Introduction

The ideal research platform for inhibitor development is able to screen for both large and small molecule inhibitors within the same assay format. Assays developed for the detection of small molecule inhibitors may not be amenable to detection of blocking antibodies. One way to overcome this is to utilize assays that probe the downstream signaling events that are impacted by both types of inhibitors. Therapeutic antibodies directed to cell surface receptors are increasingly being developed as treatments for a variety of diseases as varied as asthma, cancer, and diabetes. As such, it is important to have available simple, fast, and sensitive assays for the measurement of antibody modulation of receptors in a cellular system.

Characterizing Chemokine Receptor Inhibitors with AlphaLISA SureFire Ultra, Alpha SureFire Ultra Multiplex and LANCE Ultra cAMP Assays
The measurement of protein phosphorylation is a useful tool for measuring the modulation of receptor activation by both antibodies and small molecules. AlphaLISA® SureFire® Ultra™ and Alpha SureFire® Ultra™ Multiplex are mix-and-read assays that use the highly sensitive Alpha bead-based proximity detection system, coupled with CaptSure™ immobilization technology to assess relative levels of both phosphorylated and total states for a variety of proteins. These assays do not suffer from interference from exogenous antibodies. As there are no wash steps, these assays are automation friendly, may be applied to both small and large-scale screens, and are used to assess phosphorylation status in complex matrices including diverse cell culture models and serum samples (see Figure 1 for Assay models).

Another secondary messenger that is widely used to measure downstream signaling of receptor activation is cAMP. The LANCE Ultra cAMP assay is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay designed to measure cyclic AMP (cAMP) produced upon modulation of adenylyl cyclase activity by G-protein coupled receptors (GPCRs). The assay is based on the competition between a europium (Eu) chelate-labeled cAMP tracer and endogenous cAMP for binding sites on cAMP-specific monoclonal antibodies labeled with the ULight™ dye (Figure 2). When antibodies are bound to the Eu-labeled cAMP tracer, a light pulse at 320 or 340 nm excites the Eu chelate molecule of the tracer. The energy emitted by the Eu chelate is transferred by FRET to ULight molecules on the

Figure 1. General AlphaLISA SureFire Ultra and Alpha Surefire Ultra Multiplex assay schematics.

Figure 2. LANCE Ultra cAMP assay schematic. The assay detects cAMP on the basis of a competition immunoassay with Eu-labeled cAMP, such that an increase in endogenous cAMP results in a 665 nm TR-FRET signal decrease.

The CCR7 chemokine receptor is expressed in B and T lymphocytes and in dendritic cells and its primary ligands are MIP-3β (CCL19) and 6CKine (CCL21). CCR7 plays a role in immune cell migration and the development and control of the immune response, making it a therapeutic target for such disorders as lupus erythematosus and adult leukemia and lymphomas. The CXCR2 receptor, also known as IL8RB, is a receptor for interleukin 8 (IL8). It is expressed at high levels on circulating neutrophils and is critical for directing their migration to sites of inflammation, making it a therapeutic target for disorders like chronic obstructive pulmonary disease (COPD) and sepsis. Multiple functional assays can be used to detect antibody modulation of activity of both these receptors, including through the measurement of kinase signaling cascades and cAMP formation. To demonstrate the utility of the SureFire Ultra and LANCE Ultra cAMP assays for measuring a variety of inhibitors to different cell surface receptors, we chose to examine both CCR7 and CXCR2 pathways in a cellular model system whereby these receptors are overexpressed in CHO cells. Assay conditions were optimized to measure the blockage of these receptors (activated with appropriate agonists). Receptor activity modulation was assayed by the detection of ERK and AKT phosphorylation status.
Materials and Methods

Cell Culture and Treatment

Two PerkinElmer ValiScreen® GPCR cell lines were utilized to examine the downstream effects of chemokine receptor modulation using multiple blocking antibodies and a small molecule. The Human Chemokine CCR7 Receptor Cell Line (PerkinElmer #ES-140-C) and Human Chemokine CXCR2 Receptor Cell Line (PerkinElmer #ES-145-C) are both CHO-K1 cells transfected with a proprietary bicistronic expression plasmid. Cells were thawed from a frozen vial stock, were then expanded for a couple of passages with culture media following the procedure described in detail in the Technical Data Sheet for each cell line.

