DETECTION OF DIPEPTIDYL PEPTIDASE ACTIVITY WITH DPPIV-GLO™ ASSAY

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Ranbaxy Laboratories Ltd., is a research-based, international pharmaceutical company located in India. In addition to being a dominant player in the area of developing generic medicines, Ranbaxy has committed to investing in drug discovery. Keeping in mind the highly complex, cost-intensive and failure-prone nature of drug discovery, Ranbaxy has evolved an alliance-centric business model to bring sustainability to its drug discovery effort. Ranbaxy has entered into alliance with partners at different stages of drug discovery and development. A partnership with Promega is an effort to access robust assays that can assist Ranbaxy in hit identification and lead optimization during early drug discovery stages. Here we describe our characterization of the DPPIV-GLO™ Protease Assay.

Introduction

Dipeptidyl peptidase IV (DPPIV) has emerged as a validated and druggable target for type 2 diabetes mellitus. DPPIV inactivates incretin hormone GLP-1. GLP-1 is a 30-amino-acid polypeptide that promotes blood glucose homeostasis by stimulating insulin secretion from pancreatic β cells in a glucose-dependent manner (1). DPPIV cleaves GLP-1 at the N-terminal end to release a dipeptide, thereby converting active GLP-1 (*GLP-1[7-36]amide*) to inactive GLP-1*(GLP-1[9-36]amide*; 1). An inhibitor of DPPIV is likely to lower blood sugar levels by increasing the level of active GLP-1 (2). Screening small-molecule DPPIV inhibitors requires a robust assay that is sensitive, reproducible, homogeneous and detects the target enzyme in body fluids. In the following article, we describe the characterization of the DPPIV-Glo™ Protease Assay developed by Promega.

Biochemically, DPPIV belongs to the family of prolyl oligopeptidase (POP) members that include dipeptidyl peptidase VIII (DPP8), dipeptidyl peptidase IX (DPP9), dipeptidyl peptidase II (DPP2), post-prolyl cleaving enzyme (PPCE) and fibroblast activating protein (FAP) (3). DPPIV acts by cleaving N-terminal peptides from polypeptides with L-alanine or L-proline at the penultimate position (Figure 1). Cleavage of L-alanine results in inactivation of biologically active GLP-1 polypeptide. Commercial DPPIV substrates use the dipeptide Gly-Pro, in which L-proline has replaced L-alanine and amino methyl coumarin (AMC) is coupled to the C-terminus of proline. Cleavage of the peptide bond by DPPIV releases AMC, which emits fluorescent signal (Figure 1). Scientists at Promega have coupled aminoluciferin to L-proline to create the DPPIV-Glo™ reagent. Upon cleavage by DPPIV, aminoluciferin is released and serves as a substrate for a coupled luciferase reaction that emits detectable luminescence (Figure 1).

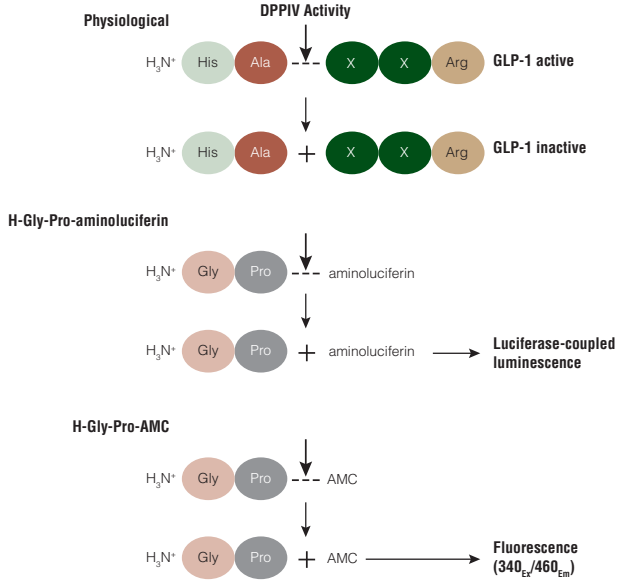

Figure 1. Active GLP-1 (*GLP-1[7-36]amide*) is secreted by L cells of the intestine in a glucose-dependent manner. DPPIV converts active GLP-1 to inactive GLP-1 (*GLP-1[9-36]amide*) by cleaving a dipeptide from the N-terminus of active GLP-1. Commercial DPPIV substrates have the dipeptide (mainly Gly-Pro) attached to a probe. H-Gly-Pro-aminoluciferin is acted upon by DPPIV to produce aminoluciferin. Aminoluciferin in turn serves as a substrate for a coupled luciferase reaction to emit luminescence that can be measured by a luminometer. H-Gly-Pro-AMC is acted upon by DPPIV yielding fluorescent AMC, which can be measured by a spectrofluorometer at $340_{Ex}/460_{Em}$.

