universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Natural products to fight insulin resistance and hyperglycemia – establishment and application of relevant *in vitro* and cell-based screening models“

Verfasserin

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Abstract

This diploma thesis deals with two different approaches to examine and ameliorate existing treatments for diabetes mellitus and its concomitant afflictions.

On one hand I investigated the possibility to alleviate diabetes mellitus and its accompanying symptoms like insulin resistance and metabolic syndrome with the aid of natural products on cellular level by direct intervention with the insulin signaling pathway. Therefore I used ethanol extracts and pure compounds of different plants, predominantly of *Morus alba*, the white mulberry tree. This special plant has been used in Chinese medicine since ancient times because of its hypoglycaemic, antibacterial and astringent effects.

Over the course of this study I tried to inhibit the protein tyrosine phosphatase 1B (PTP1B), which is a major negative regulator of insulin signaling. The inhibition of this enzyme is thought to alleviate insulin resistance and increase the sensitivity and capacity of the insulinreceptor for its substrate, thereby lowering the glucose level in the blood.

To achieve this goal I performed colorimetric enzyme inhibition assays with ethanol extracts and isolated constituents of *Morus alba.* The most promising hits were further investigated in cell based assays with hepatocytes to determine whether they were able to block PTP1B activity in living cells.

On the other hand I tried to establish a test system for measuring the glucose uptake in adipocytes with a fluorescence-labelled glucose in 96-well plates. So far the glucose uptake assay is performed in 24-well plates with a radiotracer in the lab. A switch to the fluorescence based assay would increase efficiency of glucose uptake measurements because in 96 wells more putative insulin sensitizers

could be tested at the same time and the non-radioactive assay constitutes a safer and environmentally friendly method.

Zusammenfassung

Diese Diplomarbeit beschäftigt sich mit zwei unterschiedlichen Herangehensweisen um neue Ansätze für bestehende Diabetes Mellitus Therapien zu untersuchen und diese möglicherweise zu verbessern.

Zum einen wurde die Möglichkeit untersucht, Diabetes Mellitus und deren Begleitsymptome wie Insulinresistenz und das metabolische Syndrom mit pflanzlichen Stoffen durch Eingriffe auf zellulärer Ebene in den Insulin Signalweg zu mildern. Dafür wurden Extrakte und Verbindungen verschiedener Pflanzen, vorwiegend von *Morus Alba*, dem weißen Maulbeerbaum ausgewählt, da diese Pflanze in der chinesischen Medizin schon seit dem Altertum verwendet wird und die hypoglycaemisch aber auch antibakteriell und adstringierend wirken soll.

Im Zuge der Untersuchung sollte die Protein-Tyrosin-Phosphatase PTB1B, die ein wichtiger Negativregulator des Insulin Signalsystems ist, inhibiert werden und so die Insulinresistenz abgeschwächt und die Sensitivität und Aufnahmefähigkeit des Insulinrezeptors für sein Substrat erhöht werden.

Dazu wurden colorimetrische Enzymtests durchgeführt, um zu klären, ob die *Morus Alba*-Extrakte und compounds den gewünschten Effekt zeigen und das Enzym inhibieren. In weiterer Folge wurden zellbasierte Tests mit Leberzellen (Hepatocellular carcinoma) durchgeführt, um die PTP1B Aktivität in lebenden Zellen zu untersuchen.

Zum anderen wurde ein Testsystem etabliert um die Glucoseaufnahme in Adipozyten auf 96-well-Platten mit einem fluoreszenz-markierten Glucosederivat zu testen, statt lediglich auf 24-well-Platten und radioaktiv wie bisher. Diese Alternative mit einem Fluoreszenz-basierten Assay im 96 well Format könnte die Effizienz der Messung der Glucose Aufnahme steigern, da somit mehr

Stoffe in kürzerer Zeit auf ihre Fähigkeit die Glucose Aufnahme zu erhöhen. Ausserdem ist der nicht-radioaktive Assay umweltschonender und sicherer für den Experimentator.

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1 Background

1.1 General

The expansion of type 2 diabetes (T2DM) and impaired glucose tolerance is one of the main causes of disease and mortality worldwide. The cells of muscle, adipose and liver tissue become less responsive or even resistant to insulin in the course of both disorders. This can lead to other common health problems such as obesity and/or the metabolic syndrome [1]. Although it is still unclear where the development of T2DM originates, one early deficiency in the pathogenesis of type 2 diabetes is determined as defective insulin action in skeletal muscle [10].

The insulin signaling system involves a complex network of pathways, activated by the insulin receptor, which regulates intermediary metabolism and its organization in cells. Recent studies have shown that diverse other enzymes and signaling events play a part in insulin action, and are crucial in type 2 diabetes [1].

PTP1B (Protein Tyrosine Phosphatase 1B) has been found to be a negative regulator of insulin signaling. It attenuates the insulin pathway by dephosphorylating the insulin receptor (IR). Inhibiting the phosphatase would be expected to prolong insulin signaling and thereby facilitate glucose uptake and thus result in a lowering of blood sugar [2].

1.1.1 Type II Diabetes mellitus

After food intake the blood glucose levels rise. When this is sensed by the body, insulin is secreted by the pancreatic beta-cells and binds to insulin receptors (IR) which are present on every cell. This initiates the insulin signaling pathway which results in glucose uptake into the cell hence decrease of blood glucose levels. Insulin sensitive cells can get less and less sensitive to insulin which can lead to a status called insulin resistance. The term Diabetes mellitus, or simply diabetes, comprises a group of metabolic diseases which all have high blood sugar in common. As determined by the Austrian Diabetes Association (ÖDG) diabetes is diagnosed when fasting glucose is ≥ 126 mg/dL or non-fasting glucose is ≥ 200 mg/dL on two following days.

There are two main types of diabetes:

Type I diabetes mellitus or insulin dependent diabetes mellitus results from the body´s disability to produce insulin. Patients suffering from this kind of diabetes are obliged to inject insulin or to wear an insulin pump.

Type II diabetes mellitus or non-insulin dependent diabetes mellitus is characterized by hyperglycemia caused by insulin resistance and relative insulin deficiency and makes up about 90% of all diabetes cases.

According to the international diabetes federation (IDF) the rate of type 2 diabetes cases has increased markedly over the last 50 years. As of 2010 there are approximately 285 million people with the disease compared to around 30 million in 1985. As a possible first line therapy regular exercise and a diet consisting of 40% to 50% complex carbohydrates, 10% to 20% protein and monounsaturated fats such as olive oil is recommended. If non-pharmacologic therapy does not work, an oral antidiabetic is needed. To date the existing medication comprises sulfonylureas, which work by stimulating insulin secretion, metformin, which inhibits excessive hepatic glucose

production, acarbose, which delays the absorption of carbohydrates in the gut and thioazolidinediones like pioglitazone and rosiglitazone, which reduces insulin resistance primarily in skeletal muscle [3].

1.1.2 Metabolic Syndrome

In 1988, Reaven [5] observed that several risk factors, like hypertension, dislipidemia and hyperglycemia tend to cluster together and he defined this clustering as syndrome X. Also he identified it as a multiplex risk factor for CVD and assumed that insulin resistance underlies syndrome X [6].

The term “metabolic syndrome” has been used over the past few years to describe the symptoms of increased risk for cardiovascular disease (CVD) and type 2 diabetes. The metabolic syndrome consists of a clustering of risk factors, above all abdominal obesity, hyperglycemia, low HDL, hypertriglyceridemia and hypertension. The causes of this syndrome are still unclear but insulin resistance seems to be closely connected with it. Regrettably there is no clear definition or statement of diagnostic criteria for the metabolic syndrome, yet. But the syndrome plays an important role in identifying individuals at an increased risk for of T2DM and CVD, thus several large organizations have come up with formulations of the diagnostic criteria.

The World Health Organization (WHO) made the first definition in 1998 which is premised on the importance of insulin resistance, as well as two other criteria (Table 1). The most widely used definition is the Third Report of the National Cholesterol Education Program´s Adult Treatment Panel (ATP III) which focuses on CVD risk and requires three of five criteria. At last, the International Diabetes Federation (IDF) created a practical definition that helps identify people worldwide hit by high risk for diabetes and CVD [4].

WHO	ATP III	IDF
Required: Diabetes, impaired fasting glucose, impaired glucose tolerance OR insulin resistance (assessed by clamp studies)		**Required:** Waist circumference, ethnicity specific
AND at least two of the following:	**ANY three of the following:**	**AND at least two of the following:**
Waist-to-hip ratio >0.90 in men and >0.85 in women	Waist circumference >102 cm in men and >88 cm in women	
Serum triglycerides > 150 mg/dL (1.7 mmol/l) OR HDL cholesterol <0.9 mmol/l in men and <1.0 mmol/l in women	Serum triglycerides >150 mg/dL (1.7 mmol/L)	Raised triglycerides >150 mg/dL (1.7 mmol/L) OR specific treatment for this lipid abnormality
Blood pressure > 140/90 mmHg	Blood pressure >130/85 mmHg	Raised blood pressure: systolic >130 mmHg OR diastolic >85 mmHg OR treatment of previously diagnosed hypertension
Urinary albumin excretion rate >20 µg/min or albumin-to-creatinine ratio >30 mg/g	HDL cholesterol <40 mg/dL (1.03 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women	Reduced HDL cholesterol <40 mg/dL (1.03 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women OR specific treatment for this lipid abnormality
	Serum glucose >110 mg/L (6.1 mmol/L) (>100 mg/dL or 5.6 mmol/L may be applicable)	Fasting plasma glucose >100 mg/dL (5.6 mmol/L) OR previously diagnosed Type 2 Diabetes

Table 1: definitions of the metabolic syndrome [4]

1.1.3 Insulin signaling & Glucose

Insulin is the principal regulatory hormone involved in the regulation of fuel metabolism. It is secreted by the β cells of the pancreas in response to blood glucose levels. The primary function of insulin is to maintain glucose homeostasis, which is achieved via the insulin dependent translocation of the glucose transporter, GLUT4, from intracellular vesicles to the cell surface. It acutely promotes glucose uptake in muscle and adipose tissue, while suppressing hepatic glucose production. Practically all cell types are responsive to insulin, although the term 'insulin sensitive tissue' often refers to liver, muscle and adipose tissue.

At a molecular level, a postreceptor defect of insulin signaling is thought to underlie the basis of insulin resistance in type II diabetes. The insulin signaling pathway, once activated, regulates many events involving glucose transport and metabolism, protein synthesis, and gene expression. Several phosphorylation events mediate the insulin signal transduction and link the initial activation of the insulin receptor (IR) tyrosine kinase to downstream substrates [10].

1.1.3.1 The insulin receptor

The insulin receptor is indispensable to mediate insulin action. It is present in almost all vertebrate tissues. The concentration varies from as few as 40 receptors on circulating erythrocytes to up to more than 200 000 receptors on adipocytes and hepatocytes [11]. Like the receptors for other protein hormones, the receptor for insulin is embedded in the plasma membrane. The insulin receptor is a glycoprotein composed of two alpha subunits and two beta subunits. Both types of subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of 4 basic amino acids . The alpha chains are entirely extracellular and contain insulin

binding domains, the intracellular section of the β-subunit contains the tyrosine protein kinase which is regulated by insulin. The two alpha subunits, as well as the beta subunits are linked by disulfide bonds [11].

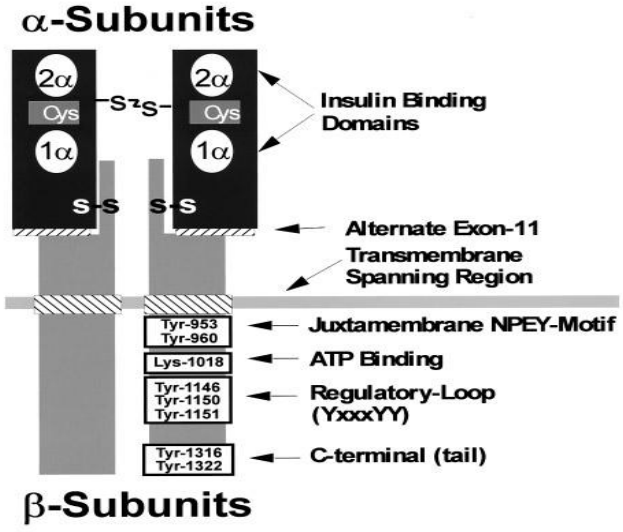


Figure 1: Schematic diagram of the insulin receptor tetramer. [11]
The diagram represents the insulin binding sites of the receptor (α-subunits) as well as the autophosphorylation sites (β-subunits)

Following its release by the beta cells of the pancreas, insulin binds to the receptor´s extracellular α-subunit. Binding of insulin brings the two α-subunits closer together. This conformational change makes it possible that ATP can bind to the β-subunit´s intracellular domain. This ATP-binding activates the autophosphorylation of the receptor, which in turn enables the receptor´s kinase activity for intracellular protein substrates [12].

Shortly after insulin is released, it inhibits glucose production in the liver and stimulates peripheral glucose utilization. The most important effect of insulin is represented by resultant decrease in blood glucose concentration. Insulin is a pleiotropic hormone. Its influence on carbohydrate, protein and lipid metabolism is significant. On one hand it stimulates amino acid uptake and protein and lipid synthesis, on the other hand it inhibits lipolysis, proteolysis

and ketogenesis [13]. In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis. Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation and dephosphorylation events. The versatile effects of insulin action start at the moment when it binds to its receptor on the surface of cells.

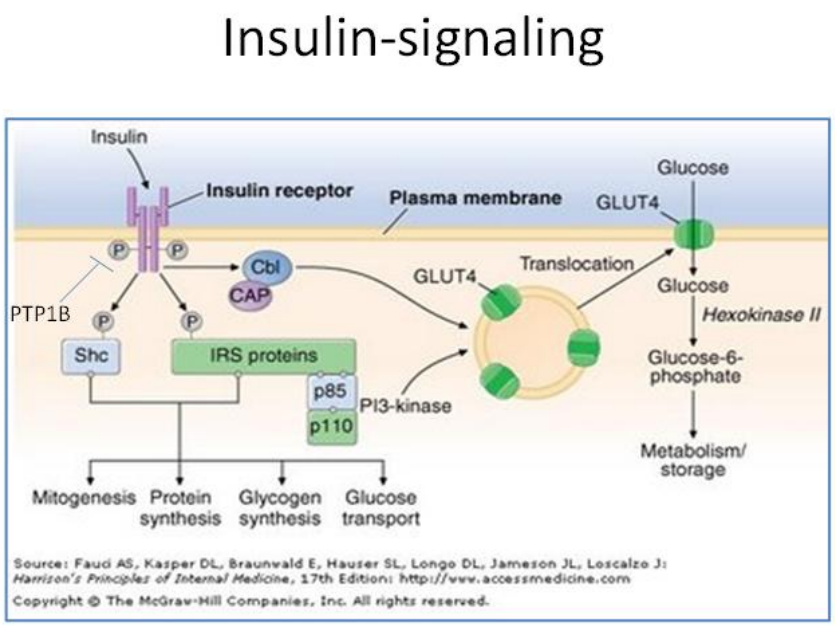


Figure 2: Insulin Signaling - picture modified [14]
Insulin is secreted by pancreatic β-cells in response to high blood glucose concentrations. Binding of the hormone to its receptor, a tetrameric complex consisting of two extracellular α-subunits and two intracellular β-subunits, triggers a signaling cascade which culminates in the uptake of glucose into the cell by the glucose transporter GLUT4. PTP1B, amongst other PTPs, deactivates the receptor by dephosphorylating the β-subunits.

Because of the essential role of insulin signaling in various biological processes it has to be tightly regulated. The task of negative regulation of insulin receptor signaling is undertaken by Protein tyrosine phosphatases (PTPs), which catalyze the dephosphorylation of tyrosyl-phosphorylated proteins [15].

