CHAPTER 3

Identification and characterisation of carnostatine (SAN9812), a potent and selective carnosinase (CN1) inhibitor with in-vivo activity

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Abstract

Carnosinase 1 (CN1) has been postulated to be a susceptibility factor for developing diabetic nephropathy (DN). Although its major substrate, carnosine, is beneficial in rodent models of DN, translation of these findings to humans has been hampered by high CN1 activity in human serum resulting in rapid degradation of carnosine.

To overcome this hurdle, we screened a protease-directed small molecule library for inhibitors of human recombinant CN1.

We identified SAN9812 as a potent and highly selective inhibitor of CN1 activity with a Ki of 11 nM. It also inhibited CN1 activity in human serum and serum of transgenic mice overexpressing human CN1. Subcutaneous administration of 30 mg/kg SAN9812 led to a sustained reduction in circulating CN1 activity in human CN1 transgenic (TG) mice. Simultaneous administration of carnosine and SAN9812 increased carnosine levels in plasma and kidney by up to 100fold compared to treatment-naïve CN1 overexpressing mice.

To our knowledge, this is the first study reporting on a potent and selective CN1 inhibitor with in-vivo activity. SAN9812, also called carnostatine, may be used to increase renal carnosine concentration as a potential therapeutic modality for renal diseases linked to glycoxidative conditions.

Introduction

The rising global prevalence of type 2 diabetes (T2D) is accompanied by an increase in diabetic complications, such as diabetic nephropathy (DN), the single most common cause of end-stage renal disease (ESRD). Despite our continuously evolving understanding of the pathogenesis of DN and of potential interventions current standard of care has largely failed to achieve stable long-term results, and a strong unmet need for novel and more efficacious therapies remains [1].

Carnosine (beta-alanyl-L-histidine) is a physiological dipeptide first discovered in meat extract more than 100 years ago [2]. It is primarily found in skeletal muscle where its content can be as high as 2 g/kg wet tissue or 0.2 % of overall skeletal muscle mass [3]. It is also present in other tissues like brain, kidney or spleen albeit at 10- to 100-fold lower concentrations [4]. Properties pertaining to the physiological roles of carnosine include its pH-buffering capacity, scavenging of reactive oxygen species (ROS) and peroxyl radicals, metal-ion chelation and protection against advanced glycation and lipoxidation end products (AGE and ALE) [3]. Notably, carnosine has also been demonstrated to reverse pre-formed glycated proteins [5]. In line with its role in muscle physiology, food supplements containing carnosine or its precursor beta-alanine improve exercise performance in untrained individuals [6] as well as in elite athletes [7,8]. Due to its metal-ion chelating and anti-oxidative properties, carnosine can ameliorate transition metal catalysed or hyperglycaemia mediated oxidative stress which may occur in several (pathologic) conditions such as diabetes and its complications [9]. Apart from nutritional intake, tissue carnosine concentration also depends on the activity of carnosine synthase, an ATP-dependent enzyme expressed in a variety of tissues, e.g. skeletal muscle, brain or kidney [10,11].

Carnosinase (CN1, EC 3.4.13.20) is the major carnosine-degrading enzyme. The human gene was cloned in 2003 and identified as the gene encoding the secreted form of carnosinase [12]. By positional cloning in a large Turkish family affected by DN, the *Carnosine Dipeptidase 1 gene (CNDP1)* was identified as a susceptibility gene for developing DN [13,14]. The leucine repeat in the signal sequence of *CNDP1* gene is essential for CN1 secretion with the short allelic variant $(CTG)_5$ associated with low serum CN1 concentrations. Importantly, patients with T2D without DN are significantly more frequently homozygous for the $(CTG)_5$ allele [15–18]. Since it was postulated that low CN1 activity might favour high tissue carnosine concentrations resulting in protection from DN, carnosine supplementation studies were carried out in diabetic rodent models. Indeed, these studies demonstrated therapeutic efficacy of carnosine to ameliorate diabetes and DN, as albuminuria and histological lesions were reduced after supplementation [19–26]. However, rodents – with the exception of Syrian hamster - lack the signal peptide in the CN1 gene and consequently do not have CN1 in the circulation [3]. This results in increased carnosine levels after oral carnosine supplementation. In contrast, carnosine supplementation in humans leads only to a small increase because of its fast degradation by serum CN1. Because of this difference, extrapolation of rodent findings to humans is difficult. As T2D patients have even higher serum CN1 activity levels compared to healthy controls [27], CN1 inhibition could be a potential strategy to increase the serum concentration of carnosine, especially in combination with carnosine supplementation. So far, this possibility has not been explored due to the lack of a specific CN1 inhibitor. Here we describe the identification and characterisation of a potent and selective CN1 inhibitor,

carnostatine, and its effect on serum CN1 activity and carnosine levels in serum and renal tissue after co-administration with carnosine.

Research Design and Methods

CN1 activity

CN1 activity was measured using a modified method described previously by Teufel et al. Details can be found in supplementary methods.

Transgenic Mice

Human CNDP1 transgenic mice with BTBR wt/ob background were used for the experiments (Jackson Laboratory Stock number: 004824) . These mice are expressing human CN1 mainly in the liver from where it is secreted into the blood. The generation and characterisation of these hCN1 over-expressing mice will be described in a separate paper. The animals were housed in a 12h light/dark cycle at 22 °C and sacrificed under isoflurane anaesthesia by neck dislocation.