DPPIV-Glo™ Reagent is a Sensitive Substrate

In our first series of experiments we compared sensitivity of DPPIV-Glo™ reagent to H-Gly-Pro-AMC substrate by varying the concentration of human recombinant DPPIV enzyme. As shown in Figure 2, we detected a robust signal of Gly-Pro cleaving activity with as little as at 0.5 ng of recombinant DPPIV. Under similar conditions, the signal obtained with the GP-AMC substrate was much less intense. Optimal signal was seen with 10 ng of recombinant human DPPIV for GP-AMC, whereas 0.5 ng of human recombinant DPPIV was enough to produce a good signal with the DPPIV-Glo™ Reagent, indicating that it is at least 20-fold more sensitive than the fluorescent substrate. This sensitivity may enable undertaking studies using much less enzyme when performing a screening assay for DPPIV activity.

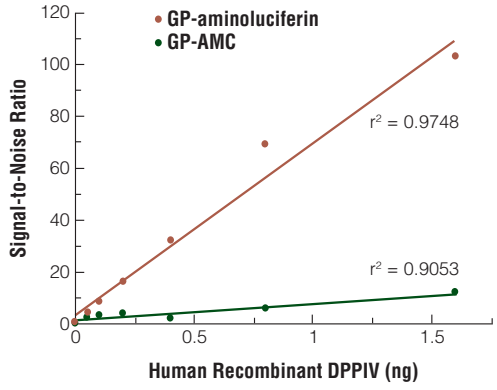

Figure 2. Protein titration of human recombinant DPPIV with GP-aminoluciferin and GP-AMC substrate. Human recombinant DPPIV protein was titrated at 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 ng in 50 mM HEPES buffer (pH 7.8) containing 80 mM $MgCl_2$, 140 mM NaCl, and 1% BSA. Samples and buffer were added to 96-well white plates (25 µl/well) followed by 25 µl of DPPIV-Glo™ reagent (final reaction volume 50 µl). For GP-AMC, the enzyme and buffer were added (50 µl) followed by the substrate (50 µl) in 96-well black plates; GP-AMC final concentration was 40 µM. Reaction volume was 100 µl. In an end-point assay, luminescence was measured using a Safire²™ readerat 15 minutes. Fluorescence was measured using a PolarStar Galaxy instrument after 20 minutes at $340_{Ex}/460_{Em}$.

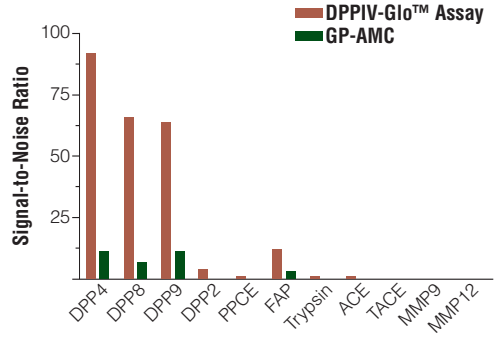

Figure 3. Various proteases were checked for their ability to cleave DPPIV-Glo™ substrate. Standard assay conditions were followed for all the assays except that the fluorescent substrate was replaced with DPPIV-Glo™ Reagent. Reconstituted substrate (25 µl) was used in all the assays. Recombinant human enzymes were used in the case of DPP4 (0.5 ng), DPP8 (10 ng), DPP9 (10 ng), DPP2 (10 ng), PPCE (10 ng), FAP (10 ng), ACE (20 ng), TACE (10 ng), MMP-9 (20 ng) and MMP-12 (6 ng). Trypsin is from Sigma. DPP4, DPP8 and DPP9 have significant DPPIV-Glo™ cleaving activity compared to GP-AMC.