1.1.4 Insulin resistance

More and more studies have shown that a large variety of physiological and pathophysiological states, like obesity, noninsulin-dependent diabetes mellitus (NIDDM), polycystic ovary syndrome

(PCOS) and the combination of central obesity, hypertension, glucose intolerance, and hyperlipidemia known as metabolic syndrome or syndrome X, are accompanied by insulin resistance. Insulin resistance is defined as a state “where a normal or elevated insulin level produces an attenuated biological response” [7] or “in which a greater than normal amount of insulin is required to elicit a quantitatively normal response” [8]. The detection of insulin resistance relies on a variety of tests for example the determination of insulin levels either at baseline (fasting) or after oral glucose tolerance testing (OGTT). Quantifying insulin secretion and resistance by the glucose clamp technique is also a common method to measure how sensitive an individual is to insulin. According to the OGTT method, fasting insulin levels above 50-70 μU/mL or peak insulin levels above 350 μU/mL indicate severe insulin resistance. The fasting serum insulin levels observed in normal individuals should be below 20 μU/mL and peak insulin levels below 150 μU/mL [8].

To date the principles of therapy for diabetes mellitus have been diet and exercise, in addition to drug therapy, albeit the treatment of patients with severe insulin resistance syndromes with drugs is presently unsatisfactory because of undesirable side effects [8]. Two classes of drugs are currently available that reduce insulin resistance. These are metformin and insulin sensitizers, such as the thioazolidinediones (TZDs). Metformin is a biguanide that suppresses hepatic glucose output and increases insulin mediated glucose disposal [8]. Thioazolidinediones (TZDs), which include pioglitazone and rosiglitazone (and troglitazone which meanwhile is withdrawn from market), are shown to reduce hyperglycemia and improve insulin sensitivity in patients with T2DM [9]. Moreover treatment of patients suffering from NIDDM with vanadate or vanadium salts has led to amelioration of glycemic profile and peripheral insulin resistance [8]. Also sulfonylureas, which stimulate insulin secretion, and acarbose, a starch blocker, are used as antidiabetics [3]. Due to severe side effects like gain of weight and other complications, novel

therapies are needed and drug design now leads to testing natural products to find specific agents to fight insulin resistance.

1.1.5 Protein tyrosine Phosphatases

The control of signaling pathways that guide a broad spectrum of elemental physiological processes is shared by the protein tyrosine phosphatase (PTP) superfamily of enzymes and protein tyrosine kinases. Recent studies about the function of these enzymes implicate that the kinases control the amplitude of a signaling response while the rate and duration of the response is dependent on the phosphatases. Protein tyrosine phosphatases are encoded by the largest family of phosphatase genes and can be divided into classical PTPs and dual specificity PTPs. The classical protein tyrosine phosphatases can be categorized as transmembrane receptor-like (e.g. LAR) and non-transmembrane (e.g. PTP1B, TCPTP, SHP2) proteins. Receptor-like phosphatases regulate signaling through ligand-controlled protein tyrosine dephosphorylation. The non-transmembrane, cytoplasmic PTPs contain regulatory sequences that flank the catalytic domain and control activity directly, or by controlling substrate specificity [16].

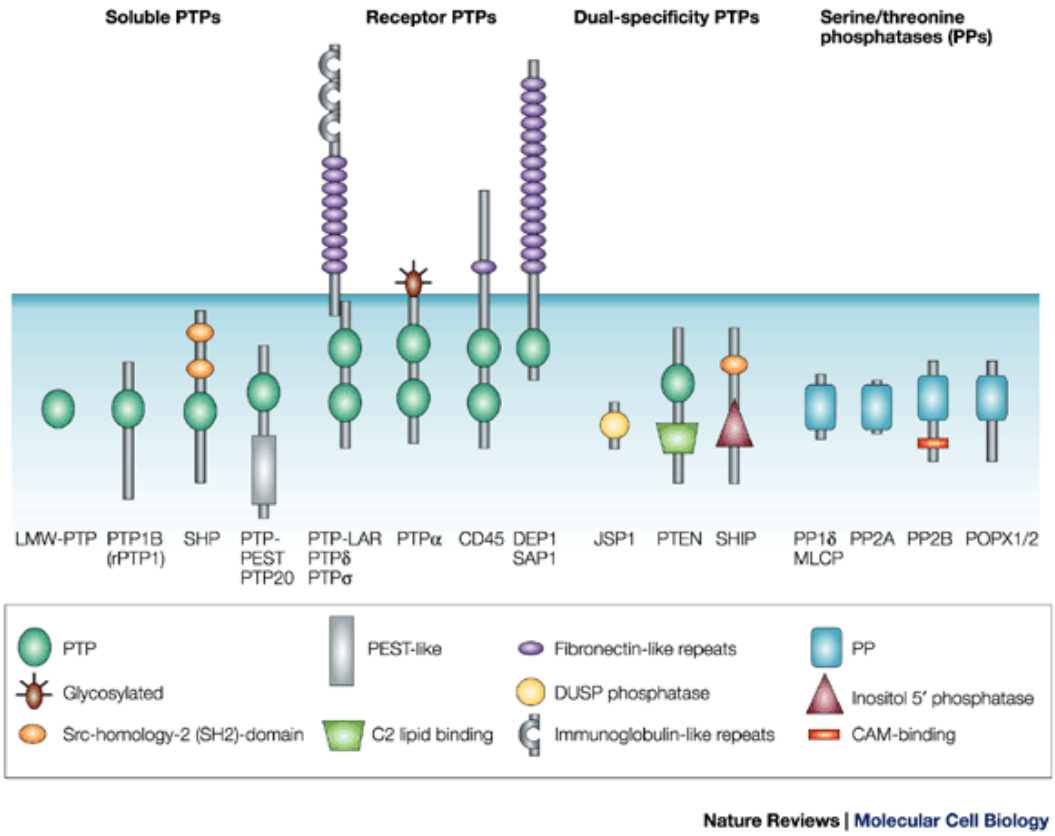


Figure 3: Superfamily of protein tyrosine phosphatases, an overview. [17]
Soluble and receptor-like PTPs represent the classical subset of the family.

1.1.5.1 Mode of action of protein-tyrosine phosphatases

Classical PTPs contain a catalytic domain of about 280 residues which is defined by several short sequence motifs. The most important is the signature sequence that functions as a phosphate binding loop at the active site. The enzymes are defined by the active-site signature motif HCX_5R [16]. This signature sequence motif includes the cysteine residue that catalyses the hydrolysis of protein-phosphotyrosine residues by the formation of a cysteinyl-phosphate intermediate [18]. The action of insulin throughout the insulin signaling pathway is facilitated by a cascade of tyrosine phosphorylation events, initiated by the binding of insulin to the insulin receptor [19]. Termination of Insulin signaling is achieved by the dephosphorylation of signal molecules by PTPs [16]. While the action of insulin is mediated by a cascade of tyrosine phosphorylation

events, PTPs have the potential to terminate insulin signaling by the dephosphorylation of signal molecules above all the insulin receptor [16].
In this work I focused on the non-transmembrane phosphatases PTP1B, TCPTP and SHP2, and on the receptor-like phosphatase LAR, which are known to act directly on the insulin receptor.

1.1.5.2 PTP1B

On major key element in the negative regulation of the insulin signaling pathway is the protein tyrosine phosphatase 1B. It is thought to play an important role in diabetes and metabolic syndrome [19].

PTP1B is a widely expressed intracellular enzyme which is localized mainly in the endoplasmic reticulum. It is associated with the ER of cells by attachment to a noncatalytic subunit or through hydrophobic interaction but it is also found in the cytosol of various tissues. Several biochemical *in vitro* studies have shown that PTP1B is active against autophosphorylated IR and that it also dephosphorylates

IRS-1 [20].

Through substrate trapping methods the direct association of PTP1B and IR has been proven. Further it was shown that in cells overexpressing PTP1B glucose uptake and GLUT-4 translocation to the cell membrane was reduced. Also the regulation of insulin signaling by PTP1B appears to be tissue specific. The overexpression of PTP1B in 3T3L1 adipocytes show a decrease in IR and IRS-1 tyrosine phosphorylation whereas in L6 myocytes and Fao hepatoma cells the overexpression led to complete blocking of insulin stimulated tyrosine phosphorylation of IR and IRS-1. Also the insulin stimulated glycogen synthesis in the latter two cell types was inhibited. This led to the suggestion that PTP1B plays an important role in the regulation of insulin action in muscle and liver. Through *in vivo*

studies it was found that PTP1B KO mice (PTB1B$^{-/-}$) showed enhanced insulin sensitivity. Furthermore, the PTP1B deficient mice were resistant to weight gain and remained insulin sensitive when set on a high-fat diet. Altogether these results implicate that PTP1B is a major regulator of insulin signaling and makes a potentially excellent therapeutic target in obesity and type II diabetes. Therefore the pharmaceutical industry showed increased interest in the development of PTPB1 inhibitors, because targeting of PTP1B raises the opportunity to establish insulin sensitizers without unwanted side effects like weight gain as seen with current sensitizers e.g. PPARγ activators (Thiazolidinediones). For it is difficult to identify a selective, save and effective PTP1B inhibitor, there are two major challenges in developing a potential therapeutic targeting PTP1B: selectivity over other similar PTPs (e.g. TCPTP) and cell permeability [21].

1.1.5.3 TCPTP

The protein tyrosine phosphatase TCPTP is expressed predominantly in hematopoietic tissues and exists as endoplasmic reticulum targeted form as well as a nuclear form. Its main action is thought to be the regulation of hematopoiesis and cytokine response. This discovery is based on TCPTP-knock out experiments - the TCPTP deficient mice died within 3 to 5 weeks from defects in hematopoiesis and immune function. A further role of TCPTP, regulation of insulin signaling, was found by a screening approach to identify PTPs that interact with the insulin receptor [21]. It is responsible for the downregulation of insulin receptor signaling [22]. Human TCPTP is the PTP most closely related to PTP1B (over 70 % structure identity) [15]. Due to this structural similarity to PTP1B it is crucial to find specific phosphatase inhibitors that do not interfere with TCPTP so

that by inhibiting PTP1B the important activities of this phosphatase are not turned down at the same time.

1.1.5.4 LAR

The human leukocyte antigen-related protein tyrosine phosphatase (LAR) is a receptor-like PTP consisting of two cytoplasmic phosphatase domains linked via a single hydrophobic transmembrane stretch to a large extracellular segment. The association between LAR and IR was demonstrated by co-immuno-precipitation studies of lysates of CHO cells overexpressing IR. In these studies the amount of IR/LAR complexes was increased by insulin treatment. Further the overexpression of human LAR in transgenic mice specifically in the muscle led to muscle insulin resistance which suggests a possible role of LAR as negative regulator of IR. LAR is highly expressed in adipose tissue which makes it a possible target for tissue specific regulation of insulin signaling [21].

1.1.5.5 SHP-2

SHP-2 is a cytoplasmic PTP that contains two amino-terminal src homology 2 (SH2) domains, a catalytic domain and a carboxy-terminal segment which contains two tyrosyl phosphorylation sites. It was shown to bind to both, the tyrosyl-phosphorylated IR and IRS-1 but it is not clear yet if it is either a negative or a positive regulator of insulin signaling. Rat adipose cells that were transfected with catalytic inactive mutants of SHP-2 featured slight changes in insulin stimulated GLUT-4 translocation. That suggested a minor role as a positive modulator of the metabolic effects of insulin. Also the absence of SHP-2 in KO mice was embryonic lethal although the heterocygote SHP-2 deficient mice appeared normal [21]. Those

findings suggested that SHP-2 does not play a significant role in the metabolic actions of insulin but other studies suggest that it has a major role on mitogenic signaling [20].

1.2 The Enzyme Inhibition Approach

1.2.1 Natural products as source for PTP1B inhibitors

The following substances have been tested on PTP1B inhibitory activity by Renate Baumgartner and showed promising results.

Substance	IC 50
Docosandioic acid (C22)	1.23 µM
Octadecandioic acid (C18)	12.87 µM
Riboflavin-5´-phosphate (RP)	6.91 µM
Folic acid (F)	79.56 µM
Pentagalloylglucose (from paeonia radix rubra)	4.75 µM
Oleic acid (from phellodendri cortex)	12.89 µM

These substances were utilized as controls and standards in the course of the establishment of the enzyme inhibition assays. Once the assay was built up, compounds and extracts from *Morus alba* were deployed.

1.2.1.1 Morus alba

Common name: white mulberry

Family: moraceae

Figure 4: Picture outlining the leaves and fruit of *Morus alba* [23]

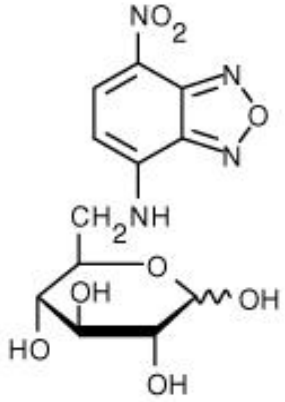
Since ancient times people have been using herbs and spices not only to improve the flavor of food but also to prevent and treat maladies. While the scientific evidence for the use of such common herbs and medicinal plants is rare or lacking, the beneficial effects that had been observed are generally encouraging. Because the interest in understanding the versatile effects of herbs is increasing, several studies have been focusing on the cellular and molecular modes of action of the active chemical components in herbs and their biological properties. Beneficial actions of herbs/medicinal plants include anti-inflammatory, anti-oxidant, anti-hypertensive, gluco-regulatory and anti-thrombotic effects. One major active chemical component of such plants are polyphenols, of which there are some who are associated with attenuating the metabolic syndrome. [24]

One of such plants which deserve more investigation is *Morus alba*, the white mulberry tree. It has been widely cultivated in China where it was used as food source for silk worms and to treat various maladies like diabetes, arthritis and rheumatism for thousands of years. Many flavones, stilbenes and benzofuran derivatives were isolated from the root bark or the stem bark of *Morus alba* presently [25].

In traditional Chinese medicine the white mulberry tree (*Morus alba*) has been useful in treating various health conditions for centuries. The white Mulberry is considered to be antibacterial, astringent, diaphoretic, hypoglycaemic. Recent researches in Japan showed that leaves contain substances that inhibit intestinal enzymes from passing sugar into the bloodstream [26]. The plant parts used in Chinese herbology include the fruit, the leaves and the root bark.

1.3 The Glucose Uptake Measuring Approach

6-NBDG (6-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose (6-NBDG)) is a fluorescent glucose analog that has been used to visualize glucose uptake and transport in live cells. NBD fluorescence typically displays excitation/emission maxima of ~465/540 nm [32].



$C_{12}H_{14}N_4O_8$

Figure 5: structural and molecular formula of 6-NBDG [32]

The glucose uptake into peripheral tissues as response to insulin is disturbed in diabetes which leads to chronically elevated levels of glucose in the circulation. Therefore in diabetes mellitus research measuring the ability of cells to take up glucose is fundamental. Current anti-diabetic medicine research focuses on the development and screening of compounds with potential insulinomimetic effects to stimulate the rate of cell glucose uptake. Most studies on glucose uptake are carried out using radiotracers such as 2-deoxy-D-[^{14}C] glucose or 2-deoxy-D-[^{3}H] glucose, which is accompanied by several disadvantages like disposal of radioactive waste or radioactive cleanup. Also with radiotracers it is not possible to measure glucose uptake in single, living cells.

Measuring the translocation of the GLUT-4 glucose transporter constitutes another common method used in diabetes research.

2 Aim of work

2.1 The Enzyme Inhibition Approach

Our therapeutical approach is to block the tyrosine phosphatase PTP1B in order to increase IR phosphorylation. The inhibitors must show significant selectivity over the other phosphatases (TCPTP, LAR, SHP-2).