SAN9812 in human and mice serum

To determine the concentration-response curve of SAN9812, serum from 2 healthy donors and 3 CN1 transgenic mice were used. SAN9812 was diluted with PBS and added to the serum before measuring the CN1 activity.

Pharmacokinetics in mice

Pharmacokinetic parameters were determined in female C57BL/6 mice (Harlan, Germany). After a single administration of either 3 mg/kg i.v. or 30 mg/kg s.c. of test compound dissolved in phosphate-buffered saline, pH 7.4, plasma samples were taken at eight time points over 24 hours. Three animals per time point were used. Concentrations of the test compounds were determined with an exploratory LC-MS/MS method using an internal standard. Pharmacokinetic parameters were calculated using the program WinNonlin 6.4 (Pharsight Corporation) assuming a non-compartmental model and linear trapezoidal interpolation.

Pharmacodynamics

The mice are injected with 30 mg/kg SAN9812 in a 0,9% NaCl solution subcutaneously. Before and after the injection blood was taken at different time points. The blood samples were incubated for 30min at room temperature before being centrifuged at 2500 g for 10min. The supernatant as serum was used afterwards to determine the CN1 activity. Two groups of animals with 3 animals each were used, one with activity levels all below 45 µmol/(ml*h) defined as the low activity group and one with activity levels all above 250 µmol/(ml*h) defined as high activity group. These two groups are depicted separately in the Figures.

Pharmacodynamics for one week

The mice are injected with 30 mg/kg SAN9812 in a 0.9% NaCl solution subcutaneously daily for one week. Before and after the injection serum was taken and CN1 activity was measured. 5 mice per group were used.

Carnosine concentration

CN1 overexpressing mice were supplemented with a bolus of 200 mg carnosine alone or in combination with 30mg/kg SAN9812 in a 0.9% NaCl solution by s.c. administration. hCN1-negative and hCN1-overexpressing mice served as controls (n = 5-8). Plasma samples were taken after 4h and after 8h. After 8h, mice were sacrificed and kidneys were harvested. Carnosine concentration was measured by high-performance liquid chromatography as previously described [33].

Figures

Figures were created using Microsoft Excel and Graphpad Prism 7.0. Error bars denote either standard deviation (SD) or standard error of the mean (SEM) as described in the individual figure legends.

Results

We screened a protease-directed small molecule library containing 6080 compounds for inhibitors of human recombinant CN1 using a screening assay based on the detection of histidine generated by proteolytic cleavage of carnosine (**supplementary methods**). The human recombinant CNDP1 showed a Km value of 190 µM (**supplementary figure 1**), similar to the previously reported value of 175 µM [28]. The average Z' value (±SD) of all 38 test plates was 0.85 ± 0.06 (**supplementary figure 2**), indicating that the assay was of excellent robustness [29]. Results are summarised in **Figure 1**. In total, we identified 133 compounds with >50 % inhibition. These compounds were re-tested in duplicates (**supplementary figure 3**)

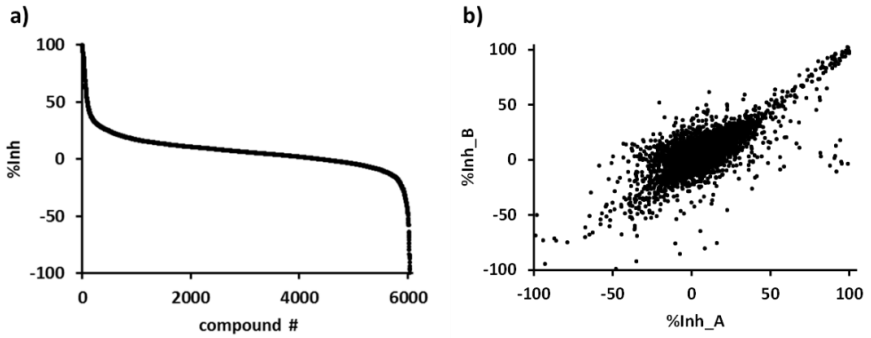

Figure 1: (a) Distribution of mean percent-inhibition values in the screen of 6080 compounds for inhibitors of human recombinant CN1. (b) Comparison of percent-inhibition values obtained in duplicate experiments.

One of the most potent compounds identified was SAN9812 (**Figure 2**), with an IC50 value of 18 nM on human recombinant CN1 at a carnosine concentration of 200 µM, i.e. close to the Km of 190 µM. SAN9812 was found to be a competitive inhibitor, showing a right-shift in its IC50 value with increasing carnosine concentration (**Figure 3a**). The Ki value of SAN9812 was determined as 11 nM (**Figure 3b**). The synthesis of SAN9812 is described in **supplementary figure 4** and **supplementary methods**.

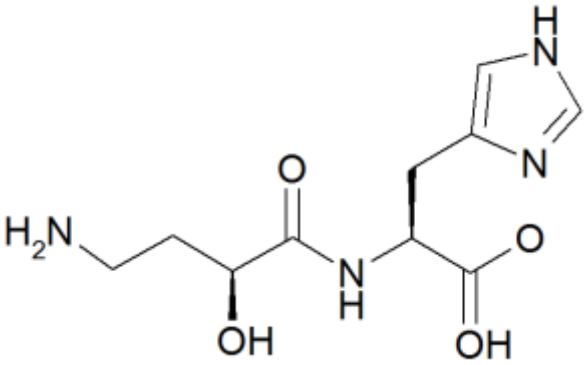

Figure 2 Structure of SAN9812

The inhibitory activity of SAN9812 on human CN1 was confirmed in serum from TG mice expressing human CN1 and serum from healthy human volunteers (**Figure 3c, d**). IC50 values were determined as 650 and 340 nM, respectively, at a carnosine concentration of 0.2 mg/ml or 880 µM. The average baseline hCN1 activities in transgenic mice and healthy human volunteers were 137 ± 25 µmol/ml/h and 1.1 ± 0.25 µmol/ml/h, respectively.

