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A fluorescent assay suitable for inhibitor screening and vanin tissue quantification

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ABSTRACT

Vanin-1 is a pantetheinase that catalyzes the hydrolysis of pantetheine to produce pantothenic acid (vitamin B5) and cysteamine. Reported here is a highly sensitive fluorescent assay using a novel fluorescently labeled pantothenate derivative. The assay has been used for characterization of a soluble version of human vanin-1 recombinant protein, identification and characterization of hits from high-throughput screening (HTS), and quantification of vanin pantothenase activity in cell lines and tissues. Under optimized assay conditions, we quantified vanin pantothenase activity in tissue lysate and found low activity in lung and liver but high activity in kidney. We demonstrated that the purified recombinant vanin-1 consisting of the extracellular portion without the glycosylphosphatidylinositol (GPI) linker was highly active with an apparent K_m of 28 μM for pantothenate–7-amino-4-methylcoumarin (pantothenate–AMC), which was converted to pantothenic acid and AMC based on liquid chromatography–mass spectrometry (LC–MS) analysis. The assay also performed well in a 384-well microplate format under initial rate conditions (10% conversion) with a signal-to-background ratio (S/B) of 7 and a *Z* factor of 0.75. Preliminary screening of a library of 1280 pharmaceutically active compounds identified inhibitors with novel chemical scaffolds. This assay will be a powerful tool for target validation and drug lead identification and characterization.

Pantetheinase activity has been correlated to oxidative stress and inflammation [1–7], suggesting a possible role for vanin-1 as a tissue sensor in pathological conditions associated with a large oxidative stress component. Vanin-1 is a member of the biotinidase family and is expressed at the cell surface via a glycosylphosphatidylinositol (GPI)[1] anchor in epithelial cells [3]. Vanin-1 knockout mice are resistant to systemic oxidative stress and intestinal inflammation, as evidenced by the decreased presence of inflammatory mediators and increased antioxidative responses [1,8]. Vanin-1 knockout mice lack detectable cysteamine in tissues, suggesting that catabolism of pantetheine by vanin-1 is the primary source of endogenous cysteamine. Interestingly, cystamine (oxidized form of cysteamine) administration to vanin-1 knockout animals reverses the protection observed in intestinal and oxidative stress models [6]. Vanin-1, via its pantetheinase activity, regulates the specific intracellular pathways that lead to generation of pro-inflammatory mediators. Therefore, inhibiting vanin-1 pantetheinase activity might be beneficial in treating inflammatory bowel disease.

Pantetheinase assays reported so far are not suitable for high-throughput screening (HTS) because the assays either require radioactive materials or result in a product that is insensitive to detection. For example, early assay formats measured cysteamine production by derivatizing cysteamine followed by chromatographic separation [9–13] or measured pantothenic acid (vitamin B5) formation using ^{14}C-labeled pantetheine followed by paper chromatography [14]. Later, an assay using *S*-pantetheine-3-pyruvate as substrate was developed. Hydrolysis gives pantothenic acid and *S*-cysteamine-3-pyruvate, which then spontaneously cyclizes to aminoethylcysteine ketamine that can be quantitated by ultraviolet (UV) absorbance at 296 nm [15]. However, *S*-pantetheine-3-pyruvate is unstable, and UV absorbance measurement at 296 nm is not suitable for HTS because many small molecules absorb at this wavelength and will

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[1] *Abbreviations used:* GPI, glycosylphosphatidylinositol; HTS, high-throughput screening; UV, ultraviolet; pantothenate–AMC, pantothenate–7-amino-4-methylcoumarin; LOPAC, library of pharmacologically active compounds; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance; cDNA, complementary DNA; PCR, polymerase chain reaction; CMV, cytomegalovirus; DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC–MALS, size exclusion chromatography–multiangular light scattering; DTT, dithiothreitol; BSA, bovine serum albumin; EX, excitation; EM, emission; t_R, retention time; S/B, signal-to-background ratio; SD, standard deviation; IQR, interquartile range; BCA, bisinchoninic acid; qRT, quantitative reverse transcription; BME, β-mercaptoethanol; mRNA, messenger RNA.