The plant of choice in this project was *Morus alba*. Ethanol extracts and pure compounds of the plant were tested on their capability to inhibit the protein tyrosine phosphatase PTP1B to leave the insulin receptor active and responsive for insulin. The family of protein tyrosine phosphatases comprises a lot of similar enzymes with different and important functions hence the chosen compounds must not inhibit one of them.

Aim of this project was to screen *Morus alba* compounds for potent PTP1B inhibitors and investigate their selectivity over the other protein tyrosine phosphatases (TCPTP, LAR and SHP2).

The promising compounds were further tested in cell-based assays (western blotting, luciferase assays).

2.2 The Glucose Uptake Measuring Approach

An alternative way of examining glucose uptake is by direct incubation of mammalian cells with a fluorescent D-glucose analog like 2-NBDG [33] or, as in my project 6-NBDG, followed by flow cytometric detection of fluorescence produced by the cells. This represents a health-protective method and as it uses living cells it provides insight into the mechanisms of glucose accumulation and metabolism in cells.

I tried to establish an assay for testing substances on their ability to increase glucose uptake in adipocytes (3T3-L1). In order to test many

substances for their insulin sensitizing ability at a time it would be a great advantage to have 96-well plates coated with fully differentiated adipocytes.

3 3. Material & Methods

3.1 The Enzyme Inhibition Approach

3.1.1 Enzyme assays

The colorimetic enzyme assays were established in 96 well format using para-nitrophenylphosphate (pNPP) as the substrate for the phosphatase. If the enzyme is inhibited pNPP cannot be cleaved and no change of colour is visible. If the phosphatase is active, it dephosphorylates pNPP and forms pNP (para-nitrophenol), which results in a yellow colour. With adding NaOH 10M the p-nitrophenol is converted to its salt sodium-p-nitrophenolat, the reaction is stopped and the colour intensified. The absorption was measured with a photometer (Tecan/SUNRISE™) at 405 nm.

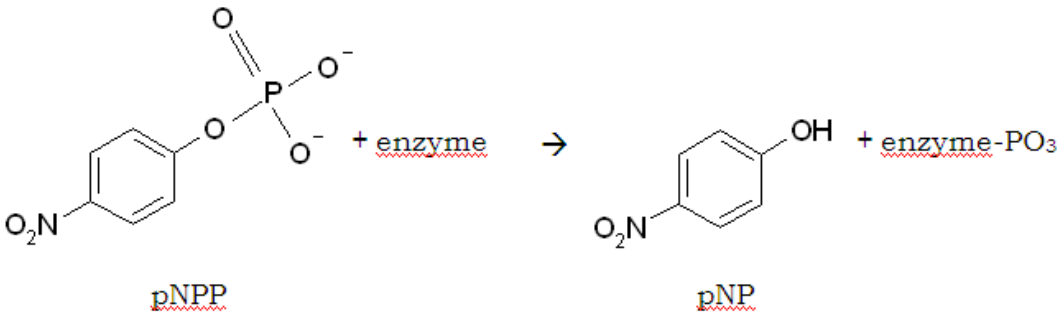


Figure 6: The principle of the colorimetric assay

Test compounds or extracts were solved in dimethyl sulfoxide (DMSO) to a final concentration of 1% DMSO in a total volume of 100 µL/well. All dilutions were done in MOPS buffer (N-morpholino-propanesulphonic acid), 50 mM, pH 6.5.

DMSO was used as negative control. Two known PTP1B inhibitors, ursolic acid and sodium orthovanadate (SOV) were used as positive controls. Furthermore DTT (dithiothreitol) 1mM was used to protect the enzyme as it is quite unstable when exposed to oxygen. Also, regarding the instability of the enzyme, it was pipetted left to right of the plate once and the repetition from right to left.

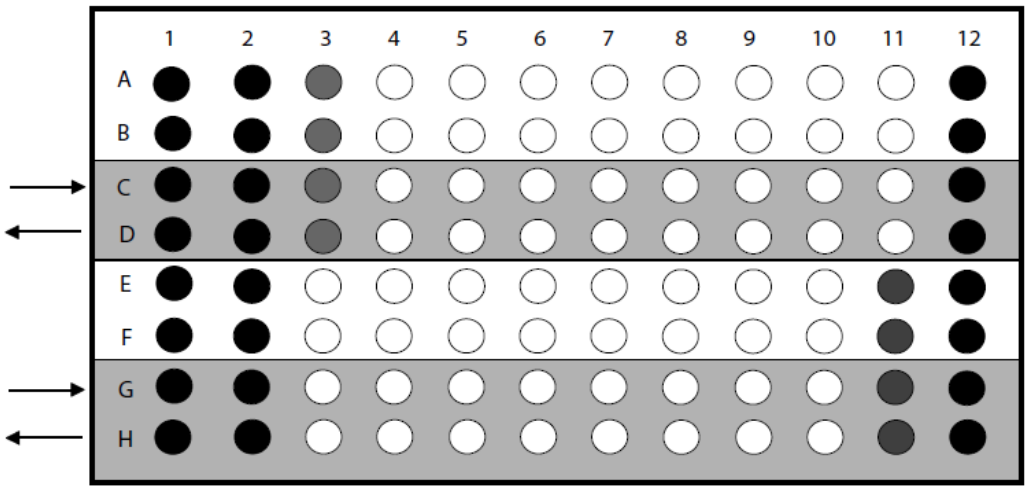


Figure 7: Example of a 96-well plate assay for enzyme inhibition

Order of pipetting:

1. test substances and controls in MOPS buffer (without pNPP) (50 µL, 45 µL in the lower two lanes, respectively)
2. 5 µL of freshly diluted enzyme (only in the lower two lanes)
3. pNPP double concentrated in MOPS buffer (50 µL)

Then the plate was subjected to a kinetic measurement in the photometer (11 cycles, measurement every 3 minutes, 5 seconds of shaking between cycles). Afterwards 25 µL of NaOH 10M was added to every well and one more measurement was taken. Only values of the last measurement were used for evaluation.

protein	concentration used	supplier
PTP1B	0.025 µg in 100 µL	R&D Systems (Minneapolis, MN, USA)
TC-PTP	0.015 µg in 100 µL	Enzo Life Science (Farmingdale, NY)
LAR	0.39 µg in 100 µL	Enzo Life Science (Farmingdale, NY)
SHP-2	0.5 µg in 100µL	Enzo Life Science (Farmingdale, NY)

Table 2: Phosphatases used in this thesis

Phosphatase inhibitors	concentration	supplier
Sodium orthovanadate (SOV)	1-1000 µM	Sigma (St. Louis, MO, USA)
Ursolic acid (UA)	30 µM	Sigma (St. Louis, MO, USA)
Sanggenon C	diff.	J. Rollinger (University of Innsbruck, AUT)
Sanggenon B	diff.	J. Rollinger (University of Innsbruck, AUT)
Sanggenon D	diff.	J. Rollinger (University of Innsbruck, AUT)
Kuwanon L	diff.	J. Rollinger (University of Innsbruck, AUT)

Table 3: Controls and test compounds

buffername	reagents	concentrations
Reconstitutionbuffer for PTP1B pH 7.5	HEPES EGTA EDTA DTT BSA	10 mM 0.1 mM 0.1 mM 1 mM 0.5 mg/mL
Reconstitutionbuffer for TCPTP	TRIS/HCL NaCl EDTA DTT Brij Glycerol	50 mM 100 mM 2 mM 5 mM 35 % 15 %
Reconstitutionbuffer for LAR pH 7.0	TRIS/HCL NaCl EDTA DTT Brij35 BSA	25 mM 50 mM 2 mM 5 mM 0.01 % 1 mg/mL
Assay buffer for PTP1B, TCPTP, LAR pH 6.5 with or without	3-(N-Morpholino)-Propanesulfonic acid (MOPS) pNPP	50 mM 4 mM
Assay buffer for SHP-2 pH 7.0 with or without	TRIS NaCl EDTA DTT Brij35 BSA pNPP	25 mM 50 mM 2 mM 5 mM 0.01 % 1 mg/mL 4 mM

Table 4: PTP buffers

3.1.2 Cell culture

The following cell lines have been used

For Western blotting:

HCC → Hepatocellular carcinoma cells
A kind gift from M. Eisenbauer from the Institute of Cancer Research, Medical University of Vienna, Austria

MEF → Mouse embryonic fibroblasts (PTP1B rec and PTP1B ko) provided by Benjamin Neel, Division of Stem Cell and Developmental Biology, Ontario Cancer Institute, Toronto

For PPAR transfection and luciferase based assay:

HEK 293 → Human embryonic kidney cells were purchased from the American Tissue Culture Collection.

3.1.2.1 Cultivation of HCC

Cells were maintained in RPMI 1640 medium supplemented with 10 % FBS, Glutamine (2 mM) and Penicillin (100 U/mL) / Streptomycin (100 µg/mL) in 5 % CO2 environment at 37 °C. Cells were passaged once a week and grown to near confluence.

3.1.2.2 Cultivation of MEF

Cells were cultivated in DMEM medium without phenol red, with 4.5 g/L Glucose, 10 % FBS, Glutamine and Penicilline/Streptomycin as indicated above. They were passaged twice a week.

3.1.3 Western blots

Preparation of samples:

Lysis buffer is prepared and supplements added freshly (important!). Cell lysates are prepared by washing the cells with cold PBS, adding lysis buffer into the well, and incubating the plate on ice. After 10 minutes, lysates are scraped into Eppendorf tubes and subjected to sonification. Lysates are cleared in the table top centrifuge at >10,000*g* for 10 minutes at +4 °C. Protein concentrations are determined after the method of Bradford [34].

Protein quantification (Bradford Assay [34])

For protein quantification samples were diluted 1:10 in aqua bidest. and 10 µL of this dilution was pipetted to a 96 well plate. Samples were applicated in triplicate beside a standard curve of BSA 50 - 500 µg/mL. Before the measurement 190 µL of Bradford reagent (Roti® -Quant) was added per well. The absorbance of the plate was measured at 595 nm in a Tecan Sunrise microplate reader.

SDS-PAGE

30 µg of protein is mixed with 3xSDS sample buffer and denatured at 95 °C for 5 minutes. Polyacrylamid gels are casted at appropriate concentrations (depending on protein sizes) and loaded with the prepared samples and molecular weight markers. Gels are run at 25mA per gel until the dye reaches the lower end.

Western blot and detection

After the gel run, proteins are transferred onto polyvinyldifluoride (PVDF) membranes, using a tank blot system with cooling at 100 V for 100 minutes. After the transfer, free binding sites are saturated with BSA in TBS/T (5 % w/v) as blocking agent. Primary antibodies are diluted in TBS/T ± BSA (depending on the antibody) and

incubated with the membrane over night at 4 °C. Membranes are washed 3x10 minutes with TBS/T to remove unbound antibody. The secondary antibody is incubated with the membrane for at least one hour at room temperature. Membranes are washed again 3x10 minutes and the ECL solution prepared. Membranes are incubated at least for 4 minutes with the ECL solution before detection in a chemiluminescence detector. Quantification of the bands was done with AIDA-software.

Stripping of membranes

Mostly, more than one antibody was applied to the same membrane. Therefore, membranes were stripped of the already detected antibody. For this purpose a 0.5 M solution of NaOH was applied for 10 min. Afterwards, membranes were washed 2 times for 10 min with TBS-T. Then, the membranes could be probed with another antibody.

Medium/solutions	reagents	concentration/volume
Growth medium (HEK 293 and MEF)	DMEM FCS Penicillin Streptomycin Glutamine	500 µL 10 % 100 U/mL 100 µg/mL 2 mM
Growth medium (HCC)	RPMI FCS Penicillin/ Streptomycin Glutamine	500 µL 10 % 100 U/mL / 100 µg/mL 2 mM
Starvation medium (MEF)	DMEM FCS Penicillin/ Streptomycin Glutamine	500 µL 0.5 % 100 U/mL / 100 µg/mL 2 mM
Starvation medium (HCC)	RPMI Penicillin/ Streptomycin Glutamine	500 µL 100 U/mL / 100 µg/mL 2 mM
PBS pH 7.4	NaCl KCl Na_2HPO_4 KH_2PO_4 $MgCl_2$ x 6 H_2O $CaCl_2$ x 6 H_2O Aqua dest.	0.8 g 0.2 g 1. 15 g 0.2 g 0.1 g 0.1 g ad 1000 mL
Trypsin/EDTA	Trypsin EDTA in PBS	0.05 % 0.02 %

Table 5: Solutions cell culture of the Enzyme Inhibition Approach

Starvation medium (HCC)	RPMI Penicillin/ Streptomycin Glutamine	500 µL 100 U/mL / 100 µg/mL 2 mM
PBS pH 7.4	NaCl KCl Na_2HPO_4 KH_2PO_4 $MgCl_2$ x 6 H_2O $CaCl_2$ x 6 H_2O Aqua dest.	0.8 g 0.2 g 1. 15 g 0.2 g 0.1 g 0.1 g ad 1000 mL
Trypsin/EDTA	Trypsin EDTA in PBS	0.05 % 0.02 %

Table 6: Solutions cell culture of the Enzyme Inhibition Approach

solutions	reagents	concentration/volume
SDS Sample Buffer (3x stock)	TRIS-HCl, 0.5 M, pH 6.8 SDS Glycerol Bromophenol blue ddH_2O 2-Mercaptoethanol (add fresh each time)	37.5 mL 6.0 g 30.0 mL 15.0 mg 100.0 mL 15 % (v/v)
Electrophoresis Buffer (5x stock) (dilute with ddH_2O)	TRIS-base Glycine SDS ddH_2O	15.0 g 72.0 g 5.0 g 1000 mL
Blotting Buffer (5x stock) working solution	TRIS-base Glycine ddH2O 5x stock Methanol ddH2O	15.0 g 72.0 g 1000 mL 20 % (v/v) 20 % (v/V) 60 % (v/v)
TBS-T (10x stock)	Tris base NaCl Tween20 dissolve in ddH2O, adjust pH to 8 and dilute with ddH2O	250 µM 1.90 µM 10 mL
ECL Solution	Luminol (44 mg/mL) para-coumaric acid (15 mg/mL) Tris-base, 1M, pH 8.5 ddH2O	50 µL 50 µL 1 mL 9 mL

Table 7: Solutions for Western blot

Recipe PAGE-Gels

	resolving gel [µL]				stacking gel [µL]
	7.5 %	10 %	12 %	15 %	
bis-/acrylamid 0.8/30 %	1875	2500	3000	3750	640
Tris-HCl 1.5 M pH=8.8	1875				
Tris-HCl 1.25 M pH=6.8					375
SDS 10 % (w/v)	75				37.5
ddH_2O	3675	3050	2550	1800	2620
APS 10% (w/v)	37.5				37.5
TEMED	7.5				7.5
sum (approx.)	7500				3750

Table 8: Composition of PAGE-Gels for Western blot

Antibodies

Insulin receptor β (rabbit mAb)	cell signal # 3025
Phospho IR-Tyr 1158, 1162, 1163	Sigma
Rabbit IgG	New England Biolabs

Antibodies were diluted 1:1000 in TBS-T/BSA

3.1.4 Transfection and Luciferase-based assay

We also examined the effects of the promising *Morus alba* compounds on the transcriptional activity of PPARs in a luciferase-based screening assay in HEK293 cells. The cells were transfected with mouse PPAR*a* or PPARγ, a luciferase construct under the control of the PPAR response element (PPRE), and EGFP.

PPAR (peroxisome proliferator activated receptor) activators are in use for treatment of T2DM. Pioglitazone and other TZDs are known to activate PPARγ and are used as insulin sensitizers. PPARα activators are lipid-lowering drugs. Those *Morus alba* substances which showed enzyme inhibitory activity were also investigated for their capability of

increasing the transcriptional activity of PPARs. This was investigated in a luciferase-based assay where increased activity can be measured through bioluminescence.

