High-Throughput Screening with HyperCyt® Flow Cytometry to Detect Small Molecule Formylpeptide Receptor Ligands

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High-throughput flow cytometry (HTFC), enabled by faster automated sample processing, represents a promising high-content approach for compound library screening. HyperCyt® is a recently developed automated HTFC analysis system by which cell samples are rapidly aspirated from microplate wells and delivered to the flow cytometer. The formylpeptide receptor (FPR) family of G protein–coupled receptors contributes to the localization and activation of tissue-damaging leukocytes at sites of chronic inflammation. Here, the authors describe development and application of an HTFC screening approach to detect potential anti-inflammatory compounds that block ligand binding to FPR. Using a homogeneous no-wash assay, samples were routinely processed at 1.5 s/well (~2500 cells analyzed/sample), allowing a 96-well plate to be processed in less than 2.5 min. Assay sensitivity and accuracy were validated by detection of a previously documented active compound with relatively low FPR affinity (sulfinpyrazone, inhibition constant [K_i] = 14 μM) from among a collection of 880 compounds in the Prestwick Chemical Library. The HyperCyt® system was therefore demonstrated to be a robust, sensitive, and highly quantitative method with which to screen lead compound libraries in a 96-well format. (*Journal of Biomolecular Screening* 2005:374-382)

Key words: drug discovery, flow cytometry, automation, ligand binding assay, fluorescence

MODERN DRUG DISCOVERY involves testing cellular targets against millions of potentially valuable compounds that may bind cellular receptors to effect clinically therapeutic cellular responses. The need for high-throughput analytical screening technologies is therefore paramount. With respect to the serial analysis of individual cells or beads, the flow cytometer has always been considered a high-throughput analysis instrument, routinely analyzing from thousands to tens of thousands of particles per second. However, for automated analysis of multiple discrete samples of cells, the throughput of flow cytometry has been severely limited, with commercial systems capable of processing only about 2 samples per minute. This is a significant bottleneck when the objective is to screen a large collection of compounds against replicate cell samples.

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Received Jul 15, 2004, and in revised form Nov 18, 2004. Accepted for publication Nov 20, 2004.

Journal of Biomolecular Screening 10(4); 2005
DOI: 10.1177/1087057105274532

Flow cytometry is an inherently high-content methodology, capable of simultaneous quantitative analysis of multiple optical markers of biochemical expression or physiological response. Even the least expensive modern instruments can measure 5 optical parameters at once (3 fluorescence and 2 light scatter signals), and specially configured research instruments may simultaneously measure as many as 14.[1] High-throughput flow cytometry (HTFC), enabled by faster automated sample processing, therefore represents a potentially valuable high-content approach for compound library screening. Microfluidic analysis systems and microliter sample volumes are of particular value due to the cost and often limited availability of test compounds. HyperCyt® is a recently developed automated HTFC analysis system by which cell samples are rapidly aspirated from microplate wells and delivered to the flow cytometer. Accurate quantitative measurements have been demonstrated in endpoint assays at rates of 20 to 40 samples/min over a 4-decade range of fluorescence intensity. Intact cells may be used at concentrations of 1 to 20 million/ml in assay volumes of 8 to 15 μl.[2] Typical sample volumes of 1 to 2 μl from each assay volume allow scarce quantities of test cells or reagents to be efficiently utilized.

G protein–coupled receptors (GPCRs) constitute the largest known family of cell membrane receptors and are involved in the recognition and transduction of messages as diverse as light, Ca++, odorants, amino acids, nucleotides, peptides, and proteins.

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Compounds that can stimulate (agonist) or inhibit GPCR function (antagonists) are a major focus of drug discovery efforts, as indicated by the large proportion of drugs on the market (>50%) that target GPCRs.[3] We have now initiated a HyperCyt®-based HTFC screening effort to detect potential anti-inflammatory compounds that block ligand binding to a prototypic GPCR, the leukocyte formylpeptide receptor (FPR). Here, we characterize basic features of the HTFC FPR assay and validate its application to screening of the Prestwick Chemical Library, a collection of off-patent drugs and alkaloids.

MATERIALS AND METHODS

Cells and reagents

Fluorescein-labeled formylmethionine-leucine-phenylalanine-lysine (fMLFK-FITC) and unlabeled formylmethionine-leucine-phenylalanine-phenylalanine (fMLFF, 4PeP), fML (2Pep), fMLF (3Pep), and formylnorleucine-leucine-phenylalanine-norleucine-tyrosine-lysine (fNleLFNleYK) were obtained from Penninsula Labs (San Carlos, CA). The fNleLFNleYK was tagged with Alexa-633 (Molecular Probes, Eugene, OR) as previously described.[4] Myeloid U937 cells transfected with the human FPR were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 10 mM HEPES, 10 U/ml penicillin, and 10 µg/ml streptomycin, 4 µg/ml CIPRO, and 500 µg/ml Geneticin (Invitrogen, Japan). Cultures were grown at 37° C in a 5% CO_2 atmosphere and passaged every 3 days. Unless otherwise indicated, U937 cells were used that expressed a mutant FPR with glycine and alanine substituted for serine and threonine residues in the C-terminal tail (DeltaST).[5] DeltaST cells do not internalize the receptor when stimulated with fMLF.[5] Chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

