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Targets and Off-Targets of Kinase Inhibitors in Diabetes

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ABSTRACT

Various tyrosine kinase inhibitors (TKIs) developed for the treatment of cancer have been reported to exert a protective effect in diabetic patients, which manifested in improved glycemic control and insulin sensitivity, favorable regulation of lipid metabolism and increased insulin secretion. In our studies we focused on the insulinotropic property of sunitinib and inspired by the TKI associated clinical investigations our aim was to discover kinase targets and better compound analogues that on one hand can help us to understand the mechanism how these TKIs induce insulin secretion, furthermore could open a new field for the development of anti-diabetic therapy. We assembled a library of 558 compounds for a phenotypic screen in order to look for candidates having insulinotropic effect. The members of this compound library were rationally picked and built upon similarity with the target profile of sunitinib. By insulin ELISA assay we could identify 55 compounds that effectively stimulated insulin secretion in RIN-5AH beta cells. These compounds could be clustered into different core structure families and for some of them we were able to set up qualitative structure activity relationships (SAR). Ten active compounds with 8 different core structures were profiled for their kinase affinities and screened on a kinase panel of 392 kinases. Four of these compounds were validated by affinity chromatography followed by mass spectrometry, wherein RIN-5AH beta cell lysates and special linker derivatives of the active compounds coupled to affinity matrices were employed. We hypothesized that there are common targets for these compounds which upon inhibition can induce insulin secretion. We identified three kinases by siRNA that induced insulin release in RIN-5AH and INS-1E beta cell lines. The compounds were also investigated for other potential mechanisms, including cAMP or Ca²⁺ level upregulation, membrane potential changes and related pathways. We have found and characterized interesting candidate compounds, furthermore based on the available SAR and assay results we could conclude that kinase inhibitor molecules can be optimized for insulinotropic effect. These observations also support the fact that we should not ignore the blood glucose lowering effect of some small molecule kinase inhibitors used in cancer therapy.

ABBREVIATIONS

| | |
|-------------------|---|
| 2-NBDG | 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose |
| ACS | Acyl Coenzyme Synthase |
| ADME | Absorption Distribution Metabolism Excretion |
| AMPK | 5'-AMP - Activated Protein Kinase |
| ATP | Adenosine Triphosphate |
| AUC | Area Under The Curve |
| BCA | Bicinchoninic Acid |
| BOC | Tert-Butyloxycarbonyl |
| CAMK2 | Calcium/Calmodulin-Dependent Protein Kinase II |
| cAMP | Cyclic Adenosine Monophosphate |
| CC | Current Clamp |
| CPD | Compound |
| CPT-1 | Carnitine-Palmitoyl Transferase |
| CREB | Camp Responsive Element Binding Protein |
| CSNK2A1 | Casein Kinase 2, Alpha 1 Polypeptide Pseudogene |
| DAO | Diazoxide |
| DNTP | Deoxynucleotide Triphosphates |
| DPP-4 | Dipeptidyl Peptidase-4 |
| DTT | Dithiothreitol |
| EC ₅₀ | Median Effective Concentration |
| ECL | Enhanced Chemiluminescence |
| EFD | Efonidipine |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ERK | Extracellular Signal-Regulated Kinase, Mitogen-Activated Protein Kinase |
| FACS | Fluorescence Activated Cell Sorting |
| FADH ₂ | Flavin Adenine Dinucleotide |
| FFA | Free Fatty Acid |
| FLT3 | Fms-Related Tyrosine Kinase 3 |
| G6PASE | Glucose 6-phosphatase |
| GBA | Glibenclamide |
| GDH | Glutamate Dehydrogenase |
| GDIS | Glucose Dependent Insulin Secretion |
| GIP | Glucose-Dependent Insulinotropic Polypeptide |
| GLP-1 | Glucagon-Like Peptide-1 |
| GLUT | Glucose Transporter |
| GPCR | G Protein-Coupled Receptor |
| GSIS | Glucose Stimulated Insulin Secretion |
| HBSS-- | Hank'S Balanced Salt Solution Without Ca ²⁺ And Mg ²⁺ |
| HBSS++ | Hank'S Balanced Salt Solution With Ca ²⁺ And Mg ²⁺ |
| HFD | High Fat Diet |
| HRP | Horseradish Peroxidase |
| IBMX | 3-Isobutyl-1-methylxanthine |

| | |
|------------------|--|
| IC ₅₀ | Median Inhibition Concentration |
| IP3R | IP3 receptor |
| JNK2 | Mapk9 - Mitogen-Activated Protein Kinase 9, Jun Kinase |
| KATP | Potassium Atp Channel |
| KRBH | Krebs Ringer Buffer With Hepes |
| MARK2 | Map/Microtubule Affinity-Regulating Kinase 2 |
| MAST1 | Microtubule Associated Serine/Threonine Kinase 1 |
| MCF | Metabolic Coupling Factor |
| MEK | Mapk Kinase |
| MOA | Mode Of Action / Mechanism Of Action |
| MS | Mass Spectrometry |
| MTOR | Mechanistic Target Of Rapamycin |
| MTT | Thiazolyl Blue Tetrazolium Bromide |
| NADH | Nicotinamide Adenine Dinucleotide |
| NaOV | Sodium Orthovanadate |
| NDP | Nifedipine |
| NHS | N-Hydroxysuccinimide |
| ON | Overnight |
| PDE | Phosphodiesterase |
| PEPCK | Phosphoenolpyruvate Carboxykinase |
| PIN | Pinacidil |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |
| PMSF | Phenylmethylsulfonyl Fluoride |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| ROS | Reactive Oxygen Species |
| RT | Room Temperature |
| SAR | Structure Activity Relationship |
| SGLT-2 | Sodium-Glucose Cotransporter 2 |
| SLK | Ste20-Like Kinase |
| STK16 | Serine/Threonine Kinase 16 |
| T1D/T1DM | Diabetes Type I |
| T2D/T2DM | Diabetes Type II |
| TBST | Tris-Buffered Saline With Tween 20 |
| TG | Thapsigargin |
| TI | Therapeutic Index |
| TKI | Tyrosine Kinase Inhibitor |
| TUM | Technical University München |
| TZD | Thiazolidinedione |
| VC | Voltage Clamp |
| VDCC | Voltage Dependent Calcium Channel |
| VER | Verapamil |

1 INTRODUCTION

1.1 Diabetes

Diabetes is a chronic disease and it shows an increasing incidence in the world. There are 347 million people worldwide diagnosed with diabetes¹ and globally 6% of deaths are caused by diabetes². The causes that result in diabetes are that either the beta cells can't produce enough insulin or the body can't use the hormone efficiently. Untreated diabetes can lead to serious side effects and consequences including increased risk of heart failure and stroke, retinopathy, nephropathy and neuropathy. Diabetes onset can be prevented or delayed with regular physical activity, healthy diet and maintaining normal body weight³.

1.1.1 Type I diabetes

Type I diabetes (insulin-dependent diabetes or juvenile diabetes, T1D) is an autoimmune disease, where beta cells are destroyed and insulin cannot be produced anymore. Typical symptoms of type I diabetes are: excessive thirst and hunger, unusually high levels of urination, extreme fatigue, body weight loss, and blurry vision. The first sign of development of autoimmunity is the presence of any of these antibodies associated with the disease: IA-2Ab, IAA, GAD65Ab, and ZnT8Ab. Later on the decrease of these antibodies may indicate the progression of T1D. The treatment of T1D is currently possible by insulin only.

1.1.2 Type II diabetes

Type II diabetes (non-insulin-dependent or adult-onset diabetes, T2D) occurs in 90% of the people diagnosed with diabetes. T2D is the result of ineffectively utilized insulin in the body. Symptoms are similar to T1D but the diagnosis can be more difficult because of the slow progression of the disease. Medically uncontrolled diabetes can lead to severe complications including neuropathy, ketoacidosis, nephropathy, high blood pressure, stroke and complication on skin and eyes. Therefore it is necessary to recognize the symptoms as early as possible. Oral glucose tolerance test (OGTT) is one of the possibilities to diagnose pre-diabetes or diabetes. The pre-diabetic state may indicate a risk for the development of T2D, which later on leads to beta cell dysfunction. Factors that can contribute to the progressive deterioration of the beta cells and insulin resistance include genetic⁴⁻⁶ and acquired (glucotoxicity, lipotoxicity, alterations in GLP-1 and GIP, small membrane permeant islet amyloid polypeptide formation,

pro-inflammatory cytokines) causes⁷⁻¹². Reactive oxygen species (ROS) are produced when cells are chronically exposed to glucose or fat, and their excessive accumulation can damage beta cells. This phenomenon often occurs in diabetes. Nevertheless ROS are necessary to maintain physiological function of beta cells as well. Not surprisingly it was also observed that the levels of antioxidant enzymes in pancreatic islets were lower compared to other tissues¹³. It is also proposed that not only the excessive ROS generation, but the insufficient oxidative redox potential and therefore decreased S-S bond formation can contribute to the development of T2D¹⁴. It is interesting to notice that in contrary to the inhibitory effect of physiological ROS scavenger proteins, the small molecule polyphenolic flavonoid antioxidants can activate Ca^{2+} channels and induce insulin release. These flavonoids may also improve beta cell health by regulating the expression of important survival genes in beta cells^{15,16}. Although there are a big variety of medicines available for the treatment of T2D, notwithstanding every therapy has its side effects and contraindications or inconvenience. Antidiabetic medication needs to be customized for every patient and combinations of drugs are sometimes necessary to sustain normoglycemia. Currently insulin secretagogues, insulin sensitizers and some other medications (alpha glucosidase inhibitors, incretin-based therapy, DPP-4 inhibitors and SGLT2 inhibitors) including insulin products are available for the treatment of type II diabetes.

1.2 General mechanisms of insulin secretion in beta cells

Beta cells are important fuel sensors of the body. Under normal physiological circumstances insulin secretion is regulated by the metabolism of carbohydrates, fatty acids and amino acids (*Figure 1.4.1*). During the process different metabolic coupling factors (MCFs) are produced (e.g. ATP, cAMP, NADH, Acyl-CoA, glutamate) that take part in the regulation of insulin secretion. The ATP is one of the most important that can directly modulate the activity of ATP dependent potassium channels (K_{ATP} channels) in beta cells. Pancreatic K_{ATP} channels have crucial role in the regulation of insulin secretion and membrane potential. They consist of a hetero-octamer structure, where four Kir6.2 pore forming units and four SUR1 regulatory subunits connect each other¹⁷. At elevated ATP concentration K_{ATP} channels are inhibited by binding ATPs at the Kir6.2 subunits and as a result it induces a shift in the membrane potential ($\Delta\Psi$)¹⁸⁻²⁰. This gives rise to the opening of voltage dependent calcium channels (VDCC) which leads to an increased intracellular calcium level, that stimulates exocytosis of insulin vesicles²¹. Besides inactivation of K_{ATP} channels, other factors and ion channels e.g. TRP channels might be involved to reach the necessary rate of membrane potential shift required for exocytosis^{22,23}. It is important to mention that glucose can also increase insulin secretion independently from

K_{ATP} channels. This is often referred as metabolic amplifying pathway and could be linked to the second phase of insulin secretion²⁴⁻²⁶. ATP is also needed for the generation of cAMP that is catalyzed by the adenylyl cyclase (AC) enzyme. AC can be stimulated by the activation of some G protein (G_s) coupled receptors (GPCRs) that are found in beta cells (e.g. GLP-1 receptor, beta adrenergic receptor, GPR119) or inhibited by G_i coupled GPCRs (e.g. ADRA2A, GALR1, SSTR)²⁷⁻²⁹, additionally other regulators of AC (e.g. glucose, CAMK2) also exist³⁰⁻³². The negative regulation and degradation of cAMP is mediated by the phosphodiesterase (PDE) enzymes. The increased intracellular cAMP level potentiates exocytosis through its effector molecules, PKA and Epac2. PKA can phosphorylate various substrates related to exocytosis (e.g. phosphorylation of SNAP-25, Snapin, K_{ATP} , and VDCC). Epac can initiate the Epac-Rap-1-Raf-MEK-ERK pathway, furthermore it probably activates small GTPases such as Cdc42 and Rac which regulate cytoskeleton realignment therefore promote vesicle transport. Similarly to PKA it is also connected to intracellular calcium mobilization through IP3R. Sulphonylureas may also act in an Epac sensitive way; however the direct mechanism is not clarified yet³³⁻³⁷. Free fatty acids (FFA) enter the cells by diffusion and they are converted to long chain acyl-CoA in the cytosol. Accumulation of long chain acyl-CoA in the cytoplasm can acutely stimulate Ca^{2+} influx and insulin exocytosis, furthermore may assist secretory vesicle fusion with the plasma membrane³⁸⁻⁴⁰. During beta-oxidation (fasting state) it is degraded to acetyl-CoA, NADH and FADH2 in the mitochondria. The generated NADH and FADH2 will be used in the electron transport chain (oxidative phosphorylation) to generate ATPs, while the acetyl CoA can join the TCA cycle, where additional NADH is generated. Glycolysis and lipid signaling can intercross (FA-CoA and G3-P) and send further exocytotic related signals (FFA, Munc13-1 activation, PKC, PKD)⁴¹. It is worth to mention that a loss function of HADH (Hydroxyacyl-CoA, the enzyme in beta oxidation that catalyzes the transformation of hydroxyacyl-CoA to ketoacyl-CoA) is associated with hyperinsulinemia⁴²⁻⁴⁵. FFAs can also stimulate GPR40, hence induce IP3/DAG/PKC pathway which also leads to the release of insulin^{46,47}. In the case of amino acid induced secretion, glutamine is transported into the cell and converted to glutamate, then to α -ketoglutarate (by glutamate dehydrogenase, GDH). Afterwards α -ketoglutarate enters TCA cycle and participates in the generation of NADH. The importance of this process was confirmed by inspecting the gain of function mutations of GDH in animal models and in patients harboring the mutant form, which caused hyperinsulinemic hypoglycemic syndrome, triggered by protein meals⁴⁸⁻⁵⁰. Leucine induced insulin secretion can happen due to activation of GDH and the production of its metabolite, α -ketoisocaproate which enters TCA cycle as well. Reportedly, Leu induced hypoglycemia was only observed in patients

harboring HADH mutations, but not in the control group⁵¹⁻⁵³. Arginine as it has a cationic character at physiological pH, after entering the cell, it induces insulin secretion directly through membrane depolarization. It is also often used as a control substance in clinics to test beta cell function and capacity^{54,55}. Alanine and glycine induced insulin secretion can be attributed to the co-transport with Na⁺ and the consequent changes in membrane potential similarly to arginine⁵⁶⁻⁵⁸.

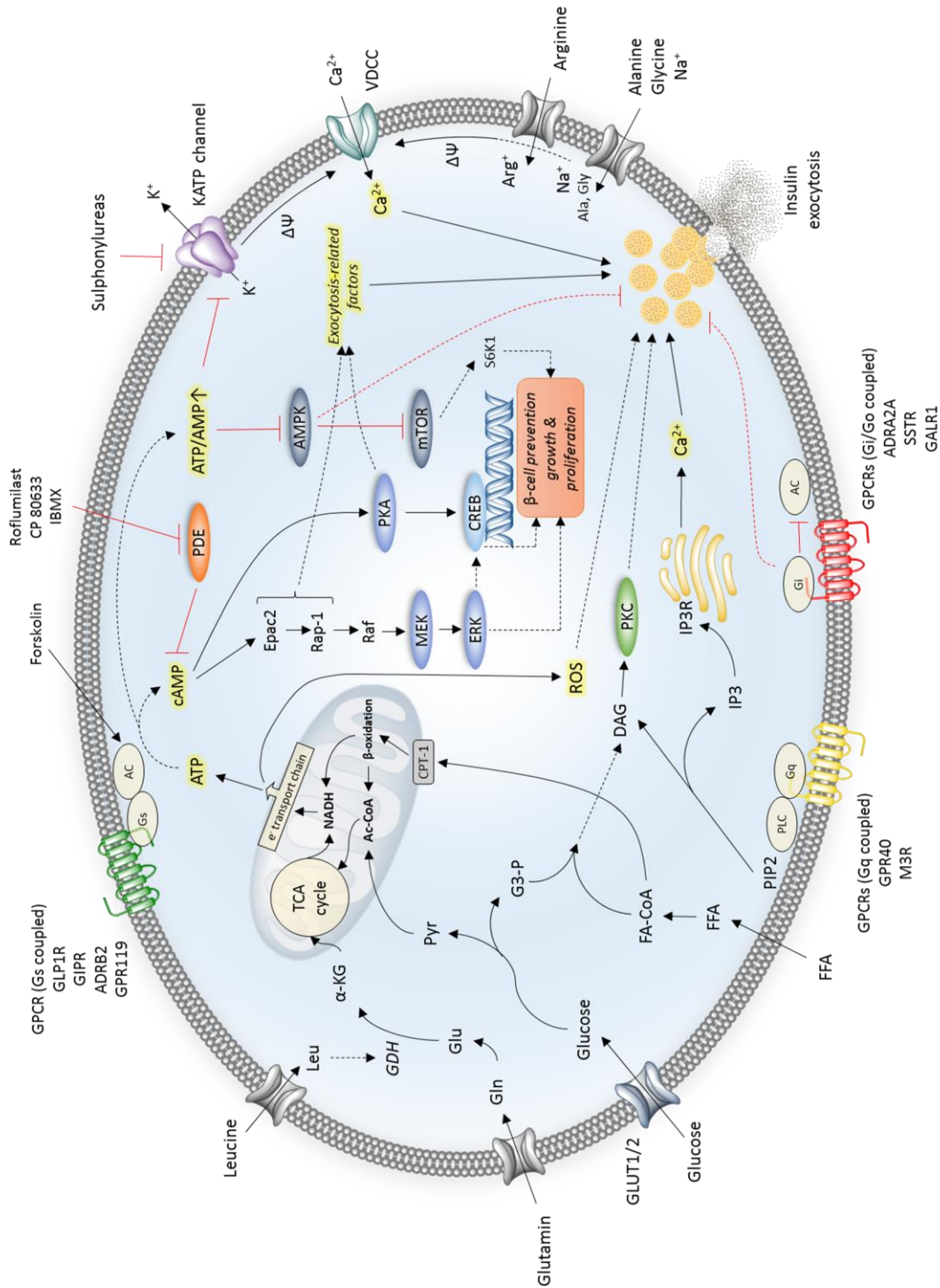


Figure 1.4.1 Schematic view of a beta cell, including metabolic pathways and some investigated elements related to insulin secretion. Effector molecules are highlighted with yellow. Please find the detailed description in the text. α -KG (α -ketoglutarate); ADRA2A (α adreneg receptor); ADRA2A (α adreneg receptor); DAG (diacyl-glycerol); FFA (free fatty acid); FA-CoA (fatty acid CoA); G3-P (glycerol-3P); GALR1 (Galanin receptor); GDH (Glutamate dehydrogenase); Gln (glutamine); GLP1R (GLP-1 receptor); Glu (glutamate); GLUT1/2 (glucose transporters GLUT1 in human, GLUT2 in rodents); IP3 (inositol 1,4,5-trisphosphate); M3R (muscarinic acetylcholine receptor); PDE (Phosphodiesterase); Phosphatidylinositol 4,5-bisphosphate (PIP2); Pyr (Pyruvate); ROS (reactive oxygen species); TCA cycle (citric acid cycle/Krebs cycle); VDCC (Voltage dependent Ca²⁺ channel); $\Delta\Psi$ (membrane potential)

1.3 Medicaments used in the therapy of diabetes

1.3.1 Insulin secretagogues

1.3.1.1 *Sulfonylureas (SU)*

The insulin secretagogue sulfonylurea drug family inhibits K_{ATP} channels found in beta cells (Kir6.2/SUR1) by binding with high affinity to SUR1 regulatory subunit. Nevertheless they have a low affinity binding site on Kir6.2 subunit as well. Blocking K_{ATP} channels induces membrane depolarization which promotes Ca^{2+} influx into the cells. The increased intracellular Ca^{2+} level stimulates the exocytosis of insulin vesicles. Interestingly certain SU may also directly activate Epac2, an important protein that senses cAMP level and regulate PKA independent insulin secretion⁵⁹. By application of these drugs there is an increased risk of hypoglycemia, therefore it is advised to take them during meal. Weight gain is also associated with the long term usage of these medicaments. Some SU might be potentially teratogenic. Chronic treatment with SU can be associated with apoptosis and therefore loss of beta cell mass⁶⁰. First generation SU-s are chlorpropamide, tolbutamide, tolazamide. They are not recommended and rarely used because of more side effects compared to newer SU. Second generation (glipizide, glibenclamide/glyburide, glimepiride, glycopyramide) and third generation (glimepiride) SU-s are still widely used for the treatment of type II diabetes.

1.3.1.2 *Meglitinides*

Repaglinide, nateglinide and mitiglinide belong to the meglitinide drug family. They promote insulin secretion by the same mechanism as SU-s. However they have a shorter effect (they act quickly within 30 min, but their effect can last for several hours) and hypoglycemia is less frequent compared to the treatment with SU-s. Another mechanism by which they increase insulin secretion is the activation of ryanodine receptors (RyR). Side effects are weight gain, headache, muscle and joint ache and sinus infections.

1.3.2 Insulin sensitizers

1.3.2.1 *Biguanides*

Metformin is often prescribed as initial or combination therapy (with insulin or SU/glinides). It lowers blood glucose level by decreasing the glucose production in the liver. This is often a preferred therapy because of several benefits. There is no risk of hypoglycemia, low side effect profile; furthermore some anticancer effects are also reported during the treatment of metformin. It is currently under investigation in clinical trials and used in neoadjuvant therapy, it may also selectively kill cancer stem cells. Confirmation is needed for whether it can enhance the treatment outcome of various cancer types⁶¹⁻⁶³. Metformin can suppress appetite and compared to other members of the family it does not cause lactic acidosis. Metformin blocks the synthesis of NfkB which is responsible for inflammatory processes, furthermore activates Nrf2, a transcription factor that regulates many antioxidant enzymes. These enzymes can stop the activity of reactive oxygen species, thus may prevent the destruction of pancreatic beta cells⁶⁴. Metformin indirectly activates AMPK (AMP-activated protein kinase) by targeting and inhibiting the complex I of the respiratory chain in the mitochondria. As a result it is lowering the mitochondrial ATP/AMP ratio that's sufficient for activation of AMPK⁶⁵. AMPK has a central role in the regulation of metabolism, some of its function will be discussed later⁶⁶.

1.3.2.2 *Thiazolidinediones (TZD)*

Thiazolidinediones (pioglitazone, rosiglitazone) are peroxisome proliferator-activated receptor- γ (PPAR γ) agonists and are approved for the treatment of type II diabetes (T2D). PPARs are nuclear receptors that are mainly expressed in brown adipose tissue and play a decisive role in the development of adipocytes, therefore weight gain can be a substantial side effect of PPAR γ activation^{67,68}. After activation, it forms a heterodimer with the retinoid receptor (RXR), and exerts antidiabetic effects by binding to DNA and regulating genes which are involved in glucose homeostasis and lipid metabolism^{69,70}. TZDs have a complex effect that in overall leads to an improved insulin sensitivity of muscle and fat cells, increased lipolysis and reduced FFA secretion⁷¹. It also stimulates the translocation of GLUT4 transporters to the membrane, thus promoting glucose uptake from blood⁷². TZDs can improve the inflammatory state of fat cells in T2D by inhibiting the expression of cytokines (TNF α)^{73,74}. Furthermore they induce adiponectin secretion that is an important hormone responsible for fatty acid oxidation and can reverse hyperglycemic effects^{75,76}. A recent study suggests that they also act by increasing the expression level of GPR40⁷⁷. In spite of these, safety issues raised big concerns by application of these drugs. According to some studies, the treatment with rosiglitazone can be associated

with increased risk of getting heart attack and stroke, however this correlation was not significant in another analysis⁷⁸. Rivoglitazone, a new TZD under development has a greater potential compared to pioglitazone, however it produced serious adverse effects (cardiovascular events, renal failures)⁷⁹. Another drawback of the TZDs is that they can induce osteoporosis under some circumstances⁸⁰. Patients who can't take metformin because of renal insufficiency may take TZDs^{81,82}. INT-131, a new non-TZD PPAR agonist is under development and it is expected to be better tolerable and less harmful for the cardiovascular system compared to available TZDs⁸³⁻⁸⁵.

1.3.2.3 Dual PPAR agonists

Saroglitazar, the first compound of a new class of drugs is approved for the treatment of diabetic dyslipidemia. It acts on both PPAR α and PPAR γ , therefore an improved, joint effect is expected from this group of compounds that might also lower the cardiovascular risks caused by TZDs^{67,86-91}.

1.3.3 Incretin-based therapies

The physiologically acting incretin hormones, glucagon like peptide-1 (GLP-1) and gastric inhibitory peptide or glucose dependent insulintropic polypeptide (GIP) are produced by the intestinal K and L cells. They both act on G-protein coupled receptors (GPCRs) and exert their effects in a glucose dependent manner. Therefore patients treated with incretin analogues (exenatide, liraglutide, lixisenatide, albiglutide and the recently approved dulaglutide) are less affected by hypoglycemia. For reaching their final effect, activation of MEK/ERK1/2 pathway and Ca²⁺ influx through L-type Ca²⁺ channels is necessary⁹². Exenatide shows 50% homology with human GLP-1. It slows down the gluconeogenesis, slows stomach emptying and regulates hunger. Since it causes weight loss as well this is a suitable treatment for patients who are overweight^{93,94}. In some cases severe side effects can develop by the treatment of incretin-based therapies, however the incidence was low. Hemorrhagic or necrotizing pancreatitis were reported in some patients and currently there is a big discussion and several studies ongoing about comparing the benefits and disadvantages of this therapy⁹⁵⁻¹⁰¹. An additional benefit could be the protective effect produced on the heart. However this might be negligible according to a study, because of the more dangerous and damaging effect produced in the pancreas¹⁰². Liraglutide also possess cardio-protective effect¹⁰³. It was however reported to increase the risk of development of thyroid cancer in rats and mice, but this couldn't be confirmed in humans yet¹⁰⁴⁻¹⁰⁶. Incretin analogues are administered subcutaneously either daily (exenatide, liraglutide) or weekly (albiglutide, dulaglutide).

1.3.4 Alpha-glucosidase inhibitors

Acarbose and miglitol inhibit the alpha glucosidase enzyme that breaks down oligosaccharides. They slow down the digestion of starch and other carbohydrates which helps to keep the blood glucose level low. They do not cause hypoglycemia, weight gain and have only minor side effects (flatulence). It must be taken after the first bite during meal.

1.3.5 DPP-4 inhibitors

Inhibition of dipeptidyl peptidase-4 (DPP-4) leads to decreased degradation of glucagon-like peptide-1 (GLP-1). It significantly reduces HbA1c level, there is no induction of weight gain and there is only a low risk of hypoglycemia under the treatment of these medicines. The first FDA approved drug in this class is sitagliptine, so far 8 derivatives are available in the market (Sitagliptin, Vildagliptin, Saxagliptin, Linagliptin, Anagliptin, Teneagliptin, Alogliptin, Gemigliptin). However many studies and meta-analyses revealed that there are no increased risks for cardiovascular events, pancreatic cancer and pancreatitis for this class of drugs¹⁰⁷⁻¹¹⁰, according to a recent study, sitagliptin was highlighted for increased risk of heart failure compared to other members of DPP4 inhibitors. The adverse cardiovascular effects of sitagliptine might be due to the reduced level of a cardio active GLP-1 metabolite and increased level of the native, intact GLP-1¹¹¹. Further studies raised concerns for Saxagliptin and Alogliptin, which are now under FDA investigation¹¹²⁻¹¹⁴.

1.3.6 SGLT2 inhibitors

This new class of diabetes medication lowers blood glucose level through inhibition of sodium-glucose co-transporter 2 (SGLT-2) that is located in the proximal tubule of kidneys. It is a low affinity and high capacity glucose transporter, which is responsible for 90% of the reabsorption. Inhibiting the SGLT-2 will lower blood glucose level by facilitating the renal glucose excretion. This new mechanism of action further improves glucose control by increasing the glucose sensitivity and the glucose uptake in the muscle cells, decrease gluconeogenesis in liver and improve insulin release from beta cells. So far no serious side effects (no cardiovascular risk) were observed by treatment of these drugs, however there is an increased risk of getting urinary and genital infections, nasopharyngitis and mycotic infections for people who are taking it^{115,116}. The medication is not indicated in type 1 diabetes and it is also not recommended for patients with severe renal impairment, but recent studies suggest that they might improve glycemic control in combination with insulin in T1DM as well^{117,118}. Currently dapagliflozin and canagliflozin are approved by FDA, ipragliflozin approved in Japan and empagliflozin completed phase III in Dec 2013.

1.4 Preclinical developments for the treatment of diabetes

1.4.1 G protein-coupled receptors (GPCRs)

The GPCR family consists of around 1000 members and they are targeted by approximately 50% of the available drugs today. They regulate many of the most important physiological processes and are good targets because they show different expression patterns in various tissues and organs as well (e.g. adrenergic receptors). There are GPCRs in beta cells which main roles are the potentiation and regulation of glucose stimulated insulin release (GSIS). Targeting these receptors could be a big advantage compared to other available medications that often cause hypoglycemia. Therefore proper agonist and antagonist development for these receptors is currently a quickly emerging field giving hope for a novel therapy against diabetes with a potentially reduced risk of getting hypoglycemia from the treatment^{27,34}. From numerous targetable GPCRs I would highlight the GPR119, but there are many other candidates as well¹¹⁹⁻¹²⁷. Several GPR119 agonists were studied in preclinical and clinical experiments, unfortunately with poor outcomes yet. The main handicaps associated to them are tachyphylaxis and therefore low potency of glucose lowering effect in vivo^{128,129}.

1.4.2 FGF21 and its analog LY2405319

The fibroblast growth factor family consists of 22 members. FGF21 is known to induce favorable metabolic effects in rodents and in humans as well. Its serum level is significantly increased after exercise in humans¹³⁰. In vitro it increased glucose uptake and upregulated GLUT1 in 3T3-L1 cells. In vivo it lowers blood glucose level and it was free of the common side effects of insulin therapy, like increased weight gain and hypoglycemia. However FGF21 activates FGFR related pathways, it does not bind to FGFR directly. It requires the co-receptor β Klotho (KLB) to exert its action via phosphorylation of FRS2 and activation of MAPK pathway. LY2405319 is an improved and more stable analog of FGF21. In clinical trials it decreased serum triglyceride, LDL, insulin levels in consequence reduced body weight was observed as well. Importantly adiponectin level – a protein hormone secreted by adipose tissue, which regulates glucose level and fatty acid oxidation - was elevated after treatment.¹³¹⁻¹³⁴

1.4.3 Glucokinase activators (GKA)

The principle of mechanism of action of these drugs is that they activate the glucokinase (GK), an enzyme that facilitates glucose metabolism by phosphorylating glucose. Presumably they lower blood glucose level by two mechanisms: by increasing insulin secretion from beta cells and by increasing glucose uptake and modulating its metabolism in hepatocytes¹³⁵⁻¹³⁷. They

might also have a protective effect on beta cells¹³⁸. There were many clinical trials conducted with GK activators, but most of them were terminated in phase I, some of them reached phase II: AZD1656¹³⁹ (Japan, phase I), LY2608204, LY2599506 (Eli Lilly, phase I); ZYGK1 (ZyduS Cadila, India, phase I); HMS5552 (Hua Medicine Limited, China, phase I); ARRY-403 (Array BioPharma / Amgen, phase II); MK-0941¹⁴⁰ (Merck Sharp & Dohme Corp., phase II); GK Activator (2) (Hoffmann-La Roche, phase II). The cause of discontinuation of these trials might be due to their potential side effects including hyperlipidemia, hypoglycemia and fatty liver¹⁴¹.

1.4.4 11beta-hydroxysteroid dehydrogenase type 1 (11 β -HSD-1) inhibitors

Overexpression of 11 β -HSD-1 in adipose tissues can lead to the development of metabolic syndrome and obesity. The enzyme catalyzes the conversion of inactive cortisone to active cortisol, which primary function is to increase gluconeogenesis. Thus it has been proposed to have a potential benefit for T2DM patients, since it significantly reduced haemoglobin A1c (HbA1c) levels. INCB013739 (Incyte Corporation, phase II), P2202 (Piramal Enterprises Limited, terminated).

1.4.5 Glycogen phosphorylase inhibitors (GPIs)

Hepatic glycogenolysis is the physiological process that forms glucose-6-phosphate from glycogen, by this blood glucose level raises. Glycogen phosphorylase catalyzes the formation of glucose-1-phosphate from glycogen which is converted later into glucose-6-phosphate. Two endogenous hormones that can induce glycogenolysis are adrenalin and glucagon. They are produced in response to stress or low blood glucose level. Theoretically as these physiological compensatory mechanisms are present, hypoglycemia can also be prevented by application of GPIs. So far there were only a few studies with low success in this field: GSK1362885 (GlaxoSmithKline, phase I); CP-91149 and CP-316819 (Pfizer, preclinical studies).

1.5 Roles of sunitinib and other kinase inhibitors in diabetes

Sunitinib and imatinib both were able to reverse type 1 diabetes in nonobese diabetes (NOD) mice model. The study is based on previously observed positive outcomes achieved with imatinib in the treatment of different autoimmune diseases and underlines its anti-inflammatory effect. The possible mechanisms of imatinib and sunitinib treatments were investigated and found that on-set prevention could be linked to the neutralization of PDGFR. It was also concluded that c-Kit, c-Fms or Lck was not associated with this effect¹⁴². For demonstration of this principle in human, a clinical trial has been started recently which purpose is to study the potential role of short-term therapy with imatinib to induce T1DM remission^{143,144}. Additional evidences confirm that tyrosine kinase inhibitors (TKIs) might have beneficial effects in T2DM patients as well, such as normoglycemia upon treatment, increased insulin secretion, favorable regulation of lipid metabolism and improved insulin sensitivity. Templeton et al. reported an interesting case of a patient with renal cell carcinoma (RCC) who received sunitinib treatment. The patient got diabetes therefore needed insulin treatment, but this could be gradually reduced and later on completely stopped during the administration of sunitinib. Interestingly sunitinib was able to sustain normoglycemia even after terminating the therapy as well¹⁴⁵. A follow-up clinical study conducted on more patients with both RCC and T2D receiving sunitinib treatment, ended with similar results. Sunitinib induced remission in T2D patients and lowered blood glucose level. In addition it should be noted that blood glucose level was not dropped significantly in non-diabetic patients, so it cannot be excluded that the effect of sunitinib is glucose dependent¹⁴⁶. VEGFR, PDGFR, c-Kit, Flt3, RET, CSF-1R are considered as main targets for sunitinib but apart from these, it is binding to many kinases and may inhibit others as well¹⁴⁷. A recent review about TKIs proposed some mechanisms and theories for the general action of them in diabetes and insulin resistance. These involve the following effects: reversing adipose tissue macrophage phenotype from M1 to the non-inflammatory M2 type (PD153035); reducing ER stress and JNK/IRS1 Ser307 phosphorylation (imatinib); inhibiting c-Abl that can prevent beta cell death. Additionally erlotinib, nilotinib, pazopanib, dasatinib and sorafenib treated patients were also reported to produce lowered fasting plasma glucose level^{148,149}. In overall the TKIs seem to be able to reduce beta cell apoptosis as well in T1D and T2D^{150,151}. A recent study performed on rabbits revealed that sunitinib was able to reduce plasma glucose level in this model as well. The drop in the plasma glucose level was higher in diabetic animals, which seems to correlate with the plasma level of sunitinib. This could be attributed to the decreased function of CYP3A4 in diabetes, therefore allowing the drug to act longer and produce a stronger effect¹⁵². Sunitinib could also prevent beta cell loss and cure hyperglycemia

in spontaneously diabetic Torii rats¹⁵³. Although TKIs are proposed to carry out their anti-diabetic actions mostly by improving insulin resistance and preventing beta cell failure, in our experiments we demonstrated that sunitinib was able to increase insulin secretion from different beta cell lines. The mechanism of this process was not studied by others yet, therefore we hypothesized that some of the “off-target kinases” or potentially other proteins may negatively regulate the insulin release in their active form, which can be inhibited by TKIs, including sunitinib. This hypothesis has been partly proven previously by an siRNA-based target discovery screening in beta-TC6 cells¹⁵⁴.

2 AIMS AND SCOPE

Our main goal was to use a diverse compound library as a tool to identify targets based on a phenotypic screening method, which are likely responsible for negative regulation of insulin secretion. Furthermore we were looking for less toxic compounds with comparable or better insulinotropic effect than sunitinib. The mechanisms of action of these compounds were further studied in *in vitro* systems.

3 MATERIALS AND METHODS

3.1 Cell lines

Culture medium and supplements were purchased from Life Technologies. RIN-5AH insulinoma beta cell line and the BRIN-BD11 were cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine. For propagation of the glucose responsive INS-1E cells the same RPMI 1640 medium was used, further supplemented with 10mM HEPES, 1mM Sodium pyruvate and 50 μ M beta-mercaptoethanol. The Beta-TC-6 cells were grown in DMEM (glucose: 4.5g/l) with 15% FBS, 2mM glutamine and 1mM pyruvate. C2C12 mouse myoblast cells were cultured in high glucose DMEM (4.5g/l) with 10% FBS, 2mM glutamine. 3T3-L1 adipocytes were propagated in low glucose DMEM (1g/l) supplemented with 10% NBCS and 2mM glutamine. All culture medium contained 1x Penicillin-Streptomycin. Cells were grown on 15cm culture plates in 37°C, 5% CO₂ in a high humidity atmosphere.

| CELL lines and culture media | | | | | | |
|---|--------------------------------|--------------------------------|--------------------------------------|--|--|------------------|
| <i>Cell line</i> | RIN-5AH | TC-6 | BRIN-BD11 | INS-1E | C2C12 | 3T3-L1 |
| <i>Description</i> | rat beta cell line | mouse beta cell line | primary beta cells- RINm5F hybrid | glucose sensitive rat beta cell line | mouse myoblasts | mouse adipocytes |
| <i>Origin</i> | | ATCC | PHE | Kindly provided by prof. Claes Wollheim | ATCC | ATCC |
| <i>Medium</i> | RPMI 1640 | DMEM Hi | RPMI 1640 | RPMI 1640 | DMEM Hi | DMEM Lo |
| <i>Serum</i> | FBS 10% | FBS 15% | FBS 10% | FBS 10% | FBS 10% | NBCS 10% |
| <i>Glutamine</i> | 2mM | 2mM | 2mM | 2mM | 2mM | 2mM |
| <i>Pen/Strep</i> | 1x | 1x | 1x | 1x | 1x | 1x |
| <i>Pyruvate</i> | | 1mM | | 1mM | | |
| <i>β-mercaptoethanol</i> | | | | 50 μ M | | |
| <i>HEPES</i> | | | | 10mM | | |
| <i>Splitting</i> | 2x10 ⁶ / 15cm plate | 2x10 ⁶ / 15cm plate | 1,5x10 ⁶ / 15cm plate | 1,5x10 ⁶ / 15cm plate | 0,4x10 ⁶ / 15cm plate or 1 to 10 | 1 to 5-8 |

INS-1E cells were kindly provided by prof. Claes Wollheim and Pierre Maechler (Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland)

ATCC - American Type Culture Collection

PHE – Public Health England

3.1.1 Differentiation of 3T3-L1 cells

100.000 cells were seeded in 12 well plates and incubated for 1-2 days at 37°C, 5% CO₂. Medium was carefully removed and replaced with differentiation medium I (10% NCS in high glucose DMEM containing 500 μ M IBMX, 1 μ M Dexamethasone and 1 μ g/ml insulin). After 2 days of incubation, medium was changed for differentiation medium II (high glucose DMEM supplemented with 10% NCS and 1 μ g/ml insulin). Then after further incubation for 2-4 days

in differentiation medium II cells were ready for the experiment. Differentiation medium II was changed every second day if necessary.

3.2 Insulin ELISA

To determine the insulin level from the cell supernatants we used the insulin ELISA kits from Millipore or Merckodia. Cells were seeded in 96 well or 12 well plates and grown overnight (RIN-5AH, TC-6 cells) or for 2 days (INS-1E, BRIN-BD11). They never reached more than 80% confluency before treatment. Cells were either washed 1x with PBS (RIN-5AH, TC-6, and BRIN-BD11) or preincubated for 40min in KRBH containing 1mM glucose (INS-1E cells) before treatment. Cells were treated in medium or KRBH buffer with 1mM or 11mM glucose (INS-1E cells) for 2 hours and 5uM if otherwise not stated. Samples were assayed immediately or stored at -20°C until measurement. Dilutions were made if necessary then ELISA was performed according to the user's manual. Plates were read on BioTek Elx800 Absorbance reader at 450 or 490nm.

3.3 CellTiter-Glo assay

Cells were plated on flat bottom white 96 well plates (Costar). Treatment was performed after overnight incubation for the desired time. CellTiter-Glo component (Promega) was added in a 1:1 volume ratio to the wells and then plate was incubated at RT in dark for 15min. Luminescence intensity was measured on TECAN Evolution microplate reader.

3.4 IC₅₀ and EC₅₀ determination

IC₅₀ values for viability were determined with CellTiter-Glo and treatment was performed for 72h. EC₅₀ values for insulin secretion were determined with insulin ELISA kit and normalized to the 2h treatment MTT absorbance value. Values were calculated in GraphPad Prism using four parameters fit. Top and Bottom constrains were set to 100 and 0. IC₅₀ values were calculated for curves with R²>0.85.

3.5 MTT assay

After 2h treatment of RIN-5AH beta cells, their viability was checked by MTT assay in order to normalize their insulin secretion capacity. A stock solution of 5mg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma, M5655) was prepared in PBS and filtered. MTT reagent was added in 1:5 ratio to the wells and incubated in 37°C for 45min. Later on 1:1 stop solution (200ml 10% SDS; 12ml iso-butanol; 1.3ml 2N HCl) was added and incubated overnight in dark at RT. Absorbance reading was done on BioTek Elx800 plate reader at 570nm.

3.6 Protein concentration determination

Protein concentrations were determined by BCA assay kit in 96 well plates (Thermo Scientific, 23225). This method was used to measure the protein concentrations for western blot experiments and for normalizing the insulin secretion data to protein amount of INS-1E cells as well. Assay was performed according to the manual, using 5 – 12.5µl sample amounts. After 30min - 1h incubation time absorbance was read at 570nm on BioTek Elx800 plate reader.

3.7 Calcium influx measurements

For Fluo-4 AM (molecular probes, F14201) staining a 3µM stock solution was prepared in HBSS with Ca²⁺ and Mg²⁺ (HBSS++) with addition of a final amount of 0.02% Pluronic-F127 (molecular probes, P6867). Probenecid was not necessary for loading and displayed toxicity on cells as well. Cells were incubated with the dye for 1h at 37°C, in 5% CO₂. They were washed 2x with Ca²⁺ and Mg²⁺ free HBSS (HBSS--) (Gibco, 14170) before moving to the next step of the protocol (see below). Washing steps could be reduced for Fluo-4 Direct (molecular probes, F10471) and Calcium-6 (Molecular Devices, R8194) staining, since these formulas contain a secondary masking dye that does not enter the cell and quenches the unspecific signal of residual extracellular calcium indicators. These dyes were used when performing the calcium measurements on Flexstation plate reader. Moreover it does not disturb the fluorescent read-out as they have different excitation and emission wavelengths.

3.7.1 Calcium influx measurement by FACS

Similar protocols were already described using this method for Ca²⁺ influx determination¹⁵⁵⁻¹⁵⁷. RIN-5AH, TC-6, INS-1E (30.000 cells/well) or C2C12 cells (80.000 cells/well) were plated in 12 well plates and incubated ON at 37°C, 5% CO₂. After washing with HBSS--, cells were trypsinized for 3min, at 37C, 5% CO₂. Then diluted with HBSS-- and centrifuged for 3min at 1200 rpm. Cells were suspended in 1ml of HBSS++ or HBSS-- before starting the measurement. Base fluorescent signal was recorded for 30s, afterwards treatments with the various compounds were done, by quickly suspending them into FACS tubes, while the acquiring was not interrupted. Final DMSO concentration did not exceed 0.1%. Then samples were measured continuously for the desired time and all measurements completed within 40 min. Unstained cells were used as negative controls for each treatment. Compounds used in the calcium influx experiments were purchased from various sources (Chloroquine diphosphate - Sigma, C6628; Quinidine - Sigma, Q3625; Verapamil - Santa Cruz, sc-3590A; Efonidipine HCl monoethanolate - Sigma, E0159; NNC 55-0396 - Sigma, N0287; Glibenclamide - Santa Cruz, sc-200982; Nickel(II) chloride hexahydrate - Sigma, N6136; Gatifloxacin - Santa Cruz, sc-

204762; Levofloxacin HCl - Santa Cruz, sc-202693; Thapsigargin - Serva, 36000; A23187 Calcium ionophore - Sigma, C7522; Ryanodine - Santa Cruz, sc-201523; Caffeine - Sigma, C0750; 4-Chloro-3-methylphenol - Sigma, C55402; 9-Phenanthrol - Sigma, 211281). Data were acquired on BD-FACSCalibur flow cytometer from at least 15.000 cells, data visualizations were prepared by FlowJo software.

3.7.2 Calcium influx measurement on Flexstation

The increased throughput capacity of the Flexstation (Molecular Devices) made it available to test more compounds with more replicates. Further advantages of this system were that using the built in pipetting system 8 samples could be treated and measured almost at the same time. RIN-5AH cells were seeded (50.000 cells/200µl/well) and incubated overnight at 37°C, 5% CO₂ then loaded either with Fluo-4 Direct or Calcium-6 (100µl/well). Buffer was replaced with 200µl of HBSS++ and plates were stored at RT in dark for max. 30 minutes until measurement. Plates were treated by adding 50µl of 5x stock solution of compounds prepared in the appropriate buffer to each well using the built in pipetting system. Measurements were performed with Flexstation 3 in FLEX mode using the shortest reading interval time. Evaluation and calculation of AUC values were made with SoftMax Pro 5.4.5 software.

3.7.3 Calcium influx measurement by spinning disc confocal microscopy

RIN-5AH cells were plated (30.00 cells) in 35mm u-Dishes (IBIDI, 80136) and incubated overnight at 37°C, in 5% CO₂. Cells were loaded with Fluo-4 AM and washed 2x with HBSS-. Volume was adjusted with HBSS++ to 1200µl / dish. Base fluorescence signal was recorded for 30s and treatment was done by using a perfusion pump (Ismatec) with a final concentration of 5µM for VCC981125:09, VCC673193:01, VCC718565:01 and 250µM for glibenclamide. Monitoring and recording of fluorescence intensity was carried out on an UltraVIEW Vox spinning disk microscope system (PerkinElmer), which was attached to a HAMAMATSU ImagEM camera (cooled down to -80°C). Calculation of results was done by Volocity software (PerkinElmer).

3.8 Patch clamp experiments

In order to confirm the calcium influx results for the quinoline molecule VCC981125:09, whole cell patch clamp experiments were carried out under the supervision and kind help of Dr. Jana Hartmann from TUM (Institute for Neurosciences).

RIN-5AH cells were trypsinized and suspended in medium. Cells were transferred into the recording chamber and allowed to settle down before beginning a slow perfusion with the

external buffer. Cells were sealed and patched by the application of a gentle negative pressure. Holding membrane potential was set up to -70mV for voltage clamp experiments. After patching the cells, either 10s or 20s of base signal was recorded for both voltage clamp and current clamp measurements. External stimulation of cells was done by using Picospritzer II instrument (Parker) with 5psi for 30s. Total recording time was set to 2-4 minutes. AUC of the curves were analyzed in GraphPad Prism Software.

3.9 2-NBDG (glucose analog) uptake

The 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) is a fluorescent analogue of D-glucose, commonly used for monitoring glucose uptake in in vitro experiments.

