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Discovery of Small Molecule Vanin Inhibitors: New Tools To Study Metabolism and Disease

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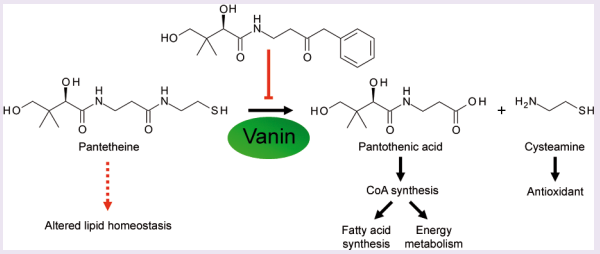
Supporting Information

ABSTRACT: Vanins are enzymes with pantetheinase activity and are presumed to play a role in the recycling of pantothenic acid (vitamin B5) from pantetheine. Pantothenic acid is an essential nutrient required to synthesize coenzyme A, a cofactor involved in many biological processes such as fatty acid synthesis and oxidation of pyruvate to fuel the citric acid cycle. Hydrolysis of pantetheine also liberates cysteamine, a known antioxidant. Vanin-1 is highly expressed in liver and is under transcriptional control of PPAR-α and nutritional status, suggesting a role in energy metabolism. The lack of potent and specific inhibitors of vanins has hampered detailed investigation of their function. We hereby report the design, synthesis, and characterization of a novel pantetheine analogue, RR6, that acts as a selective, reversible, and competitive vanin inhibitor at nanomolar concentration. Oral administration of RR6 in rats completely inhibited plasma vanin activity and caused alterations of plasma lipid concentrations upon fasting, thereby illustrating its potential use in chemical biology research.



Members of the vanin (*VNN*) gene family encode proteins with pantetheinase activity.[1,2] In humans, three vanins have been described, two of which have confirmed enzymatic activity (vanin-1 and vanin-2).[3,4] The natural substrate of vanins is pantetheine, which is hydrolyzed to pantothenic acid (also called vitamin B5 or pantothenate) and cysteamine. On the basis of sequence similarities, vanins were classified as members of the biotinidase branch of the nitrilase superfamily.[5] Nitrilases are thiol enzymes that perform their hydrolytic reactions through a catalytic triad consisting of Glu, Lys, and Cys, where the cysteine nucleophile produces a covalent intermediate. Although vanins are poorly characterized at the functional level, their primary function is assumed to be in pantothenate recycling, which is a necessary factor in the synthesis of Coenzyme A (CoA).[6] Recent investigations, however, have shown that at least murine vanin-1 has additional functions.[7] *Vnn1* knockout mice did not have an obvious spontaneous phenotype but were resistant to intestinal inflammation, oxidative stress, and experimental colitis.[7−9] Other conditions with a putative role of vanins include malaria susceptibility,[10] psoriasis,[11] carcinogenesis,[12] and cardiovascular disease.[13] Vanins are the only known source of pantetheinase activity in mammalian tissues. Interestingly, the *Vnn1* gene was shown to be one of the major targets of PPAR-α in mouse liver,[14] suggesting a role for vanin-1 in metabolism.[15]

The lack of selective high-affinity vanin inhibitors has so far hampered investigation of vanin biology *in vitro* and *in vivo*. Recently, an inhibitor screen of Sigma's Library of Pharmacologically Active Compounds (LOPAC library) identified six compounds with anti-pantetheinase activity. These comprised widely different chemotypes with IC_{50} values toward recombinant human vanin-1 in a range of 4−20 μM.[16] All of these compounds had other known pharmacological activities or cytotoxic properties that would preclude their use as specific vanin inhibitors. These observations emphasize the need for selective and potent vanin inhibitors as tools in chemical biology, to study the role of vanins in human biology and to evaluate these enzymes as potentially druggable targets. Herein, we report the design of pantetheine analogues as reversible, competitive inhibitors of mammalian vanins. It is shown that RR6, our most potent vanin inhibitor, is selective and displays excellent bioavailability and potency *in vivo*.

