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Synthesis and Characterization of Valyloxy Methoxy Luciferin for the Detection of Valacyclovirase and Peptide Transporter

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Abstract

An amino acid ester derivative of luciferin (valoluc) was synthesized to mimic the transport and activation of valacyclovir. This molecule was characterized *in vitro* for specificity and enzymatic constants, and then assayed in two different, physiologically-relevant conditions. It was demonstrated that valoluc activation is sensitive to the same cellular factors as valacyclovir and thus has the potential to elucidate the dynamics of amino acid ester prodrug therapies in a functional, high-throughput manner.

Valacyclovir is an antiviral prodrug used for the treatment of Herpesvirus infections. It is the valyl ester derivative of the nucleoside analog acyclovir, which is preferentially phosphorylated by viral kinases and leads to chain termination during DNA synthesis.¹ Acyclovir has poor bioavailability and is of limited utility, but valacyclovir can be transported across biological membranes by the oligopeptide transporter (PEPT1), granting it much greater utility *in vivo*.² Valacyclovirase has been identified as the enzyme responsible for hydrolysis of valacyclovir to acyclovir, and while much has been resolved regarding its biochemistry and specificity, comparatively little is known about its

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distribution and dynamics *in vivo*.³⁻⁶ In this respect, a surrogate molecule with a functional component could be highly advantageous.

Luciferin is the small molecule substrate for luciferase, an oxidizing enzyme found in many terrestrial organisms such as the common eastern firefly, *Photinus pyralis*. A significant byproduct of luciferin oxidation is bioluminescence, and this phenomenon has been capitalized upon for a host of various assays in biological research.⁷ It has been shown in several instances that derivatization of luciferin at either its hydroxyl or carboxyl groups prohibits its oxidation by luciferase.^{8, 9} This results in a “caged” luciferin molecule that must first be hydrolyzed by an enzyme before oxidation by luciferase, thus producing a bioluminescent assay for specific enzymatic activity.

Using the caged luciferin strategy, a valyl ester derivative of luciferin (Figure 1a) was designed as a functional reporter for valacyclovirase activity. The *in vitro* stability of the luciferin derivative, however, was found to be quite poor. HPLC analysis of valyl ester luciferin revealed a half-life ($t_{1/2}$) of $12 (\pm 2)$ min at pH 7.4. It was hypothesized that the μ -amino group and aromatic ring structure destabilized the ester bond making it labile to chemical hydrolysis. Due to its prohibitive impermanence under physiologically relevant conditions, valyl ester luciferin was abandoned for further studies in favor of a more chemically steadfast analogue.

To improve the stability of valyl ester luciferin, a methylene bridge was inserted between the aromatic ring and ester linker. This type of linker has been used previously in the design of poorly permeable anti-HIV drugs to improve stability.¹⁰ Valyloxy methoxy luciferin (Figure 1b) was synthesized as shown in Scheme 1. Boc-protected valine **1** was converted to the iodomethyl ester of valine **2** by first converting it to a chloromethyl ester intermediate using chloromethyl chlorosulfate and sodium bicarbonate along with tetrabutylammonium hydrogen sulfate in dichloromethane:water (1:1) and then by reaction with sodium iodide in acetone.¹¹ 2-cyano-6-hydroxybenzothiazole **4** was generated by combining pyridine hydrochloride and 2-cyano-6-methoxybenzothiazole **3** in the presence of heat. Intermediate **5** was synthesized by reacting **2** and **4** in the presence of cesium carbonate in acetone. In the absence of light, cysteine was then cyclized to produce intermediate **6** in the presence of sodium carbonate and DMF (dimethylformamide). The final compound **7** was deprotected by dissolving **6** in dichloromethane and 20% trifluoroacetic acid at 0°C for one hour. HPLC analysis of valyloxy methoxy luciferin demonstrated that the half-life was dramatically improved by the addition of the methylene bridge, exhibiting an experimentally-determined half-life of 495 ± 23 minutes in 50mM HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) buffer, pH 7.4.

