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Discovery and pharmacological characterization of a novel small molecule inhibitor of phosphatidylinositol-5-phosphate 4-kinase, type II, beta

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ABSTRACT

Phosphatidylinositol-5-phosphate 4-kinase, type II, beta (PIP5K2B) is linked to the pathogenesis of obesity, insulin resistance and diabetes. Here, we describe the identification of a novel pyrimidine-2, 4-diamine PIP5K2B inhibitor, designated SAR088. The compound was identified by high-throughput screening and subsequently characterized in vitro and in vivo. SAR088 showed reasonable potency, selectivity and physicochemical properties in enzymatic and cellular assays. In vivo, SAR088 lowered blood glucose levels of obese and hyperglycemic male Zucker diabetic fatty rats treated for 3 weeks. Thus, SAR088 represents the first orally available and in vivo active PIP5K2B inhibitor and provides an excellent starting point for the development of potent and selective PIP5K2B inhibitors for the treatment of insulin resistance and diabetes.

1. Introduction

Obesity and associated metabolic disorders such as insulin resistance and type 2 diabetes represent an increasing burden for societies and highlight the necessity to develop new pharmaceutical therapies [1]. Insulin resistance is characterized by a relative impairment of insulin-induced activation of the phosphatidylinositol-3-phosphate kinase (PI3K) signaling pathway downstream the insulin receptor, subsequently decreased levels of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) and eventually impaired membrane translocation of glucose transporter 4 and uptake of glucose into the cells. Phosphatidylinositol-5-phosphate 4-kinase, type II, beta (PIP5K2B) catalyzes the generation of phosphatidylinositol-4,5-bisphosphate PtdIns(4,5)P2, the major substrate for PI3K signaling. However, PIP5K2B activity has been shown to negatively impact on PI3K signaling in insulin action on the level of Akt/protein kinase B (PKB) phosphorylation and glucose uptake in cellular assays [2]. This implies that the relative level of the PIP5K2B substrate, phosphatidylinositol-5-phosphate (PtdIns(5)P), seems to impact on PtdIns(3,4,5)P3 levels and Akt/PKB phosphorylation. In support of this hypothesis, levels of PtdIns(5)P have been reported to be induced by insulin stimulation and that injection of PtdIns(5)P into cells could mimic insulin stimulation at the level of Akt/PKB phosphorylation, Glut4 translocation as well as F-Actin stress fiber breakdown in different cell systems [3,4]. Genetic ablation of PIP5K2B in mice resulted in increased insulin sensitivity, improved weight and lipid profiles. This phenotype was independent from body fat content even under a high fat diet and likely mediated by an increased insulin sensitivity in muscle and liver that was reflected in increased insulin signal transduction in those tissues [5,6]. Here, we disclose the discovery of a representative compound of a completely novel class of pyrimidine-2,4-diamine PIP5K2B inhibitors, designated SAR088. For the first time, we provide in vivo evidence for the beneficial phenotype of pharmacological PIP5K2B inhibition. In hyperglycemic male Zucker diabetic fatty rats treated for three weeks, SAR088 reduced blood glucose levels. These findings could provide a base for future development of PIP5K2B inhibitors for the treatment of metabolic disorders such as insulin resistance and type 2 diabetes.

2. Materials and methods

2.1. Screening of compound libraries and determination of compound in vitro properties

PIP5K2B and PIP5K2A proteins were produced in *Escherichia coli* BL21(DE3/pLysS) transformed with pET16 plasmids (Merck-Millipore, Darmstadt, Germany) encoding the full length proteins

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(NM_003559 and NM_005028, respectively), purified and diluted in a buffer containing 50 mM Tris/pH 7.5 and 500 mM NaCl and 10 mM DTT. Synthesis of pyrimidine-2,4-diamines has been previously described [7]. Screening assays were developed using Transcreener™ fluorescence polarization and Transcreener™ time-resolved fluorescence electron transfer (TR-FRET) kinase assay formats in 384-well format (BellBrook Labs/Mobitech GmbH, Goettingen, Germany). Reactions were probed in a buffer containing 50 mM HEPES pH7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$; 0.015% Brij 35, 1 mM DTT, 85 nM PIP5K2B or A enzyme and with the respective amount of compound in a final concentration of 1.4% DMSO and were pre-incubated at 26 °C for 15 min. 9 μM D-myo di8-PtdIns(5)P substrate and 20 μM ATP were added and incubated for 1 h at 26 °C. After addition of ADP Antibody and ADP Far-red Tracers (or ADP Tb-Antibody and ADP FAM Tracer for the TR-FRET format) the reaction was incubated for 90 min at 26 °C before the respective measurement in a Tecan Ultra™ reader. Part of the structure activity relationships were determined in an ADP-Glow™ assay (Promega, Mannheim, Germany), here designated hPIP5K2B ATP luminescence assay, as recommended by the manufacturer using 50 mM HEPES pH7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$; 0.015% Brij 35, 1 mM DTT, 20 nM enzyme and 10 μM substrate and 10 μM ATP. The assay was used to determine the IC_{50}s of substitutions at R1 and yielded results comparable to the Transcreener™ fluorescence polarization or TR-FRET assay formats in a more robust and easier to handle assay format. Phosphorylation of Akt/PKB and glucose uptake was determined in L6 cells, as previously described [8].