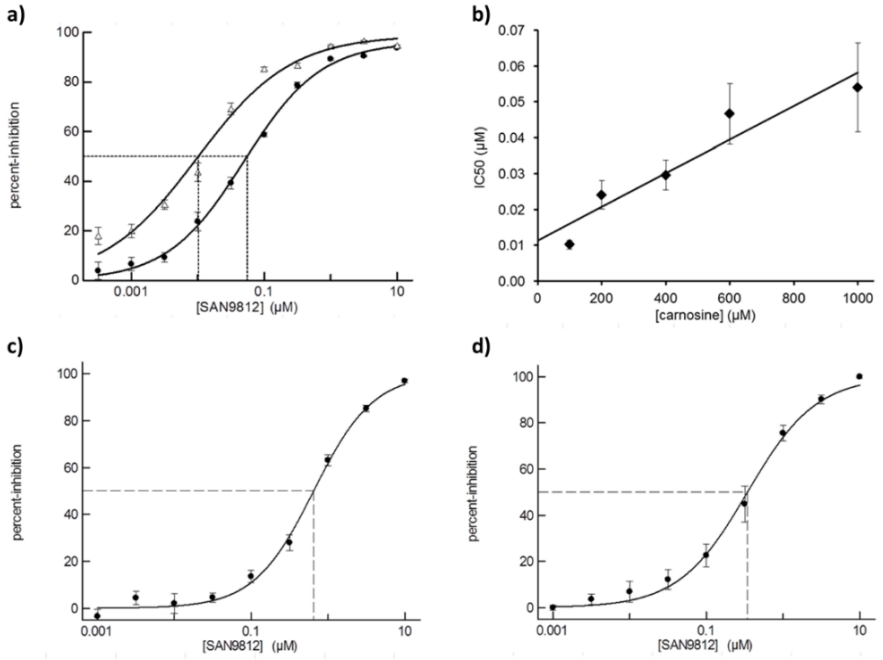

Figure 3: (a) Concentration-response curves for SAN9812 determined at 100 µM (open triangles) and 1000 µM (filled squares) carnosine with human recombinant CN1 in PBS (n=6 each), corresponding IC50 values were determined as 10 and 54 nM, respectively. (b) Dependence of SAN9812 IC50 values on carnosine concentration (n=6 each). The Ki values defined as the intersection of the linear regression line (R2=0.91) with the y-axis was determined as 11 nM. (c) Concentration-response curve of SAN9812 in serum from transgenic mice expressing human CN1 (3 replicates from three mice), and (d) in human serum (3 replicates from 2 subjects) at a carnosine concentration of 880 µM (0.2 mg/ml). IC50 values were determined as 650 and 340 nM, respectively. Data are presented as mean ± SEM.

Of note, SAN9812 was found to be inactive on a panel of 33 different receptors, channels transporters and enzymes (supplementary table 1) when tested at a concentration of 10 µM. The ADME/PK (absorption, distribution, metabolism, excretion and pharmacokinetics) profile of SAN9812 is summarised in table 1. The compound is very stable in human, rat and mouse liver microsomes and shows moderate clearance in primary human hepatocytes. Permeability in the CaCo-2 assay as a measurement of intestinal permeability was determined as 17.9 nm/sec, indicating moderate oral absorption potential.

Plasma levels of SAN9812 after single bolus administration of either 3 mg/kg i.v. or 30 mg/kg s.c. are shown in **Figure 4a**. After subcutaneous injection, maximal plasma concentrations were achieved already at the first sampling time point after administration (15 min). The maximal compound concentration after s.c. administration was 80 µg/ml, corresponding to approximately 300 µM. Based on the area under the curve, subcutaneous bioavailability was determined as 42.5 % (table 1). Plasma levels quickly decrease to 30 µg/ml (120 µM) after 30 min, 5.7 µg/ml (22 µM) after 1h, and 350 ng/ml (1.4 µM) after 2h. The lower level of quantification for the detection method was 10 ng/ml (40 nM).

In a subsequent pharmacodynamics study, CN1 activity was measured after a single subcutaneous bolus of 30 mg/kg SAN9812, at the same time points as previously in the PK experiment. Results are shown in **Figure 4b** and **4c**. Animals were stratified according to their baseline serum carnosinase activity into a high activity (>40 µmol/ml/h) and a low activity (<40 µmol/ml/h) group. Mean baseline activities in the two groups were 259 ± 13 and 7.4 ± 4.9 µmol/ml/h, respectively. Administration of SAN9812 led to a rapid reduction in serum carnosinase activity by more than 90% in both groups which was sustained for >8 hours. At the last sampling time point 16 hours after compound administration, serum carnosinase activity was still reduced by 40 % in the low-activity group and by 75 % in the high activity group.