C2C12 cells (80.000 cells/well) and RIN-5AH cells (300.000 cells/well) were seeded in 12w plates and incubated overnight (ON) at 37°C, in 5% CO₂. 3T3-L1 cells were first differentiated before starting the experiment. Medium was removed and cells were washed 1x with PBS. Treatment was done with various compounds at 5µM for 1h or 3h in glucose free medium or in medium containing 100µM 2-NBDG (molecular probes, N13195). Cells were washed 1x with PBS and trypsinized for 3min, then the volume was completed to 1ml with cold PBS and centrifuged at 4°C, 1400 rpm for 3min. Cells were resuspended in cold PBS with 5% FBS and stored at 4°C until measurement. Acquisition was done with BD-FACSCalibur flow cytometer from at least 15.000 cells. Data were analyzed with FlowJo software (Tree Star Inc.). Glucose uptake rate was normalized for all samples according to the following equation:

$$I_{1-wo} / I_{2-wo} * I_{2-w} / I_{1-w}$$

I_{1-wo}: cells in glucose free medium treated with vehicle

I_{2-wo}: cells in glucose free medium treated with compound

I_{2-w}: cells in 2-NBDG medium treated with compound

I_{1-w}: cells in 2-NBDG medium treated with vehicle

3.10 Intracellular cAMP determination

RIN-5AH (25.000 cells/well) were seeded in U-shaped bottom 96 well plates (Nunc, 163320) and incubated for 2 days at 37°C, in 5% CO₂. Cells were washed with KRBH without glucose and treatment was performed in medium (RIN-5AH) or in KRBH (INS-1E) supplemented with 11.1mM glucose. Supernatant was taken for insulin assays, then cells were lysed in 140µl of 0.1N HCl + 0.5% Triton-X for 10min at RT. Samples were centrifuged and stored at -20°C or assayed immediately by using the acetylation method described in the protocol of cAMP Complete ELISA kit (Enzo, ADI-900-163).

3.11 Gene silencing

In all experiments for each target, specific siRNA pools (pool of 3-4 siRNA sequences) were used and purchased from Dharmacon.

3.11.1 siRNA resuspension protocol

The siRNAs were resuspended and stored as described in the basic siRNA resuspension protocol of GE Dharmacon. Briefly, tubes were centrifuged and siRNA were resuspended in 1x siRNA buffer (Dharmacon, Cat. #B-002000-UB-100) to a convenient stock concentration (100 μ M for Accell siRNA and 5 μ M for DharmaFECT ON-TARGETplus protocol). The siRNA stocks were aliquoted and stored at -20°C or -80°C.

3.11.2 Accell siRNA transfection

RIN-5AH cells were seeded in 96 well (12.000 cells/well) or 12 well (100.000 cells/well) plates and incubated for 24h allowing them to attach well to the surface. Medium was removed and cells were transfected with 1 μ M of Accell siRNAs in 1:1 RPMI/Accell Delivery medium (Thermo, Cat #B-005000) mixture supplemented with 1% FBS, without antibiotics. Knock-down efficiency and insulin release was determined after 72 hours of incubation.

3.11.3 Transfection with DharmaFECT ON-TARGETplus

The siRNAs and DharmaFECT 4 reagent were incubated separately in serum and antibiotic free medium for 5min then the 1:1 ratio of siRNA:transfection reagent was incubated for 20 minutes at RT. 20-20 μ l of siRNA:transfection reagent mixture were pipetted into the wells of a 96 well plate then 80 μ l of RIN-5AH cells (12.000 cells/well) and INS-1E cells (20.000 cells/well) were added. Knock-down efficiency was determined by RT-PCR and insulin release was checked after 48h-72h KD by washing the cells and replacing the medium or buffer for 2h. Results are displayed in normalized values to protein amount or MTT absorbance.

3.12 Preparation of affinity matrices for MS experiment

Phenotypically active linker derivatives with primary amino groups were dissolved in the appropriate coupling buffers. They were then covalently bound to NHS- or EPOXY-activated sepharose beads (GE Healthcare, 17-0906-01; GE Healthcare, 17-0480-01). According to our theory, choosing the proper beads can be a critical point. Subsequently upon coupling compounds to the matrix, the altered basicity may change the binding properties of the immobilized compound to the proteins (*Figures 3.1 and 3.2*). The beads were washed 1x with water and 1x with coupling buffer. Compounds in coupling buffer were incubated with the beads in 2:1 ratio overnight on a rotating wheel at RT in dark. NHS-beads in coupling buffer were used as negative and EAH-beads (GE Healthcare, 17-0569-01) in coupling buffer with compounds as positive control. Supernatants were taken and coupling efficiencies were determined by recording the UV-absorbance maximums of NHS-coupled and EAH control samples. Coupling efficiency was normalized to the signal of free NHS by the following equation:

$$\text{NHS normalized coupling efficiency} = (1 - ((\text{ABS.SMPL} - \text{ABS.NHS}) / \text{ABS.EAH})) * f * c$$

ABS.SMPL = max. absorbance of the compound in the supernatant of NHS beads+compound

ABS.EAH = max. absorbance of the compound in the supernatant of EAH beads+compound

ABS.NHS = max. absorbance of NHS in the supernatant of NHS beads+coupling buffer

f = dilution factor = 2 (beads:coupling solution ratio = 1:2)

c = concentration equals the conc. used in the coupling solution in mM

Beads were then washed 1x with water, and 1x with coupling buffer. After that 1M ethanolamine (Aldrich, 411000) in coupling buffer was added and incubation was continued for 6h at RT in order to block unreacted sites on NHS-beads. Then beads were washed 1x with DMSO (or DMF) / ethanol (50-50%), 1x with water and 1x with NaCl. The affinity matrices were then stored in 20% ethanol (1:1) at 4°C until use. The coupling densities were calibrated as described in chapter 3.13. and the optimized affinity matrices were used in MS experiments (*Table 3.1*).

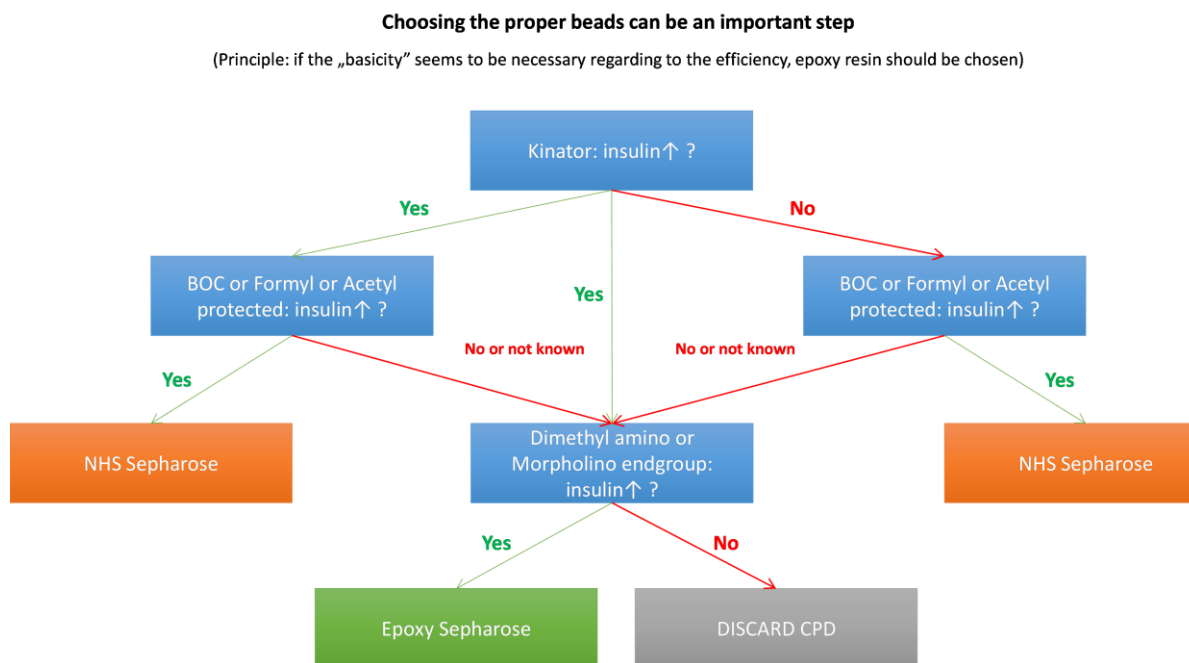


Figure 3.1 All linkers have their amino protected derivate for the purpose of modelling the coupled compound in the cellular assay. If BOC, Formyl or Acetyl protected cpd is active in the insulin ELISA assay, NHS beads can be chosen. If compound with dimethyl or morpholino endgroup is effective and no BOC/Formyl/Acetyl derivate was available, epoxy beads should be chosen.

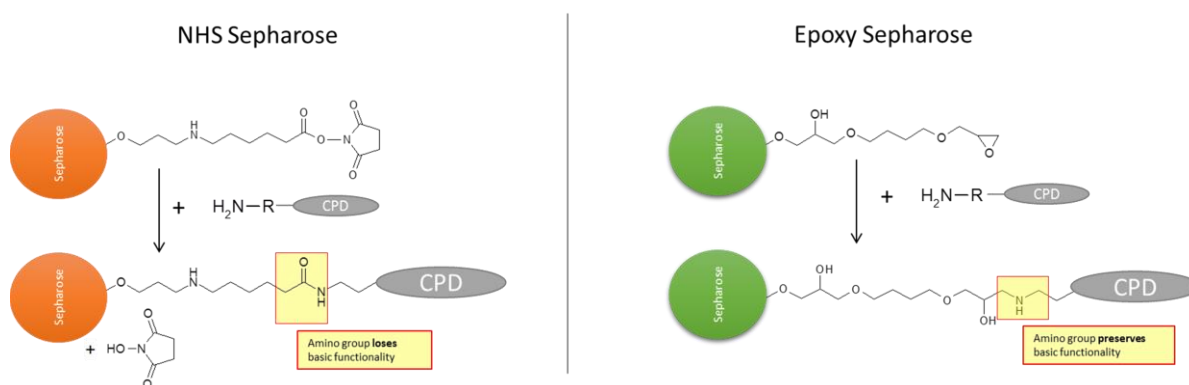


Figure 3.2 Linker derivatives and their amino protected versions were tested for insulin release before coupling. Based on the protective group, different beads should be chosen.

3.13 Binding and competition pre-experiments

The coupling densities were optimized for the final experiments by preparing affinity matrices with different coupling concentrations. After that samples were run on SDS gel and silver staining was employed to detect specific protein-matrix interactions.

For binding experiments 30µl beads were washed 2x with HEPES lysis buffer then 750µl of RIN-5AH cell lysate (4mg/ml) at 4°C for 2.5h at 4°C on a rotating wheel. For pull-down experiments (competition) prior to the 2.5h incubation, 30-100µM compound was mixed with lysate then incubated for 30min at 4°C, on a rotating wheel. (*Figure 3.3*)

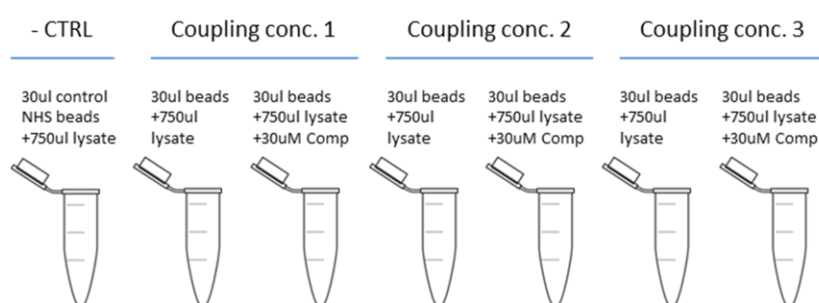


Figure 3.3 Example layout for binding/competition experiments.

After incubation beads were washed 3x with HEPES lysis buffer and residual supernatants were carefully removed from the top. 40µl LDS sample buffer (Invitrogen, NP0007) was added to the samples and boiled for 3min at 95°C. 5µl of eluates and 3-5µg of total cell lysates were loaded in SDS-PAGE gel. Gels were run in MES buffer (life technologies, NP0002) at 150V for 1h then silver-stained according to the user's manual (Invitrogen, LC6070).

3.14 SILAC labeling of RIN-5AH cells for mass spectrometry (MS) experiments

Cells were metabolically labeled with Arg and Lys isotopes in SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture) medium. The advantage of this technique is that the differentially labeled samples could be mixed and analyzed together by LC-MS/MS. The peptide peaks can be quantified very accurately relative to each other to determine the peptide and protein ratios^{158,159}. SILAC RPMI 1640 medium (Thermo, 89984) supplied with 10% dialyzed FCS and penicillin streptomycin was prepared. For triple labeling of cells, three different compositions were prepared with the addition of the proper amino acid isotopes (Sigma, L8662; CIL, DLM-2640-0; Isotec, 608041; Sigma, A6969; CIL, CLM-2265-0; CIL, CNLM-539-0). For light labeled cells: Arg0/Lys0, for medium labeled cells: Arg6/Lys4 and

for heavy labeled cells: Arg10/Lys8 was employed. The concentrations used were: 42mg/l of Arg0 and 72mg/l of Lys0 and the molarity of all amino acids were equal. For efficient labeling of cells (>97% incorporation of amino acid isotopes) 14 days of culturing was necessary, which equals to ~10 doublings. Cells were then harvested by trypsinization, washed 2x with cold PBS and pellets were snap frozen in liquid nitrogen, then transferred to -80°C for long term storage.

3.15 Cellular Target Profiling by MS

We obtained the specific Kd values (Kd_{free}) of each target protein that are present in the proteome of RIN-5AH cells by employing the Cellular Target Profiling technology¹⁶⁰. This method combines chemical proteomics with SILAC-based quantitative MS^{161,162}. The LC/MS-MS measurements and proteomics analysis were done in collaboration with Evotec AG (Martinsried). The generated affinity matrices were used to capture potential target proteins from the cell lysate in a dose-dependent manner by using a serial dilution of affinity matrices. Thus binding constants (Kd_{imm}) specific to immobilized compound could be determined. These specifically enriched proteins were competed off by the free compound (mother compound) with 6 different concentrations (up to 30µM) and the concentration at 50% competition (CC_{50}) was determined. Kd_{free} for each interacting protein target were then calculated by the Cheng-Prusoff equation, based on the Kd_{imm} , the CC_{50} and the known concentration of the immobilized compound (C_{comp})¹⁶³:

$$Kd_{free} = \frac{Kd_{imm}}{Kd_{imm} + C_{comp}} * CC_{50}$$

Specifically bound and competed proteins were filtered by a specific algorithm, among others: at least two fold higher protein enrichment on affinity matrix compared to negative control beads. At least a twofold difference between bound and competed proteins was considered as specific interaction partner for the free compound. Furthermore a ratio of 5h and 2.5h incubation time was used, where 5h/2.5h should be > 0.7 for further gating of relevant targets. Other criteria were taken into account as well, including reasonable number of peptide numbers identified in the measurements (sequence coverage >20%) and number of bound proteins should be more than in total lysate. Samples were analyzed in an LTQ-Orbitrap system.

Four target deconvolution experiments were performed:

| Linker derivative | Mother compound | Competition type | Beads | Coupling buffer | Coupling density used |
|---|---------------------------|--------------------|---------------------|--|-----------------------|
| VCC938609:01 (Styryl quinazolines) | VCC350485:02 | - | NHS- Sepharose | 50mM NaHCO ₃ +DMSO (50-50%) | 2mM |
| VCC757274:08 (Oxindoles) | sunitinib VCC512891:01 | Direct Indirect | NHS- Sepharose | 50mM NaHCO ₃ +DMSO (50-50%) | 2.5mM |
| VCC607440:01 (2-Amino-pyrimidines I.) | VCC512891:01 sunitinib | Direct Indirect | EPOXY- Sepharose | 50mM Na ₂ CO ₃ +DMSO (50-50%) | 4.2mM |
| VCC124075:02 (2-Amino-pyrimidines II.) | VCC124075:02 | Direct | NHS- Sepharose | 50mM NaHCO ₃ +DMSO (50-50%) | 8mM |

Table 3.1 Linker derivatives were covalently coupled to sepharose beads with the indicated coupling densities. Competition type refers to the core structure difference between the linker compound and the mother compound. Direct competition means that the core structure is identical to its linker derivative, in turn indirect competition means that compounds do not belong to the same structure class.

3.16 Kinase affinity experiments

Ten active compounds in the insulin ELISA were selected and tested for their binding affinity to kinases in a full kinome panel assay. The service was provided by DiscoverX¹⁶⁴. The screen was performed against 451 kinases (total of 392 non-mutant kinases) at 5μM. Briefly, the unknown test compounds were used to compete with immobilized control ligands against DNA labeled kinases. The identification of hits is based on detecting DNA-labeled kinases by qPCR, where lower signal intensity means that targets are bound and competed off by the test compound. The exact calculation for each target was:

$$\frac{\text{test cpd signal} - \text{positive ctrl signal}}{\text{negative ctrl signal} - \text{positive ctrl signal}} \times 100$$

test cpd signal = compounds submitted for testing

negative ctrl = DMSO (100% control)

positive ctrl = positive control compound (0% control)

Selectivity scores are calculated by dividing the number of kinases that compounds bind to by the total number of distinct kinases tested, excluding mutant variants:

$$S = (\text{number of non-mutant kinases with \%Ctrl} < 35) / (\text{number of non-mutant kinases tested})$$

3.17 Real-time QPCR (RT-PCR)

Total RNA was isolated using RNeasy Mini kit (Qiagen, 74104) including a DNase digestion step according to the user's manual. The cDNA was transcribed from RNA based on the following protocol:

1-1µl of "K1" annealing primer (5'-AAGCAGTGGTATCAACGCAGAGTACT(30)V N-3' where N = A, C, G, or T; V = A, G, or C) was added to 9µl of 3-5µg RNA (mix1) and heated at 65°C for 2min, then incubated at RT for 5min and 20s on ice. Mix2 was prepared: 4µl RT-puffer (Roche, 11 465 368 001); 2µl dNTPs (10mM); 1.5µl AMV Reverse transcriptase (Roche, 10 109 118 001); 1.5 µl DTT (0.1M); 1µl RNase inhibitor (Fermentas, EO0381) and added to mix1, then incubated for 2h at 42°C. Reaction was stopped by incubating the sample at 95°C for 2minutes. The cDNA was purified using the QIAquick PCR Purification Kit (Qiagen, 28104).

PCR reaction mix was prepared as follows: 6.25µl Fast SYBR® Green Master Mix (Life Technologies, 4385612) + 2.5µl primer mix (1.5µl+1.5µl of 100pmol stocks in 100µl water) + 3.75µl of cDNA (20ng). PCR reaction was run for 40 cycles on Abi StepOnePlus™.

Primers used in RT-PCR:

| | | | |
|-------------------------|---------------------------|--------------------------|----------------------------|
| SUR1_Fw | CAGGACCAAGAGCTGGAGAAGGA | Cyclophilin B_Rev | GCCAAATCCTTTCTCTCCTGTAGC |
| SUR1_Rev | CATCCAGCAGAAGGCCATCTCTT | MAST1_Fw | TACCTAGGTGCCTCGTCACA |
| SUR2A_Fw | GGAGTGCGATACTGGTCCAAACCT | MAST1_Rev | TGTAGTTTCGGTGACAGCCC |
| SUR2A_Rev | CCCAGTGCAGAGAACGAGACT | STK16_Fw | TTCAAGAGAGGTACTACTGTGGAATG |
| SUR2B_Fw | CATAGCTCATCGGGTTCACACCATT | STK16_Rev | TTCAGGTCCTGTGTGCATAAC |
| SUR2B_Rev | GCATCGAGACACAGGTGCTGTTGT | CSNK2A1_Fw | CCAAGGTTCTGGGAACGGAA |
| Kir6.1_Fw | AGCTGGCTGCTCTTCGCTATCA | CSNK2A1_Rev | TTACGGGAGTGTCTGCCCAA |
| Kir6.1_Rev | CCCTCCAAACCAATGGTCACT | AAK1_Fw | AAGTTTTCTCGACTCCAGGCGG |
| Kir6.2_Fw | CAACGTCGCCCCACAAGAACATC | AAK1_Rev | GACGAGAGCGAATCCACCTTC |
| Kir6.2_Rev | CCAGCTGCACAGGAAGGACATG | SLK_Fw | ATCTTCAAGTTGGGGAGCGAG |
| β-Actin_Fw | CCTCTATGCCAACACAGTGCTGTCT | SLK_Rev | CTCCTTATTCTGGGCCTTATACACT |
| β-Actin_Rev | GCTCAGGAGGAGCAATGATCTTGA | MARK2_Fw | TAAGTCCAACATGCTGCGGG |
| GAPDH_Fw | AGACAGCCGCATCTTCTGTG | MARK2_Rev | ACAGCTACTCTTTCCCGTC |
| GAPDH_Rev | TGATGGCAACAATGTCCACT | JNK2_Fw | TGGGCTACAAGGAGAATGTTGA |
| Cyclophilin B_Fw | CTCCGTGGCCAACGATAAGAA | JNK2_Rev | AACTCTGCGGATGGTGTTC |

3.18 Western Blot

Cells were lysed in CellLytic M (Sigma, C2978) lysis buffer supplemented with aprotinin (4µg/ml), NaF (10mM), PMSF (1mM), NaOV (1mM), Phosphatase inhibitor cocktail 2 (Sigma, P5726) and using cell scrapers lysate was collected and centrifuged at 4°C, 12.000 rpm for 20 minutes. 15-20µg protein was loaded in 8-10% SDS-gels. Gel electrophoresis was performed at 150V for 1h. Proteins were then transferred onto nitrocellulose membrane (GE) for 1.5 h at 0.8 mA/cm² using transblot-SD buffer and semi dry blotting technique. Afterwards, protein bands were stained on the membrane with Ponceau S (2 g/L in 2 % TCA) if necessary. The membrane was destained in TBST or NET-gelatine and blocked in the appropriate buffer containing 5% milk or 5% BCA for 1h at RT. Then antibody was diluted according to the manufacturer's instruction and added to the membrane for overnight incubation at 4 °C. On the following day HRP-conjugated secondary antibody was applied for 1h at RT. Luminescent signal was developed with Enhanced ChemiLuminescent (ECL) Kit (PerkinElmer/NEN, Köln, NEL104001EA) before detecting signal on Hyperfilm TM MP (Amersham Pharmacia, Freiburg). Between steps membrane was washed 3x15min with TBST or NET-Gelatine. Membranes were stripped (strip buffer, 50°C, 1h) and checked for sample loading accuracy with α -tubulin antibody. The following antibodies were used: p-AMPK- α (2531); p-CREB (9196); p-PKAc (4781); p-mTOR (2971); p-MEK1/2 (9154); p-ERK1/2 (9101); p-PKC-pan (9371); p-PKC α / β II (Thr638/641) (9375); p-PKC ζ / λ (Thr410/403) (9378); p-Tyr (9411); p-Thr (9381) from Cell Signaling; pSer (804-164-C100) from Alexis; Goat Anti-Mouse IgG secondary Ab (115-035-003) from Jackson Immuno Research; Goat Anti-Rabbit IgG Ab (172-1019) from Biorad.

3.19 Excel macro for the analysis of more than 2 sets of protein targets

As it was planned to perform 6 target deconvolutions, I have prepared an excel macro to compare more than two sets of targets. By this method the overlapping target hits for each compound could be quickly identified using their UniProt identifiers. The full macro code (MS Excel 2010) is included in *Appendix*.

3.20 In silico prediction of ADME properties of the compounds

In silico Pharmacokinetic (PK) and ADME parameters were predicted with the Schrödinger software package Qikprop in cooperation with F. Baska (Vichem Chemie Ltd.). Predicted descriptor values and ranges are included in *Appendix*.

3.21 Buffers and solutions

Coupling solutions for preparation of affinity matrices: depending on the solubility of linker derivatives, either DMSO or DMF was used mixed with 50mM NaHCO₃ (pH=8.5-9 for NHS beads) or Na₂CO₃ (pH=12-13 for Epoxy beads) in 1:1 ratio. In certain cases EtOH/DMF or EtOH/DMSO pH adjusted with TMEA (tetramethylammonium hydroxide, Acros Organics, AC42053) was used as coupling solution.

Extracellular solution for patch clamping: 138.0 mM; NaCl; 5.6 mM KCl; 11.1 mM Glucose; 1.2 mM; MgCl₂; 2.6 mM CaCl₂; 10.0 mM HEPES

HEPES Lysis Buffer for MS experiments and pre-experiments: 50mM HEPES (pH 7.5); 150mM NaCl; 0.5 % Triton X-100; 1mM EDTA; 1mM EGTA; 10mM NaF; 2.5mM Na₃VO₄; 1% Phosphatase Inhibitor Cocktail 3 (Sigma, P0044); 1 cComplete Protease Inhibitor Cocktail Tablet (Roche,) / 50ml ; 10µg/ml aprotinin; 10µg/ml leupeptin; 1mM PMSF

Intracellular solution for patch clamp experiments: 185 mM Potassium-gluconate (Sigma); 13 mM HEPES; 13 mM NaCl; 1 mM MgCl₂; 1 mM ATP (Sigma); 1 mM GTP (Sigma)

Krebs Ringer Buffer (KRBH): 134mM NaCl; 3.5mM KCl; 1.2mM KH₂PO₄; 0.5mM MgSO₄; 1.5mM CaCl₂; 5mM NaHCO₃; 10mM HEPES; 0.05% BSA (Sigma)

Laemmli buffer (3x): 100 mM Tris/HCl pH 6.8; 3 % SDS; 45 % Glycerol; 0.01 % Bromphenol blue; 7.5 % beta-mercaptoethanol

MTT stop solution: 9.5 % SDS; 5 % 2-Butanol; 0.012 M HCl

NET-gelatine: 50 mM Tris/HCl pH 7.4; 5 mM EDTA; 0.05 % Triton X-100; 150 mM NaCl

PBS: 137 mM NaCl; 27 mM KCl; 81 mM Na₂HPO₄; 1.5 mM KH₂PO₄

Running buffer for western blot: 25 mM Tris/HCl pH 7.5; 200 mM Glycine; 0.1 % SDS

Strip buffer: 62.5 mM Tris/HCl pH 6.8; 2 % SDS; 100 mM beta-mercaptoethanol

TBST: 24.2g Tris HCl; 80g NaCl; pH adjusted to 7.6 with HCl and total volume to 1000ml;
+0.05% Tween 20

Transblot SD: 50 mM Tris/HCl pH 7.5; 40 mM Glycine; 20 % Methanol; 0.004 % SDS

4 RESULTS

4.1 A phenotype-based drug and target discovery project

Out of the several beneficial effects of kinase inhibitors observed in diabetes, we focused on the insulin secretagogue effect of sunitinib. We tested sunitinib in different beta cell lines and found that it induced insulin secretion. With this object a compound library (558 members) was prepared by Vichem Chemie Ltd. (D. Erős). Their kinase inhibitor profiles were predicted to be analogous/overlapping with that of sunitinib, however they were structurally diverse and different. A semi-high throughput insulin ELISA assay was set up to start a phenotypic compound screening, looking for candidates with strong insulinotropic effect. Nevertheless many of them are derived from known cores of approved drugs, modifications in their structures (side group replacement, position change, extension or simplification of the structures) led to significant changes in their target profiles and phenotypic effects. For our primary screen we used the RIN-5AH rat insulinoma beta cell line. In further experiments other rodent derived beta cell lines (BRIN-BD11, INS-1E, TC6) were also included. These models are often used to study beta cell functions, since there are no immortalized human beta cell lines available yet. However recently a few human hybrid beta cell lines were established and some of them are stated to produce insulin¹⁶⁵. The steps of our target discovery strategy are displayed in *Figure 4.1*.



Figure 4.1 Steps of phenotypic based drug discovery. Sunitinib increased insulin secretion in different beta cells lines and insulin dosing could be eliminated in diabetic patients. Based on this observation we selected 558 compounds to use them as tools to identify common targets and/or mechanisms responsible for insulin secretion.

4.2 Results of the primary screen of insulin secretion in RIN-5AH cells

Since initial experiments were conducted with RIN-5AH (and beta-TC6) cell lines¹⁵⁴, therefore RIN-5AH was selected to screen compounds for insulin secretion. Insulin ELISA was used for the measurement of insulin secretion of an initial library consisting of 558 compounds. Treatment was performed at 5 μ M & for 2h.

4.2.1 Efficient compounds

An excellent hit rate of ~10% was observed in the assay. Some compounds induced insulin release from the beta cells (there were 128 compounds with higher than 20% insulin secretion) and some of them showed a negative effect, which could be either due to toxicity or because of negative modulation of insulin secretion (*Figure 4.2.1*). The *Figure 4.2.2* displays hit distributions. From this set of compounds we have chosen the best 80 candidates for further confirmation. 55 compounds could be confirmed and finally clustered into different core structure families (Chapter 4.4).

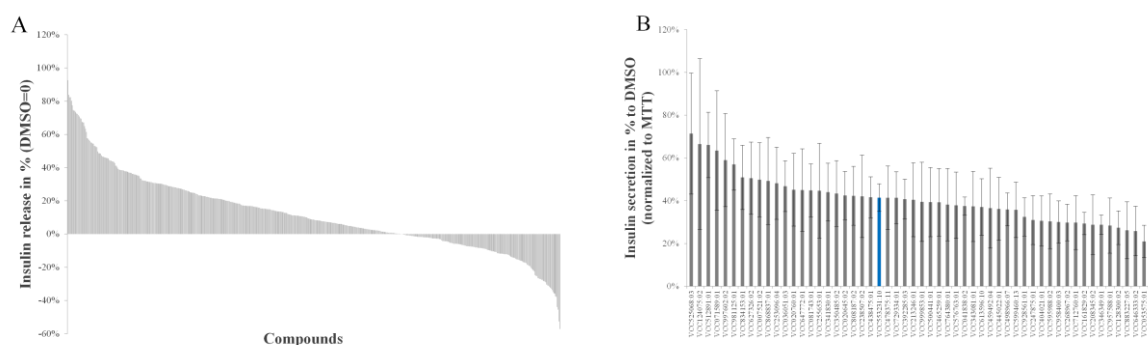


Figure 4.2.1 **A.** Displaying the effect on insulin secretion of 558 compounds. Sunitinib has an effect of ~60%. Compounds with more than 20% percent of insulin secretion were considered active. **B.** 55 compounds were confirmed later on for insulin secretion. Sunitinib is highlighted with blue color. (n=4)

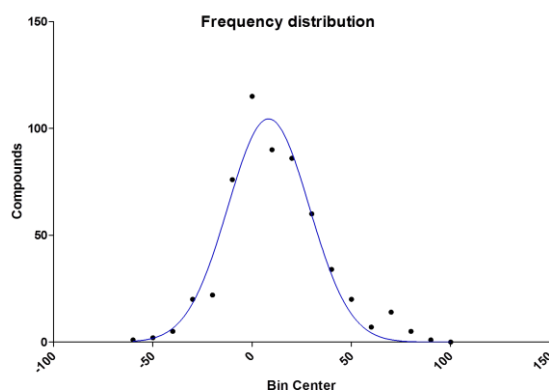


Figure 4.2.2

Gaussian distribution displays the efficiency (insulinotropic action) of compounds. The median of the curve is slightly shifted to the direction of higher insulin secretion (x-axis, median = 8,6), which reflects to the relatively high hit rate in the assay. Moreover since many of the compounds were originally designed to inhibit kinases and the median of the curve is positive, it is also likely that kinases are playing role in the mechanism of action.

4.2.2 In vitro toxicity studies

Cell viabilities were checked either by MTT assay (after treatment with compounds for 2h at 5 μ M) or with CellTiter-Glo for longer incubation times. In the primary screen we ruled out 40 highly toxic compounds (out of 558) that showed decreased MTT activity after 2h and 5 μ M treatment. These compounds have reduced cell viability in CellTiterGlo assay by at least 30%. We found some structure-activity relationships responsible for “acute toxicity” in some of the core structure groups (see Appendix). The 24h treatment revealed additional 2 toxic compounds (*Figure 4.2.3*) It should be noted that since MTT assay readout is proportional with mitochondrial activity and with functional NADPH oxidoreductase enzymes¹⁶⁶⁻¹⁶⁸, diminished reduction of the dye is associated with decreased metabolism of the cell, which in turn could also affect glucose metabolism not only viability. Similar is the mechanism for CellTiter-Glo assay, which detects the ATP level.

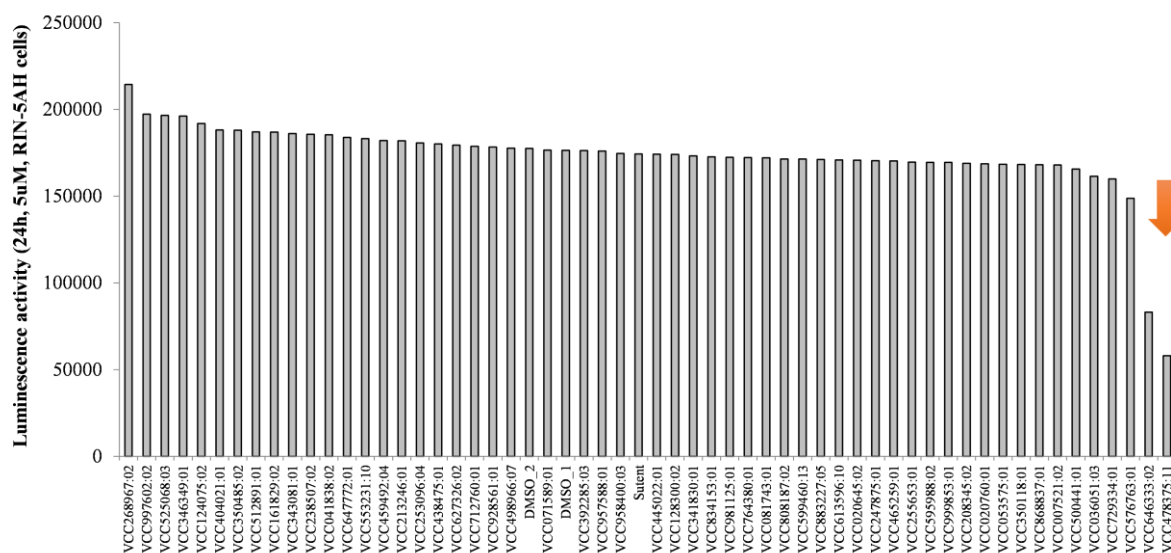
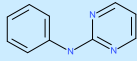




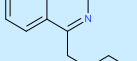


Figure 4.2.3 CellTiter-Glo. Most of the “55 efficient compounds” did not decrease the viability after 24h, 5 μ M treatment in RIN-5AH cells. Two, probably toxic compounds are indicated with the arrow.

4.2.3 Clustering the hits into different core structures

The most efficient compounds were clustered and their core structures are summarized in *Table 4.2.1*. The initial compound library of 558 compounds was extended with additional members from the styryl-quinazoline, quinoline and thieno-pyrimidine groups in order to set up qualitative structure-activity relationships.

| | Core structure family | Core structure | Insulin release >25 % | Insulin release <25% or probably toxic | Total No. Of tested cpds | Hit rate |
|----|---|---|-----------------------|--|--------------------------|----------|
| 1 | N-phenylpyrimidin-2-amine |  | 20 | 22 | 42 | 47,6% |
| 2 | 7,8-dihydropyrido[2,3-d]pyrimidine |  | 5 | 20 | 25 | 20,0% |
| 3 | 1,6-naphthyridine |  | 5 | 11 | 16 | 31,3% |
| 4 | tetrahydrothiopyran-4-one |  | 0 | 3 | 3 | 0,0% |
| 5 | thieno[3,2-c]pyridine |  | 1 | 1 | 2 | 50,0% |
| 6 | quinoline |  | 25 | 22 | 47 | 53,2% |
| 7 | benzothiophene |  | 3 | 16 | 19 | 15,8% |
| 8 | 5,6,7,8-tetrahydrobenzothiopheno[2,3-d]pyrimidine ("Thieno-pyrimidine") |  | 29 | 46 | 75 | 38,7% |
| 9 | quinazoline |  | 3 | 12 | 15 | 20,0% |
| 10 | 4-quinazolin-4-ylbutyl ("Styryl quinazoline") |  | 51 | 3 | 54 | 94,4% |


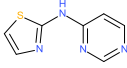

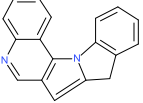
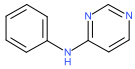

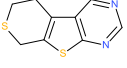

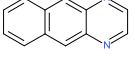
| | | | | | | |
|----|---------------------------------------|---|----|----|----|-----------|
| 11 | 1,6-dihydroimidazo[4,5-c]pyrazole |  | 1 | 1 | 2 | 50,0% |
| 12 | N-pyrimidin-4-ylthiazol-2-amine |  | 0 | 1 | 1 | Dasatinib |
| 13 | indoline (including "oxindols") |  | 17 | 14 | 31 | 54,8% |
| 14 | Misc. 1 |  | 1 | 0 | 1 | 100,0% |
| 15 | N-phenylpyrimidin-4-amine |  | 4 | 22 | 26 | 15,4% |
| 16 | 5,7-dihydro-4H-thieno[2,3-c]thiopyran |  | 1 | 1 | 2 | 50,0% |
| 17 | Misc. 2 |  | 2 | 7 | 9 | 22,2% |
| 18 | quinoxaline |  | 7 | 8 | 15 | 46,7% |
| 19 | benzo[g]quinoxaline |  | 2 | 22 | 24 | 8,3% |
| 20 | +Other misc. structures | | | | | |

Table 4.2.1 Example core structures of insulin secretion inducer compounds including the highlighted core structures. Compounds with these core structures (highlighted in blue) were more extensively studied later on.

4.2.3.1 Highlighted Core structures and structure-activity relationships (SAR)

Based on the confirmation of 55 efficient compounds, we clustered the hits and highlighted the most promising core structures: oxindoles, thieno-pyrimidines, styryl-quinazolines, other quinazolines, quinolines, quinoxalines, 1,6-naphthyridines, 2-amino-pyrimidines, and some additional efficient compounds were included in miscellaneous structural groups. We established qualitative structure activity relationships in quinoline, thieno-pyrimidine and styryl-quinazoline groups (Figures 4.2.4a-c).

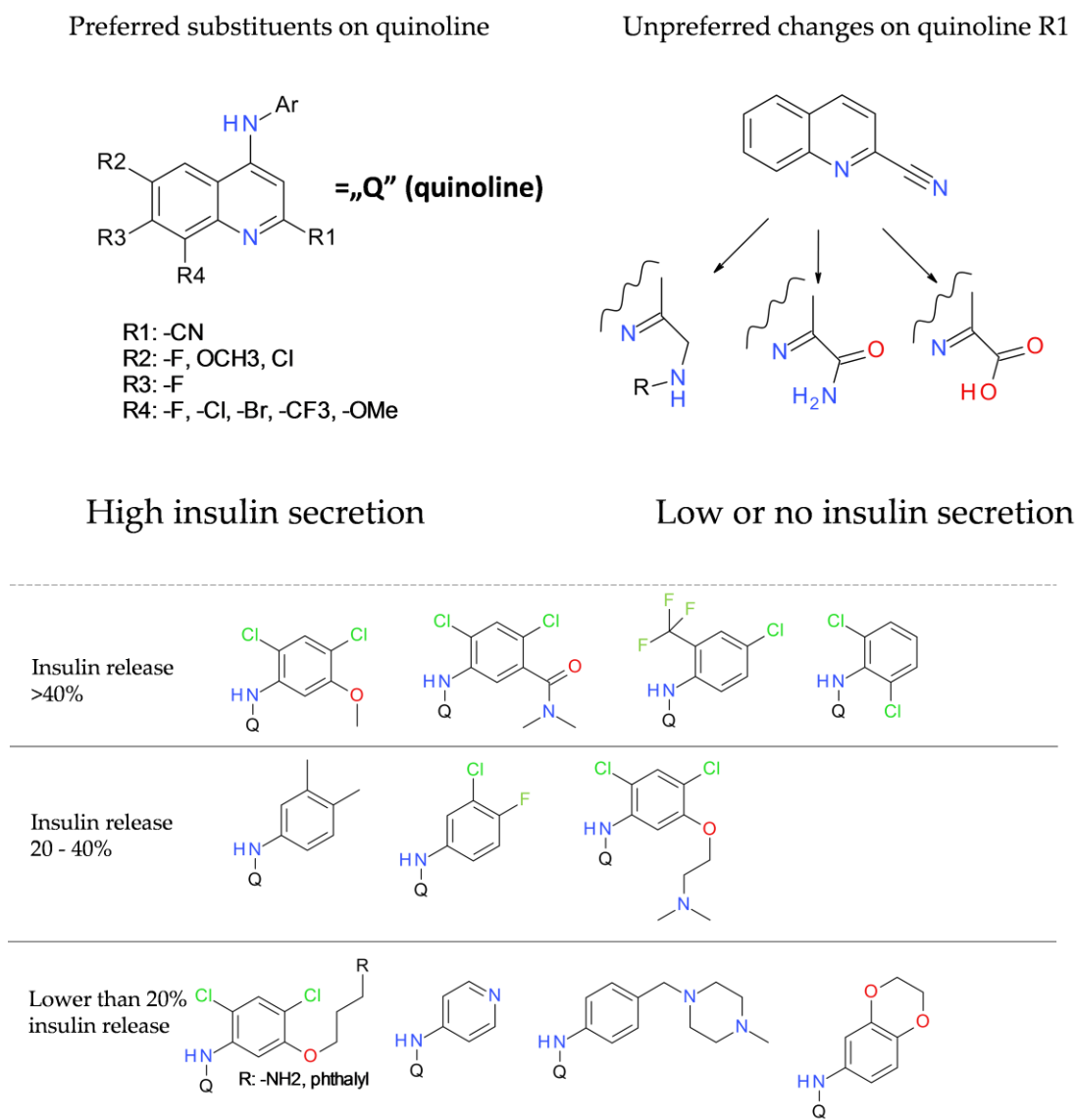


Figure 4.2.4a Quinoline SAR. Core structure is displayed separately for the quinoline (top) and aryl (bottom) ring of the molecule. Preferred and unpreferred substituents and the rate of insulin secretion in RIN-5AH beta cells are displayed.

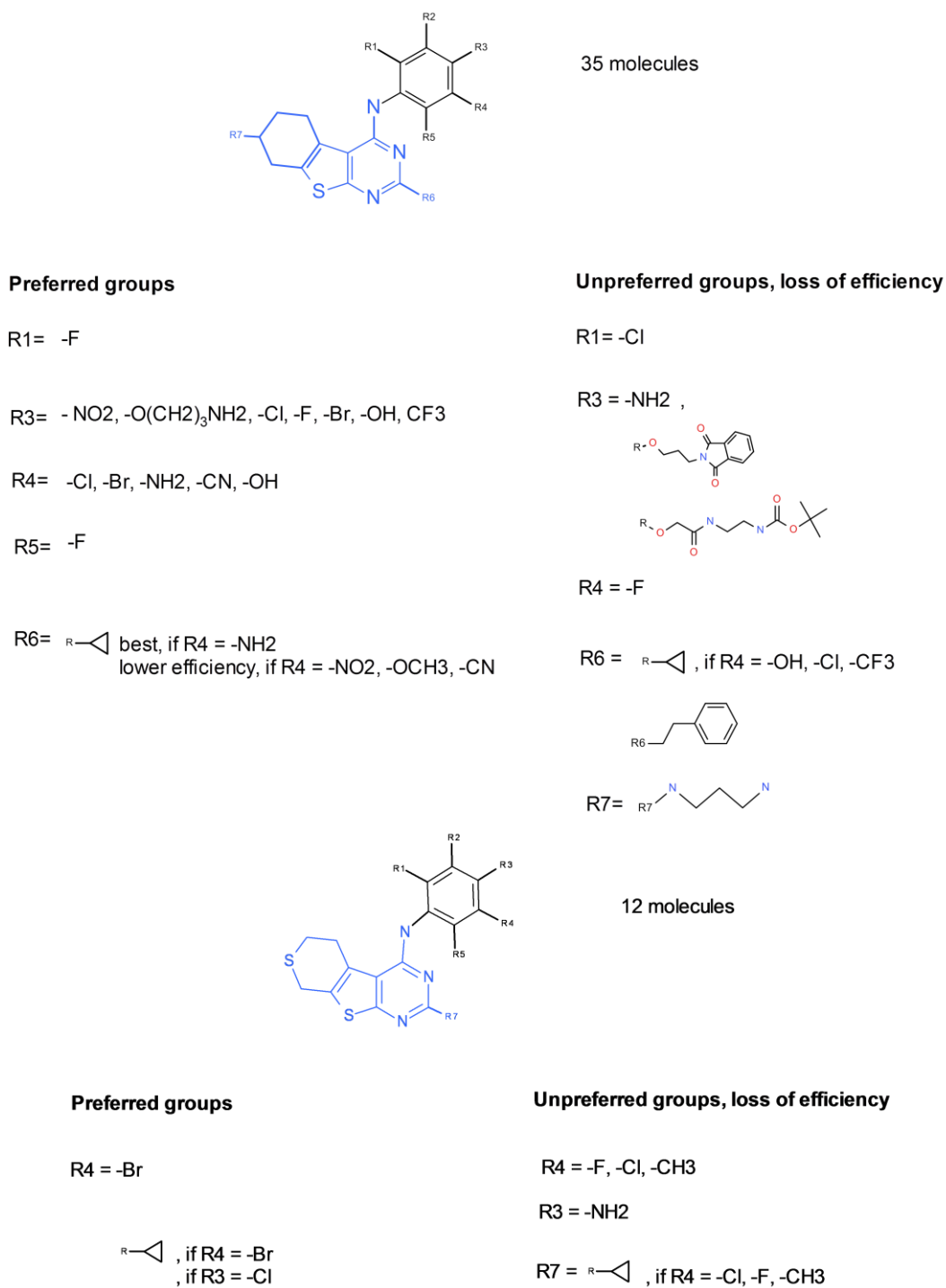


Figure 4.2.4b Thieno-pyrimidine SAR. The core structures and functional groups are displayed where SAR could be defined. Preferred substituents produced >20% insulin secretion in RIN-5AH cells.