FPR assay

The FPR assay measured the ability of test compounds to compete with a high-affinity fluorescent ligand, fMLFK-FITC, for binding to cell membrane FPR (Fig. 1). The assay response range was defined by replicate control wells containing unlabeled fMLFF peptide (4Pep; Fig. 1B) or buffer alone (Fig. 1A). Unlabeled fMLFF was at a 100-fold higher concentration that completely blocked binding of the fluorescent ligand; hence, wells containing this were designated blocked controls. Control wells containing buffer alone were designated unblocked controls. Test compound inhibition of fluorescent peptide binding (Fig. 1C) was calculated as $100 \times [1 - (MFI_{Test} - MFI_{Blocked})/(MFI_{Unblocked} - MFI_{Blocked})]$, in which MFI was the median fluorescence intensity of cells in wells containing test compounds, blocked control wells, and unblocked control wells as indicated by subscripts of each MFI term in the equation. Compounds inhibiting 70% or more were considered "hits."

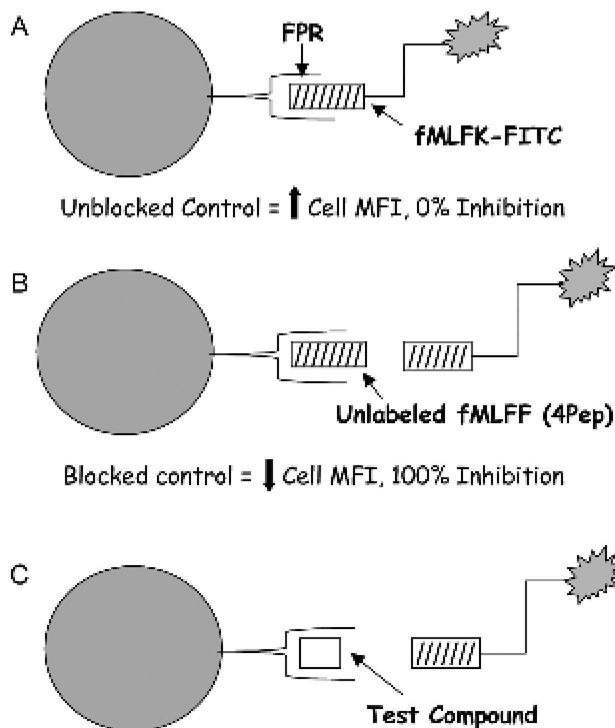

FIG. 1. Formylpeptide receptor (FPR) competitive inhibition assay. The assay measures the ability of a compound to compete with a fluorescent high-affinity peptide for binding to the human FPR. **(A)** Unblocked control wells containing no competitor result in maximum fMLFK-FITC binding and cell fluorescence (0% inhibition), expressed as the median fluorescence intensity (MFI). **(B)** Blocked control wells contain a high-affinity unlabeled peptide (4Pep) at high concentration to define the minimum fluorescence associated with blocked FPR (100% inhibition). **(C)** The assay measures test compound effects on cell MFI relative to the range defined by blocked and unblocked controls.

For assay performance, additions to wells were in sequence as follows: 1) test compounds and control reagents (5 µl/well), 2) cells (10^7/ml, 5 µl/well), and 3) (after 30 min, 4° C incubation) fMLFK-FITC (5 µl/well). After an additional 45 min, 4° C incubation, plates were immediately analyzed by flow cytometry with the HyperCyt® system.

MFI measurements from control wells were also used to calculate a Z′ factor for each assay. The Z′ factor is a measure of screening assay quality that reflects both assay signal dynamic range and data variation associated with the signal measurements.[6] The Z′ factor for 268 plates analyzed in this and other studies was 0.62 ± 0.16 (mean ± *SD*).

Cell viability, determined by uptake of trypan blue or ethidium bromide, was routinely 90% or greater in all assays. Cell count and concentration references pertain to total viable cells. In the flow cytometer, viable cells were effectively distinguished from nonviable cells and debris on the basis of a combination of 2 light-scatter signatures (forward vs. side scatter). Only cells with appropriate light-scatter characteristics were evaluated for fluorescent

ligand binding. An expected phenotype of nonviable cells, in addition to aberrant light-scatter characteristics, would be high nonspecific uptake of fluorescent ligand that was unaffected by the presence of blocking peptide controls. Thus, conditions of poor cell viability would be indicated by a lower than expected range of fluorescence intensity between positive and negative controls as well as a low calculated assay Z′ factor.

Prestwick Chemical Library compounds were provided as DMSO stock solutions and were diluted 1:50 in assay dilution buffer (ADB; 110 mM NaCl, 30 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 0.1% bovine serum albumin) to attain DMSO concentrations of 2% prior to addition to wells. Final DMSO concentration in the assay was 1%. Test peptides were diluted in ADB to the indicated final concentrations. fMLFF (4Pep blocked control) and fMLFK-FITC peptides were used at final concentrations of 150 and 1.5 nM, respectively. A starting cell stock of 10^7 cells/ml (in ADB) was diluted 1:3 in the final assay mixture (~3.3×10^6 cells/ml, 15 µl total volume). This resulted in analysis of ~2500 cells from each well when sampling at 40 wells/min (aspirated sample volume ~2 µl) with HyperCyt®.