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interfere. Recently, a nitroanilide derivative of pantothenate was used as a vanin-1 substrate with UV/visible readout at 400 nm, although no chemical structure was disclosed [7]. Again, UV/visible absorbance measurement at 400 nm might not be suitable for HTS because small molecules may interfere. Therefore, we investigated other pantothenate derivatives as potential vanin-1 substrates to develop an assay that is suitable for HTS and for the quantitation of vanin-1 in various cell types and tissues. Reported here is the development of a vanin-1 HTS-amenable assay using pantothenate–7-amino-4-methylcoumarin (pantothenate–AMC) as a substrate and application of this novel fluorescent assay to screen a LOPAC (library of pharmacologically active compounds) to identify small molecule vanin-1 inhibitors and to quantify vanin-1 in mouse tissue extracts.

Materials and methods

Materials

β-Alanine 7-amido-4-methylcoumarin was purchased from Chem-Impex International (Wood Dale, IL, USA), and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Protein purification reagents were obtained from Pierce (Rockford, IL, USA). Assays were run in Matrix 384-well polypropylene plates using a PlateMate 2 × 2 robot by Matrix (Hudson, NH, USA). Fluorescent assays were carried out on an Envision plate reader (PerkinElmer, Waltham, MA, USA) or a Safire multidetection monochromator microplate reader (Tecan, Durham, NC, USA). NIC-H292 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

Chemical synthesis of pantothenate–AMC

A solution of β-alanine 7-amido-4-methylcoumarin trifluoroacetic acid (TFA) salt (H-β-Ala-AMC.TFA, 36 mg, 1 eq) and *R*-(−)-pantolactone (45 mg, 3 eq) was heated to 60 °C in ethanol (5 ml) for 2 days. The solvent was evaporated, and the residue was dissolved in dimethyl sulfoxide (DMSO)/water and purified by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column with 5% to 95% acetonitrile in water containing 0.05% TFA buffer to give pantothenate–AMC (32 mg, >99% purity) as a white powder. MS (ESI) m/z 377.1 $(M+H)^+$, ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.41 (br s, 1H), 8.04 to 7.62 (m, 3H), 7.47 (dd, J = 9.0, 3.0 Hz, 1H), 6.27 (s, 1H), 5.39 (d, J = 5.7 Hz, 1H), 4.47 (5, J = 5.7 Hz, 1H), 3.71 (d, J = 5.7 Hz, 1H), 3.48 to 3.14 (m, 4H), 2.58 (t, J = 6.9 Hz, 2H), 2.39 (s, 3H), 0.80 (s, 3H), 0.77 (s, 3H).

Vanin-1 plasmid constructs

Human vanin-1 complementary DNAs (cDNAs) were purchased from Origin and subcloned into pDEST12.2 vector by homologous recombination using Gateway technology. Human vanin-1 ectodomain construct (amino acid position 22–483) was modified by replacing the endogenous vanin-1 leader with the honeybee melanin prepro leader followed by a Gly-Ser-Gly-His6× tag–Gly-Ser-Gly-Flag tag by overlap polymerase chain reaction (PCR) of 45 to 50 bp synthetic oligonucleotides and cloned by InFusion cloning (Clontech, Mountain View, CA, USA) into pDONR221, which was performed by Dragonfly Sciences (Wellesley, MA, USA). The insert was Gateway subcloned into a mammalian expression vector with a cytomegalovirus (CMV) promoter. All PCR-derived products were sequenced to ensure sequence fidelity.