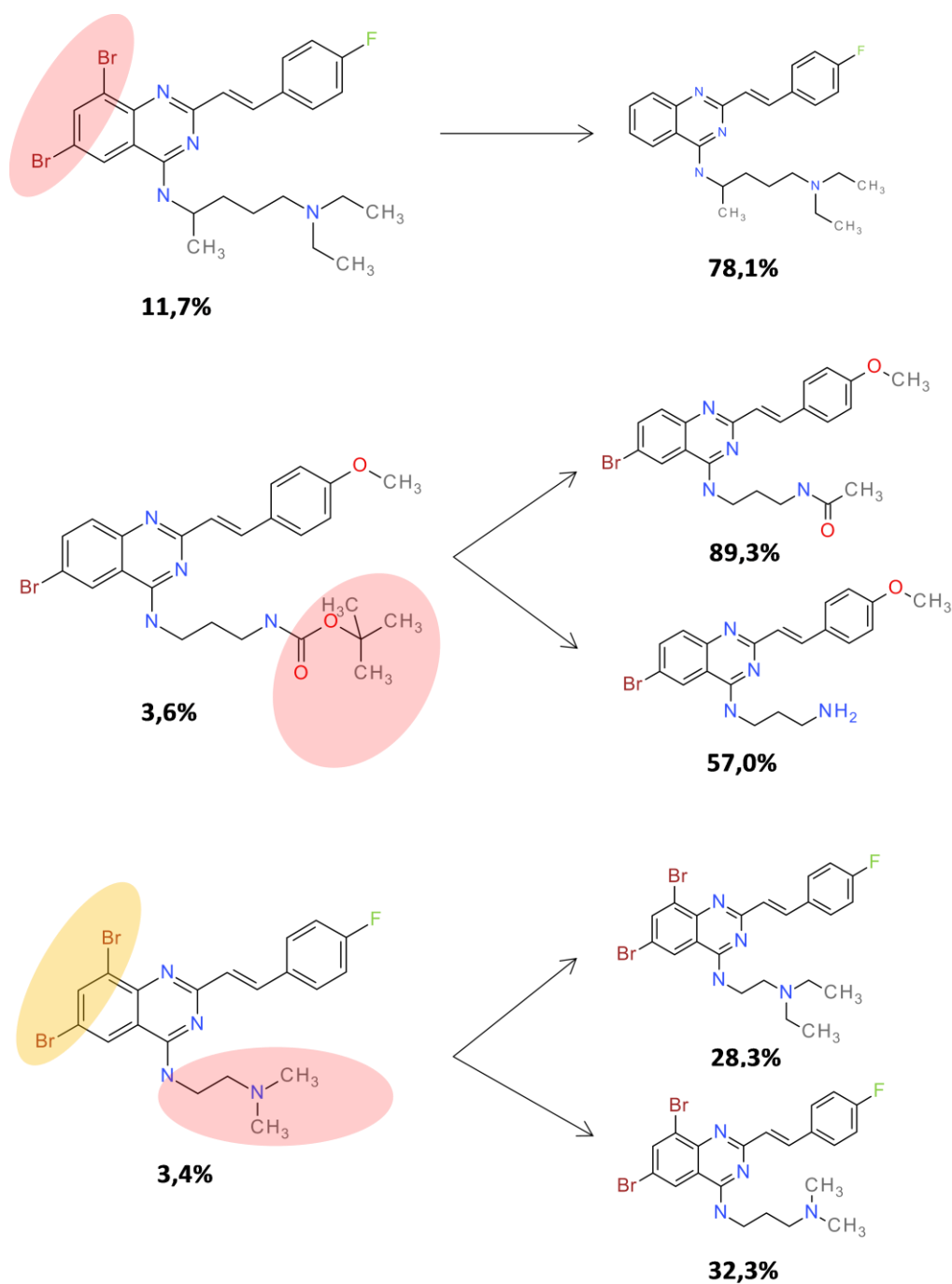


Figure 4.2.4c Styryl-quinazoline SAR. 3 ineffective compounds were found out of total 54 tested derivatives. Removing the 6,8 positioned Br substituents from the quinazoline ring significantly improves the efficiency of the compound (up), as well as removing BOC group from the aliphatic primer amino part of the molecule increases the insulinotropic effect (middle). A slight improvement in insulin secretion could be achieved when modifying the aliphatic part of the molecule (bottom). The insulin secretion in RIN-5AH cells is displayed in % under the structures.

4.2.4 Insulinotropic action of some marketed kinase inhibitors in RIN-5AH cells

Several clinically approved kinase inhibitors were also included in our ELISA screen as reference compounds. Based on the results of the primary screen we found no correlation between general kinase inhibitor property and insulin release. In our assay sunitinib and the imatinib derivate, missing the “magic methyl group” had stable insulinotropic property. This methyl group on the diaminophenyl ring serves also as a selectivity switch for PKC or Bcr-Abl kinases^{169,170} and probably it regulates the overall selectivity as well. Further kinase inhibitors showing weak or no insulinotropic effect in the primary screen were: dasatinib, sorafenib, tivantinib (*Figure 4.2.5*). Besides sunitinib, the small molecular inhibitors imatinib, dasatinib and sorafenib were also reported to have glycemic effects in clinical patients^{149,171,172}.

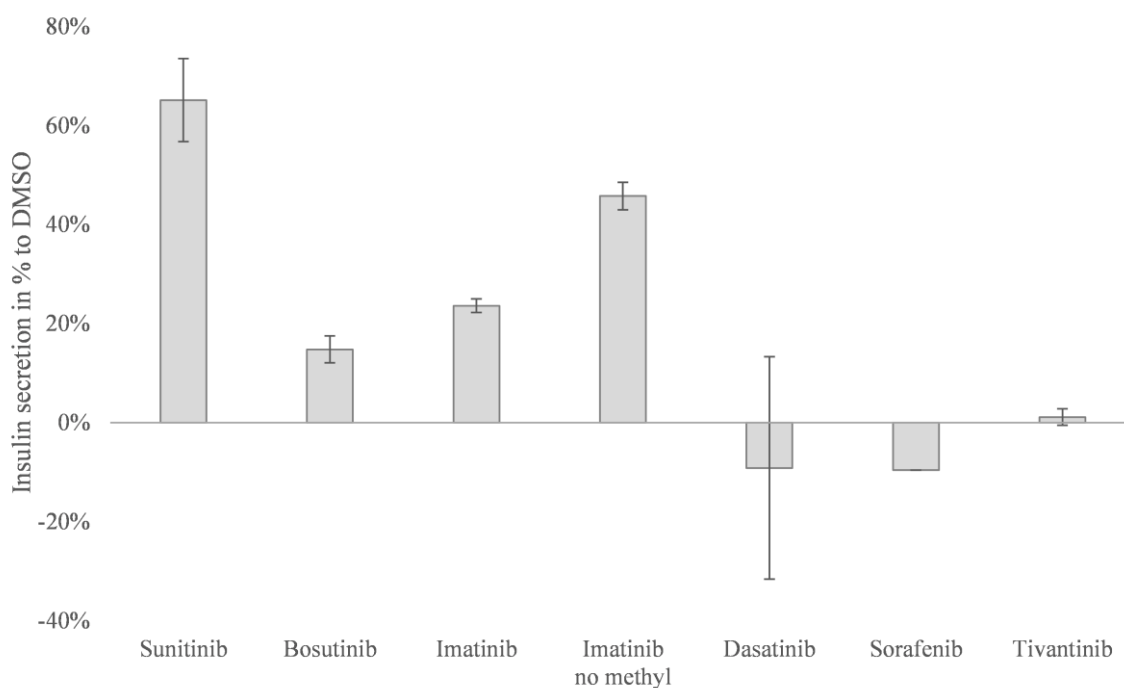


Figure 4.2.5 Some commercially available kinase inhibitors were included in the primary screen. It is worth to note that the derivative of imatinib lacking the methyl group was more active in insulin ELISA assay.

4.3 Glucose sensitivity of different beta cell lines

Glucose stimulated insulin secretion (GSIS) is one of the main physiological regulation mechanisms of insulin secretion, therefore it was important to include a cell line that secrete insulin in a glucose concentration dependent manner in our in vitro experiments. INS-1E cells proved to be the most sensitive and reliable model for testing GSIS¹⁷³. In other cells lines (RIN-5AH, beta-TC6, BRIN-BD11) GSIS was not observed, however BRIN-BD11 is also claimed to have this property^{174,175}. GSIS of different cell lines was tested in medium or KRBH buffer and the increase of insulin release was glucose dependent only in INS-1E cells (*Figure 4.3.1*).

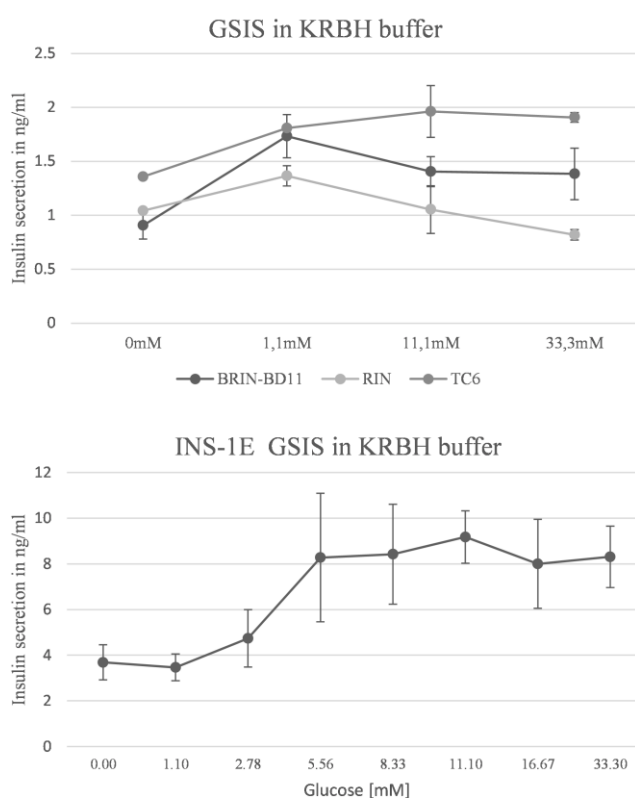


Figure 4.3.1 GSIS in different beta cell lines. Only INS-1E seems to be sensitive to GSIS, the RIN-5AH, TC6 and BRIN-BD11 didn't show any difference in insulin secretion between 1mM and higher concentrations of glucose.

4.4 Characterization of the most effective compounds

Altogether 12 compounds (including sunitinib) were selected as best hit compounds, at least one from each structure family. Compounds from this list were more extensively studied later on (*Table 4.4.1*). They increased insulin secretion in a concentration dependent manner and showed >25% insulin secretion in RIN-5AH cells. EC₅₀ values were calculated for the best candidates (*Figure 4.4.1*). Cell viability was determined after 72h treatment with the compounds in RIN-5AH cells and an “in vitro therapeutic index” (TI) was calculated, which reflects the relatively lower toxicity of the compounds compared to sunitinib (*Table 4.4.2a*). The TI was above 2 for most of the compounds, the highest TIs were observed for VCC036051:03 (8.54) and VCC981125:009 (6.19). The compounds were tested for in vitro toxicity in different cell lines as well (*Table 4.4.2b*). None of the compounds showed any decrease in viability up to 10µM treatment in C2C12 cells. Some compounds had a lower than 5µM IC₅₀ value in 3T3-L1, HepG2 and TC6 cells (VCC512178:08, sunitinib and VCC346349:01), however only one compound proved to be more toxic than sunitinib (VCC512178:08).

| <i>Core structures</i> | <i>Compound ID</i> | <i>Insulin</i> | <i>properties already known / drugs with same core</i> |
|--------------------------------|---------------------|----------------|--|
| <i>Oxindoles</i> | sunitinib | 55.9% | broad spectrum affinity to kinases |
| <i>Thieno pyrimidines</i> | VCC341830:01 | 38.6% | Selective EGFR inhibitor |
| | VCC834153:01 | 77.7% | |
| <i>Styryl-quinazolines</i> | VCC350485:02 | 40.5% | Derivative is a p53 inducer in HCT116 cells |
| <i>Other quinazolines</i> | VCC036051:03 | 35.7% | Contains core structure of erlotinib, gefitinib, lapatinib |
| | VCC981125:09 | 71.9% | Contains core structure of bosutinib, quinidine, chloroquine |
| <i>Quinolines</i> | VCC718565:01 | 101.4% | |
| | VCC673193:01 | 92.8% | |
| | VCC225733:01 | 72.7% | |
| <i>Bis-quinolines</i> | VCC405984:01 | 46.3% | |
| <i>Quinoxalines</i> | VCC958400:03 | 38.5% | |
| <i>1,6-Naphthyridines</i> | VCC459492:04 | 31.2% | |
| | VCC124075:02 | 32.7% | |
| <i>2-Amino-pyrimidines I.</i> | VCC512891:01 | 53.3% | Contains core structure of imatinib |
| | VCC346349:01 | 48.2% | |
| <i>2-Amino-pyrimidines II.</i> | VCC525068:03 | 82.4% | Inhibits PKC |
| | VCC161829:02 | 59.3% | Inhibits PAK |
| | VCC512178:08 | 72.2% | Inhibits PAK |

Table 4.4.1 List of compounds that were selected for further research. Compounds in bold: kinase affinity profile is available. Insulin release is displayed in % to DMSO, 5 μ M and 2h treatment in RIN-5AH cells.

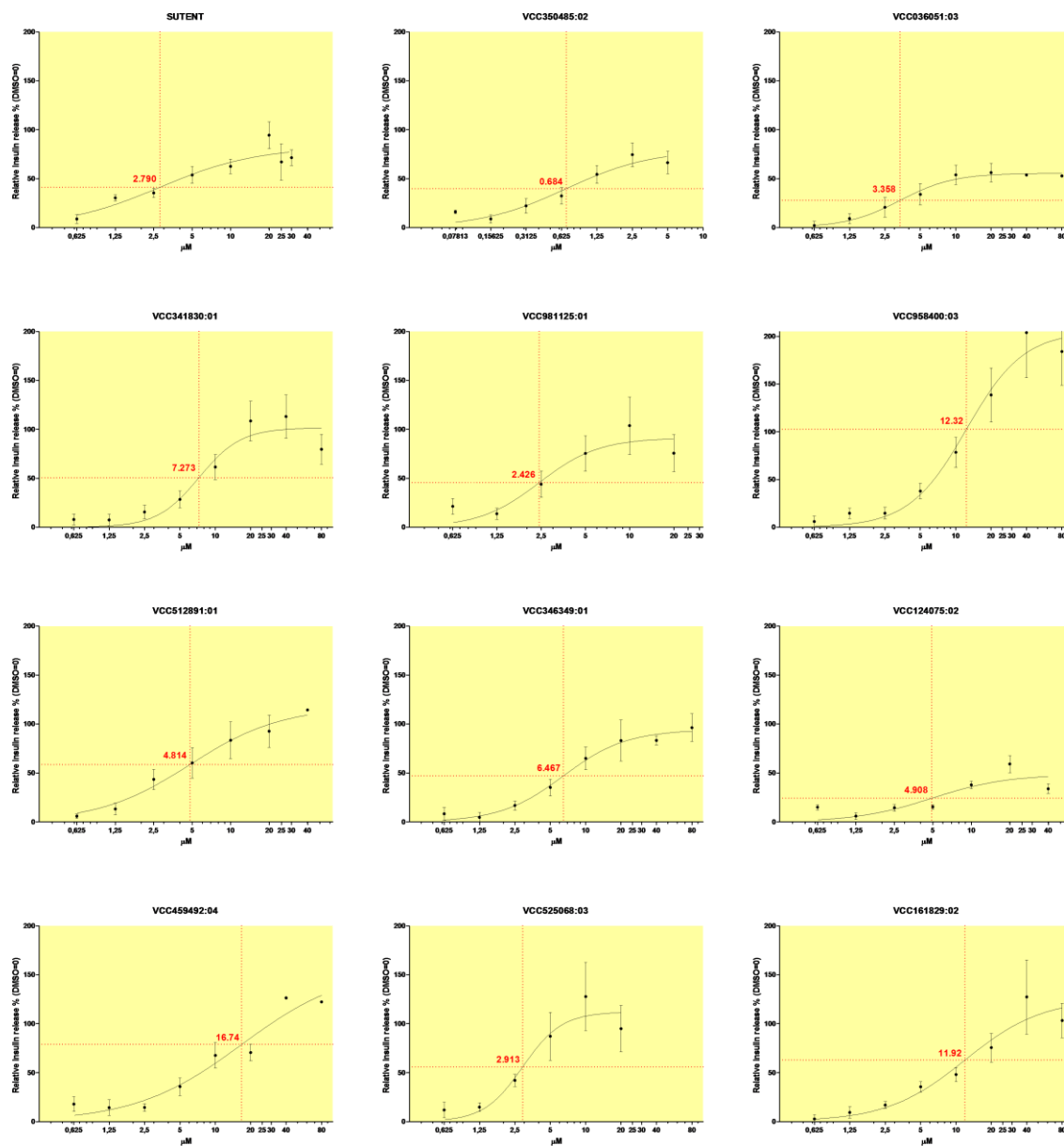


Figure 4.4.1 Concentration dependent effect on insulin secretion. EC50 values are displayed on the graphs for each compound. It is worth to mention that the total activity where the plateau is reached is different for the compounds.

| Compound | EC50 | IC50 | "In vitro" TI |
|--------------|-------|-------|---------------|
| SUTENT | 2.79 | 6.43 | 2.30 |
| VCC350485:02 | 0.68 | 2.96 | 4.32 |
| VCC036051:03 | 3.36 | >20 | 8.54 |
| VCC341830:01 | 9.84 | >20 | 2.84 |
| VCC981125:09 | 2.43 | 15.02 | 6.19 |
| VCC958400:03 | 12.32 | >20 | >1.67 |
| VCC512891:01 | 4.81 | 12.08 | 2.51 |
| VCC346349:01 | 6.47 | 13.99 | 2.16 |
| VCC124075:02 | 5.58 | >20 | 4.60 |
| VCC459492:04 | 16.74 | >20 | 3.13 |
| VCC525068:03 | 3.59 | 12.22 | 3.40 |
| VCC161829:02 | 11.92 | >20 | >1.67 |

Table 4.4.2a Summarized EC₅₀ for insulin release, IC₅₀ viability values for 72h CellTiterGlo experiments and calculated "in vitro therapeutic indexes" (TI) values for RIN-5AH cells.

| Compound | 3T3-L1 | C2C12 | HepG2 | TC6 |
|--------------|--------------|-------|--------------|--------------|
| VCC350485:02 | 5.942 | >10 | 5.65 | 5.403 |
| VCC036051:03 | >10 | >10 | >10 | >10 |
| VCC834153:01 | >10 | >10 | 14.39 | 14.54 |
| VCC341830:01 | >10 | >10 | >10 | 11.9 |
| VCC981125:09 | >10 | >10 | >10 | >10 |
| VCC958400:03 | >10 | >10 | >10 | >10 |
| VCC512891:01 | 6.802 | >10 | 12.27 | 10.02 |
| SUTENT | 2.138 | >10 | 5.742 | 6.056 |
| VCC346349:01 | 7.734 | >10 | >10 | 3.99 |
| VCC124075:02 | 6.286 | >10 | 10.9 | 10.34 |
| VCC459492:04 | >10 | >10 | >10 | >10 |
| VCC525068:03 | 13.11 | >10 | >10 | 8.326 |
| VCC512178:08 | 3.469 | >10 | 3.979 | 2.288 |

Table 4.4.2b CellTiterGlo. IC₅₀ values (uM) of compounds are shown on different cell lines for 72h treatment. IC₅₀ values <5uM are highlighted in bold.

4.5 Selected best compounds were more selective than sunitinib

Since these compounds were originally designed as kinase inhibitors, their affinity and selectivity were tested against 392 kinases¹⁶⁴. The service was provided by DiscoverX. Most of the compounds were more selective than sunitinib and had better (VCC981125:09) but at least comparable impact on insulin secretion in RIN-5AH cells (*Figure 4.5.1*). The VCC124075:02 was the least selective of all compounds beside sunitinib ($S > 0.5$). The VCC512891:01, VCC346349:01 and VCC459492:04 shared moderate selectivity ($0.5 > S > 0.1$). The VCC350485:02, VCC036051:03 and VCC341830:01 were highly selective ($S < 0.1$). The VCC350485:02 binds FLT3 kinase and its numerous mutant forms selectively with high affinity (%Ctrl <20). It binds other kinases as well but with much lower affinity: CSNK1A1, RIOK3, GAK ($35 > \%Ctrl > 20$). VCC341830:01 is highly specific for EGFR, HER2, MKNK2 and KIT (%Ctrl <20) and binds several other kinases with lower affinity ($35 \geq \%Ctrl > 20$; IRAK3, RIPK2, GAK, TYK2, LTK). The VCC036051:03 binds two kinases selectively but with relatively low affinity ($30 < \%Ctrl < 40$; BMPR1B, MAP3K1). The VCC981125:01 and VCC958400:03 seemed to have no affinity to kinases (no hits at %Ctrl <35). The full profiling data is available in the *Appendix* separately for each compound.

Regarding to our main hypothesis that compounds probably share common kinase targets which can induce insulin secretion upon their inhibition, we planned to create a pool of overlapping kinase hits, but first, these targets had to be validated by MS to get information also about their presence in the RIN-5AH cell lysate (Chapter 4.6).

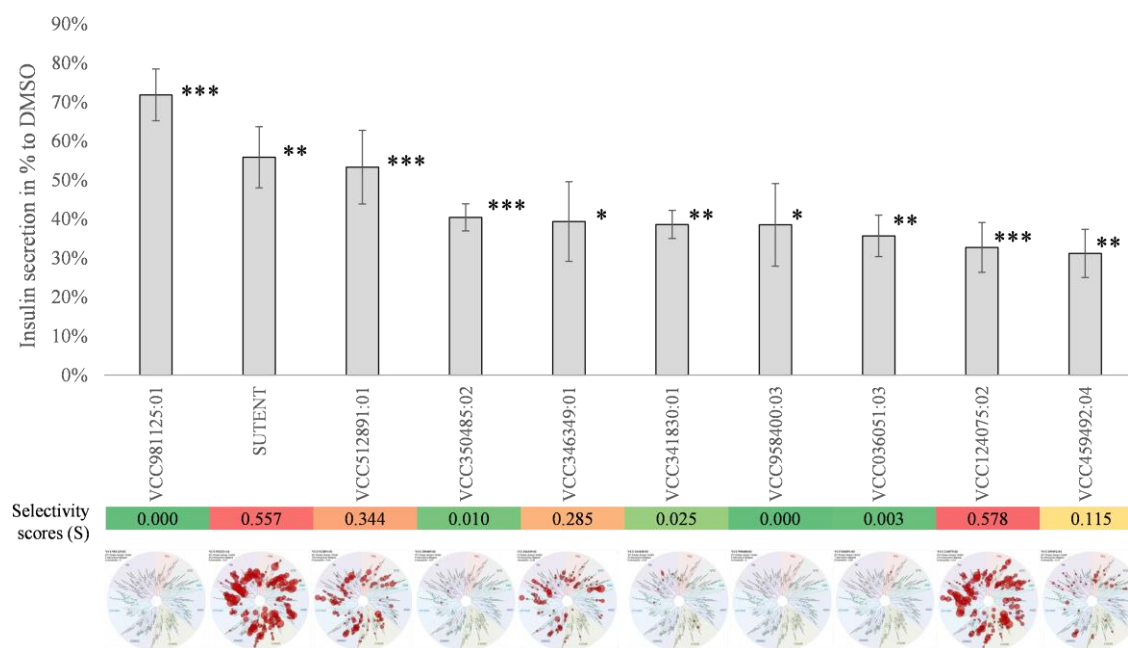
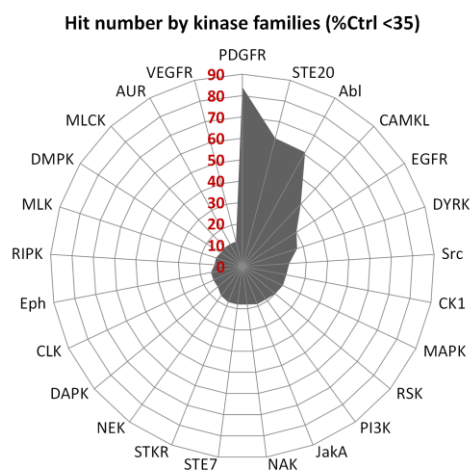
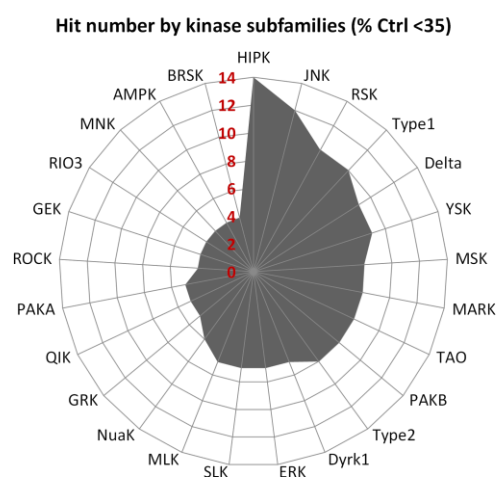


Figure 4.5.1 Insulin secretion is displayed on the diagram in % to DMSO in RIN-5AH cells ($n=4$; $*p<0.01$; $**p<0.005$; $***p<0.001$). Selectivity scores and kinome-trees are displayed under the bars (service by DiscoverX). Selectivity score (S) = (number of non-mutant kinases with %Ctrl < 35) / (392 non-mutant kinases tested)

The following figures highlight the kinase families and subfamilies that were most frequently hit by the compounds in the kinase affinity screen, however it does not mean that they are responsible for insulin secretion, indeed we think that off-targets (not main targets of the compound) or a combination of targets are more important in the matter of enhanced insulin secretion.



Frequent hits for all compounds were sorted by *kinase families*. 25 kinase families with most hits are shown (most hits were found in the PDGFR family = 84 kinases).



Frequent hits for all compounds were sorted by *kinase subfamilies*. 25 kinase subfamilies with most hits are shown (most hits were found in the HIPK subfamily = 14 kinases).

4.6 MS affinity chromatography using RIN-5AH cell lysate

Using the MS-based target deconvolution we could confirm targets from kinase affinity assay and their expression in the RIN-5AH cells. This technique is based on the binding affinity of compounds to protein targets which is independent of ATP concentration, therefore we can identify compounds with different binding modes. Altogether 20 amino-linker derivatives (“kinators”) were prepared and according to the criteria (see materials and methods) some of them were selected to perform the coupling experiments. Compounds were synthesized for oxindole, thieno-pyrimidine, styryl-quinazoline, quinoline, quinoxaline, 1,6-naphthyridine, amino-pyrimidine and misc. families (for some core structures more linker derivatives were available with distinct linker positions on the molecule). Four candidates were selected and analyzed by MS in the final experiments. These compounds showed specific binding or specific competition (target pull-down) in the pre-experiments as well.

4.6.1 Pre-experiments for MS- based target deconvolution

Linker derivatives and their amino-protected versions were tested for insulin secretion (5 μ M, 2h) and then affinity matrices were generated so far as the derivatized compounds remained active. Affinity matrices were incubated with protein lysate and free compound, after that samples were run in SDS gel and silver staining was performed as described in materials and methods.

4.6.1.1 VCC350485:02 (Styryl-quinazoline)

Its acetyl-protected derivative and the unprotected kinator were also effective in RIN-5AH cells. Different coupling densities were tested (2mM, 6mM and 10mM) and based on these results, 2mM was chosen for the final experiment. At higher densities the chance of unspecific binders is increased. Specific binders could be detected with silver-staining, however apparently no proteins could be competed off from the matrix (*Figure 4.6.1*)

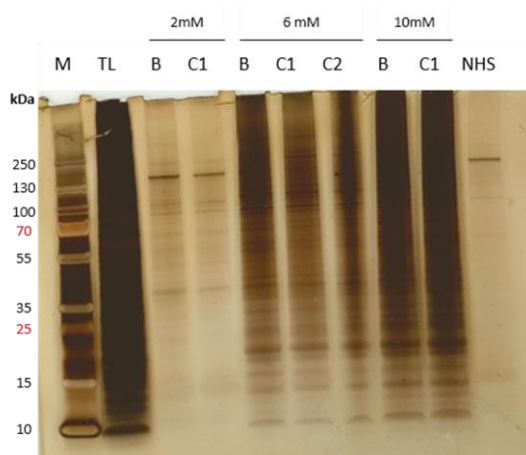


Figure 4.6.1

Silver gel staining of protein targets for VCC350485:02 matrix (2mM, 6mM and 10mM coupling densities).

M: Protein marker (Fermentas, #26619)

TL: Total lysate

B: Binders to the affinity matrix

C1: Competition with 50uM VCC350485:02

C2: Competition with 50uM Sunitinib

NHS: Empty NHS beads blocked with ethanolamine

No protein could be competed off that was visible by eye, however specific binders were detected.

4.6.1.2 Sunitinib (Oxindoles)

Acetyl protected derivative and kinator with the free primary amino group were effective (20%) but not the BOC protected. Reduced efficiency of the linker variant can be explained due its altered membrane permeability properties. This hypothesis is also supported by Schrödinger QikProp measurements using different prediction algorithms for testing pharmaceutically relevant properties of the compound structures, including Caco-permeability test, octanol/water logP, logS values. According to the Caco test, the linker derivative was ~4-fold less permeable (*See Appendix*). We assume that the binding affinity is changing because BOC is a “bulky group”, therefore it can strongly affect binding because of sterical hindrance. The effect of the kinator itself plays less importance in the cellular assay, because of its free amino group, its properties differ very much compared to the original lead molecule and the beads-coupled one. Linker derivative was tested in different coupling densities (2mM, 6mM and 8mM), for final experiment 2.5mM density was chosen (NHS beads). After silver staining, specific binders and competed off targets were also observed for sunitinib. Compounds possessing different core structures apparently did not compete off any proteins from the matrix, probably because of their binding modes or affinities are different (*Figure 4.6.2*).

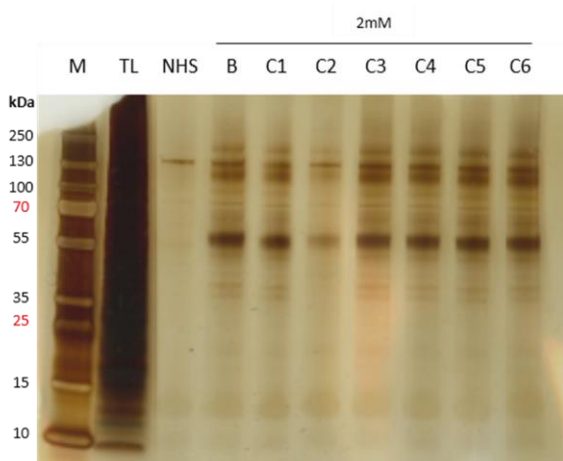


Figure 4.6.2

Silver gel staining of protein targets for Sunitinib affinity matrix (2mM coupling density) Direct and indirect pulldowns with different compounds.

M: Marker
TL: Total lysate
NHS: Empty NHS beads blocked with ethanolamine
B: Binders to affinity matrix of Sunitinib
C1: Competition with 50uM VCC341830:01
C2: Competition with 50uM Sunitinib (disappearing bands)
C3: Competition with 50uM VCC981125:01
C4: Competition with 50uM VCC346349:01
C5: Competition with 30uM VCC036051:03
C6: Competition with 50uM VCC350485:02

4.6.1.3 VCC512891:01 (Amino pyrimidines I.)

The protected amino derivative was effective in RIN-5AH cells (27%), weaker effect can be explained due to its lower permeability compared to the free linker derivative (see Appendix). After silver staining of gels specific protein enrichment and competition could be observed (Figure 4.6.3) Different coupling densities were tested on NHS beads (1.5mM, 3mM and 6mM) and 4.2mM density on epoxy beads. Coupling density used for final experiment was 4.2mM (epoxy beads).

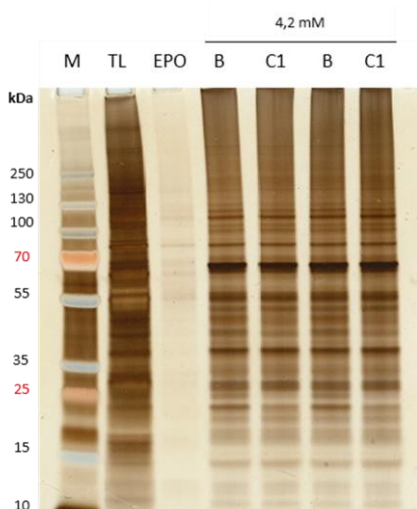


Figure 4.6.3

Silver gel staining of protein targets for epoxy coupled VCC512891:01 affinity matrix (4.2mM density).

M: Marker
TL: Total lysate
EPO: empty epoxy beads blocked with ethanolamine
B: Binders to affinity matrix of VCC512891:01
C1: Competition with 50uM VCC512891:01

Targets competed off at ~45kDa and ~25kDa

4.6.1.4 VCC124075:02 (Amino pyrimidines II.)

Formyl protected derivative was effective (23.8%) for insulin release, therefore NHS beads were used for coupling. Specific protein enrichment (binders) and competed targets were observed on silver stained gels (Figure 4.6.4). Coupling densities tested: 2mM, 4mM and 8mM. Coupling density used for final experiment was 8mM.

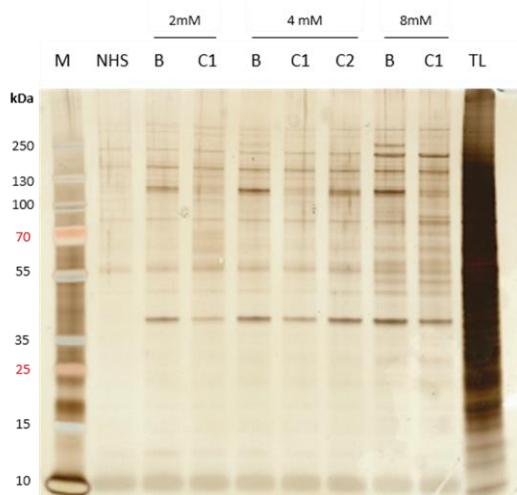


Figure 4.6.4

Silver staining of protein targets for VCC124075:02 matrix (densities: 2mM, 4mM, 8mM).

M: Marker

NHS: Empty NHS beads blocked with ethanolamine

B: Binders to affinity matrix

C1: Competition with 50uM VCC124075:02

C2: Competition with 50uM Sunitinib

TL: Total lysate

Targets competed off at ~120kDa, ~40kDa, ~250kDa

4.6.2 Target deconvolution results

The results and targets displayed in this chapter were selected by their $K_{d_{free}}$ and $K_{d_{imm}}$ values. The LC/MS-MS analysis and K_d values were calculated by an algorithm developed by Evotec AG.

VCC350485:02 linker derivative. Unfortunately no targets were competed off from the matrix, therefore $K_{d_{free}}$ values could not be determined. However specifically enriched binders ($K_{d_{imm}}$) were found which can still give a hint about the real targets of the mother compound. Hits with $K_{d_{imm}} < 90 \mu\text{M}$, peptide numbers ≥ 2 and sequence coverage $> 20\%$ were chosen. The main kinase target FLT3, from the kinase affinity screen was not enriched on the affinity matrix, probably because of its relatively low expression level in beta cells. FLT3 is mainly expressed in hematopoietic cells, thymus, lymph nodes, brain and gonads¹⁷⁶. We were focusing mainly on kinases but it is worth to mention other possibilities as well. Theoretically possible mechanisms and pathways related to insulin secretion were analyzed using the IPA software from Ingenuity, Qiagen, which is based on available literature findings. These potential targets include glycolytic enzymes (e.g.: aldolase A and pyruvate kinase) as well and were also described in a study to associate with K_{ATP} channels. Patch clamp measurements also showed that upon stimulation with their substrates (phosphoenolpyruvate or fructose-1,6-bisphosphate) K_{ATP} channel could be reversibly blocked¹⁷⁷, which in effect led to insulin secretion (*more detail in Figure 4.6.5*).

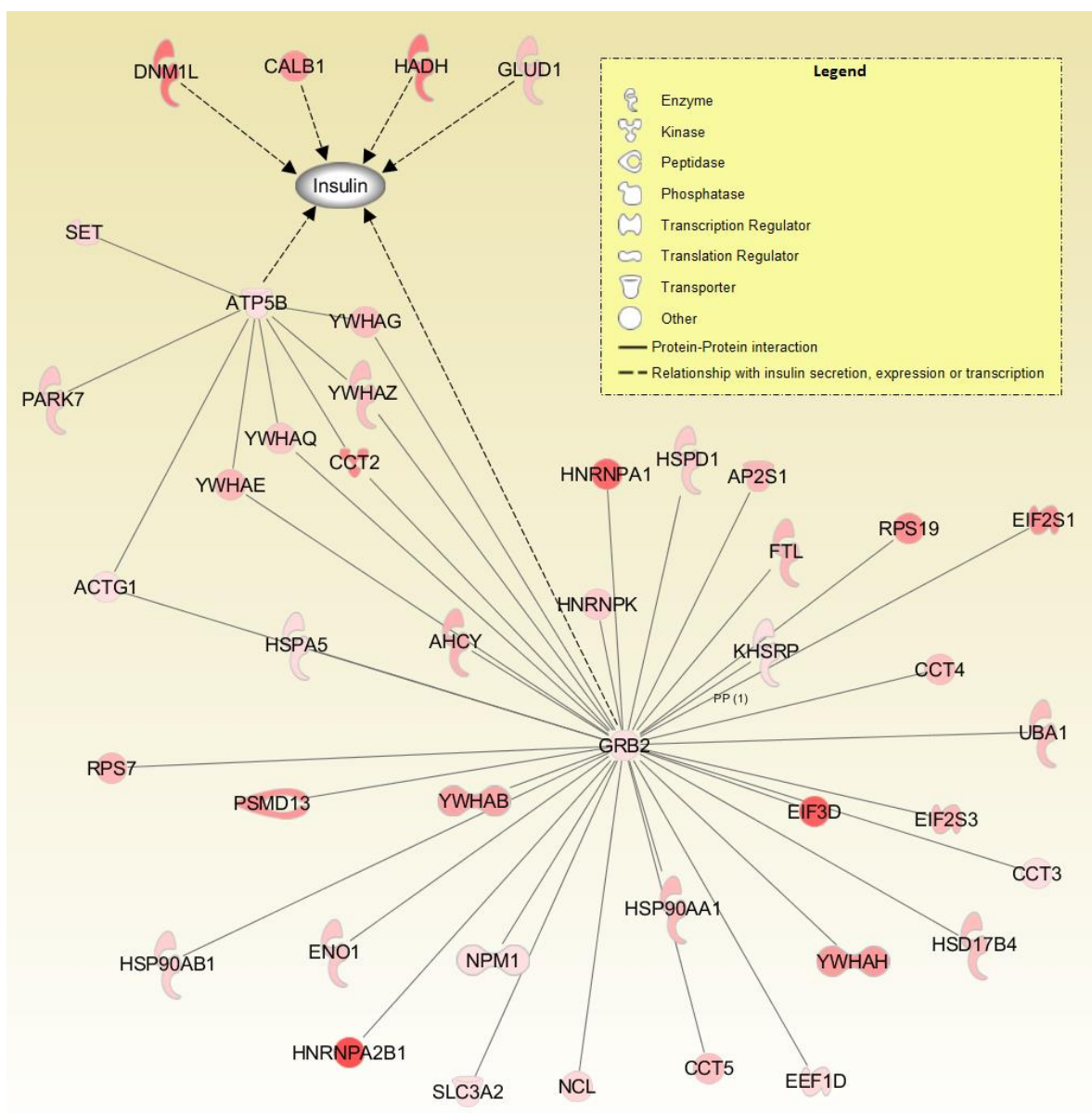


Figure 4.6.5 IPA Ingenuity pathway analysis (Qiagen). Binders for VCC350485 matrix and their relationship to insulin are depicted in the picture. Six targets were found by IPA analysis to play a role in the regulation of insulin secretion and are marked with dashed lines. Dominant negative mutant of DNM1L (Dynamin-1-like protein) was found to decrease GSIS insulin secretion in INS-1E cells¹⁷⁸; Calb1 (Calbindin) overexpression increased insulin mRNA level in RIN 1046-38 cells¹⁷⁹, another study showed that in knockout mice it induced insulin secretion¹⁸⁰; HADH (mitochondrial hydroxyacyl-coenzyme A dehydrogenase) was described to negatively regulate insulin secretion in INS832/13 β -cells and its loss of function mutation is associated with hyperinsulinemia⁴²⁻⁴⁵. In mice harboring the dysfunctional mutant an increased insulin level in blood was observed due to activation of glutamate dehydrogenase (GDH; GLUD1)¹⁸¹⁻¹⁸⁴. GDH stimulates glutamate conversion to α -ketoglutarate and as a consequence ATP is generated, which can induce insulin secretion⁴⁸. Interestingly GDH was also enriched on the matrix. Its activation promotes insulin secretion, conversely the knockdown reduces the effect. GRB2 and ATP5B is a binding partner of many other protein hits as well, therefore it seems that they are prone to form complexes thus they might not directly interact with the compound.

For the other compounds stronger evidence is available, since targets competed by the free compound could be identified. These targets were then compared to kinase affinity screen results.

Sunitinib. 124 targets were competed off (see $K_{d_{free}}$ values in the Appendix) from the sunitinib matrix and 70 of them are overlapping with the kinase affinity assay hits (Table 4.6.1). Threshold for kinase affinity screen were set to %Ctrl<55 and these potential targets were pooled together to compare the two methods and find the overlaps between them. The indirect competition with linker free derivate of sunitinib and VCC607440:01 matrix (VCC512891:01 linker derivative) resulted 49 hits. By comparing the direct and indirect competition experiments, 23 overlaps were found.

| | | | | | | |
|---------------|----------------|---------------|----------------|-------------|----------------|--------------|
| <i>Aak1</i> | <i>Camk2b</i> | <i>Gsk3b</i> | <i>Map3k15</i> | <i>Mek2</i> | <i>Pctk3</i> | <i>Src</i> |
| <i>Abl1</i> | <i>Camk2d</i> | <i>Insr</i> | <i>Map3k2</i> | <i>Mek5</i> | <i>Pdpk1</i> | <i>Stk16</i> |
| <i>Ampk1</i> | <i>Camkk2</i> | <i>Irak4</i> | <i>Map3k3</i> | <i>Melk</i> | <i>Phkg2</i> | <i>Tbk1</i> |
| <i>Ampk2</i> | <i>Chek1</i> | <i>Jak1</i> | <i>Map4k3</i> | <i>Plk3</i> | <i>Pip5k2c</i> | <i>Tlk2</i> |
| <i>Aurka</i> | <i>Csnk1a1</i> | <i>Jak2</i> | <i>Map4k5</i> | <i>Mst1</i> | <i>Plk4</i> | <i>Tnik</i> |
| <i>Aurkb</i> | <i>Csnk1d</i> | <i>Jnk2</i> | <i>Mark1</i> | <i>Mst2</i> | <i>Rps6ka4</i> | <i>Tnk1</i> |
| <i>Bike</i> | <i>Fak</i> | <i>Kit</i> | <i>Mark2</i> | <i>Mst3</i> | <i>Rps6ka5</i> | <i>Trka</i> |
| <i>Bmpr2</i> | <i>Fer</i> | <i>Lats1</i> | <i>Mark3</i> | <i>Nek2</i> | <i>Rsk1</i> | <i>Tyk2</i> |
| <i>Brsk2</i> | <i>Fes</i> | <i>Lyn</i> | <i>Mast1</i> | <i>Pak4</i> | <i>Rsk2</i> | <i>Ulk3</i> |
| <i>Camk2a</i> | <i>Gak</i> | <i>Map3k1</i> | <i>Mek1</i> | <i>Pak7</i> | <i>Slk</i> | <i>Yes</i> |

Table 4.6.1

VCC512891:01. With this compound both direct and indirect competition was performed. We found 84 hits by competing off targets indirectly from the sunitinib matrix. Direct competition from its matrix analogue resulted 88 hits (see $K_{d_{free}}$ values in Appendix) and 25 of them overlapped with kinase affinity screen hits (Table 4.6.2). Targets from the kinase affinity screen were pooled where %Ctrl<55. Again, by indirect pull-down, the identified targets were different and only 12 overlaps have been found. This incoherence can be explained by the different affinities of sunitinib and VCC512891:01 to the proteins. Probably the binding modes of the two compounds also differ from each other.

| | | | | | | |
|---------------|----------------|--------------|---------------|----------------|--------------|--------------|
| <i>Aak1</i> | <i>Csnk2a1</i> | <i>Gsk3b</i> | <i>Map4k3</i> | <i>Pip5k1a</i> | <i>Slk</i> | <i>Taok3</i> |
| <i>Aurka</i> | <i>Dapk3</i> | <i>Jnk1</i> | <i>Mark2</i> | <i>Pip5k2b</i> | <i>Src</i> | |
| <i>Camk1</i> | <i>Fyn</i> | <i>Jnk2</i> | <i>Mast1</i> | <i>Pip5k2c</i> | <i>Stk16</i> | |
| <i>Camk2a</i> | <i>Gak</i> | <i>Limk2</i> | <i>Mink</i> | <i>Rock1</i> | <i>Syk</i> | |

Table 4.6.2

VCC124075:02. We found 28 hits by directly competing off targets from its matrix analogue (see $K_{d\text{free}}$ values in Appendix), 14 of them overlapped with kinase affinity screen results (Table 4.6.3). Targets from the kinase affinity screen were pooled where %Ctrl<55.

| | | | | | | |
|--------------|--------------|--------------|-------------|---------------|--------------|--------------|
| <i>Aak1</i> | <i>Aurka</i> | <i>Gsk3b</i> | <i>Jnk1</i> | <i>Map3k1</i> | <i>Mark2</i> | <i>Sik</i> |
| <i>Ampk1</i> | <i>Gak</i> | <i>Hipk3</i> | <i>Jnk2</i> | <i>Mark1</i> | <i>Mark3</i> | <i>Stk16</i> |

Table 4.6.3

4.6.3 Selection of common kinase hits

I have used an excel macro to analyze the big datasets of the MS experiments (Appendix). This made it possible to compare up to 10 different sets of protein IDs and at the end an arbitrary number of intersections could be selected and visualized. When pooling together all hits from the five MS competition experiments (see materials and methods) we got a list of overlapping targets wherein all the directly or indirectly found ones were included (Table 4.6.4). We hypothesized that some of these common kinase targets are involved in the negative regulation of insulin secretion.

| | | | |
|---------------|-------|--------------|--------------|
| Aak1 | Aurka | Gsk3b | Jnk2 |
| Prkab1 | Azi2 | Mark2 | Stk16 |
| Prkag1 | Epha7 | Mark3 | |
| Prkaa1 | Gak | Mast1 | |

Table 4.6.4

Overlapping hits from MS experiments. Common targets of 4 (regular) or of 5 (**bold**) competition experiments.

A few direct correlations were found in the literature that describes the roles of these kinases in the regulation of insulin secretion in beta cells. GSK3b was described to phosphorylate MARK2¹⁸⁵, therefore assuming a direct interaction it might be also possible that MARK2 and GSK3b were enriched in a complex form on the compound matrices. Selective knockdown of GSK3b in skeletal muscle cells in mice model decreased blood glucose and insulin level, while insulin sensitivity increased¹⁸⁶. Furthermore loss of GSK3b preserved beta cell mass in *Irs2*^{-/-} mice¹⁸⁷. MARK2 null mice became insulin hypersensitive and resistant to high fat-induced weight gain, while insulin level decreased¹⁸⁸. Another study also identified MARK2 as a potential target in diabetes, as it blocks TORC2/CREB activity, which is critical for beta cell survival¹⁸⁹. MARK3 null mice showed different phenotype to MARK2 null mice (protection against high-fat diet-induced obesity and improved glycemic control, but glycogen synthase level was increased)¹⁹⁰. The AMPK constitutes of three subunits (Prkab1, Prkag1, Prkaa1) that form a heterotrimer, where Prkag1 is essential for the activity of the enzyme¹⁹¹. AMPK1, which activity depends on the cytoplasmic ratio of AMP/ATP has a negative regulator role in insulin

secretion. This was observed in a study where constitutively active AMPK1 (Prkaa1) could inhibit insulin secretion^{192,193}. Nevertheless it is worth to mention that AMPK is activated by metformin in other tissues and has a capital role in the metabolism of fatty acids, cholesterol and triglycerides and it also improves glucose uptake in muscle cells and adipocytes^{194,195}. Insulin secretion could also be stimulated by the AMPK activator AICAR however it develops its effect likely through an AMPK independent pathway¹⁹⁶. In overall, recent knowledge suggests that the objective in the treatment of diabetes is rather AMPK activation than inhibition¹⁹⁷, although its deactivation may promote insulin secretion.

