Effects of 5FU and Sorafenib on Proliferation and Biomarker Expression in a Colorectal Cancer Model Using AlphaLISA and EnSight Solutions

Introduction
Colorectal cancer (CRC) is the third most common type of cancer that affects both men and women in the United States according to the Centers for Disease Control and Prevention\(^1\). A variety of chemotherapeutic drugs with different modes of action have been developed and tested as potential therapies for CRC. One of the most commonly used chemotherapies for treating many different cancer types is 5-Fluorouracil (5FU), an anti-metabolic drug that came into medical use in the 1960s and is now often used in combination with other targeted chemotherapeutic agents. A fluoropyrimidine that inhibits thymidylate synthase, 5FU blocks the synthesis of thymidine, which is required for DNA synthesis\(^2\). Sorafenib is a small molecule inhibitor of several tyrosine kinases (such as VEGFR, PDGFR, and Raf family kinases) and works by inducing autophagy, which can suppress tumor growth. It is currently indicated as a treatment for certain types of renal, liver and thyroid cancers and has been examined in recent years as a potential last line therapy for CRC\(^3\). These two drugs with varied modes of action have been recently tested in combination in Phase I clinical trials to treat advanced liver cancer\(^4\).
AlphaLISA technology enables the rapid detection of biomolecules of interest in a homogeneous, no-wash format. In a standard AlphaLISA detection assay (illustrated in Figure 1A) a biotinylated antibody binds to streptavidin-coated Donor beads while another antibody is conjugated directly to AlphaLISA Acceptor beads. Both antibodies bind to the analyte of interest and, when that analyte is present, they bring the Donor and Acceptor beads into close proximity. Upon excitation with light at 680 nm, the Donor beads emit singlet oxygen molecules that travel up to approximately 200 nm in solution to activate nearby Acceptor beads, which then emit light at 615 nm. This light emission, proportional to the concentration of analyte, can be detected on an Alpha-enabled reader, such as the EnSight™ multimode plate reader (Figure 1B).

In addition to being equipped with all the standard detection technologies for detection of absorbance, fluorescence, TRF, Alpha and ultra-sensitive luminescence, the EnSight offers a well-imaging detection module for performing automated cellular-imaging that includes brightfield, digital phase and up to four color fluorescence imaging. The EnSight is an extremely useful tool for cell-based assay development, allowing the user to assess cellular viability and growth dynamics through image-based cytometry as well as for the sensitive detection of luminescence signals from AlphaLISA assays.

We demonstrate here the utility and benefits of using AlphaLISA for characterizing the effects of potential drugs with different modes of action on a simple cell culture model of human colorectal cancer. For this, HCT116 cells were grown in microplates and treated with varying concentrations 5FU or sorafenib. We used the EnSight multimode plate reader to measure the effects of drug treatments on cellular proliferation by automated well-imaging and cell counting. In addition, the secretion of chemokines (IL-8, GROα/CXCL1), growth factors (VEGF), and other tumor-associated biomarkers (TIMP1, TIMP2, EGFR, CD276/B7-H3), as well as the activation of the MEK/ERK downstream signaling pathway were assessed using AlphaLISA detection assays. We show how sensitive and simple it is to rapidly assay for changes in biomarker expression and downstream signaling pathways using AlphaLISA assay technology detected using the EnSight multimode plate reader.

Materials and Methods

Cell Culture and Treatment

A vial of cells from PerkinElmer’s HCT116-Red-Fluc Bioware® Brite cell line (#BW124318) was thawed, resuspended in McCoy’s 5A Media (ATCC, #30-2007) supplemented with 10% FBS (ThermoFisher, #26140079) and maintained in 75 ml cell culture flasks. For experiments, cells were seeded at a density of 5,000 cells per well (in 100 μL) into black PerkinElmer 96-well ViewPlates™ (#6005182) and allowed to attach and grow overnight before treatment with drugs. Cultures were treated the next day with up to nine concentrations (2X titrations) of 5-Fluorouracil (5FU, Sigma, #F6627) or sorafenib (LC Laboratories, #S-8502) and grown for up to three more days. The drug compounds were diluted in DMSO and further diluted to 2X the desired final concentration in cell culture media and 100 μL was added per well. The final DMSO concentration in each well was 0.2%. To investigate the effect of drug treatments on the ERK1/2 signaling pathway, some assay plates included wells in which recombinant human Epidermal Growth Factor (EGF, R&D Systems, #236-EG) was added just prior to drug treatment (100 nM final).

