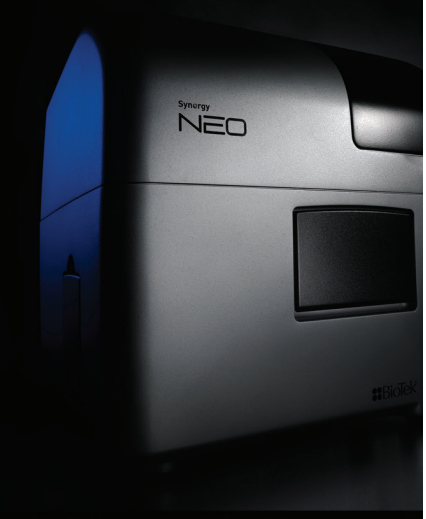
Rapid Screening of a Cell-based Assay for GLP-1 Receptor Using a Natural Product Library

Brad Larson and Peter Banks, BioTek Instruments, Inc., Winooski, VT
Nicolas Pierre, Suzanne Graham, Jean-Luc Tardieu, François Degorce Cisbio US, Inc., Bedford, MA



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BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: customercare@biotek.com
www.biotek.com

Introduction

Glucagon-like peptide-1 receptor (GLP-1R) is a G-protein coupled receptor present in insulin-secreting beta cells. GLP-1R augments glucose-induced insulin secretion following intake of carbohydrates and lipids, and also inhibits glucagon secretion and food intake. These mechanisms of action, and the potential for fewer side effects versus insulin treatments, make GLP-1R a target of interest for Type-2 diabetes research.

Intact GLP-1(1-37), the natural ligand for GLP-1R, is produced by posttranslational processing of proglucagon precursor and converted into active forms of GLP-1 [(7-36) amide and (7-37)] with N-terminal truncation. Active forms of GLP-1 are then deactivated by further fragmentation with peptidases[1,2].

Here we demonstrate the detection of GLP-1R binding through the incorporation of a homogeneous cell-based binding assay using HTRF Tag-lite® technology. The assay resembles classical radiometric ligand receptor binding, except that it uses a red-emitting labeled ligand and transfected cells. When the ligand binds to the receptor, an increase in HTRF signal may be measured. Competitive binding of non-labeled ligands or inhibitors causes a subsequent decrease.

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 a high performance xenon flash lamp and the dual photomultiplier tubes (PMTs) of a novel HTS multi-mode microplate reader. Optimization experiments were performed to validate the proper conditions for use in 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

The Glucagon GLP-1 Receptor Ligand Binding Assay used HEK293 cells (Figure 1) transfected with the pSNAP-GLP-1 plasmid (PSNAPGLP1) for twenty-four hours, then subsequently labeled with the small fusion tag, SNAP-Lumi4 Tb (SSNPTBC). The labeled cells were then frozen in liquid nitrogen and 10% DMSO. In use, the cells were dispensed into assay plates along with sequential additions of compounds to be tested and Exendin 4-red (L0030RED), a fluorescent derivative of Exendin 4 (Figure 2). When the fluorescent ligand was bound to the GLP-1 receptor, a time-resolved fluorescence resonance energy transfer (TR-FRET) occurred between the Lumi4 Tb donor bound to the GPCR and the red emitting labeled ligand. Competition between non-labeled compounds and the Exendin 4-red (at 4 nM) diminishes energy transfer, thus leading to a decrease in HTRF signal.

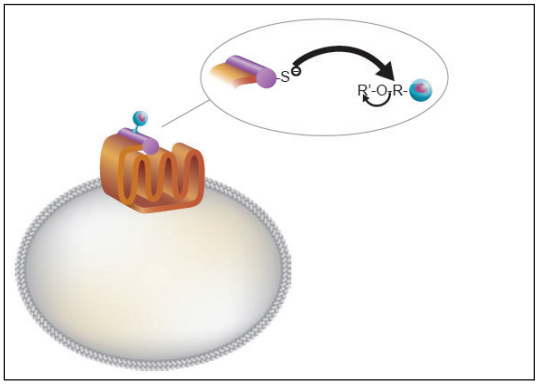

Figure 1. HEK293 cells were transiently transfected and labeled, then frozen in liquid nitrogen and DMSO.