4.7 Designing a pool of targets from affinity screen results and MS target deconvolution

To summarize the target discovery results acquired by MS and kinase affinity assays, we took overlapping targets from both experiments and selected 14 kinase hits (*Table 4.7.1*). By processing the data the following rules were applied: the threshold was set to %Ctrl<55 and compound overlaps \geq 3 in the kinase affinity screen; from the MS data the best 57 targets were selected by applying the Excel macro on 5 datasets. These 57 targets are hits for at least 3 compounds (out of 5). These 14 kinase hits that were selected, unite the targets from MS experiments mentioned in the previous chapter (GAK, JNK2, MARK1, MARK2, MAST1 and STK16) and additional hits due to the increased threshold (overlaps for 5,4 or 3 competition experiments were also included, thus the list was extended with the following kinases: AAK1, AURKA, CAMK2A, CSNK1D, CSNK2A1, MAP3K1, MAP4K3 and SLK) furthermore it includes kinases that were identified in the kinase affinity screen. Hits highlighted in bold were selected for siRNA validation.

| | | | | | | |
|-------|--------|----------------|-------------|--------|--------------|--------------|
| AAK1 | CAMK2A | CSNK2A1 | JNK2 | MAP4K3 | MARK2 | SLK |
| AURKA | CSNK1D | GAK | MAP3K1 | MARK1 | MAST1 | STK16 |

Table 4.7.1 Overlapping targets from MS and kinase affinity screen experiments

AAK1 is associated with clathrin-dependent endocytosis by phosphorylating the mu subunit of AP-2 adaptor protein¹⁹⁸⁻²⁰⁰, which triggers vesicle formation. Insulin is also stored in clathrin coated vesicles, however clathrin does not stimulate the formation or exocytosis of the granules, but it likely decreases protease activity in rat beta cells²⁰¹. GAK is probably another component of the clathrin coated vesicle, since its structure is homologous to auxilin, a protein that mediates the disassembly of the complex^{202,203}. CSNK2A1 is the alpha subunit of casein kinase II (CK2). CK2 is known to regulate insulin secretion and carbohydrate metabolism furthermore its inhibition by quinalizarin led to elevated insulin level in pancreatic beta cells^{204,205}. This target is used as a positive reference in the siRNA experiments. CK2 also plays an important role in cancer, cell viability and invasion due to its high expression level in rapidly proliferating cells, therefore it is a potentially new target against cancer. Additionally CK2 has an angiogenic effect as well. Two CK2 inhibitors are already in clinical trials for cancer treatment²⁰⁶⁻²⁰⁸. SLK is a microtubule associated protein and it belongs to the STE-20 kinase subfamily and transduces proapoptotic signal. It mediates apoptosis, adhesion, cell-component localization and migration²⁰⁹⁻²¹¹. Since it is a microtubule associated protein it might also regulate movement of secretory vesicles, i.e. vesicles loaded with insulin. Interestingly its activity can be down-regulated by CK2, in the course of direct phosphorylation²¹². The role of MARK2 in insulin level regulation was described previously, nevertheless it is also a possible target in tauopathies (e.g. Alzheimer disease). It regulates cell polarity and overexpression can be assigned with negative qualities in different cell types²¹³. MAST1 seems to be a recurrent partner of gene fusions in breast cancer^{214,215}. It includes a PDZ domain, which participates in organizing signaling complexes and anchoring transmembrane proteins to the cytoskeleton. In general these motifs bind to the C-terminus of other proteins²¹⁶. The well-studied JNK2 that is a member of the MAP kinase family and was also identified as hit of some compounds. It is involved in cell proliferation, differentiation, migration and programmed cell death. After stress stimuli the SAP/JNK2 pathway gets activated, which in turn activates transcription factor components of the AP-1 complex, such as c-Jun and ATF^{217,218}. JNK2 also promotes cell apoptosis by directly phosphorylating p53 at Ser6 and YAP1 protein^{219,220}. Activation of different isoforms of JNKs is also observed in insulin resistance and obesity models, for instance stimulation with fatty acids and high concentration of glucose and can be associated with apoptosis or increased level of proapoptotic genes in INS-1 beta cells²²¹. Consequently overexpression of dominant-negative JNK in adipose tissue and selective suppression of both JNK1 and JNK2 have a protective effect in preventing obesity, apoptosis and insulinitis in different models²²²⁻²²⁴. Deactivation of JNK resulted in increased insulin-stimulated glucose

uptake in skeletal muscles, furthermore decreased mRNA levels of PEPCK and G6Pase (enzymes responsible for gluconeogenesis) were observed, therefore glucose production was also reduced in dominant-negative HFD mice models compared to lean controls²²². The exact function of STK16 is not defined yet, but it is known that it undergoes myristylation and palmitoylation which is one of the important events regulating the subcellular localization of proteins. These changes in the protein structures can influence and regulate signaling pathways inside the cell. The acetylation by fatty acids of other proteins and enzymes (Ras, Src families, heterotrimeric G proteins and GRK6) were also shown to be important for their function^{225,226}. STK16 is a Golgi-resident protein and its structure and function is conserved amongst different species²²⁷. Env7, a strongly homologous protein to the human STK16 in yeast negatively regulates membrane/vacuole fusion^{227,228}. Regarding to these observations STK16 likely has an important role in the regulation and organization of the endomembrane structure in cells (ER, Golgi, endosomes, lysosomes and plasma membrane). A few studies revealed its involvement in different secretory events as well²²⁹⁻²³¹. The calmodulin-dependent kinase 2 (CAMK2) mediate the effects of Ca²⁺ in the cells. It takes part in the regulation of insulin exocytosis, as many insulin secretagogue can activate the kinase and thus induce insulin secretion^{232,233}. Furthermore a recent study reported that CAMK2 modulates insulin secretion by inhibiting K_{ATP} channels²³⁴ but it is also claimed to reduce the adenylyl cyclase III activity (AC-III) as well and by this it may downregulate cAMP level, albeit these studies were done not in beta cells^{32,235,236}. AC-III is expressed in beta cells and can induce the generation of cAMP²³⁷. A recent study showed that, under diabetic conditions the activation of hepatic CAMK2 can be associated with insulin resistance²³⁸. In overall CAMK2 exerts a complex regulatory effect in different tissues and its uncontrolled functioning can lead to phenotypical changes.

4.8 Validation of potentially negatively regulating kinases of insulin secretion by siRNA

The role of CK2 (CSNK2A1) in the negative regulation of insulin secretion is already known from some publications. This property of CSNK2A1 seems to exist in RIN-5AH and INS-1E cells too. Other kinases such as AAK1, GAK and STK16 are thought to modulate vesicle formation or trafficking, but their direct role in insulin secretion was not investigated yet by others. The function of MAST1 and SLK in insulin release was not studied yet either. MARK2 was described to have favorable impacts on insulin resistance in knockout mice, however its targeted knockdown was not tested in beta cells. The results achieved with ON-TARGETplus siRNAs show that knockdown of STK16, CSNK2A1 and MARK2 shows an increased insulin secretion in RIN-5AH cells (*Figure 4.8.1*). In INS-1E cells by silencing MAST1 and JNK2 an increased insulin secretion was observed besides knockdown of STK16, CSNK2A1 and MARK2 (*Figure 4.8.2*). However at 72h knockdown of MARK2 showed a reduced effect (*See Appendix*). By using Accell siRNAs, a different assay method to generate knockdown models, reasonable efficiencies that correlated with an increased insulin release could only be determined for STK16 and MAST1 samples. The stimulatory effects and results of the Accell siRNA experiments are displayed in *Appendix*.

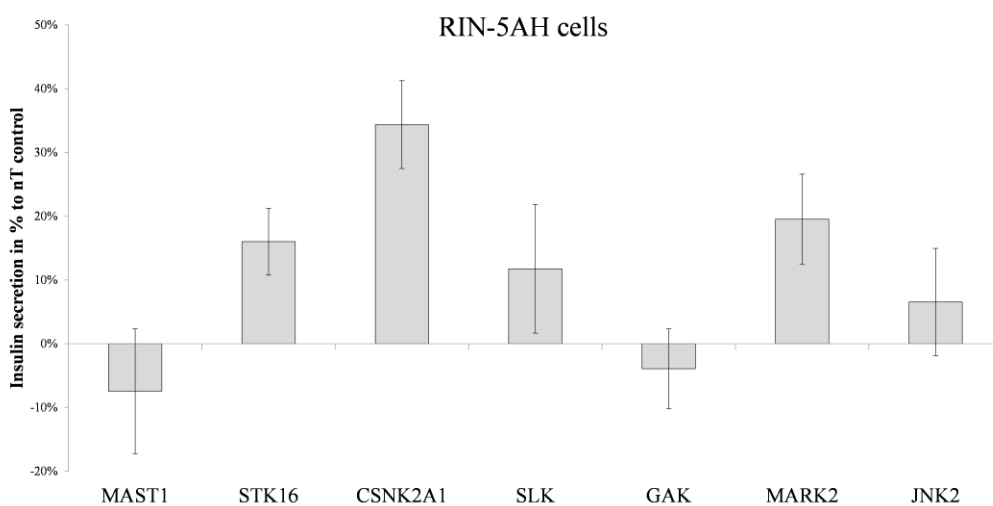


Figure 4.8.1 Knockdown effect on insulin secretion in RIN-5AH cells using Dharmacon ON-TARGETplus SMARTpool siRNAs. Cells were reverse transfected for 48h and insulin level was checked after 2h by replacing the medium (n=5)

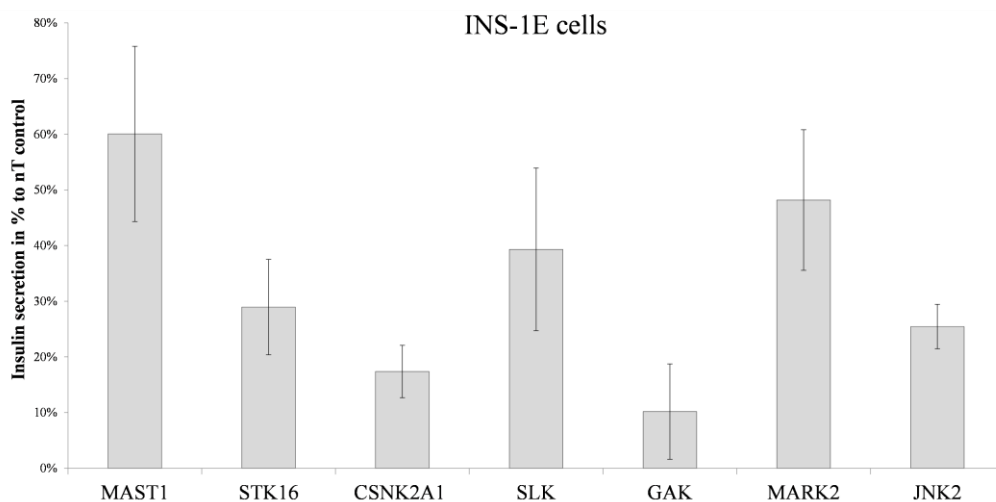


Figure 4.8.2 Knockdown effect on insulin secretion in INS-1E cells. Cells were reverse transfected for 48h and insulin level was measured after replacing the medium to KRBH and 11mM glucose (n=4)

4.9 Other potential non-kinase hits

Basically it is possible that the selected tool compounds induce insulin secretion through other pathways and targets than kinases. It is also assumed that they are acting on multiple targets and the observed result is an outcome of a complex effect. Many proteins other than kinases were described in the literature to have negative (or positive) key regulatory effect in insulin release, including G-proteins, PDEs, metabolic enzymes, ATPases and other proteins. Some non-kinase additional hits were identified by MS affinity chromatography pull-down or binding experiments, including enzymes participating in glucose metabolism, ATPases, PDEs, SNARE building elements and GDIs (GDP-dissociation inhibitors) (*Table 4.9.1*). The role of PDEs were further investigated later. However they are not validated compound targets, but can be possible intervention points for the regulation of secretion and can contribute to the MOA of compounds. Several G proteins were also found (mostly only binding and not pulled down) by MS approach (in collaboration with Evotec AG), which might encroach into GPCR signaling. A recent publication discovered the important role of MST1 kinase in beta cell apoptosis and insulin secretion as well²³⁹. In the kinase affinity assay MST1 was identified as a binding partner for sunitinib with high affinity, while other compounds did not bind it. Probably another interesting family is SGK, which main properties are that they regulate many different ion channels²⁴⁰⁻²⁴⁸ which could interfere with insulin secretion as well. SGK1 was identified as hit in kinase affinity assay for VCC124075, VCC346349, VCC512891 and sunitinib. Because of lack of validation, these two additional hits should be considered only as hints, furthermore we do not know anything about the nature of the compound-protein interaction yet.

| | Bound to affinity matrix | | | | Competed from affinity matrix | | | |
|--|--------------------------|-------------------|--------------|--------------|-------------------------------|-------------------|--------------|--------------|
| | VCC350485:02 | VCC553231:08 (SU) | VCC512891:01 | VCC124075:08 | VCC350485:02 | VCC553231:08 (SU) | VCC512891:01 | VCC124075:08 |
| Negative modulators of Insulin release and Glucose metabolism | | | | | | | | |
| AMPK (5-AMP activated kinase) ^{193,249} | | X | X | X | | X | X | X |
| Cpt-1a (Carnitine O-palmitoyltransferase 1) ²⁵⁰ | | X | | | | | | |
| G6PC3 (Glucose-6-phosphatase 3) ²⁵¹ | | X | | | | | | |
| HADH (Long chain 3-hydroxyacyl-CoA dehydrogenase) ^{51,252,253} | X | X | X | X | | | | |
| IDH1 (Isocitrate dehydrogenase [NAD] cytoplasmic) ^{254,255} | X | X | | | | | | |
| IDH2 or IDH3 (Isocitrate dehydrogenase [NAD] mitochondrial) | X | X | X | X | | | | |
| Other regulators of metabolism | | | | | | | | |
| ATPases – influence the amount of ATP | | | | | | | | |
| Vcp | X | X | X | X | | | | |
| Atp6v1e1 | X | X | | | | | | |
| Atp6v1b2 | X | X | | X | | | | |
| Ola1 | | | X | X | | | | |
| Atp1a1 | | X | | | | | | |
| Ahsa1 | | X | | | | | | |
| Atp2a2 | | X | | X | | | | |
| Atp6v1a | | X | | | | | | |
| Psm13 | X | X | | | | | | |
| Psm11 | | X | | | | | | |
| Psm6 | | X | | | | | | |
| Psm1 | | X | X | X | | | | |
| Atp9a | | X | | | | | | |
| Psm2 | | X | X | X | | | | |
| Psm8 | | X | | | | | | |
| Psm7 | | X | | | | | | |
| Cyclic nucleotide phosphodiesterases (PDEs) ²⁵⁶⁻²⁶¹ | | | | | | | | |
| PDE2A | | | | X | | | | X |
| PDE1B | | | | X | | | | X |
| PDE10A | | | | X | | | | |
| PDE4C | | X | X | | | X | X | |
| SNAREs - required for vesicle fusion and exocytosis ²⁶² | | | | | | | | |
| Vti1b (Vesicle transport through interaction with t-SNAREs homolog 1B) | | | | X | | | | X |
| Ckap5 (Cytoskeleton-associated protein 5) | | X | | | | | | |
| G protein regulators responsible for secretion ^{263,264} | | | | | | | | |
| Rho-GDI (Arhgdia) | | X | X | | | | | |
| Gdi1 | X | | | | | | | |
| Gdi2 | X | X | | X | | | | |
| Activated in obesity ²³⁸ | | | | | | | | |
| CAMK2 | | X | X | | | X | X | |
| Can regulate ion channels ²⁴¹ | | | | | | | | |
| Sgk2 | | X | X | | | X | X | |

x = enriched on affinity matrix or pulled down from affinity matrix directly or indirectly

4.9.1 Effect of PDE4 and PDE5 inhibitors in RIN-5AH and TC6 cells

Some of the PDEs (cyclic nucleotide phosphodiesterases) were identified by MS as interactors for compounds, therefore they looked interesting target candidates to test. PDEs are enzymes that hydrolyze the phosphodiester bond of cyclic GMP (cGMP) or cyclic AMP (cAMP), which are important secondary messengers and play decisive role in the regulation of insulin release^{33,36,265-268}. PDEs can be classified into 11 different families and have several isoforms in each family that show different substrate specificities^{257,269}. By inhibition of certain PDEs in beta cells, cAMP level raises and insulin is secreted. On the other hand cGMP can also have a function in regulating PDE activity. During metabolism the produced nitric oxide (for example produced from L-Arginine) can increase cGMP level and augment insulin level, thus as a consequence the cGMP sensitive PDE3B is deactivated and thus cAMP concentration simultaneously increase. Expression pattern of different PDEs in INS-1 cells can correlate with their role played in insulin secretion²⁷⁰. Further research support the fact that in different beta cell lines different PDEs can be dominant. PDE1C was found to play the relevant role in TC3 beta cells, but in primary pancreatic islets both PDE1 and PDE3 inhibition stimulated the secretion. Zaprinast a PDE1/5A inhibitor with higher affinity to PDE1 potentiated insulin release only when co-administered with guanylyl cyclase activator YP-1, but not alone in BRIN-BD11 cells²⁷¹. Its inefficiency was confirmed on human beta cell islets in another study²⁷². PDE3B and PDE4C were found to play a major role in stimulation of insulin secretion by lowering the blood glucose level in INS-1 cells and in mice as well^{260,270,273,274}. Several PDE10A inhibitors could also effectively stimulate insulin secretion²⁵⁶. The PDE5 enzyme hydrolyzes cGMP and may have other favorable effects in diabetes. The chronic treatment with PDE5 inhibitors can be advantageous, because they reversed and improved the condition of oxidative stress in epithelial cells occurring in insulin resistance²⁷⁵⁻²⁷⁷ and could also improve the insulin sensitivity in mice on high fat diet, but did not increase the fasting insulin level²⁷⁸. Long term tadalafil treatment also improved fasting glucose level and reduce infarct size in db/db mice models²⁷⁹. By insulin ELISA assay we demonstrated that the acute treatment with some of the PDE5 inhibitors (sildenafil, vardenafil but not tadalafil) resulted in an enhanced insulin level in TC6 cells but not in RIN-5AH cells (*Figure 4.9.1*). Taken together PDE4C seems to be an important cyclic nucleotide phosphodiesterase enzyme in beta cells and putatively in pancreatic islets as well²⁷⁴. We could confirm this in our experiments by the treatment with selective PDE4 inhibitors (Roflumilast, CP 80633) in RIN-5AH and TC6 cells (*Figure 4.9.1*). Furthermore the knockdown of PDE4C in INS-1E cells caused a moderate but significant effect on insulin release (12%) as well (data not shown). The application of PDE

inhibitors can also suppress the transcription of iNOS and therefore may prevent beta cell dysfunction²⁷².

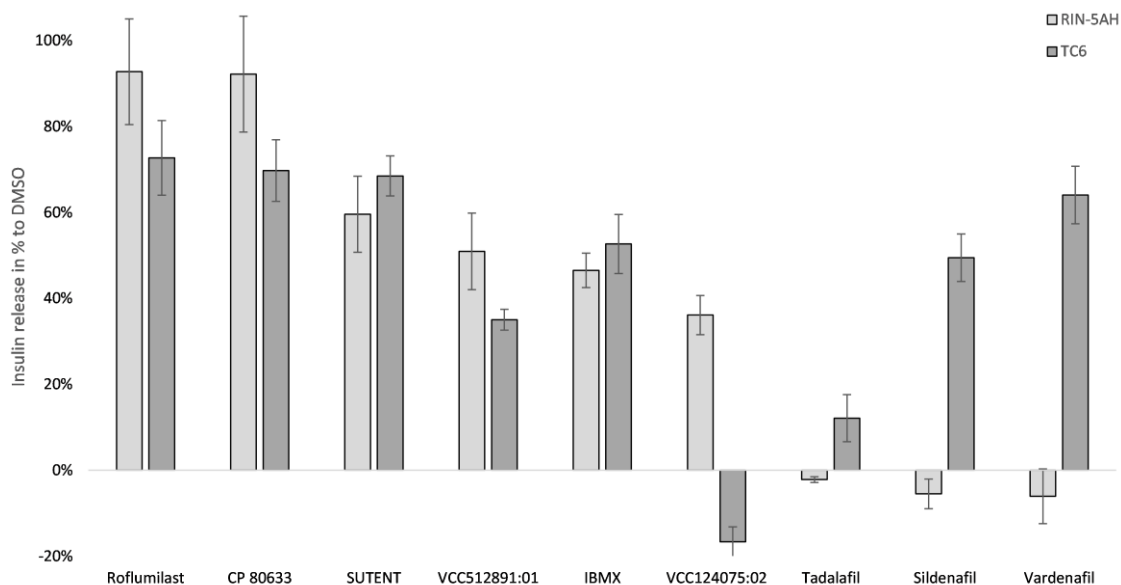


Figure 4.9.1 Effect of PDE4 and PDE5 inhibitors on insulin secretion in RIN-5AH (rat) and TC6 (mouse) cells (2h and 5uM treatment, SEM, n=4-6). Since PDEs were identified as hits for certain compounds (Sunitinib, VCC124075, VCC512891), officially available PDE4 (Roflumilast, CP 80633) and PDE5 (sildenafil, tadalafil, vardenafil) and PDE pan-inhibitors (IBMX) were tested. The importance of PDE4C could be confirmed in RIN-5AH and TC6 cells, that is in correlation with published data in other models. Interestingly PDE5 inhibitors affected insulin production in TC6 cells only. Furthermore it should be taken into account that tadalafil is structurally very much different compared to sildenafil and vardenafil. The expression level of PDE5 was not checked in the cells, therefore it is not clear whether the absence of PDE5 in RIN cells is the cause of the inefficiency of PDE5 inhibitors or is it an off-target effect.

4.10 Insulin secretion and GDIS in different beta cells

The potencies of the selected best compounds were tested in different beta cell line models. Many of them could maintain a generic insulinotropic effect, however one of the most effective compound in RIN-5AH cells (VCC981125:09 and other members from quinoline family) did not induce insulin release in TC6 and INS-1E cells (*Figure 4.10.1*). Only some of the VCC981125:09 derivatives were able to induce insulin secretion in BRIN-BD11 cells. (*see Appendix*). The cell line specific effect of some of the compounds (VCC981125:01, VCC124075:02 and VCC036051:03) can be explained with the heterogeneity of the different clonal insulinoma beta cell lines. The glucose sensitive INS-1E cell line provided a good model for testing the compounds in high (11.1mM) and low (1.1mM) glucose environments. Most of the compounds increased insulin release in high glucose but not in low glucose content buffer. Interestingly a compound from the thieno-pyrimidine family (VCC834153:01) showed a significant induction in low glucose compared to high glucose. On the other hand the bis-quinoline compound VCC405984:01 has produced an ideal result, it had negligible insulinotropic effect in buffer supplemented with 1.1mM glucose (*Figure 4.10.2*).

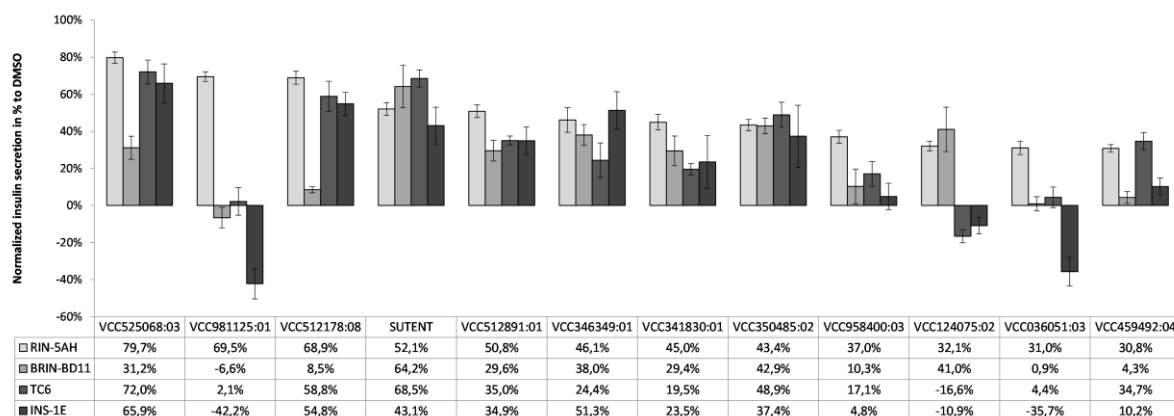


Figure 4.10.1 Efficacy of compounds in different beta cell lines. Most of the compounds maintained their potency and were effective on all beta cell lines. These compounds are probably targeting key regulators of insulin secretion which are present in all models. On the other hand VCC981125, VCC124075 and VCC036051 seem to have a cell line specific effect. (n=5-6; SE, 5µM; 2h treatment)

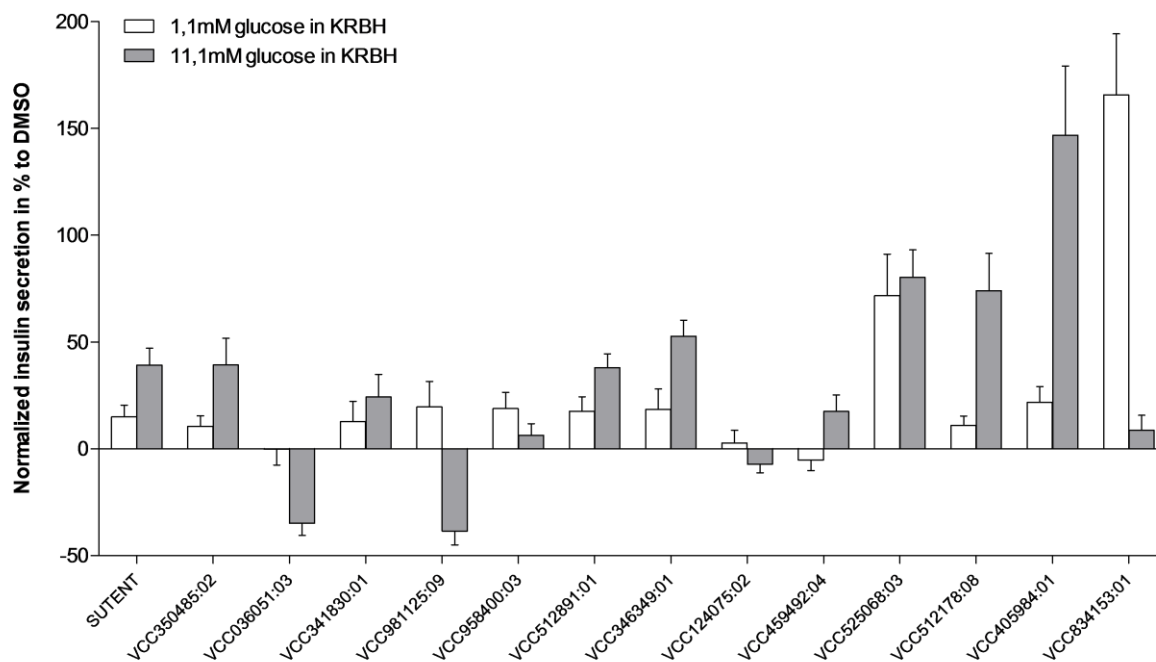


Figure 4.10.2 Glucose dependent insulin secretion (GDIS) by treatment of VCC compounds. Most of the compounds increased insulin in high glucose (11.1mM) but not in low glucose (1.1mM) environment. This property can be an important factor for an ideal treatment of diabetes as well, since most of the medicaments cause hypoglycaemia. Interestingly VCC981125 (quinoline) and VCC036051 (quinazoline) had an opposite effect in INS-1E cells. Nevertheless a bis-quinoline derivative VCC405984:01 had a massive impact on insulin secretion in INS-1E cells in high glucose compared to low glucose. It is worth to notice that its effect was not significant in low glucose compared to DMSO treatment. (n=5-8; SE; 2h and 5µM treatments)

4.11 Elevated cAMP levels can be a possible mechanism of action for some compounds

Intracellular cAMP level is one of the main driving mechanisms of insulin release. Certain GPCR ligands can activate the generation of cAMP. Upon activation of receptors a series of downstream signals stimulate the adenylyl cyclase enzymes which convert ATP to cAMP. Then cAMP can activate and/or phosphorylate effector proteins, i.e. PKA and Epac, which transmit several exocytotic stimulatory signals^{33,36}. Subsequently different enzymes that are regulating insulin secretion and/or cAMP sensitive transcriptional activators can be affected, e.g. CREB, CREM and ATF1. The breakdown of cAMP to AMP is mediated by PDEs as it was reviewed in the previous chapter, therefore Roflumilast a PDE4C inhibitor was also included in the cAMP assay. We found that after compound treatments (including not only those that bound PDEs in the affinity chromatography experiments) the intracellular cAMP level significantly increased in most cases (*Figure 4.11.1*), therefore supposedly this could be an important factor in the mode of action for the majority of tested VCC compounds.

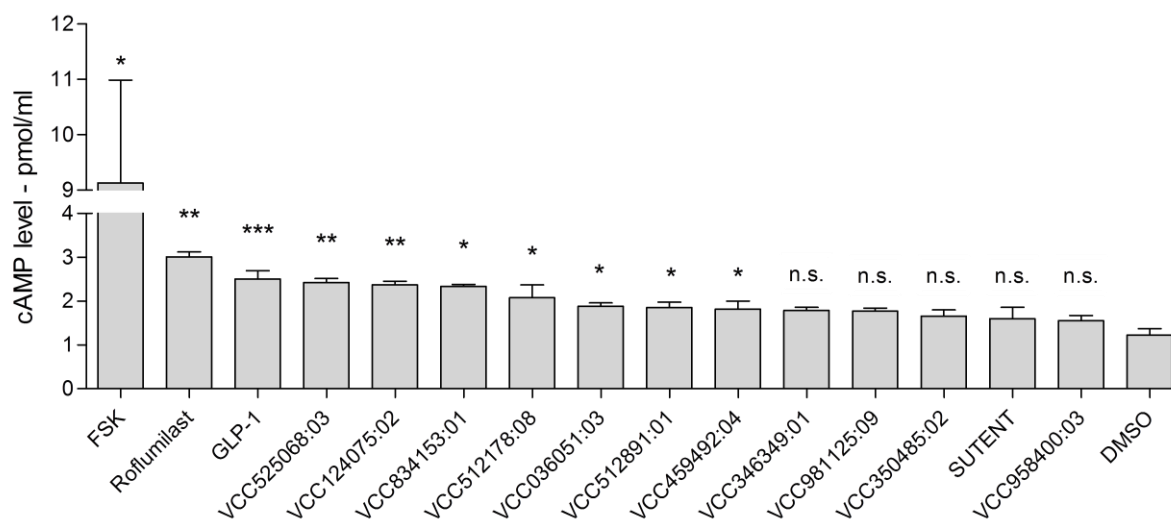


Figure 4.11.1 Changes in intracellular cAMP level in RIN-5AH cells. ($n=4$; 2h and 10 μ M treatment; paired t -test results: * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

4.12 Increased Ca^{2+} influx is another mechanism of action for some compounds

Voltage dependent calcium channels play an important role in beta cell physiology and are critical regulators of insulin secretion in beta cells. Membrane depolarization or other stimuli that induce calcium channel opening and therefore calcium influx, triggers the exocytosis of insulin granules²¹. Several VDCCs are involved in this mechanism in human beta cells^{280,281}. In rat beta cells similarly to human beta cells the same set of calcium channels are also expressed (L-type, T-type, N-type and P/Q type Ca^{2+} channels), however it was found that P/Q type channels likely do not participate in insulin secretion in INS-1 cells^{282,283}. Interestingly in mouse beta cells the N-type and T-type Ca^{2+} currents are not present²⁸⁴⁻²⁸⁷. The L-, P/Q- and N- type currents are high voltage activated (HVA), while T-type current is considered as low voltage activated (LVA). Furthermore L-type channels inactivate slowly (500msec), the inactivation of T-type channels are faster (20-50msec). The involvement of the dihydropyridine (DHP) sensitive (nifedipine, efonidipine) L-type Ca^{2+} channels (Cav1.2 and Cav1.3) are probably the most crucial for GSIS secretion, they are responsible for ~80% of the Ca^{2+} current in human and rat beta cells as well²⁸⁸. Another important participant in Ca^{2+} influx is the T-type Ca^{2+} channel²⁸⁷. As VCC981125:01 was not hitting any kinases, it was suspected to have a direct effect on Ca^{2+} influx probably through ion channels or GPCRs. The Ca^{2+} influx measurements done by FACS have revealed that the compound and some of its derivatives rapidly increased calcium influx in RIN-5AH cells. Non-insulinotropic compounds from the same core family did not show any effect (*Figure 4.12.1*).

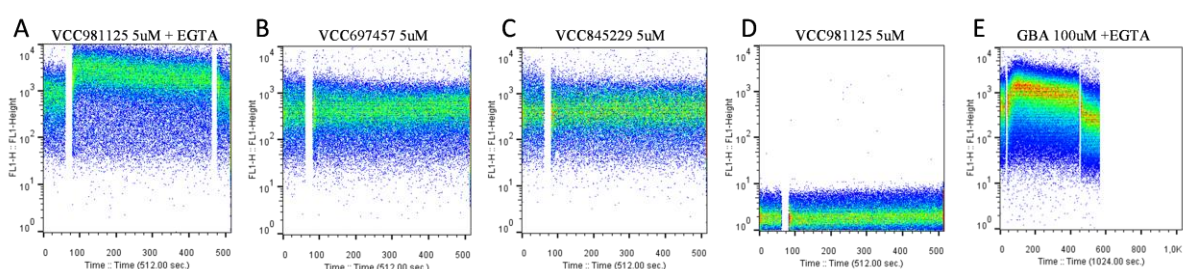


Figure 4.12.1 Calcium influx experiments by FACS. **A**, Addition of the compound VCC981125 (1st gap) induced Ca^{2+} influx in RIN-5AH cells when extracellular Ca^{2+} was present in the buffer. The long sustained signal could be quenched by EGTA (2nd gap). **B-C**, Non-insulinotropic compounds had no effect on Ca^{2+} influx. **D**, VCC981125 did not change the fluorescence signal in Fluo-4 unstained cells. **E**, Glibenclamide (GBA) effect.

It is important to notice that the effect of VCC981125:01 could only be observed in HBSS++ (HBSS with CaCl₂ and MgCl₂), which accounts for that the compound is unlikely to release Ca²⁺ from intracellular stores. Furthermore similarly to the insulinotropic effect Ca²⁺ inducing effect was also cell line specific. For positive control thapsigargin (SERCA inhibitor) was used, which increased [Ca²⁺]_i in HBSS-- (CaCl₂ and MgCl₂ free HBSS) as well as in HBSS++. The ionophore A23187 and thapsigargin (TG) showed a different characteristics for Ca²⁺ increase compared to VCC981125, furthermore they were not able to induce insulin secretion in the beta cells. The augmented Ca²⁺ influx could be attenuated by verapamil (L-type calcium channel blocker), efonidipine (L- and T-type inhibitor) and high concentration of NiCl₂ (T-Type inhibitor). Additionally some known drugs with quinoline cores (bosutinib, quinidine) were also investigated for Ca²⁺ level modulation (*Figure 4.12.2*).

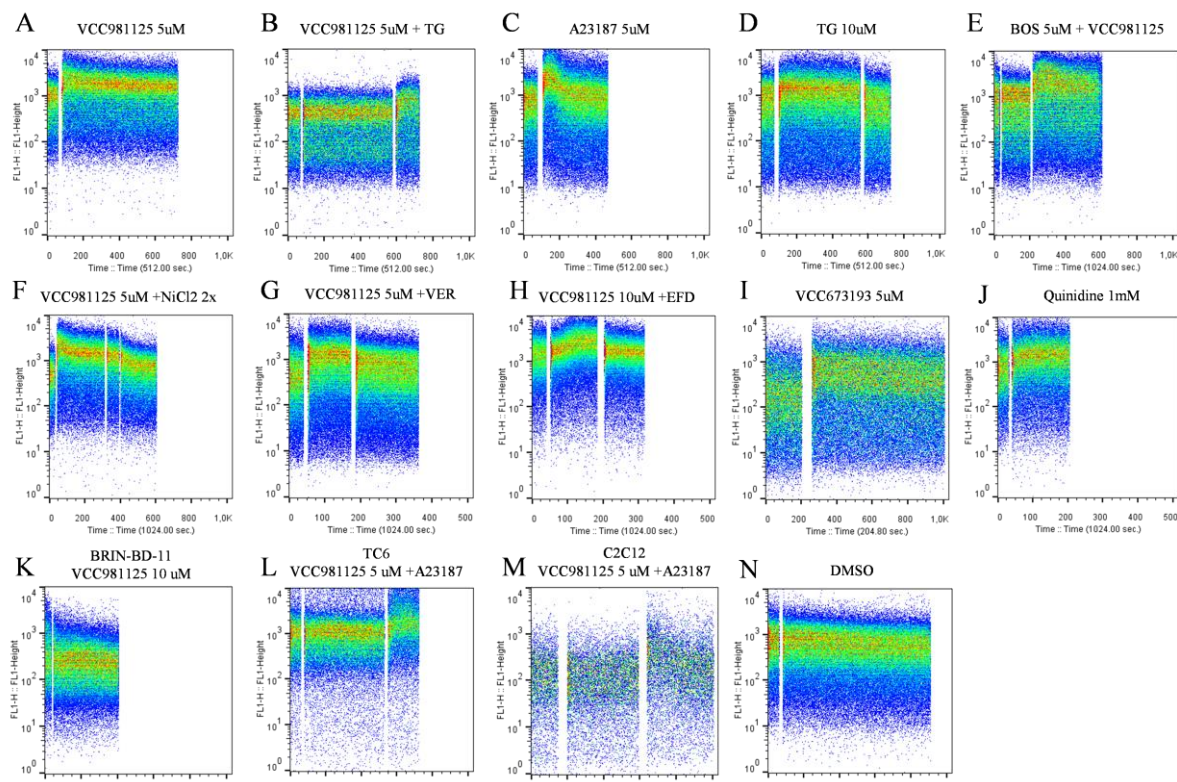


Figure 4.12.2 **A**, VCC981125 induces Ca^{2+} influx in HBSS++ buffer. **B**, In HBSS-- buffer it is unable to develop its effect. The addition of TG (2nd gap) confirms that cells were still healthy. **C-D**, Demonstrating the effect of positive control compounds A23187 and TG. The increased signal by TG could be quenched by EGTA (2nd gap). **E**, Bosutinib did not stimulate Ca^{2+} influx. **F-H**, The action of VCC981125 could be attenuated by Ca^{2+} channel blockers, $NiCl_2$, verapamil (VER) and efonidipine (EFD) respectively. **I-J**, Additional compounds sharing quinoline cores which induced Ca^{2+} influx in RIN-5AH cells. VCC673193 an analogous structure of VCC98112. Quinidine and VCC673193 have also induced insulin secretion in RIN-5H cells. **K-M**, The effect of VCC981125 seems to be cell line specific. **N**, DMSO control.

Ca²⁺ influx for the other selected VCC compounds was not detectable by FACS (data not shown). Effect on Ca²⁺ influx of quinoline molecules were confirmed in the same cells by spinning disc confocal microscope as well (*Figure 4.12.3a and 4.12.3b*). By using the Flexstation 3 microplate reader, additional relevant compounds were screened and the AUCs could be calculated for the Ca²⁺ influx curves (*Figure 4.12.4*).

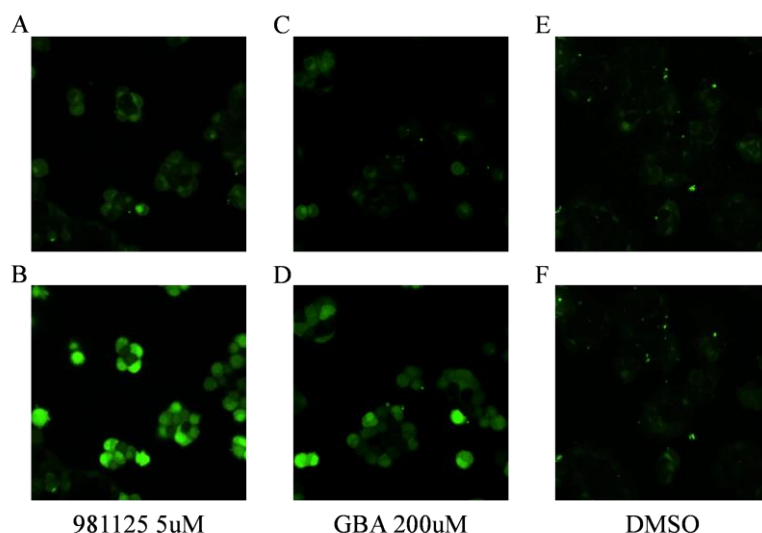


Figure 4.12.3a

Spinning disc confocal microscopy measurements in Fluo-4 AM loaded RIN-5AH beta cells. A-B, Before and after stimulation with 5uM VCC981125. C-D, Before and after stimulation with 200uM glibenclamide (GBA). E-F, Negative control with DMSO, before and after stimulation.

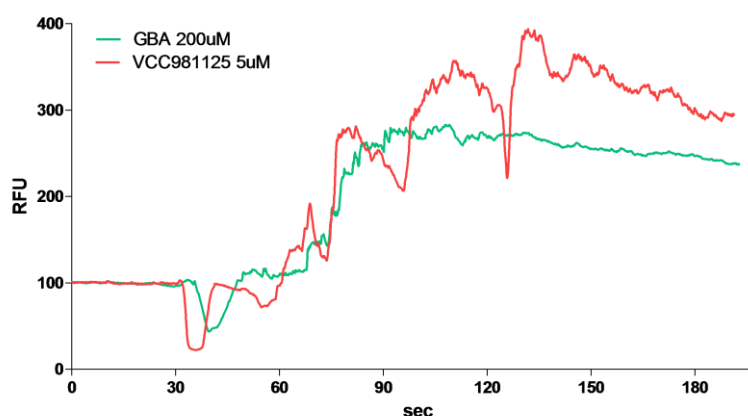


Figure 4.12.3b

Relative fluorescence signal registered by confocal microscope. Overlaid graphs for 200uM GBA and 5uM VCC981125. Compounds added at 30s. 6-9 cells were gated and their relative mean fluorescence values were averaged and plotted. The base line fluorescence level was calculated by taking the average fluorescence of the first unstimulated 20s.

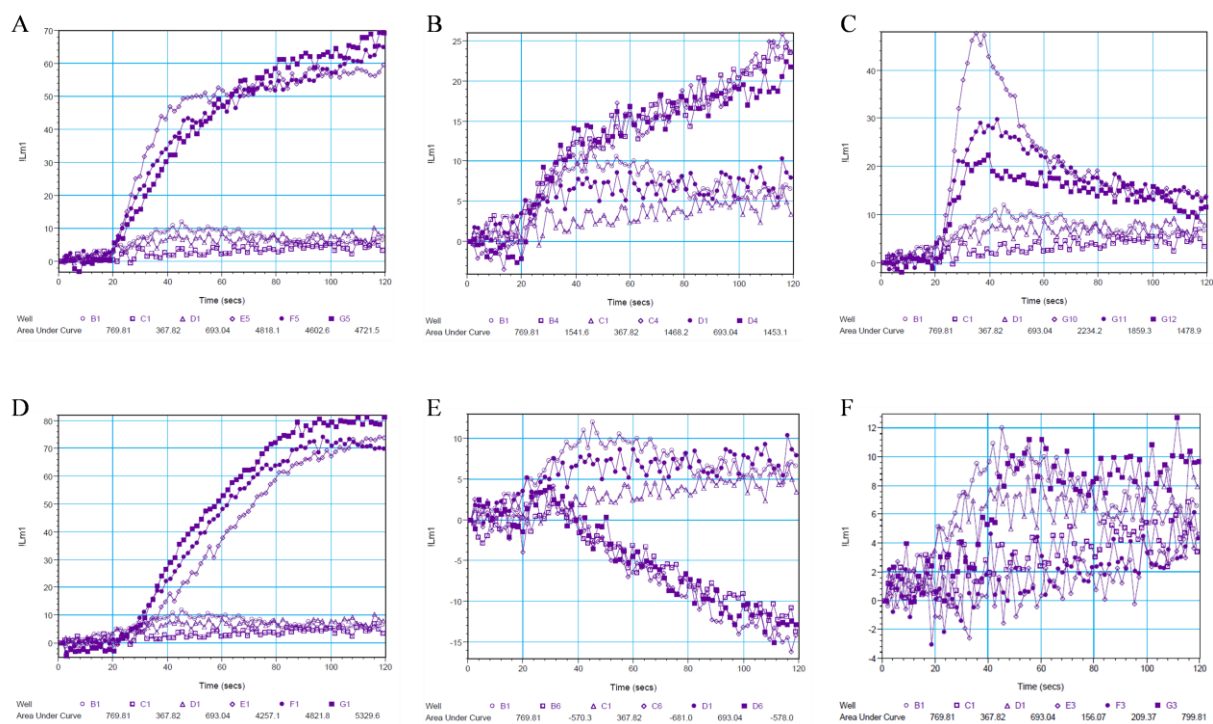


Figure 4.12.4 Confirmation of Ca^{2+} influx results on Flexstation 3 (demo instrument, Molecular Devices). Treatments with different compounds are displayed in comparison to DMSO ($n=3$) **A**, 5 μM VCC633768:01 (quinoline derivative); increased fluorescence **B**, 5 μM VCC981125 (quinoline); increased fluorescence **C**, 4.7 μM Glibenclamide; increased fluorescence **D**, 10 μM Thapsigargin; increased fluorescence **E**, 10 μM Verapamil; decreased fluorescence **F**, 1 $\mu\text{g/ml}$ Exendin-4; no change

4.12.1 Validation experiments with patch clamp for VCC981125

In order to validate if VCC981125 induces Ca^{2+} influx via membrane depolarization in RIN-5AH beta cells, whole cell patch clamp studies were performed under the supervision and help of Dr. Jana Hartmann (TUM, Institute of Neuroscience). 500 μM glibenclamide (GBA) was used as control compound in the experiments. After applying either 50 μM or 500 μM “puffs” for 30s of the quinoline compound on the cells, a shift in membrane potential was registered from -70mV up to -20mV in current clamp mode (CC). An increase in inward current could also be detected in voltage clamp mode (VC), which lasted longer compared to GBA stimulation. This effect correlated with the Ca^{2+} influx results seen on FACS. When comparing VC and CC curves for VCC981125 treatment, it was clear that the effect in VC is shorter than in CC, thus the compound keeps the membrane potential for a prolonged time in depolarized state even when it probably does not act on its target anymore. On the other hand treatment with VCC981125 has increased the inward current immediately, unlike GBA where a small delayed response could be observed. Interestingly lower concentration of the drug could generate almost the same rate of inward current as with higher concentration (*Figures 4.12.5-7*). We also experienced a change in the membrane capacitance after treatment with the compounds, which reflects to the exocytotic events happening in the cell²⁸⁹.

