Rapid Screening of a Cell-based Assay for GLP-1 Receptor Using a Natural Product Library

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Introduction

Glucagon-like peptide-1 receptor (GLP-1R) is a G-protein coupled receptor that is present in insulin-secreting beta cells. Intact GLP-1(1-37) is produced by post-translational processing of proglucagon precursor and converted into active forms of GLP-1 [(7-36) amide and (7-37)] with N-terminal truncation. Then, active forms of GLP-1 are deactivated by the further fragmentation with peptidases[1,2].

Its defining action is augmentation of glucose-induced insulin secretion following intake of carbohydrates and lipids. It also inhibits glucagon secretion and food intake. For this reason, GLP-1R is an interesting target for type-2 diabetes intervention.

Here we will demonstrate the detection of GLP-1R binding through the incorporation of a homogeneous cell-based binding assay using Tag-lite® technology. The assay was automated using a non-contact liquid dispenser commonly used in high-throughput settings. Detection of the two fluorescent emissions was accomplished simultaneously using the matched PMTs of an HTS multi-mode microplate reader. Optimization experiments were performed to validate the proper conditions to use for automated assay processing. A small primary screen of natural products was then performed under high throughput screening conditions, followed by the creation of dose-response curves using known GLP-1R binders.

Glucagon GLP-1 Receptor Ligand Binding Assay

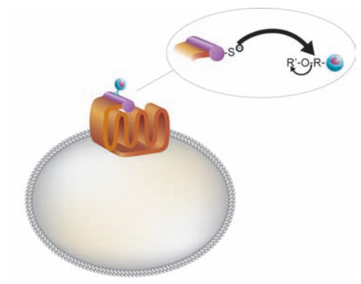

Figure 1 – *HEK293 cells were transiently transfected with a pSNAP-GLP-1 plasmid (PSNAPGLP1) for 24 hours prior to being labeled with SNAP-Lumi4Tb (SSNPTBC). Then, labeled cells were frozen in liquid nitrogen in the presence of 10% DMSO.*

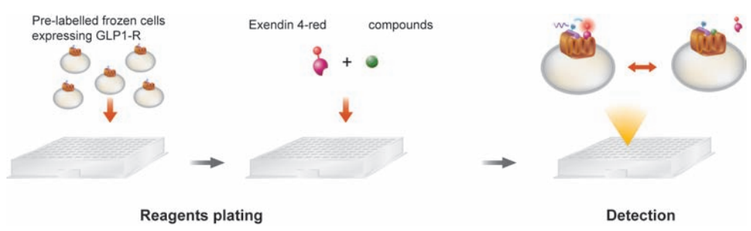

Figure 2 – *GLP-1 receptor cells are dispensed into 384-well plates. Compounds to be tested and a fluorescent derivative of Exendin 4, Exendin 4-red (L0030RED), are then added sequentially to the plate. Upon binding of the fluorescent ligand to the GLP-1 receptor, a time resolved fluorescence resonance energy transfer (TR-FRET) occurs between the Lumi4 Tb donor attached to the GPCR and the red emitting labeled ligand. Competition between non-labeled compounds and the Exendin 4-red (at 4 nM) leads to a decrease in HTRF signal.*

BioTek Instrumentation

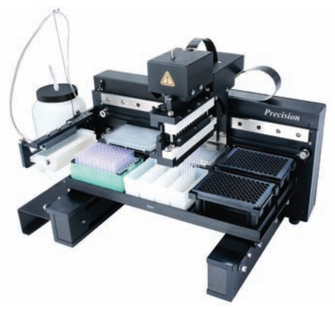

Figure 3 – Precision™ Microplate Pipetting System. *The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dilute the Natural Product library and transfer the final 4X concentrations to the LV384-well assay plates.*

Figure 4 – MultiFlo™ Microplate Dispenser. *The dispenser offers fast, accurate non-contact plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 µL. The peristaltic pump on the instrument was used to dispense cryopreserved cells, and detection solution to the LV384-well assay plates.*

Figure 5 – Synergy™ NEO HTS Multi-Mode Microplate Reader. *The reader combines a filter-based and monochromator-based detection system in one unit. This HTRF certified reader uses a filter-based system, high performance Xenon flash lamp, as well as dual matched PMTs to simultaneously detect the 665 nm and 620 nm fluorescent emissions from this chemistry.*

Materials

Glucagon GLP-1 Receptor Ligand Binding Assay: Tag-lite GLP-1 transformed, labeled, ready-to-use cryopreserved cells (Catalog No. C1TT1GLP1), Tag-lite buffer (TLB) (Catalog No. LABMED), and GLP-1 receptor red agonist were provided by Cisbio Bioassays (Codolet, France).