DPPIV-Glo™ Reagent Detects Dipeptidyl Peptidases

As a POP family member, DPPIV shares a certain degree of sequence homology and functionality with other members of the family (i.e., cleaving N-terminal dipeptides from polypeptides). Thus, it is possible that some of the POP family members may share the substrate specificity. We tested the Gly-Pro cleaving activity of various proteases using DPPIV-Glo™ reagent. As shown in Figure 3, the dipeptidyl peptidase class of enzymes DPP8 and DPP9 at 10 ng showed greater Gly-Pro cleaving activity when assayed with the DPPIV-Glo™ reagent compared to the GP-AMC substrate. However, matrix metalloproteinases like gelatinase at 20 ng (MMP9), elastase at 6 ng (MMP12), and angiotensin-converting enzyme (ACE) at 20 ng did not show any Gly-Pro cleaving activity. Other related and unrelated proteases tested, DPP2 and FAP at 10 ng, TACE at 10 ng and trypsin at 10 ng, were detected with much less sensitivity than the dipeptidyl peptidases. These specificity data suggest that DPPIV-Glo™ reagent is suitable for detecting dipeptidyl peptidase activity, including DPPIV activity.

Table 1. IC_{50} Values Obtained for DPPIV Inhibitors.

DPPIV Source	Substrate		Reported
Human Recombinant	DPPIV-Glo™	GP-AMC	
LAF237	5.1 nM	1.3 nM	
MK431	31.8 nM	16.5 nM	
Plasma			
LAF237	1.2 nM		2.7 nM (4)
MK431	45.7 nM		18 nM (5)
Saliva			
LAF237	5.6 nM		
MK431	15.3 nM		

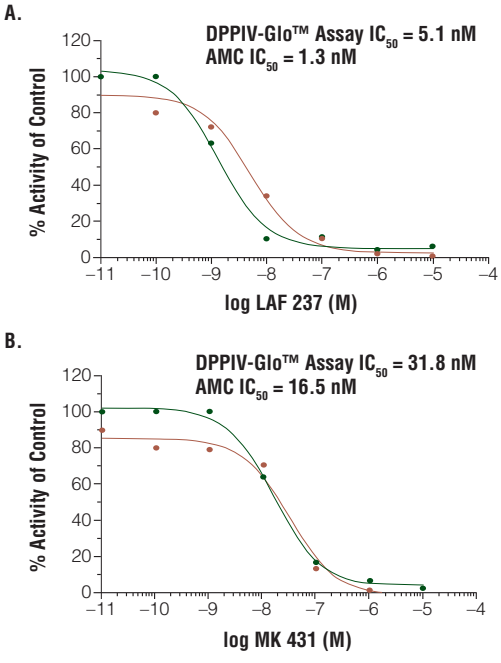

Figure 4. Inhibition of human recombinant DPPIV with inhibitors. Human recombinant protein at 0.5 ng and 10 ng/well in assay buffer was added to 2 sets of 96-well plates. DPPIV inhibitors LAF237 and MK431 were added at 100 nM to 10 µM. One plate of inhibited and control samples was tested with DPPIV-Glo™ reagent; the other was tested with GP-AMC substrate. Luminescence was recorded after 15 minutes on a Safire²™ instrument Fluorescence was recorded after 20 minutes on a PolarStar Galaxy at $340_{Ex}/460_{Em}$. **Panel A.** LAF 237; **Panel B.** MK 431; Red lines: DPPIV-Glo™ reagent; Green lines: GP-AMC substrate.

DPPIV-Glo™ Reagent Retains Inhibitor Potency

To establish that using DPPIV-Glo™ reagent does not affect potency of inhibitors for DPPIV enzyme, we computed potency of two standard DPPIV inhibitors. LAF 237 (vildagliptin, tradename Galvus®; 4) and MK 431 (sitagliptin, tradename Januvia®; 5). Concentration response curves to these inhibitors were obtained using DPPIV-Glo™ and

GP-AMC reagents in the presence of human recombinant DPPIV at 0.5 (DPPIV-Glo™) and 10 ng (GP-AMC), respectively. As shown in Figure 4 and Table I, both inhibitors showed comparable IC_{50} values in the present study with DPPIV-Glo™ and GP-AMC substrates. The IC_{50} values are summarized in Table 1. IC_{50} values were also comparable to values reported by previous authors (4,5).

DPPIV-Glo™ Reagent Retains Sensitivity in Biological Fluids

DPPIV/CD26 is a glycoprotein that exists both as a membrane-bound and soluble form. The soluble form is reported to be present at very high concentrations in plasma. The level of soluble DPPIV in saliva has not been previously reported. Some of the other plasma proteases may show Gly-Pro cleaving activity. However, the majority of plasma activity is due to DPPIV (6).