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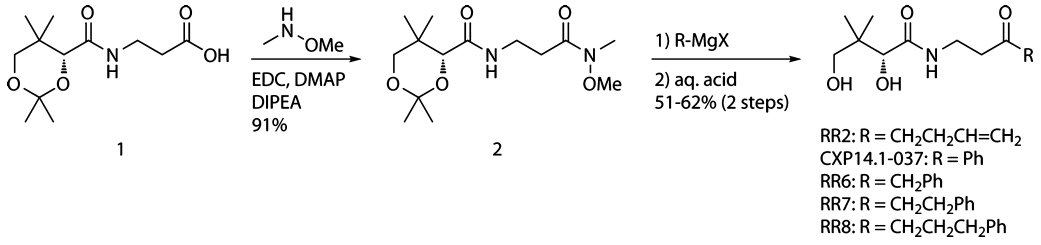
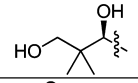
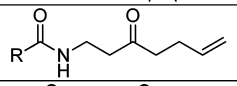
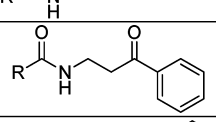
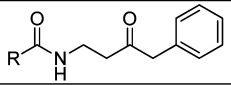
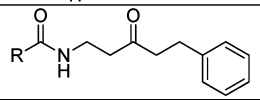
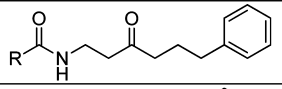
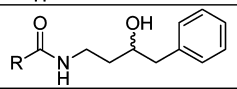


Figure 1. Weinreb ketone synthesis strategy including yields.

Table 1. Structures of Pantothenate Derivatives and IC_{50} Values for Pantetheinase and Biotinidase Inhibition

Compound	Structure R = 	Rec. hVNN1 pantetheinase IC_{50} (μM)[a]	Human serum pantetheinase IC_{50} (μM)[a]	Fetal bovine serum pantetheinase IC_{50} (μM)[a]	Rat serum pantetheinase IC_{50} (μM)[a]	Human serum Biotinidase IC_{50} (μM)
RR2		16 (8.8 - 27)	2.8 (2.4 - 3.4)	1.0 (0.86 - 1.3)	1.0 (0.80 - 1.3)	30
CXP14.1-037		70 (40 - 122)	18 (15 - 20)	10 (7.9 - 14)	4.7 (3.7 - 5.9)	>200
RR6		0.54 (0.43 - 0.68)	0.040 (0.037 - 0.043)	0.041 (0.029 - 0.057)	0.087 (0.064 - 0.12)	>200
RR7		2.6 (1.8 - 3.6)	0.11 (0.088 - 0.13)	0.33 (0.22 - 0.48)	0.25 (0.16 - 0.41)	>200
RR8		1.6 (0.93 - 2.8)	0.27 (0.23 - 0.32)	0.17 (0.13 - 0.23)	0.22 (0.16 - 0.30)	>200
CXP14.1-034		9.9 (6.6 - 15)	1.7 (1.4 - 2.1)	3.5 (2.5 - 4.8)	4.7 (0.35 - 0.99)	>200