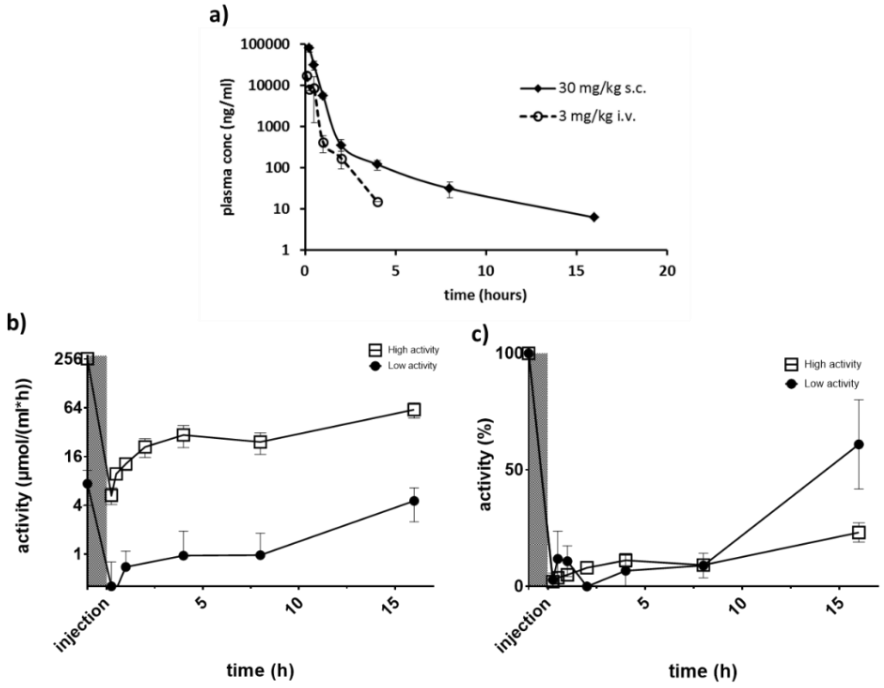

Figure 4: (a) Plasma levels of SAN9812 after single bolus administration of either 3 mg/kg i.v. or 30 mg/kg s.c. (n=3 each). The lower level of quantification for the detection method was 10 ng/ml (40 nM). Data are shown as mean ± standard deviation. (b), (c) Serum carnosinase activity before and after a single s.c. bolus injection of 30 mg/kg SAN9812. Absolute activities are shown in (b) for mice with low serum carnosinase activity (<45 µmol/ml/min, n=3) or high serum carnosinase activity (>250 µmol/ml/min, n=3). Percent-activity values from the same set of experiments are shown in (c). Data are depicted as mean ± SEM.

SAN9812 was then subcutaneously administered over seven days once-daily at a dose of 30 mg/kg. Blood samples were taken on days 1, 4 and 7 before, 6 h and 12 h after the injection to measure CN1 activity. The results are depicted in **Figure 5**. Again, CN1 activity was strongly and rapidly reduced after the first SAN9812 injection on day 1 and remained low over a period of >12 hours. Importantly, baseline CN1 activities on days 4 and 7, measured 24 hours after the previous SAN9812 administration on days 3 and 6, respectively, were considerably lower compared to CN1 activity on day 1 before the first injection, indicating sustained carnosinase inhibition over 24h in both the low-activity and high-activity groups.

The same average inhibition in percent was achieved in the group with low baseline activity as in the group with high baseline activity. In mice with low baseline activity, CN1 activity were efficaciously inhibited over the whole period with an activity level kept below 10 µmol/(ml*h). In the group with high baseline activity levels, absolute CN1 activity could sometimes exceed 100 µmol/(ml*h) between injections, although percent-inhibition values were comparable in all animals (**Figure 5**).

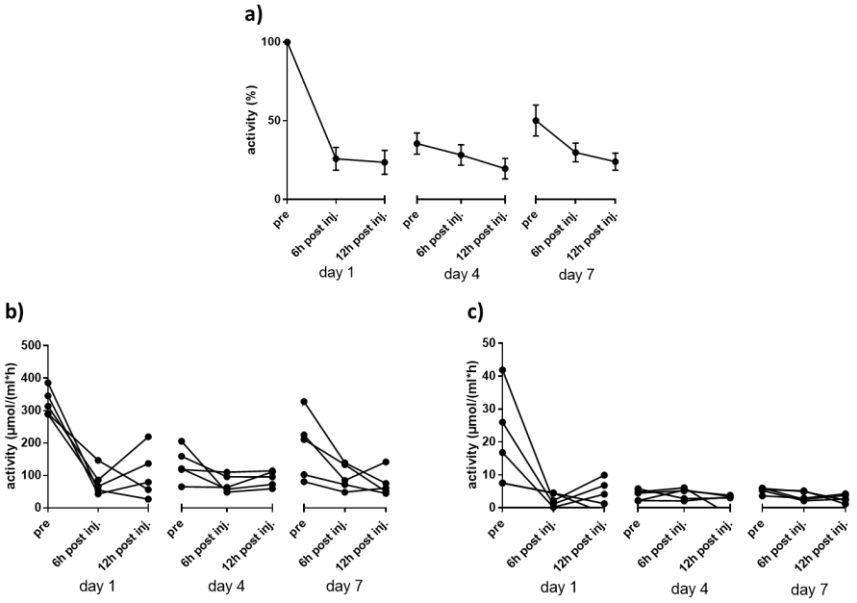

Figure 5: Subchronic (7d) administration of SAN9812 (30 mg/kg s.c. q.d.) inhibits serum CN1 activity. (a) Serum CN1 activity (relative to day 1 baseline) prior to, 6 and 12 hours after compound administration on days 1, 4 or 7 for all mice investigated (n=10, mean ± SEM). (b, c) Individual absolute CN1 activities for the high-activity (b, n=5) and the low-activity (c, n=5) group of hCN1-expressing transgenic mice.