We evaluated how the DPPIV-Glo™ Assay performs in the presence of biological fluids like plasma and saliva. A robust assay may help track enzyme inhibitory activity of compounds in human trials. Human plasma and saliva were titrated at different concentrations using DPPIV-Glo™ reagent and GP-AMC; both reagents showed a linear response up to 3 µg of protein. DPPIV-Glo™ showed an excellent signal-to-noise ratio of 12.5 at 250 ng protein with both plasma and saliva (Figure 5, Panel A). However, at 250 ng protein, the signal-to-noise ratio using GP-AMC was threefold lower for plasma and at the limit of detection for saliva (Figure 5, Panel B). These data suggest that when biological samples are limited, the DPPIV-Glo™ assay may exhibit better signal-to-noise ratio compared to an assay using the AMC substrate.

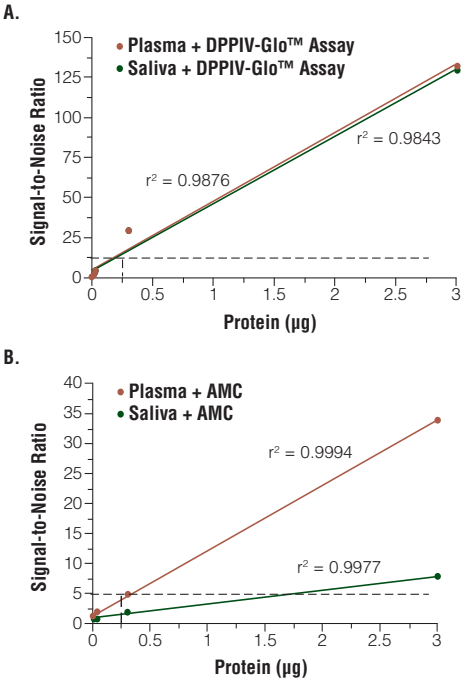

Figure 5. Protein titration of biological fluids. Human plasma and saliva were diluted in the assay buffer at 300 pg, 3 ng, 30 ng, 300 ng and 3 µg. Duplicate 96-well plates were prepared, and either DPPIV-Glo™ reagent (**Panel A**) or GP-AMC (**Panel B**) was added. Luminescence was recorded after 15 minutes on a Safire²™ reader. Fluorescence was recorded after 20 minutes on a PolarStar Galaxy instrument at $340_{Ex}/460_{Em}$. The responses were converted into signal-to-noise ratio and plotted against protein concentration for comparison.

DPPIV-Glo™ Reagent Can Be Used with Biological Fluids to Detect Enzyme Inhibition

It has been reported that more than 95% of the circulating Gly-Pro cleaving activity is attributed to DPPIV (6). We have already shown that 250 ng is optimal protein concentration to give significant signal-to-noise ratio in human plasma as well as saliva (Figure 5). With this background, inhibition studies with standard inhibitors were carried out with human plasma and saliva as the DPPIV source (250 ng protein). For plasma tested with DPPIV-Glo™ reagent, a dose-response curve (DRC) of both LAF237 and MK431 showed a very good dose response, and IC_{50} values were consistent with those obtained for recombinant DPPIV (Table 1; Figure 6, Panel A). Similar studies were carried out with saliva. The response was similar to plasma and the IC_{50} values of the inhibitors were again comparable to those obtained for recombinant DPPIV (Table 1; Figure 6, Panel B). These inhibition studies have demonstrated that the sensitive DPPIV-Glo™ reagent is maintaining the inhibitory profile of the standard DPPIV inhibitors using plasma and saliva as the DPPIV source (Table 1).

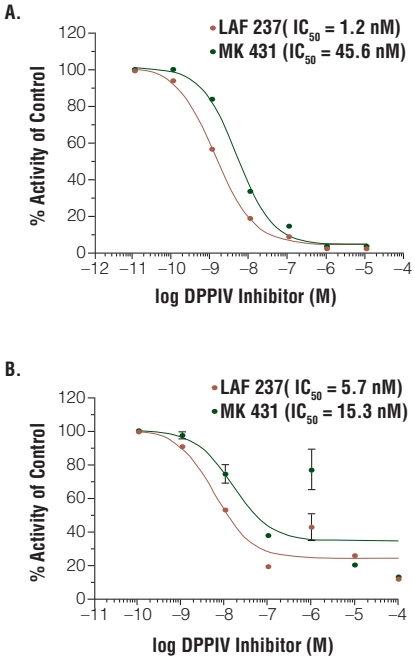

Figure 6. Inhibition of DPPIV activity in human plasma and saliva using inhibitors. Plasma and saliva were diluted in 50 mM HEPES buffer (pH 7.8) containing 80 mM $MgCl_2$, 140 mM NaCl and 1% BSA. Diluted samples were added to two 96-well plates at 250 ng/well. DPPIV inhibitors LAF237 and MK431 were added at 100 nM to 100 µM. To the plates, DPPIV-Glo™ reagent was added to both plasma and saliva dilutions, and luminescence was recorded after 15 minutes on a Safire²™ reader. **Panel A.** DRC of LAF237 and MK431 in plasma using the DPPIV-Glo™ reagent; **Panel B.** DRC of LAF237 and MK431 in saliva using the DPPIV-Glo™ reagent. Data were analyzed using GraphPad Prism® Software, version 4.0.