The best flow cytometry sampling and analysis results are obtained with uniform cell suspensions. Significant cell settling may occur after 5 to 10 min in undisturbed wells.[2] To minimize cell settling, test cells and fluorescent peptide ligand were added quickly (1-2 min) under automated control of a Titertek MAP-C liquid dispensing system. Specialized 96-well microplates were used (Greiner Imp@ct plate, Intermountain Scientific) that allowed retention of samples in wells (by surface tension) when the microplates were inverted. To maintain uniform cell suspensions during the two 4° C incubations, microplates were placed on a rotating cell suspension system device (in a refrigerator) that continuously rotated them between upright and inverted positions at 4 rpm.[2] HyperCyt® sampling was completed in less than 3 min.

HyperCyt®

The HyperCyt® system[2,7] interfaces a flow cytometer and autosampler. As the sampling probe of the autosampler moves from one well to the next of a multiwell microplate, a peristaltic pump sequentially aspirates sample particle suspensions from each well. Between wells, the continuously running pump draws a bubble of air into the sample line. This results in the generation of a tandem series of bubble-separated samples for delivery to the flow cytometer. Sample and bubble volumes are determined by the time that the autosampler probe is in a microplate well or above a well intaking air. We have validated cell-based high-throughput end point assays for ligand binding, surface antigen expression, and immunophenotyping.[2]

Sample fluorescence was excited with a 488-nM laser in a BD Biosciences FacScan flow cytometer. The assay response data were measured in the FL1 green fluorescence emission channel (515-545 nM). The FL3 red fluorescence emission channel (>650 nm) was used for detection of Cytoplex L9 or L10 beads (Duke Scientific, Palo Alto, CA) that were added to a subset of wells to facilitate proper registration of flow cytometry data with source wells.

Data analysis

In HyperCyt®, the air bubble–separated samples are delivered in a continuous stream to the flow cytometer. Likewise, the data are collected in a continuous stream, with the accumulated data from all wells of a microplate representing a single data file. The time-resolved data, with periodic gaps corresponding to the passage of the sample-separating air bubbles, were analyzed by proprietary software (FCSQuery). The program automatically detects the time-resolved data clusters, ensures that there are 96, and analyzes each to determine the MFI of bound peptide. These reduced data are automatically exported to a Microsoft Excel spreadsheet template that immediately calculates the assay quality control Z′ factor and peptide binding inhibition percentage for each well. Thus, comprehensive assay results are available within 1 to 2 min after assay plate sampling is completed. Because each sample consists of 2 µl taken from a 15-µl volume in each well, we routinely sampled and analyzed each plate twice and averaged the results in the Excel spreadsheet.

Ligand competition curves were fit by nonlinear least-squares regression using a 1-site competition model with Prism® software (GraphPad Software, Inc., San Diego, CA) to determine the concentration of added competitor that inhibited fluorescent ligand binding by 50% (IC_{50}). FPR expression ranged from 100,000 to 400,000 receptors per cell in different assays as determined by comparison to standard curves generated with Fluorescein Reference Standard Microbeads (Bangs Laboratories, Fishers, IN). This corresponded to total FPR concentrations of 0.6 to 2.2 nM. To account for effects of ligand depletion at the higher receptor concentrations, inhibition constants (K_i) were calculated from the IC_{50} by the method of Munson and Rodbard[8]:

$$K_i = K_d \times [y_0/(y_0 + 2)] + IC_{50}/\{1 + [p^* \times (y_0 + 2)]/[2 \times K_d \times (y_0 + 1)] + y_0\},$$

in which y_0 is the initial bound-to-free concentration ratio for the fluorescent ligand, p^* is the added concentration of fluorescent ligand (1.5 nM), and K_d is the dissociation constant of the fluorescent ligand (3 nM).

RESULTS

Characterization of assay sensitivity

In a representative experiment, a 12-well sequence of samples (Fig. 2A: 2 unblocked controls, 1 4Pep-blocked control, 4 concentrations of 2Pep, 4 concentrations of 3Pep, and 1 additional unblocked control) was replicated 8 times in a 96-well plate. HyperCyt® analysis at 40 samples/min produced 96 time-resolved data clusters, 1 for each well (Fig. 2B). The MFI was determined for each data cluster (Fig. 2C) and used to generate dose-response