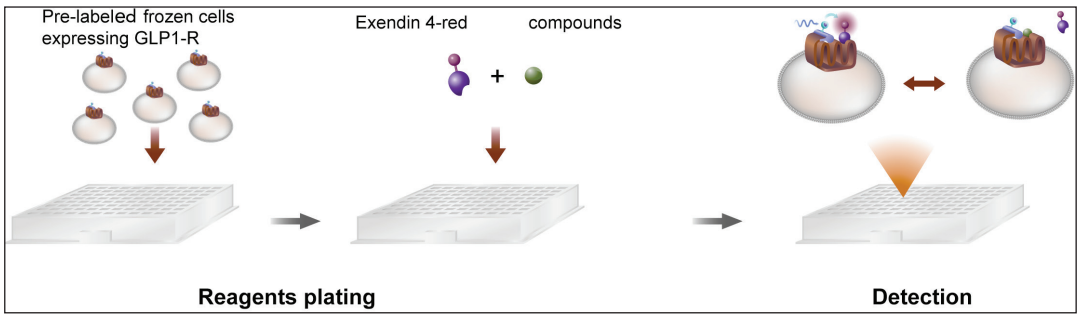

Figure 2. GLP-1 receptor cells were dispensed into 384-well plates. Test compounds and Exendin 4-red were then added sequentially to the plate. Upon binding of the fluorescent ligand to the GLP-1 receptor, energy was transferred and detected on a microplate reader.

Materials and Methods

Materials

Cells and Reagents

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) as part of the Glugagon GLP-1 Receptor Ligand Binding Assay kit. 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).

Instrumentation

The Precision™ Microplate Pipetting System (BioTek Instruments, Winooski, VT) combines an eight-channel pipetting head and an eight-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 low-volume (LV) 384-well assay plates. The MultiFlo™ Microplate Dispenser (BioTek Instruments, Winooski, VT) offers fast, accurate, non-contact microplate-based liquid dispensing through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 µL. The peristaltic pump on the MultiFlo was used to dispense cryopreserved cells and detection solution to the LV 384-well assay plates. Synergy™ NEO HTS Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) combines a filter- and monochromator-based detection system in one compact unit. The HTRF® certified reader used a high performance xenon flash lamp and dual PMTs in the filter-based optics to simultaneously detect the assay's 665 nm and 620 nm fluorescent emissions when the excitation was set to 340 nm.

Methods

Assay Component Preparation

Cells: Frozen cells were thawed at 37 °C and transferred to a vial containing 5 mL 1X TLB. The vial was centrifuged for 5 minutes at 1200xG at 4 °C. Supernatant was aspirated, and the pellet resuspended in 2.7 mL 1X TLB. GLP-1 Receptor Red Agonist: Per K_d determination, 400 nM red agonist was prepared by diluting the stock concentration in 1X TLB per the manufacturer's instructions. Six additional 1:2 dilutions were then prepared using 1X TLB. For all other tests, the 400 nM concentration was further diluted with 1X TLB to create a 4X 16 nM concentration. This was further diluted during the assay to create a final 4 nM concentration. Compound Library: Compounds in the Natural Product Library were supplied as 100% DMSO stocks at variable concentrations and were diluted using the Precision Microplate Pipetting System. Each 1000X compound concentration was initially diluted 1:25 by transferring 5 µL of the stock concentration into 120 µL of 1X TLB. The compounds were further diluted 1:10 by transferring 10 µL of the intermediate concentration into 90 µL of 1X TLB. Five microliters of the 4X stock were then transferred in duplicate to the appropriate assay plate. A 0.1% DMSO, 1X concentration was then created during the assay procedure.

Experimental Assay Procedures
GLP-1 Red Agonist Binding Kinetics

Binding kinetic determination of the red-labeled Exendin-4 compound is essential to calculate the proper incubation time following the test compound and Competition Solution additions. 10 µL of cells and 5 µL of 1X TLB were added to the assayplate in two separate wells. Five microliters of red-labeled agonist was then added to both wells, and kinetic reading began immediately on the Synergy NEO, with reads taken every two seconds for one hour.

GLP-1 Red Agonist K_d Determination

Incorporating the proper GLP-1 red-labeled agonist concentration is also essential when testing for potential receptor ligands. The red agonist was titrated manually, and 5 µL of each concentration was added to 10 µL of cells and 5 µL of 1X TLB. The assay plate was incubated at room temperature for two hours, then read using the previously mentioned parameters.