For all SureFire Ultra assays, CHO cells were plated in white 96-well CulturPlates (PerkinElmer #6005680) and allowed to attach and grow overnight (18-24 hours) in a standard humidity controlled 37 °C incubator with 5% CO2. For initial optimization experiments, the culture media was removed from wells, and replaced by agonist dilutions prepared in 1X HBSS buffer with 25 mM HEPES and 0.1% BSA (to minimize potential sticking to plastic surfaces). Plates were then incubated at room temperature (−22 °C) for 10 minutes for ERK or 15 minutes for AKT assays prior to removal of stimulation buffer. For blocking antibody/inhibitor titrations, antagonist (inhibitor) dilutions were added in 1X HBSS buffer with 25 mM HEPES and 0.1% BSA. Cultures were then incubated at 37 °C for 60 minutes, and then allowed to come to room temperature (−22 °C). Agonists were then added (achieving final desired concentrations) and incubated for 10 minutes for ERK or 15 minutes for AKT prior to buffer removal and cell lysis. For SureFire Ultra assays, cultures were then lysed with 50 µL / well of 1X SureFire Ultra Lysis buffer (provided in the kit) and the AlphaLISA SureFire Ultra or MultiPlex assay performed as instructed in the Technical Data Sheets. For LANCE Ultra cAMP assays, cells from other wells were lysed according to the protocol of the LANCE Ultra cAMP assay kit.

Agonists, Blocking Antibodies and Small Molecule Inhibitors

All chemokines used for CXCR2 and CCR7 receptor stimulation were purchased from R&D Systems: recombinant Human CCL19/MIP-3β Protein (#361-MI/CF) and recombinant Human IL-8/CXCL8 Protein (#208-IL/CF).

For blocking CCR7 and CXCR2 activation, the anti-CCR7 antibody (#MAB197) and anti-CXCR2 antibody (#MAB331) used were purchased from R&D Systems. The unrelated antibody used as a negative control, mlgG2a isotype control (#MA8003), was also from R&D Systems. SB 225002, the CXCR2 antagonist (SB 225002) used, was purchased from Tocris (#2725).

AlphaLISA SureFire Ultra and AlphaSureFire Ultra Multiplex Assays

The AlphaLISA SureFire Ultra p-ERK1/2 (Thr202/Tyr204) Assay Kit (PerkinElmer #ALSU-ERK2-A500) was used as described in the kit manual to measure ERK1/2 phosphorylation at Thr202 and Tyr204 in ERK1 and at Thr185/Tyr187 in ERK2. The AlphaSureFire Ultra Multiplex p-Akt Kit (1/2/3 (Ser473) (Eu) + Total Akt1 (Tb) Kit (#MPSU-PTAKT-K500) protocol was followed as described in the kit manual to measure AKT phosphorylation and total AKT levels. The buffer used for agonist and antagonist dilutions for SureFire Ultra assays was composed of 1X Hank’s Balanced Salts Solution (HBSS, ThermoFisher #14025-092), 25 mM HEPES (ThermoFisher #15630-080), and 0.1% Bovine Serum Albumin (BSA, Sigma #A7284).