[a] IC_{50} (95% CI), n = 3

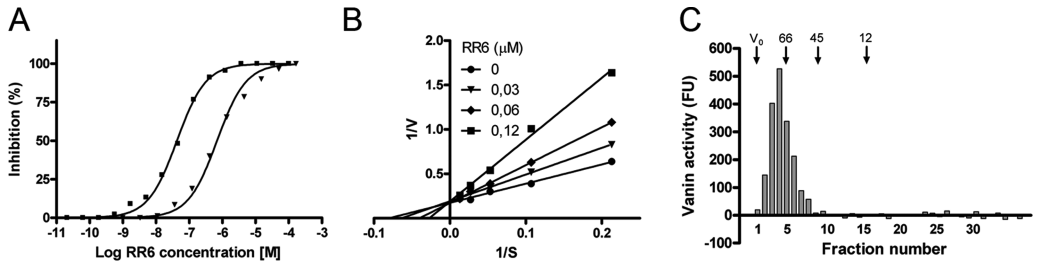


Figure 2. Characterization of the pantetheinase/vanin inhibitor RR6. (A) Inhibition of pantetheinase activity of recombinant human vanin-1 (▼) and bovine serum vanin (■) by RR6, using the synthetic substrate AMC-Pan. (B) A Lineweaver–Burk plot of reaction velocities at different substrate concentrations and inhibitor concentrations indicates that RR6 is a competitive inhibitor. The kinetic constants K_m (12 μM) and the V_{max} (6 pmol/min) were read from this graph. Bovine serum was used as source of vanin activity. (C) Superdex-75 chromatography of fetal bovine serum in the presence of excess RR6. Complete recovery of vanin activity in the high molecular weight fraction was obtained, indicating a reversible binding of RR6. V_0: void volume of the column. Molecular mass markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome c (12 kDa). FU: fluorescence units.

We reasoned that pantetheine analogues lacking the amide bond, but containing a warhead that would form a reversible covalent bond with the cysteine residue of the catalytic triad, could potentially interfere with vanin activity. We synthesized compounds substituting the amide bond by a keto group, a known warhead for proteases,[17] by applying the Weinreb ketone synthesis strategy (Figure 1). Conversion of partially protected pantothenic acid **1** into Weinreb amide **2** allowed the addition of a variety of Grignard reagents. After acid-mediated deprotection of the pantoic acid moiety a series of potential vanin inhibitors was obtained. (Table 1 and Supplementary Methods for details of chemical structures and synthetic procedures). Compounds were tested for vanin inhibition using pantothenate-7-amino-4-methylcoumarin (AMC-Pan) as a highly sensitive fluorogenic substrate.[16] As sources of vanin activity we used recombinant human vanin-1 as well as serum. Vanin-1, which is highly expressed in epithelia as a membrane-bound GPI-anchored ectoenzyme, was cloned without the C-terminal GPI-anchoring site and expressed in a eukaryotic system as a 6-His tagged, secreted protein (Supplementary

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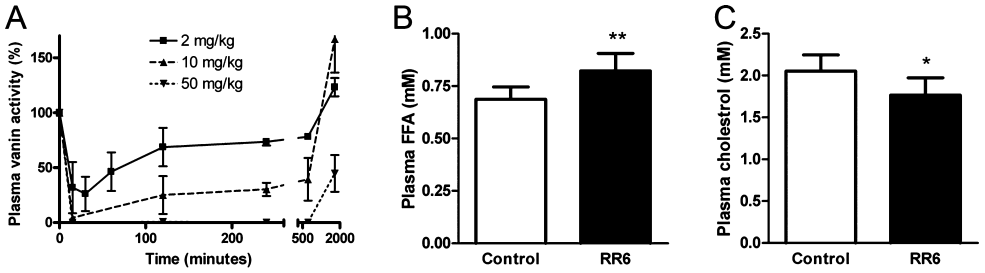

Figure 3. Pharmacodynamics of RR6 in rats. (A) Inhibition of plasma pantetheinase activity by RR6 following oral administration to rats at the indicated doses. Plasma vanin activity is dose-dependently inhibited, as determined by hydrolysis of the synthetic AMC-Pan substrate *ex vivo*. (B) RR6 in drinking water (3 mg mL^{-1}) increases plasma free fatty acid levels in rats after a 24-h fast as compared with rats without RR6 in the drinking water (control). Values are means ± SD (n = 7). (C) RR6 in drinking water (3 mg mL^{-1}) reduced plasma cholesterol levels after a 24-h fast in rats as compared with rats without RR6 in the drinking water (control). Values are means ± SD (n = 7). *P < 0.05; **P < 0.01.