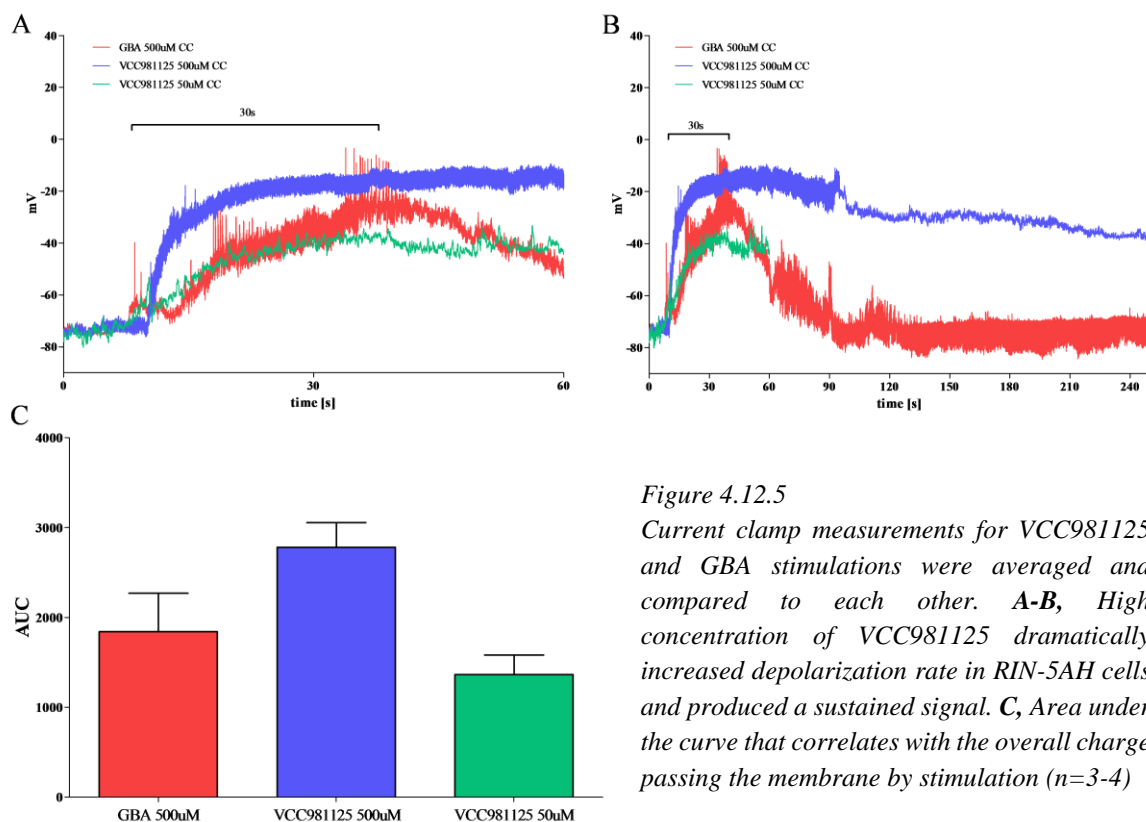


Figure 4.12.5

Current clamp measurements for VCC981125 and GBA stimulations were averaged and compared to each other. **A-B**, High concentration of VCC981125 dramatically increased depolarization rate in RIN-5AH cells and produced a sustained signal. **C**, Area under the curve that correlates with the overall charge passing the membrane by stimulation ($n=3-4$)

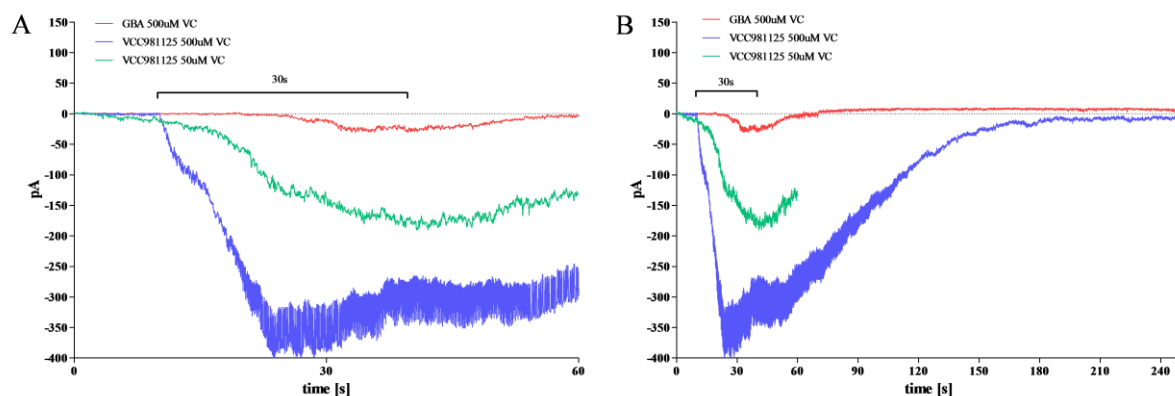


Figure 4.12.6 **A-B**, Averaged voltage clamp measurements. VCC981125 has markedly increased the inward current in RIN-5AH cells and flow returns back to baseline after 3 minutes. VCC981125 had a longer lasting effect compared to GBA. ($n=3-4$)

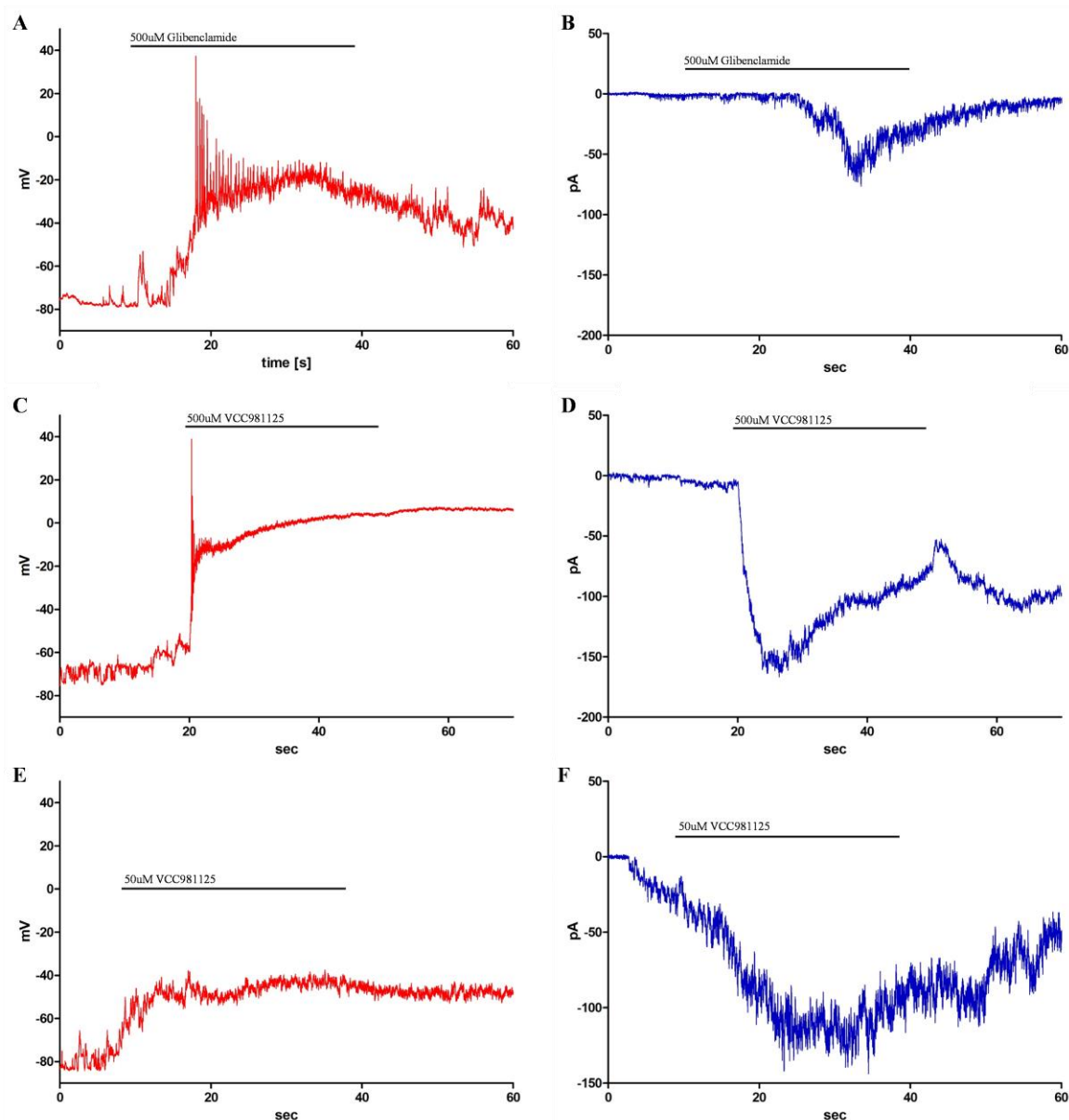


Figure 4.12.7 Representative non-averaged whole cell patch clamp curves of RIN-5AH cells. **A-B**, Current clamp and voltage clamp measurements respectively for 500uM glibenclamide was used as control in the experiments **C-D**, Current clamp and voltage clamp curves for 500uM VCC981125. It is worth to notice that the effect in voltage clamp is immediate and not delayed like at glibenclamide treatment **E-F**, Effect of 50uM VCC981125.

The exact target of VCC981125 is still unknown, however there is a high chance that it acts on any of the Ca^{2+} channels. Further possible targets could be other ion channels or maybe GPCRs that interact with ion channels²⁹⁰.

4.13 Combination treatments with ion channel modulators suggest different mechanism of actions for certain compounds

From previous experiments it turned out that Ca^{2+} signaling has a crucial role in the MOA of quinoline compounds in RIN-5AH cells. Therefore VCC981125 and Ca^{2+} channel inhibitors were combined together and checked for insulin secretion. Additionally the same combinations for sunitinib, glibenclamide (GBA), GLP-1 and Exendin-4 were done as well. All tested Ca^{2+} channel inhibitors could lower the VCC981125- or GBA-induced insulin secretion. Sunitinib, GLP-1 and Exendin-4 were not sensitive to verapamil and nifedipine, only to efonidipine. Furthermore, combinations with K_{ATP} channel openers diazoxide (DAO) and pinacidil (PIN) were also studied. DAO had no impact on GBA effect, which is probably due to the low K_{ATP} expression level in the cell line and therefore relatively low sensitivity to sulphonylureas (*see Appendix*) or because the affinity of GBA to the ion channel is higher than DAO. The K_{ATP} channel openers PIN and DAO should normally lower the insulin secretion, however PIN did not have any effect. It can be explained with the differences in selectivity of the compounds, since PIN has a much greater affinity to SUR2 subunit, which is expressed at a very low level compared to SUR1 in RIN-5AH cells (*see Appendix*). This correlates with published data also, where PIN did not influence insulin or calcium levels (along with diazoxide) in SUR1 knockout mice, presuming that no other isoforms of $\text{K}_{\text{ATP-S}}$ are expressed in the pancreatic islets²⁹¹. DAO targets both SUR1 and SUR2 subunits, therefore a diminished insulin level could be registered after treatment in RIN-5AH cells. DAO could significantly reduce the effect of VCC981125, sunitinib, GLP-1 and Exendin-4. These results revealed that Ca^{2+} (T-type) and K^{+} currents strongly modulate the potencies of VCC981125, glibenclamide, sunitinib, GLP-1 and exendin-4 in RIN-5AH cells (*Figure 4.13.1*).

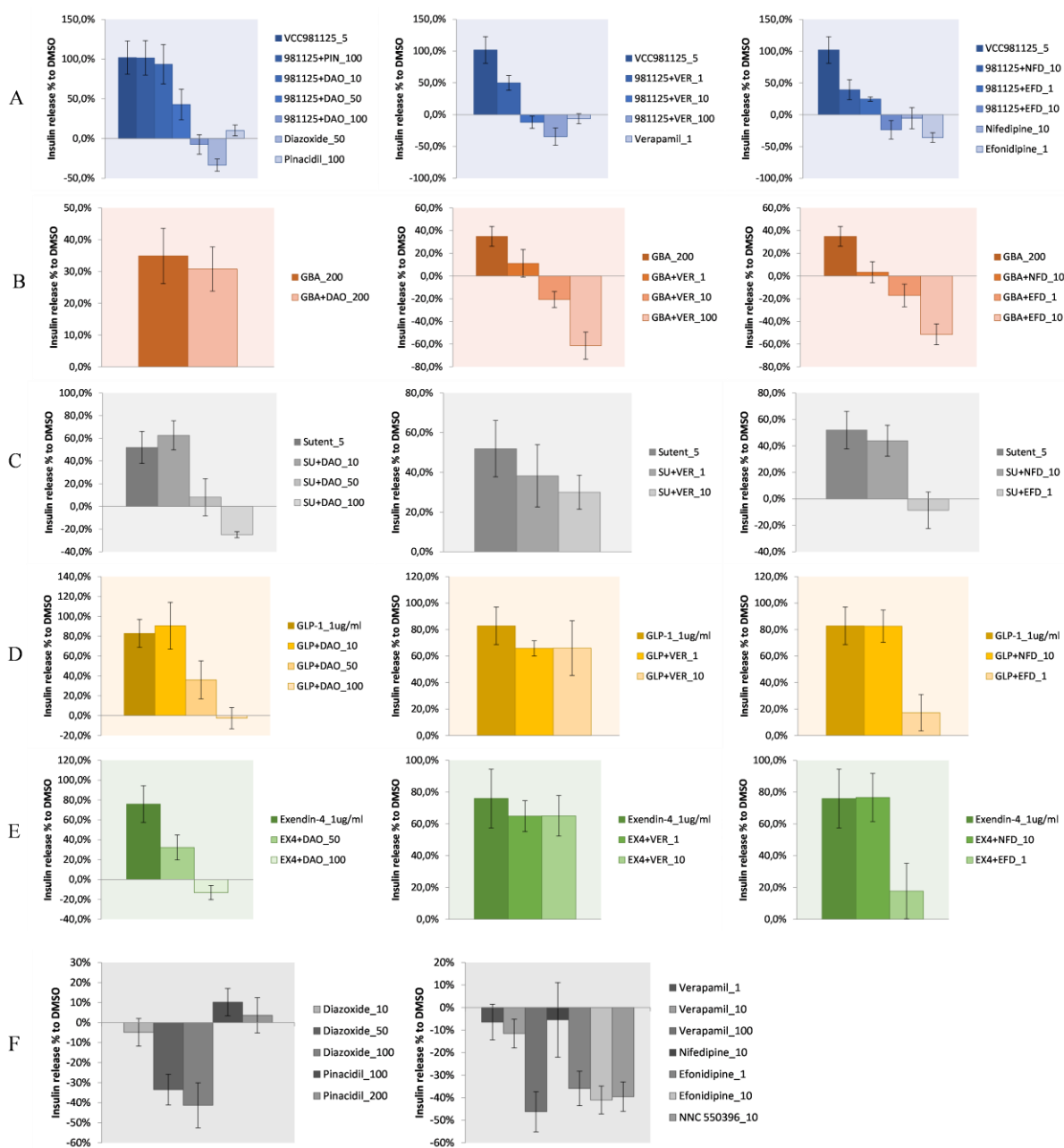


Figure 4.13.1 **A-B**, Combination treatment results for VCC981125 and glibenclamide are similar. They were both sensitive to L- and T-type Ca^{2+} channel inhibitors. The cell line shows low sensitivity to glibenclamide and probably due to the high concentration applied on the cells, its effect was not attenuated by DAO. **C**, Sunitinib treatment was sensitive to DAO and EFD. **D-E**, GLP-1 and Exendin-4 induced secretion was sensitive also to DAO and EFD. **F**, Effects of Ca^{2+} channel inhibitors and K^{+} channel openers on insulin secretion. PIN, pinacidil; DAO, diazoxide (K_{ATP} openers); VER, verapamil (L-type inhibitor); NFD, nifedipine (L-type inhibitor); EFD, efonidipine (L- and T-type inhibitor); EX-4, exendin-4

4.14 Compounds can increase glucose analog (2-NBDG) uptake in different cells

Glucose serves as one of the main energy source for the cells and is needed for their healthy functioning. In beta cells extracellular glucose is taken up particularly by the GLUT2 transporters. In healthy muscle cells and adipose tissue, insulin regulates glucose uptake by inducing the translocation of GLUT4 transporters to the plasma membrane. The failure or decreased utilization of glucose from the blood is one of the main issues in diabetic patients. The presence of excessive amount of glucose can lead to glucotoxicity and insulin resistance. We investigated how do the selected compounds influence glucose uptake in vitro in beta cells (RIN-5AH), myoblasts (C2C12) and differentiated adipocytes (3T3-L1). We utilized flow cytometry and the fluorescent glucose analogue, 2-NBDG for the measurements. Most of the compounds increased 2-NBDG uptake in C2C12 myoblasts. Four compounds significantly increased 2-NBDG uptake in differentiated 3T3-L1 cells together with insulin. This might suggest that they have an insulin independent mechanism for glucose uptake stimulation. In RIN-5AH cells three compounds caused a significant increase and the effect of VCC341830:01 could be alleviated by the GLUT4/2 inhibitor Cytochalasin B. By summarizing the results, VCC341830:01 and VCC346349:01 seem to be the most promising candidates for further investigation of their role played in the regulation of glucose uptake (*Figures 4.14.1-4*)

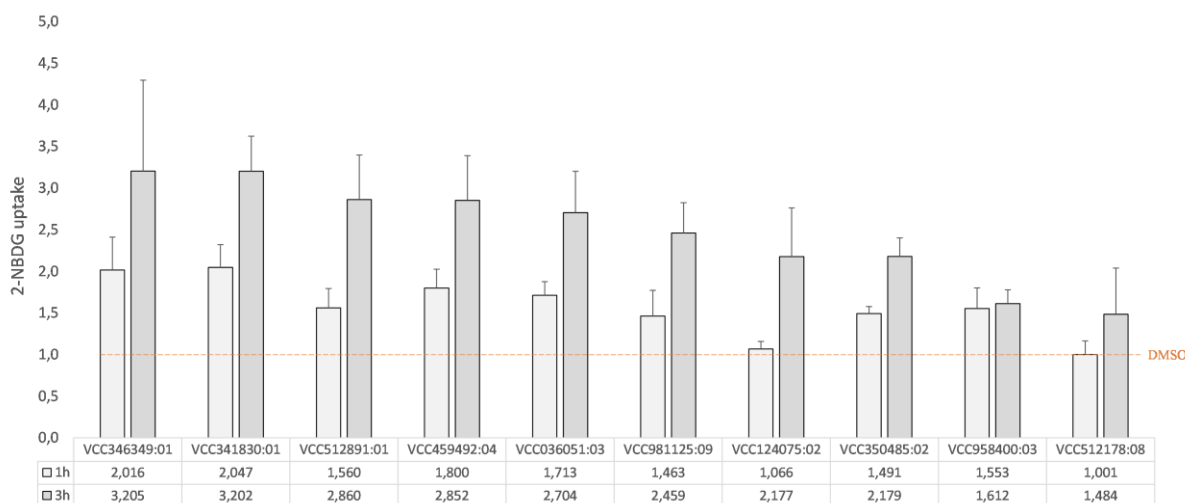


Figure 4.14.1 Normalized 2-NBDG uptake in C2C12 myoblasts. DMSO=1. All compounds had significantly increased 2-NBDG uptake after 1h stimulation, except VCC124075:02 and VCC512178:08. 2-NBDG uptake couldn't be measured for sunitinib and VCC525068:03, because of autofluorescence. ($n \geq 4$)

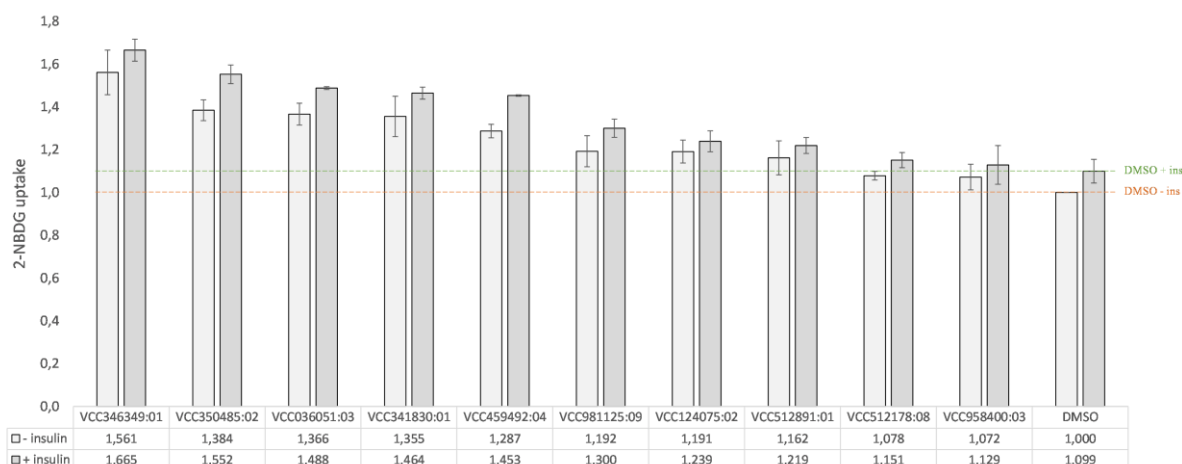


Figure 4.14.2 Normalized 2-NBDG uptake in differentiated 3T3-L1 adipocytes. DMSO=1. Five compounds produced a significant increase ($p < 0.05$) in 2-NBDG uptake with the addition of 0.5ug/ml insulin (VCC346349:01, VCC350485:02, VCC036051:03, VCC341830:01 and VCC459492:04) compared to non-treated samples (DMSO). It's likely that the compounds possess an insulin independent mechanism for the stimulation of 2-NBDG uptake. ($n=3$)

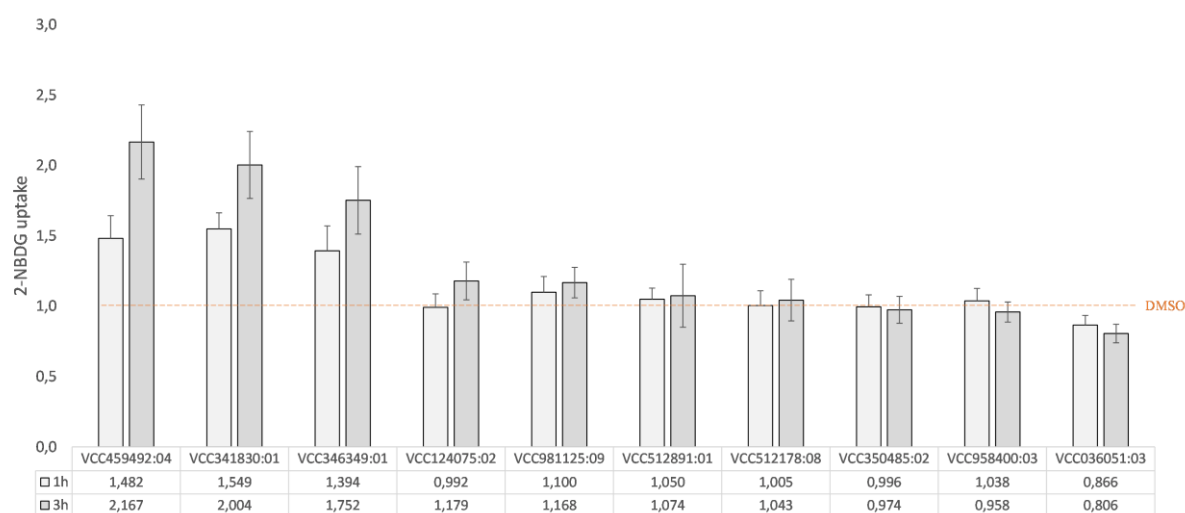


Figure 4.14.3 Normalized 2-NBDG uptake in RIN-5AH beta cells. DMSO=1. The 2-NBDG uptake was also checked in beta cells, since insulin secretion depends on glucose level. 3 compounds (VCC459492:04, VCC341830:01 and VCC346349:01) showed an increase in 2-NBDG uptake ($n=4$).

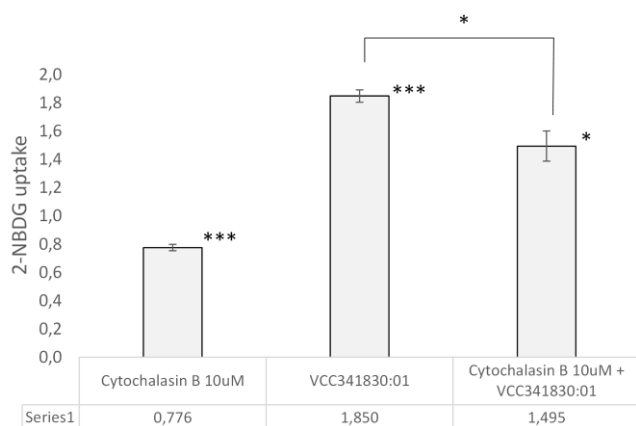


Figure 4.14.4

Normalized 2-NBDG uptake (DMSO=1). In RIN-5AH cells cytochalasin B significantly decreased 2-NBDG uptake compared to untreated DMSO control. It could also alleviate but not abolish the effect of VCC341830:01, which suggests that its action is partly dependent on glucose transporters (GLUT-2). (** $p < 0.001$; * $p < 0.05$ to DMSO or to combination treatment; $n=4-5$)

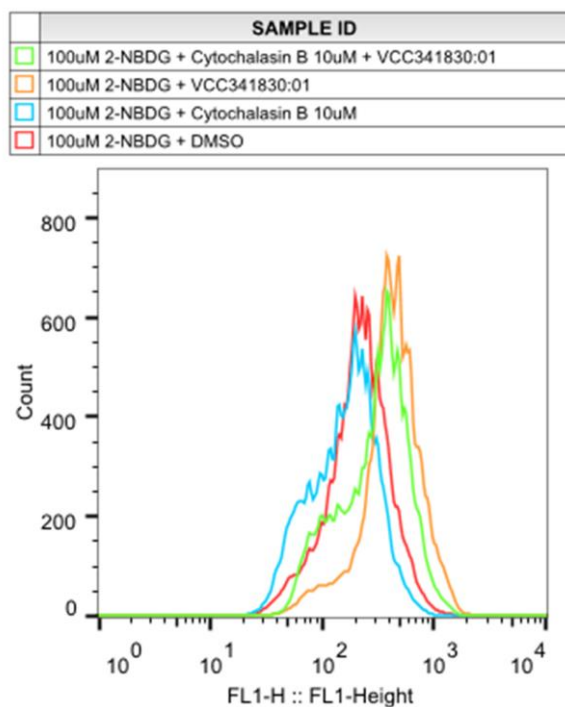


Figure 4.14.5

Representative histograms for 2-NBDG uptake assay. 5uM VCC341830 evoked a positive shift in FL-1 compared to DMSO treated cells, which could be attenuated by the co-treatment with 10uM Cytochalasin B.

4.15 Pathway analysis in RIN-5AH beta cells

AMPK, PKCs and ERK1/2 phosphorylation were tested for the seeded insulinotropic compounds and PKA, CREB, mTOR and ERK1/2 activities for the cAMP inducer compounds in RIN cells.

AMP-activated protein kinase (AMPK) plays central role in metabolic processes and its activated form negatively regulates nutrient-induced insulin secretion. By food uptake and metabolism, the AMP/ATP ratio is decreased, as a consequence AMPK activity is also lowered. It is thought that activated form negatively regulates insulin secretion from beta cells, thus its inhibition can be a MOA for insulin secretion in beta cells^{249,292}. On the other hand glucose starvation induced AMPK stimulation can be responsible for beta cell apoptosis²⁹³. It is important to notice that AMPK activation might be preferential in other tissues (in diabetes)¹⁹⁷ and blocking can produce cardio toxic effects²⁹⁴. The western blot results showed that AMPK phosphorylation was mildly induced by VCC036051:03 and markedly inhibited by sunitinib but not changed by the treatment of other compounds in RIN-5AH cells (*Figure 4.15.1*).

Protein kinase C (PKC) proteins can be classified into different subgroups: conventional PKCs (α, βI, βII and γ), novel PKCs (δ, ε, η and θ) and atypical PKCs (ζ, λ/ι). PKCs are often referred that they evoke exocytosis upon activation, they may induce a low rate Ca²⁺ influx as well²⁹⁵. I would like to highlight some of them which are associated with diabetes, insulin resistance or insulin release. PKCβ deletion or inhibition may be beneficial in the treatment of diabetic nephropathy; furthermore in PKCβ -/- mice GLUT-4 translocation was induced in different tissues and a slightly reduced blood glucose level was observed, however serum insulin level was not changed compared to PKCβ +/+ or +/- mice²⁹⁶. During exercise PKCs are regulated differentially, PKCβ was downregulated in mouse muscle and liver cells²⁹⁷ and upregulated PKCζ was detected in human muscle cells²⁹⁸. The atypical PKCs, PKC ζ and λ may regulate exercise-induced glucose mobilization through AMPK activation and interestingly can induce beta cell proliferation²⁹⁸⁻³⁰⁰. In beta cells the atypical PKCs regulate insulin secretion. Studies performed with selective PKC inhibitors and on knockout mice showed that atypical PKCs, most likely PKC λ plays an important role in insulin secretion. Down regulation of this isoform produced interesting results. It reduced the expression levels of GLUT2, Kir6.2 and SUR1, making the cells less sensitive to glucose and tolbutamide treatment, hexokinases (enzymes that initiate glycolysis) were unregulated. In PKC λ -/- mice showed impaired glucose tolerance and decreased insulin level. The involvement of atypical PKCs in insulin release was also shown in previous publications by using small molecule PKC

inhibitors^{301,302}. Additionally GLP-1 and PKC ζ is likely to share a common pathway, which induces beta cell proliferation³⁰³. PKC ζ activation could promote the proliferation of insulinoma cells and islets, indeed a transgenic mice model harboring the constitutively active PKC ζ showed an increased glucose tolerance and enhanced insulin secretion. This effect was concomitant with the activation of mTOR pathway^{304,305}. The conventional PKC α is necessary and plays an important role in GSIS. Interestingly beta cell stimulation with GLP-1 and GIP or glucose can also involve PKC α activation or its translocation to the membrane^{306,307}. Studies that investigated the cholinergic pathway or the effects of neurotensin or iduronate-2-sulfatase, also support the need of PKC α translocation or activation in the stimulation of exocytosis in beta cells³⁰⁸⁻³¹¹. The PKC α/β , PKC ζ/λ and unspecific PKC (PKC-pan) phosphorylation were tested after compound treatments in RIN-5AH cells (*Figure 4.15.1*).

It is known that activation of MEK/ERK1/2 pathway and the phosphorylation of ERK1/2 is implicated by the treatment of various insulinotropic drugs including GLP-1 and GIP. The beta cell proliferative effects of incretins might happen partly due to the activation of this signaling pathway^{92,312,313}. The clear correlation and the full mechanism of this process is not clear and numerous pathways were associated with the increased ERK phosphorylation. In MIN6 beta cells stimulated by GLP-1 or carbachol ERK activation was found to be dependent on L-type calcium channels or IP3Rs^{314,315}. An interesting study revealed that ERK2 activation may directly regulate PDE4 activity. Upon stimulation with EGF, Erk2 was able to bind and inhibit the long isoforms of PDE4C and PDE4B enzymes, thus increased the cAMP level in transfected COS-1 cells³¹⁶. A positive feedback mechanism is also possible for Erk1/2 activation via secreted insulin³¹⁷. ERK1/2 phosphorylation could also be stimulated through increased calcium level and PKA activation in INS-1 cells³¹⁸. However a recent study done with different MEK inhibitors on MIN6 cells revealed that ERK1/2 phosphorylation might not be necessary for insulin secretion³¹⁹. In overall the phosphorylation of ERK1/2 is always a stable consequence of most of the insulinotropic drug treatments and it is associated with insulin secretion as well. The p-ERK1/2 activity was markedly increased after treatment with the selected VCC compounds in RIN-5AH cells, although VCC124075:01 was an exception (*Figures 4.15.1-2*). Unfortunately the upstream signaling elements of ERK1/2 couldn't be completely unveiled so far.

The cyclic AMP-dependent protein kinase (PKA) is one of the key proteins that regulate the process of insulin exocytosis when intracellular cAMP is elevated. The protein is composed of two catalytic (PKA_C) and two regulatory (PKA_R) domains. When cAMP molecules bind to the regulatory domain, the catalytic domains dissociate and exert their effects by phosphorylating

different targets, including a lot of proteins that are associated with exocytosis as well. PKA_R may also mediate biological action apart from modulating the catalytic subunits activity. The PKA_C can be phosphorylated at two highlighted sites, Ser338 and Thr197. The p-Thr197 which is located at the activation loop of PKA_C is required for its enzymatic activity^{33,320}. This phosphorylation site is considered very stable, since it resists strongly to phosphatases. Interestingly, in mammals the phosphorylation of Thr197 is likely to happen by PDK-1 and it is not an autophosphorylation process as in bacteria^{321,322}. Taken together the detection of PKA activity by looking at p-PKA_C levels might be difficult due to its persistent phosphorylated state. This was also revealed for some compound treatments in western blot. Nevertheless testing the phosphorylation of one of its substrate (e.g. CREB Ser133) can be a good indicator for this purpose (*Figure 4.15.2*).

The cAMP response element binding protein (CREB) is a nuclear factor, which is a phosphorylation target for multiple kinases. When phosphorylated it binds to the conserved gene promoter element CRE (cAMP response element) and regulates the expression of many genes. It can be phosphorylated and activated by many kinases³²³. The cyclic AMP-dependent protein kinase (PKA) is one of them which is able to activate CREB when intracellular cAMP level is increased; furthermore CAMK-II and CAMK-IV are also important regulators for CREB, which activity depends on Ca²⁺ level³²⁴. Additional CREB kinases are e.g. the p90RSK, MAPKAP K2 and MSK via p38 kinase³²⁵. It was described by others that ERKs are also able to phosphorylate CREB through the Rap1-Raf-MEK-ERK pathway in PC12 cells, when Ca²⁺ level is increased³²⁶. In beta cells CREB provides an anti-apoptotic signal, it contributes for the preservation of normal beta cell mass and it also prevents beta cell dysfunction associated with glucotoxicity by stimulating the transcription of different pro-survival genes^{324,327-330}. Based on these data, I suspected that CREB might be concerned in the mechanism of action of the compounds, especially the ones that increased intracellular cAMP level. It was shown that some of the compounds induced CREB phosphorylation at Ser133, which provided further information in the understanding of the mechanism of action of the compounds that elevated cAMP or Ca²⁺ levels in RIN-5AH cells (*Figure 4.15.2*).

The mammalian target of rapamycin (mTOR) is a key metabolic regulator protein in the body and its impaired or loss of function can cause significant phenotypic changes in different tissues³³¹. In beta cells it senses ATP level; furthermore promotes proliferation signals through activation of S6K1. Upstream it can be inhibited by AMPK probably indirectly involving the Tsc1/2 - Rheb-GTP pathway. The normal functioning of mTOR is necessary for beta cell mass prevention and for insulin synthesis as well^{332,333}. Another potential regulator of mTOR activity

is cAMP. In the endocrine thyroid³³⁴ and beta cells cAMP activates the mTOR pathway. In beta cells this process was described by the treatment with GLP-1, thus it indicates another link for the proliferative effect of incretins³³⁵. Compound treatment in RIN-5AH cells, did not change mTOR activity, however a mild effect was detected for some compounds on mTOR phosphorylation (*Figure 4.15.2*). α -tubulin was used for loading control and are included in the Appendix.

The summarized western blot results and qualitative evaluation is displayed in *Table 4.15.1*.

| VCC Compounds Phosphorylation | VCC525068:03 | VCC512178:08 | VCC981125:01 | SUTENT | VCC512891:01 | VCC350485:02 | VCC346349:01 | VCC341830:01 | VCC834153:01 | VCC958400:03 | VCC036051:03 | VCC124075:02 | VCC459492:04 | Forskolin | Roflumilast | GLP-1 | Effects When Activated | Effects When Inactivated/ Knockdown / Knock out |
|----------------------------------|--------------|--------------|--------------|--------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|-----------|-------------|-------|------------------------|---|
| | | | | | | | | | | | | | | | | | | |
| AMPK (2h) | / | / | / | - | / | / | / | / | / | + | / | / | | | | | Suppression of IS | Induction of IS |
| PKC a/b (2h) | / | + | / | + | + | + | + | + | | + | + | / | / | | | | PKCa: may induce IS | PKCb: no effect on IS? |
| PKC z/la (2h) | - | + | / | / | / | / | / | / | | / | + | / | / | | | | PKCz: induced IS | PKCa: decreased IS PKCz: decreased proliferation |
| PKC-pan (2h) | - | + | / | + | / | + | + | / | | / | + | / | / | | | | | |
| ERK (2h) | + | + | + | + | + | + | + | + | + | + | + | + | + | | | | Proliferation | Suppressed proliferation |
| MEK (30min) | + | + | | + | + | | / | | + | | | / | | + | + | + | Proliferation | Suppressed proliferation |
| ERK (30min) | + | + | | + | + | | / | | + | | | / | + | + | + | + | Proliferation | Suppressed proliferation |
| PKAc (30min) | + | / | | + | + | | + | | + | | | + | | + | + | + | Induction of IS | Suppression of IS |
| CREB (30min) | + | / | | + | + | | + | | + | | | + | | + | + | + | Anti-apoptotic | Beta cell dysfunction |
| mTOR (30min) | + | / | | + | + | | + | | + | | | + | | / | / | + | Proliferation | Decreased insulin synthesis, loss of beta cell mass |

Table 4.15.1 Summarized results and qualitative evaluation of western blot results of treatment with VCC compounds and indicating the known effects in beta cells. Detailed description in the text. IS: insulin secretion; + : phosphorylated; - : dephosphorylated; / : no change

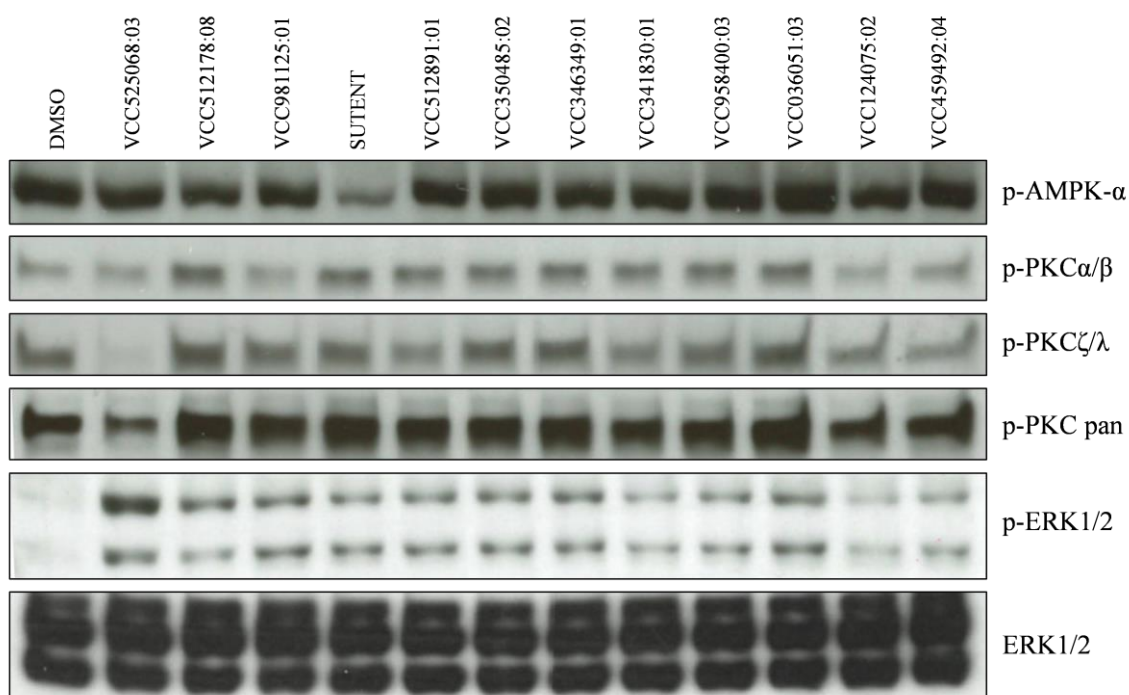


Figure 4.15.1 Western blot studies after compound treatments (5 μ M, 2h) in RIN-5AH cells.

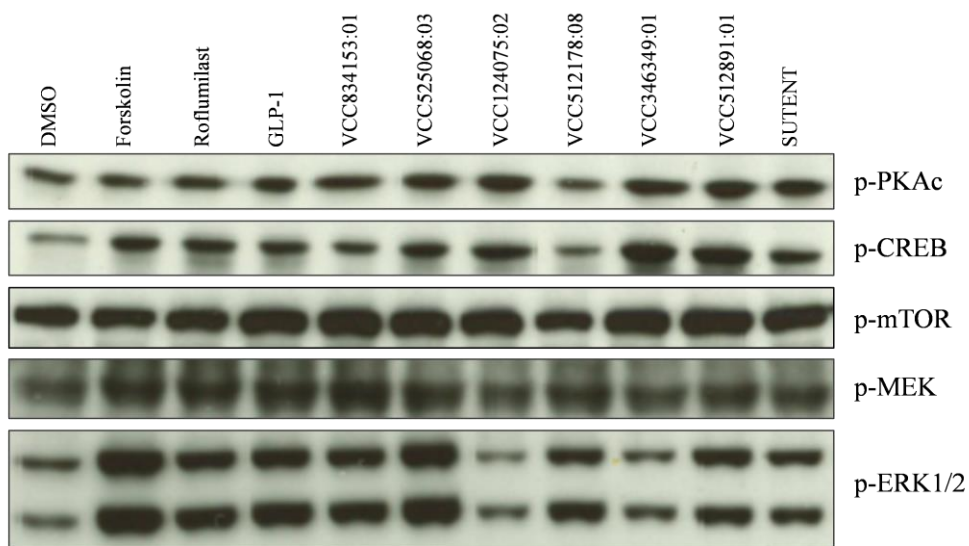


Figure 4.15.2 Western blot studies of cAMP inducer compounds in RIN-5AH cells (30min, 10 μ M). In order to confirm cAMP level stimulation, some of the "cAMP sensitive" proteins and pathways were studied.

5 **DISCUSSION**

According to human clinical data and experiments performed on different diabetic animal models, several TKIs were proven to have a capital role in improving the primary symptoms of diabetes. The following abilities were observed: they could restore normoglycemic state and treat hyperglycemia, they increased insulin sensitivity in peripheral tissues, also improved lipid metabolism and prevented beta cell loss by reducing the rate of apoptosis. Amongst these beneficial effects of TKIs, sunitinib was also able to elevate serum insulin level in vivo. We were focusing on this latter property of sunitinib and investigated the mechanisms that could be responsible for this phenomenon in in vitro systems. In the first instance the roles of kinases were investigated and for this purpose we composed a diverse compound library. The kinase inhibitor profiles of the members of this compound library were predicted to be analogous with the profile of sunitinib. These compounds were later on used as tools to identify kinases that may negatively regulate insulin secretion in beta cells. In RIN-5AH cells insulin secretion was induced by many of these compounds achieving a 10% hit rate in the insulin ELISA assay and the acutely toxic compounds were eliminated by MTT. The 55 active compounds could be clustered into different core structure families and kinase selectivity/affinity were tested for one member from each compound family. However there were differences in compound selectivity, a pool of common targets could be established. Moreover for VCC981125:09 (quinoline) and VCC958400:03 (quinoxaline) no kinase targets could be assigned (using threshold %Ctrl<35). 14 kinases could be validated by MS affinity chromatography of four substances, these were also identified in the kinase affinity panel test. I would also like to note, that in spite of the powerful capabilities of the target profiling done by MS, it has limitations and the synthesis of a proper and active linker derivative in cellular assays for these experiments is indispensable.

The results of the siRNA knockdown (KD) experiments performed in RIN-5AH and INS-1E beta cells lead to the identification of three kinases (STK16, CSNK2A1 and MARK2) that possibly negatively regulate insulin secretion in their active state in both cell lines. STK16 shares structure homology with Env7 a protein identified in yeast that negatively regulates membrane/vacuole fusion. In this regard it can't be excluded that it plays a role in insulin exocytosis as well. CSNK2A1 was already described as a regulator of insulin secretion, here we could validate this property by targeted KD in beta cells. KD effect of MARK2 in beta cells was not studied before, although in knockout mice experiments it improved insulin sensitivity and reduced high fat-induced weight gain. According to beta cell KD experiments it can be

concluded that MARK2 might have a short term effect on inhibiting insulin secretion, as longer KD has attenuated the insulin secretagogue effect. Double KD experiments and compound + KD combinations would be necessary for further validation of these targets.

By mass spectrometry additional non-kinase proteins were also identified as potential targets. Out of these PDE4C were studied in more detail and its importance was confirmed by selective PDE4 inhibitors (Roflumilast, CP 80633). As another family of PDE inhibitors (PDE5) proved to be advantageous in chronic applications in several experiments, we were curious to see whether these inhibitors can regulate insulin release in beta cells. It turned out that sildenafil and vardenafil but not tadalafil could increase insulin secretion from TC6 cells. The inefficiency of PDE5 inhibitor compounds on RIN-5AH cells can be explained with the different heterogeneity of these clonal beta cell lines.

The dissimilarity of the beta cell lines could be an important factor for the mode of action for the VCC compounds, therefore they were tested in different cell lines. Three compounds showed cell line specific insulinotropic effect (VCC981125:01, VCC124075:02 and VCC036051:03).

The cAMP molecules have crucial role in the transmission of insulinotropic signals. The cAMP level can be elevated by diverse impacts, including PDE inhibition, induction of AC enzymes e.g. by receptor signaling, and of course as a result of metabolism and conversion from ATP. In our assay the intracellular cAMP level was increased by several compounds in RIN-5AH cells. This additional effect seem to contribute to the mode of action of VCC compounds.

Another important criteria for stimulation of insulin secretion is the increased Ca^{2+} influx into the cells. Short term experiments with VCC compounds done in Fluo-4 stained cells revealed that several derivatives from the quinoline family (including VCC981125:09) induced Ca^{2+} influx in RIN-5AH cells. I hypothesized that the rapid Ca^{2+} influx might be a consequence of membrane depolarization. This idea was confirmed with whole cell patch clamp measurements. The application of higher concentration from VCC981125:09 may open the high voltage activated L-type Ca^{2+} channels, which get activated at -30mV. Moreover the 10-fold lower concentration which also depolarized the membrane, was probably enough to activate the T-type currents (threshold: between positive and -70mV). Most likely both L-type and T-type channels are involved in insulin secretion and this hypothesis was supported by other measurements: efonidipine (L- and T-type channel blocker), NiCl_2 (blocks T-type current) and verapamil (L-type channel blocker) could attenuate VCC981125-induced Ca^{2+} influx and/or insulin secretion. However the direct target of VCC981125:09 is yet unknown but most likely

it interacts with ion channels that are differently expressed in RIN-5AH (and maybe in BRIN-BD11) cells compared to TC6 and INS-1E cells.

The VCC compounds may exert some effect on peripheral tissues as well, since many of them could induce 2-NBDG uptake in myoblasts or differentiated adipocytes and they did it apparently independently to insulin. However it would be worth to check whether they can induce GLUT4 translocation in differentiated myoblasts or adipocytes, since the GLUT4/GLUT2 inhibitor Cytochalasin B could significantly attenuate the effect of VCC341830:01 in RIN-5AH cells.