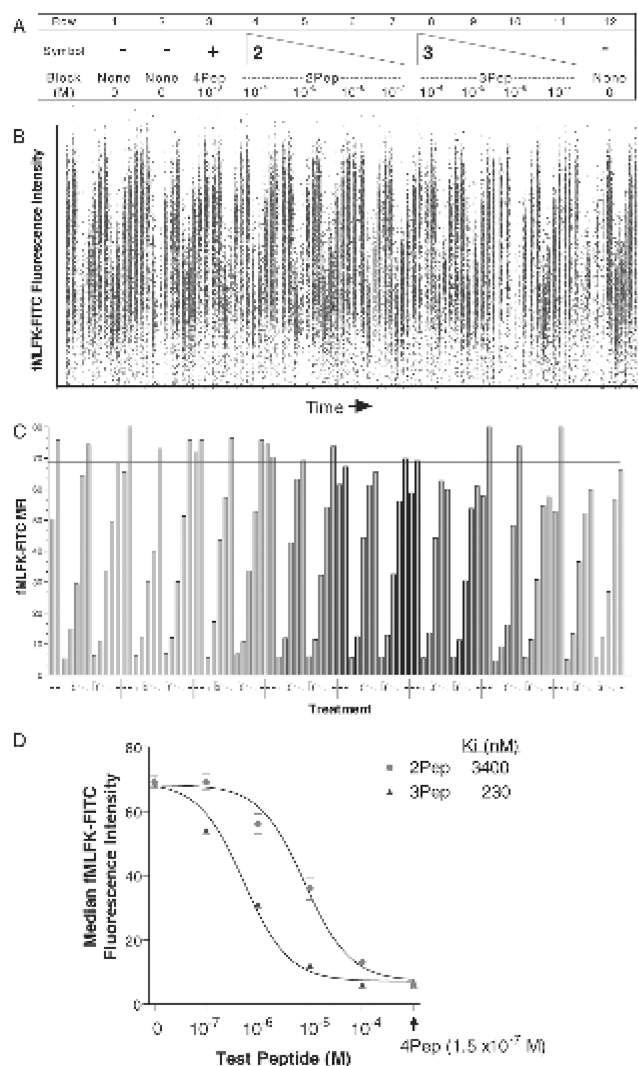

FIG. 2. Analysis of assay sensitivity and performance. Two test peptides with low (2Pep, fML) and intermediate (3Pep, fMLF) formylpeptide receptor (FPR) binding affinity were assessed for competitive binding in the FPR assay. **(A)** The 12-well series of samples (3 unblocked controls, 1 blocked control, and 4 concentrations of each test peptide) that was replicated 8 times in a 96-well plate. **(B)** Time-resolved clusters of cell fluorescence data, 1 for each well, that were produced in conjunction with HyperCyt® analysis. **(C)** Plot of the median fluorescence intensity (MFI) of cells from each of the wells. **(D)** Calculated dose-response curves and inhibition constants (K_i) for the 2 peptides. Error bars represent standard errors of the mean for each measurement (n = 24 for the point at 0 M and 8 for each of the others).

curves for the 2 peptides (Fig. 2D). The estimated K_i values of 3400 nM for 2Pep and 230 nM for 3Pep were consistent with previous manual measurements with formylpeptides of similar size (fMF and fNLF, K_d ~3000 and 300 nM, respectively).[9] Both peptides consistently inhibited ligand binding >70% at a 100 μM concentration (Figs. 2C, 2D). The assay was thus fast (1.5 s/well), reproducible, homogeneous, and quantitative over a 4-decade range of test compound concentration.

FPR screening assay

A standard screening assay protocol was developed in which 96-well microplates were configured with 16 control wells (columns 1 and 12 for unblocked and blocked controls, respectively, of Fig. 3A) and 80 wells to which test compounds were added (columns 2-11 of Fig. 3A). Time-resolved data were simultaneously monitored in 2 fluorescence channels. The FL1 green fluorescence channel was used to quantify fluorescent peptide binding to cells (Fig. 3B). The FL3 red fluorescence channel was used to detect beads that were added to each control well in column 1 of the microplate (Fig. 3C).

The clusters of red bead fluorescence in the FL3 channel data display were used as a means to verify that the data clusters in the FL1 channel were properly matched with source microplate wells. Twelve FL1 data clusters were expected in the interval from each FL3 data cluster to the next (i.e., 12 wells/row), and any deviation indicated an error condition due, for example, to aberrant sample uptake (e.g., empty well) or sample delivery (e.g., transient plug in the nozzle). However, in the present study and in all subsequent screening assays done to date, we have yet to encounter a nozzle blockage problem. A common basis of nozzle blockage, cell aggregation, can be minimized, if necessary, by filtering cells through nylon mesh prior to the assay, addition of chelating agents such as EDTA that prevent many types of cell-cell adhesion, or the selective use of cells that are not prone to aggregation under the assay conditions of concern.

The bead data also served as an internal control to assess particle carryover between wells, as indicated by the number of beads detected in downstream samples from bead-free wells. Particle carryover typically ranged from <1% to 2% in the no-wash format of the screening assay.

In air-segmented sample streams such as employed by the HyperCyt® system, a thin film of fluid lining the walls of the transport tubing is a basis of intersample fluid contamination.[10] Fluid carryover is more pronounced than particle carryover, requiring 1 or more postsampling rinse steps to eliminate.[10,11] However, in end point assays, fluid compound carryover occurs only during the time that samples are being transported from microplate to flow cytometer. The 4Pep blocking peptide, in well 12 of each microplate row, had a K_d similar to the fluorescent peptide (~3 nM) and was used at a 100-fold higher concentration (150 nM). To assess potential 4Pep carryover effects, we systematically analyzed wells sampled downstream of each 4Pep-blocked control well. To generate the data illustrated in Figure 3D, each row of wells was sampled from left to right (columns 1-12), progressing from the top row to the bottom (rows A-H). The cell fluorescence intensity in all wells downstream of the 4Pep wells (columns 1-11 in rows B-H) was comparable to that in the carryover-free wells sampled before the first 4Pep well (wells 1-11 in row A; Fig. 3D). Thus, any 4Pep carryover that occurred was insufficient to perturb assay results during sample transport.