Figure 1). Affinity-purified vanin-1 was used for subsequent inhibitor assays. Attempts to express enzymatically active human vanin-2 were unsuccessful. We used serum (human, rat, and bovine) as a natural, physiological source of pantetheinase activity. Serum pantetheinase activity likely represents vanin-2 (originally called GPI-80),[18] which is known to be present in a secreted form.[19] From our first series of compounds, RR2 was identified as a hit compound, showing IC_{50} values in the low micromolar range. Limited optimization of RR2 (Table 1), replacing the alkenyl moiety by an aromatic residue (RR6 compound), yielded a 30-fold improvement of the IC_{50} value towards recombinant vanin-1 down to the nanomolar range. When serum was used, an even lower IC_{50} (40 nM for human serum) was observed (Figure 2A). Reduction of the keto group to a hydroxyl (CXP14.1−034) caused a strong decrease of the potency, supporting the idea that the keto group has a warhead function. The chain length between the keto group and the phenyl moiety clearly affected the potency, as indicated by the reduced potency of CXP14.1-037, RR7, and RR8. One carbon atom turned out to be optimal, as increasing the chain length showed a decrease in potency, while leaving out the linker led to a similar result (Table 1).

In a next step we sought to characterize the mechanism of binding. As the inhibitor is an analogue of its natural substrate we assumed that inhibition would be competitive. Figure 2B shows that, using RR6 as an inhibitor, this is indeed the case. A Lineweaver−Burk plot of the data indicated that RR6 did not change V_{max} but caused an increase of the apparent K_m. To investigate the reversibility of binding, we used serum diluted in PBS with excess RR6 (100 μM) to achieve complete inhibition of vanin activity. Gel permeation chromatography of this sample showed complete recovery of serum vanin activity in the high molecular weight fractions, indicating a reversible binding of RR6 (Figure 2C).

To characterize the selectivity of RR6, we first tested biotinidase, an enzyme that has the highest structural similarity to vanins. Only RR2 has significant biotinidase inhibiting activity (IC_{50} = 30 μM), whereas none of the other compounds showed activity up to the highest concentration tested (200 μM) (Table 1). As the scissile bond between pantothenate and the AMC group resembles a peptide bond, and knowing that nitrilases use a cysteine in their active site, we considered the possibility that cysteine proteases could hydrolyze the AMC-Pan substrate. None of the ubiquitous human cysteine proteases cathepsin B and L nor the plant cysteine protease papain hydrolyzed the AMC-pan substrate. RR6, up to the highest concentration tested (200 μM) did not inhibit the activity of any of these cysteine proteases toward their own specific synthetic substrates. Similarly, we did not observe any inhibition of serum vanin activity by the generic cysteine protease inhibitor E-64 or the generic serine protease inhibitor PMSF.

To investigate the *in vivo* potency of our lead compound RR6 on vanin inhibition and subsequent downstream biological effects, we first investigated its pharmacodynamic properties. Oral administration in rats showed a strong dose-dependent inhibition of plasma vanin activity (Figure 3A). After a single oral dose of 50 mg/kg, a prolonged complete inhibition of plasma vanin activity was achieved that lasted up to 8 h after the initial dose. No adverse effects on the animals were noted at any dose given. These data suggested that RR6 could be used as a tool to study *in vivo* biological processes that involved vanin activity. In order to facilitate animal-friendly and easy experimentation, we investigated whether RR6 could be given in the drinking water of rats to achieve prolonged complete vanin inhibition without the need of multiple daily drug administrations. We found that dosing of RR6 at 3 mg mL^{-1} in rats caused a nearly complete inhibition of plasma vanin activity (Supplementary Figure 2).