2.2. Determination of compound in vivo properties

All experimental procedures were conducted in accordance to the German Animal Protection Law, as well as according to international animal welfare legislation and rules. Male lean (ZDF/Gmi-Fa/?) and obese (ZDF/Gmi-fa/fa) ZDF rats (8-weeks old, Charles River Laboratories, Belgium) were once daily administered orally with vehicle or 30 mg/kg SAR088 in 5% Solutol/0.5% hydroxyethylcellulose for 3 weeks. They were housed six per group and two per cage in an environmentally controlled room with a 12:00 h:12:00 h light–dark circle (light on at 06:00) and *ad libitum* access to food (standard rat chow R/M−H, V1535, Ssniff, Soest, Germany) and water. Compound levels were determined in the respective tissues 120 min after the last compound application [9]. All clinical blood parameters were determined by commercially available diagnostic kits on a Hitachi 912 device or as previously described [10,11].

2.3. Statistical analysis

IC_{50} values were calculated from 8-point dose–response curves using the internal software Biost@t-SPEED v1.3 using the 4-parameter logistic model according to Ratkovsky and Reedy [12]. The adjustment was obtained by non-linear regression using the Marquardt algorithm in SAS v8.2 software under UNIX. Statistical analysis was performed with one-way ANOVA, followed by a Bonferroni comparison. All values are expressed as mean ± s.e.m. p values <0.05 were considered as statistically significant.

3. Results and discussion

3.1. Screening and identification of SAR088 as PIP5K2B-Inhibitor

To identify specific inhibitors of PIP5K2B, a library of 115,000 substances was screened at a concentration of 20 μM using a Transcreener™fluorescence polarization kinase assay, D-myo-PtdIns(5)P as substrate and recombinant PIP5K2B. This yielded a number of 1760 primary positives that were reconfirmed in triplicates in the primary assay with and without PIP5K2B kinase as control, resulting in 236 confirmed specific actives. An alternative assay format, the Transcreener™ TR-FRET kinase assay, was used for confirmation of the single-dose values by determination of IC_{50} values in 8-concentration curves. This approach eventually identified 132 specific actives from 4 different chemical series that were further analyzed in different enzymatic and cellular assays. Of these, a series of pyrimidine-2,4-diamine derivatives was selected for the

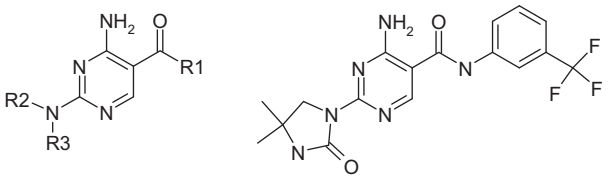
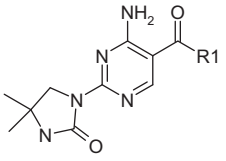


Fig. 1. General structure of pyrimidine-2,4-diamine derived PIP5K2B inhibitors (**I**) and SAR088.

Table 1a
Structure–activity relationship resulting from variation of residues R1 around pyrimidine-2,4-diamine chemical series of general structure **I** (Fig. 1), IC_{50}s derived from ATP-Luminescence assay.

		R1	IC_{50} Hu_ATPLum Assay (μM)
SAR088			3.8
1			5.4
2			8.1
3			13
4			13
5			25
6			>30
7			>30

identification of the structure–activity relationship (SAR) around this chemical series and for further compound optimization. Fig. 1 shows the general chemical structure of the pyrimidine-2,4-diamine derivatives (**I**) and the individual chemical structure of SAR088, a representative of this compound class identified by testing structural analogs of the initial screening hits of general structure **I**.