For AlphaLISA biomarker detection assays, supernatants were collected (125 μL/well) from three separate assay plates after one, two, and three days of drug treatment into 96-well (polypropylene) StorPlates™ (PerkinElmer, #6008290) and frozen at -20 °C for later assessment. The remainder of the supernatants were removed from each plate and, and cells were lysed for up to 30 minutes for the standard AlphaLISA assay by adding 50 μL of 1X AlphaLISA Lysis Buffer (#AL003C). Lysates were transferred to StorPlates and then frozen. Once all samples were collected from each timepoint, supernatants and lysates were thawed and used for testing with AlphaLISA detection assays. For investigation of ERK1/2 phosphorylation, two separate culture plates were prepared and treated with each drug for two or 24 hours, then lysed with 1X SureFire® Ultra® Lysis buffer (for 15 minutes). Lysate samples were frozen in StorPlates until assays could be run on all samples.

AlphaLISA Assays

All AlphaLISA assays were run according to manufacturer’s specifications. Supernatant and lysate sample plates were thawed and divided into separate 384-well AlphaPlate™ microplates (PerkinElmer, #6005350). AlphaLISA assays were run in parallel on 5 μL samples taken from the same wells of the original culture plate to measure expression levels of various chemokines and tumor markers. Biomarker data were collected using PerkinElmer’s AlphaLISA human biomarker detection kits for IL-8 (#AL328C), VEGF (#AL201C), CXCL1/GROα (#AL349C), TIMP-1 (#AL309C), TIMP-2 (#AL3091C), EGFR (#AL340C), and CD276 (#AL3060C). Titrations of recombinant protein standards provided in each kit were run alongside samples being tested in the same sample.
matrix (culture media or lysis buffer) and raw data produced were interpolated to the standard curves to determine endogenous protein concentrations. AlphaLISA SureFire Ultra p-ERK1/2 (Thr202/Tyr204) (#ALSU-PERK-A500) and ERK1/2 Total (#ALSU-TERK-A500) Assay kits were run on 10 μL lysate samples collected previously and frozen. The assays were performed as prescribed in the manual for adherent cells with the 2-plate protocol. All assays run had individual incubation steps of an hour or less, allowing each assay to be completed in less than three hours. In addition, all AlphaLISA biomarker detection and AlphaLISA SureFire Ultra assays were measured using the PerkinElmer EnSight multimode plate reader using standard Alpha detection settings.

Figure 2. Treatment effects of 5FU and Sorafenib on cellular proliferation over three days. A) Representative images of HCT116 cells in a well cultured for three days post treatment with 6.25 μM 5FU using brightfield (top image) and UV optics for Hoechst (blue stained image). The middle image shows an enlarged subregion of the same well and illustrates the power of the object detection function on the EnSight (pink outlines around blue nuclei). The brightfield images below illustrate the effects of high concentrations of each drug after three days on cell density and morphology. B, C) Average cell number per well (# of nuclei) at each time point and concentration. The data from B and C plotted as inhibition curves at each timepoint for 5FU (D) and sorafenib (E) with IC₅₀ value reported from best fit line; n=3 wells, error bars=StDev.
Standard Curve and Data Analysis

Standard curves were plotted in GraphPad Prism® using nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting. Protein concentrations were determined by interpolating the counts measured onto the standard curve and all data shown are the average of at least three individual wells. Interpolated analyte concentrations represent the amount of protein in pg/mL in 5 μL of sample. The lower detection limit (LDL) of the assay was calculated by taking three times the standard deviation of the average of the background and interpolating from the standard curve. All data shown are the average of triplicate measurements (samples from three adjacent wells from the same culture plate).