Expression and purification of human vanin-1 protein

Dihydrofolate reductase (DHFR)-negative Chinese hamster ovary (CHO) DUKX cells were transfected with the above-described vanin-1 ectodomain plasmid construct using the transfection reagent TransIT LT1 (Mirus Bio, Madison, WI, USA) and subsequently put into 20 nM methotrexate for selection for 2 weeks. Clones were picked and analyzed for expression level by anti-His6× Western blot analysis. The best clone was expanded into HYPERFlask (Corning, Lowell, MA, USA). Once the cells were confluent, the medium was changed to serum-free medium R1CD1 and the temperature was shifted to 32 °C. Three harvests of conditioned medium were collected after 3 days each.

The recombinant His- and Flag-tagged vanin-1 was purified over a 10-ml HisTrap Fast Flow column (GE Healthcare, Piscataway, NJ, USA). Bound protein was washed with 50 mM Tris, 1 M NaCl, and 15 mM imidazole buffer (pH 8.0). The recombinant vanin-1 was eluted with 50 mM Tris, 1 M NaCl, and 250 mM imidazole buffer (pH 8.0), dialyzed against phosphate-buffered saline (PBS, pH 7.2), and characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and Western blot analysis using 10% tricine gels (Invitrogen, Carlsbad, CA, USA). The molecular weight was determined by size exclusion chromatography–multiangular light scattering (SEC–MALS) analysis as followings. The recombinant vanin-1 was injected onto a YMC-Pack Diol-300 column (500 × 8.0 mm i.d., Waters) using a Waters HPLC unit. The column was developed with 50 mM phosphate buffer with 300 mM NaCl (pH 7.2) at a flow rate of 1 ml/min. The eluted proteins were detected by a miniDAWN Tristar multiangular light scattering device connected in tandem with an OptiLab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA) to determine the homogeneity and molecular weight of the protein as it migrates through the SEC column. The purified recombinant vanin-1 was greater than 95% pure by Coomassie blue stain analysis, and the endotoxin level was less than 1 EU/ml. The protein (1.75 mg/ml) was stored in PBS at −80 °C.

LC–MS analysis of pantothenate–AMC hydrolysis by vanin-1

Pantothenate–AMC (200 μM, 100 μl) was incubated in phosphate buffer (100 mM potassium phosphate buffer [pH 8.0], 5 mM dithiothreitol [DTT], 0.01% bovine serum albumin [BSA], and 0.0025% Brij-35) in the presence or absence of vanin-1 protein (100 nM) at 37 °C for 1 h. The reaction products were detected by fluorescent analysis using an excitation (EX) wavelength of 350 nm and an emission (EM) wavelength of 460 nm by a Safire plate reader. The products were characterized by LC–MS analysis performed on a Waters LCT mass spectrometer coupled with an Agilent 1100 HPLC device. The HPLC column (Waters Symmetry 2.1 × 50 mm, 3.5 μm) was developed in a gradient of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 0.6 ml/min. The gradient started at 2% solvent B in solvent A for 3 min, linearly ramped to 5% solvent B in 4 min and then to 100% solvent B in 4 min, and finally held at 100% solvent B for another 2 min. The eluted fractions were analyzed by MS with ESI using the following parameters: capillary voltage, 3500 V; cone voltage, 25 V; desolvation temperature, 350 °C; source temperature, 120 °C; scan speed, 100 to 2000 Da in 1 s. Data acquisition was made by alternating between positive ion mode and negative ion mode with an interscan delay of 0.7 s.

Assay optimization

The optimal assay conditions were obtained by varying the individual buffer components—DTT (0 μM–50 mM), DMSO (0–50%), pH (3–10), and vanin-1 (10 pM–32 nM) in 50 mM potassium phosphate buffer containing 0.01% BSA, 0.0025% Brij-35, and pantothenate–AMC (1 or 2 μM) in a black 384-well at 25 °C for 1 h. The progress of the reaction was followed every 2 min by fluorescence (Safire, EX 350 ± 2.5 nm, EM 460 ± 2.5 nm), and the conversion rate was calculated using standard titration curves of AMC and pantothenate–