To determine whether serum CN1 inhibition by SAN9812 can increase carnosine concentration in vivo in hCN1 overexpressing mice, animals were given a single bolus of 200 mg carnosine alone or in combination with 3 0mg/kg SAN9812 per s.c. injection. Plasma samples were taken 4h and 8h after injection. Kidneys were harvested 8 h after injection. CN1-negative wildtype mice and hCN1 mice without treatment served as controls.

Compared to wildtype animals, hCN1 TG mice had significantly lower plasma carnosine levels (**Figure 6a, 6b**). A similar, but non-significant trend was also seen for the kidney (**Figure 6c**). As shown in **Figure 6a**, in hCN1 mice after a single subcutaneous bolus of carnosine, the plasma carnosine concentration increased compared to hCN1 overexpressing controls (p=.049) 4h after injection but was still lower compared to wildtype mice (p=.058). In contrast, a concurrent administration of carnosine and SAN9812 led to a further increase in carnosine plasma concentration, up to the levels of hCN1-negative controls 4h after administration. 8h after concurrent administration of carnosine and SAN9812, plasma carnosine levels had returned to levels of hCN1 controls probably due to residual CN1 activity (mean=14.4µmol/ml/h). Mean CN1 activity in hCN1 mice was 55.3 µmol/ml/h.

As shown in **Figure 6c**, in the kidney concurrent administration of carnosine and SAN9812 significantly increased carnosine concentration (mean ± SD = 3.5 ± 2.3 nmol/mg) compared to wildtype controls (0.32 ± 0.46 nmol/mg), hCN1 overexpressing mice (0.034 ± 0.028 nmol/mg) and also hCN1 overexpressing mice supplemented with carnosine (0.52 ± 1.2) (p<.005). Of note, kidneys were removed 8h after injection when plasma carnosine levels had already returned to normal.

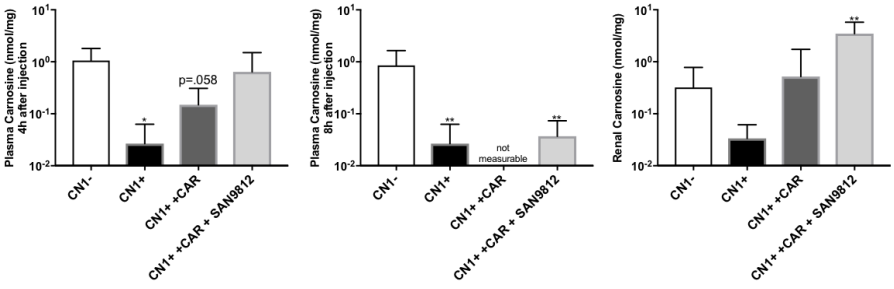

Figure 6: Plasma & renal tissue carnosine concentration upon subcutaneous carnosine supplementation (CAR) with and without SAN9812 administration in hCN1 overexpressing (CN1+) mice (n = 5-8 per group). hCN1- negative wildtype mice (CN1-) served as controls. The lower level of quantification for the detection method was 0.0001 nmol/mg. Data are shown as mean ± SD. (a) Plasma carnosine concentration 4h and (b) 8h after administration of carnosine +/- SAN9812. (c) Carnosine concentration in the kidney harvested 8h after injection. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism.

Discussion

In this work, we discovered carnostatine (SAN9812) as a potent and selective CN1 inhibitor with a Ki of 11 nM for human recombinant CN1. Subcutaneous injection of SAN9812 led to a sustained reduction in serum carnosinase activity in transgenic mice overexpressing human CN1, both acutely as well as upon subchronic treatment. Additionally, a combination of carnosine and carnostatine caused a further increase in serum carnosine concentration and, for a disease such as DN most importantly, an increase of carnosine levels in renal tissue.

CN1 has attracted considerable interest through the discovery in humans that a polymorphism of CN1 associated with lower serum CN1 concentrations protects against DN. In keeping with the hypothesis that low serum CN1 concentration may protect patients with type 2 diabetes from developing DN, the rationale of the current study was to generate a potent CN1 inhibitor as a potential drug candidate for preventing or slowing down the progression of DN.

The protective effect of a genotype with lower CN1 activity, as shown in large cross-sectional studies, is likely mediated by an increase in serum carnosine concentration. To increase serum carnosine, many carnosine supplementation studies have been conducted in rodent models. However, as rodents lack the signal peptide in the CN1 gene and consequently do not have CN1 in the circulation [3], the translation of these findings to humans is difficult. Unlike rodents, humans have considerable serum CN1 activity which may even be higher in particular disease states, e.g., in diabetic individuals compared to healthy controls [27]. Thus, as observed in healthy volunteers, carnosine supplementation only leads to a moderate increase in plasma carnosine for a limited period of time between 1h and 2h because of its rapid degradation by CN1 [30] that may markedly limit carnosine's beneficial effects in humans.

Several human trials in the field of sports medicine have been conducted, either with carnosine or beta-alanine, the rate-limiting precursor for endogenous carnosine synthesis and showed beneficial effects on exercise performance [6] [7,8]. However, effects were moderate which is likely due to rapid carnosine turnover and thus a limited increase in tissue carnosine levels. Although muscle carnosine was not measured in our study, the marked increase in renal tissue concentration of carnosine by simultaneous administration of carnosine and a CN1 specific inhibitor suggests that our approach may also enhance the beneficial effects of carnosine in other tissues.