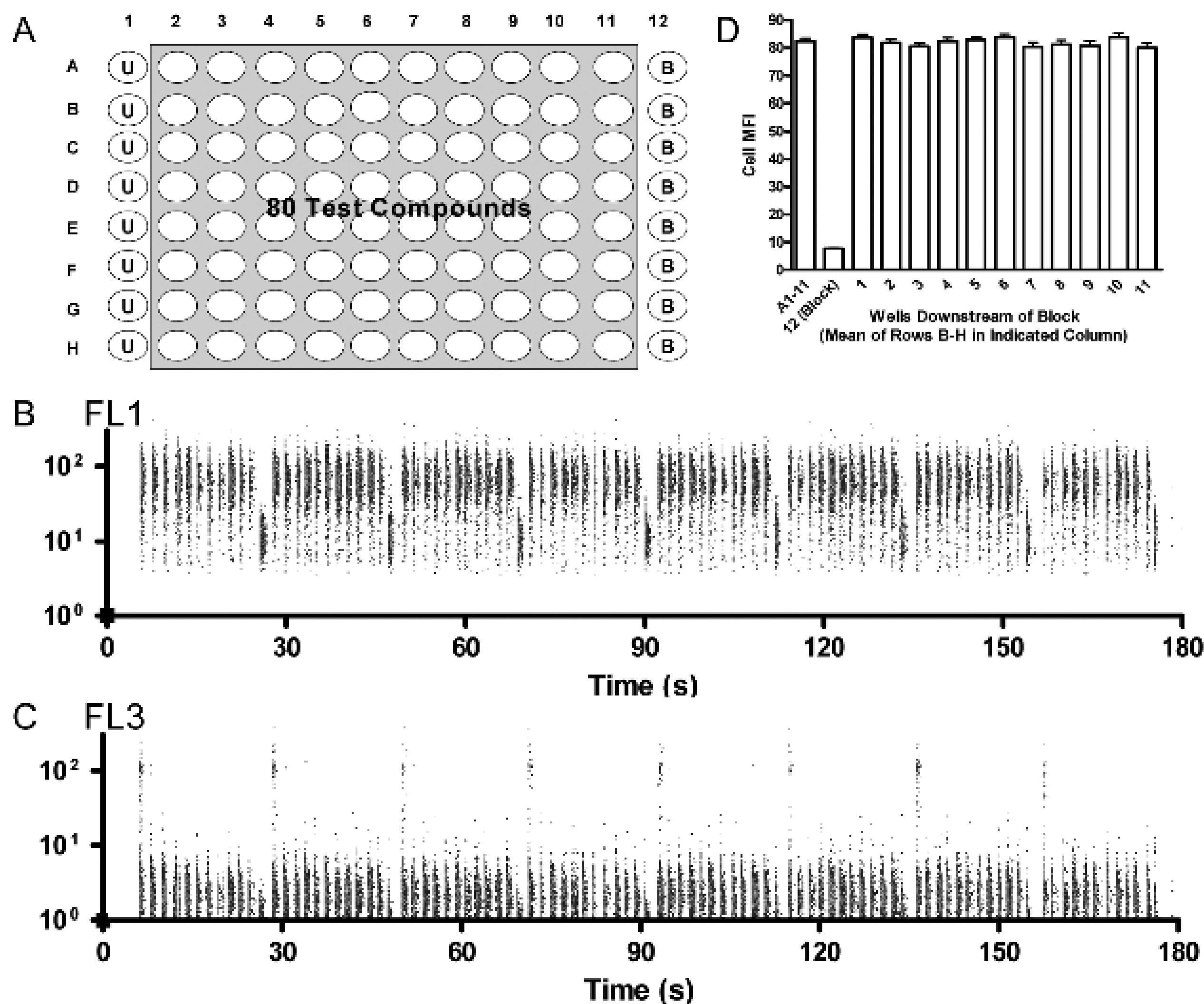

FIG. 3. Screening assay configuration and performance. **(A)** Each 96-well screening plate contained a column of 8 unblocked control wells (U), a column of blocked control wells (B), and 80 wells containing test compounds. (**B**) Time-resolved data collected in the FL1 green fluorescence channel. Rows A to H were sampled from left to right, 1 row at a time from top to bottom. Every 12th data cluster shows the decreased cell fluorescence associated with blocked control wells. (**C**) Time-resolved data collected in the FL3 red fluorescence channel. Red fluorescent beads added to each unblocked control well resulted in a bright red fluorescence signal detected in the 1st well and at 12-well intervals thereafter. **(D)** Analysis of the effects of compound carryover from wells containing 4Pep blocking peptide. The first 11 wells in row A were considered carryover free because they were sampled before the 1st blocked-control well, A12. In rows B to H, all wells which were potentially exposed to 4Pep carryover, cell median fluorescence intensity (MFI) was averaged on a column-by-column basis. Illustrated are means and standard deviations of MFI from the indicated groups. Results from all wells sampled downstream of 4Pep wells were comparable to carryover-free wells.

Correlation of within-plate and between-plate assay measurements

Five sets of duplicate plates were prepared and analyzed in series to assess between-plate measurement variation. Each individual plate was also analyzed twice to determine within-plate variation associated with the HyperCyt® sampling and analysis component. The R^2 correlation coefficient for within-plate replicate measurements made on 10 plates (96 duplicate measurements/plate) was 0.950 ± 0.020. After averaging the within-plate duplicate measurements for each plate, between-plate R^2 for the 5 plate pairs was 0.796 ± 0.068. The lower R^2 for between-plate comparisons reflected additional components of variance associated with plate preparation and biological response.