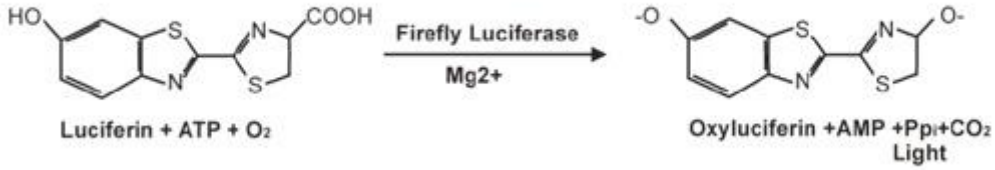


Figure 8: Reaction of luciferin breakdown catalysed by luciferase

Fluorescence and luminescence measurements in a Tecan GENios. Pro plate reader (Fluorescence Top, Excitation wavelength 485nm, Emission wavelength 520 nm; and Luminescence, injection mode standard) [31].

HEK 293 cells were transfected with mouse PPARγ and PPARα, a luciferase construct under control of the PPAR response element (PPRE) and EGFP through the calcium phosphate precipitation method. Cells were re-seeded and treated with compounds from the phosphatase inhibitor hitlist. Then a luciferase based assay was performed to measure PPAR activity.

3.1.4.1 Cultivation of HEK 293 cells

Cells were cultivated in DMEM medium with 10 % FBS, Glutamine and Penicilline/Streptomycin in 150 cm² flasks.
Medium was changed every third day. Confluent cells were passaged twice a week, then 2 x 106 - 4 x 106 cells were seeded in a new flask.

3.1.4.2 PPAR Transfection and Luciferase Assay

HEK293 cells are seeded in petri dishes and, after 6 hours, transfected by the calcium-phosphate method over night. Then cells are re-seeded in 96-well plates and incubated for 6 hours. Cells are treated with solvent and drugs diluted in culture medium over night

(18 hours). The supernatant is aspirated and cells are lysed with a luciferase assay lysis buffer for 10 minutes on a plate shaker.
Meanwhile, the injector system of the plate reader is filled with the ATP buffer and Luciferin buffer. The cell lysates are transferred to a black 96-well plate and the measurement protocol started: first, fluorescence for EGFP (excitation 485 nm, emission 535 nm) is measured for the whole plate, then luminescence in each well is measured after injection of both assay solutions. EGFP serves as an indicator of transfection efficiency. Luminescence values were evaluated by subtracting background measurements (no transfection) and divided by their respective EGFP fluorescence values for normalization.

solutions	reagents	concentration/volume
2 x HBS	NaCl	8.0 g
pH 7.0	HEPES	6.5 g
	NaH_2PO_4	10 mL
$CaCl_2$ solution	$CaCl_2$	2 M
Plasmids	PPAR (α or γ)	
	PPRE	
	EGFP	
	pSG5	

Table 9: Solutions for transfection

solutions	reagents	concentration/volume
Luciferin Buffer	Luciferin Tricine Buffer pH 7.8 in ddH2O	0.32 mg/mL 21 mM
ATP Buffer	ATP Tricine Buffer pH 7.8 MgCl2 in ddH2O	3.8 mM 20 mM 21.5 mM
Lysis Buffer	Luciferase Cell Culture Lysis 5X Reagent (Promega) in ddH2O add fresh each time used DTT CoenzymeA	 1 µM 270 µM

Table 10: Solutions for Luciferase assay

3.2 The Glucose Uptake Measuring Approach

3.2.1 Cell culture

Used cell line: 3T3-L1→ murine preadipocytes, subclone of 3T3 fibroblasts

Pre-adipocytes were seeded in 96 well plates and differentiated with DMEM/FCS (with glutamine, insulin, dexamethasone and IBMX). Cells are fully differentiated after 12 days, when lipid droplets are visible. Cells were then treated with different concentrations of pioglitazone. Pioglitazone is a known insulin sensitizer and belongs to the class of thiazolidinediones (TZD). After treatment with pioglitazone, the adipocytes were stimulated with different concentrations of insulin and then DOG-uptake was measured.

3.2.1.1 Cultivation and differentiation of 3T3L1

3T3-L1 cultivation in T75 flasks, $12x10^4$ cells for 3 days in approx. 13 ml medium. Cells must be split before reaching confluence. After 3 days the preadipocytes were harvested and seeded in 96well plates, 5000 cells/well, a total of 200µl/well. Cells were grown to confluence and then treated with differentiation medium for 4 days, medium was changed every 2 days. Day 4 to 6 the differentiating cells were treated with adipocyte cultivation medium with or without insulin.

Cells were treated with putative insulin sensitizers (enzyme inhibitors) in different concentrations, with insulin and glucose, also in different concentrations. Then the glucose uptake was measured.

medium	reagents	concentration
Cultivation medium (3T3-L1)	DMEM white	500 mL
	NBS (newborn bovine serum)	10 %
	Penicillin/ Streptomycin	100 U/mL / 100 µg/mL
	Glutamine	2 mM
Differentiation medium (3T3-L1)	DMEM white	500 mL
	FCS (fetal calf serum)	10 %
	Penicillin/ Streptomycin	100 U/mL / 100 µg/mL
	Glutamine	2 mM
	Insulin	1 µg/mL
	Dexamethasone	500 nM
	IBMX	500 µM
Adipocyte cultivation medium	DMEM white	500 mL
	FCS (fetal calf serum)	10 %
	Penicillin/ Streptomycin	100 U/mL / 100 µg/mL
with or without	Glutamine	2 mM
	Insulin	1 µg/mL

Table 11: Solutions cell culture of the Glucose Uptake Measuring Approach

Order of pipetting of the 6-NBDG-assay

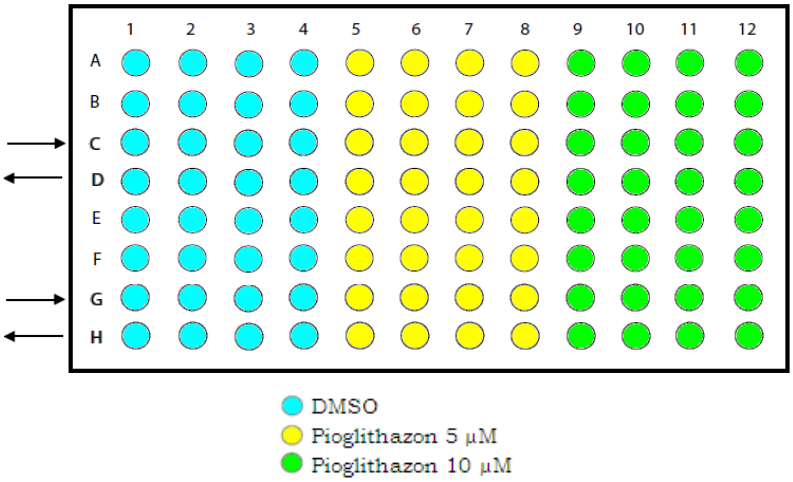


Figure 9: Treatment with DMSO and Pioglitazon

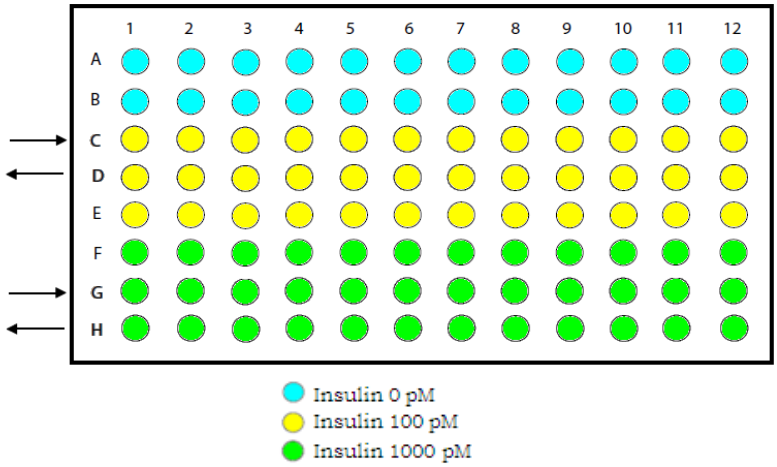


Figure 10: Stimulation with insulin

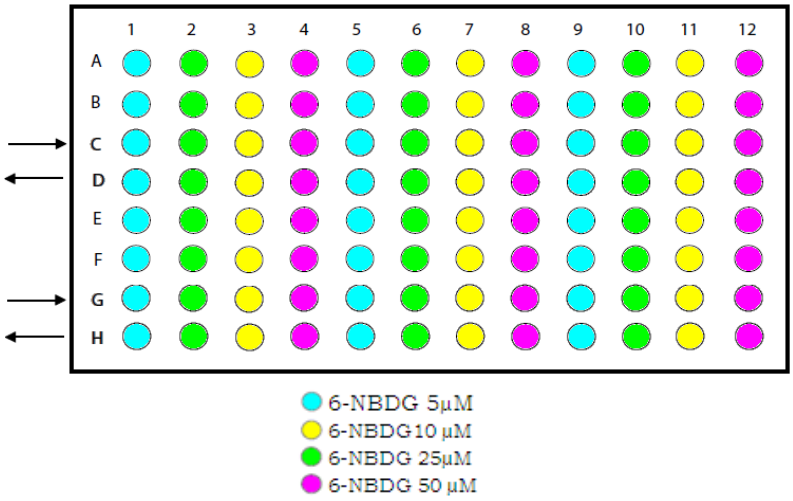


Figure 11: 6-NBDG uptake

4 Results & Discussion

4.1 The Enzyme Inhibition Approach

As blocking agents for enzyme inhibition different fractions and pure compounds of the mulberry tree *Morus alba* were tested *in vitro* in a colorimetric enzyme assay. The most promising compounds were then tested on different cell lines to show the increased uptake of insulin and glucose.

The following pure compounds were proven effective: Sanggenon B, C, D and Kuwanon L. They were able to inhibit PTP1B even at low concentrations (3.8 µM-13.8 µM), without blocking otherphosphatases from the same superfamily like TCPTP, LAR and SHP 2. LAR and SHP 2 were not affected at all while TCPTP was inhibited only at concentrations significantly higher than PTP1B (10.4 µM-27 µM).

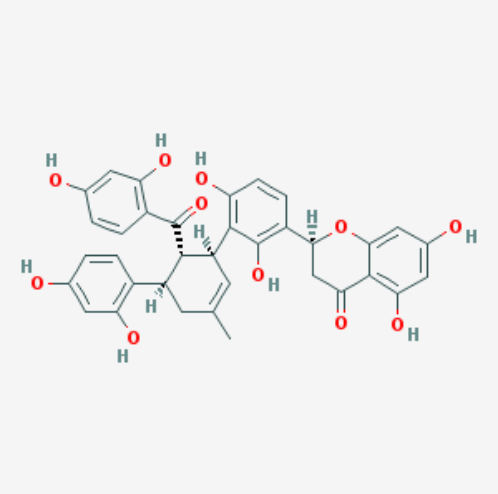


Figure 12: Kuwanon L [27]

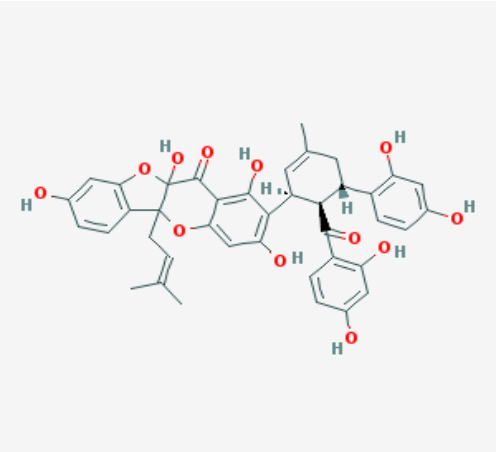


Figure 13: Sanggenon C [28]

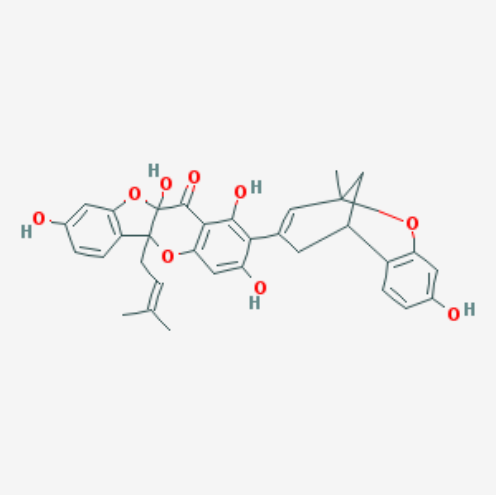


Figure 14: Sanggenon B [29]

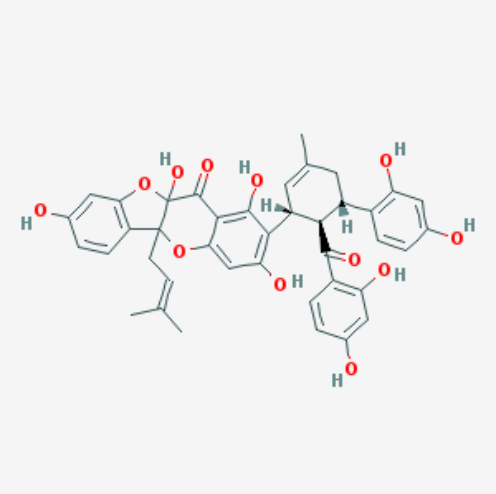


Figure 15: Sanggenon D [30]

Figure 5-8 show the structural formulas of the pure *Morus alba* compounds that were further investigated

4.1.1 Enzyme Assays

Phosphatase Inhibition

Substances that are known PTP1B inhibitors were tested for inhibitory effects on the phosphatases TCPTP, LAR and SHP-2. Then pure compounds and extracts of *Morus alba* in different concentrations were tested on these enzymes.

First, all the fractions and pure compounds of *Morus alba* were tested on their possible inhibitory effect on PTP1B. Then the IC50 values of the promising hits were determined. The IC50 of a compound or substance is a measure for the effectiveness of this substance to inhibit a biological process or component of a process such as an enzyme. The value of IC50 indicates the half maximal inhibitory concentration of the substance.

We wanted to achieve maximum inhibitory effects for PTPB1 accompanied by non-inhibition of the other tested phosphatases.