Natural Product Library Screen

A total of 384 compounds from the Screen-Well Natural Product Library plates 1-4 were screened in duplicate. Compounds were diluted 1:1000 from the original 100% DMSO stocks using the aforementioned automated compound dilution process. MultiFlo™ was used to dispense 10 µL of cells, and Precision™ was used to add 5 µL of 1X TLB and 5 µL diluted compound to the assay plate. MultiFlo was then used again to dispense 5 µL of the 16 nM red-labeled agonist. A no compound (0% inhibition) was included as well as Exendin-4, the unlabeled GLP-1 receptor agonist, and the known antagonists GLP-1 (9-36) and Exendin-3 (9-39).

Positive Inhibitor Validation

Dose response curves were generated, using Precision, for those compounds exhibiting positive red agonist binding inhibition with no effect on the donor molecule fluorescent signal in the primary compound library screen. Five microliters of each compound was added to 10 µL cells along with 5 µL fluorescent solution, using the previously mentioned automated procedure. After a two hour, room temperature incubation, the plates were read using the aforementioned parameters. Eight-point 1:10 titration curves were created for each compound, starting with a 10 µM concentration. K_i data were calculated from IC_{50} results using the Cheng-Prusoff equation[3].

Results and Discussion

GLP-1 Red Agonist Binding Kinetics

As previously stated, the correct incubation time following test compound and competition solution additions was dependent on the red-labeled Exendin-4 compound binding kinetics calculation. Once added to the well, the labeled agonist was bound to the GLP-1 receptor, but does not remain permanently bound. Rather, binding and unbinding occurs until equilibrium was established. Per Figure 3, HTRF ratios were calculated for each data point, and a Michaelis-Menten curve was created to calculate the molecule's observed on-rate.

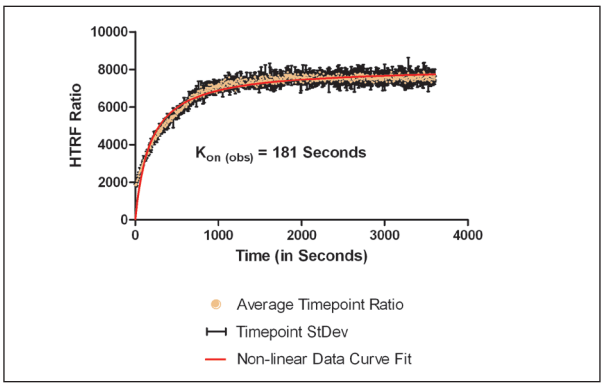

Figure 3. GLP-1 Red-Labeled Agonist Kinetic Binding Curve. Average and standard deviation values were plotted versus time. A Michaelis-Menten curve fit and $K_{on\ (obs)}$ (observed on-rate) was then generated from the data, where $K_{on(obs)}$ = 181 sec.

GLP-1 Red Agonist K_d Determination

GLP-1 red-labeled agonist concentrations at, or slightly below, the K_d value are preferred when assaying potential receptor ligands. Higher concentrations may cause a right-shift in the test compound results, making them appear less potent than true *in vivo* behavior. Using the labeled agonist dose response curve (Figure 4), the K_d value was calculated as 1.5 nM. This compares well to the manufacturer's previously derived value of 5 nM. A final 1X concentration of 4 nM was used for the library screen and subsequent testing.

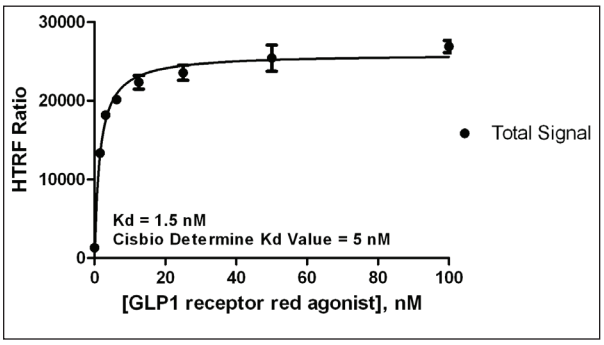

Figure 4. GLP-1 Red Labeled Agonist Dose Response Curve. A serial 1:2 titration curve was generated with concentrations ranging from 100-0 nM. Average and standard deviation was plotted for the wells tested at each concentration (n=3). The Michaelis-Menten curve fit and K_d value were then generated from the observed results, where K_d = 1.5 nM.