In order to extend the knowledge about the mode of action of the VCC compounds in RIN-5AH cells, western blot studies were performed, which revealed some additional information. These results will be reviewed separately for each substance. The complete summarized data about the substances is displayed in *Table 5.1*.

| Highlighted VCC compounds | SUTENT | VCC350485-02 | VCC036051-03 | VCC341830-01 | VCC834153-01 | VCC981125-01 | VCC405984-01 | VCC958400-03 | VCC512891-01 | VCC346349-01 | VCC124075-02 | VCC459492-04 | VCC525068-03 | VCC512178-08 | VCC161829-02 |
|----------------------------|-----------------------|---------------------|--------------------|--------------------|--------------|----------------|--------------|------------------------|-------------------------|-------------------------|--------------------|--------------|--------------|--------------|--------------|
| Compound family | Indolines / oxindoles | Styryl quinazolines | Other quinazolines | Thieno pyrimidines | Quinolines | Bis-quinolines | Quinoxalines | 2-Amino-pyrimidines I. | 2-Amino-pyrimidines II. | 2-Amino-pyrimidines II. | 1,6-Naphthyridines | Misc. | Misc. | Misc. | Misc. |
| Kinase selectivity panel | + | + | + | + | + | + | + | + | + | + | + | + | | | |
| MS affinity chromatography | + | only binders | | unsuccessful | unsuccessful | unsuccessful | unsuccessful | | | | | | | | |
| RIN-5AH cells | / | / | + | + | / | / | / | + | + | / | + | + | + | + | + |
| RIN-5AH cells | | | + | + | | | | + | + | | | | | | |
| RIN-5AH cells | / | / | / | / | / | / | / | / | / | / | / | / | / | / | / |
| RIN-5AH cells | | / | / | + | / | / | / | / | / | ? | / | + | / | / | / |
| RIN-5AH cells | | | + | + | / | / | / | + | + | + | / | + | / | / | / |
| C2C12 cells | | + | + | + | / | + | + | + | + | + | + | + | + | + | + |
| 3T3L1 cells | | ++ | ++ | ++ | + | + | / | / | ++ | ++ | ++ | ++ | / | / | / |
| RIN-5AH cells | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| BRIN-BD11 cells | + | + | / | + | / | / | / | / | + | + | + | + | + | + | + |
| TC6 cells | + | + | / | + | / | / | + | + | + | + | + | + | + | + | + |
| INS-IE cells | + | + | - | + | / | - | + | + | + | + | - | / | + | + | + |
| INS-IE cells | + | + | | + | - | + | - | + | + | + | + | + | / | + | + |
| RIN-5AH cells | 2,3 | 4,32 | 8,54 | 2,84 | 6,19 | 2,51 | >1,67 | 2,51 | 2,16 | 4,6 | 3,13 | 3,4 | >1,67 | >1,67 | |
| RIN-5AH cells | | + | | + | + | | | | | | | | | | |
| ADME prediction (QRP-Prop) | 198,789 | 1423,62 | 2609,734 | 1381,72 | 4540,374 | 336,759 | 918,284 | 69,798 | 459,745 | 135,273 | 3246,84 | 117,319 | 66,625 | 66,625 | |
| | 3,907 | 5,668 | 6,338 | 3,374 | 4,721 | 4,505 | 4,899 | 2,585 | 3,51 | 2,538 | 4,125 | 4,299 | 2,495 | 0,993 | |
| No. of tested CPPDs | 17 | 51 | 3 | 29 | 25 | 2 | 7 | 20 | 20 | 5 | 5 | | | | |
| | 14 | 3 | 12 | 46 | 22 | 0 | 8 | 22 | 22 | 11 | 11 | | | | |
| | 31 | 54 | 15 | 75 | 47 | 2 | 15 | 42 | 42 | 16 | 16 | | | | |
| Proposed mechanism | kinases | enzymes? / FLT3? | kinases | kinases | ion channel? | GPCR | ? | kinases | kinases | kinases | kinases | kinases | kinases | kinases | kinases |

+: positive effect; -: negative effect; /: no change
 *: ** analogues structures (difference is one functional group or substituent)
 ? ambiguous

Table 5.1

Sunitinib (oxindole). Several studies confirmed that sunitinib has clearly the potential to efficiently lower the blood glucose level. Although its main targets are already known, there is a lack of evidence underlying the complete mechanism. It was proposed that c-Kit and PDGFR is responsible for the glucose concentration lowering effect, but we hypothesized that this is a more complex effect. Since sunitinib was our primary tool compound, a comprehensive target profiling was performed. Not surprisingly it was shown binding affinity to many kinases and other proteins (MS), making it difficult to select the relevant targets responsible for insulin secretion. Nevertheless some of its targets (CSNK2A1, STK16, and MARK2) could be validated by siRNAs. The sunitinib-induced insulin secretion in RIN-1AH cells may also involve the inhibition of AMPK. AMPK was identified as a target for sunitinib in the affinity assays and it also inhibited the phosphorylation of AMPK in RIN-5AH cells. Sunitinib activated the MEK/ERK pathway in RIN-5AH cells, furthermore it has induced PKC-pan phosphorylation, notably most likely PKC α phosphorylation. However we couldn't demonstrate a significant increase in cAMP level, a slight activation of CREB was also detected. Sunitinib effectively increased the insulin level in TC6, BRIN-BD11 and INS-1E beta cells as well, which means that it has a "general effect" independently from their heterogeneity. In the glucose sensitive INS-1E cells it raised insulin level in a glucose dependent manner, similarly to other compounds. Combination treatments done with Ca²⁺ channel inhibitors showed that its insulinotropic effect could be significantly reduced only by efonidipine, the L- and T-type Ca²⁺ channel inhibitor DHP drug. Combinations with verapamil and nifedipine (blocking the L-type currents) couldn't alleviate the insulin secretion, similarly to GLP-1 and Exendin-4 treatments. In conclusion it seems that T-type channels are critical for sunitinib-induced insulin secretion in RIN-5AH cells. Moreover application of high concentration diazoxide could also reduce the effect of sunitinib, same way as incretin treatments. Since sunitinib is a multi-TKI with remarkable side effects in clinic, it is proposed that the same complex mechanisms are also present which contribute to its insulinotropic effect in beta cells.

VCC341830:01 and VCC834153:01 (thieno-pyrimidine). This compound family was originally designed to inhibit EGFR. Compound VCC341830:01 showed a high selectivity for EGFR, HER2, MKNK2 and KIT in the kinase affinity assay. Numerous kinator derivatives were tested (data not shown) by silver stained gel electrophoresis, but none of them produced any affinity to proteins. Furthermore these linker derivatives have also lost their efficiency in cellular assays. Therefore MS affinity chromatography was not performed. Nevertheless, the detection of membrane proteins by MS is one of the limitations of this technique (and needs

special protocols), which could also explain the negative results. Later on by testing more compounds from this family, the structure activity relationship data showed that most likely it is not the EGFR inhibition that is responsible for increased secretion. Some substances (e.g. VCC855801:01 and VCC321171:07) did not inhibit EGFR, but had significant insulinotropic effect (see Appendix) in RIN-5AH. The VCC834153:01 significantly increased cytoplasmic cAMP level in RIN-5AH cells and activated PKA, CREB and mTOR. It also had a great impact on ERK1/2 phosphorylation. The VCC834153:01 produced an extraordinary effect in INS-1E cells compared to the other compounds. It strongly increased insulin level in low glucose buffer, but interestingly not in high glucose buffer. The other derivative VCC341830:01 showed no glucose dependent effect but it was active in the insulin ELISA assay of all four beta cell line models. In 2-NBDG uptake assay VCC341830:01 was one of the most efficient compound and its effect was Cytochalasin B dependent. Thus it might be possible that it facilitates glucose metabolism. The VCC341830:01 and VCC834153:01 showed very low toxicity on C2C12, 3T3-L1, HepG2, TC6 and RIN-5AH cells.

VCC981125 and derivatives (quinoline). The VCC981125:09 is one of the most efficient compounds in RIN-5AH cells which exerts its effect by enhancing Ca^{2+} influx. Although VCC981125:09 shares the same core structure with bosutinib, the chemical modification in the molecule has resulted in a loss of affinity to kinases. This was confirmed by the kinase affinity screen, by MS pre-experiments and silver gel staining (data not shown), where no targets could be identified. There were numerous linker derivatives designed, but unfortunately none of them were potent enough in the cellular assay to perform an MS experiment using the target deconvolution technique. The compound did not affect p-Tyr, p-Ser and p-Thr levels (see Appendix); this further confirms that it is not a kinase inhibitor. Other officially available drugs that resemble to VCC981125:09 are quinidine, chloroquine and fluoroquinolones. Quinidine was shown to inhibit K_{ATP} channels in MIN6 cells³³⁶ and it also increased insulin level in our assay in RIN-5AH cells (data not shown). Reported data suggests that fluoroquinolones also inhibit K_{ATP} channels and can induce Ca^{2+} release from internal stores as well, dependently on ryanodine receptors³³⁷. These data supports the fact that VCC981125:09 and close derivatives may also influence ion channel or Ca^{2+} signaling. Nevertheless, the mechanism of this substance couldn't be compared to any of these drugs, neither to the action of glibenclamide, since it needs extracellular calcium to exert its stimulatory effect, furthermore glibenclamide which is targeting K_{ATP} channels, was effective on both TC6 and RIN-5AH cells, while VCC981125:09 wasn't (see Appendix). Thapsigargin a drug which works by emptying intracellular calcium

stores through inhibition of SERCA and the ionophore drug A23187 had an inhibitory effect on insulin secretion, therefore VCC981125:09 does not possess any of these mechanisms. Bosutinib did not induce Ca^{2+} influx as expected. Combinatorial treatments with calcium channel blockers and K_{ATP} opener diazoxide proved that its potency is dependent on L-type and T-type currents, furthermore the repolarizing effect of diazoxide could abrogate its activity. The influx of calcium is most likely the consequence of membrane depolarization that was validated by whole cell patch clamp measurements. By patch clamp, we could also register the changes in membrane capacitance, which reflects to an increased insulin exocytosis^{289,338}. Although a clear structure activity relationship could be established for this family of compounds in RIN-5AH cells, unfortunately these compounds were ineffective in INS-1E and TC6 cells, indeed in INS-1E cells it produced a negative shift. Conversely in BRIN-BD11 cells some of its derivatives could potentiate insulin secretion (see Appendix). The VCC981125:09 did not have any significant impact on 2-NBDG uptake. It activated ERK1/2 and did not change PKC phosphorylation.

VCC405984:01 (bis-quinoline). This compound increased insulin secretion in RIN-5AH and in INS-1E cells. In INS-1E cells the activity was highly dependent on glucose concentration and more importantly in low glucose buffer it had no significant effect compared to DMSO. This property could be very important in the treatment of diabetes and this is characteristic resembles to GPCR agonist drugs. Moreover it produced a significantly better response compared to sunitinib treatment. Since it did not inhibit p-Tyr, p-Ser and p-Thr, we can assume that it's not a kinase inhibitor. By the treatment of RIN-5AH cells the ERK1/2 phosphorylation was upregulated similarly to other efficient compounds (see Appendix). Preliminary data suggests that it is a GPCR antagonist coupled to a Gq/Gi mechanism however it did not increase Ca^{2+} influx by acute treatment. More research is needed to identify and validate the GPCR target and to discover whether this compound or its derivatives could be relevant candidates for the treatment of diabetes.

VCC350485:02 (Styryl-quinazoline) is a derivative of a compound that induces p53 activation³³⁹. It is a very potent compound in RIN-5AH cells with the lowest EC_{50} for insulin, but the lowest IC_{50} as well. By utilizing the target deconvolution method we couldn't pull down any targets, but several specific binders were detected, which could be correlated with insulin secretion or insulin gene transcription. The compound was effective on four different beta cell lines and a structure activity relationship could be prepared based on RIN-5AH insulin ELISA results. In differentiated adipocytes it increased 2-NBDG uptake significantly but not in RIN-5AH or C2C12 cells. The p-ERK1/2 level was upregulated and slightly the p-PKC α as well.

Taken together the substance showed a general insulin secretagogue effect however we should reckon with the increased toxicity, but this property can be likely eliminated by the synthesis of new derivatives.

VCC036051:03 (quinazoline) shares core structure with the EGFR inhibitor gefitinib, erlotinib or lapatinib, although it has no EGFR inhibitory feature (see Appendix). Anyway as it was proposed for the thieno-pyrimidine family, the EGFR inhibition is not feasible for the enhanced insulin release. The compound had the highest “in vitro therapeutic index”, thus it proved to be the least toxic in RIN-5AH cells (and other cells too). On the other hand along with VCC981125 it decreased secretion from INS-1E cells and was inactive on other beta cells. In mouse myoblasts (C2C12) and differentiated adipocytes (3T3-L1) it increased 2-NBDG uptake. The decreased insulin secretion could be associated with its activating effect on AMPK but on the other hand if this property is also present in peripheral tissues (liver, muscle or fat) this could give an explanation for its glucose uptake inducing effect. In overall AMPK activation is preferable and advantageous in the treatment of diabetes¹⁹⁷.

VCC459492:04 (1,6-Naphthyridine) was active for insulin secretion in RIN-5AH and TC6 cells, and less efficient on INS-1E cell. It was a strong 2-NBDG uptake inducer in RIN-5AH beta cells, myoblasts and differentiated adipocytes.

VCC346349:01 (2-Amino-pyrimidine II.) is also a highly effective 2-NBDG uptake inducer and possess a general insulinotropic effect on all four beta cell lines. It produced a delayed effect on ERK1/2 phosphorylation, compared to other compounds (VCC834153, VCC525068, VCC512178, VCC512891 and sunitinib) that already stimulated ERK1/2 activation after 30min treatment. The postliminary effect might be attributed to the positive feedback of insulin. On the other hand it raised the intracellular cAMP level in RIN-5AH cells and activated the PKA/CREB pathway, including mTOR.

VCC512891:01 (2-Amino-pyrimidine Ia.) shares kinase targets with sunitinib and was used as a tool to identify negative regulators of insulin release. It contains the core structure of imatinib, however it was more potent insulin secretion inducer in RIN-5AH cells. It significantly increased insulin secretion from all four beta cell lines, it slightly elevated cAMP level in RIN-5AH cells and activated PKA/CREB and mTOR.

VCC124075:02 (2-Amino-pyrimidine Ib.) was used as the fourth tool compound in the target deconvolution MS experiment. According to its kinases selectivity panel it was the least selective compound after sunitinib and showed a cell line specific effect for RIN-5AH and BRIN-BD11 cells in insulin ELISA assay. The slight negative effect achieved on TC6 and INS-1E cells can be attributed to the different expression patterns and heterogeneity of the clonal

beta cells. In RIN-5AH cells it activated PKA/CREB and mTOR, which correlates with the increased cAMP level caused by the drug.

VCC958400:03 (Quinoxaline) was a moderately active compound in insulin assay, although it showed some activity on p-PKC α/β . It has no, or very low affinity to kinases.

VCC512178:08 and VCC161829:02 (Misc. I.) are analogous structures. VCC512178:08 is a potent insulin secretagogue molecule which is known to inhibit PAK. It was active in RIN-5AH, TC6 and INS-1E cells too. It increased insulin secretion in a glucose dependent manner in INS-1E cells. Treatment with this compound caused a slightly increased cAMP level in RIN-5AH, however interestingly it seems that VCC512178:08 did not affect the PKA/CREB pathway, but involved the activation of ERK1/2. One possibility for ERK1/2 phosphorylation could be the Epac/Rap1/Raf/MEK/ERK pathway activation³⁴⁰, but other factors may play role as well.

VCC525068:03 (Misc. II.) is a PKC inhibitors molecule, but in spite of that it markedly induced insulin secretion in all cell lines. In RIN-5AH cells we demonstrated that PKA/CREB and mTOR, respectively the MEK/ERK pathway was strongly activated. It is worth to notice that despite its high activity it did not regulate the secretion glucose dependently from INS-1E cells.

In this study numerous small molecule kinase inhibitors and their derivatives were investigated for their insulinotropic effect. We found that not only Sutent but other kinase inhibitors stimulated the insulin secretion in beta cells and therefore may lower blood glucose level as well. The utilized VCC compounds in our assays provided efficient tools to investigate beta cell function and other mechanisms related to diabetes. Hopefully these data will also contribute to the development of novel therapies and to a better understanding of how TKIs can modulate beta cell function.

SUMMARY

We found that sunitinib increased insulin secretion *in vitro* in different beta cell lines. Based on this observation we prepared a compound library consisting of 558 members, which were predicted to overlap with the kinase inhibition profile of sunitinib. Using this library we performed a phenotypic screen for insulin secretion, utilizing an insulin ELISA assay. Then the compounds were clustered into different core structure families and from each family one compound was further investigated in different assays.

Summarizing our results, the following conclusions were made:

- We identified compounds that stimulated insulin secretion and were less toxic compared to sunitinib. The action of most of the compounds was dependent on glucose concentration in INS-1E cells
- Structure activity relationships could be set up for quinoline, styryl-quinazoline and thienopyrimidine compound families in RIN-5AH cells, therefore it seems to be possible that TKI compounds can be optimized for insulinotropic effects
- We could define and validate kinases (MARK2, STK16 and CSNK2A1) by siRNA that negatively regulate insulin secretion in RIN-5AH and INS-1E cells, however we can't rule out multitarget effects of compounds
- We could assign partial mechanisms of actions for most of the compounds, these involve the activation of cAMP and Ca²⁺ pathways
- Some VCC compounds were also able to increase 2-NBDG (glucose analogue) uptake into beta-cells, myoblasts and differentiated adipocytes, which could be also an attractive viewpoint in the treatment of diabetes

In conclusion we found potential lead candidates that should be further investigated. Amongst these, there are also compounds with a more selective kinase profile compared to sunitinib. An interesting group of compounds are the quinolines/bis-quinolines that are not targeting kinases, but can cause a rapid Ca²⁺ influx (quinoline) in RIN-5AH cells or act glucose dependently (bis-quinoline) in INS-1E cells. On the other hand, since many of the tested compounds are kinase inhibitors, our results further attract the attention to the insulinotropic effect of TKIs used in clinical treatments and this should be taken into account in clinical practice as well.

ZUSAMMENFASSUNG

Wir haben festgestellt, dass das Krebsmedikament Sutent die Insulinsekretion in vitro in verschiedenen pankreatischen beta-Zelllinien erhöhte. Basierend auf dieser Beobachtung wurde eine chemische Substanzbibliothek erstellt, die aus 558 Verbindungen bestand die mit dem Kinase Inhibitions Profil von Sunitinib korrelierte. Diese Bibliothek wurde mit einem „phänotypischen drug screen“ auf Insulin Induktion geprüft. Positive Verbindungen wurden nach Core-Struktur Familien geordnet und aus jeder Strukturgruppe wurde eine repräsentative Verbindung weiter untersucht.

Die Zusammenfassung unserer Ergebnisse erlaubten folgende Schlussfolgerungen:

- Es wurden mehrere Moleküle identifiziert, die in den verwendeten Zellen die Insulinsekretion induzierten und im Vergleich mit Sunitinib weniger toxisch waren. Die Wirkung der meisten Substanzen waren in INS-1E Zellen Glukosekonzentration-abhängig.
- Struktur-Aktivitäts-Beziehungen wurden bestimmt für die Qinolin, Styryl-quinazolin und Thieno-Pyrimidin Substanzfamilien nach den Insulin-ELISA Ergebnissen in RIN-5AH Zellen. Es scheint also möglich zu sein, dass Kinase Inhibitoren auf insulinotrope Wirkung optimiert werden können
- Wir konnten einige Ziel- Kinasen mit siRNA identifizieren und validieren (MARK2, STK16 and CSNK2A1), die die Insulinsekretion in RIN-5AH und INS-1E Zellen negativ regulierten, obwohl Multi-Target-Effekte der Verbindungen nicht ausgeschlossen werden können
- Für die meisten Substanzen konnten Wirkungsmechanismen etabliert werden welche, die Aktivierung von cAMP und Ca^{2+} Signalübertragungswegen betrafen.
- Einige Compounds konnten auch die Aufnahme von 2-NBDG (Glukose-Analog) in beta-Zellen, Myoblasten und differenzierte Adipozyten stimulieren was ein interessanter Aspekt bei der Behandlung von Diabetes sein könnte

Wir fanden auch potenzielle Substanzen, die weiteres Entwicklungspotenzial aufweisen. Darunter sind Moleküle, die im Vergleich zu Sunitinib selektivere Kinase-Profile aufweisen. Eine interessante Substanz Gruppe sind die Quinoline/Bis-quinoline die keine Kinase Bindung aufwiesen wurden, aber „ Ca^{2+} influx“ (Quinoline) in RIN-5AH Zellen oder Glukose-abhängige Wirkung in INS-1E Zellen stimulierten.

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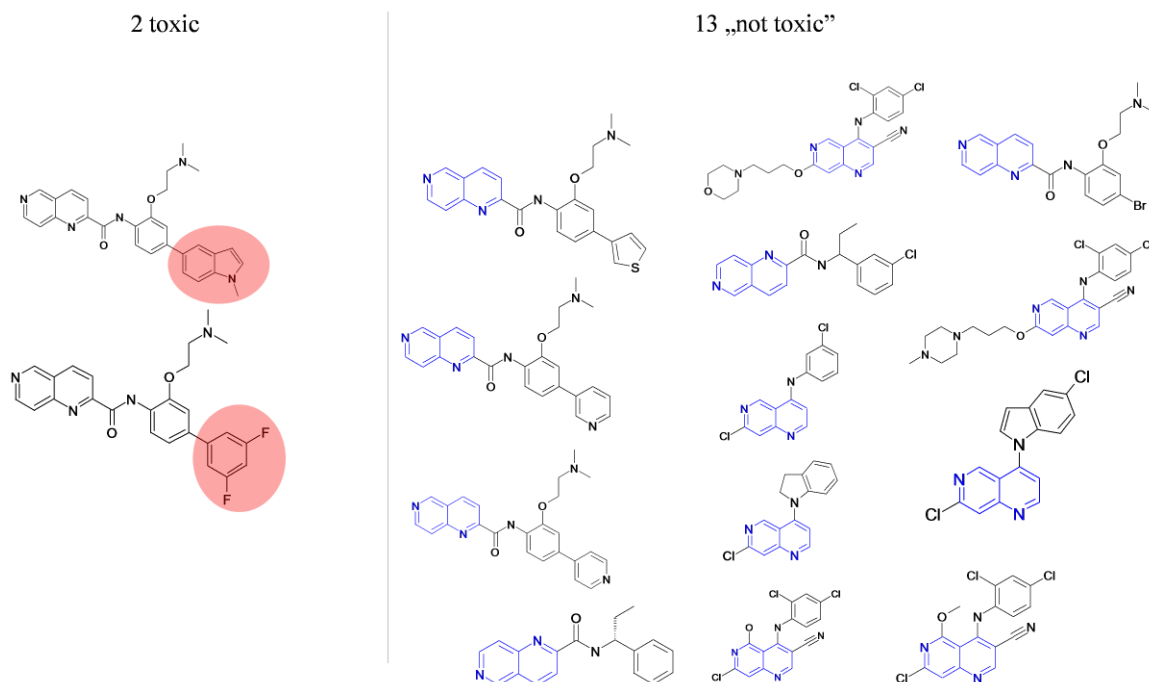
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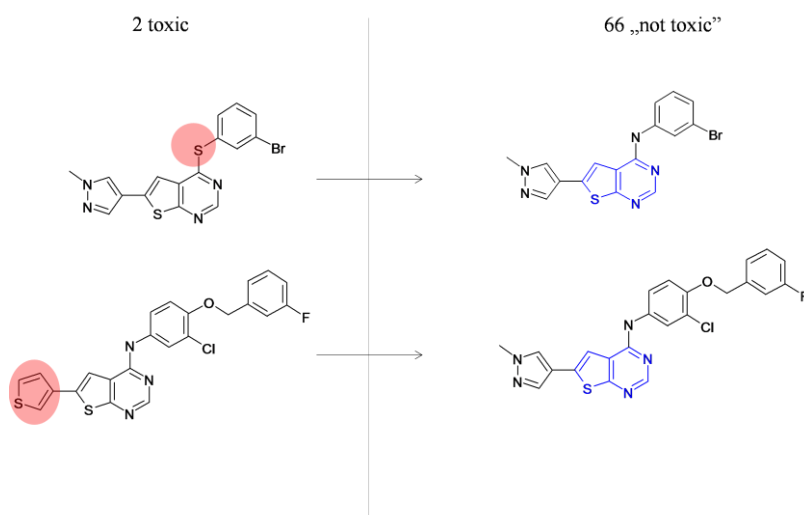
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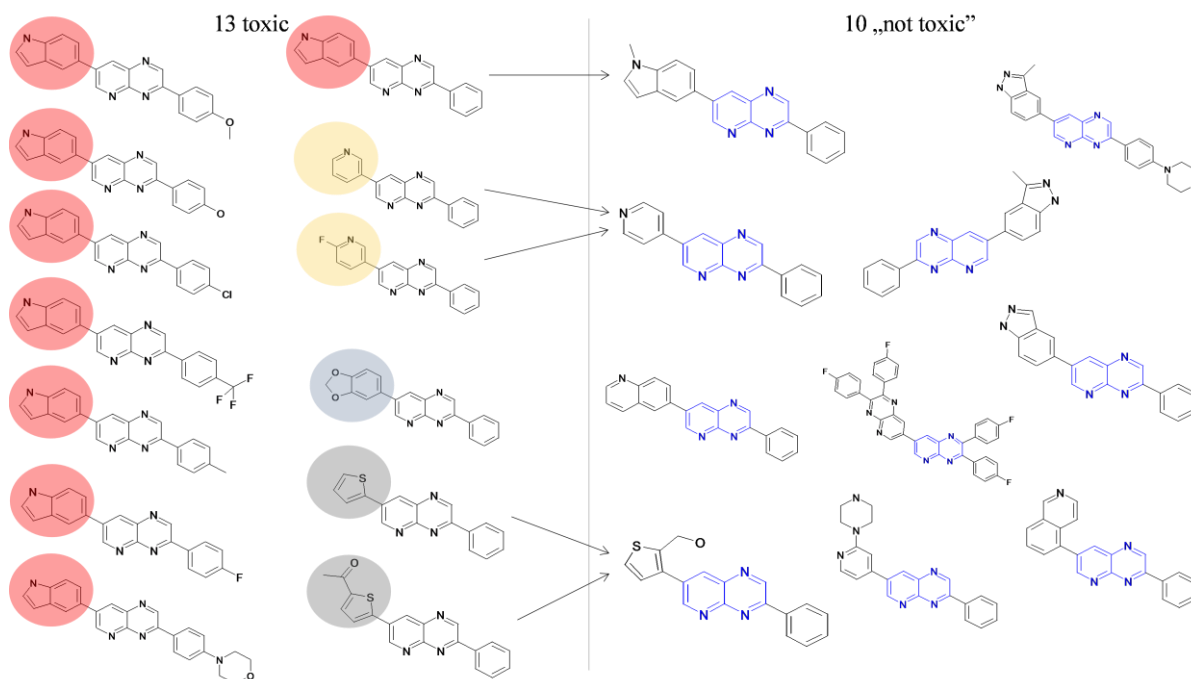
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APPENDIX**Toxic compounds**

1,6-Naphthyridine derivatives (core structure highlighted in blue). To the left: Two derivatives that decreased viability in MTT assay, after 2h treatment, probably because of the highlighted differences in their structures. To the right: 13 derivatives that were not toxic in the RIN-5AH cells. These structures include effective and non-effective compounds as well.

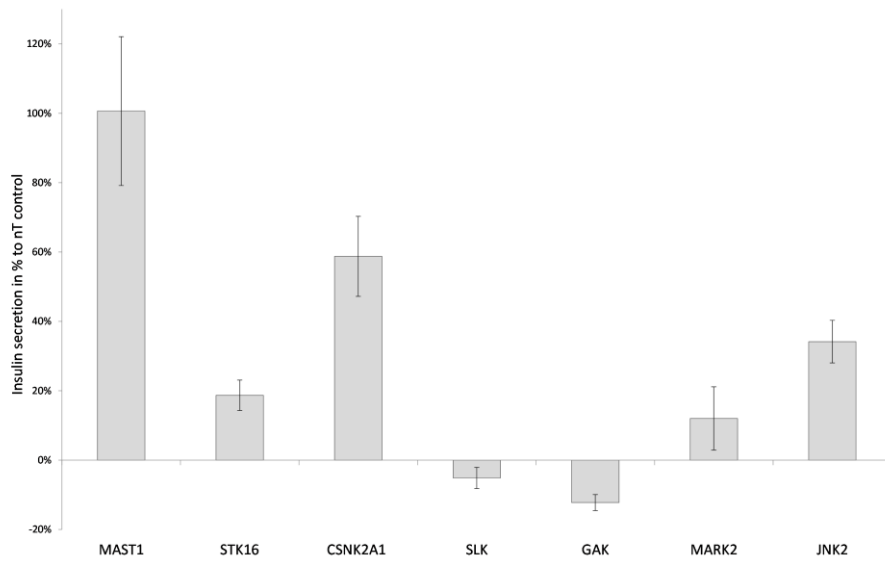


Structure activity relationships of toxic derivatives on RIN-5AH cells. Modifications in the cores structures decreased cell viability (to the left). Within the same core structure 66 non-toxic compounds were found. The displayed structures include effective and non-effective compounds as well.



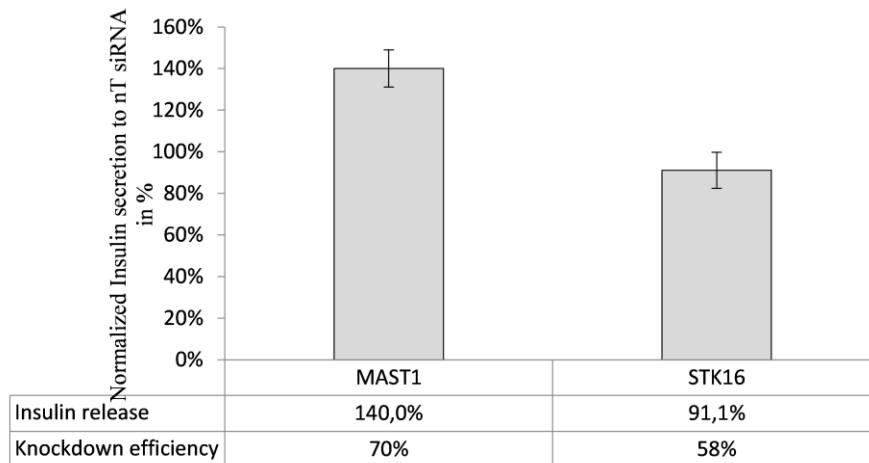
Structure activity relationships of toxic derivatives on RIN-5AH cells. Modifications in the cores structures decreased cell viability (to the left). Within the same core structure 10 non-toxic compounds were found. The displayed structures include effective and non-effective compounds as well.

Supplementary info for knockdown experiments



The 72h knockdown effect with DharmaFECT ON-TARGETplus in INS-1E cells

Accell siRNA knockdown - RIN-5AH cells



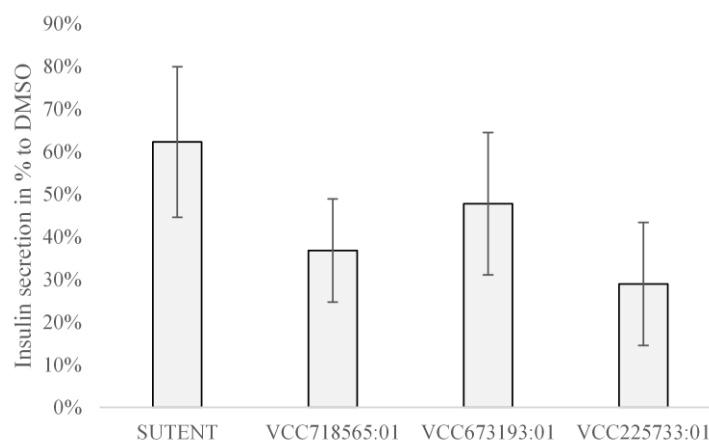
The siRNA effect of MAST1 and STK16 on insulin release and knockdown efficiency by Accell siRNA in RIN-5AH cells

RIN-5AH KD efficiencies with Accell siRNA

| knockdown | sample | primer | Ct | Ct2 | AV_Ct | SD_Ct | delta Ct | dd Ct | 2 ⁻ -(dd Ct) | % KD |
|-----------|--------------|--------|-------------|-------------|-------------|-------------|-------------|-------------|-------------------------|-------|
| MAST1 | KD_spl_MAST1 | MAST1 | 30,75922012 | 31,21061897 | 30,98491955 | 0,319187187 | 9,165354729 | 1,747289658 | 0,297860835 | 70,2% |
| STK16 | KD_spl_STK16 | STK16 | 25,47702599 | 25,55031586 | 25,51367092 | 0,051823765 | 5,858898163 | 1,243083954 | 0,422468607 | 57,8% |

RIN-5AH KD efficiencies with ON-TARGETplus siRNA

| Sample Name | Target Name | Ct Mean | Ct SD | Δ Ct Mean | Δ Ct SE | $\Delta\Delta$ Ct | RQ Min | RQ Max | RQ | % KD |
|-------------|-------------|-------------|-------------|------------------|----------------|-------------------|-------------|-------------|-------------|------|
| KD_MAST1 | MAST1 | 28,69954681 | 0,362191826 | 9,129863739 | 0,245223388 | 2,188987017 | 0,14468357 | 0,332414031 | 0,219305381 | 78% |
| KD_STK16 | STK16 | 24,78972435 | 0,296184301 | 5,326443195 | 0,205360219 | 2,313143969 | 0,142038867 | 0,285063326 | 0,201221451 | 80% |
| KD_CSNK2A1 | CSNK2A1 | 31,7316227 | 0,197242051 | 11,7581234 | 0,147837013 | 1,920359373 | 0,205597773 | 0,339476764 | 0,264188707 | 74% |
| KD_SLK | SLK | 28,49589729 | 0,519297957 | 8,879658699 | 0,329549074 | 2,697406292 | 0,088156834 | 0,269614756 | 0,154169977 | 85% |
| KD_GAK | GAK | 26,33444405 | 0,37216711 | 7,08211565 | 0,261951506 | 1,6872859 | 0,199124381 | 0,484203815 | 0,310510516 | 69% |
| KD_MARK2 | MARK2 | 24,42905617 | 0,450565636 | 5,135225773 | 0,322582752 | 1,707970977 | 0,177107528 | 0,529007673 | 0,306090266 | 69% |
| KD_JNK2 | JNK2 | 26,56005859 | 0,023358146 | 7,083334923 | 0,024443628 | 2,518489122 | 0,16743806 | 0,181913212 | 0,174525633 | 83% |

Efficient quinoline derivatives in BRIN-BD11 cells

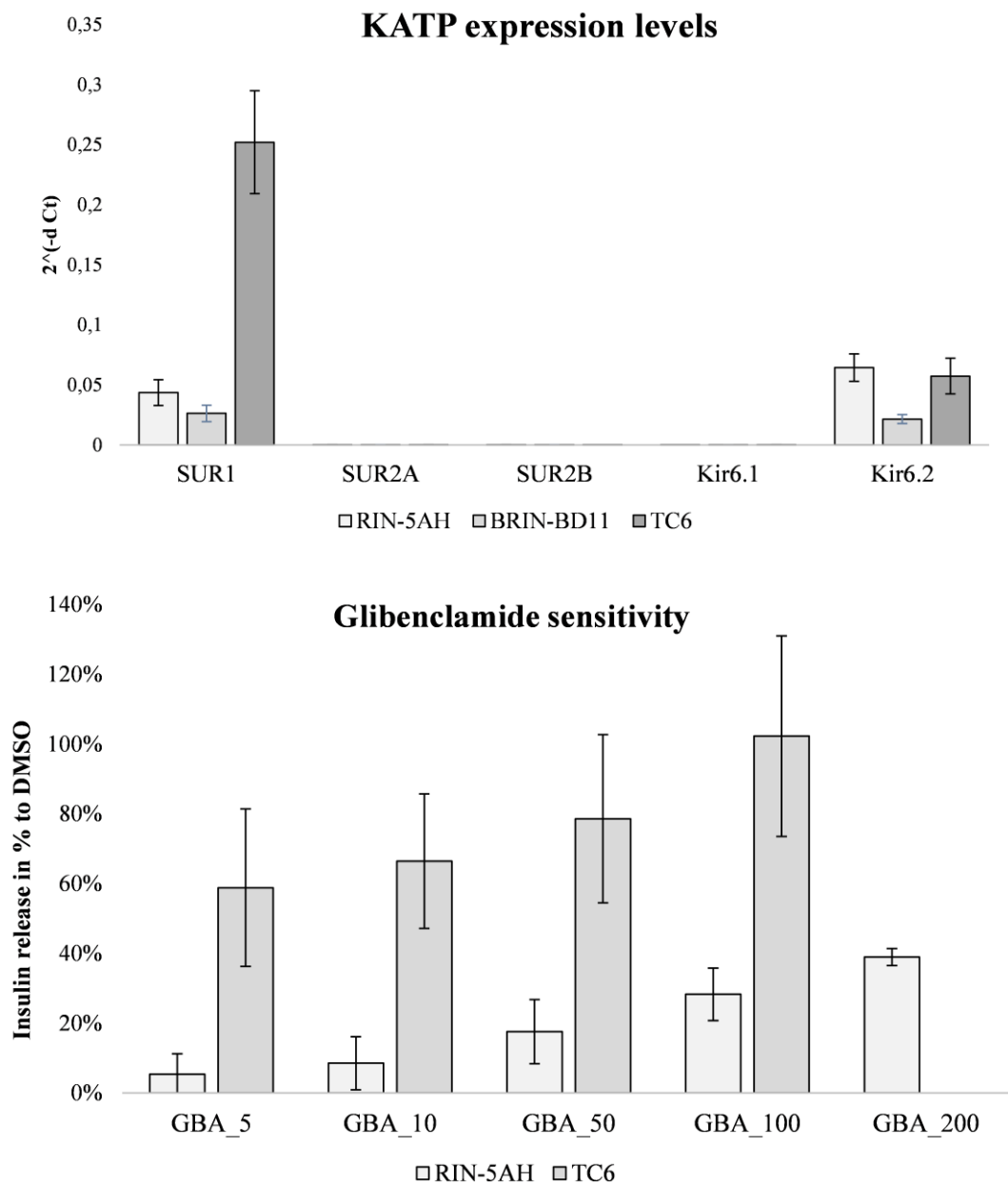
In BRIN-BD11 cells some derivatives of VCC981125 increased insulin secretion. (n=2)

Thieno-pyrimidines supplementary data

| ID | Insulin RIN-5AH | Kinase inhibition | | |
|--------------|--------------------|-------------------|--------|--------|
| | | EGFR | HER2 | KIT |
| VCC613596:10 | 80,7% | 86,4% | 55,7% | 18,1% |
| VCC855801:01 | 60,5% | 10,9% | -0,9% | 1,8% |
| VCC919477:01 | 37,7% | 64,8% | 17,9% | -10,0% |
| VCC341830:01 | 35% | 87% | 40% | 74% |
| VCC912492:01 | 31,7% | 95,4% | 62,4% | 86,1% |
| VCC321171:07 | 30,4% | 11,8% | -13,4% | -15,5% |
| VCC045928:01 | 22,7% | -10,3% | 1,9% | -11,3% |
| VCC212350:01 | 22,0% | -7,1% | 2,4% | -23,4% |
| VCC921037:01 | 15,9% | 3,8% | 0,3% | 4,9% |
| VCC786494:01 | 5,2% | 4,1% | 4,6% | 4,8% |

Highlighted values above 30%. All compounds from the same family, but different selectivities for kinases. Data provided by Vichem Chemie Ltd. for kinase inhibition

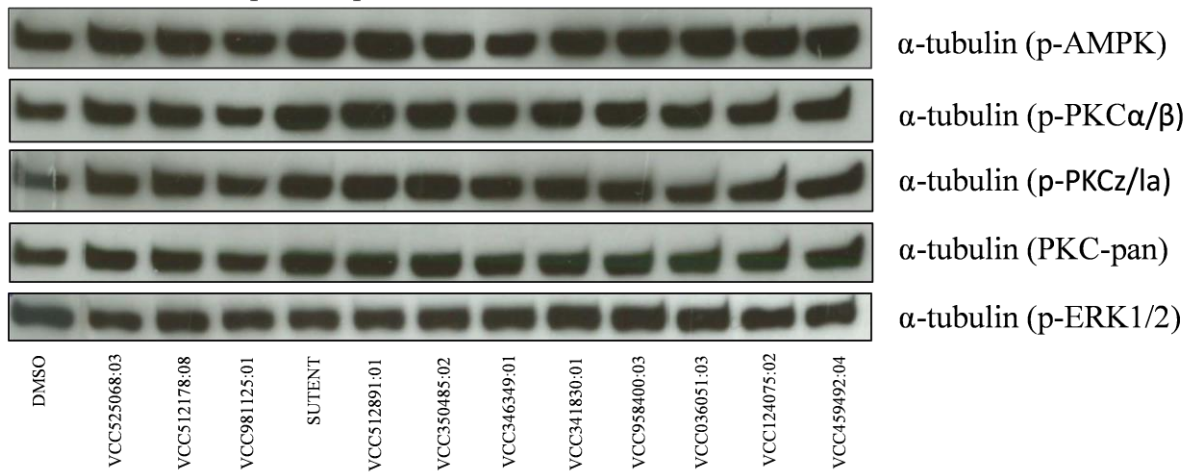
Altered K_{ATP} expression levels in different beta cell lines and the correlation with GBA sensitivity



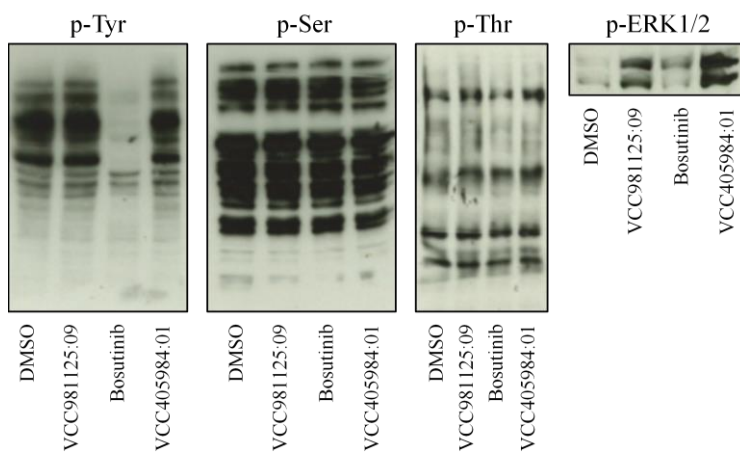
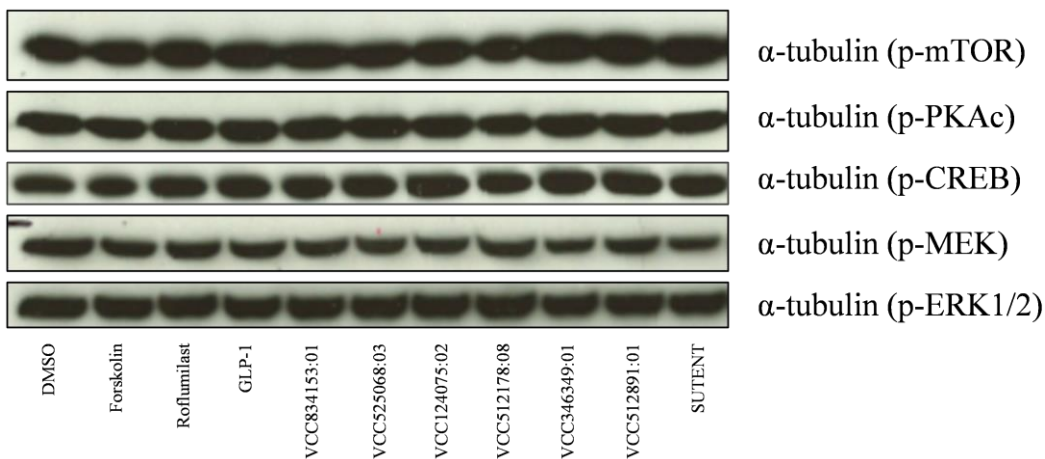
K_{ATP} subunits are expressed differentially in beta cells, which correlates with altered sensitivity of glibenclamide treatment as well. ($n=3$; housekeeping genes: *b-actin*, *GAPD*, *Cyclophilin B*)

Western blot supplementary info

„VCC insulinotropic compounds”



„cAMP inducer VCC compounds”



Top and middle: α-tubulin loading controls for VCC compound treatments and cAMP inducer compounds.

Bottom: Quinoline, bosutinib and bis-quinoline treatments.

ADME QikProp (Schrödinger)

| ID | VCC350485:02 - mother compound | VCC350485:02 - acetyl protected | VCC350485:02 - dimethyl endgroup | VCC350485:02 - kinator | SUNITINIB | SUNITINIB linker | VCC512891:01 - mother compound | VCC512891:01 - linker | VCC512891:01 - BOC protected linker | VCC124075:02 - kinator/mother compound | VCC124075:02 - formyl protected linker |
|--------------------------|--------------------------------|---------------------------------|----------------------------------|------------------------|-----------|------------------|--------------------------------|-----------------------|-------------------------------------|--|--|
| tautomer probability | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 | 1,00 |
| mol MW | 469,42 | 455,35 | 362,47 | 413,32 | 398,48 | 398,48 | 437,93 | 461,57 | 561,69 | 396,45 | 438,49 |
| dipole | 2,59 | 4,15 | 3,25 | 4,06 | 7,16 | 8,31 | 5,74 | 2,90 | 3,29 | 7,08 | 9,16 |
| SASA | 742,15 | 767,46 | 719,43 | 702,31 | 740,85 | 763,85 | 791,64 | 868,97 | 998,74 | 718,87 | 764,31 |
| FOSA | 362,75 | 300,82 | 339,80 | 231,16 | 403,90 | 365,61 | 259,35 | 352,28 | 502,50 | 334,64 | 405,74 |
| FISA | 25,25 | 100,51 | 46,17 | 82,42 | 112,81 | 174,18 | 105,70 | 162,46 | 161,45 | 133,04 | 130,63 |
| PISA | 276,85 | 290,57 | 333,47 | 311,42 | 177,23 | 177,16 | 352,57 | 354,24 | 334,79 | 251,19 | 227,94 |
| WPSA | 77,31 | 75,56 | 0,00 | 77,31 | 46,91 | 46,91 | 74,01 | 0,00 | 0,00 | 0,00 | 0,00 |
| volume | 1379,2 | 1331,9 | 1262,3 | 1207,5 | 1311,9 | 1329,8 | 1383,7 | 1523,5 | 1817,7 | 1261,8 | 1372,9 |
| donorHB | 1,00 | 2,00 | 1,00 | 3,00 | 2,00 | 4,00 | 2,00 | 5,00 | 4,00 | 4,00 | 3,00 |
| acctptHB | 5,25 | 5,75 | 5,25 | 4,25 | 6,00 | 5,00 | 10,00 | 11,50 | 13,00 | 7,25 | 8,75 |
| dip ² /V | 0,00 | 0,01 | 0,01 | 0,01 | 0,04 | 0,05 | 0,02 | 0,01 | 0,01 | 0,04 | 0,06 |
| ACxDN [^] ,5/SA | 0,01 | 0,01 | 0,01 | 0,01 | 0,01 | 0,01 | 0,02 | 0,03 | 0,03 | 0,02 | 0,02 |
| glob | 0,81 | 0,76 | 0,79 | 0,78 | 0,78 | 0,77 | 0,76 | 0,74 | 0,72 | 0,79 | 0,78 |
| QPpolrz | 44,62 | 44,14 | 41,77 | 39,37 | 42,91 | 42,34 | 48,74 | 51,78 | 62,72 | 40,32 | 44,55 |
| QPlogPC16 | 13,95 | 14,54 | 12,93 | 13,51 | 12,71 | 13,84 | 15,53 | 17,63 | 20,24 | 13,64 | 14,30 |
| QPlogPoct | 18,85 | 20,74 | 17,64 | 19,73 | 20,66 | 23,16 | 25,00 | 30,69 | 33,80 | 23,27 | 24,36 |
| QPlogPw | 7,80 | 13,29 | 8,68 | 10,71 | 10,57 | 12,54 | 16,22 | 21,77 | 21,74 | 14,86 | 16,74 |
| QPlogPo/w | 5,67 | 4,36 | 4,54 | 4,23 | 3,92 | 3,57 | 2,56 | 1,16 | 3,00 | 2,54 | 2,71 |
| QPlogS | -5,16 | -5,78 | -4,75 | -4,80 | -5,14 | -5,13 | -3,87 | -2,33 | -5,10 | -3,61 | -4,41 |
| CIQPlogS | -6,59 | -6,42 | -4,40 | -5,61 | -4,65 | -4,80 | -3,20 | -1,91 | -4,37 | -4,23 | -4,99 |
| QPlogHERG | -6,70 | -5,38 | -7,33 | -7,29 | -6,63 | -6,90 | -8,57 | -9,66 | -9,09 | -6,93 | -4,78 |
| QPlogCaco | 1423,62 | 604,71 | 901,48 | 408,51 | 210,40 | 55,09 | 61,28 | 4,43 | 18,14 | 135,27 | 354,29 |
| QPlogBB | 0,23 | -0,95 | -0,08 | -0,29 | -0,63 | -1,51 | -0,04 | -1,24 | -1,37 | -1,08 | -1,47 |
| QPlogMDCK | 2125,88 | 1427,34 | 489,27 | 551,43 | 183,44 | 43,10 | 75,30 | 1,91 | 7,94 | 62,98 | 270,29 |
| QPlogKp | -2,02 | -1,48 | -2,39 | -3,14 | -4,27 | -5,21 | -5,78 | -6,43 | -6,39 | -4,19 | -2,16 |
| IP(eV) | 8,33 | 8,44 | 8,31 | 8,35 | 8,72 | 8,71 | 8,40 | 8,14 | 8,21 | 8,20 | 8,14 |
| EA(eV) | 1,02 | 1,20 | 0,98 | 1,02 | 1,30 | 1,30 | 1,08 | 0,51 | 0,61 | 0,66 | 0,65 |
| #metab | 2,00 | 1,00 | 2,00 | 3,00 | 3,00 | 4,00 | 7,00 | 9,00 | 7,00 | 7,00 | 5,00 |
| QPlogKhsa | 0,87 | 0,33 | 0,63 | 0,51 | 0,61 | 0,55 | 0,11 | -0,10 | 0,34 | 0,05 | -0,18 |

| | | | | | | | | | | | |
|-----------------------------------|--------|--------|--------|--------|-------|--------|-------|--------|--------|--------|--------|
| HumanOralAbsorption | 3,00 | 3,00 | 3,00 | 3,00 | 3,00 | 3,00 | 2,00 | 2,00 | 2,00 | 2,00 | 3,00 |
| PercentHumanOralAbsorption | 100,00 | 100,00 | 100,00 | 100,00 | 91,47 | 79,02 | 73,89 | 45,28 | 41,11 | 79,95 | 88,43 |
| SAfluorine | 0,00 | 0,00 | 0,00 | 0,00 | 46,91 | 46,91 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| SAamideO | 0,00 | 32,82 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 26,10 |
| PSA | 41,55 | 82,18 | 47,96 | 65,76 | 89,50 | 112,42 | 89,23 | 126,09 | 141,07 | 104,42 | 117,04 |
| #NandO | 5 | 6 | 5 | 5 | 6 | 6 | 8 | 10 | 12 | 9 | 10 |
| RuleOffFive | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| RuleOfThree | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 1 | 0 |
| #ringatoms | 16 | 16 | 16 | 16 | 14 | 14 | 24 | 24 | 24 | 18 | 18 |
| #in34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| #in56 | 16 | 16 | 16 | 16 | 14 | 14 | 24 | 24 | 24 | 18 | 18 |
| #noncon | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 4 | 0 | 0 |
| #nonHatm | 30 | 29 | 27 | 26 | 29 | 29 | 31 | 34 | 41 | 29 | 32 |

Descriptors predicted:

mol_MW Molecular weight of the molecule. 130.0 – 725.0

dipole† Computed dipole moment of the molecule. 1.0 – 12.5

SASA Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 Å radius. 300.0 – 1000.0

FOSA Hydrophobic component of the SASA (saturated carbon and attached hydrogen). 0.0 – 750.0

FISA Hydrophilic component of the SASA (SASA on N, O, and H on heteroatoms). 7.0 – 330.0

PISA π (carbon and attached hydrogen) component of the SASA. 0.0 – 450.0

WPASA Weakly polar component of the SASA (halogens, P, and S). 0.0 – 175.0

volume Total solvent-accessible volume in cubic angstroms using a probe with a 1.4 Å radius. 500.0 – 2000.0

donorHB Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer. 0.0 – 6.0

acceptHB Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer. 2.0 – 20.0

dip²/V† Square of the dipole moment divided by the molecular volume. This is the key term in the Kirkwood-Onsager equation for the free energy of solvation of a dipole with volume V. 0.0 – 0.13

ACxDN⁵/SA Index of cohesive interaction in solids. This term represents the relationship; see Ref. [2]. 0.0 – 0.05

glob Globularity descriptor, $\frac{r}{R}$, where r is the radius of a sphere with a volume equal to the molecular volume. Globularity is 1.0 for a spherical molecule. 0.75 – 0.95

QPpolarz Predicted polarizability in cubic angstroms. 13.0 – 70.0

QPlogPC16 Predicted hexadecane/gas partition coefficient. 4.0 – 18.0

QPlogPoct‡ Predicted octanol/gas partition coefficient. 8.0 – 35.0

QPlogPw Predicted water/gas partition coefficient. 4.0 – 45.0

QPlogPo/w Predicted octanol/water partition coefficient. –2.0 – 6.5; should be <5 according to Lipinski rules

QPlogS Predicted aqueous solubility, log S. S in mol dm⁻³ is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid. –6.5 – 0.5;

CIQPlogS Conformation-independent predicted aqueous solubility, log S. S in mol dm⁻³ is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid. –6.5 – 0.5

QPlogHERG Predicted IC₅₀ value for blockage of HERG K⁺ channels. concern below –5 ($\frac{acceptHB}{donorHB}$) / (SA) $4\pi r^2$ / (SASA)

QPPCaco Predicted apparent Caco-2 cell permeability in nm/sec. Caco-2 cells are a model for the gut-blood barrier. QikProp predictions are for non-active transport.

