavaged with THBA dissolved in sun flower oil (100µl) 6 days per week (Monday-Saturday) for 4 to 8 weeks starting at 8 weeks of age. The following doses of THBA were administered: weeks 1-4 (3mg), week 5 (3.75mg), week 6 (4.5mg), week 7 (5.25mg), week 8 (6mg). These dosages correspond to 150mg THBA/kg to 260mg THBA/kg body weight. Un-supplemented sun flower oil served as control. During the study animals were closely monitored for signs of impairment or clinical disease. Following parameters were monitored: body weight, food and water consumption, respiratory pattern, gait, posture, fur (e.g. pilorection), eyes & nose (e.g. secretions, lacrimations) and behavior (e.g. excessive grooming, self-mutilation, response to handling, clonic or tonic movements). Mice were sacrificed after 4 and 8 weeks for histopathological examination of liver, kidney and spleen. Tissue samples were fixated in 5% paraformaldehyde solution for 24h at RT before paraffin embedding and sectioning.

**Acute oral toxicity**

An acute toxicity study was performed according to OECD guideline 423. Briefly, eight weeks old female Wistar rats received a single oral administration of THBA dissolved in corn oil at the limit dose of 2000mg/kg body weight and were closely monitored for signs of toxicity. After 2 weeks rats were sacrificed and a gross necropsy as well as a histopathological analysis of the liver and the kidneys (including adrenals) was performed (The study was performed in collaboration with ETAP, France).

**Statistical analysis**

Results are presented as Mean ± SD or SEM. Statistical analyses were performed using a 2-tailed Student’s t-test or parametric analysis of variance (ANOVA) followed by Fisher’s LSD post-hoc comparisons whenever appropriate. A statistical significance of *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 was used.
7. Conclusion

The increasing global epidemic of diabetes and pre-diabetes, requires novel strategies to halt this trend. To date, 14 drug classes are available to treat type 2 diabetes, but the majority of diabetic patients do not achieve glycemic control [94]. In addition, targeting pre-diabetes has been recognized to be key in preventing secondary complications and reversing the diabetes epidemic. However, current guidelines mainly focus on life-style interventions, which are mostly not crowned with long-term success [22, 90]. Therefore, new treatment approaches for pre-diabetes and type 2 diabetes are urgently needed. After thiazolidinediones have recently fallen into disuse due to safety concerns, there is in particular a demand for safe and efficacious insulin sensitizers.

This thesis focused on the identification and pre-clinical development of novel compounds for the treatment of pre-diabetes and the prevention and treatment of type 2 diabetes. S100A4 protein led to a significant improvement in insulin sensitivity, body weight and metabolic parameters in several in vivo models. S100A4 secretion by wild type epididymal fat transplants was sufficient to rescue the impaired metabolic parameters of S100A4 knockout mice. The insulin sensitizing effect of S100A4 seemed to be at least partially mediated by enhanced adipogenesis and increased insulin sensitivity of mature adipocytes. However, exact mechanisms and kinetics of S100A4 remain elusive and will be subject to future studies. As S100A4 acted as insulin sensitizer and led to weight reduction it represents an interesting agent for the treatment of type 2 diabetes and the metabolic syndrome. But the association of S100A4 with metastasis and cancer progression represents a major obstacle for the development as a drug. Nevertheless, detailed understanding of the mechanisms, by which S100A4 causes insulin sensitization and in particular weight loss, might be very helpful for the development of future anti-diabetic agents.