Other human trials have shown a positive effect of carnosine supplementation on insulin sensitivity in obese human individuals [31,32]. Because of the reasons given above, an additional CN1 inhibition may further increase these beneficial effects. So far, this has not been studied because of the lack of a specific CN1 inhibitor. Here we describe SAN9812, a specific CN1 inhibitor as a new tool for such studies. As SAN9812 can stabilise carnosine levels

both in plasma and kidney tissue after co-administration with carnosine, we propose to use the name carnostatine for SAN9812.

To our knowledge, this is the first in-vivo active carnosinase inhibitor described. This CN1 inhibitor is potent with a Ki of 11nm and specific with no reactivity on a panel of 33 different receptors, channels transporters and enzymes. So far, our studies were limited to healthy animals and short treatment periods. It will be interesting to see whether chronic carnosinase inhibition, especially in combination with carnosine supplementation, can protect from diabetic nephropathy as seen in carnosine supplementation studies in mice devoid of plasma CN1 activity. Such studies are currently underway.

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Conflict of interest statement

A.K., S.R. and M.T. are or were employed by Sanofi Aventis Deutschland GmbH.

Ethics statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The experiments were approved by the local Ethical Committee.

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Table 1: ADME and PK profile of SAN9812

Metabolic stability h/r/m microsomes (% remaining)	100/94/99
Human hepatocyte clearance (ml/h/10^6 cells)	0.032
Caco-2 permeability (nm/sec)	17.9
Clearance (mL/min/kg)	0.344
Vdss (L/kg)	0.122
F% (s.c.)	42.5
Selectivity panel	Inactive[a]

Supplementary Methods

CN1 high-throughput-screening (HTS)

The carnosinase assay described in Bando et al. 1984 and Teufel et al. 2003 was adapted to high-throughput conditions with 10 µl reaction volume in 384-well small volume microtiter plates. In brief, the assay was carried out as follows: 3 µl of human recombinant CNDP1 (ThermoFisher Scientific, Germany) was added to 3 µl solution containing the test compound. The reaction was started by addition of 4 µl carnosine (Sigma-Aldrich, Germany). The reaction was carried out in 100 mM sodium phosphate, pH 7.1, 0.01 % bovine serum albumin. For the high-throughput screen, concentrations of carnosine and carnosinase in the reaction volume were 200 µM and 0.1 µg/ml, respectively. After incubation at 37 °C for 45 min, the reaction was stopped by addition of 5 µl 1% trichloroacetic acid, and histidine generated in the enzymatic reaction was derivatised by addition of 5 µl o-phthaldialdehyde and incubation for 30 min at room temperature. The concentration of the resulting Schiff Base was determined fluorometrically (ex=365 nm, em=440 nm) using a Tecan Infinite plate reader. The compound screening was done in duplicate. High controls with reaction buffer instead of compound solution (0 % inhibition) and low controls reaction buffer instead of compound and carnosinase (100 % inhibition) were present on every assay plate. Robustness of the assay was assessed by calculating Z' values as described by Zhang et al.

CN1 activity

Briefly, carnosine dissolved in 50mM pH=7,5 Tris buffer was added to serum to an end concentration of 0,2 mg/ml (880µM) on ice. The final solution was divided into three aliquots. To stop the enzymatic reaction, 10% sulfosalicylic acid was added to one aliquot instantly to an end concentration of 2 % and mixed by vortexing. The other two aliquots were incubated in the water bath at 30 Celsius for 5min and 15min before the addition of sulfosalicylic acid. All the aliquots are put on ice immediately after the addition. All aliquots were incubated on ice for 30min, followed by centrifugation at 12000g for 5min. The supernatant with the released histidine was mixed with a 1:1:1 solution of 50mM pH=7,5 Tris buffer, 1% trichloroacetic acid in distilled water and 5mg/ml o-phtaldialdehyd in 2N NaOH. Together with a standard row of known histidine concentrations, the samples are incubated again in the water bath for 30min at 30 Celsius before the fluorescence is measured at an excitation length of 360nm and an emission length of 465nm with an integration time of 40 microseconds. Enzyme activity was calculated using a linear regression curve based on the three time points (0min, 5min and 15min).

SAN9812 synthesis

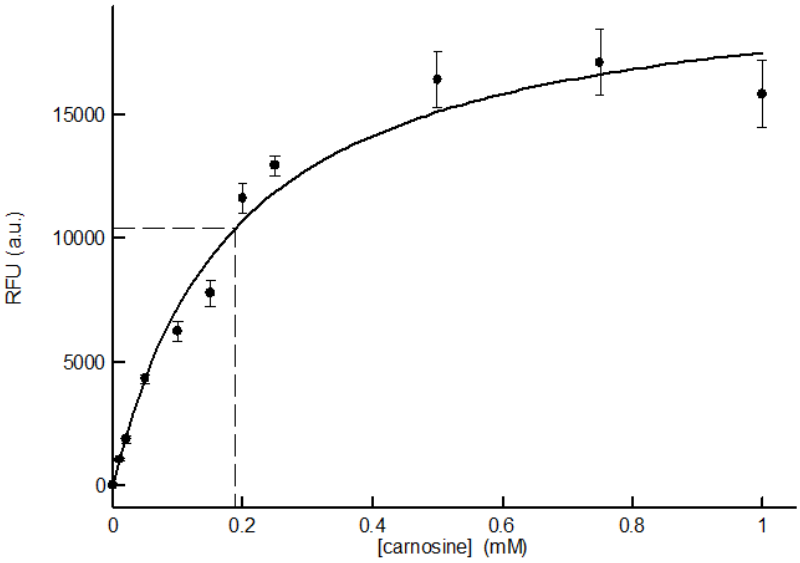
DCM (500 mL) was added to a vessel containing Fmoc-His(Trt)-OH (80 mmol, 50 g) and CTC resin (80 mmol scale, 80 g) to swell, DIEA (320 mmol, 56 mL) was added subsequently. The resin solution was mixed for 2 hours and washed with DMF three times. 20% piperidine in DMF was used for deblocking.