<25 poor,

>500 great

QLogBB Predicted brain/blood partition coefficient. Note: QikProp predictions are for orally delivered drugs so, for example, dopamine and serotonin are CNS negative because they are too polar to cross the blood-brain barrier
-3.0 – 1.2

QPPMDCK Predicted apparent MDCK cell permeability in nm/sec. MDCK cells are considered to be a good mimic for the bloodbrain barrier. QikProp predictions are for non-active transport.

<25 poor,

>500 great

QLogKp Predicted skin permeability, log K_p . -8.0 – -1.0

IP(ev)† PM3 calculated ionization potential. 7.9 – 10.5

EA(eV)† PM3 calculated electron affinity. -0.9 – 1.7

#metab‡ Number of likely metabolic reactions

QLogKhsa Prediction of binding to human serum albumin. -1.5 – 1.5

HumanOralAbsorption

Predicted qualitative human oral absorption: 1, 2, or 3 for low, medium, or high. The text version is reported in the output. The assessment uses a knowledge-based set of rules, including checking for suitable values of PercentHumanOralAbsorption, number of metabolites, number of rotatable bonds, logP, solubility and cell permeability.

PercentHuman-OralAbsorption

Predicted human oral absorption on 0 to 100% scale. The prediction is based on a quantitative multiple linear regression model. This property usually correlates well with HumanOral-Absorption, as both measure the same property.

>80% is high

<25% is poor

SAFluorine Solvent-accessible surface area of fluorine atoms. 0.0 – 100.0

SAamideO Solvent-accessible surface area of amide oxygen atoms. 0.0 – 35.0

PSA Van der Waals surface area of polar nitrogen and oxygen atoms. 7.0 – 200.0

#NandO Number of nitrogen and oxygen atoms. 2 – 15

RuleOfFive: Number of violations of Lipinski's rule of five [3]. The rules are: mol_MW < 500, QLogPo/w < 5, donorHB ≤ 5, accptHB ≤ 10. Compounds that satisfy these rules are considered druglike (The "five" refers to the limits, which are multiples of 5.) maximum is 4

RuleOfThree: Number of violations of Jorgensen's rule of three. The three rules are: QLogS > -5.7, QP PCaco > 22 nm/s, # Primary Metabolites < 7. Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available. maximum is 3

#ringatoms: Number of atoms in a ring

#in34: Number of atoms in 3- or 4-membered rings

#in56: Number of atoms in 5- or 6-membered rings

#noncon: number of ring atoms not able to form conjugated aromatic systems (e.g. sp³ C).

#nonHatm: Number of heavy atoms (nonhydrogen atoms)

Kinase affinity assay results

Binding affinities for kinases at 5 μ M in Ctrl%.

| Kinase | VCC036051:03 | VCC124075:02 | VCC341830:01 | VCC346349:01 | VCC350485:02 | VCC459492:04 | VCC512891:01 | VCC553231:16 | VCC958400:03 | VCC981125:01 |
|-------------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| AAK1 | 89 | 2,9 | 66 | 14 | 100 | 76 | 3,8 | 0,85 | 97 | 94 |
| ABL1(E255K)-phosphorylated | 70 | 0,85 | 100 | 39 | 84 | 11 | 10 | 1,3 | 98 | 70 |
| ABL1(F317I)-nonphosphorylated | 69 | 47 | 94 | 72 | 64 | 74 | 35 | 17 | 100 | 80 |
| ABL1(F317I)-phosphorylated | 82 | 28 | 80 | 78 | 67 | 51 | 46 | 12 | 92 | 85 |
| ABL1(F317L)-nonphosphorylated | 57 | 30 | 75 | 58 | 64 | 44 | 17 | 17 | 81 | 78 |
| ABL1(F317L)-phosphorylated | 48 | 7,3 | 88 | 48 | 76 | 21 | 23 | 3,1 | 70 | 47 |
| ABL1(H396P)-nonphosphorylated | 71 | 0,1 | 65 | 9,1 | 69 | 4 | 1,6 | 0,2 | 100 | 100 |
| ABL1(H396P)-phosphorylated | 67 | 0,25 | 80 | 39 | 81 | 13 | 7,6 | 1,8 | 100 | 84 |
| ABL1(M351T)-phosphorylated | 65 | 1,1 | 65 | 31 | 88 | 21 | 15 | 0,2 | 77 | 69 |
| ABL1(Q252H)-nonphosphorylated | 85 | 1,2 | 100 | 17 | 98 | 13 | 2,6 | 0,65 | 94 | 64 |
| ABL1(Q252H)-phosphorylated | 74 | 1,2 | 100 | 37 | 100 | 21 | 10 | 2,4 | 100 | 100 |
| ABL1(T315I)-nonphosphorylated | 56 | 1,7 | 87 | 35 | 85 | 69 | 3,6 | 0,05 | 91 | 69 |
| ABL1(T315I)-phosphorylated | 51 | 0,8 | 100 | 29 | 85 | 70 | 10 | 0 | 82 | 61 |
| ABL1(Y253F)-phosphorylated | 61 | 0,4 | 92 | 30 | 97 | 15 | 7,8 | 1,2 | 99 | 75 |
| ABL1-nonphosphorylated | 58 | 2,2 | 85 | 26 | 89 | 21 | 3,9 | 1,4 | 95 | 70 |
| ABL1-phosphorylated | 56 | 0,3 | 97 | 41 | 79 | 14 | 5,8 | 1,6 | 100 | 76 |
| ABL2 | 92 | 30 | 100 | 84 | 95 | 60 | 47 | 67 | 100 | 82 |
| ACVR1 | 100 | 40 | 100 | 14 | 100 | 7,8 | 6,2 | 71 | 87 | 100 |
| ACVR1B | 100 | 100 | 100 | 100 | 100 | 89 | 100 | 100 | 100 | 100 |
| ACVR2A | 100 | 55 | 100 | 46 | 100 | 16 | 58 | 98 | 71 | 84 |
| ACVR2B | 94 | 76 | 76 | 32 | 96 | 1,8 | 43 | 100 | 73 | 88 |
| ACVRL1 | 97 | 100 | 100 | 100 | 100 | 12 | 91 | 100 | 93 | 92 |
| ADCK3 | 100 | 49 | 71 | 9,8 | 85 | 62 | 75 | 87 | 86 | 88 |
| ADCK4 | 98 | 57 | 96 | 3,4 | 100 | 24 | 96 | 95 | 100 | 88 |
| AKT1 | 77 | 62 | 73 | 75 | 78 | 91 | 91 | 65 | 51 | 64 |
| AKT2 | 87 | 100 | 100 | 100 | 100 | 98 | 71 | 38 | 91 | 100 |
| AKT3 | 100 | 100 | 86 | 100 | 83 | 97 | 100 | 100 | 76 | 98 |
| ALK | 99 | 45 | 46 | 19 | 60 | 55 | 100 | 0,15 | 87 | 82 |
| AMPK-alpha1 | 100 | 12 | 100 | 96 | 100 | 90 | 57 | 0,75 | 92 | 100 |
| AMPK-alpha2 | 100 | 17 | 100 | 86 | 100 | 87 | 50 | 0,75 | 81 | 98 |
| ANKK1 | 98 | 1,6 | 98 | 91 | 100 | 85 | 46 | 1,5 | 94 | 90 |
| ARK5 | 74 | 1,4 | 98 | 53 | 100 | 98 | 59 | 0,95 | 87 | 80 |
| ASK1 | 92 | 100 | 97 | 94 | 87 | 76 | 85 | 100 | 69 | 82 |
| ASK2 | 77 | 50 | 100 | 100 | 100 | 96 | 100 | 51 | 95 | 81 |
| AURKA | 77 | 0,05 | 100 | 4 | 95 | 68 | 7,7 | 14 | 100 | 87 |
| AURKB | 68 | 1,4 | 100 | 18 | 100 | 55 | 18 | 1,9 | 77 | 69 |

| | | | | | | | | | | |
|---------------|-----|------|-----|-----|-----|-----|------|------|-----|-----|
| AURKC | 100 | 1,2 | 97 | 11 | 93 | 51 | 5,6 | 1,9 | 100 | 88 |
| AXL | 100 | 2,4 | 84 | 19 | 84 | 78 | 54 | 0 | 100 | 100 |
| BIKE | 100 | 0,05 | 83 | 41 | 65 | 67 | 0,15 | 0 | 100 | 80 |
| BLK | 93 | 3,6 | 78 | 54 | 65 | 41 | 69 | 0,45 | 76 | 73 |
| BMPR1A | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| BMPR1B | 31 | 1,4 | 36 | 11 | 41 | 8 | 0,25 | 24 | 37 | 38 |
| BMPR2 | 100 | 16 | 100 | 91 | 100 | 100 | 13 | 2 | 100 | 100 |
| BMX | 100 | 72 | 77 | 81 | 72 | 75 | 64 | 74 | 77 | 92 |
| BRAF | 100 | 29 | 100 | 36 | 100 | 16 | 64 | 100 | 100 | 100 |
| BRAF(V600E) | 75 | 25 | 100 | 27 | 100 | 11 | 57 | 93 | 100 | 100 |
| BRK | 91 | 78 | 82 | 57 | 98 | 53 | 64 | 68 | 93 | 86 |
| BRSK1 | 100 | 64 | 69 | 83 | 96 | 8,5 | 100 | 26 | 100 | 100 |
| BRSK2 | 98 | 59 | 58 | 63 | 100 | 11 | 63 | 12 | 66 | 88 |
| BTK | 100 | 47 | 100 | 100 | 100 | 90 | 60 | 39 | 100 | 100 |
| BUB1 | 100 | 0 | 100 | 41 | 100 | 19 | 39 | 12 | 100 | 100 |
| CAMK1 | 100 | 15 | 100 | 18 | 100 | 89 | 41 | 11 | 91 | 100 |
| CAMK1D | 99 | 9,2 | 98 | 59 | 87 | 91 | 16 | 5,2 | 84 | 100 |
| CAMK1G | 100 | 47 | 100 | 42 | 100 | 92 | 59 | 7 | 100 | 100 |
| CAMK2A | 88 | 16 | 94 | 74 | 100 | 96 | 49 | 2,8 | 92 | 100 |
| CAMK2B | 100 | 40 | 97 | 75 | 100 | 98 | 66 | 16 | 100 | 100 |
| CAMK2D | 96 | 19 | 100 | 71 | 100 | 100 | 76 | 12 | 100 | 100 |
| CAMK2G | 93 | 57 | 100 | 91 | 100 | 83 | 71 | 11 | 100 | 100 |
| CAMK4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 2 | 99 | 89 |
| CAMKK1 | 95 | 81 | 100 | 60 | 100 | 100 | 38 | 11 | 100 | 100 |
| CAMKK2 | 96 | 76 | 100 | 47 | 100 | 98 | 13 | 22 | 100 | 100 |
| CASK | 83 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 93 |
| CDC2L1 | 98 | 99 | 100 | 100 | 100 | 84 | 74 | 91 | 100 | 87 |
| CDC2L2 | 89 | 81 | 96 | 100 | 85 | 77 | 77 | 79 | 100 | 89 |
| CDC2L5 | 88 | 99 | 93 | 61 | 99 | 99 | 65 | 90 | 94 | 99 |
| CDK11 | 68 | 100 | 98 | 100 | 100 | 64 | 100 | 100 | 100 | 100 |
| CDK2 | 100 | 100 | 98 | 58 | 100 | 100 | 100 | 100 | 95 | 100 |
| CDK3 | 72 | 100 | 100 | 84 | 98 | 93 | 96 | 82 | 87 | 73 |
| CDK4-cyclinD1 | 92 | 97 | 100 | 43 | 99 | 85 | 63 | 48 | 97 | 98 |
| CDK4-cyclinD3 | 43 | 76 | 84 | 36 | 94 | 95 | 33 | 46 | 85 | 70 |
| CDK5 | 98 | 100 | 100 | 88 | 100 | 95 | 100 | 86 | 100 | 94 |
| CDK7 | 86 | 13 | 100 | 71 | 100 | 88 | 26 | 0,2 | 100 | 86 |
| CDK8 | 70 | 100 | 85 | 68 | 100 | 80 | 99 | 66 | 98 | 97 |
| CDK9 | 100 | 99 | 100 | 83 | 100 | 84 | 83 | 100 | 100 | 86 |
| CDKL1 | 94 | 18 | 100 | 83 | 100 | 100 | 28 | 75 | 100 | 82 |
| CDKL2 | 83 | 62 | 97 | 9,5 | 100 | 87 | 2,6 | 20 | 95 | 100 |
| CDKL3 | 97 | 100 | 91 | 100 | 100 | 82 | 6,2 | 83 | 100 | 84 |
| CDKL5 | 69 | 100 | 100 | 27 | 98 | 100 | 5,8 | 97 | 80 | 82 |
| CHEK1 | 100 | 23 | 100 | 100 | 100 | 100 | 100 | 2,7 | 100 | 100 |
| CHEK2 | 75 | 1,4 | 73 | 59 | 91 | 79 | 46 | 0,05 | 76 | 82 |
| CIT | 100 | 0,1 | 64 | 70 | 100 | 34 | 20 | 22 | 94 | 92 |

| | | | | | | | | | | |
|---------------------------|-----|------|-----|------|-----|-----|------|------|-----|-----|
| CLK1 | 96 | 2,6 | 86 | 5,4 | 100 | 93 | 0,75 | 0,65 | 67 | 100 |
| CLK2 | 90 | 1,4 | 85 | 9,2 | 100 | 93 | 1 | 0,2 | 98 | 100 |
| CLK3 | 100 | 18 | 100 | 14 | 100 | 88 | 30 | 82 | 76 | 100 |
| CLK4 | 100 | 2 | 51 | 3,6 | 94 | 81 | 0,75 | 0,6 | 74 | 91 |
| CSF1R | 100 | 2,1 | 100 | 63 | 100 | 100 | 24 | 0 | 100 | 77 |
| CSF1R-autoinhibited | 69 | 55 | 93 | 79 | 85 | 94 | 20 | 0,35 | 83 | 77 |
| CSK | 93 | 61 | 100 | 75 | 100 | 79 | 85 | 69 | 89 | 86 |
| CSNK1A1 | 79 | 1,8 | 100 | 5 | 23 | 52 | 58 | 0,35 | 91 | 89 |
| CSNK1A1L | 97 | 17 | 100 | 3,8 | 55 | 69 | 100 | 5,7 | 100 | 100 |
| CSNK1D | 100 | 10 | 76 | 0,55 | 51 | 30 | 100 | 0,05 | 84 | 90 |
| CSNK1E | 98 | 19 | 90 | 0 | 48 | 5,9 | 100 | 0,1 | 74 | 97 |
| CSNK1G1 | 80 | 46 | 74 | 5,4 | 81 | 60 | 73 | 7,1 | 70 | 78 |
| CSNK1G2 | 100 | 96 | 100 | 0,6 | 100 | 92 | 100 | 1,6 | 100 | 100 |
| CSNK1G3 | 100 | 89 | 100 | 1,4 | 86 | 74 | 85 | 2,2 | 64 | 94 |
| CSNK2A1 | 100 | 0,9 | 100 | 62 | 100 | 100 | 34 | 16 | 100 | 100 |
| CSNK2A2 | 71 | 0,05 | 64 | 7,4 | 51 | 81 | 2,9 | 2,8 | 77 | 69 |
| CTK | 89 | 24 | 89 | 74 | 78 | 69 | 92 | 6,1 | 100 | 82 |
| DAPK1 | 100 | 6,1 | 68 | 73 | 91 | 93 | 13 | 1,2 | 100 | 100 |
| DAPK2 | 100 | 8,8 | 75 | 76 | 100 | 84 | 19 | 0,95 | 84 | 100 |
| DAPK3 | 85 | 1,4 | 67 | 55 | 89 | 85 | 4,4 | 0,7 | 87 | 82 |
| DCAMKL1 | 62 | 20 | 74 | 72 | 61 | 71 | 40 | 3,9 | 76 | 64 |
| DCAMKL2 | 100 | 98 | 100 | 100 | 100 | 95 | 86 | 40 | 100 | 98 |
| DCAMKL3 | 92 | 19 | 68 | 100 | 100 | 84 | 100 | 0,25 | 95 | 83 |
| DDR1 | 99 | 100 | 100 | 54 | 100 | 24 | 42 | 43 | 100 | 73 |
| DDR2 | 90 | 24 | 100 | 69 | 92 | 54 | 44 | 15 | 100 | 87 |
| DLK | 95 | 68 | 100 | 94 | 100 | 100 | 77 | 4,2 | 97 | 96 |
| DMPK | 100 | 34 | 90 | 88 | 90 | 83 | 74 | 100 | 80 | 100 |
| DMPK2 | 91 | 73 | 76 | 14 | 96 | 36 | 85 | 73 | 95 | 98 |
| DRAK1 | 99 | 0 | 40 | 44 | 94 | 74 | 0,1 | 0 | 99 | 93 |
| DRAK2 | 92 | 0,15 | 81 | 80 | 100 | 80 | 2 | 1 | 91 | 83 |
| DYRK1A | 64 | 5,7 | 48 | 8,2 | 91 | 90 | 3,2 | 3,4 | 81 | 79 |
| DYRK1B | 100 | 49 | 64 | 12 | 100 | 82 | 25 | 24 | 100 | 96 |
| DYRK2 | 100 | 3 | 100 | 12 | 100 | 100 | 37 | 17 | 100 | 100 |
| EGFR | 91 | 79 | 7,1 | 96 | 100 | 33 | 98 | 75 | 89 | 85 |
| EGFR(E746-A750del) | 93 | 38 | 4 | 78 | 100 | 13 | 83 | 74 | 74 | 100 |
| EGFR(G719C) | 93 | 15 | 1,8 | 84 | 77 | 33 | 56 | 46 | 96 | 91 |
| EGFR(G719S) | 100 | 57 | 1,4 | 95 | 100 | 24 | 86 | 62 | 97 | 100 |
| EGFR(L747-E749del, A750P) | 100 | 30 | 0,9 | 75 | 96 | 12 | 84 | 79 | 88 | 83 |
| EGFR(L747-S752del, P753S) | 100 | 43 | 3,7 | 92 | 84 | 12 | 80 | 94 | 82 | 100 |
| EGFR(L747-T751del,Sins) | 100 | 52 | 2,2 | 81 | 100 | 12 | 72 | 67 | 89 | 100 |
| EGFR(L858R) | 100 | 68 | 5,6 | 100 | 100 | 34 | 100 | 73 | 89 | 87 |
| EGFR(L858R,T790M) | 83 | 23 | 71 | 84 | 66 | 79 | 71 | 48 | 90 | 83 |
| EGFR(L861Q) | 100 | 63 | 4,7 | 89 | 100 | 32 | 78 | 68 | 99 | 100 |
| EGFR(S752-I759del) | 100 | 27 | 4,4 | 92 | 82 | 16 | 78 | 75 | 100 | 100 |
| EGFR(T790M) | 62 | 5,1 | 18 | 45 | 44 | 29 | 37 | 17 | 61 | 62 |

| | | | | | | | | | | |
|--------------------|-----|------|-----|------|-----|-----|-----|------|-----|-----|
| EIF2AK1 | 100 | 63 | 100 | 79 | 100 | 77 | 100 | 91 | 100 | 100 |
| EPHA1 | 95 | 19 | 83 | 7,3 | 100 | 19 | 25 | 62 | 78 | 68 |
| EPHA2 | 100 | 70 | 87 | 36 | 91 | 53 | 61 | 62 | 78 | 71 |
| EPHA3 | 55 | 15 | 59 | 40 | 69 | 7,2 | 45 | 22 | 58 | 60 |
| EPHA4 | 100 | 73 | 89 | 64 | 100 | 49 | 90 | 69 | 98 | 79 |
| EPHA5 | 89 | 93 | 100 | 81 | 100 | 55 | 92 | 81 | 90 | 87 |
| EPHA6 | 100 | 50 | 87 | 51 | 100 | 22 | 100 | 47 | 100 | 100 |
| EPHA7 | 100 | 70 | 100 | 89 | 100 | 95 | 92 | 77 | 100 | 98 |
| EPHA8 | 92 | 98 | 100 | 100 | 100 | 60 | 88 | 96 | 100 | 91 |
| EPHB1 | 96 | 54 | 100 | 25 | 100 | 66 | 67 | 52 | 100 | 93 |
| EPHB2 | 100 | 93 | 100 | 42 | 100 | 68 | 81 | 62 | 100 | 91 |
| EPHB3 | 95 | 100 | 100 | 53 | 100 | 72 | 100 | 91 | 99 | 99 |
| EPHB4 | 83 | 72 | 100 | 32 | 100 | 25 | 85 | 56 | 100 | 92 |
| EPHB6 | 80 | 1,8 | 84 | 0,8 | 100 | 37 | 2,7 | 16 | 100 | 70 |
| ERBB2 | 83 | 75 | 8,4 | 88 | 100 | 60 | 86 | 85 | 100 | 98 |
| ERBB3 | 93 | 78 | 87 | 79 | 100 | 15 | 92 | 80 | 100 | 80 |
| ERBB4 | 100 | 100 | 100 | 100 | 100 | 84 | 100 | 100 | 100 | 100 |
| ERK1 | 100 | 100 | 100 | 100 | 100 | 93 | 89 | 100 | 100 | 100 |
| ERK2 | 84 | 100 | 100 | 100 | 100 | 89 | 86 | 91 | 87 | 85 |
| ERK3 | 88 | 16 | 73 | 11 | 100 | 95 | 3,6 | 75 | 98 | 100 |
| ERK4 | 100 | 95 | 100 | 100 | 100 | 83 | 28 | 100 | 100 | 87 |
| ERK5 | 89 | 89 | 100 | 100 | 98 | 96 | 97 | 81 | 100 | 83 |
| ERK8 | 90 | 34 | 100 | 0,95 | 91 | 92 | 3,7 | 80 | 70 | 98 |
| ERN1 | 74 | 61 | 100 | 66 | 100 | 77 | 71 | 6,6 | 80 | 76 |
| FAK | 99 | 44 | 100 | 59 | 98 | 100 | 76 | 11 | 86 | 92 |
| FER | 100 | 100 | 100 | 99 | 100 | 100 | 95 | 38 | 98 | 100 |
| FES | 70 | 63 | 99 | 44 | 100 | 79 | 39 | 53 | 84 | 95 |
| FGFR1 | 100 | 35 | 94 | 7,4 | 100 | 69 | 61 | 8,2 | 87 | 100 |
| FGFR2 | 80 | 31 | 85 | 2,4 | 72 | 74 | 54 | 14 | 85 | 80 |
| FGFR3 | 89 | 44 | 70 | 1,4 | 88 | 76 | 69 | 7,3 | 84 | 84 |
| FGFR3(G697C) | 90 | 72 | 79 | 1,6 | 100 | 87 | 71 | 8,8 | 78 | 73 |
| FGFR4 | 96 | 88 | 84 | 9,1 | 100 | 76 | 82 | 60 | 95 | 91 |
| FGR | 86 | 19 | 92 | 51 | 87 | 49 | 86 | 8,8 | 100 | 93 |
| FLT1 | 92 | 17 | 100 | 22 | 89 | 77 | 32 | 0 | 88 | 80 |
| FLT3 | 78 | 0,1 | 64 | 4 | 19 | 90 | 14 | 0 | 98 | 99 |
| FLT3(D835H) | 79 | 0,05 | 45 | 5,4 | 2,9 | 72 | 2,7 | 0 | 91 | 71 |
| FLT3(D835Y) | 84 | 0,1 | 52 | 8,6 | 5,4 | 68 | 4,8 | 0 | 100 | 93 |
| FLT3(ITD) | 98 | 0 | 72 | 10 | 4,2 | 85 | 14 | 0 | 96 | 96 |
| FLT3(K663Q) | 100 | 0,35 | 64 | 8,4 | 20 | 82 | 13 | 0,05 | 82 | 90 |
| FLT3(N841I) | 98 | 0 | 75 | 16 | 5 | 92 | 2,2 | 0 | 100 | 95 |
| FLT3(R834Q) | 80 | 2,5 | 100 | 90 | 48 | 86 | 26 | 1,5 | 88 | 87 |
| FLT3-autoinhibited | 54 | 1,4 | 94 | 100 | 66 | 83 | 32 | 0 | 70 | 59 |
| FLT4 | 88 | 0,45 | 100 | 16 | 94 | 96 | 26 | 0,1 | 100 | 83 |
| FRK | 99 | 78 | 95 | 47 | 93 | 34 | 58 | 20 | 86 | 83 |
| FYN | 100 | 8,5 | 95 | 52 | 71 | 43 | 53 | 24 | 100 | 88 |

| | | | | | | | | | | |
|------------------------------|-----|------|-----|------|-----|-----|------|------|-----|-----|
| GAK | 97 | 0,7 | 34 | 2,8 | 35 | 1,5 | 10 | 0,45 | 88 | 100 |
| GCN2(Kin.Dom.2,S808G) | 85 | 0,2 | 100 | 77 | 100 | 82 | 31 | 0,55 | 82 | 100 |
| GRK1 | 59 | 46 | 81 | 70 | 68 | 71 | 72 | 7,3 | 75 | 63 |
| GRK4 | 100 | 0,6 | 100 | 77 | 100 | 100 | 2,2 | 0,95 | 100 | 100 |
| GRK7 | 76 | 39 | 100 | 100 | 85 | 94 | 42 | 2,5 | 100 | 99 |
| GSK3A | 91 | 100 | 100 | 2 | 100 | 100 | 29 | 95 | 100 | 100 |
| GSK3B | 52 | 54 | 100 | 9,6 | 100 | 65 | 23 | 56 | 67 | 61 |
| HASPIN | 88 | 15 | 38 | 41 | 81 | 84 | 34 | 1,8 | 100 | 100 |
| HCK | 100 | 27 | 100 | 85 | 96 | 43 | 100 | 2,9 | 100 | 100 |
| HIPK1 | 68 | 1 | 62 | 28 | 100 | 94 | 3,4 | 0,45 | 98 | 73 |
| HIPK2 | 100 | 1,5 | 86 | 40 | 100 | 88 | 5,4 | 0,25 | 100 | 100 |
| HIPK3 | 66 | 0,5 | 100 | 37 | 100 | 65 | 8,8 | 0,2 | 100 | 100 |
| HIPK4 | 98 | 6,6 | 61 | 4,6 | 100 | 85 | 3,2 | 2,5 | 98 | 100 |
| HPK1 | 96 | 9 | 96 | 28 | 96 | 90 | 51 | 2,3 | 100 | 88 |
| HUNK | 90 | 100 | 100 | 84 | 83 | 55 | 99 | 1,6 | 71 | 72 |
| ICK | 100 | 100 | 100 | 61 | 100 | 100 | 35 | 44 | 100 | 100 |
| IGF1R | 100 | 100 | 100 | 100 | 100 | 93 | 100 | 64 | 100 | 100 |
| IKK-alpha | 57 | 0,3 | 88 | 67 | 100 | 73 | 6,2 | 46 | 92 | 72 |
| IKK-beta | 92 | 0 | 100 | 83 | 100 | 100 | 7,4 | 91 | 100 | 79 |
| IKK-epsilon | 54 | 4 | 89 | 61 | 57 | 80 | 29 | 0 | 93 | 75 |
| INSR | 58 | 35 | 62 | 40 | 62 | 64 | 54 | 1,8 | 78 | 66 |
| INSRR | 79 | 100 | 100 | 100 | 100 | 97 | 100 | 4,8 | 84 | 72 |
| IRAK1 | 100 | 0,3 | 100 | 1,2 | 100 | 100 | 3,4 | 0,05 | 100 | 100 |
| IRAK3 | 97 | 4,2 | 28 | 14 | 100 | 47 | 17 | 18 | 100 | 100 |
| IRAK4 | 100 | 3,6 | 100 | 100 | 100 | 100 | 100 | 2,4 | 100 | 100 |
| ITK | 87 | 100 | 100 | 100 | 92 | 94 | 87 | 3,8 | 100 | 82 |
| JAK1(JH1domain-catalytic) | 85 | 22 | 100 | 72 | 100 | 95 | 100 | 63 | 90 | 100 |
| JAK1(JH2domain-pseudokinase) | 69 | 0,25 | 100 | 99 | 100 | 100 | 0,3 | 0 | 98 | 84 |
| JAK2(JH1domain-catalytic) | 69 | 0,1 | 69 | 21 | 82 | 61 | 0,7 | 0,05 | 75 | 72 |
| JAK3(JH1domain-catalytic) | 96 | 0 | 83 | 30 | 100 | 76 | 0,1 | 0 | 100 | 85 |
| JNK1 | 76 | 0,35 | 69 | 3,4 | 81 | 7,1 | 0,85 | 68 | 96 | 93 |
| JNK2 | 65 | 23 | 100 | 11 | 100 | 1,2 | 7,4 | 39 | 97 | 86 |
| JNK3 | 58 | 1,1 | 91 | 3,4 | 91 | 0,9 | 0,7 | 47 | 78 | 69 |
| KIT | 100 | 0,7 | 16 | 0,15 | 72 | 79 | 1,2 | 0 | 91 | 89 |
| KIT(A829P) | 75 | 0 | 55 | 57 | 40 | 63 | 11 | 0,35 | 88 | 84 |
| KIT(D816H) | 77 | 2,2 | 100 | 78 | 85 | 74 | 15 | 7 | 87 | 75 |
| KIT(D816V) | 86 | 0,15 | 62 | 12 | 57 | 64 | 3,4 | 2,4 | 70 | 75 |
| KIT(L576P) | 100 | 0 | 20 | 0,9 | 69 | 69 | 1,6 | 0 | 100 | 100 |
| KIT(V559D) | 90 | 0,05 | 9,7 | 0,1 | 54 | 70 | 0,35 | 0 | 88 | 86 |
| KIT(V559D,T670I) | 100 | 3,1 | 80 | 4,1 | 100 | 100 | 19 | 0 | 100 | 89 |
| KIT(V559D,V654A) | 100 | 23 | 17 | 22 | 100 | 82 | 24 | 1 | 92 | 98 |
| KIT-autoinhibited | 85 | 71 | 100 | 80 | 100 | 100 | 15 | 0 | 100 | 77 |
| LATS1 | 100 | 10 | 96 | 100 | 100 | 97 | 100 | 4 | 100 | 100 |
| LATS2 | 63 | 4,2 | 75 | 77 | 92 | 68 | 61 | 0,35 | 73 | 66 |
| LCK | 100 | 23 | 100 | 85 | 100 | 21 | 33 | 2,4 | 89 | 100 |

| | | | | | | | | | | |
|---------------|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| LIMK1 | 100 | 71 | 97 | 20 | 100 | 95 | 55 | 100 | 86 | 100 |
| LIMK2 | 91 | 62 | 73 | 5,7 | 87 | 100 | 39 | 76 | 100 | 80 |
| LKB1 | 70 | 1,5 | 73 | 78 | 85 | 63 | 12 | 0,9 | 66 | 86 |
| LOK | 100 | 8,3 | 85 | 87 | 100 | 23 | 74 | 3,8 | 96 | 79 |
| LRRK2 | 95 | 2,6 | 98 | 74 | 100 | 100 | 65 | 2,4 | 100 | 100 |
| LRRK2(G2019S) | 64 | 0,75 | 80 | 85 | 100 | 92 | 75 | 1,1 | 82 | 83 |
| LTK | 100 | 32 | 35 | 67 | 100 | 83 | 100 | 16 | 99 | 100 |
| LYN | 100 | 36 | 92 | 38 | 100 | 29 | 86 | 13 | 100 | 95 |
| LZK | 100 | 52 | 100 | 100 | 100 | 100 | 83 | 11 | 100 | 97 |
| MAK | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 95 |
| MAP3K1 | 37 | 26 | 100 | 43 | 93 | 42 | 42 | 35 | 48 | 56 |
| MAP3K15 | 100 | 21 | 100 | 78 | 100 | 100 | 79 | 9,8 | 100 | 100 |
| MAP3K2 | 59 | 0,9 | 58 | 60 | 71 | 63 | 16 | 0,1 | 64 | 63 |
| MAP3K3 | 75 | 2,5 | 83 | 83 | 100 | 61 | 52 | 0,1 | 100 | 78 |
| MAP3K4 | 97 | 36 | 92 | 54 | 100 | 87 | 46 | 59 | 84 | 84 |
| MAP4K2 | 61 | 0,05 | 38 | 23 | 74 | 32 | 1,1 | 0,3 | 61 | 64 |
| MAP4K3 | 95 | 20 | 100 | 36 | 100 | 61 | 34 | 0,4 | 100 | 100 |
| MAP4K4 | 87 | 6,2 | 93 | 30 | 100 | 38 | 4,2 | 0,85 | 94 | 87 |
| MAP4K5 | 95 | 89 | 99 | 52 | 100 | 54 | 54 | 0,6 | 91 | 94 |
| MAPKAPK2 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 94 | 100 |
| MAPKAPK5 | 81 | 48 | 82 | 93 | 88 | 96 | 80 | 59 | 96 | 94 |
| MARK1 | 100 | 37 | 69 | 69 | 100 | 65 | 46 | 11 | 73 | 100 |
| MARK2 | 55 | 5,6 | 65 | 37 | 69 | 56 | 24 | 5,1 | 62 | 45 |
| MARK3 | 100 | 14 | 100 | 100 | 100 | 100 | 69 | 5,8 | 100 | 100 |
| MARK4 | 100 | 15 | 82 | 71 | 100 | 76 | 47 | 7,8 | 76 | 96 |
| MAST1 | 100 | 19 | 96 | 80 | 100 | 79 | 17 | 3,2 | 100 | 100 |
| MEK1 | 92 | 4,8 | 70 | 98 | 89 | 68 | 64 | 0,25 | 98 | 100 |
| MEK2 | 94 | 4 | 84 | 94 | 100 | 70 | 56 | 0,1 | 100 | 100 |
| MEK3 | 81 | 1 | 67 | 31 | 100 | 41 | 12 | 38 | 89 | 99 |
| MEK4 | 50 | 1,9 | 67 | 29 | 56 | 19 | 3,6 | 11 | 66 | 64 |
| MEK5 | 100 | 0,1 | 36 | 11 | 36 | 3,8 | 4,6 | 0,15 | 100 | 100 |
| MEK6 | 76 | 28 | 77 | 41 | 80 | 55 | 51 | 42 | 73 | 94 |
| MELK | 100 | 16 | 86 | 56 | 79 | 52 | 20 | 1,2 | 76 | 63 |
| MERTK | 100 | 1,6 | 91 | 2,2 | 78 | 76 | 46 | 0 | 89 | 84 |
| MET | 100 | 68 | 97 | 100 | 100 | 77 | 73 | 58 | 86 | 92 |
| MET(M1250T) | 100 | 27 | 100 | 100 | 100 | 100 | 100 | 24 | 100 | 100 |
| MET(Y1235D) | 100 | 65 | 100 | 100 | 100 | 75 | 47 | 49 | 74 | 100 |
| MINK | 100 | 1,8 | 100 | 54 | 100 | 27 | 17 | 0,65 | 93 | 91 |
| MKK7 | 85 | 71 | 83 | 73 | 72 | 82 | 50 | 78 | 87 | 79 |
| MKNK1 | 99 | 0,55 | 42 | 100 | 100 | 99 | 82 | 44 | 100 | 87 |
| MKNK2 | 100 | 0 | 10 | 51 | 60 | 55 | 5,8 | 56 | 100 | 100 |
| MLCK | 100 | 0 | 86 | 75 | 100 | 80 | 7,5 | 0,15 | 100 | 90 |
| MLK1 | 100 | 0,3 | 100 | 100 | 100 | 92 | 73 | 56 | 97 | 100 |
| MLK2 | 100 | 1,6 | 100 | 69 | 100 | 91 | 48 | 57 | 86 | 87 |
| MLK3 | 100 | 3,1 | 100 | 62 | 100 | 99 | 50 | 15 | 94 | 100 |

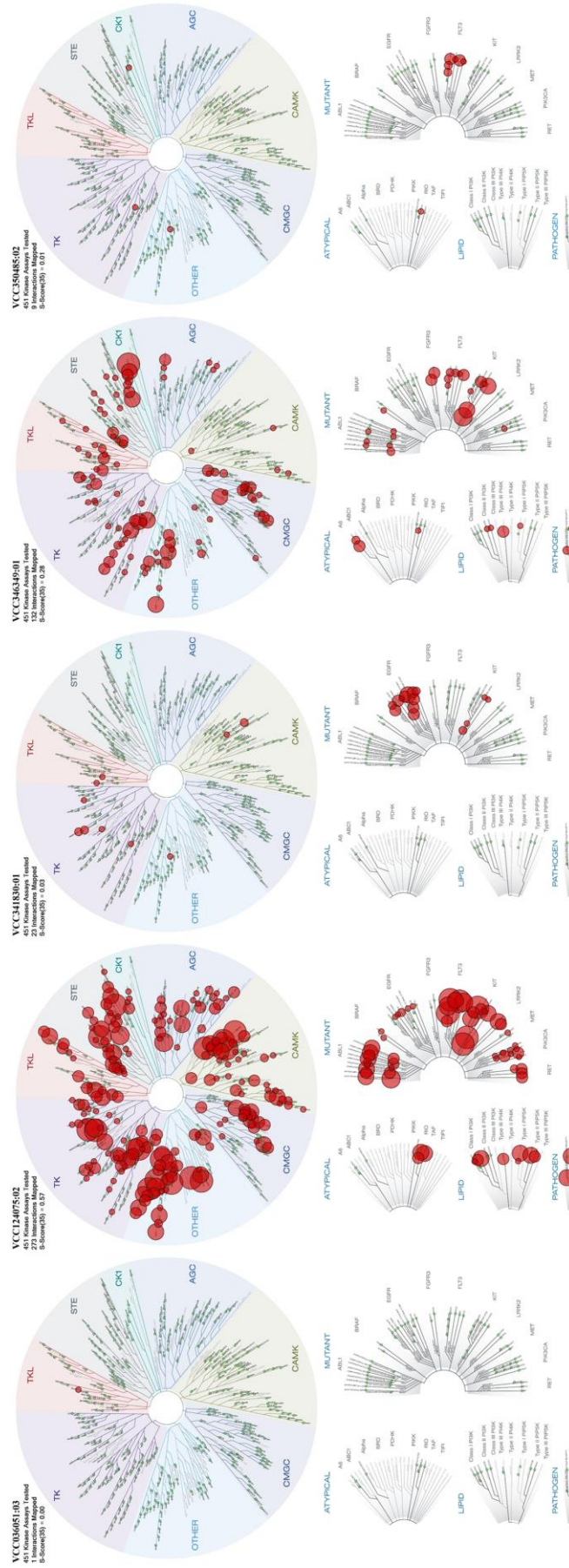
| | | | | | | | | | | |
|-----------------------|-----|------|-----|------|-----|-----|-----|------|-----|-----|
| MRCKA | 100 | 97 | 78 | 4,4 | 100 | 83 | 100 | 100 | 100 | 90 |
| MRCKB | 89 | 78 | 99 | 22 | 100 | 71 | 98 | 87 | 85 | 87 |
| MST1 | 100 | 100 | 100 | 100 | 100 | 91 | 100 | 2,2 | 100 | 100 |
| MST1R | 97 | 15 | 100 | 100 | 100 | 91 | 100 | 100 | 90 | 95 |
| MST2 | 78 | 3,2 | 100 | 100 | 100 | 69 | 100 | 0 | 82 | 73 |
| MST3 | 95 | 57 | 99 | 88 | 89 | 88 | 80 | 0,8 | 90 | 98 |
| MST4 | 67 | 17 | 100 | 89 | 100 | 91 | 82 | 2,4 | 100 | 76 |
| MTOR | 100 | 65 | 100 | 71 | 100 | 100 | 98 | 91 | 100 | 100 |
| MUSK | 81 | 99 | 100 | 100 | 100 | 98 | 33 | 4,4 | 89 | 78 |
| MYLK | 85 | 6,8 | 78 | 75 | 74 | 87 | 21 | 0 | 100 | 74 |
| MYLK2 | 100 | 0,75 | 100 | 100 | 100 | 100 | 20 | 5,6 | 100 | 100 |
| MYLK4 | 100 | 0 | 100 | 56 | 100 | 100 | 17 | 0 | 100 | 90 |
| MYO3A | 100 | 70 | 96 | 100 | 100 | 52 | 93 | 50 | 100 | 100 |
| MYO3B | 84 | 100 | 96 | 100 | 100 | 87 | 100 | 56 | 100 | 100 |
| NDR1 | 100 | 4,8 | 100 | 83 | 100 | 100 | 100 | 12 | 100 | 100 |
| NDR2 | 100 | 15 | 100 | 61 | 86 | 78 | 96 | 36 | 100 | 100 |
| NEK1 | 100 | 9,2 | 70 | 46 | 90 | 56 | 30 | 74 | 100 | 100 |
| NEK11 | 100 | 49 | 100 | 95 | 100 | 95 | 39 | 100 | 98 | 100 |
| NEK2 | 86 | 14 | 96 | 55 | 92 | 60 | 50 | 24 | 87 | 97 |
| NEK3 | 100 | 0,15 | 100 | 31 | 100 | 94 | 0,6 | 87 | 100 | 100 |
| NEK4 | 100 | 15 | 100 | 46 | 95 | 91 | 73 | 82 | 100 | 91 |
| NEK5 | 100 | 0,2 | 61 | 0,45 | 89 | 83 | 0,5 | 87 | 100 | 92 |
| NEK6 | 88 | 1,4 | 100 | 0,25 | 97 | 100 | 0,2 | 91 | 93 | 94 |
| NEK7 | 88 | 43 | 100 | 55 | 100 | 85 | 52 | 83 | 100 | 100 |
| NEK9 | 97 | 15 | 100 | 1,6 | 100 | 100 | 1 | 100 | 100 | 100 |
| NIM1 | 100 | 3,6 | 100 | 100 | 100 | 100 | 100 | 45 | 100 | 100 |
| NLK | 93 | 86 | 100 | 31 | 100 | 30 | 53 | 87 | 99 | 96 |
| OSR1 | 81 | 28 | 84 | 61 | 82 | 89 | 98 | 10 | 96 | 100 |
| p38-alpha | 100 | 100 | 99 | 90 | 100 | 66 | 100 | 86 | 96 | 88 |
| p38-beta | 100 | 100 | 100 | 83 | 100 | 90 | 100 | 100 | 100 | 100 |
| p38-delta | 81 | 100 | 100 | 100 | 90 | 98 | 100 | 100 | 85 | 83 |
| p38-gamma | 73 | 42 | 68 | 56 | 60 | 86 | 62 | 38 | 67 | 61 |
| PAK1 | 84 | 5,8 | 100 | 86 | 100 | 100 | 81 | 96 | 100 | 85 |
| PAK2 | 95 | 34 | 97 | 76 | 99 | 100 | 79 | 71 | 100 | 96 |
| PAK3 | 90 | 2,7 | 89 | 65 | 83 | 76 | 29 | 0,45 | 67 | 100 |
| PAK4 | 82 | 3,6 | 100 | 34 | 100 | 90 | 67 | 32 | 89 | 73 |
| PAK6 | 100 | 15 | 89 | 8,4 | 100 | 77 | 77 | 65 | 100 | 100 |
| PAK7 | 68 | 0,35 | 78 | 24 | 89 | 69 | 53 | 18 | 78 | 75 |
| PCK1 | 65 | 95 | 100 | 33 | 100 | 100 | 82 | 14 | 78 | 82 |
| PCK2 | 100 | 82 | 100 | 73 | 99 | 100 | 100 | 69 | 100 | 93 |
| PCK3 | 100 | 100 | 100 | 94 | 100 | 84 | 91 | 36 | 100 | 90 |
| PDGFRA | 100 | 1,2 | 68 | 51 | 88 | 100 | 10 | 0 | 100 | 100 |
| PDGFRB | 100 | 0 | 37 | 0,2 | 55 | 75 | 2,8 | 0 | 89 | 89 |
| PDPK1 | 97 | 28 | 68 | 76 | 62 | 76 | 60 | 23 | 58 | 68 |
| PFCDPK1(P.falciparum) | 80 | 0,35 | 43 | 8,8 | 50 | 40 | 34 | 24 | 83 | 79 |

| | | | | | | | | | | |
|----------------------|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| PFPK5(P.falciparum) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| PFTAIRE2 | 97 | 97 | 100 | 100 | 100 | 90 | 100 | 79 | 100 | 100 |
| PFTK1 | 95 | 98 | 100 | 86 | 93 | 97 | 89 | 38 | 100 | 87 |
| PHKG1 | 100 | 17 | 100 | 73 | 100 | 100 | 67 | 1 | 100 | 100 |
| PHKG2 | 99 | 22 | 90 | 55 | 100 | 100 | 13 | 0,6 | 100 | 100 |
| PIK3C2B | 100 | 40 | 100 | 35 | 64 | 49 | 100 | 100 | 100 | 100 |
| PIK3C2G | 78 | 94 | 95 | 21 | 80 | 44 | 84 | 100 | 97 | 97 |
| PIK3CA | 100 | 30 | 100 | 91 | 89 | 100 | 88 | 100 | 100 | 89 |
| PIK3CA(C420R) | 93 | 30 | 74 | 70 | 69 | 96 | 76 | 100 | 99 | 96 |
| PIK3CA(E542K) | 100 | 32 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| PIK3CA(E545A) | 100 | 30 | 74 | 90 | 61 | 100 | 98 | 100 | 100 | 100 |
| PIK3CA(E545K) | 100 | 21 | 100 | 81 | 100 | 100 | 69 | 100 | 100 | 99 |
| PIK3CA(H1047L) | 96 | 4,3 | 100 | 64 | 100 | 100 | 16 | 92 | 100 | 100 |
| PIK3CA(H1047Y) | 71 | 30 | 69 | 89 | 59 | 87 | 52 | 54 | 73 | 60 |
| PIK3CA(I800L) | 85 | 3,2 | 62 | 14 | 91 | 72 | 22 | 42 | 88 | 98 |
| PIK3CA(M1043I) | 75 | 15 | 62 | 62 | 58 | 88 | 56 | 95 | 98 | 100 |
| PIK3CA(Q546K) | 67 | 21 | 92 | 71 | 74 | 93 | 64 | 87 | 76 | 81 |
| PIK3CB | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| PIK3CD | 95 | 1,4 | 100 | 43 | 65 | 87 | 21 | 90 | 100 | 100 |
| PIK3CG | 91 | 0,6 | 100 | 40 | 92 | 75 | 0,6 | 86 | 100 | 95 |
| PIK4CB | 100 | 2,2 | 36 | 3,2 | 100 | 92 | 52 | 100 | 100 | 100 |
| PIM1 | 99 | 100 | 67 | 100 | 95 | 100 | 100 | 71 | 100 | 96 |
| PIM2 | 61 | 100 | 37 | 69 | 54 | 53 | 56 | 61 | 37 | 37 |
| PIM3 | 85 | 85 | 60 | 91 | 100 | 100 | 65 | 27 | 94 | 82 |
| PIP5K1A | 95 | 0,75 | 100 | 31 | 71 | 73 | 6,2 | 0,15 | 78 | 67 |
| PIP5K1C | 86 | 13 | 100 | 65 | 100 | 90 | 100 | 2,1 | 95 | 100 |
| PIP5K2B | 100 | 0,45 | 97 | 74 | 100 | 100 | 2,6 | 0,15 | 100 | 100 |
| PIP5K2C | 78 | 4,7 | 93 | 48 | 98 | 55 | 50 | 50 | 83 | 85 |
| PKAC-alpha | 78 | 24 | 75 | 83 | 69 | 80 | 76 | 66 | 58 | 71 |
| PKAC-beta | 91 | 25 | 100 | 100 | 91 | 80 | 100 | 84 | 71 | 99 |
| PKMYT1 | 88 | 62 | 90 | 52 | 100 | 58 | 100 | 93 | 79 | 76 |
| PKN1 | 97 | 1,2 | 100 | 100 | 92 | 90 | 61 | 15 | 100 | 100 |
| PKN2 | 100 | 4,7 | 100 | 58 | 100 | 87 | 36 | 14 | 85 | 100 |
| PKNB(M.tuberculosis) | 79 | 0,8 | 100 | 29 | 80 | 100 | 4,8 | 0,2 | 100 | 100 |
| PLK1 | 90 | 100 | 100 | 100 | 100 | 100 | 96 | 100 | 96 | 98 |
| PLK2 | 69 | 1,6 | 77 | 74 | 80 | 92 | 29 | 46 | 74 | 67 |
| PLK3 | 68 | 0,75 | 81 | 74 | 78 | 90 | 16 | 87 | 77 | 79 |
| PLK4 | 69 | 0,35 | 99 | 13 | 87 | 96 | 40 | 3,2 | 80 | 81 |
| PRKCD | 100 | 12 | 74 | 68 | 100 | 82 | 77 | 58 | 79 | 100 |
| PRKCE | 100 | 3,2 | 70 | 92 | 100 | 98 | 66 | 56 | 100 | 100 |
| PRKCH | 100 | 37 | 73 | 59 | 100 | 91 | 100 | 97 | 78 | 96 |
| PRKCI | 100 | 42 | 100 | 87 | 100 | 88 | 100 | 66 | 100 | 90 |
| PRKCQ | 100 | 9,6 | 100 | 100 | 94 | 98 | 100 | 52 | 100 | 100 |
| PRKD1 | 100 | 40 | 100 | 100 | 100 | 100 | 30 | 3,6 | 100 | 100 |
| PRKD2 | 76 | 26 | 100 | 100 | 91 | 87 | 60 | 2,6 | 82 | 100 |