Table 1a summarizes the SAR trends elaborated by modification of residues R1 in the pyrimidine-2,4-diamine chemical series of general structure **I** (Fig. 1). IC_{50} data are derived from the hPIP5K2B ATP luminescence assay. SAR088, the most potent compound within this chemical series, incorporates a m-CF3 phenylamino residue as R1, leading to an IC_{50} of 3.8 μM. Several m-substituted, lipophilic phenylamino moieties showed similar IC_{50} values (e.g., compounds **2**, **3**), while o- and p-substituted phenylamino R1 residues as well as compounds with additional substitution on the phenyl residue such as compounds **3**–**5** showed a loss of potency as compared to SAR088. N-Methylation of the m-CF3 phenylamino residue as well as substitution of the m-CF3 phenylamino residue by a m-CF3 phenoxy residue led to a complete loss of potency (IC_{50} > 30 μM) (compounds **6**, **7**). In addition, hundreds of analogous aliphatic amides with R1s derived from primary, secondary or benzylic amines led to IC_{50}s > 30 μM.

Table 1b summarizes SAR trends elaborated by modification of R2 and R3 around the exocylic N-C(2) of the pyrimidine moiety of general structure **I** (Fig. 1). IC_{50} data are derived from the above described hPIP5K2B Transcreener™ fluorescence polarization kinase assay.

Dozens of variations of R2 and R3 were investigated, including replacements of the imidazolidinone moiety of SAR088 by H, OH, NH_2, primary and secondary amines, aromatic and heteroaromatic benzylic amines, urea-derived motives, etc. The imidazolidinone-derived left hand side residue of SAR088 was clearly favoured with an IC_{50} of 2.2 μM in this assay, while all variations (e.g., resembled by compounds **11**–**22**) led to a significant or complete loss of potency.

3.2. In vitro characterization of SAR088

SAR088 inhibited recombinant PIP5K2B with an IC_{50} value of 2.18 μM in a Transcreener™ fluorescence polarization kinase assay in vitro (Table 2). Selectivity towards the iso-enzyme PIP5K2A was determined to be 9-fold. Treatment of L6 muscle cells with 10 μM SAR088 for 24 h decreased the EC_{50} of insulin by 47% in subsequent glucose uptake experiments from 15 to 7 nM and led to a 21% increase of insulin-stimulated Akt/PKB-phosphorylation. To assess SAR088 overall selectivity, an internal 35 kinase selectivity panel including different PI3K isoforms and the CEREP™ diversity panel were tested. In addition, an AMES II bacterial reverse mutation test was performed. No significant effects were observed in either assay, with the exception of a 27% remaining binding of human dopamine transporter in the CEREP™ diversity panel (data not shown). These tests on a large panel of unrelated drug targets and specificity assays reveal that SAR088 is a relatively specific PIP5K2B inhibitor. In addition, SAR088 exhibited reasonable physico-chemical properties, no liver CYP34A inhibition, as well as intermediate cell-permeability and high metabolic stability (Table 2).

3.3. In vivo characterization of SAR088

Substance tissue exposure levels and pharmacodynamic effects on different metabolic and physiological parameters were determined in vivo in male obese ZDF (ZDF/Gmi-fa/fa) rats treated once daily with vehicle or 30 mg/kg SAR088 *per os* for 3 weeks. In addition, male lean ZDF rats (ZDF/Gmi-Fa/?) served as healthy controls.

The serum and tissue exposure levels of SAR088 were determined 120 min after the last 30 mg/kg *per os* application in serum (962 ± 100 ng/ml), liver (4482 ± 524 ng/g) and skeletal muscle (606 ± 83 ng/g), thus providing evidence for a high exposure in these target tissues.

The pharmacological efficacy of SAR088 was assessed in parallel (Fig. 2). After once daily treatment at 30 mg/kg *per os*, the

Table 1b
Structure–activity relationship resulting from variation of residues R2, R3 around pyrimidine-2,4-diamine chemical series of general structure **I** (Fig. 1), IC_{50}s derived from PIP5K2B fluorescence polarization (FP)assay.