(2S)-4-(tert-butoxycarbonylamino)-2-hydroxy-butanoic acid (26.31 g, 120.00 mmol, 2.00 Eq) was coupled using HATU (43.35 g, 114.00 mmol, 1.90 Eq) and DIPEA (4 eq) as activator reagents for 1 hour. Then the resin was washed with DMF (400 mL) three times. Ninhydrin test was used to verify the reaction.

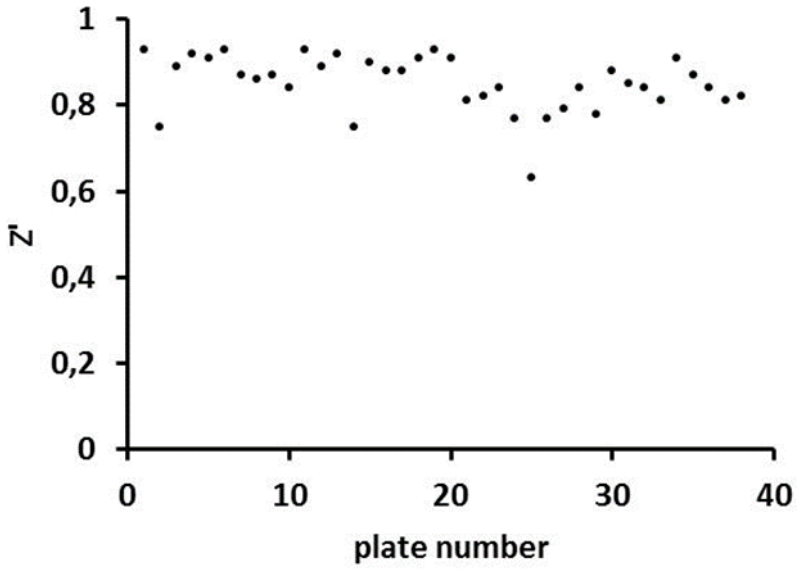
After washing with MeOH (500 mL, 3 times) and drying under vacuum, the resin was treated with 1%TFA in DCM (2000 mL) for 30 min (10 min, 3 times). The pH was adjusted to 7-8 by progressively adding DIPEA. The mixture was concentrated on a rotary evaporator to give the side chain protecting crude peptide. The crude was purified by preparative reverse phase HPLC (A: 0.1%TFA in H2O, B: ACN).

The side chain protecting peptide was treated with cleavage cocktail (TFA: TIS: H2O=90:5:5, 150 mL) for 2 h. The peptide was precipitated with cold 2-isopropoxypropane (1.5 L) and centrifuged (6000 rpm, 2 min). The supernatant was decanted and the precipitate was washed one more time (100mL). The peptide was dried under vacuum for 2 hours, and then dissolved in 0.5% HCl in H2O, and lyophilized to give the final product (2.16 g, 34.8% yield).