Finally, we investigated if vanin inhibition by RR6 could affect a biological process. It is not known if vanins have nonredundant biological functions in normal physiology, as the *Vnn1* knockout mouse displays a phenotype only upon challenge (infection, inflammation, cancer). Previous studies have shown that pantethine, the dimer of pantetheine, the presumed natural substrate of vanins is involved in lipid metabolism.[20,21] This suggested to us that pharmacological modulation of vanin activity could have an effect on lipid homeostasis. Similary, a number of studies have shown that vanin-1 expression in liver is induced by peroxisome proliferator activated receptor (PPAR)-α agonists. PPARs are ligand activated transcription factors and play an important role in nutrient homeostasis. Oral administration of triglycerides or PPAR-α ligands such as WY14643 and fenofibrate caused a strong increase of vanin-1 expression in mouse liver.[14] Although the functional effect of vanin-1 upregulation in PPAR-α dependent metabolic changes is completely unknown and unexplored, these findings also suggested a possible role of vanin-1 in lipid homeostasis. Moreover, a recent study has shown that fasting induces a huge increase in liver vanin-1 expression in wild type mice.[15] We therefore investigated the effect of RR6 administration on lipid metabolism following fasting. Rats were given RR6 for 4 days in the drinking water (3

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mg mL^{-1}) and were subjected to fasting for 24 h at day 4. Analysis of a limited set of metabolites in plasma showed modest but statistically significant changes such as an increase in plasma free fatty acids (FFA) and a decrease of plasma cholesterol (Figure 3B and C) as compared with rats without RR6 in drinking water (control). Plasma glucose levels were not altered. Upon fasting, lipids are mobilized from tissue fat stores as a source of energy, thereby causing an increase of plasma FFA and liver steatosis. At the same time fasting will induce high levels of liver vanin-1 activity in a PPAR-α-dependent manner. This suggests a role of vanin-1 in mobilization and/or conversion of triglycerides, fatty acids, and cholesterol. Detailed investigation of these metabolic changes was beyond the scope of this report, but these findings clearly offer a starting point to study the physiological role of vanins in lipid metabolism.

On the basis of its potency, selectivity, and bioavailability, RR6 represents a novel biological chemistry tool that will greatly facilitate the study of vanin function in health and disease. It is assumed that pantetheine is the major vanin substrate, and hence a role in vitamin B5 recycling (pantothenate release) and antioxidant production (cysteamine release) has been proposed. The fact that vanin-1 is a PPAR-α target and appears to have a role in nutrient or lipid metabolism as witnessed by the current study suggests that enzymatic activity of vanins may not be restricted to pantetheine alone. We propose that RR6 and optimized versions of this novel chemotype constitute a promising starting point for a platform of vanin inhibitors that may open up a new field of research.

METHODS

Synthesis of Probes. The detailed description of the synthesis of the vanin inhibitors can be found in the Supporting Information.

Pantothenate-7-amino-4-methylcoumarin (AMC-Pan). AMC-Pan was synthesized according to the published method by Ruan et al.[22]

Assay of Pantetheinase Activity and Pantetheinase Inhibition. Pantetheinase activity was measured largely as described by Ruan et al.[22] Samples (10 μL volume) with pantetheinase activity (recombinant human vanin-1 or diluted serum from different origin) were added to the substrate AMC-Pan (10 μM final concentration) in a 100 μL final volume, in PBS at pH 7.4. Five hundred nanograms of recombinant vanin-1 was used per reaction. Human serum was diluted 2.5 times, FBS was diluted 100 times, and rat serum was diluted 2 times before addition to the reaction (all in PBS at pH 7.4). In the case that inhibition was measured, 10 μL of inhibitor solution was added to 10 μL of sample (recombinant vanin or diluted serum) and allowed to equilibrate for 10 min, whereafter substrate was added. From these reaction mixtures 10 μL was taken after 10 min of incubation, diluted 10-fold in PBS, and measured in a 96-well plate fluorescence reader (Perkin-Elmer LS55) at excitation 360 nm and emission 450 nm. IC_{50} determinations were made in duplicate, by measuring the inhibition of inhibitors in a 10^{-10} to 10^{-3} M dilution range. IC_{50} values were calculated from the fit of the % inhibition of the dose–response curve (Graphpad Prism version 4.0). The mode of inhibition was investigated by assaying RR6 inhibition of plasma vanin activity in the presence of varying substrate concentrations, from 300 to 1 μM (Graphpad Prism version 4.0). When we wished to determine pantetheinase activity under near physiological conditions, as in undiluted rat plasma taken from animals that received oral RR6, we used the following procedure. A buffered substrate solution (20 μL) was put in 96-well plates and evaporated to dryness. The rat plasma samples were added, and substrate hydrolysis was allowed to proceed. Substrate hydrolysis and inhibition by our experimental compounds was measured as described above.