Prestwick Compound Library screening

The Prestwick Chemical Library is a collection of 880 high-purity off-patent drugs and alkaloids selected for structural diversity, broad spectrum covering a range of therapeutic areas, and

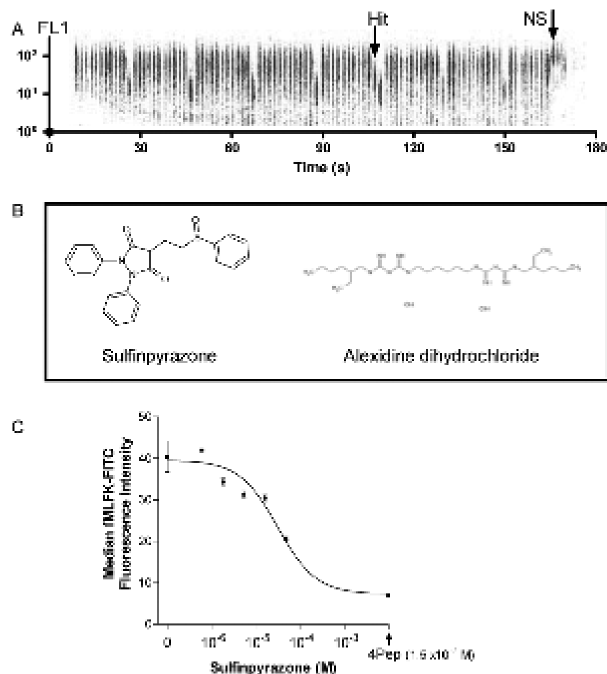

FIG. 4. Screening of the Prestwick Chemical Library. Eleven plates containing 880 compounds were screened. **(A)** Analysis of compounds from source plate 4 illustrating >70% inhibition by sulfinpyrazone in well E11 (Hit) and nonspecific fluorescence resulting from quinacrine dihydrochloride in well H9 (NS). **(B)** Compounds that reproducibly reduced fluorescent ligand binding in the formylpeptide receptor assay. Sulfinpyrazone, K_i = 14 µM, was determined to be an antagonist. Alexidine dihydrochloride exhibited an apparent K_i of 20 µM, but this was subsequently attributed to toxic effects on the test cells. **(C)** Dose-response profile of sulfinpyrazone. Error bars represent standard errors of the mean for each measurement (n = 10 for the point at 0 M and 5 for each of the others).

known safety and bioavailability in humans (http://www.prestwick.com). This library was screened at final compound concentrations ranging from 20 to 100 µM (1:100 dilutions of compound stocks). One active compound was detected that produced >70% inhibition in the screening assay (Figs. 4A, 4B). This was sulfinpyrazone, a compound structurally related to the anti-inflammatory drug phenylbutazone. Sulfinpyrazone was previously reported to bind FPR with a K_i of 26 µM.[12] Our estimated K_i was 14 µM. Sulfinpyrazone acted as an antagonist in that it failed to elicit an elevation in intracellular Ca^{2+} in DeltaST cells and blocked the Ca^{2+} response elicited by fMLFF peptide (data not shown).

Another test compound, alexidine dihydrochloride (Fig. 4B), resulted in ~50% inhibition in the screen and an apparent K_i of 20 µM in subsequent dose-response analysis. However, it elicited strong Ca^{2+} responses and toxicity (membrane permeability to trypan blue or ethidium bromide) in parental U937 cells that lacked FPR as well as in FPR-expressing DeltaST cells. A structurally related compound, dimethylbiguanide (Metformin), failed to block fluorescent ligand binding to FPR and was nontoxic at concentrations up to 10 mM (data not shown). Alexidine dihydrochloride, and the related bisbiguanide compound chlorhexidine, are commonly used antimicrobial agents in mouth rinses that are thought to act by interfering with bacterial membrane function.[13] It seems likely that the apparent FPR-blocking activity of alexidine dihydrochloride was an artifact of nonspecific membrane perturbation.

Table 1. Compounds Conferring Nonspecific Cell Fluorescence

Compound	*Color in DMSO*[a]	*FL1*	*FL3*
Hydrastinine hydrochloride	Yellow	Yes	No
Quinacrine dihydrochloride dihydrate	Yellow	No	Yes
Isotretinoin	Yellow	No	Yes
Dantroline sodium salt dihydrate	Red/orange	Yes	Yes
Hycanthone	Yellow	No	Yes
Celestine blue	Blue/black	No	Yes
Clofazimine	Red	No	Yes
Mitoxantrone dihydrochloride	Blue/black	No	Yes
Anthraquinone, 1,5-diamino	Blue/brown	No	Yes
Apigenin	Yellow	No	Yes
Chicago sky blue 6B	Blue	No	Yes
Doxorubicin hydrochloride	Red	No	Yes
Methylene blue	Blue/black	No	Yes
Myricetin	Yellow	Yes	Yes
Daunorubicin hydrochloride	Red	Yes	Yes
Quercetin dihydrate	Yellow	Yes	Yes
Tetrahydroalstonine	None	Yes	Yes
Harmalol hydrochloride dihydrate	Yellow/green	Yes	Yes
Elipticine	Yellow	Yes	Yes
Riboflavin	Yellow	Yes	Yes
Ergocryptine-alpha	Orange	No	Yes
Demeclocycline hydrochloride	Yellow	No	Yes
Merbromin	Red/orange	Yes	Yes
Propidium iodide	Violet	Yes	Yes

Significant increase in cell fluorescence intensity in the FL1 or FL3 channel as indicated.
a. Innate color of compound in the stock DMSO solution.