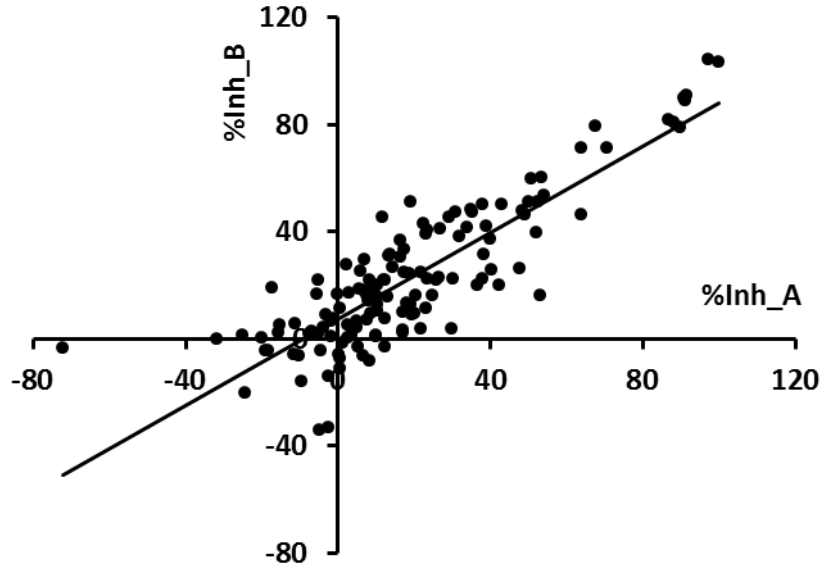
Supplementary Figures



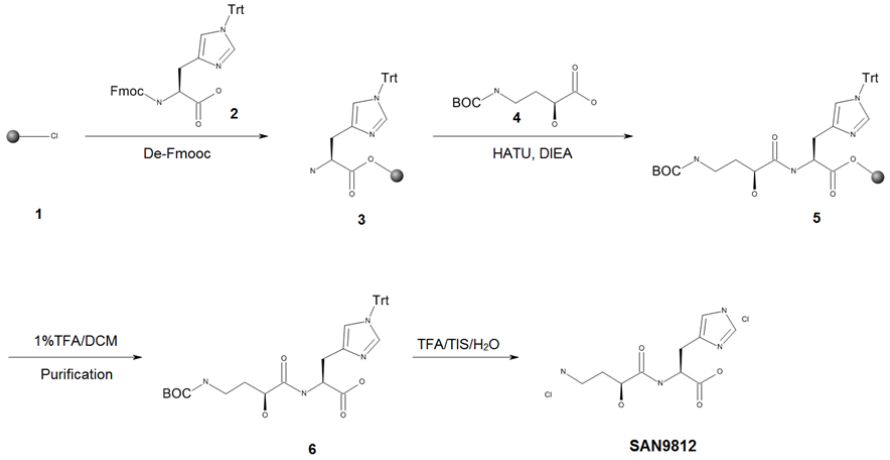
Supplementary figure 1: Enzyme kinetics for human recombinant CNDP1. The Km value is indicated by the dashed line and was determined as 190 µM.



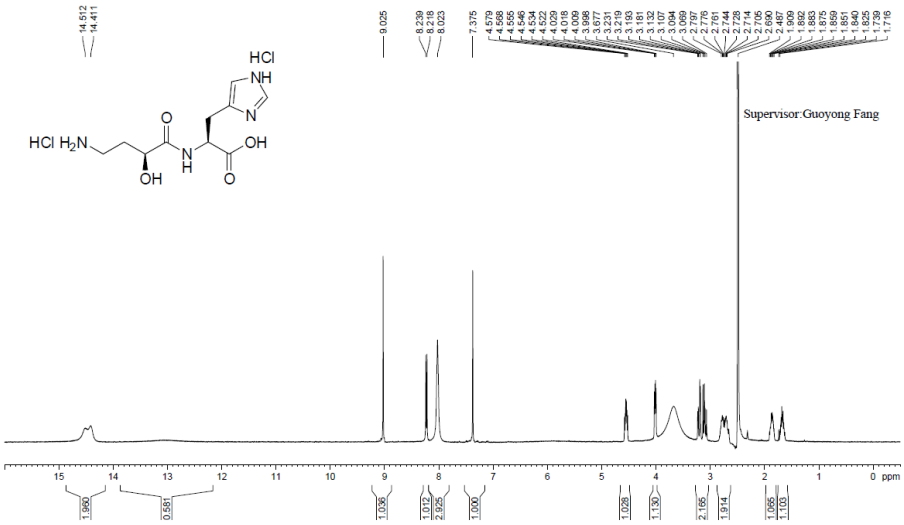
Supplementary figure 2: The average z-value of the plates was 0.85 +/- 0.06 indicating an excellent quality of the assay.



Supplementary figure 3: Re-test of HTS hits in duplicate.



Supplementary figure 4: Synthesis scheme for SAN9812



Supplementary figure 5: 1H-NMR spectrum of SAN9812 (400 MHz) in DMSO-d6

Supplementary Tables

Supplementary table 1: Selectivity profile of SAN9812 (at 10 µM) vs a panel of 33 receptors, channels, transporters and enzymes

	E% (agonistic)	I% (antagonistic or inhibitory)
cGMP-inhibited 3,5-cyclic phosphodiesterase A (PDEA3)		-14.5
Acetylcholinesterase (AChE)		-8.1
Adenosine Receptor 1 (ADORA1)	-0.7	-0.6
Adenosine Receptor 2A (ADORA2A)	-1.5	10.0
adrenergic, alpha-1A-, receptor (ADRA1A)	-3.0	-10.2
Adrenoreceptor alpha 2A (ADRA2A)	1.1	-8.9
Adrenoreceptor beta 1 (ADRB1)	1.4	3.0
Adrenoreceptor beta 2 (ADRB2)	-2.6	-1.3
Ca2+ Channel (L-Type DHP Site)		-0.3
Cannabinoid Receptor 1 (CB1)	12.9	1.6
Cannabinoid Receptor 2 (CB2)	18.6	-4.8
Cl- Channel		6.3
Dopamine Receptor 1 (D1)	8.7	0.2
Dopamine Receptor 2 Short (D2S)	5.1	9.2
Dopamine Transporter		1.8
Glycine Receptor (strychnine-sensitive)		-3.8
Histamine Receptor 1 (H1)	-1.7	-8.7
Histamine Receptor 2 (H2)	-1.0	1.7
K+ Channel Voltage Dependent		-2.0
Mono Amine Oxidase A (MAO-A)		-14.8
Muscarinic Receptor 1 (CHRM1)	-2.8	-1.1
Muscarinic Receptor 2 (CHRM2)	1.2	-8.8
Muscarinic Receptor 3 (CHRM3)	-0.9	-3.8
Na+/K+ ATPase Pump		-0.5
Nicotinic AChR alpha4 beta2 (nAChRa4b2)		-9.1
Nicotinic AChR Receptor Muscle-Type		-0.6
Norepinephrin (Noradrenalin) Transporter		-4.6
Opioid Receptor mu (OPRM1)	-20.8	-8.2
Phencyclidine Receptor (PCP)		-8.5
Serotonin Receptor 1A (5HT1A)		
Serotonin Receptor 2A (5HT2A)	-0.4	-2.5
Serotonin receptor 2B (5HT2B	-0.5	5.5
SK+Ca channel-		2.7

Part II

Carnosinase 1 in patients with diabetes mellitus