Cellular Imaging and Automated Cell Counting

For cellular imaging and automated cell counting, cells were labeled with the nuclear dye Hoechst 33342 (Life Technologies, #H3570) by dilution in culture media and addition to each well (1:2000 final dilution). After a 30-minute incubation (at 37 °C), images of each well were rapidly acquired using the cellular imaging module of the EnSight multimode plate reader using Brightfield and the UV fluorescence filter. The number of cells per well was automatically measured in these applications using a built-in object identification and nuclei counting function in the Kaleido™ 3.0 software.

Results

Drug Treatment Effects on Cellular Proliferation Over Three Days

HCT116 cells are a fast-growing, adherent cell line with epithelial morphology and are a well-established cellular model of human CRC5. Since the HCT116 cell line has been previously shown to proliferate rapidly, a preliminary experiment was performed where cells were titrated to determine an optimal starting cell number that resulted in untreated wells achieving confluency without overcrowding (data not shown). The starting cell concentration chosen for subsequent experiments was 5,000 cells per well in 96-well ViewPlates. After allowing the cells to attach and grow overnight, wells were treated with increasing concentrations of either 5FU or sorafenib.

To assess the effects of drug treatment on cellular proliferation, the number of cells per well was measured in three separate plates at each timepoint and drug concentration by labeling cells with the Hoechst 33342 nuclear stain and imaging. Hoechst is a cell-permeable dye that binds to the minor groove of double-stranded DNA and can be imaged using UV fluorescence filters. Each well was imaged, and cell numbers were quantified using the EnSight's well imaging mode (Figure 2). According to cell counts, after one day of treatment (two days in culture), control wells (without drug) contained more than 50,000 cells per well, an exponential increase in cell numbers from the initial 5,000 cells plated two days before.

Figure 3. Detection of human IL-8 protein secretion from HCT116 cells. A) Raw AlphaLISA signals generated from supernatant samples taken from cultures grown and treated with varying concentrations of 5FU for 1, 2, or 3 days. To determine concentrations of endogenous IL-8, raw AlphaLISA signals were interpolated from the (B) standard curve generated from a titration of recombinant human IL-8. The effects of treatment at each timepoint are shown for (C) 5FU and (D) Sorafenib; n=3 wells, error = StDev.
AlphaLISA Human IL-8 Detection in Cellular Supernatant

Tumor cells secrete small, soluble, chemotactic cytokines and chemokines that can recruit proinflammatory cells to the tumor site that interact through both cell-cell contact and by secreting additional factors that form a complex tumor microenvironment. In this microenvironment, additional cytokines, chemokines, and other factors work through both paracrine and autocrine stimulation on tumor maturation and metastases. Tumor cells have also been shown to secrete a variety of cytokines and chemokines in response to cellular stress caused by chemotherapeutic agents\(^6\). Interleukin-8 (IL-8) is a proinflammatory chemokine that has been shown to be regulated by a number of different stimuli, including inflammatory signals and chemical stresses like exposure to chemotherapy agents\(^7\). To measure IL-8 secretion in our cultures, supernatants were collected from cultures after one, two, or three days of drug treatments and samples frozen. After all samples were collected, supernatants were examined for the presence of IL-8 protein, as well as several other secreted factors using AlphaLISA detection assays (next section). The AlphaLISA signals were read on the EnSight multimode plate reader and raw data from wells treated with 5FU are presented in Figure 3A. A standard curve generated from titrating recombinant human IL-8 alongside the samples (Figure 3B) was used to interpolate the raw AlphaLISA signal (Figure 3A) to actual concentrations of protein in pg/mL (Figure 3C-D). The data from this first experiment indicate that HCT116 cells do secrete significant levels of IL-8 and that these levels increase over time with increasing concentrations of 5FU and, to a lesser extent, sorafenib. This suggests that IL-8 protein secretion increases with cellular stress.