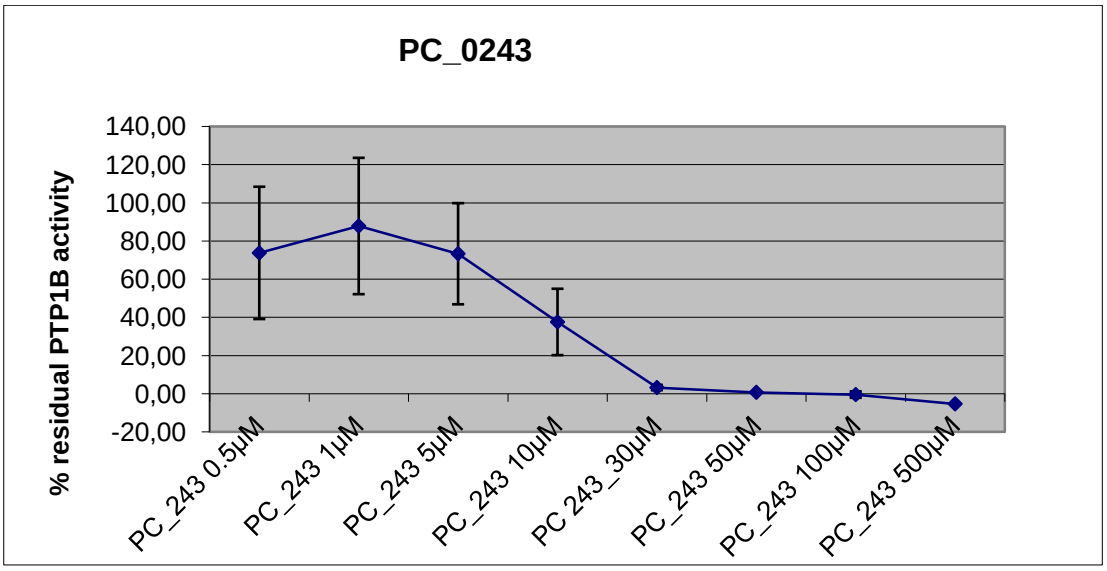


Figure 16: Inhibition of PTB1B by PC_0243
PC 243 (oleic acid) is known to inhibit PTP1B. The kinetic measurement of the colorimetric inhibition assay showed that at a concentration of 10 µM PTP1B shows only 40 % residual activity, at 30 µM no more activity is measurable

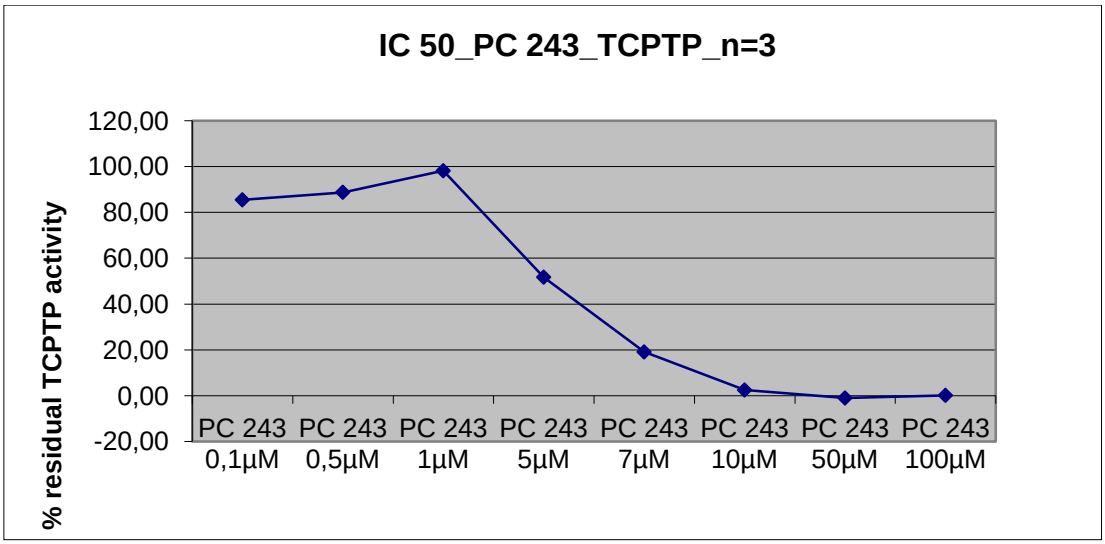


Figure 17: Inhibition of TCPTP by PC_0243 (oleic acid)
After blocking the reaction with NaOH 6M the IC50 value of PC243 is 5 µM, total inhibition of the enzyme is measured at 10 µM

Obviously oleic acid is a strong inhibitor of both phosphatases. It was important to construct this inhibition curve to know what it looks like when TCPTP is completely inhibited. Next, the *Morus alba* compounds and extracts were tested in detail.

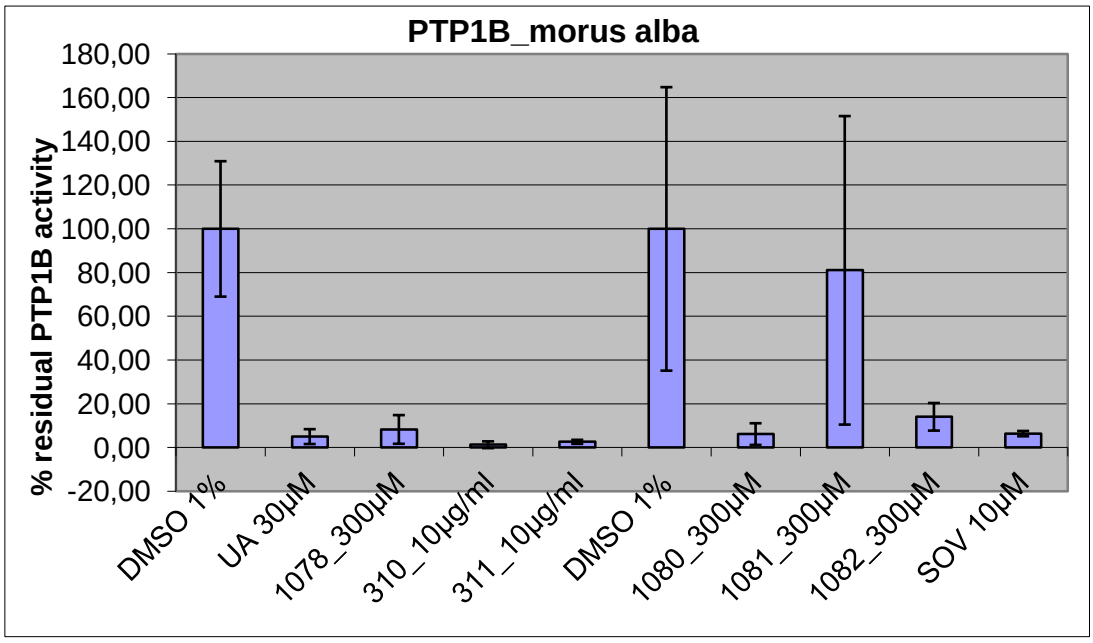


Figure 18: Inhibition of PTB1B by different *Morus alba* extracts
Morus Alba (310, 311; 10µg/mL) and pure compounds: 1078 (Sanggenon D, 300µM), 1080 (Sanggenon C, 300µM), 1081 300µM, 1082 (Sanggenon B, 300µM).Tested all together on one plate to find out the most promising hits.

Sanggenon D (MA 1078)

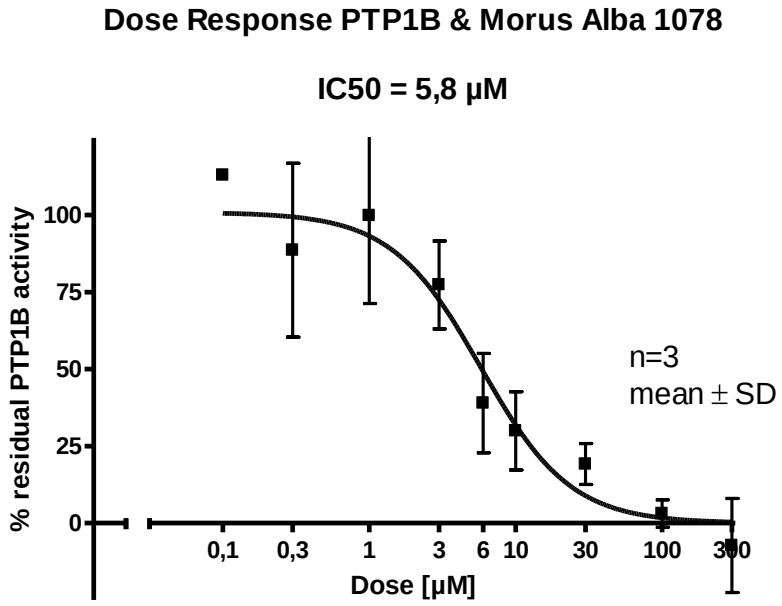


Figure 19: Inhibition of PTB1B by Sanggenon D
Different concentrations (0.1 – 300 µM) of Sanggenon D (MA 1078) were applied to the PTP1B assay to obtain a sigmoidal dose response curve. At a concentration of 5.8 µM only 50% residual activity of PTP1B is measured.

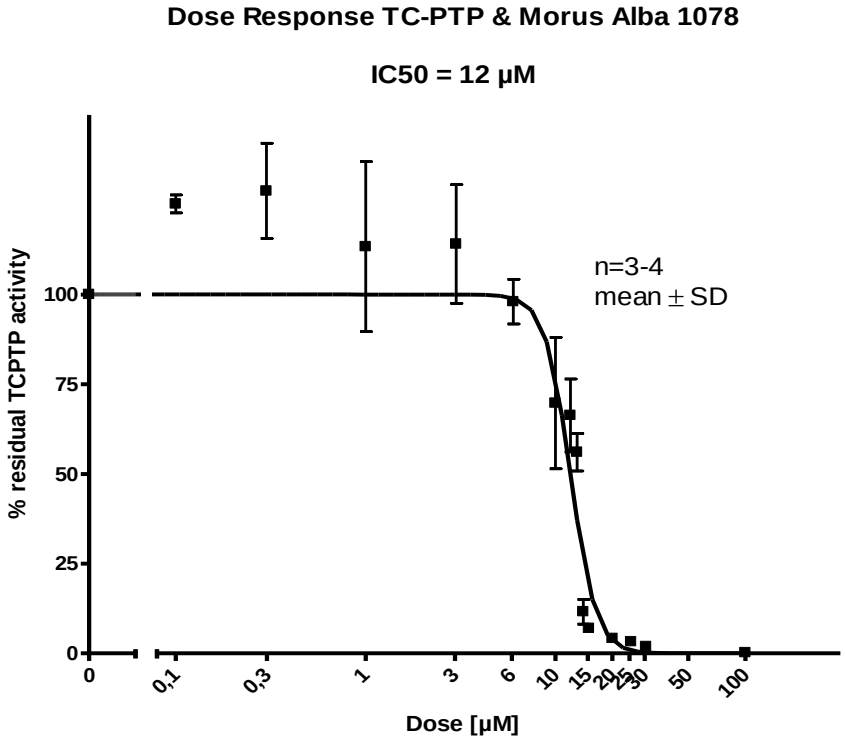


Figure 20: Inhibition of TCPTP by Sanggenon D
Different concentrations (0.1 – 100 µM) of Sanggenon D (MA 1078) were applied to the TCPTP assay to obtain a sigmoidal dose response curve. At a concentration of 12 µM only 50 residual activity of TCPTP is measured.

The comparison of the dose response of PTP1B and TCPTP to Sanggenon D showed that this compound is able to block half the activity of PTP1B at a concentration of 5.8 µM. At this concentration TCPTP is not inhibited.

Sanggenon C (MA1080):

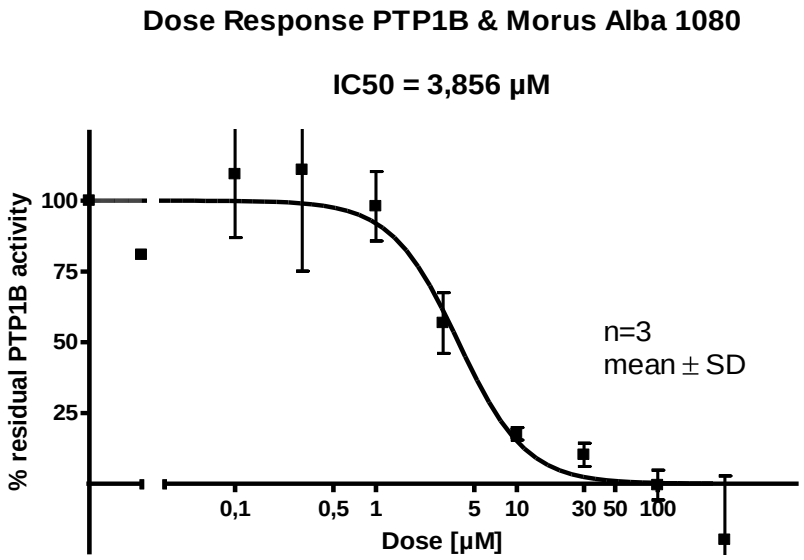


Figure 21: Inhibition of PTB1B by Sanggenon C
Different concentrations (0.1 – 100 µM) of Sanggenon C (MA1080) were applied to the PTP1B assay to obtain a sigmoidal dose response curve. At a concentration of 3.9 µM PTP1B showed only 50% residual activity.

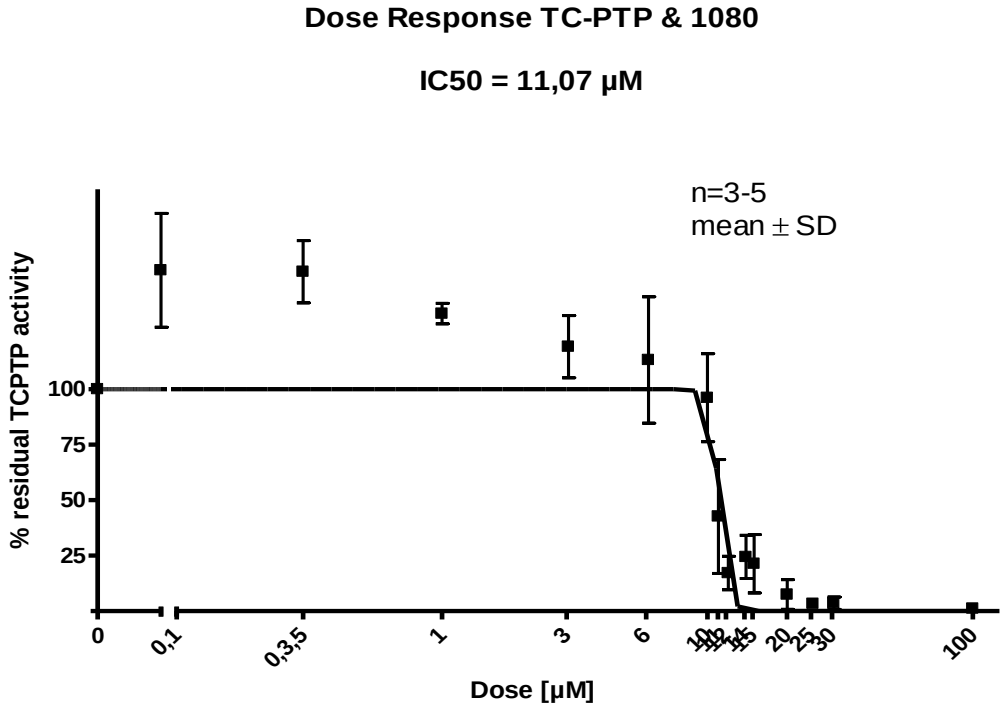


Figure 22: Inhibition of TCPTP by Sanggenon C
Different concentrations (0.1 – 100 µM) of Sanggenon C (MA 1080)were applied to the TCPTP assay to obtain a sigmoidal dose response curve. At a concentration of 11.7µM TCPTP showed only 50% residual activity.

The comparison of the dose response of PTP1B and TCPTP to Sanggenon C showed that this compound is able to block half the activity of PTP1B at a concentration of 3.9µM. At this concentration TCPTP is not inhibited.

Kuwanon L (MA1079):

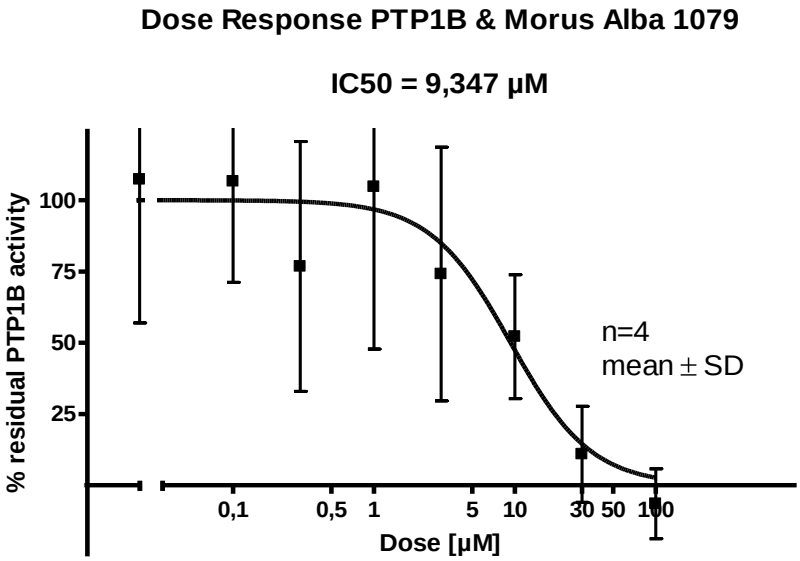


Figure 23: Inhibition of PTB1B by Kuwanon L
Different concentrations (0.1 – 100 µM) of Kuwanon L (MA1079) were applied to the PTP1B assay to obtain a sigmoidal dose response curve. At a concentration of 9.3µM PTP1B showed only 50% residual activity.