Compounds: The Screen-Well® Natural Product Library, v. 7.2 (Catalog No. BML-2865) was generously donated by Enzo Life Sciences (Farmingdale, NY). GLP-1 (9-36) amide (Catalog No. 3266), Exendin-3 (9-39) amide (Catalog No. 2081), and Exendin-4 (Catalog No. 1933) were purchased from R&D Systems (Minneapolis, MN).

Methods

Cell Preparation: Frozen cells are thawed at 37 °C and transferred to a vial containing 5 mL of 1X TLB. The vial is then centrifuged for 5 minutes at 1200xG at 4 °C. Supernatant is then aspirated and the pellet is resuspended in 2.7 mL of 1X TLB.

GLP-1 Receptor Red Agonist Preparation: A. K_d Determination – The 400 nM concentration of the red agonist is prepared by diluting the stock concentration in 1X TLB (See kit insert for stock concentration). Six additional 1:2 dilutions were then prepared using 1X TLB. B. Red Agonist Binding and Competition Assays – The 400 nM agonist concentration was further diluted with 1X TLB to create a 4X 16 nM concentration. This is further diluted during the assay to create the final 4 nM concentration.

Compound Library Dilution: Compounds in the Natural Product Library come as 100% DMSO stocks at variable concentrations. Each 1000X compound concentration was initially diluted 1:25 using the Precision by transferring 5 µL of the stock concentration into 120 µL of 1X TLB. The compounds were further diluted 1:10 by the Precision by transferring 10 µL of the intermediate concentration into 90 µL of 1X TLB. Five microliters of the 4X stock was then transferred in duplicate to the appropriate assay plate, again using the Precision. A 0.1% DMSO, 1X concentration is then created during the assay procedure.

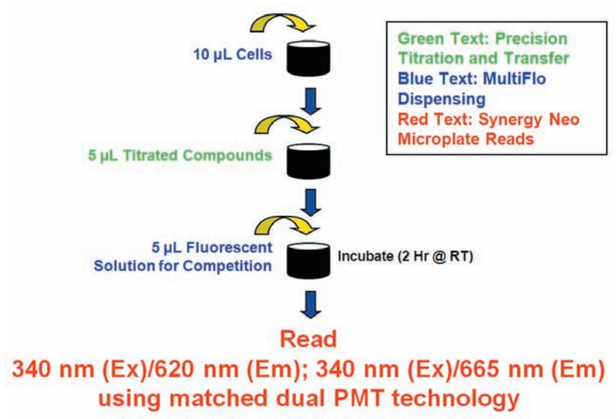

Figure 6 – *Automated Assay Procedure. Dilution and transfer of compounds accomplished via the Precision. Cells and GLP-1 red agonist solution dispensed using MultiFlo. Detection of 620 and 665 nm signals accomplished simultaneously using Synergy NEO.*

GLP-1 Red Agonist Binding Kinetics

Determination of the binding kinetics of the red labeled Exendin-4 compound is essential in order to calculate the proper incubation time following the addition of test compounds and Competition Solution. Cells and 1X TLB were added to the assay plate in two separate wells. The red labeled agonist was then added to both wells and a kinetic read of the two wells began immediately. Reads were taken every two seconds over a sixty minute period.