Also illustrated in Figure 4A is an example of the nonspecific fluorescence effects that were occasionally observed (labeled NS). The compound responsible in this instance was quinicrine dihydrochloride dihydrate, one of 11 compounds (1.3%) that significantly affected cell fluorescence measured in the FL1 channel (Table 1). Thirteen additional compounds (1.5%) exclusively affected cell fluorescence in the FL3 channel (Table 1). As illustrated in Figure 4A, some of the compounds that fluoresced in the FL1 channel caused nonspecific FL1 fluorescence artifacts in cells sampled from wells immediately downstream. Likewise, compounds that fluoresced in the FL3 channel sometimes affected FL3 fluorescence measurements in downstream wells (data not shown). This was due to fluid compound carryover. Up to 6 downstream wells were affected in some instances, although 0 to 2 wells was the more typical range observed. Compound carryover between plates was not observed because the sampling probe was routinely rinsed in the rinse station for 20 s or more after sampling of each plate was completed.

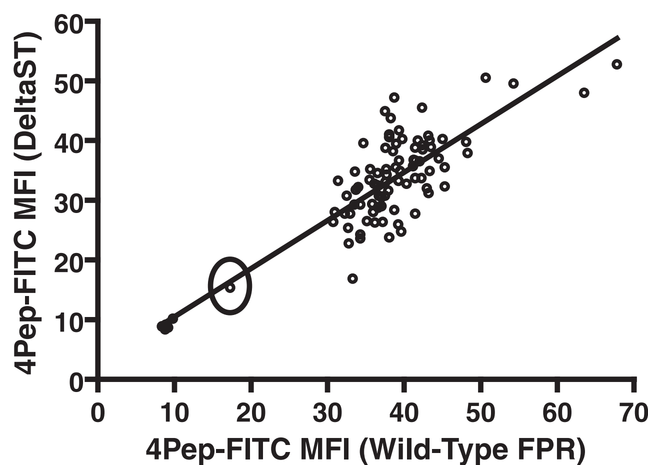

FIG. 5. Comparison of screening assay performance with cells expressing mutant and wild-type formylpeptide receptor (FPR). The FPR screening assay was performed on 2 screening plates containing the same set of compounds in which 1 plate was loaded with DeltaST cells expressing mutant FPR and the other with U937 cells expressing wild-type FPR. Illustrated is a plot of median fluorescence intensity (MFI) data resulting from the binding of fMLFK-FITC to cells in matching wells from each plate. Regression analysis resulted in an R^2 value of 0.74, comparable to results with matched plates containing the same cells. An antagonist present in one of the wells was detected similarly in each plate (circled point).

To determine if such carryover was masking inhibition signals from compounds in downstream wells, we simply removed the interfering compounds from the test plate and reanalyzed the remaining compounds in their absence. To determine if inhibition signals from the fluorescent compounds themselves might have been masked, we modified the assay to use a hexapeptide FPR ligand (fNleLFNleYK, K_d ~30 nM) tagged with Alexa-633, a fluorescent probe excited by the red diode laser of a FacsCalibur flow cytometer. When the 11 compounds with autofluorescence in the FL1 channel (Table 1) were reanalyzed with the Alexa-tagged peptide, there was minimal or no compound autofluorescence observed in the FL4 fluorescence emission channel (>670 nM). In several replicate assays with Z′ scores of 0.6 or higher, none of the 11 compounds exhibited significant inhibitory activity (data not shown).

Comparison of mutant and wild-type FPR

To avoid the possibility of receptor internalization in the FPR assay, we routinely used DeltaST cells expressing a mutant FPR that does not internalize when stimulated with fMLF.[5] However, because the screening assay was maintained at 4° C after ligand addition, a condition unfavorable to wild-type FPR internalization, experiments were done to determine if wild-type FPR might perform comparably in the assay. In a representative experiment (Fig. 5), DeltaST cells and U937 cells expressing wild-type FPR were tested in separate plates that contained the same set of compounds. The assay response points were evenly distributed on either side of the computed regression line (Fig. 5). The R^2 coefficient was 0.743, falling within the normal range of R^2 observed in between-plate comparisons of duplicate plates described above. Moreover, an FPR antagonist was detected comparably on each plate (circled point in Fig. 5). The FPR antagonist used in this example was one of more than 30 that have been identified in screening of additional compound libraries (manuscript in preparation). These results indicated that under appropriate assay conditions, an internalization-deficient FPR was not necessary for good assay performance.

DISCUSSION

The HyperCyt® system for HTFC screening was demonstrated to be a robust, sensitive, and highly quantitative method with which to screen lead compound libraries in a 96-well format. Samples were routinely processed at 1.5 s/well, allowing a plate to be processed in less than 2.5 min. Assay sensitivity was validated by detection of an active structure with relatively low FPR affinity (sulfinpyrazone, K_i = 14 µM) from among a collection of 880 compounds in the Prestwick Chemical Library. At the time of screening, we were unaware of the previously reported FPR activity of sulfinpyrazone.