Natural Product Library Screen

When plotting the compound percent inhibition data (Figure 5), a distribution profile similar to that seen in larger compound library primary screens was observed. Most compounds exhibited little to no inhibition, while a small percentage demonstrated high positive or negative inhibition. The donor molecule fluorescent signal assessment (Figure 6) was used to ensure that compounds exhibited true positive or negative red-labeled agonist binding inhibition.

This control, inherent in HTRF technologies, provided a rapid method to remove false hits. An example of a false result seen in Figure 6 is Chelerythrine. This compound exhibited high negative inhibition, but also decreased the donor molecule signal by approximately 50%. In contrast, Exendin-3, Exendin-4 and GLP-1 all showed high positive inhibition with no negative effect on the donor molecule's fluorescent signal.

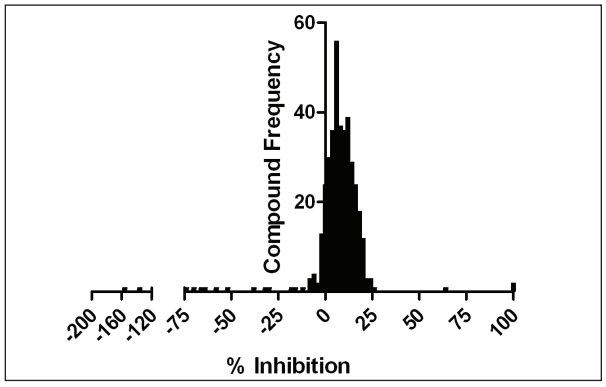

Figure 5. Natural Product Compound Library screen percent inhibition distribution.

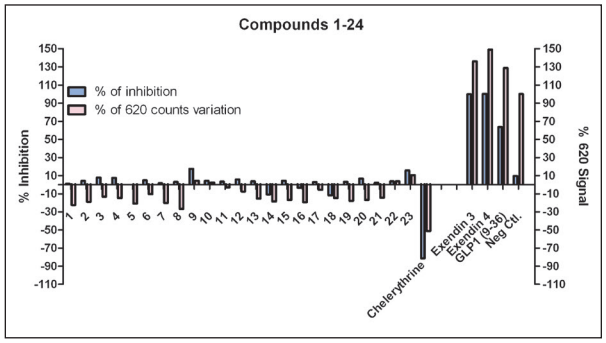

Figure 6. Representative GLP-1 Receptor and Assay Inhibition Data. Percent inhibition of red labeled Exendin-4 agonist and percent of donor molecule fluorescent values from 0% inhibition wells shown for Natural Product Library compounds 1-24.

Positive Inhibitor Validation

Dose response curves were created for the positive inhibitors Exendin-3, Exendin-4 and GLP-1 (Figure 7), which were then used to generate K_i values (Table 1). Data agreed with previously published values.

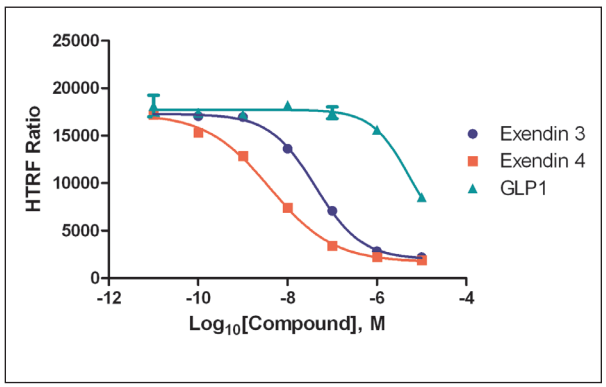

Figure 7. Dose response curves generated for positive inhibitor compounds.

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. K_i values for positive inhibitor compounds.

Conclusions

As shown, the Tag-lite Glucagon GLP-1 Receptor Ligand Binding Assay provided an easy-to-use, cell-based format for detecting GLP-1 receptor ligands. Concurrently, the Synergy NEO HTS Multi-Mode Microplate Reader provided fast, accurate, simultaneous detection of the dual-emission signal from the assay in kinetic or end-point formats. Combining assay and instrumentation to create an automated system created a simple process for, primary, and secondary screening, and the Natural Product Library was efficiently screened in an automated high-throughput manner. Overall, combining assay, automation, and rapid, high-quality detection created a robust process for high-throughput screening of potential GLP-1 receptor modulators.

References

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