THBA, the endogenous ligand of RORγ, strongly increased insulin sensitivity and glucose homoestasis by potentially stimulating adipogenesis in obese mice. In contrast to thiazolidinediones, which have been described to enhance adipocyte differentiation and mature adipocyte function, THBA did not cause weight gain. The effect on glucose homeostasis of the low-dose treatment (approximately 0.01mg/day) was lower compared to the medium- and high-dose treatment in the intermediate evaluation after 6 weeks but almost equal after 12 weeks of treatment. This might be due to the half-life of THBA of around 5 days and dose-dependent accumulation of THBA after repeated daily administration. Hence it might be feasible to consider a higher dose at the beginning of treatment and a lower maintenance dose in THBA treatment. THBA raised no safety concerns during assessment of genotoxicity and cardiotoxicity in vitro and acute and chronic toxicity in vivo. Because
of its natural occurrence in food and its favourable risk-benefit profile, we decided to develop THBA as a medical food/Food for Special Medical Purposes. Further safety studies, such as long term toxicity studies, will be required before THBA can enter planned clinical trials in 2016 and the application for regulatory novel food approval can be submitted. Because of the lack of efficacious and safe therapeutic agents for pre-diabetes treatment and diabetes prevention, pre-diabetic patients represent an interesting first target group for THBA. In addition, due to the mode of action of THBA and its safety profile, a combination therapy with current anti-diabetic drugs, in particular metformin seems feasible and will be evaluated in future studies. In 2012, the American Diabetes Association and the European Association for the Study of Diabetes released a statement, underlining the importance of patient centered care in diabetes treatment [59]. In addition, market research has reported an increasing consumer demand in natural products and alternatives to drugs in diabetes treatment. This development as well as the increasing regulatory hurdles for drug development in diabetes, are expected to drive the demand for anti-diabetic medical food products [91]. Hence, THBA might have an interesting profile from a medical as well as a socioeconomic perspective, and represents a highly promising novel solution to combat the type 2 diabetes epidemic.
8. Appendix

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>0.1% THBA</th>
<th>0.1% Cholic acid</th>
<th>High Fat Diet</th>
<th>Chow Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>1.53 ± 0.59</td>
<td>1.48 ± 0.5</td>
<td>1.91 ± 0.48*</td>
<td>1.29 ± 0.24</td>
</tr>
<tr>
<td>IL-10</td>
<td>22.2 ± 7.8</td>
<td>26.1 ± 11.0</td>
<td>33.5 ± 6.7</td>
<td>29.7 ± 5.5</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>103.6 ± 27.2</td>
<td>89.5 ± 40.0</td>
<td>99.1 ± 16.6</td>
<td>84.7 ± 19.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>6.66 ± 2.41</td>
<td>6.03 ± 2.45</td>
<td>7.34 ± 1.03</td>
<td>7.30 ± 1.24</td>
</tr>
<tr>
<td>IL-6</td>
<td>24.71 ± 8.73</td>
<td>29.27 ± 16.17</td>
<td>43.04 ± 19.17</td>
<td>26.42 ± 6.22</td>
</tr>
<tr>
<td>KC</td>
<td>61.01 ± 13.89</td>
<td>50.14 ± 6.35</td>
<td>49.98 ± 14.18</td>
<td>55.66 ± 18.09</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.89 ± 1.26</td>
<td>2.72 ± 0.91</td>
<td>2.56 ± 1.69</td>
<td>2.56 ± 0.51</td>
</tr>
</tbody>
</table>

**Appendix Table 1 Plasma Levels of Inflammatory cytokines.** Levels of the indicated inflammatory cytokines were measured in plasma taken from C57BL/6 mice being fed a 60% high fat diet for six weeks starting at four weeks of age. The diet was given un-supplemented or admixed with 0.1% of THBA or cholic acid as bile acid control. Chow diet fed mice served as control. The asterisks indicate significance with * representing p<0.05.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Skin</th>
<th>Fur</th>
<th>Eyes &amp; Nose</th>
<th>Respiratory pattern</th>
<th>Gait</th>
<th>Posture</th>
<th>Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Gross Necropsy</td>
<td>Liver</td>
<td>Kidney</td>
<td>Spleen</td>
<td>Brain</td>
<td>Uterus &amp; ovaries</td>
<td>Thymus</td>
<td>Heart</td>
</tr>
<tr>
<td></td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Liver</td>
<td>Kidney</td>
<td>Spleen</td>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Appendix Table 2 Evaluation of toxicity chronic toxicity study.** THBA was administered daily to female C57BL/6 mice (6 days per week) for 8 weeks starting at 8 weeks of age. Mice were closely monitored for sings of impairment or clinical disease. Mice were sacrificed after eight weeks for gross necropsy or histopathological examination. Nothing abnormal detected (NAD).
## Appendix Table 3 Summary of key results regarding efficacy, kinetic and safety of THBA