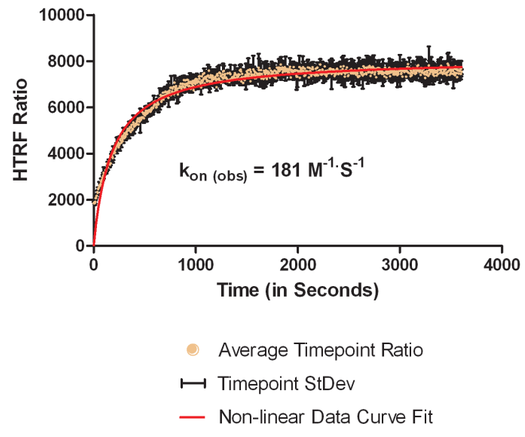

Figure 7 – GLP-1 Red Labeled Agonist Kinetic Binding Curve. *Average and standard deviation values were plotted versus time. A Michaelis-Menten curve fit and $K_{on\ (obs)}$ (observed on-rate) was then generated from the data.*

The labeled agonist will begin binding to the GLP-1 receptor upon addition, but will not remain permanently bound. Therefore binding and unbinding will take place until an equilibrium is established. HTRF ratios were calculated for each datapoint. The Michaelis-Menten curve that was created from the plot of the data allows for the calculation of the observed on-rate for the molecule.

GLP-1 Red Agonist K_d Determination

Incorporating the proper concentration of the GLP-1 red labeled agonist is also essential when testing for potential receptor ligands. Typically a concentration that is at, or slightly below, the K_d value is acceptable. Use of a concentration that is higher than the K_d value can cause a right-shift in the results from test compounds, causing them to appear less potent than what is may be seen *in vivo*. The red agonist was titrated, and the various concentrations added to cells and 1X TLB. An endpoint assay was run using the incubation times previously described.

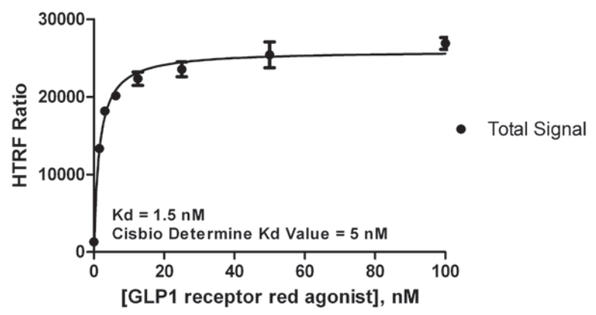

Figure 8 – GLP-1 Red Labeled Agonist Dose Response Curve. *A serial 1:2 titration curve was generated with concentrations ranging from 100-0 nM. Average and standard deviation was plotted for the wells tested at each concentration (n=3). The Michaelis-Menten curve fit and K_d value were then generated from the observed results.*

A K_d value of 1.5 nM was calculated from the labeled agonist dose response curve. This value compares well to the 5 nM value previously generated. A final 1X concentration of 4 nM was used for the library screen and subsequent testing.

Natural Product Library Screen

A total of 384 compounds from plates 1-4 of the Screen-Well Natural Product Library were screened in duplicate. Compounds were diluted 1:1000 from the original 100% DMSO stocks as previously described. A no compound (0% inhibition) control was included on each assay plate. The unlabeled form of the known GLP-1 receptor agonist, Exendin-4, was also included in the library, as well as two known antagonists, GLP-1 (9-36) and Exendin-3 (9-39).

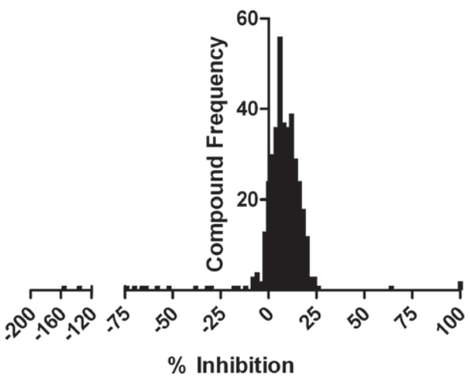

Figure 9 – Compound Library % Inhibition Distribution. *Distribution of percent inhibition data from Natural Product Library compound screen.*

The inhibition data from the compounds tested show a distribution typically seen from primary screens using larger compound libraries. Most compounds exhibited little to no inhibition, while a small percentage demonstrated a high level of positive or negative inhibition.