Biotinidase and Cysteine Protease Assays. Biotinidase activity was assayed by the hydrolysis of 6-amidoquinoline (Apollo Scientific) as a fluorimetric substrate,[23] and IC_{50} for biotinidase was determined as described for vanin activity. Cathepsins B and L (R&D Systems) and papain (Sigma) were assayed as described previously, using the substrates Z-Leu-Arg-AMC (R&D Systems) for the cathepsins and Z-Phe-Arg-AMC (Bachem) for papain.[24]

Cloning and Expression of Recombinant Human Vanin-1. Human vanin-1 cDNA was obtained from image clone 40006204 (Geneservice).[25] Using PCR the vanin-1 ORF (aa 1–483) was amplified without its GPI-anchor and cloned into the eukaryotic expression vector pHLsec.[26] Transfection of pHLsec-VNN1 into 293T cells using PEI transfection reagent results in the producion of His-tagged recombinant human vanin-1 protein. Media containing the secreted recombinant protein were collected 72 h post transfection, and recombinant protein was affinity purified using Ni-NTA agarose beads. After the protein was eluted from the column, it was concentrated and dialyzed to PBS using Microcon YM-10 filters (Millipore).

Gel Permeation Chromatography. Bovine serum diluted 1:3 with PBS was used and incubated with 100 μM RR6 for 2 h. At this concentration a complete inhibition of serum vanin activity is achieved. A 50 μL sample was injected on a Superdex-75 HR 10/30 SMART column (GE Health Sciences) in PBS at a flow rate of 40 μL per min. The column was calibrated with bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome *c* (12 kDa), all obtained from Sigma. Eluted proteins were monitored at 280 nm using a microflow cell. Fractions (40 μL) were collected and measured for pantetheinase activity.

Pharmacodynamic Studies. Female Wistar rats (Harlan) weighing 200–250 g were housed in groups of two or three animals in the Central Animal Laboratory of the Radboud University and given water and chow ad libitum. Protocols were approved by our local committee for animal experiments (DEC). Rats (2–3 per group) were given RR6 by a single oral administration (dissolved in 10% DMSO in PBS at doses of 2, 10, and 50 mg/kg) or *via* their drinking water (dissolved at doses of 0.3, 1, and 3 mg mL^{-1} in water). Heparinized blood samples were drawn at several time points, and plasma was obtained by centrifugation of the blood samples for 20 min at 465*g* at 4 °C, for pantetheinase activity determination as indicated above.

Metabolic Studies. Male Wistar rats (Harlan) weighing 150–200 g were housed in groups of two or three animals in the Central Animal Laboratory of the Radboud University and given water and chow ad libitum. Protocols were approved by our local committee for animal experiments (DEC). Rats were given RR6 *via* drinking water (3 mg mL^{-1}) for 4 days. Blood was collected after a 24 h period of fasting, placed on ice, and centrifuged, and plasma was assayed for free fatty acids (FFA) using the NEFA-C kit (Wako Diagnostics). Glucose and cholesterol were determined using kits from Human Diagnostics.

Data Analysis. Data were analyzed using Graphpad Prism version 4. Kinetic constants were determined with the nonlinear regression analysis module. Statistical analysis of FFA and cholesterol measurements was performed using Student's *t* test (SPSS Inc.). $P < 0.05$ was considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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