Homogeneous assays

One feature of the FPR assay highlights an aspect of flow cytometry that has been historically underappreciated. Because of the optical configuration, the laser in a flow cytometer excites only a very small volume of the sample fluid immediately surrounding the cell. This allows distinction of free and particle-bound fluorescent probe over a wide range of probe concentrations. As a consequence, homogeneous no-wash assays may be easily implemented to streamline sample processing. In the present study, we demonstrated high signal-to-background assay responses in the presence of a 1.5-nM fluorescent peptide. At the receptor expression levels used (100,000 to 400,000/cell), our experience has been that good signal resolution may be obtained with FITC-conjugated peptides (1 FITC/peptide) at free peptide concentrations as high as 200 nM. Titration experiments determined that maximal competitor sensitivity occurred when the fluorescent ligand concentration was at or below the K_d (data not shown). Thus, homogeneous competition assays should be feasible using fluorescent ligands with K_ds of up to 200 nM or more.

High-throughput ligand-binding assays

The FPR assay detects active structures on the basis of their ability to block binding of a high-affinity ligand to the FPR. Because ligand binding is measured directly, the assay is likely to detect active compounds independently of potential complexities in cell physiological response patterns. Molecules are detected independently of whether they act as agonists or antagonists or mediate full, partial, or selective (e.g., signaling pathway-specific) activity. Binding assays are also preferable for structure-activity relation-

ship determinations as they produce more quantitative information about compound binding thermodynamics.

Other fluorescence-based methods for high-throughput ligand-binding analysis include fluorescence anisotropy (FA), fluorescence intensity distribution analysis (FIDA), and laser scanning imaging (LSI). FA measurements are confounded by light scatter and are thus most appropriate for analysis of cell lysates rather than intact cells. Solution-based methods such as these require receptor concentrations in the range of the K_d for signal detection. Under such conditions, ligand depletion may be a significant concern. FIDA uses confocal technology to probe very small volumes (~1 fl) as a means to allow distinction between bound and unbound fluorescent ligand in homogeneous assays. FIDA is also most typically used for probing cell lysates because the small interrogation volume is more suitable for measuring ligand interactions with membrane vesicles or small diameter (<200 nm) beads.[14,15]

LSI is in essence an implementation of FIDA in which the excitation laser is continuously scanned through the sample volume during the measurement period. This results in an extended series of confocal volume measurements that comprises a fluorescence image. Like flow cytometry, LSI is capable of robust fluorescence measurements of intact cells. In a recent study, LSI analysis was accomplished at a rate of ~5 s/well in which 1-µl sample volumes containing up to 3000 cells were scanned in each well.[16] In the present study, we routinely analyzed comparable numbers of cells from each well with an average sampling time of ~1.5 s. Both technologies are capable of higher throughput at the expense of decreased optical resolution or increased cell density requirements.

Assay volumes in the present study were 15 µl/well from which 2-µl samples were aspirated for each measurement. We have determined that a minimum assay volume of 8 µl is required to allow accurate sample pick up (data not shown). Thus, HyperCyt® HTFC is presently amenable to assay miniaturization down to the sub-10-µl range but not as low as the 1-µl format reported for LSI. Both flow cytometry and LSI are capable of simultaneous multiparameter measurements,[1,17] thus offering the promise of high-content multiplex screening assays in which multiple receptor-ligand interactions might be measured simultaneously. LSI may be used to analyze cells attached to surfaces, whereas HyperCyt® HTFC requires single-cell suspensions for analysis. An advantage of HyperCyt® is the ubiquity in laboratories worldwide of relatively inexpensive flow cytometers with which it is compatible and the broad diversity of bioassays that have been developed and validated for flow cytometry.

Fluid compound carryover

Compound carryover is an important consideration in the design of high-throughput flow cytometry assays. This is an inherent feature of air-segmented sample streams and results from a thin film of fluid lining the walls of the transport tubing.[10] One solution to this problem is to rinse the sampling probe 1 or more times between each sample. We have documented optimal HyperCyt® rinsing protocols in recent studies in which cells were mixed with compounds online just prior to analysis and subsequently sorted into culture wells on the basis of compound-induced intracellular Ca^{2+} responses.[11] In the present study, we demonstrated an alternate approach that should be generally applicable to properly configured end point assays. Fluid compound carryover effects are manifested only during the time that samples are being transported from microplate to flow cytometer. In a competition assay, the effects of carryover are determined by the off rate of the fluorescent ligand, the initial concentration and affinity of the contaminating competitor relative to fluorescent ligand, and the length of the transport tubing. The existence of compound carryover in our screening studies was indicated by contamination of downstream samples with compounds that caused nonspecific cell fluorescence (e.g., in Fig. 4A). However, although the 4Pep control peptide had an initial concentration 100-fold higher than the fMLFK-FITC ligand and a similar affinity for the FPR, it exerted no detectable inhibitory effects on fluorescent ligand binding to cell samples from downstream wells (Fig. 3D). Thus, the FPR assay as performed is insensitive to carryover effects from this relatively extreme condition of an upstream high-affinity competitor. Compound carryover is unlikely to be a significant issue for general screening because high-affinity compounds are likely to be rare and secondary screens can quickly resolve ambiguities. For unique circumstances such as serial analysis of a collection of high-affinity compounds, it may be prudent to use low compound concentrations or to rinse the sampling probe between samples.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants AI48517, R24-GM60799 (now EB00264), and R24-CA88339 and the University of New Mexico Cancer Research and Treatment Center. Technical and instrument support was provided by the University of New Mexico Shared Flow Cytometry Resource. This work was performed at the Cancer Research Facility, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque. This work was presented in part at the G-Protein-Coupled Receptors Keystone Symposium, Taos, NM, February 2004, and the 22nd Congress of the International Society for Analytical Cytology, Montpellier, France, May 2004.

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