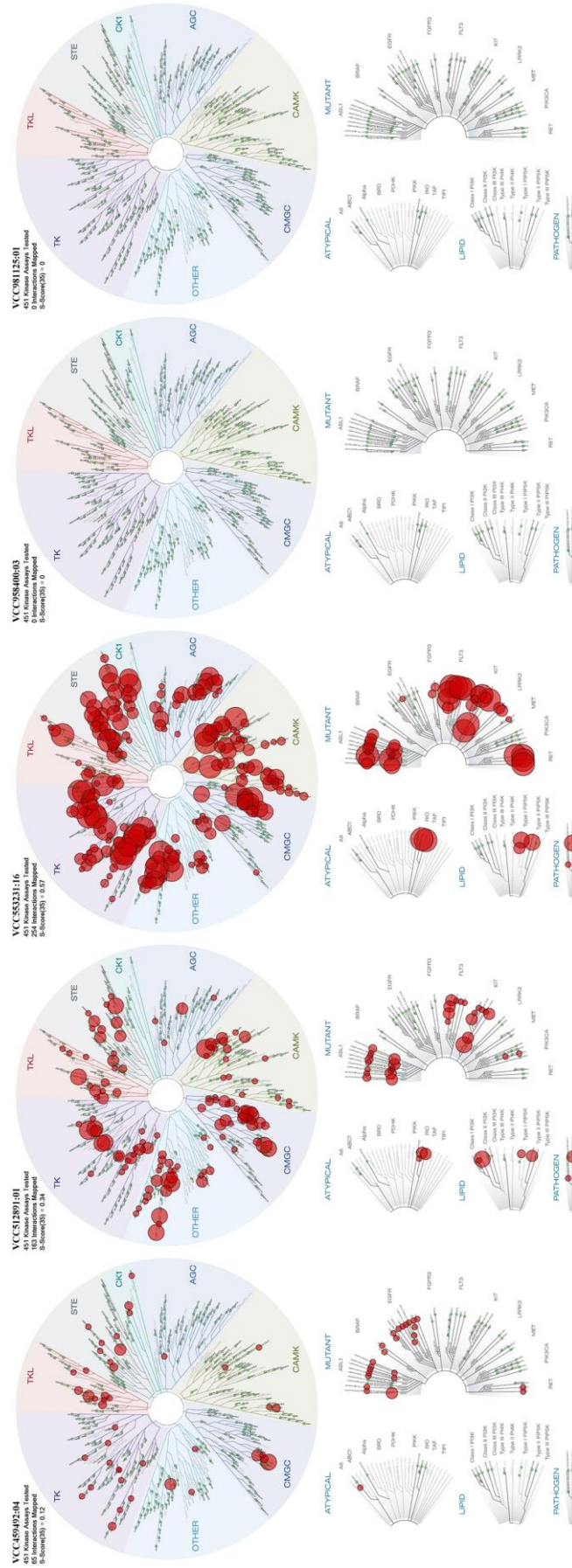
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|-------------------------------|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| PRKD3 | 100 | 33 | 100 | 100 | 100 | 91 | 20 | 3,8 | 100 | 100 |
| PRKG1 | 98 | 53 | 100 | 77 | 84 | 87 | 92 | 42 | 90 | 97 |
| PRKG2 | 84 | 0,95 | 100 | 69 | 100 | 89 | 97 | 57 | 94 | 79 |
| PRKR | 80 | 7,7 | 78 | 27 | 93 | 71 | 22 | 4,6 | 66 | 73 |
| PRKX | 90 | 36 | 100 | 92 | 100 | 100 | 100 | 74 | 100 | 100 |
| PRP4 | 100 | 1,2 | 100 | 100 | 100 | 100 | 6,4 | 0,3 | 100 | 95 |
| PYK2 | 92 | 37 | 100 | 60 | 100 | 93 | 69 | 4,4 | 100 | 100 |
| QSK | 67 | 8,2 | 100 | 100 | 100 | 93 | 77 | 56 | 79 | 66 |
| RAF1 | 100 | 76 | 99 | 38 | 100 | 38 | 69 | 96 | 100 | 92 |
| RET | 90 | 4,6 | 68 | 98 | 96 | 33 | 89 | 0 | 94 | 100 |
| RET(M918T) | 75 | 1,4 | 51 | 80 | 85 | 21 | 67 | 0 | 92 | 80 |
| RET(V804L) | 77 | 4,8 | 95 | 85 | 88 | 90 | 82 | 0 | 91 | 84 |
| RET(V804M) | 95 | 6,3 | 100 | 98 | 99 | 87 | 77 | 0 | 77 | 84 |
| RIOK1 | 80 | 0,65 | 89 | 75 | 37 | 91 | 1,8 | 0 | 83 | 88 |
| RIOK2 | 81 | 2,5 | 92 | 12 | 78 | 79 | 15 | 47 | 97 | 83 |
| RIOK3 | 100 | 0,2 | 54 | 66 | 26 | 81 | 1,3 | 0,05 | 93 | 78 |
| RIPK1 | 81 | 73 | 82 | 11 | 91 | 90 | 8 | 5,6 | 100 | 85 |
| RIPK2 | 87 | 5,3 | 32 | 40 | 93 | 7,6 | 31 | 54 | 100 | 75 |
| RIPK4 | 69 | 0,3 | 80 | 73 | 80 | 85 | 4,8 | 52 | 84 | 83 |
| RIPK5 | 80 | 20 | 73 | 77 | 84 | 82 | 63 | 14 | 91 | 99 |
| ROCK1 | 100 | 7,4 | 100 | 100 | 94 | 100 | 52 | 13 | 100 | 100 |
| ROCK2 | 100 | 1,4 | 90 | 100 | 80 | 100 | 40 | 5 | 100 | 100 |
| ROS1 | 100 | 33 | 100 | 4,6 | 100 | 88 | 47 | 100 | 92 | 100 |
| RPS6KA4(Kin.Dom.1-N-terminal) | 84 | 71 | 99 | 77 | 100 | 89 | 100 | 0,65 | 69 | 91 |
| RPS6KA4(Kin.Dom.2-C-terminal) | 56 | 1,4 | 8,7 | 57 | 58 | 63 | 1,8 | 5,1 | 67 | 64 |
| RPS6KA5(Kin.Dom.1-N-terminal) | 98 | 100 | 82 | 93 | 100 | 94 | 100 | 12 | 73 | 88 |
| RPS6KA5(Kin.Dom.2-C-terminal) | 100 | 7,6 | 100 | 99 | 98 | 90 | 39 | 22 | 100 | 99 |
| RSK1(Kin.Dom.1-N-terminal) | 93 | 88 | 100 | 84 | 100 | 100 | 100 | 0,2 | 100 | 90 |
| RSK1(Kin.Dom.2-C-terminal) | 93 | 59 | 48 | 36 | 79 | 18 | 60 | 38 | 90 | 94 |
| RSK2(Kin.Dom.1-N-terminal) | 64 | 8,8 | 72 | 28 | 63 | 58 | 68 | 0,1 | 69 | 65 |
| RSK2(Kin.Dom.2-C-terminal) | 100 | 79 | 100 | 100 | 100 | 95 | 100 | 100 | 100 | 100 |
| RSK3(Kin.Dom.1-N-terminal) | 78 | 65 | 100 | 74 | 81 | 74 | 100 | 0,5 | 100 | 96 |
| RSK3(Kin.Dom.2-C-terminal) | 100 | 92 | 100 | 99 | 100 | 85 | 90 | 28 | 78 | 92 |
| RSK4(Kin.Dom.1-N-terminal) | 55 | 1,5 | 60 | 46 | 88 | 65 | 70 | 1 | 72 | 61 |
| RSK4(Kin.Dom.2-C-terminal) | 98 | 74 | 83 | 75 | 86 | 17 | 64 | 58 | 87 | 93 |
| S6K1 | 100 | 19 | 100 | 71 | 100 | 97 | 100 | 1 | 100 | 100 |
| SBK1 | 93 | 7,2 | 100 | 95 | 100 | 94 | 69 | 8,2 | 100 | 100 |
| SGK | 76 | 9,6 | 72 | 11 | 56 | 58 | 30 | 0,2 | 92 | 88 |
| SgK110 | 100 | 1,7 | 98 | 100 | 100 | 92 | 26 | 18 | 98 | 100 |
| SGK3 | 72 | 24 | 100 | 21 | 100 | 78 | 69 | 2,8 | 81 | 86 |
| SIK | 88 | 44 | 100 | 92 | 92 | 85 | 48 | 58 | 100 | 91 |
| SIK2 | 86 | 9 | 78 | 33 | 100 | 53 | 32 | 14 | 92 | 88 |
| SLK | 97 | 0,9 | 77 | 55 | 100 | 7,2 | 14 | 0,95 | 91 | 98 |
| SNARK | 87 | 0,95 | 100 | 28 | 100 | 83 | 13 | 1,1 | 86 | 95 |
| SNRK | 78 | 55 | 100 | 84 | 100 | 77 | 76 | 22 | 93 | 76 |

| | | | | | | | | | | |
|------------------------------|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| SRC | 80 | 1,4 | 80 | 58 | 68 | 49 | 38 | 10 | 56 | 69 |
| SRMS | 59 | 34 | 42 | 34 | 38 | 52 | 46 | 33 | 54 | 51 |
| SRPK1 | 100 | 0,45 | 94 | 100 | 100 | 84 | 11 | 0 | 92 | 75 |
| SRPK2 | 100 | 2 | 100 | 89 | 100 | 84 | 9 | 0,8 | 100 | 88 |
| SRPK3 | 100 | 0,35 | 100 | 67 | 100 | 84 | 1,8 | 0 | 100 | 98 |
| STK16 | 98 | 0,75 | 100 | 1,2 | 100 | 79 | 5,4 | 5,8 | 97 | 95 |
| STK33 | 82 | 1,4 | 97 | 29 | 76 | 71 | 40 | 0,4 | 50 | 81 |
| STK35 | 100 | 3,6 | 100 | 31 | 89 | 15 | 68 | 20 | 100 | 100 |
| STK36 | 92 | 80 | 100 | 20 | 100 | 92 | 37 | 76 | 100 | 88 |
| STK39 | 100 | 16 | 99 | 51 | 100 | 79 | 50 | 8,2 | 97 | 87 |
| SYK | 100 | 2,4 | 100 | 100 | 98 | 91 | 16 | 49 | 89 | 100 |
| TAK1 | 78 | 1,2 | 100 | 25 | 97 | 66 | 14 | 0 | 100 | 85 |
| TAOK1 | 48 | 7,4 | 51 | 1,7 | 59 | 36 | 1,6 | 9,4 | 45 | 48 |
| TAOK2 | 100 | 69 | 100 | 37 | 96 | 100 | 44 | 94 | 100 | 100 |
| TAOK3 | 64 | 20 | 54 | 2 | 57 | 71 | 5,7 | 8,2 | 64 | 64 |
| TBK1 | 77 | 3 | 100 | 53 | 88 | 100 | 19 | 4,6 | 90 | 70 |
| TEC | 88 | 100 | 94 | 100 | 100 | 100 | 100 | 100 | 100 | 91 |
| TESK1 | 95 | 42 | 92 | 63 | 100 | 91 | 88 | 86 | 85 | 84 |
| TGFBR1 | 84 | 94 | 100 | 47 | 100 | 43 | 55 | 68 | 94 | 78 |
| TGFBR2 | 100 | 100 | 100 | 3 | 100 | 17 | 37 | 100 | 100 | 84 |
| TIE1 | 89 | 4,4 | 100 | 18 | 100 | 81 | 20 | 51 | 84 | 85 |
| TIE2 | 100 | 84 | 100 | 57 | 100 | 100 | 100 | 100 | 100 | 100 |
| TLK1 | 85 | 51 | 97 | 90 | 82 | 95 | 87 | 3,9 | 90 | 96 |
| TLK2 | 90 | 59 | 97 | 72 | 100 | 85 | 85 | 1,4 | 94 | 96 |
| TNIK | 61 | 0,3 | 51 | 34 | 69 | 15 | 7,6 | 0,9 | 61 | 57 |
| TNK1 | 100 | 10 | 94 | 13 | 100 | 90 | 43 | 37 | 83 | 100 |
| TNK2 | 100 | 54 | 100 | 53 | 100 | 80 | 62 | 57 | 81 | 100 |
| TNNI3K | 100 | 19 | 82 | 57 | 97 | 59 | 31 | 90 | 98 | 95 |
| TRKA | 79 | 25 | 93 | 91 | 74 | 100 | 57 | 0,25 | 100 | 97 |
| TRKB | 100 | 8 | 100 | 75 | 100 | 96 | 28 | 0,6 | 84 | 50 |
| TRKC | 61 | 28 | 93 | 65 | 64 | 83 | 43 | 2,2 | 72 | 63 |
| TRPM6 | 100 | 94 | 100 | 100 | 100 | 100 | 100 | 80 | 100 | 90 |
| TSSK1B | 100 | 33 | 100 | 92 | 100 | 100 | 45 | 9,3 | 100 | 100 |
| TTK | 91 | 11 | 90 | 28 | 96 | 62 | 41 | 2,6 | 94 | 99 |
| TXK | 91 | 42 | 81 | 40 | 100 | 37 | 90 | 75 | 90 | 75 |
| TYK2(JH1domain-catalytic) | 57 | 0 | 63 | 21 | 40 | 36 | 3,6 | 0 | 70 | 60 |
| TYK2(JH2domain-pseudokinase) | 100 | 91 | 34 | 100 | 100 | 100 | 0,6 | 6,6 | 100 | 100 |
| TYRO3 | 89 | 84 | 100 | 32 | 100 | 79 | 81 | 17 | 79 | 100 |
| ULK1 | 46 | 0,1 | 62 | 69 | 44 | 66 | 42 | 0 | 70 | 66 |
| ULK2 | 100 | 1,9 | 100 | 100 | 100 | 100 | 87 | 0,1 | 100 | 100 |
| ULK3 | 48 | 0 | 65 | 33 | 61 | 49 | 2,8 | 0,05 | 62 | 58 |
| VEGFR2 | 73 | 10 | 100 | 35 | 100 | 85 | 34 | 0 | 100 | 80 |
| VRK2 | 89 | 45 | 83 | 51 | 53 | 19 | 52 | 51 | 89 | 87 |
| WEE1 | 99 | 98 | 100 | 53 | 100 | 100 | 64 | 29 | 82 | 87 |
| WEE2 | 95 | 76 | 100 | 100 | 100 | 94 | 81 | 14 | 100 | 100 |

| | | | | | | | | | | |
|-------|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| WNK1 | 100 | 100 | 100 | 94 | 100 | 84 | 92 | 100 | 100 | 100 |
| WNK3 | 100 | 100 | 100 | 100 | 100 | 100 | 9,2 | 100 | 100 | 100 |
| YANK1 | 94 | 19 | 100 | 59 | 100 | 68 | 65 | 77 | 95 | 92 |
| YANK2 | 99 | 100 | 95 | 37 | 100 | 76 | 100 | 100 | 70 | 90 |
| YANK3 | 74 | 100 | 90 | 60 | 100 | 84 | 64 | 100 | 85 | 100 |
| YES | 100 | 36 | 100 | 70 | 100 | 83 | 72 | 2,7 | 88 | 98 |
| YSK1 | 100 | 25 | 88 | 63 | 100 | 91 | 100 | 2,4 | 93 | 98 |
| YSK4 | 100 | 0,05 | 100 | 12 | 100 | 46 | 0,2 | 0,25 | 100 | 100 |
| ZAK | 100 | 61 | 85 | 12 | 100 | 35 | 24 | 100 | 98 | 98 |
| ZAP70 | 100 | 3,1 | 92 | 96 | 97 | 89 | 45 | 59 | 94 | 100 |



Kinome-trees depicting compound selectivities in kinase affinity assay. (Threshold set to 35)



Kinome-trees depicting compound selectivities in kinase affinity assay. (Threshold set to 35)

Cellular Target Profiling results (MS-affinity chromatography)

| Binding/competition Affinity matrix: | Binders | | Competed off, direct from SUTENT matrix | | Competed off, indirect from VCC512891 matrix | | Competed off, direct from VCC512891 matrix | | Competed off, indirect from SUTENT matrix | | Competed off, from direct from VCC124075 matrix | |
|---|------------------|--------|--|---------|---|-----------|---|-----------|--|--------------|--|--|
| | VCC350485 linker | SUTENT | K _d free [μM] | SUTENT | K _d free [μM] | VCC512891 | K _d free [μM] | VCC512891 | K _d free [μM] | VCC124075:02 | K _d free [μM] | |
| A7VJC2 | Q66HA4 | 0,0001 | Q924I2 | 0,1376 | P0C0R5 | 0,0881 | F1LR17 | 0,0314 | P63170 | 0,0053 | | |
| B0BN93 | Q91Z96 | 0,0048 | P54645 | 0,3735 | O88763 | 0,0891 | F1LZD9 | 0,0461 | P97874 | 0,0179 | | |
| B0K020 | P05532 | 0,0101 | O35346 | 0,4073 | Q91XJ1 | 0,0939 | D4A4B2 | 0,0577 | Q9R1U5 | 0,0674 | | |
| B2RYG6 | Q3UEI1 | 0,0212 | P80386 | 0,4286 | Q6P6T4 | 0,1000 | P18266 | 0,1272 | P57760 | 0,0687 | | |
| O35303 | P52332 | 0,0318 | P80385 | 0,4451 | Q78P75 | 0,2075 | G3V9W2 | 0,2117 | O08678 | 0,0755 | | |
| O35509 | Q8BWJ3 | 0,0335 | P14644 | 0,5575 | P63170 | 0,2241 | P57760 | 0,2687 | P80386 | 0,1367 | | |
| O35567 | Q7TSH2 | 0,0367 | P57760 | 0,5921 | Q6AY80 | 0,2813 | F1LN52 | 0,4331 | P54645 | 0,1375 | | |
| O35763 | Q8BGM7 | 0,0556 | P97874 | 0,6258 | P18266 | 0,3451 | F1M7R9 | 0,6638 | P0C1X8 | 0,1378 | | |
| O35814 | O08576 | 0,0570 | F1LP90 | 0,6672 | Q6AYG3 | 0,4027 | D3ZFU9 | 0,6738 | Q9QY78 | 0,1493 | | |
| O55096 | P14644 | 0,0595 | Q8VHF0 | 0,8515 | P57760 | 0,7977 | D4A355 | 0,6849 | P80385 | 0,1580 | | |
| O88767 | P0C1X8 | 0,0652 | Q9ESV1 | 0,9665 | O88370 | 0,8815 | P49186 | 0,7066 | Q8VHF0 | 0,1959 | | |
| O88989 | Q9QX05 | 0,0665 | P11275 | 0,9754 | P53670 | 1,0588 | O55099 | 0,7152 | O88850 | 0,2448 | | |
| P00406 | Q1HKZ5 | 0,0771 | O08815 | 1,0057 | Q8VHF0 | 1,1111 | F1LMD9 | 0,7256 | O08679 | 0,2477 | | |
| P00507 | F1LM93 | 0,0786 | Q810W7 | 1,0552 | Q9ESV1 | 1,2006 | F1LUY7 | 0,7287 | P59241 | 0,3164 | | |
| P00787 | Q09137 | 0,0793 | O08679 | 1,3563 | Q99N14 | 1,3199 | F1M7M4 | 0,7314 | Q4KMA0 | 0,3275 | | |
| P02793 | Q8R4Y4 | 0,0798 | O88764 | 1,3989 | P49185 | 1,4261 | D4A7D3 | 0,7835 | Q62925 | 0,3959 | | |
| P04256 | Q4QR82 | 0,0808 | Q62844 | 1,4675 | O08679 | 1,4594 | F1LQ89 | 0,8036 | Q6DG50 | 0,4620 | | |
| P04642 | Q9WUN2 | 0,0833 | Q5XIS9 | 1,6063 | O88377 | 1,5395 | D3ZG78 | 0,8076 | P49185 | 0,6345 | | |
| P04764 | P54645 | 0,0846 | P11730 | 1,6923 | Q9R0I8 | 1,5802 | P16446 | 0,9454 | Q6AY80 | 1,6180 | | |
| P04785 | Q80UK8 | 0,0864 | P08413 | 1,7298 | P59241 | 1,7910 | D3ZHP7 | 0,9475 | P18266 | 2,2193 | | |
| P04961 | O88370 | 0,0866 | Q4KMA0 | 1,8581 | P97874 | 1,8449 | G3V8Y3 | 0,9503 | P49186 | 2,4916 | | |
| P05065 | P80386 | 0,0869 | P15791 | 1,9910 | P0C1X8 | 1,8557 | Q6DG50 | 0,9887 | Q78P75 | 3,1978 | | |
| P05197 | Q9QZH4 | 0,0873 | Q9WUD9 | 2,3062 | O88764 | 1,8712 | F1M836 | 1,0267 | P18265 | 5,0766 | | |
| P05369 | P52633 | 0,0876 | O55099 | 2,3513 | Q5XIS9 | 1,9167 | D3ZW27 | 1,0457 | Q02974 | 6,7750 | | |
| P05765 | P80385 | 0,0896 | Q91ZN7 | 2,3867 | O35346 | 3,0296 | D3Z8I4 | 1,0987 | P53670 | 7,9065 | | |
| P06302 | Q91WG5 | 0,0914 | P0C1X8 | 2,5041 | P49186 | 3,0321 | D3ZSB7 | 1,1126 | Q01062 | 8,1267 | | |
| P06761 | Q9QUL0 | 0,0976 | P59241 | 4,1354 | Q5CZZ9 | 3,2337 | F1M101 | 1,1175 | Q6MGA9 | 12,2176 | | |
| P07171 | Q924I2 | 0,1008 | P09759 | 5,1688 | Q924I2 | 3,3254 | F1LQJ7 | 1,1576 | Q01066 | 12,3303 | | |
| P07335 | P17426 | 0,1078 | Q63802 | 5,6399 | P86172 | 3,3796 | G3V715 | 1,1808 | | | | |
| P07632 | O35643 | 0,1093 | Q63450 | 5,8399 | Q53UA7 | 3,9528 | G3V8J8 | 1,2588 | | | | |
| P07943 | Q6DG50 | 0,1095 | P54759 | 6,1219 | F1LP90 | 4,6596 | Q66HA1 | 1,2795 | | | | |
| P09527 | Q07014 | 0,1256 | Q03114 | 10,7503 | Q4KMA0 | 5,6177 | P09760 | 1,2934 | | | | |
| P10111 | Q8BMK0 | 0,1282 | Q62868 | 11,6294 | P39951 | 5,8611 | F1LNP9 | 1,2991 | | | | |
| P10719 | Q4KMA0 | 0,1307 | Q06486 | 12,3763 | A0JPM9 | 7,1118 | F1LQL4 | 1,3679 | | | | |
| P10760 | P84091 | 0,1312 | P18266 | 13,3582 | Q3ZAV8 | 7,7006 | F1LR34 | 1,4002 | | | | |
| P10860 | P70347 | 0,1344 | O35331 | 15,9230 | P11275 | 7,9634 | D3ZUD6 | 1,5032 | | | | |
| P11348 | Q9QYP6 | 0,1420 | Q63644 | 15,9963 | P68255 | 8,0107 | Q66H82 | 1,7156 | | | | |
| P11598 | Q04736 | 0,1434 | Q78P75 | 16,0720 | P63102 | 8,0359 | F1LUM8 | 1,7317 | | | | |
| P11980 | Q8BPB0 | 0,1546 | A0JPM9 | 16,5459 | Q810W7 | 8,1427 | D3ZJ71 | 1,7376 | | | | |

| | | | | | | | | |
|--------|--------|--------|--------|---------|--------|---------|----------|---------|
| P13084 | P57760 | 0,1677 | P35213 | 16,6105 | P67874 | 8,2006 | F8WFI7 | 1,9256 |
| P13383 | Q3T1J9 | 0,1683 | P67874 | 18,3442 | P62260 | 8,9855 | D3ZPU9 | 2,1184 |
| P13596 | P31325 | 0,1704 | P61983 | 18,4355 | Q6MGB6 | 9,3643 | B5DF62 | 2,2389 |
| P13668 | Q61846 | 0,1714 | P84092 | 19,6090 | P35213 | 9,5302 | D3ZIC0 | 2,2567 |
| P13803 | O35346 | 0,1720 | Q6MGB6 | 21,9513 | P68511 | 9,5861 | E9PT20 | 2,4885 |
| P14668 | P62743 | 0,1850 | P49186 | 25,7620 | P19139 | 9,7418 | Q6AY80 | 2,5509 |
| P15865 | P97874 | 0,1865 | Q9ESW0 | 26,0064 | Q516B8 | 10,1568 | Q61FZ5 | 2,5763 |
| P16617 | Q3B7D5 | 0,1899 | O54874 | 26,2248 | Q510H5 | 10,9356 | F1LR45 | 2,6442 |
| P16638 | Q8BYR2 | 0,1961 | Q7TT49 | 29,4974 | P11730 | 10,9876 | D3ZID5 | 2,6640 |
| P17074 | Q9J111 | 0,2101 | P97633 | 30,0000 | P70486 | 11,1378 | D4A1V7 | 2,7056 |
| P17220 | P17427 | 0,2141 | | | P21708 | 11,2426 | D3ZYV4 | 2,8988 |
| P18297 | A2A9T0 | 0,2239 | | | P08413 | 11,3812 | D4A280 | 3,2272 |
| P18298 | O08815 | 0,2333 | | | P09759 | 11,8640 | F1MAJ0 | 3,4006 |
| P18418 | P36506 | 0,2519 | | | P15791 | 12,5323 | O08678 | 3,4166 |
| P18421 | P83510 | 0,2522 | | | P54645 | 12,6111 | Q3T1J9 | 3,9441 |
| P19804 | Q8C0P0 | 0,2574 | | | P63086 | 12,6992 | F1M2K4 | 4,2434 |
| P21670 | O35832 | 0,2685 | | | Q63802 | 12,8654 | G3V9X3 | 4,4079 |
| P22734 | Q06486 | 0,2688 | | | P52303 | 12,8799 | D3ZZQ0 | 4,5763 |
| P24155 | Q8BPM2 | 0,2731 | | | P53676 | 13,2436 | Q924I2 | 4,7869 |
| P24368 | Q9Z2B9 | 0,2846 | | | Q9JIX5 | 13,3438 | F1M0N1 | 4,8958 |
| P25113 | Q810W7 | 0,2889 | | | P14644 | 13,5722 | P80386 | 4,9335 |
| P26772 | Q8VHF0 | 0,2983 | | | Q9WUD9 | 13,7636 | Q810W7 | 5,0443 |
| P28075 | O55099 | 0,3108 | | | P80386 | 13,7673 | P54645 | 5,0503 |
| P29410 | O08678 | 0,3261 | | | O08815 | 13,8882 | Q9JJ10 | 5,1618 |
| P31044 | P08413 | 0,3466 | | | P54759 | 13,9325 | F1LQC5 | 5,1726 |
| P34058 | P16879 | 0,3503 | | | P80385 | 14,0964 | D3ZMG0 | 5,2512 |
| P34064 | P09760 | 0,3539 | | | Q68H95 | 14,5536 | P80385 | 5,2577 |
| P35213 | O08679 | 0,4032 | | | Q6TQE1 | 15,4695 | F1LM93 | 5,5213 |
| P35565 | Q9R117 | 0,4267 | | | Q64725 | 15,6545 | P31325 | 5,7239 |
| P37199 | P11275 | 0,4588 | | | Q62844 | 16,4681 | F1LLY5 | 5,8771 |
| P37285 | Q3U214 | 0,4627 | | | P46892 | 17,5497 | P63086 | 5,9757 |
| P37996 | Q8VE70 | 0,5052 | | | Q9R1Q2 | 19,6394 | D4A7E4 | 6,4885 |
| P38983 | BOLT89 | 0,5093 | | | Q63450 | 20,0855 | D4AE19 | 7,2877 |
| P40112 | Q91ZN7 | 0,5445 | | | Q6TMG5 | 21,0496 | P21708-2 | 8,0439 |
| P40307 | O35942 | 0,5858 | | | Q9QY78 | 21,1995 | P54759 | 9,6970 |
| P41498 | Q8R4U9 | 0,6188 | | | Q5RK09 | 21,3863 | P35739 | 9,7766 |
| P41562 | Q63531 | 0,6326 | | | P18484 | 22,7378 | F1M7W0 | 9,9408 |
| P42123 | P05480 | 0,6626 | | | P62944 | 23,0470 | Q8R4U9 | 10,3352 |
| P45592 | Q8R4K2 | 0,6951 | | | P84092 | 23,1050 | F1M801 | 11,2750 |
| P46462 | Q3U3Q1 | 0,7618 | | | Q1JU68 | 24,1540 | P50297 | 11,3575 |
| P47942 | P18654 | 0,7819 | | | Q6P9U8 | 24,5975 | D3ZHL6 | 11,4904 |
| P48004 | P15127 | 0,7901 | | | Q9ESW0 | 24,6805 | F1M778 | 11,5145 |
| P48500 | O54748 | 0,8013 | | | B5DFC8 | 25,2247 | Q62862 | 12,8546 |
| P48508 | Q4KSH7 | 0,8878 | | | Q6AYK8 | 25,4211 | Q5XIF0 | 16,0433 |
| P49088 | Q01986 | 0,9870 | | | Q4G061 | 25,4556 | Q06486-2 | 18,4259 |
| P50137 | O35099 | 0,9972 | | | Q641X8 | 26,1392 | | |
| P50398 | P15791 | 1,0531 | | | BOBNA7 | 28,4073 | | |
| P50399 | Q66HA1 | 1,1004 | | | Q7TT49 | 29,4974 | | |
| P52555 | P00520 | 1,1072 | | | Q63644 | 30,0000 | | |
| P52925 | Q9QYR9 | 1,1253 | | | | | | |
| P54313 | B2GUY1 | 1,1276 | | | | | | |
| P55053 | A2AQW0 | 1,1313 | | | | | | |
| P56574 | P35739 | 1,2089 | | | | | | |
| P60881 | Q61083 | 1,2395 | | | | | | |
| P61459 | Q62689 | 1,2867 | | | | | | |
| P61765 | O88831 | 1,3034 | | | | | | |
| P61980 | O55047 | 1,3222 | | | | | | |
| P61983 | Q61084 | 1,3312 | | | | | | |
| P62083 | P59241 | 1,6140 | | | | | | |

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|--------|--------|---------|
| P62161 | D3ZML2 | 1,6447 |
| P62260 | Q62862 | 1,7684 |
| P62744 | Q8C050 | 1,7736 |
| P62752 | P54759 | 1,8365 |
| P62815 | P54763 | 1,8797 |
| P62870 | Q3U1V8 | 1,9782 |
| P62898 | O55173 | 2,0491 |
| P62902 | Q8K1R7 | 2,1077 |
| P62963 | Q9WU61 | 2,6252 |
| P62994 | P63017 | 3,0354 |
| P63029 | Q60629 | 3,5052 |
| P63036 | P50297 | 3,5284 |
| P63039 | Q4JIM5 | 3,6040 |
| P63102 | O35607 | 3,7123 |
| P63159 | P63168 | 3,7851 |
| P63259 | Q02974 | 4,0635 |
| P63324 | Q99ML2 | 4,5662 |
| P68101 | Q8C015 | 4,7651 |
| P68255 | Q8BK63 | 5,1482 |
| P68511 | Q8BTW9 | 5,8363 |
| P81795 | Q62925 | 6,2128 |
| P82995 | Q9JKY5 | 6,8749 |
| P83868 | Q29RW1 | 7,3977 |
| P83941 | P18266 | 7,7715 |
| P84245 | P22315 | 12,3765 |
| P85968 | Q9WTU6 | 17,9270 |
| P85970 | | |
| P97852 | | |
| Q00981 | | |
| Q05982 | | |
| Q08163 | | |
| Q27W01 | | |
| Q2PQA9 | | |
| Q3KRD8 | | |
| Q3TIJ1 | | |
| Q4KM49 | | |
| Q4KM73 | | |
| Q5EGY4 | | |
| Q5FVL2 | | |
| Q5I034 | | |
| Q5I0G4 | | |
| Q5M7U6 | | |
| Q5M819 | | |
| Q5RJR2 | | |
| Q5RJR8 | | |
| Q5RKI1 | | |
| Q5SGE0 | | |
| Q5U300 | | |
| Q5XFX0 | | |
| Q5XHY5 | | |
| Q5XHZ0 | | |
| Q5XI73 | | |
| Q5XIA9 | | |
| Q5XIM9 | | |
| Q5XIT1 | | |
| Q62636 | | |
| Q62658 | | |
| Q62724 | | |
| Q63009 | | |

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|--------|
| Q63081 |
| Q63396 |
| Q63413 |
| Q63524 |
| Q63525 |
| Q63584 |
| Q63598 |
| Q63610 |
| Q63617 |
| Q63945 |
| Q64057 |
| Q64270 |
| Q64537 |
| Q66HD0 |
| Q66HD3 |
| Q66HR2 |
| Q66X93 |
| Q68FQ0 |
| Q68FR9 |
| Q68FS4 |
| Q68FU3 |
| Q68FY0 |
| Q68G31 |
| Q6AXX6 |
| Q6AY80 |
| Q6AYK8 |
| Q6DGG1 |
| Q6MG08 |
| Q6MG61 |
| Q6P502 |
| Q6P6V0 |
| Q6P799 |
| Q6P7B0 |
| Q6P7Q4 |
| Q6PCU2 |
| Q6R556 |
| Q6URK4 |
| Q71UF4 |
| Q794F9 |
| Q7M0E3 |
| Q7M767 |
| Q7TPB1 |
| Q8VHF5 |
| Q920D2 |
| Q920J4 |
| Q924S5 |
| Q99NA5 |
| Q99PF5 |
| Q9EPB1 |
| Q9EQS0 |
| Q9EQX9 |
| Q9ER34 |
| Q9JHB5 |
| Q9JHW0 |
| Q9JLZ1 |
| Q9JMB5 |
| Q9QUL6 |
| Q9QXQ0 |
| Q9R063 |

| |
|--------|
| Q9WVK7 |
| Q9Z0V6 |
| Q9Z0W7 |
| Q9Z1P2 |

Excel macro for the analysis of overlapping targets

```

Sub CALC_ALL()
'
' Remove duplicates from each of original max. of 10 col. and 5000 rows (Needed for displaying
the right overlap number)
'
    Sheets("Set1").Select
    Columns("A:A").Select
    ActiveSheet.Range("$A$1:$A$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("B:B").Select
    ActiveSheet.Range("$B$1:$B$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("C:C").Select
    ActiveSheet.Range("$C$1:$C$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("D:D").Select
    ActiveSheet.Range("$D$1:$D$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("E:E").Select
    ActiveSheet.Range("$E$1:$E$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("F:F").Select
    ActiveSheet.Range("$F$1:$F$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("G:G").Select
    ActiveSheet.Range("$G$1:$G$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("H:H").Select
    ActiveSheet.Range("$H$1:$H$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("I:I").Select
    ActiveSheet.Range("$I$1:$I$5000").RemoveDuplicates Columns:=1, Header:=xlNo
    Columns("J:J").Select
    ActiveSheet.Range("$J$1:$J$5000").RemoveDuplicates Columns:=1, Header:=xlNo
'
'.....
'Macro to copy columns of variable length
'into 1 continuous column in a new sheet '
'Modified 17 Feb 2006 by Bernie Dietrick
'.....

Dim iLastcol As Long
Dim iLastRow As Long
Dim jLastrow As Long
Dim ColNdx As Long
Dim ws As Worksheet
Dim myRng As Range
Dim ExcludeBlanks As Boolean
Dim mycell As Range

ExcludeBlanks = (MsgBox("Exclude Blanks", vbYesNo) = vbYes)
Set ws = ActiveSheet
iLastcol = ws.Cells(1, ws.Columns.Count).End(xlToLeft).Column
On Error Resume Next

Application.DisplayAlerts = False
Worksheets("Calculation").Delete
Application.DisplayAlerts = True

Sheets.Add.Name = "Calculation"

For ColNdx = 1 To iLastcol

iLastRow = ws.Cells(ws.Rows.Count, ColNdx).End(xlUp).Row

Set myRng = ws.Range(ws.Cells(1, ColNdx), _
ws.Cells(iLastRow, ColNdx))

If ExcludeBlanks Then
For Each mycell In myRng
If mycell.Value <> "" Then
jLastrow = Sheets("Calculation").Cells(Rows.Count, 1) _
.End(xlUp).Row
mycell.Copy
Sheets("Calculation").Cells(jLastrow + 1, 1) _
.PasteSpecial xlPasteValues
End If
Next mycell
Else
myRng.Copy

```

```
jLastrow = Sheets("Calculation").Cells(Rows.Count, 1) _
.End(xlUp).Row
mycell.Copy
Sheets("Calculation").Cells(jLastrow + 1, 1) _
.PasteSpecial xlPasteValues
End If
Next

Sheets("Calculation").Rows("1:1").EntireRow.Delete

ws.Activate

With ActiveWorkbook.Sheets("Calculation").Tab
    .Color = 65535
    .TintAndShade = 0
End With

' Remove Duplicates from combined list

    Sheets("Calculation").Select
    ActiveSheet.Range("A:A").RemoveDuplicates Columns:=1, Header:=xlNo
    Sheets("Set1").Select

'
' Counts matching names for each of all lists
'

    Sheets("Calculation").Select
    Range("B1").Select
    ActiveCell.FormulaR1C1 = "=COUNTIF('Set1'!R1C[-1]:R5000C[-1],Calculation!RC1)"
    Range("B1").Select
    Selection.AutoFill Destination:=Range("B1:B5000"), Type:=xlFillDefault
    Range(Selection, Selection.End(xlDown)).Select
    Selection.AutoFill Destination:=Range("B1:K5000"), Type:=xlFillDefault
    Range("B1:K5000").Select
    Sheets("Set1").Select

'
' Calculates overlaps / The intersections of the different datasets
'

    Sheets("Calculation").Select
    Range("L1").Select
    ActiveCell.FormulaR1C1 = "=SUM(RC[-10]:RC[-1])"
    Range("L1").Select
    Selection.AutoFill Destination:=Range("L1:L5000"), Type:=xlFillDefault
    Range("L1:L5000").Select
    Sheets("Set1").Select

'
' Displays Overlapping Compounds
'

    Sheets("Calculation").Select
    Range("M1").Select
    ActiveCell.FormulaR1C1 = "=IF(RC[-11]>0,'CPD List'!R1C[-12],\"\")"
    Range("M1").Select
    Selection.AutoFill Destination:=Range("M1:M5000"), Type:=xlFillDefault
    Range("M1:M5000").Select
    Selection.AutoFill Destination:=Range("M1:V5000"), Type:=xlFillDefault
    Sheets("Set1").Select

'
' Copies and sorts by overlapping numbers
'

    Application.DisplayAlerts = False
    Worksheets("RESULT").Delete
    Application.DisplayAlerts = True

    Sheets.Add.Name = "RESULT"

    Sheets("Calculation").Select
```

```

Columns("A:V").Select
Selection.Copy
Sheets("RESULT").Select
Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone, SkipBlanks _
:=False, Transpose:=False
Application.CutCopyMode = False
ActiveWorkbook.Worksheets("RESULT").Sort.SortFields.Clear
ActiveWorkbook.Worksheets("RESULT").Sort.SortFields.Add Key:=Range("L1:L5000") _
, SortOn:=xlSortOnValues, Order:=xlDescending, DataOption:=xlSortNormal
With ActiveWorkbook.Worksheets("RESULT").Sort
.SetRange Range("A1:V5000")
.Header = xlGuess
.MatchCase = False
.Orientation = xlTopToBottom
.SortMethod = xlPinYin
.Apply
End With
Sheets("RESULT").Select
Columns("L:L").Select
Selection.FormatConditions.Add Type:=xlCellValue, Operator:=xlBetween, _
Formula1:="=5", Formula2:="=10"
Selection.FormatConditions(Selection.FormatConditions.Count).SetFirstPriority
With Selection.FormatConditions(1).Font
.Color = -16383844
.TintAndShade = 0
End With
With Selection.FormatConditions(1).Interior
.PatternColorIndex = xlAutomatic
.Color = 13551615
.TintAndShade = 0
End With
Selection.FormatConditions(1).StopIfTrue = False
Selection.FormatConditions.Add Type:=xlCellValue, Operator:=xlBetween, _
Formula1:="=2", Formula2:="=4"
Selection.FormatConditions(Selection.FormatConditions.Count).SetFirstPriority
With Selection.FormatConditions(1).Font
.Color = -16751204
.TintAndShade = 0
End With
With Selection.FormatConditions(1).Interior
.PatternColorIndex = xlAutomatic
.Color = 10284031
.TintAndShade = 0
End With
Selection.FormatConditions(1).StopIfTrue = False
Sheets("RESULT").Select
With ActiveWorkbook.Sheets("RESULT").Tab
.Color = 5287936
.TintAndShade = 0
End With
Sheets("Set1").Select

'
' Inserts headers + "Overlap %"
'

Sheets("RESULT").Select
Rows("1:1").Select
Selection.Insert Shift:=xlDown, CopyOrigin:=xlFormatFromLeftOrAbove
Range("A1").Select
ActiveCell.FormulaR1C1 = "ID"
Range("B1:K1").Select
With Selection
.HorizontalAlignment = xlCenter
.VerticalAlignment = xlBottom
.WrapText = False
.Orientation = 0
.AddIndent = False
.IndentLevel = 0
.ShrinkToFit = False
.ReadingOrder = xlContext
.MergeCells = False
End With
Selection.Merge
ActiveCell.FormulaR1C1 = "Matching Ids"
Range("L1").Select
ActiveCell.FormulaR1C1 = "Overlaps"

```

```
Range("M1").Select
ActiveCell.FormulaR1C1 = "'CPD List'!RC[-12]"
Range("M1").Select
Selection.AutoFill Destination:=Range("M1:V1"), Type:=xlFillDefault
Range("M1:V1").Select
Columns("A:W").EntireColumn.AutoFit
Rows("1:1").Select
Selection.Font.Bold = True
With Selection
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlBottom
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .IndentLevel = 0
    .ShrinkToFit = False
    .ReadingOrder = xlContext
End With

Columns("L:L").Select
With Selection
    .HorizontalAlignment = xlCenter
    .VerticalAlignment = xlCenter
    .WrapText = False
    .Orientation = 0
    .AddIndent = False
    .IndentLevel = 0
    .ShrinkToFit = False
    .ReadingOrder = xlContext
    .MergeCells = False
End With
Selection.Font.Bold = True
Range("W1").Select

Range("W1").Select
ActiveCell.FormulaR1C1 = "% Overlap"
Range("W2").Select
ActiveCell.FormulaR1C1 = "=RC[-11]/'CPD List'!R3C1"
Range("W2").Select
Selection.Style = "Percent"
Range("W1").Select
Selection.AutoFilter
Range("W2").Select
Selection.AutoFill Destination:=Range("W2:W5000"), Type:=xlFillDefault

End Sub
```