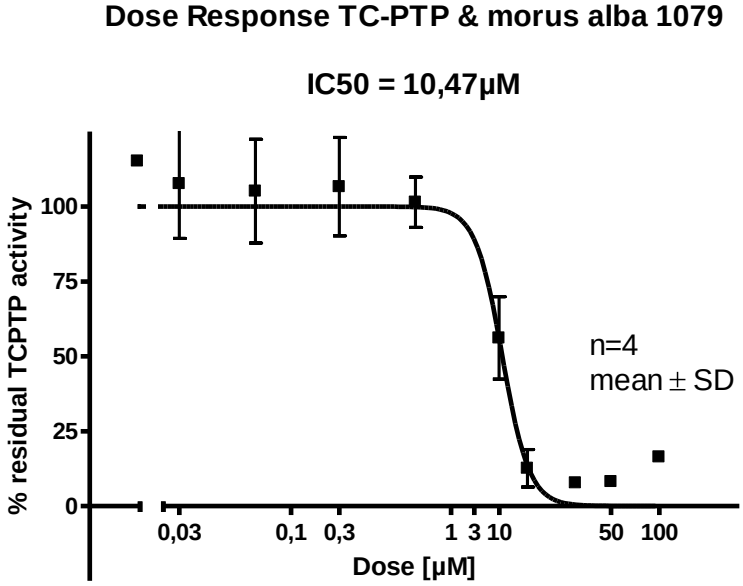


Figure 24: Inhibition of TCPTP by Kuwanon L
Different concentrations (0.1 – 100 µM) of Kuwanon L (1079) were applied to the TCPTP assay to obtain a sigmoidal dose response curve. At a concentration of 10.4µM TCPTP showed only 50% residual activity.

The comparison of the dose response of PTP1B and TCPTP to Kuwanon L showed that this compound is able to block half the activity of PTP1B at a concentration of 9.3µM. Unfortunately half the

TCPTP activity is inhibited by 10.5µM of this compound which is not significantly higher.

Sanggenon B (MA1082):

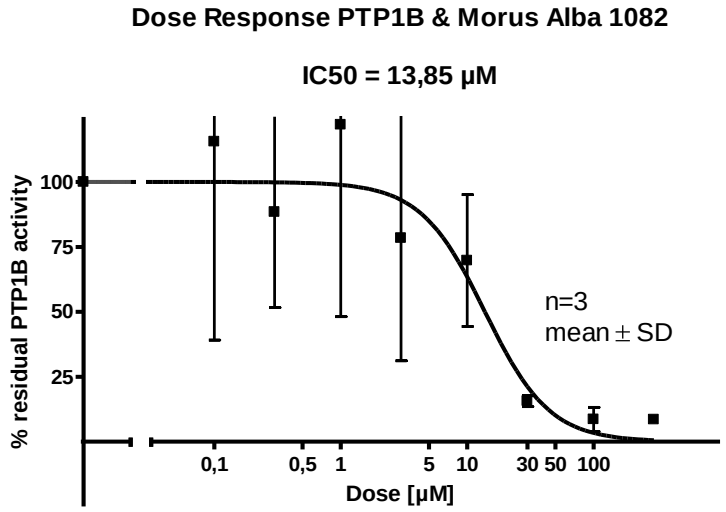


Figure 25: Inhibition of PTB1B by Sanggenon B
Different concentrations (0.1 – 100 µM) of Sanggenon B (MA1082) were applied to the PTP1B assay to obtain a sigmoidal dose response curve. At a concentration of 13.9 µM PTP1B only showed 50% residual activity.

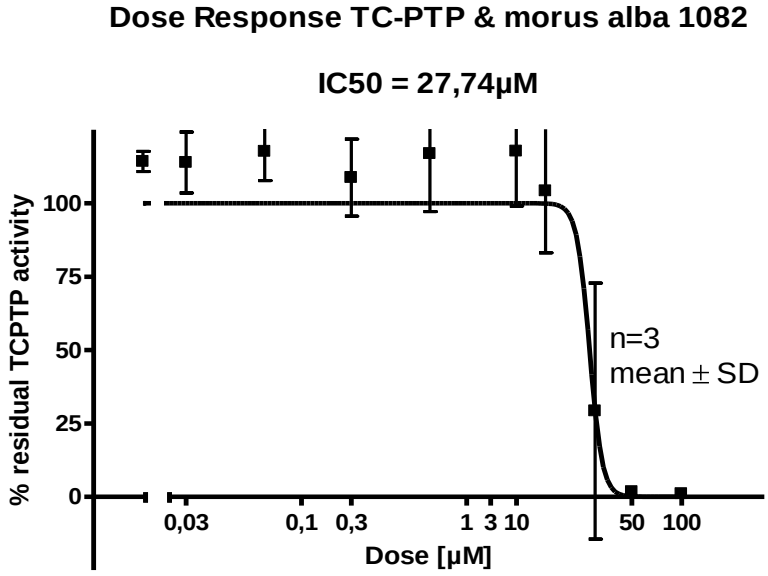


Figure 26: Inhibition of TCPTP by Sanggenon B
Different concentrations (0.1 – 100 µM) of Sanggenon B (1082) were applied to the TCPTP assay to obtain a sigmoidal dose response curve. At a concentration of 27.7% TCPTP only showed 50% residual activity.

The comparison of the dose response of PTP1B and TCPTP to Sanggenon B showed that this compound is able to block half the activity of PTP1B at a concentration of 13.9 µM. At this concentration TCPTP is not inhibited.

Former tested PTP1B inhibitors as well as *Morus alba* compounds were tested on their inhibitory effects on LAR and SHP-2.

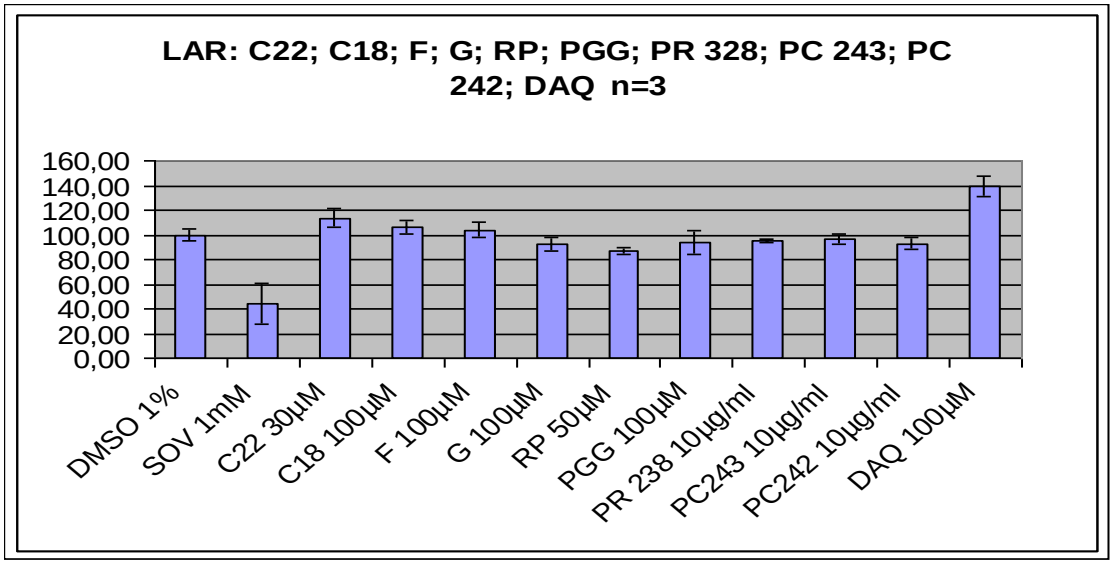


Figure 27: LAR inhibition assay with former tested PTP1B inhibitors

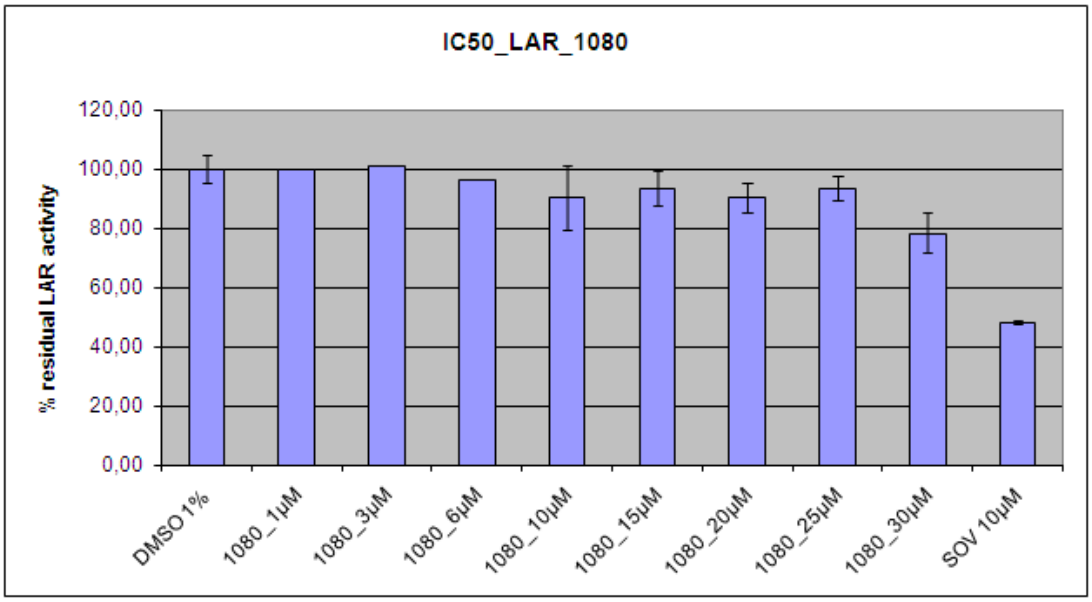


Figure 28: Inhibition of LAR by Sanggenon D
Sanggenon D, tested in different concentrations (from 1 µM to 30 µM).

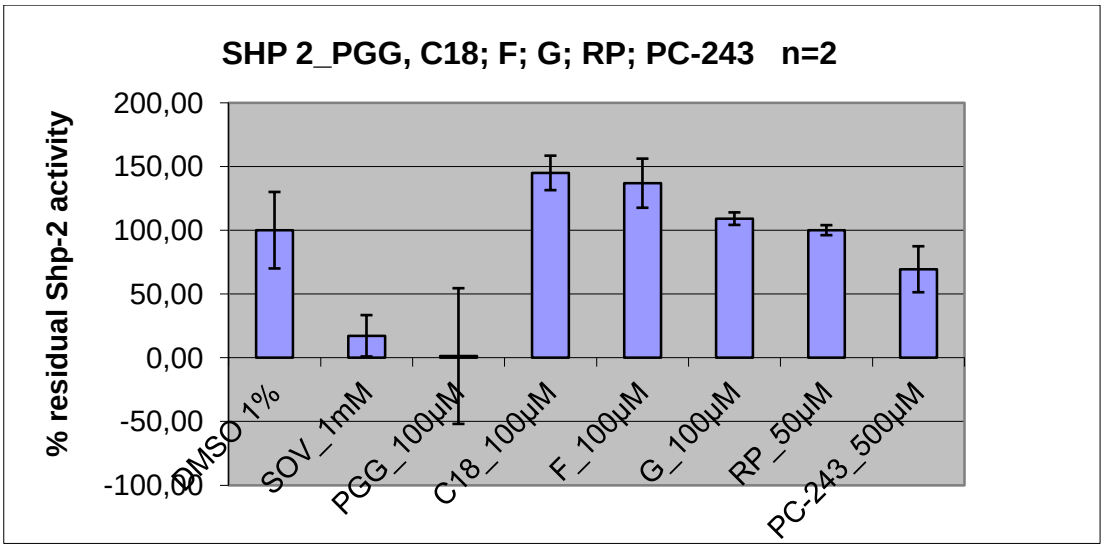


Figure 29: SHP2 inhibition assay with former tested PTP1B inhibitors

The results made clear that neither LAR nor SHP-2 were inhibited by one of those substances, even at high concentrations.

4.1.2 Western blots

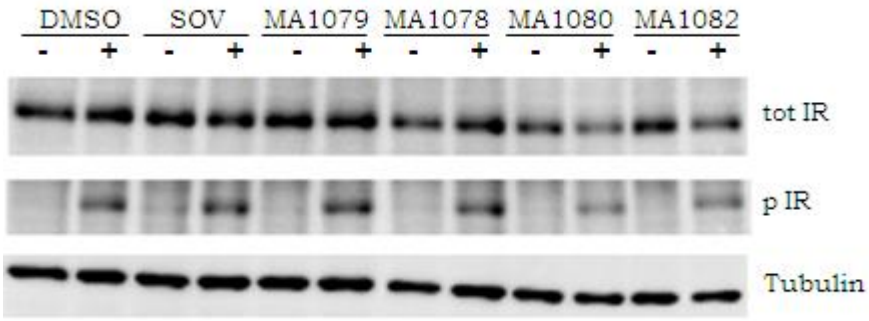
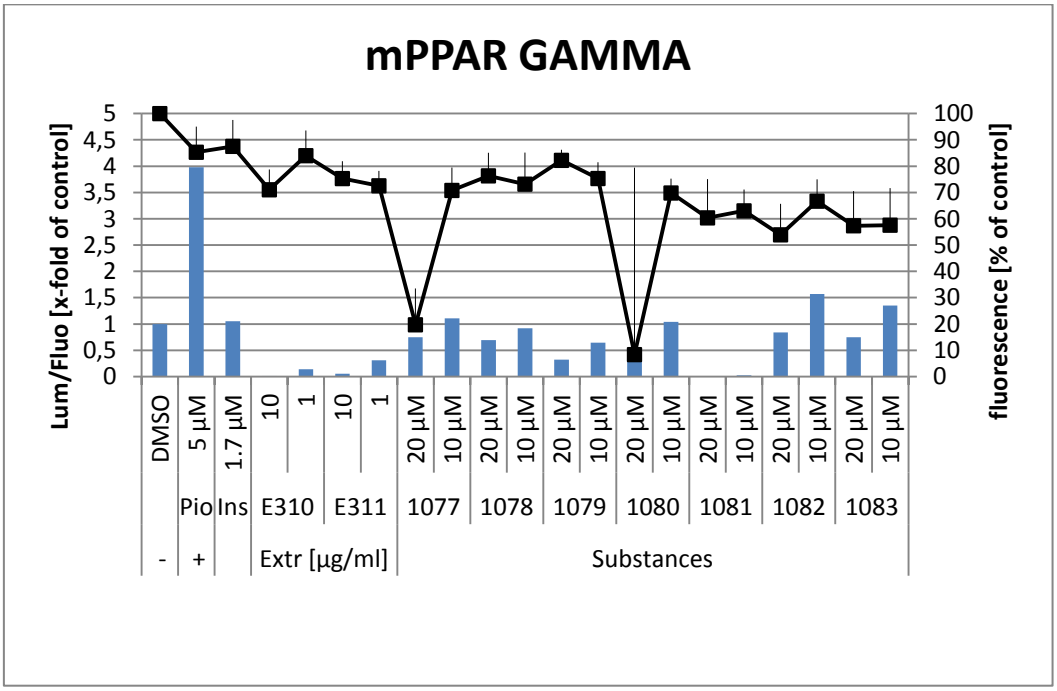


Figure 30: Representative western blot out of three experiments of insulin-dependent insulin receptor (IR) phosphorylation in HCC-1.2.
Cells were pre-incubated with *Morus alba*-compounds and stimulated with insulin 10 nM (+/-). DMSO 0.2 % was used as solvent control.