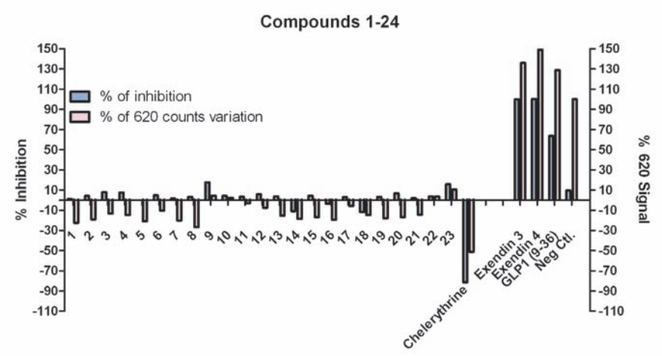

Figure 10 – Representative GLP-1 Receptor and Assay Inhibition Data. *Percent inhibition of red labeled Exendin-4 agonist and percent of donor molecule fluorescent values from 0% inhibition wells shown for compounds 1-24 of Natural Product Library.*

Assessment of the donor molecule fluorescent signal can be used to ensure that compounds are exhibiting true positive or negative inhibition of red labeled agonist binding. This control, inherent when using HTRF technology, provides a rapid method to remove false hits from lead optimization. An example from the library tested is Chelerythrine. The compound exhibits a high percentage of negative inhibition, but also decreases the donor molecule signal by approximately 50%. This combination is indicative of a false result. Exendin-3, -4, and GLP-1, in contrast show high levels of positive inhibition with no negative effect on the fluorescent signal of the donor molecule.

Positive Inhibitor Validation

Dose response curves were generated for compounds exhibiting positive inhibition of red agonist binding, as well as no effect on donor molecule fluorescent signal, in the primary compound library screen. Eight point 1:10 titration curves were created for each compound, with a starting concentration of 10 µM.

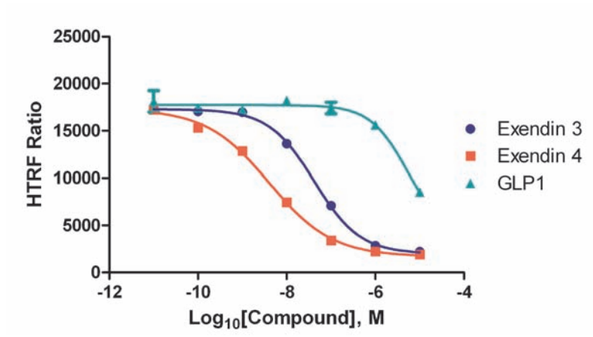

Figure 11 – *Dose response curves generated for positive inhibitor compounds.*

GLP1 Ligand K_i Values (nM)		
Compound	**Generated K_i Value**	**Literature K_i Value (using Radioactivity)**
Exendin-3 (9-39)	11.0	7.3[1]
Exendin-4	1.0	1.0[1]
GLP1 (9-36)	1444	Low Binding Affinity[2]

Table 1 – *IC_{50} values for positive inhibitor compounds.*

IC_{50} values from dose response curves generated for the three compounds demonstrating positive inhibition of red agonist receptor binding agree with values or information previously published.

Conclusions

1. The Tag-lite Glucagon GLP-1 Receptor Ligand Binding Assay provides an easy-to-use, cell-based format for detecting ligands of the GLP-1 receptor.
2. The Synergy NEO HTS Multi-Mode Microplate Reader allows for simultaneous detection of the dual-emission signal from the assay in kinetic or endpoint formats.
3. Automation of the assay procedure creates a simple process for assay optimization, primary, and secondary screening.
4. The Natural Product Library can be efficiently screened in an automated high-throughput manner.
5. The combination of assay, automation, and rapid, high-quality detection creates a robust process for high-throughput screening of potential GLP-1 receptor modulators.

[1]Jorgensen R. *et al.*: Characterization of Glucagon-Like Peptide-1 Receptor β-Arrestin 2 Interaction: A High-Affinity Receptor Phenotype. *Molecular Endocrinology* 2005, 19(3): 812-23.

[2]Knudsen, L. *et al.*: Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after in vivo administration to dogs, and it acts as an antagonist on the pancreatic receptor. *European Journal of Pharmacology* 1996, 318(2-3): 429-35.