Other Secreted Factors Modulated by Drug Treatment

Two soluble factors that have been reported to be upregulated in colon cancer are vascular endothelial growth factor (VEGF) and CXCL1\(^6\). The VEGF family of ligands (VEGF-A, -B, -C, and -D) are known to be key mediators of tumor angiogenesis. Intracrine VEGF signaling has been shown to promote CRC cell survival such that, when VEGF is deleted (knocked out), the cells become more readily impaired by 5FU treatment\(^8\). Multiple drugs to inhibit tumor neo-vascularization by targeting VEGF with biologics (anti-VEGF) have been developed\(^9\). We examined the concentrations of VEGF-A secreted by HCT116 cells using the AlphaLISA VEGF detection assay and found significant effects of drug treatment over time on the concentration of VEGF in cellular supernatants (Figure 4A-B). However, the effects of treatments appear to mostly be due to the drug effects on overall cell numbers. This is illustrated by the observation that increasing concentrations of 5FU resulted in decreased VEGF secretion with an IC\(_{50}\) of 4.78 μM after three days (Figure 4A), comparable to the effects of 5FU observed on overall on cell numbers at three days (Figure 2C).

CXCL1 (Chemokine C-X-C motif ligand 1), also known as GRO\(^{\alpha}\) (Growth regulated oncogene)-\(\alpha\), is a chemotactic cytokine known to regulate cancer progression and invasion and has been recommended as a biomarker and potential therapeutic target for CRC treatment. CXCL1 has been reported to be hypersecreted by human CRC cells\(^10\). We observe an upregulation of GRO\(\alpha\)/CXCL1 secretion over time and with increasing concentrations of 5FU. The upregulation of CXCL1 by 5FU is supported in the literature by the observation that it is upregulated in mice treated with 5FU\(^11\). In addition, GRO\(\alpha\)/CXCL1 appears to be upregulated by sorafenib at treatment dosages up to 6.25 μM, the same concentration at which sorafenib appears to be significantly inhibiting cell proliferation (Figure 2C).
Tissue Inhibitors of Metalloproteinases (TIMPs) are a family of protease inhibitors that specifically inhibit matrix metalloproteinases (MMPs), blocking their proteolytic activity and minimizing extracellular matrix degradation. They are involved in many functions, including angiogenesis, proliferation, and apoptosis, and TIMP-1 has been reported to be a prognostic marker for colon cancer progression. To examine whether TIMPs are secreted by HCT116 cells and regulated by drug treatments, supernatants from the same wells were assessed with AlphaLISA assays for the presence of TIMP-1 and TIMP-2. We find here that both TIMP-1 and TIMP-2 are secreted in large amounts by HCT116 cells (Figure 5), necessitating the dilution of samples (3X in assay buffer) to allow for the accurate measurement of concentrations at all timepoints of the assay. Much like VEGF, the effects of drug treatments on both TIMP-1 and TIMP-2 appear to be mostly correlated with the drug effects on overall cell numbers.

Assessment of Cellular Lysate Samples for EGFR and CD276

In addition to secreted factors, tumor cells are often characterized by the over-expression of a variety of cell surface receptors which are also popular therapeutic targets. The epidermal growth factor receptor (EGFR) is a central regulator of cancer cell proliferation and progression and a therapeutic target in several human cancer types, including CRC, but often only a subset of patients will respond to treatment or initially respond and then later develop acquired resistance to EGFR inhibitors. Thus, combinatorial therapy with EGFR receptors and other chemotherapeutic drugs, like 5FU and sorafenib have been, and continue to be examined. We investigated expression levels of the EGFR protein in cellular lysate samples from HCT116 cultures treated for three days with varying concentrations of 5FU or sorafenib (Figure 6A-B). Experimental results indicate a dose-dependent effect on EGFR expression at higher concentrations of both drugs.

CD276, also known as B7-H3, is an immune checkpoint protein expressed on antigen precursors and the surface of many tumors. It has been shown to play an important role in the inhibition of T cell function and, in recent years, become an important target for immunotherapy. A recent report suggests that TGF-β signaling can upregulate B7-H3 (CD276) expression to promote immune escape in HCT116 cells. We confirm here that CD276 is expressed at high concentrations in our cultures that concentrations appear to be modulated by drug treatments after three days in culture (Figure 6, C-D).