The aim of the western blot experiments was to examine whether the substances that showed PTP1B inhibitory effects in the cell-free system are equally effective in a cell-based assay. After stimulation with insulin (10 nM) the phosphorylation of the insulin receptor is stronger in cells treated with phosphatase inhibitors.

4.1.3 PPAR Transfection and Luciferase based assay

HEK 293 cells were seeded 6x10^6 per 10cm petridish and incubated over night. Transfection was performed using the calciumphosphate method. Then cells were re-seeded in 96 well plates and treated with *morus alba* substances again, over night. On the next day the supernatant is aspirated and cells are lysed with a luciferase assay lysis buffer. The cell lysates were transferred to a black 96-well plate. First, the fluorescence for EGFP (excitation 485nm, emission 535nm) is measured for the whole plate, then luminescence of each well is measured. Luminescence values were evaluated by subtracting background measurements (no transfection) and divided by their respective EGFP fluorescence values for normalization.



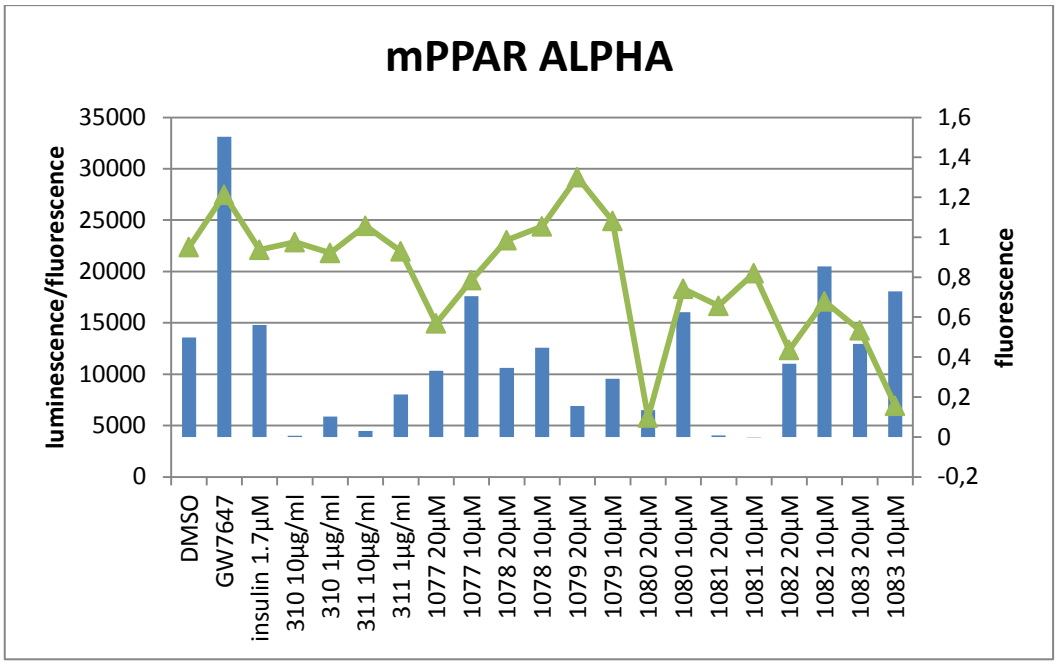


Figure 31: Evaluation of luciferase based PPAR α/γ assay
The bar diagram represents PPAR-activity (luminescence, the more luciferase is expressed, the higher the activity of PPARs); the line diagram shows EGFP fluorescence (normalization; indicator of transfection success).

None of the substances shows significant values of PPAR activation. 1081 contains a substance with is inhibiting the luciferase itself, as direct addition of 1081 to untreated but transfected cell lysate shortly before measurement completely blocks the luciferase signal (tested by Matthias Kramer).

4.1.4 Summary

Substances that are known PTP1B inhibitors were tested on TCPTP, LAR and SHP-2, to see if they also show inhibitory activity on those phosphatases. Then different fractions of *Morus alba* in different concentrations were tested on the same enzymes.

First all the fractions and pure compounds of *Morus alba* were tested on their possible inhibitory effect on PTP1B. The promising hits were further tested for their IC50, which is a measure for the effectiveness of a substance to inhibit a biological process or component of a process such as a protein. The value of this IC50 indicates the half maximal inhibitory concentration of the substance. For PTP1B we want this value to be as small as possible, making the inhibitors very strong. For the other phosphatases the IC50 value of the same compound should be significantly higher.

LAR and SHP-2 were put on a test run with former known PTP1B inhibitors. Data showed that neither LAR nor SHP-2 were inhibited by the substances that blocked PTP1B and, at significantly higher concentrations, TCPTP.

Phosphatase Inhibitor	PTP1B	TCPTP
Sanggenon D (1078)	5,8 µM	12 µM
Sanggenon C (1080)	3,856 µM	11,07 µM
Kuwanon L (1079)	9,347 µM	10,47 µM
Sanggenon B (1082)	13,85 µM	27,47 µM

Table 12: Summary of IC50 values
Comparison of the IC50 values of the most promising pure compounds for inhibiting PTP1B and TCPTP. Three of four compounds show viable data, Kuwanon L most probably is not able to inhibit PTP1B without blocking TCPTP at the same time.

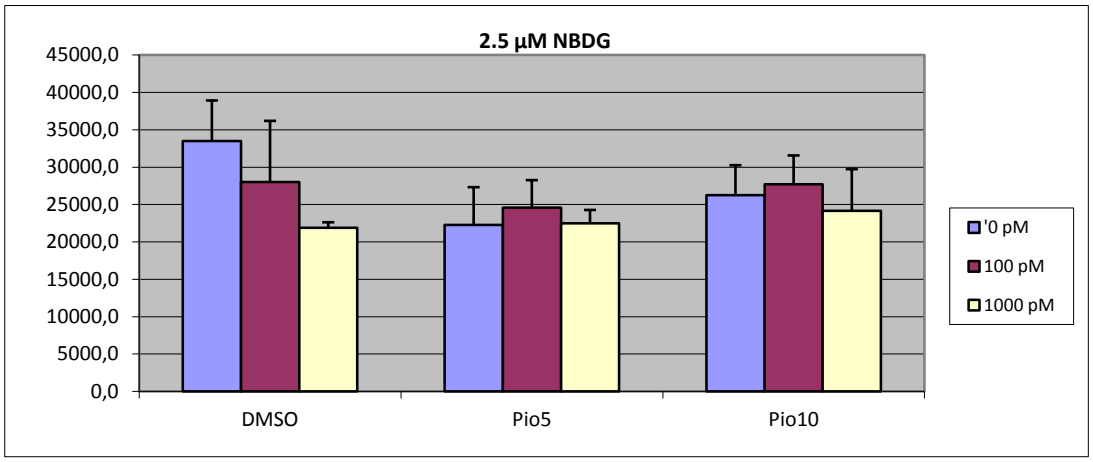
The comparison of the IC50 values makes clear that the three *Morus alba*-compounds Sanggenon D, C and B are promising candidates for

further investigation because they are able to inhibit PTP1B without affecting TCPTP activity. In the future also other members of the PTP-family should be assayed because they are needed for many important functions in different signal transduction pathway which must not be blocked by the PTP1B inhibitors. Also more experimental data of the compounds would be needed concerning their chemical properties for example toxicity or other side effects.

4.2 The Glucose Uptake Measuring Approach

4.2.1 Establishing the 6-NBDG assay

We used fully differentiated 3T3-L1 adipocytes and treated them with a known insulin sensitizer (pioglitazone) in different concentrations to establish an assay for testing other substances on their ability to increase glucose uptake. After stimulation with insulin, also in different concentrations, there should be a significant increase in the uptake of 6-NBDG measurable.



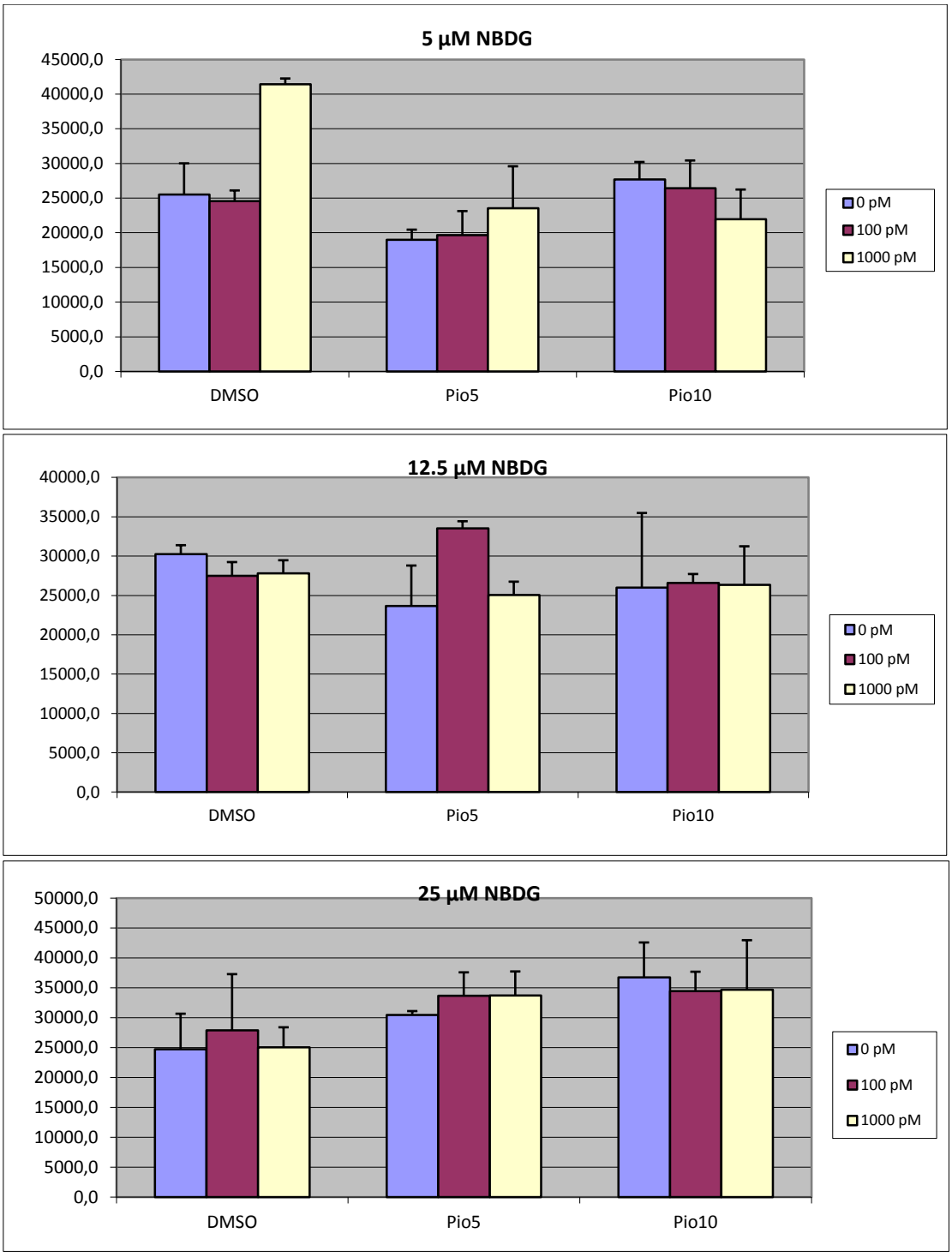


Figure 32: Evaluation of 6-NBDG-Uptake measurement
3T3-L1 cells were pretreated with insulin sensitizers (DMSO, Pioglitazone 5 µM, Pioglitazone 10 µM), then stimulated with insulin in different concentrations (0 pM, 100 pM, 1000 pM) and treated with 6-NBDG in different concentrations (2.5 µM, 5 µm, 12.5 µM, 25 µM)

With raising concentrations of pioglitazone and insulin the glucose uptake should increase significantly. The results of the pioglitazone-treated cells differ negligible from the DMSO control

4.2.2 Summary

To measure the glucose uptake in adipocytes the cells must be fully differentiated. Because the pre-adipocytes enlarge as they mature the differentiation of the cells in the 96 well format was difficult. Staining of the wells with oil red o, a dye used to make lipid droplets in adipocytes visible, showed uneven results. We concluded from that, that the differentiation in 96 well plates is not optimal with the 3T3L1 adipocytes in our hands, hence the resulting data of the glucose uptake measurement is not significant. The glucose values should rise with increasing pioglitazone concentration. The experiment was repeated twice, the protocol was altered and improved but the results were similar.

We reached the conclusion that adipocytes can not be differentiated properly in 96-well plates because of lacking space. To build up the fat droplets characteristic for fully differentiated adipocytes, the wells must be larger. As number and condition of the cells must be the same in every well to gain viable results with the glucose uptake assay, 96-well plates are not the tool of choice. Further investigations in a more consistent differentiation protocol in this format would be needed.

5 Appendix

5.1 Protocols

5.1.1 The Enzyme Inhibition Approach

5.1.1.1 Protocol PTP1B Assay

Material needed:

MOPS Buffer 50 mM, pH 6.5 "3-(N-Morpholino)-propanesulfonic acid"

Para-Nitrophenylphosphate „pNPP" in -20°C (Mr= 209.2633 g/mol)

"Dithiothreitol "DTT" 1 M, 100 µL for one plate (frozen aliquots in -20°C)

PTP1B human recombinant enzyme, concentration 0.025 µL/well. E.g. aliquots 0,025 µg in 5 µL in one well, for one lane (1-12) 60 µL, frozen in -80°C

Negative control (same as solvent, e.g. DMSO at concentration in which the substances will be used, max. 1% ! DMSO has an effect on this assay)

Positive control (e.g. Ursolic acid „UA" 3-30 µM, Sodium Orthovanadate „SOV" 10 µM)

NaOH 10 M (!)

Substances

96 well plate

Falcons

Warming hood at 30°C

UV-VIS for 96-well format

Preparation of samples and controls:

Preparation of assay buffer – 2 mM pNPP in MOPS 50 mM pH 6.5:

Store all buffers protected from light! (aluminium foil over falcon!)- do always prepare freshly!!!

Solve 21.91 mg(!!) pNPP in 5 mL MOPS buffer = 20 mM stock

Dilute this stock 1:5 in MOPS buffer (e.g. 1 mL of this stock + 4 mL buffer)

Add 100 µL DTT 1 M to pNPP-MOPS buffer

Workflow:

The assay is started by adding 50 µl/well pNPP-MOPS with 8-channel multi-pipette in wells: whole volume in one well is 100 µL: pipet 50 µL of pNPP-MOPS buffer per well.

Make 2x concentrated pre dilution of the substances and the controls in MOPS buffer and pipette 45 µl/well, in the wells "without enzyme" pipette 50 µl/well.

take PTP1B aliquot out of -80°C and lay on ice. Take a small glass with ice for better handling. Let enzyme thaw on ice, don't touch it too long with your fingers, for pipetting put it in the glass with ice to hold it. Pipet quickly 5 µL per well (one lane→ second lande ←)

shake plate gently with your fingers, put covered plate on 30°C for 30 min.

pipet 25 µL 10 M NaOH on each well, shake and measure in UV-VIS

5.1.1.2 Protocol TCPTP Assay

Material needed:

MOPS (3-morpholinopropanesulfonic acid) buffer 50 mM, pH 6.5

para-Nitrophenylphosphate (*p*NPP) in -20°C

Dithiothreitol (DTT) 1 M (frozen aliquots in -20°C)

TCPTP human recombinant enzyme, concentration 0.015 µg/well. E.g. aliquots 0.015 µg in 5 µL in one well, for one lane (1-12) 60 µL, frozen in -80°C

Negative control (same as solvent, e.g. DMSO at concentration in which the substances will be used, max. 0.5 % ! DMSO has an effect on this assay)

Positive control (ursolic acid 30 µM, sodium orthovanadate 100 µM)

NaOH 10 M

Substances

96-well plate

Falcon tubes, microfuge tubes

UV-VIS for 96-well format

Preparation of samples and controls:

Make concentrated predilutions of the substances and the controls in MOPS buffer.