<table>
<thead>
<tr>
<th>Category</th>
<th>Study</th>
<th>THBA</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physico-chemical properties</td>
<td>Permeability – CaCo-2</td>
<td>$P_{app}$: $0.38 \pm 0.09 \times 10^{-6}$ cm s⁻¹</td>
<td>&gt; 50 % uptake</td>
</tr>
<tr>
<td></td>
<td>Chemical stability at pH=2, 37°C</td>
<td>94.6 % after 120 min</td>
<td>Considered stable via oral route</td>
</tr>
<tr>
<td>Efficacy</td>
<td>Prevention of Insulin Resistance</td>
<td>Prevents blood glucose increase</td>
<td>Dose dependent effect</td>
</tr>
<tr>
<td></td>
<td>Reversion of existing Insulin R.</td>
<td>Lowers elevated blood glucose</td>
<td></td>
</tr>
<tr>
<td>Kinetic</td>
<td>Half-life in plasma</td>
<td>≈5 days</td>
<td>Excreted via faeces &amp; urine</td>
</tr>
<tr>
<td></td>
<td>Tissue distribution</td>
<td>Mostly found in metabolically active tissues</td>
<td>Taken up and secreted from various tissues</td>
</tr>
<tr>
<td>Safety</td>
<td>Genotoxicity - Ames</td>
<td>Negative</td>
<td>No safety concerns raised</td>
</tr>
<tr>
<td></td>
<td>Genotoxicity - MNT</td>
<td>Negative</td>
<td>No safety concerns raised</td>
</tr>
<tr>
<td></td>
<td>Cardiotoxicity - hERG</td>
<td>No inhibition</td>
<td>No safety concerns raised</td>
</tr>
<tr>
<td></td>
<td>Inflammatory markers</td>
<td>Normal levels</td>
<td>No safety concerns raised</td>
</tr>
<tr>
<td></td>
<td>Stability in microsomes</td>
<td>$CL_{int} = 1.51$ (µL/min/mg prot.)</td>
<td>Low turn-over in human</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t_{1/2} = 918$ minutes</td>
<td>Low turn-over in rat</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$CL_{int} = 1.06$ (µL/min/mg prot.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t_{1/2} = 1310$ minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acute Toxicity</td>
<td>No adverse effects</td>
<td>2000mg/kg (limit dose)</td>
</tr>
<tr>
<td></td>
<td>Escalating Toxicity study</td>
<td>No adverse effects</td>
<td>150-260mg/kg</td>
</tr>
</tbody>
</table>

Appendix Table 4 Thiazolidinedione prescriptions filled in US retail pharmacies, 2003-2012.

*Modified from Hampp et al. [92].*
9. References


91. The Diabetes Market Outlook to 2016. 2011, Business Insight.


Acknowledgements

To begin with, I’d like to thank Christian Wolfrum for giving me the opportunity to work on this project. His enthusiasm and dedication to science is remarkable and he was always accessible to discuss scientific questions.

Many thanks to all the past and current members of the Wolfrum group who have made the lab a great place to work. Thanks for the interesting scientific and non-scientific discussions and the great time we had within and outside the lab. Thanks to Matthias for keeping the lab running and to Elke for her help during tissue processing.

A special thanks to Myrtha for the good team work, in particular during endless surgery and glucose clamp sessions, to Suse for her great work with coordinating external studies, as well as to Daria for her constant support.

I would also like to thank our animal caretakers, Many, Michèle and Simone for their help during animal work. Thanks to the technical services team, Sylvia and Marcel.

One of the biggest thanks goes to Roland for his endless patience and constant support at all times.