LANCE Ultra cAMP Assays

The LANCE Ultra cAMP Detection Kit (PerkinElmer #TRF0262) was used to quantify levels of cAMP activation as per the description in the manual and Technical Data Sheets for both the kit and cell lines. For LANCE Ultra cAMP assays, all ligands and compounds were solubilized in stimulation buffer and cells were cultured as recommended in their respective Technical Data Sheets. The addition of 1% DMSO was used for each assay well where the small molecule SB 225002 (CXCR2 inhibitor) was tested. For both CCR7- and CXCR2-expressing cells, 2,500 cells were plated per well in a white 384-well OptiPlate (PerkinElmer #6007290), and were incubated with 10 µM Forskolin, 100 nM CCL19 or 10 nM CXCL8, and antagonist antibody dilutions for 30 min at RT. Eu-cAMP and ULight-anti-cAMP were then added for a 60 minute incubation and the standard kit protocol was followed.

Signal Detection and Data Analysis

The AlphaLISA SureFire Ultra and LANCE Ultra cAMP assays were measured using a PerkinElmer EnVision® multimode plate reader. Default values for standard Alpha detection and LANCE (TR-FRET) detection using laser excitation were used. Data is expressed as either raw AlphaLISA signal counts (luminescence output) or raw TR-FRET signal detected at 665 nm. For SureFire
Ultra Multiplex assays, raw counts were measured in the Europium and the Terbium channels for phospho-AKT and total AKT respectively (please refer to the application note# 012925A_01 for further details about the optics and filters used). Data are expressed as a Ratio of p-AKT/total AKT. Agonist and compound inhibitor titration curves were plotted and IC50 values calculated using GraphPad Prism software according to a nonlinear regression using the four-parameter logistic equation sigmoidal dose-response curve with variable slope. Data shown are the average of duplicate measurements.

Results and Discussion

Optimizing Cell Density and Agonist Concentrations

For both the CCR7 and CXCR2 overexpressing CHO cell lines, we first determined the optimal cell number and concentration of ligand/agonist to use for subsequent inhibitor competition experiments. For this, the AlphaLISA SureFire Ultra p-ERK kit was used and both cell number per well and ligand concentration were titrated. When developing an assay such as this, it is generally a good practice to examine the time course of ligand response as well. However, in this case it was not necessary, as the time course of ERK phosphorylation is well established and the peak signal is expected after 10 minutes of agonist stimulation.

For optimizing cell numbers, cells were plated at four seeding densities in white 96-well CulturPlates, starting from 20,000 cells per well titrated 2X down to 2,500 cells per well, and grown overnight. The next day, agonists specific for binding and activating CCR7 (Mip-3β/CCL19) and CXCR2 (IL-8/CXCL8) signaling were titrated to determine the optimal concentration for subsequent use. Agonists were added to wells, and plates were incubated at room temperature (~22 °C) for 10 minutes prior to removal of stimulation buffer. Cultures were then lysed and the AlphaLISA SureFire Ultra p-ERK assay performed (data presented in Figure 4). From these experiments, 2,500 cells per well for CCR7-expressing cells and 10,000 cells per well for CXCR2-expressing cells provided a sufficient assay window and were chosen as the seeding densities to be used going forward. For agonist concentrations, 10 nM of Mip-3β/CCL19 and 10 nM of IL-8/CXCL8 were chosen for use in subsequent inhibitor competition experiments.

Measuring Modulation of ERK and AKT Phosphorylation

With the cell numbers and agonist concentration chosen, the next question to address was whether agonist-induced phosphorylation could be inhibited by antibodies reported to be function-blocking for these receptors. To assess receptor inhibition for both GPCRs, we examined the effects on receptor signaling by measuring the amount of ERK1 and ERK2 protein kinase phosphorylation at Thr202/Tyr204 and measuring the amount of AKT1/2/3 phosphorylation at Ser473 relative to total AKT. For blocking antibody and compound testing, CHO cells were plated in white 96-well CulturPlates at 2,500 per well for CCR7 and 10,000 per well for CXCR2 assays and incubated overnight. Antagonists were added at dilutions through 12 points and incubated for one hour as described in the Methods section. Specific agonists for each cell line were added at the final concentrations chosen from the previous experiments and incubated for 10 minutes for p-ERK and 15 minutes for p-AKT prior to removal of media and cell lysis. Cultures were then assessed for both ERK phosphorylation (Figure 5) and AKT phosphorylation (Figure 6).