Preparation of *p*NPP-MOPS buffer – 4 mM pNPP in MOPS 50 mM, pH 6.5. Store all buffers light-free (aluminium foil over falcon!), do always prepare freshly!!! Solve 37.11 mg (!!) *p*NPP in 5 mL MOPS buffer = 20 mM stock. Dilute this stock 1:5 in MOPS buffer (e.g. 1 mL of this stock + 4 mL buffer). Add 50 µL DTT 1 M to 5 mL *p*NPP-MOPS buffer. Final concentration 2 mM pNPP in MOPS.

Workflow:

Pipet 45 µl/well of the concentrated predilutions of the substances, in the wells "without enzyme" pipet 50 µl/well.

Take TCPTP aliquot out of -80°C and lay on ice. Take a small glass with ice for better handling. Let enzyme thaw on ice, do not touch it too long with fingers, for pipetting put it in the glass with ice to hold it. Pipet quickly 5 µL per well (one lane→ second lane ←).

Pipet 50 µL of *p*NPP-MOPS buffer per well.

Put the plate on a shaker for 5 seconds. Put plate on in Sunrise™ and measure a kinetic of 11 cycles every 3 minutes.

Pipet 25 µL 10 M NaOH on each well, shake the plate in the microplate reader (for 5 seconds, stop 20 seconds before, middle) and measure the plate (405 nm).

5.1.1.3 Protein quantification according to Bradford

Workflow:
- prepare standard solutions ranging from 0 to 0.5 µg/µL
- place 10 µL of standards or 10 µL ddH2O in 96 well plate in triplicates
- add 1 to 3 µL of samples to water wells, and the same amount of lysis buffer to standards
- dilute Bradford reagent according to manual
- add reagent and incubate for 5 minutes
- measure absorption at 595 nm
- calculate protein concentration with the standard curve

5.1.1.4 PPAR Transfection and luciferase assay

Workflow:

• HEK293 cells are seeded at 6x104/cm2 in DMEM/FCS 10 %

• also seed cells for background measurements

• after 6 hours: add Transfection Mixture drop-wise to the cells

• incubate cells over night in incubator

• reseed cells in 96-well plate (5x104/well) in 50 µL

• include one column with untransfected cells

• after 6 hours: treat cells with solvent and compounds over night

• remove supernatant on measure plates

• optional: plates can be stored at -80 .after supernatant is removed

• prepare luciferase lysis buffer

• thaw ATP and Luciferin buffers

• wash and fill injector system of plate reader

• lyse cells with 50 µL lysis buffer for 10 minutes at plate shaker

• transfer 40 µL into a black 96-well plate

• start measurement protocol

– fluorescence measurement of whole plate (excitation 485 nm, emission 535 nm)

– luciferase measurement protocol

* ATP and Luciferin buffers are injected into well (100 µL each)

* after 100 miliseconds the luminescence signal is measured

* next well is injected and measured

5.1.2 The Glucose Uptake Measuring Approach

5.1.2.1 3T3-L1 Cultivation and Differentiation

Cultivation: use DMEM/NBS

Size	3 days	4 days
T75	$12x10^4$	$7x10^4$
T175	$45x10^4$	$30x10^4$

Harvest:

Size	maximum
T75	$3.75 – 4.50 \times 10^6$
T175	$8.75 – 10.5 \times 10^6$

Split cells well before reaching confluence

Differentiation:

Seed cells (Day -4)
6-well: $10 – 20 \times 10^4$
12-well: $5 – 10 \times 10^4$
24-well: 4×10^4

Grow cells to confluence, wait 2 more days, use DMEM/NBS (day -2)
Day 0:..... mc use (D)
Day 2:..... mc use (D)
Day 4:..... mc use (+)
Day 6:..... mc use (-)
Day 8/10/12: ... mc use (-)
Cells are fully differentiated: lipid droplets visible

Medium:

3T3-L1 cultivation medium: (N) DMEM/NBS

DMEM white

10% NBS (newborn bovine serum)

(Pen/Strep)

Glutamine

3T3-L1 differentiation medium: (D) DMEM/FCS

DMEM white

10% FCS (fetal calf serum)

(Pen/Strep)

Glutamine

1 µg/ml Insulin

500 nM Dexamethasone

500 µM IBMX

Adipocyte cultivation medium: (+)/(-)

DMEM white

10% FCS

(Pen/Strep)

Glutamine

+/- 1 µg/ml Insulin

5.1.2.2 Oil Red-O Staining

Workflow:

- Remove medium
- Wash cells 1x with PBS (200 µl/well)
- Add 10% formaldehyde, incubate 5 minutes at RT (100µl/well)
- Remove formaldehyde (toxic waste!)
- Add 10% formaldehyde, incubate at least 1 hour (100 µl/well)
- Prepare Oil Red O working solution
- Remove formaldehyde
- Wash cells 1x with 60% isopropanol
- Dry wells completely
- Add 150 µl Oil Red O working solution, incubate 10 minutes at RT
- Remove Oil Red O working solution
- Wash wells 3-4x with water (200µl/well)
- Take pictures
- Add 150 µL 100% isopropanol to elute dye (optional: shaker)
- Pipet 100 µL in transparent plate (isopropanol as blank)
- Measure absorption (OD) at 492nm

<u>Solutions</u>

Oil Red O stock:

0.7g Oil Red O, dissolved in 200 mL isopropanol

Stir over night, filter through 0.2 µm filter

Store at +4°C

Oil Red O working solution:

Dilute stock solution 6:10

Mix, let sit at least 20 minutes at RT

Filter through 0.2 µm filter

5.1.2.3 6-NBDG Uptake Assay

Pretreatment

-perform medium change on day before assay

-use DMEM/10% FCS± drugs

48 hrs; 200µl/well

Serum step

Wash cells 1x with DMEM/0.5% FCS

Medium change: use DMEM/0.5% FCS ± drugs

200 µL/well

Incubate for 3 hours

(prepare solutions for glucose step and stimulation)

Glucose step

Wash cells with KRH/ 1% BSA

Medium change: KRH/ 1% BSA ± drugs

100µl/well

Incubate 1 hr

(prepare solutions for lysis (on ice) and DOG Uptake, switch on heating hood)

Stimulation

Perform medium change to KRH/1% BSA ± insulin

50 µL/well

Stimulate for 15 minutes

(prepare ice col PBS in beaker glass and steppe, check fluid waste bottle)

DOG Uptake

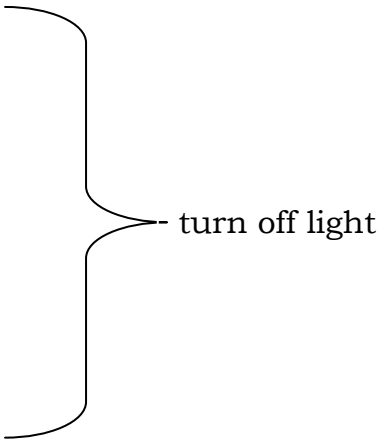
Add 50 µL/well

Incubate for 30 minutes

Add 100 µL PBS

Stop reaction by removing supernatant

Wash cells 3x with ice cold PBS (100 µL/well)



Lysis

Add 60 µL lysis buffer to each well

Incubate 10 minutes on ice (shaker optional)

Readout

Transfer 50 µL to black plate

Measure fluorescence on TecanGeniosPro at 485/520 nm

Bradford

5.1.2.4 6-NBDG Uptake Assay (modif.)

PRETREATMENT

Medium change 2 days before assay

DMEM/10% FCS+ drugs

48 hrs; 200µl/well

GLUCOSE STEP

Wash cells with KRH/ 1% BSA

Medium change: KRH/ 1% BSA + drugs

50µl/well

Incubate 3-4 hrs

STIMULATION (no washing, no aspiration)

add KRH/ 1% BSA + insulin (0 nM/ 1 nM/ 100 nM) (→ use 3 fold concentration!)

50µl/well

Stimulate 15 min

GLUCOSE UPTAKE (no washing, no aspiration)

add KRH/ 1% BSA + NBDG (0 µM/ 50 µM/ 75µM/ 100µM) (→ use 3 fold concentration!)

50µl/well

Incubate 2 hrs

Add ice cold PBS 100µl/well and remove supernatant

Wash cells 2x with ice cold PBS 100µl/well *(carefully!)*

LYSIS

Add 50µl/well lysis buffer (or luciferase lysis buffer)

Incubate 10 min on shaker

READOUT

Transfer 40µl/well in black plate

Measure fluorescence on TecanGeniosPro at 485/520 nm

Bradford

5.2 List of figures

5.3 List of tables

5.4 Abbreviations

A

ATP	Adenosintriphosphate
ATP III	Third Report of the National Cholesterol Education Program´s Adult Treatment Panel

B

BSA	bovine serum albumin

C

C18	Octadecandioic acid
C22	Docosandioic acid
$CaCl_2$	Calcium chloride
CHO	Chinese hamster ovaries
CVD	Cardio vascular disease

D

DAQ	Demethylasterriquinone
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethyl Sulfoxide
DOG	Deoxyglucose
DTT	Dithiothreitol

E

EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid

F

F	Folic acid
FCS	Fetal calf serum

G

G	Gluconapin
GLUT4	Glucose transporter 4

H

HCl	Hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

I

IBMX	3-isobutyl-1-methylxanthine
IC50	Half maximal inhibitory concentration
IDF	International Diabetes Federation
IR	Insulin receptor
IR3P	Phospho-Insulin receptor
IRS-1	Insulin receptor substrate-1

K

KCl	potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KRH	Krebs-Ringer-Hepes Buffer

L

LAR	Leukocyte antigen-related protein tyrosine phosphatase

M

MA 1078	Morus alba extract number 1078
MA 1079	Morus alba extract number 1079
MA 1080	Morus alba extract number 1080
MA 1082	Morus alba extract number 1082
$MgCl_2$	Magnesium chloride
MOPS	3-(N-Morpholino)-Propanesulfonic acid

N

Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBDG	Nitrobenz oxa diazol yl amino Deoxyglucose
NBS	Newborn bovine serum
NIDDM	Non-insulin dependent diabetes mellitus

O

ÖDG	Austrian Diabetes Association

P

PC 0243	Oleic acid (Phellodendri cortex extract number 0243)
PC 242	Phellodendri cortex No 242
PGG	Pentagalloylglucose
pIR	Phospho insulin receptor
pNPP	Para-Nitrophenylphosphate
PPAR γ/α	Peroxisome proliferator-activated receptor γ/α
PPRE	PPAR response element
PR 238	Paeonia radix compound No 238
PTP	Protein Tyrosine Phosphatase
PTP1B	Protein Tyrosine Phosphatase 1B

R

RP	Riboflavin-5‘-phosphate

S

SHP-2	SH2 domain-containing nonreceptor protein tyrosine phosphatase
SOV	Sodium ortho vanadate

T

T2DM	Type 2 Diabetes Mellitus
TCPTP	T-cell protein tyrosine phosphatase
Tub	Tubulin
TZD	Thioazolidinediones

U

UA	Ursolic acid

W

WHO	World health organization

5.5 References

1. Saltiel, Alan R.: insulin signaling and the regulation of glucose and lipid metabolism; Nature. 2001 Dec 13;414(6865):799-806.

2. Asante-Appiah, Ernest, and Kennedy, Brian P.; Protein Tyrosine Phosphatases: the quest for negative regulators of insulin action. Am J Physiol Endocrinol Metab 284, 2003

3. Edelman SV, Type 2 Diabetes Mellitus. Division of endocrinology and metabolism; advances in internal medicine, 1998

4. Wubben, D.P. et al.: Metabolic Syndrome: What´s in a name? Wisconsin medical journal, 2006

5. Reaven, GM.: Banting lecture 1988. Role of insulin resistance in human disease; Diabetes.1988 Dec; 37 (12):1595-607

6. Grundy, SM. et al.: Definition of metabolic syndrome. American heart association, 2004

7. Wilcox, G.: Insulin and Insulin Resistance; Clin Biochem Rev, Vol. 26 May 2005

8. Tritos, NA.: Clinical review 97. Syndromes of Severe Insulin Resistance; Journal of Clinical Endocrinology and Metabolism 1998, Volume 83

9. Shoelson, SE.: Inflammation and insulin resistance; The Journal of Clincal Investigaion, Vol. 116, No 7, July 2006

10. Krook, A. et al.; Sending the signal: Molecular mechanisms regulating glucose uptake. Med. Sci. Sports Exerc., 2004

11. White, M.F.: Insulin signaling system and the IRS proteins. Diabetologica 1997

12. Yokashi Kido, Jun Nakae and Domenico Accili: Clinical Review 125; The Insulin Receptor and its Cellular Targets. The Journal of Clinical Endocrinology & Metabolism, 2001

13. Goalstone, ML.: Insulin Signaling. West J Med, 1997

14. Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J: *Harrison´s Principles of Internal Medicine*, 17th Edition

15. Galic, S. et al.: Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP. Molecular and cellular biology, 2005

16. Tonks, Nicholas K.: Protein Tyrosine Phosphatases: from genes, to function, to disease. Nature reviews; molecular cell biology, volume 7, November 2006

17. Larsen, M.: Phosphatases in cell-matrix adhesion and migration; Nature reviews, Molecular Cell Biology 4, 2003

18. Goldstein, Barry J. et al.; Regulation of the insulin signaling pathway by cellular protein-tyrosine phosphatases. Molecular and cellular biochemistry, 1998

19. Zhang, Wei et al.; Ursolic acid and its derivative inhibit protein tyrosine phosphatase 1B, enhancing insulin receptor phosphorylation and stimulating glucose uptake. Biochimica et Biophysica acta, 2006

20. Goldstein, B. J.: Regulation of insulin action by protein-tyrosine phosphatases; Diabetes Mellitus: A fundamental and clinical text, 3rd edition, 2004

21. S. Koren and I. George Fantus: Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type 2 diabetes mellitus. Clinical endocrinology and metabolism, December 2007

22. Galic, S. et al.: Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. Molecular and cellular biology, 2003

23. Blanco, FM; Flora de Filipinas. 1877-1880

24. Panickar, KS.: Beneficial effects of herbs, spices, and medicinal plants on the metabolic syndrome, brain, and cognitive function; Cent Nerv Syst Agents Med Chem. 2012 Oct. 17

25. Yang, Yan et al.; Four new 2-Arylbenzofuran derivatives from leaves of *morus alba* L. Chem. Pharm. Bull. 2010

26. http://health-from-nature.net/Mulberry,White.html, dated 06/22/2013

27. PubChem Compound Database; CID=184877

28. PubChem Compound Database; CID=442458

29. PubChem Compound Database; CID=44559963

30. PubChem Compound Database; CID=442459

31. www.pjk-gmbh.com/103.html, dated 09/04/2013

32. Barros, LF et al.: Kinetic validation of 6-NBDG as a probe for the glucose transporter GLUT1 in astrocytes. J. Neurochem 2009, 109 Suppl 1:94-100

33. Zou, Chenhui et al.: 2-NBDG as a fluorescent indicator for direct glucose uptake measurement; J. Biochem. Biophys. Methods 64, 2005

34. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, vol. 72, pp. 248-254,May 1976.

5.6 Curriculum vitae

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