Summary

We have demonstrated that the DPPIV-Glo™ reagent gives robust signal-to-noise ratios in the presence of very little enzyme whether in recombinant or biological samples (Table 2). This property of the reagent makes it useful for screening assays as well as for detecting enzyme inhibition in small quantities of biological fluid. The DPPIV-Glo™ reagent does not affect inhibitor potency when used with recombinant protein or in biological fluid. In addition, the reagent can be used to screen inhibitors of other peptidyl peptidases.

References

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Table 2. Comparison of DPPIV-Glo™ Assay with Standard Fluorescent-Based Assay.

Feature	DPPIV-Glo™ Assay	Standard GP-AMC Assay
Sensitivity	0.5 ng human recombinant protein	10 ng human recombinant protein
Specificity	Good signal with DPPIV, DPP8 and 9	Moderate signal with DPPIV, DPP8 and 9
Detection of DPPIV in Saliva	250 ng protein	10 µg protein

Protocol

DPPIV-Glo™ Protease Assay Technical Bulletin #TB339 (www.promega.com/tbs/tb339/tb339.html)

Ordering Information

Product	Size	Cat.#
DPPIV-Glo™ Protease Assay	10 ml 50 ml	G8350 G8351

For Laboratory Use.

THE PROTEASE ASSAY PORTFOLIO FROM PROMEGA

All of the Promega reagents and assays listed here feature the sensitivity and robustness of bioluminescence. In addition to the DPPIV-Glo™ Assay, we offer an additional series of substrate-specific protease assays (Supplementary Table 1).

Supplementary Table 1. Protease Assays Available from Promega.

Substrate	Enzyme	Assay Available	Catalog Numbers
Z-VDVAD-aminoluciferin	Caspase-2	Caspase-Glo® 2 Assay	G0940, G0941
Z-VEID-aminoluciferin	Caspase-6	Caspase-Glo® 6 Assay	G0970, G0971
Z-DEVD-aminoluciferin	Caspases 3 & 7	Caspase-Glo® 3/7 Assay	G8090, G8091, G8092, G8093
Z-LETD-aminoluciferin	Caspase-8	Caspase-Glo® 8 Assay	G8200, G8201, G8202
Z-LEHD-aminoluciferin	Caspase-9	Caspase-Glo® 9 Assay	G8210, G8211, G8212
Z-LRR-aminoluciferin	Trypsin-like activity of the proteasome	Proteasome-Glo® Trypsin-Like Assay	G8631, G8632
Suc-LLVY-aminoluciferin	Calpain- and chymotrypsin-like activities of the proteasome	Calpain-Glo™ Assay Proteasome-Glo™ Chymotrypsin-Like Assay	G8501, G8502 G8621, G8622
Z-nLPnLD-aminoluciferin	Caspase-like activity of the proteasome	Proteasome-Glo™ Caspase-Like Assay	G8641, G8642
[NNNN]4-14	Enzyme of interest	Protease-Glo™ Assay	G9451

Additional luminescent substrates are listed in the *Cell Notes* article, Issue 18, pages 9–11, 2007 (www.promega.com/cnotes)

Custom Aminoluciferin Substrates

In addition to catalog assays containing the substrates listed above, also we have synthesized a number of substrates for custom protease assays and continue to make new ones. If you have a protease substrate of interest not listed here, visit **www.promega.com/myway** to submit your custom protease substrate inquiry.

A New Assay for P´-Requiring Proteases

The latest in protease research tools from Promega is the new Protease-Glo™ Assay, based on our GloSensor™ Technology. The Protease-Glo™ Assay is a highly sensitive, quick and easy method to interrogate proteases with or without P´ requirements through a bioluminescent readout. The assay is based upon a genetically modified firefly luciferase protein, the GloSensor™ protein, which is activated upon proteolytic cleavage. A Protease-Glo™ Assay Limited Use Label License accompanies these products.