The reported function-blocking anti-CCR7 antibody from R&D Systems has demonstrated its activity for anti-cancer therapy via ADCC and was used to block STAT3 signaling from CCL19 stimulation5, 6. Here, it was tested for its ability to inhibit CCR7 signaling through ERK (Figure 5A) and AKT (Figure 6A) pathways. To show specificity of the receptor-neutralizing antibody tested, antibodies to unrelated targets (e.g., anti-CXCR2 for CCR7-expressing cells) or isotype controls (murine IgG2a) were titrated as well to show the assays were unaffected by these negative controls. For the CXCR2 expressing cells, all three of these antibodies were tested, as well as the small molecule inhibitor SB 225002, and data are shown for p-ERK (Figure 5B) and p-AKT (Figure 6B).
Figure 5. Inhibitor titrations in the AlphaLISA SureFire Ultra p-ERK assay, from A) CCR7 expressing cells and B) CXCR2 expressing cells.

Figure 6. Inhibitor titrations in the AlphaSureFire Ultra AKT Multiplex assay, from A) CCR7 expressing cells and B) CXCR2 expressing cells.

As expected, the specific blocking antibodies and compounds reported in the literature to inhibit CCR7 and CXCR2 receptor activation show inhibitory effects downstream on the ERK and AKT intracellular signaling pathways as a reduction in phosphorylation of these kinases. The IC_{50} values determined for anti-CCR7 antibody was 2.9 nM in the p-ERK assay and 89 nM for p-AKT. For anti-CXCR2, the IC_{50} in the p-ERK assay was 2.8 nM and in the p-AKT assay was 234 nM, whereas the inhibitor SB 225002 produced IC_{50} values of 69.4 nM in p-ERK and 82.3 nM in p-AKT assays. To ascertain that these antibody and compound effects are not specific to the SureFire assay models, we next assessed the effects of these antagonists using an orthogonal assay that measures cellular cAMP levels.

Assessing cAMP Modulation With LANCE TR-FRET

The results of testing the effects of these blocking antibodies and inhibitors on cAMP expression using LANCE Ultra TR-FRET are shown in Figure 7. IC_{50} values for each antagonist are labeled on each graph. For these experiments, the anti-CCR7 antibody was the only antagonist used in CCR7-expressing cells (Figure 7A), whereas the small molecule SB 225002 (Figure 7B) and anti-CCR7 and anti-CXCR2 antibodies (Figure 7C) were all tested for their effect on cAMP in CXCR2 expressing cells. An isotype control antibody (mlgG2a) and an unrelated target antibody were used as negative controls, demonstrating the specificity of the detection of CCR7 and CXCR2 blockade (data not shown for mlgG2a).
Conclusions

As expected, the antibody reported in the literature to be function-blocking for CCR7 activation also inhibited the levels of p-ERK and p-AKT, and modulated cAMP production. The antibody reported to be function-blocking for CXCR2 showed inhibition in the CXCR2-expressing cell assays, but not in the CCR7-expressing ones. In addition, the small molecule inhibitor SB 225002 showed inhibition in the LANCE cAMP assay that agreed well with the reported IC50 value of 22 nM (Tocris Tech Data Sheet). Similar IC50s were seen in the Alpha SureFire assays (69 nM in p-ERK and 82 nM in p-AKT).

PerkinElmer provides multiple solutions for measuring many downstream functions of chemokine receptors such as CCR7 and CXCR2 in a fast, reliable, and no-wash format. The data presented in this application note show that cell-based assays utilizing the Alpha SureFire Ultra and LANCE Ultra formats are amenable to screening both biotherapeutics antibodies and small molecule compounds. Regardless of the assay technology used or the downstream messenger targeted, these assays can be used to screen and develop inhibitors for a multitude of different cell surface receptors. These assays can also be used as orthogonal assays to test for compound specificity versus off-target technology inhibition.

References