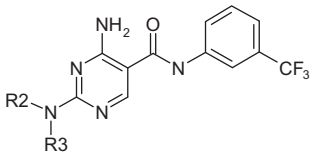
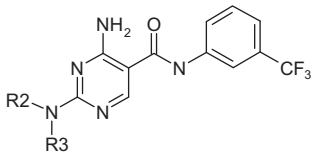
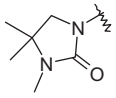
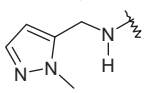
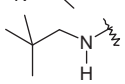
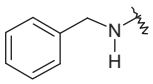
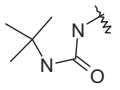
	–N(R2R3)	IC_{50} (μM)		–N(R2R3)	IC_{50} (μM)
11		>30	17		>30
12		>30	18		>30
13		4.1	19		>30
14		8.0	20		>30
15		25	21		>30
16		>30	22		>30

Table 2
Physico-chemical and in vitro properties of PIP5K2B Inhibitor SAR088. Properties and in vitro characterization data of SAR088 in different activity and selectivity assays, as described in the materials and methods section. As a compound of reasonable potency, SAR088 exhibits high specificity, high cell permeation and in vitro metabolic efficacy.

Parameter	SAR088
MW	394.36
(c) LogP/LogD	1.4
Solubility	11.1 mg/L
IC_{50} TR-FRET assay human PIP5K2B	2.1 μM
IC_{50} FP assay human PIP5K2B	3.8 μM
IC_{50} ADP-Glo assay human PIP5K2B	2.2 μM
IC_{50} TR-FRET assay human PIP5K2A	18.1 μM
Metabolic lability human liver microsomes (5 μM)	5%
Metabolic lability rat liver microsomes (5 μM)	3%
$Caco_2$ permeability	$38.41*10^{-7}$ cm/s
Inhibition of CYP34A (10 μM)	0%
pAKT/AKT L6 muscle cells	+21%
Maximum stimulation ^{14}C-deoxy glucose uptake	+26%
Insulin EC_{50} ^{14}C-deoxy glucose uptake	−47% (7 nM)

compound significantly decreased blood glucose levels as well as HbA1c compared to vehicle in the obese animals (−7.71 mmol/L and −1.32%, respectively). The anti-diabetic effect on blood glucose was already evident after 7 and 14 days of treatment (Fig. 2).

In contrast, no significant changes were observed regarding body weight and serum levels of insulin, adiponectin, triglycerides, cholesterol, as well as liver cholesterol and liver phospholipids (Table 3). However, leptin levels were increased by 2.44-fold and liver triglycerides by 1.7-fold, indicating an effect on liver fat metabolism. In line with this, a significant increase in alanine-aminotransferase by 1.58-fold and a trend, however not significant, towards increased aspartate-aminotransferase were observed in liver of the treated animals (Table 3).

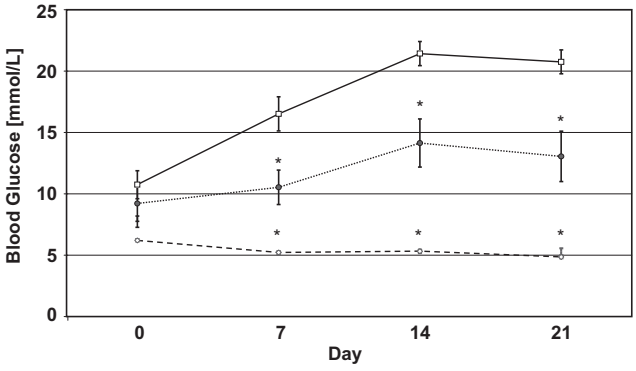


Fig. 2. SAR088 treatment improves blood glucose levels in ZDF rats. Treatment of male obese ZDF rats with 30 mg/kg SAR088 significantly (p-value <0.001) decreased blood glucose compared to the vehicle treated controls (n = 6; means ± s.e.m.).

These findings suggest that future optimization experiments have to include the assessment of possible side-effects on the liver. Recent studies, as reviewed in Reitman [13], for example established a link between elevated leptin levels, non-alcoholic fatty liver disease and steatohepatitis. An oral glucose-tolerance test, performed after 16 days of treatment with 30 mg/kg *per os*, revealed no significant changes between the SAR088 treated and the vehicle treated groups (data not shown), indicating that the effects observed for the blood glucose levels are likely not mediated by effects of SAR088 on insulin resistance. In particular, as the HOMA-index calculated from day 16 blood samples did not reveal any significant changes between the SAR088 and the vehicle treated groups (data not shown).