Valyloxy methoxy luciferin (valoluc) was first tested *in vitro* for hydrolytic specificity using purified recombinant luciferase, valacyclovirase (VACVase), and other known hydrolases (puromycin-specific aminopeptidase (PSA) and dipeptidyl peptidase 4 (DPP4)). Valoluc (0.1 μ M) was combined with thermostable luciferase (lucx4)¹² (1 μ M), ATP (0.5mM), and Mg²⁺ (5mM) in 50mM HEPES pH 7.4 and then dispensed into black microplate wells containing either VACVase, PSA, DPP4 (all at 0.1 μ M), or buffer and then measured for luminescence every 5 minutes at 37°C (Figure 2). Both the initial time point and final time

point revealed a statistical difference ($p < 0.05$) in luminescence between the VACVase-containing wells and all other negative controls, suggesting VACVase can specifically hydrolyze valoluc. To further characterize valoluc, K_m and V_{max} were determined by measuring the rate of bioluminescent production for different concentrations of valoluc (0.03 - 1.0mM) while keeping the concentration of VACVase and luciferase constant (0.2 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$, respectively). The data was fit to the Michaelis-Menten model using GraphPad Software and values for K_m and V_{max} were calculated to be 0.106 (± 0.038) mM and 20 (± 2) mmol/min/ μg , respectively, corresponding closely with reported values of other VACVase substrates.⁶

To provide a more physiological assessment of valoluc hydrolysis specificity, bacteria were transformed with dual expression vectors, encoding lucx4 and either VACVase or PSA genes, all driven by IPTG (isopropyl μ -D-1-thiogalactopyranoside)-inducible promoters. Bacterial cultures were diluted to $\text{OD}_{600}=0.6$ into black multiwell plates and then supplemented with either IPTG (10mM) or buffer. Cultures were grown at 37°C and valoluc (1nmol) was added every hour. Luminescence was measured semi-continuously at 5 minute intervals for 6 hours (Figure 3). Statistically significant ($p < 0.05$) levels of luminescence were observed for VACVase-induced wells as early as $t=1$ hour and persisted through all later time points. A small amount of hydrolysis was observed from VACVase-plasmid containing, but uninduced bacteria. This is thought to be due to the leakiness of the T7 promoter and not non-specific hydrolysis, given that the PSA-plasmid containing bacteria did not show similar levels of luminescence.

The final test of valoluc was performed in transiently transfected mammalian cells. Lucx4, VACVase, and PEPT1 (peptide transporter 1, SLC15A1) were cloned into mammalian expression vectors (CMV (cytomegalovirus)-driven) and transfected either alone or together into HEK-293 cells using Lipofectamine 2000. Intact cells were treated with valoluc (2.5nmol) 24-hours post-transfection and assayed at 5 minute intervals (Figure 4). Cells transfected with VACVase showed only a modest increase in luminescence over control cells, but cells transfected with both VACVase and PEPT1 showed substantial gains in luminescence. This suggests that PEPT1 is a significant transporter of valoluc into mammalian cells and that VACVase can mediate its hydrolysis once inside the cytosol.

Taken together, the *in vitro*, bacterial, and mammalian cell assays demonstrate that valoluc is a robust and functional determinant of VACVase activity. Furthermore, in the context of eukaryotic cells, valoluc is also sensitive to the expression of PEPT1, making it a faithful surrogate for exploring the dynamics and distribution of amino acid ester prodrug activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Valyl ester luciferin. B) Valyloxy methoxy luciferin.

Figure 2.

In vitro analysis of valoluc. Valoluc was incubated with different purified hydrolases as well as lucx4 and examined for luminescence at 5 minute intervals.

Figure 3. Valoluc hydrolysis in bacteria. Bacteria were transformed with *lux4* and either VACVase or PSA. Half of the cultures were induced to express these enzymes and all wells were treated with valoluc every hour. Wells were monitored semi-continuously for over 6 hours.

Figure 4.

Valoluc hydrolysis in mammalian cells. HEK-293 cells were transiently transfected with lucx4, VACVase, and PEPT1. 24 hours following transfection, intact cells were treated with valoluc and luminescence was measured every 5 minutes for 1 hour.

Scheme 1.