5FU and Sorafenib Have Opposing on the Effects ERK1/2 Pathway

In addition to assessment of specific protein biomarker expression, AlphaLISA assay technology can be used to assess phosphorylation of proteins in various intracellular signaling pathways with AlphaLISA SureFire Ultra assays. We examined the activation of the MEK/ERK pathway by assessing the relative phosphorylation levels of ERK1/2. In the AlphaLISA SureFire Ultra phospho-ERK1/2 assay, one antibody recognizes the phospho-Thr202/Tyr204 epitope and the second recognizes a distal epitope on ERK1/2 protein. Phosphorylation and intracellular signaling events happen traditionally on a more rapid timescale, preceding later protein expression and secretion changes, so we examined activation of ERK1/2 after 2- and 24-hour treatments with 5FU and sorafenib. As a positive control, EGF (100 nM final) was added to some wells to stimulate the MEK/ERK pathway through the EGF Receptor. Adjacent wells were lysed at each timepoint and assays for phospho-ERK1/2 as well as total-ERK1/2 were run on lysate samples from the same wells. The data from these experiments, shown in Figure 7, illustrate that increasing concentrations of 5FU result in increased ERK1/2 phosphorylation over a 24-hour period, especially when you normalize the phospho-ERK1/2 signal to total-ERK1/2 signal (Figure 7, left-side graphs). The gradual increase in ERK1/2 phosphorylation with increasing concentrations of 5FU may be in part due to increased secretion of IL-8 which can enhance the proliferation and survival of cancer cells through autocrine signaling and can regulate the activity of the MAPK signaling cascade.
Figure 6. Effects of treatment on the expression of two membrane proteins that are hallmarks of tumor progression. (A,B) EGF Receptors and (C,D) CD276 (B7-H3) checkpoint protein are both expressed at high concentrations in cellular lysate samples and are affected by high concentrations of drug after three days; n=3 wells, error bars=StDev.

Figure 7. Differential effects of two drug treatments on the MEK/ERK signaling pathway using AlphaLISA SureFire Ultra. Relative levels of ERK1/2 phosphorylation and total ERK1/2 protein were measured after two hours and 24 hours of drug treatment using SureFire Ultra assays for (A) phospho-ERK1/2 and (B) total ERK1/2 from lysate samples. C) To assess overall changes in phosphorylation levels, phospho-ERK1/2 signal was divided by total-ERK1/2 signal measured from samples taken from the same wells; n=3 wells, error bars=StDev.
The effects of increasing concentrations of sorafenib on ERK1/2 phosphorylation is more complicated to explain. It appears that treatment with lower concentrations of sorafenib induces significantly more ERK1/2 phosphorylation in wells with no drug and even wells treated with 100 nM EGF, especially over 24 hours. The observation that high concentrations of sorafenib result in reduced ERK signaling is supported by the literature showing that treatment of even tyrosine kinase inhibitor resistant strains of HCT116 cells for two days with sorafenib has been shown to down-regulate MEK phosphorylation\textsuperscript{12}, but the effects of lower concentrations that we see here are harder to explain.

Conclusions
Elevations in the expression levels of various chemokines, growth factors and cell surface markers have been observed in a variety of tumors and cancer models which can be modulated by chemotherapeutic drugs. IL-8, VEGF, CXCL1, TIMP-1, TIMP-2, EGFR and CD276 are all markers associated with colorectal cancer progression. We show here that homogeneous AlphaLISA assay technology can be used to measure multiple biomarkers in both cell culture supernatant and lysates from the same wells of a microplate to examine complex protein expression profiles from a CRC cellular model. In addition, drug treatment effects of intracellular signaling pathways can be probed by using AlphaLISA SureFire Ultra assays, as illustrated here by observed changes in ERK1/2 phosphorylation.

AlphaLISA assays provide a fast, powerful, homogeneous platform for measuring the effects of a variety of drugs on multiple biomarkers secreted by and expressed in human cells. This technology has a number of distinct advantages including high signal to background, wide dynamic range, and an extremely simple, straight-forward, no-wash protocol. Finally, all these AlphaLISA assays can be rapidly measured and treatment effects on cellular proliferation and viability can be assessed using the imaging capabilities and imaged-based cytometry of the EnSight multimode plate reader.

References
1. https://www.cdc.gov/cancer/colorectal/