In accordance with the PIP5K2B knock-out animals [5], the in vitro cell culture experiments showed improved insulin sensitivity by subacute SAR088 treatment in different cellular assays with a decreased insulin EC_{50} and an increased phospho-Akt/PKB signaling in cell culture. In contrast to the knock-out phenotype, the effect of SAR088 in vivo was mainly anti-diabetic and blood-glucose-lowering, but not targeting body-weight and insulin-sensitivity. This discrepancy might be based in developmental effects of PIP5K2B knock-down in embryogenesis and development of the knock-out animals. In addition, also the tissue distribution of SAR088 with intermediate substance levels in serum and muscle and high levels in liver are likely not reflecting the global knock-out. In particular, a possible effect on insulin secretion cannot be excluded, as insulin levels in the treated animals showed a trend to be higher than in the controls. In addition, leptin levels were significantly higher, indicating further effects on blood glucose levels, thermogenesis and fatty acid oxidation. Similar to the PIP5K2B knock-out mice, isofom PIP5K2A-specific knock-out mice on a high-fat diet were described as less obese, normoglycemic and with increased pancreatic first-phase insulin secretion on a high-fat diet [14], implying a general impact of phosphatidylinositol-5-kinase isoform on metabolic signaling pathways. Recently, Demian et al. described a screen for phosphatidylinositolphosphate-kinase inhibitors and identified a tool compound designated compound A that inhibited recombinant PIP5K2B with low micromolar activity. However, no cellular, stability, pharmacokinetic or in vivo data were disclosed for this reference in vivo [15]. Testing this compound class head-to-head with SAR088 in vivo could nevertheless provide valuable insight into the mode of action of PIP5K2B inhibitors.

Table 3
Pharmacological characterization of PIP5K2B Inhibitor SAR088 in ZDF rats. Different in vivo characterization data from male obese ZDF rats treated with vehicle or 30 mg/kg SAR088, as described in the materials and methods section. SAR088 exhibited improved metabolic parameters compared to the vehicle-treated controls (n = 6; means ± s.e.m. [*] p-value <0.05).

Parameter	Vehicle		SAR088		
Glucose	20.76 ± 0.98	mmol/L	13.05 ± 2.06	mmol/L	[*]
HbA1c	8.11 ± 0.18	%	6.79 ± 0.26	%	[*]
Insulin	1.69 ± 0.20	μg/L	4.40 ± 1.61	μg/L	
Adiponectin	3.26 ± 0.11	μg/L	4.34 ± 0.92	μg/L	
Leptin	14.66 ± 1.56	μg/L	35.81 ± 3.44	μg/l	[*]
Triglycerides	7.15 ± 0.40	mmol/L	6.72 ± 3.22	mmol/L	
Cholesterol	5.42 ± 0.19	mmol/L	6.14 ± 0.43	mmol/L	
Body weight	340.53 ± 7.36	g	340.31 ± 7.03	g	
Aspartate-aminotransferase	97.50 ± 17.45	U/L	134.50 ± 28.11	U/L	
Alanine-aminotransferase	114.33 ± 14.86	U/L	180.17 ± 39.12	U/L	[*]
Alkaline-phosphatase	333.33 ± 21.18	U/L	323.83 ± 27.50	U/L	
Liver triglycerides	24.71 ± 1.41	mg/g	42.16 ± 2.69	mg/g	[*]
Liver cholesterol	2.69 ± 0.06	mg/g	2.69 ± 0.12	mg/g	
Liver phospholipids	21.14 ± 0.43	mg/g	20.72 ± 0.51	mg/g	

In summary, these data provide evidence for the efficacy of SAR088 as anti-diabetic PIP5K2B inhibitor in vitro and in vivo. As representative of a novel class of PIP5K2B inhibitors, the 2, 4-pyrimidine-diamines, SAR088 enables initial insight into structure activity relationships of this substance class. Further future optimization steps of this series should carefully evaluate the impact of 2,4-pyrimidine-diamine PIP5K2B inhibitors on liver aminotransferases and liver triglyceride metabolism, clarify the mode of action with regard to the different tissues targeted and increase the potency on the target in vitro as well as in vivo.

In conclusion, we described the screening of a compound library, the identification of a lead series and the in vitro as well as in vivo properties of a novel, potent and selective 2,4-pyrimidine-diamine PIP5K2B inhibitor. This is, to our knowledge, the first description of a PIP5K2B inhibitor exhibiting in vivo efficacy in an animal model of insulin-resistance and type 2 Diabetes, thus providing the base for the identification and optimization of a novel class of anti-diabetic drug therapies.

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